Chemometrics and Intelligent Laboratory Systems 146 (2015) 47–54

Contents lists available at ScienceDirect

Chemometrics and Intelligent Laboratory Systems

journal homepage: www.elsevier.com/locate/chemolab

Optimization of critical parameters during antioxidants extraction from butterhead lettuce to simultaneously enhance polyphenols and antioxidant activity

Gabriela Elena Viacava [a,c], Sara Inés Roura [a,c], María Victoria Agüero [b,c,*]

[a] Grupo de Investigación en Ingeniería en Alimentos, Facultad de Ingeniería, Universidad Nacional de Mar del Plata, Juan B. Justo 4302, 7600 Mar del Plata, Argentina
[b] Laboratorio de Microbiología Industrial: Tecnología de Alimentos, Facultad de Ingeniería, Universidad de Buenos Aires, Intendente Güiraldes 2160, Pabellón de Industrias, Ciudad Universitaria, 1428 Buenos Aires, Argentina
[c] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Av. Rivadavia 1917, (1033) Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 25 September 2014
Received in revised form 24 April 2015
Accepted 4 May 2015
Available online 9 May 2015

Keywords:
Radical scavenging activity
Total phenolic content
Solvent extraction
Bioactive compounds
DPPH

ABSTRACT

The present work was undertaken to optimize critical parameters (ethanol concentration, time and temperature) for antioxidant extraction from lettuce leaves, measured through DPPH radical scavenging activity (DRSA) and total phenolics content (TPC), using Response Surface Methodology (RSM). Individual optimization of each response was carried out and compared with a simultaneous optimization that allowed maximizing the two responses at the same time. For simultaneous optimization, Desirability function with the Larger-the-Best criteria was employed. Determination coefficients (R^2) for the second-order models adjusted by RSM were above 91% and the models showed non-significant Lack of Fit. Single optimization of DRSA found conditions for extraction (70% ethanol, 32 °C and 2.5 h) that allowed obtaining 69.62 mg ascorbic acid equivalent (AAE)/100 g FW, while 43.20 mg gallic acid equivalents (GAE)/100 g FW was predicted for TPC. Meanwhile, when optimizing only TPC as a single optimization, extraction conditions changed (70% ethanol, 42 °C and 2 h) obtaining values of 46.92 mg GAE/100 g FW for TPC and 65.43 mg AAE/100 g FW for DRSA. Optimal conditions found when the Desirability function was applied to simultaneously enhance DRSA and TPC were: 70% ethanol, 32 °C and 2 h. Under these conditions, good values for both responses were predicted: 69.62 mg AAE/100 g FW and 44.37 mg GAE/100 g FW for DRSA and TPC, respectively. These results were validated and a close agreement between experimental and predicted values indicated the suitability of the model employed and the success of RSM in modeling responses to characterize their dependence with extraction conditions under evaluation. Additionally, it was demonstrated the advantage of applying the Desirability function when more than one response must be optimized finding a compromise solution without harming any response as could happen when considering the optimal conditions for only one of them.

1. Introduction

During recent years, a large number of research publications concerning bioactive compounds from vegetable tissues have been developed [1–3]. Among leafy vegetables, lettuce is of particular interest due to its high consumption level around the world and its content of phytochemicals with antioxidant properties such as caffeic acid and its derivatives, flavonols, vitamins C and E, chlorophyll, and carotenoids [4–6]. Extraction of antioxidants from its natural sources is the first step for its quantification and also for its practical application in the food industry [7]. However, there is no definitive method for extraction of lettuce antioxidants at this time.

Gomes et al. [8] optimized the extraction conditions of antioxidants from lettuce by-products using the outer and usually discarded lettuce leaves. In this way, they propose the recovery of this underutilized raw material; however it is well known that these tissues have undergone certain degree of impairment and its nutritional value is diminished. Thus, it is necessary to carry out studies using healthy leaves. In a previous work, Viacava et al. [9] working with the entire lettuce head, established the effect of the degree of sample processing, sample state (fresh and frozen), solid to solvent ratio, solvent type and mixtures with organic acids, and number of extraction steps on the extraction efficiency measured as DPPH radical scavenging activity (DRSA) of butterhead lettuce tissue. However, solvent properties and technological conditions during extraction (e.g. temperature/time combinations) as critical factors affecting extraction have not been evaluated.

* Corresponding author at: Laboratorio de Microbiología Industrial: Tecnología de Alimentos, Facultad de Ingeniería, Universidad de Buenos Aires, Intendente Güiraldes 2160, Pabellón de Industrias, Ciudad Universitaria, (1428) Buenos Aires, Argentina. Tel.: +54 11 4576 3240; fax: +54 11 4576 3241.
E-mail address: mvaguero@di.fcen.uba.ar (M.V. Agüero).

http://dx.doi.org/10.1016/j.chemolab.2015.05.002
0169-7439/

One of the most widespread methodologies to carry out processes optimizations is the widely known Response Surface Methodology (RSM). This powerful mathematical tool presents the advantage of efficiently exploring a particular region on the selected ranges of independent variables at low cost, with a small number of experimental runs [10]. However when several responses must be optimized, the independent optimization of each one can lead to conflicting results, i.e., improving one response may have an opposite effect on another one, falling in the finding of the best solution for all responses simultaneously [11]. For these cases, the Desirability function could be a complementary tool to resolve this conflict, allowing finding the optimal experimental conditions to successfully satisfy the optimization of all responses [11].

Therefore, this research was carried out with the aim of optimizing critical parameters (solvent concentration, temperature and time) affecting the extraction of antioxidants from butterhead lettuce heads using Response Surface Methodology in order to simultaneously maximize the radical scavenging activity and the total phenolics content of lettuce extracts. Single and simultaneous optimizations, through Desirability function, were applied to compare both methodologies' performances when more than one response variable must be optimized.

2. Materials and methods

2.1. Plant material

Heads of butterhead lettuce (*Lactuca sativa* var. Lores) were grown in greenhouses in Sierra de los Padres, Mar del Plata, Argentina. Lettuce heads were harvested, immediately transported to the laboratory and analyzed in the first hour after harvest. Healthy and free from defects leaves from 3 different lettuce heads were used in each experiment.

2.2. Antioxidants and phenolics extraction

Extraction was carried out following the methodology proposed by Viacava et al. [9] but varying the critical parameters: ethanol concentration in the solvent, temperature during extraction and process duration. Briefly, fresh lettuce leaves were homogenized with a tissue blender (Braun Type 4193, Spain) for 1 min. A sample (1 g) was taken from the homogenate and it was added to 10 mL of acidified solvent (with citric acid, 1% w/v). Detailed conditions for critical parameters in each run are shown in Table 1. The ranges selected for ethanol concentration (30–70%v/v), extraction time (1–3 h) and temperature (2–42 °C) were based on practical and economic aspects. Once extraction finished, a centrifugation of the homogenate at 8000 rpm and 4 °C was carried out for 15 min. The supernatant was considered the source of antioxidants.

2.3. Radical scavenging activity determination

DPPH radical scavenging activity (DRSA) was determined using the methodology adapted in our previous work [9]. Briefly, ethanol (0.25 mL) was mixed with 1 mL of DPPH (100 μM) to determine the initial absorbance. Next, 0.25 mL of lettuce extract was added to 1 mL of DPPH (100 μM). The mixture was shaken and the decrease in absorbance at 517 nm was measured after 60 min (in dark) using an UV-visible spectrophotometer (Shimadzu Corporation, Japan). Blank solutions (without DPPH) were prepared to correct any influence due to lettuce extract color.

A calibration curve of the DPPH solution ($Abs_{517nm} = 0.0117^{*}[DPPH] + 0.0086$, $R^2 = 0.9991$) and an ascorbic acid standard curve ($[DPPH] = 8.8833^{*}[\text{ascorbic acid}] - 3.2567$; $R^2 = 0.9696$) were used to express the DRSA of lettuce extracts as mg of ascorbic acid equivalents per 100 g of fresh weight of lettuce (mg AAE /100 g FW).

2.4. Total phenolics content quantification

The total phenolics content (TPC) was determined based on the method of Singleton et al. [12], using the Folin-Ciocalteu Reagent (FCR) with gallic acid as standard. 200 μL of the lettuce extract or the ethanolic solvent used for extraction was added to 1000 μL of FCR (diluted 1/10). After 3 min of incubation at ambient temperature, 800 μL of 7.5% Na_2CO_3 solution was added and the reaction mixture was incubated for 2 h at the same temperature. The absorbance was measured at 765 nm in 1 cm cuvette and TPC was calculated by using gallic acid as standard and expressed as mg of gallic acid equivalent per 100 g of fresh weight of lettuce (mg GAE/100 g FW).

2.5. Experimental design and statistical analysis

Response Surface Methodology (RSM) with a Box-Behnken (BB) design was used to study the influence of the operational conditions during extraction on DRSA and TPC of lettuce extracts. The method of least-squares regression was used to fit data to a quadratic model of the form:

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{2}\sum_{j=2.j>i}^{3}\beta_{ij}X_iX_j + \sum_{i=1}^{3}\beta_{ii}X_i^2 \quad (1)$$

where Y is the predicted response (Y_1: DRSA or Y_2: TPC), β_0 is the model constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is

Table 1
Box-Behnken experimental design matrix for antioxidants and polyphenols extraction.

Run	Independent variables			Coded independent variables			Response variables	
	x_1 Ethanol concentration (%)	x_2 Temperature (°C)	x_3 Time (h)	X_1	X_2	X_3	DRSA (mg AAE/100 g FW)	TPC (mg GAE/ 100 g FW)
1	50	2	1	0	−1	−1	50.35	27.42
2	50	2	3	0	−1	1	33.80	28.71
3	50	42	1	0	1	−1	56.08	37.75
4	50	42	3	0	1	1	48.49	39.88
5	30	22	1	−1	0	−1	53.54	34.14
6	70	22	1	1	0	−1	59.29	33.88
7	30	22	3	−1	0	1	47.66	34.27
8	70	22	3	1	0	1	64.55	38.09
9	30	2	2	−1	−1	0	35.89	28.27
10	30	42	2	−1	1	0	48.11	40.80
11	70	2	2	1	−1	0	59.73	38.98
12	70	42	2	1	1	0	66.56	45.79
13	50	22	2	0	0	0	61.33	41.27
14	50	22	2	0	0	0	60.96	36.97
15	50	22	2	0	0	0	61.49	39.97

the coefficient for the interaction effect, and X_i is a dimensionless coded value of the independent variable, x_i.

For 3 level factor BB experimental design, a total of 15 experimental runs are needed (Table 1) where each variable is tested in three different coded levels: low (−1), middle (0) and high (+1).

Once DRSA and TPC were measured for each trial, the second-order polynomial model (Eq. 1) was fitted to each response variable. Single optimizations were carried out in order to find the optimal conditions that maximize DRSA and TPC with the models found for each case.

On the other hand, a simultaneous optimization was carried out using the Desirability function (D) with the Larger-the-Best criteria. For this purpose, predicted values obtained from each model (y_n) were transformed to a dimensionless desirability scale d_n. The desirability scale ranges from 0 to 1, where $d = 0$ for an unacceptable response value, and $d = 1$ for a completely desirable one:

$$d_n = \begin{cases} 0 & if\ \ y_n \le y_n^{\min} \\ \left(\frac{y_n - y_n^{\min}}{y_n^{\max} - y_n^{\min}}\right)^r & if\ \ y_n^{\min} \le y_n \le y_n^{\max} \\ 1 & if\ \ y_n \ge y_n^{\max} \end{cases} \quad (2)$$

where y_n^{min} is the minimum acceptable value of y_n, y_n^{max} is the maximum value that is considered desirable and r is a positive constant. For our analysis r = 1, indicating that d_n increases linearly as y_n increases.

The individual desirability functions from the considered responses are then combined to obtain the overall desirability D, defined as the geometric average of the individual desirability.

$$D = (d_1, d_2, \ldots, d_n)^{1/n} \quad (3)$$

where $0 \le D \le 1$, a high value of D indicates the more desirable and best functions of the system, which is considered as the optimal solutions of this system. An algorithm is then applied to this function in order to determine the set of values that maximize it [13].

In order to test the reliability of the simultaneous optimization in predicting maximum DRSA and TPC, a new set of experiments using optimal operating conditions obtained with the Desirability function were performed. The experimental and predicted values of DRSA and TPC were compared in order to determine the validity of the model.

Data were analyzed using the SAS 9.0 software (SAS Institute Inc., Cary, U.S.A., 2002). The statistical analysis was performed using the analysis of variance (ANOVA) including the F-ratio, which established the model global significance and the determination coefficient R^2. The Lack of Fit test was performed for each model with a 95% confidence level. In addition, experimental and predicted values for each dependent variable were compared. The significant factors affecting each dependent variable were selected according to the Student t-test establishing a 95% confidence level. Simultaneous optimization was carried out using the software STATISTICA (version 7.0, StatSoft Inc., Tulsa, U.S.A., 2004).

3. Results and discussion

Table **1** shows both DRSA and TPC values for extracts obtained under conditions established by the 15-trial experimental design. As can be observed, high variations in response variables were found as a function of factors under evaluation. In this way, the minimum DRSA was obtained in experiment #2, while the maximum value (obtained in experiment #12) resulted 97% higher than the minimum. These values (Table **1**) are in the range of those reported in our previous work [9]. On the other hand, the minimum TPC was obtained in experiment #1, while the maximum value (obtained in experiment #12) resulted 67% higher than the minimum. These results are in consonance with those reported by Gomes et al. [8] who informed that the TPC of methanolic extracts of lettuce by-products ranged between 26.9 and 56.6 mg GAE/100 g FW, whereas for acetone and water extraction it ranged between 24.6 and 66.9 mg GAE/100 g FW and between 33.7 and 74.1 mg GAE/100 g FW, respectively. However these authors used higher extraction temperatures and different types of solvent for extraction. Viacava et al. [6] also reported that the TPC of lettuce ranged between 19.74 and 46.53 mg GAE/100 g FW from inner to outer leaves when the extraction was carried out with ethanol at 0 °C for 3 h.

Extraction conditions of experiment #12 (high temperature, high ethanol concentration and middle time) yielded the highest antioxidant extraction efficiency, measured as DPPH radical scavenging activity or total phenolics content.

3.1. Influence of extraction parameters on DPPH radical scavenging activity

Estimated regression coefficients for DRSA quadratic model obtained are presented in Table 2. Taking into account the significance of each term, a simplified version of the polynomial equation for DRSA can be expressed as follows:

$$Y_1 = 61.26 + 8.12 \cdot X_1 + 4.93 \cdot X_2 - 8.88 \cdot {X_2}^2 \quad (4)$$

where Y_1 is the DRSA (mg AAE/100 g FW), X_1 is the codified variable for ethanol concentration in the solvent, and X_2 is the codified variable for temperature during extraction. As it can be seen, only linear terms solvent and temperature and quadratic term of temperature resulted significant for this response variable, whereas extraction time did not affect significantly the antioxidant content measured as DRSA. Vázquez et al. [14] found a similar model when they studied the influence of temperature, ethanol concentration and extraction time for the antioxidants isolation from chestnut (*Castanea sativa*) bur. These authors reported that temperature and ethanol concentration were the only significant independent variables that affected the DPPH antioxidant activity of these extracts, but the solvent coefficient had a negative sign.

Analysis of variance showed that DRSA model was significant ($p = 0.034$) and adjusted well to experimental data with non-significant Lack of Fit (Table 3). Moreover, the predicted values for DRSA, calculated from the simplified model (Eq. 4), presented a high correlation coefficient with experimental results (Fig. 1A) confirming the capacity of the model to describe the DRSA of lettuce extracts by the fitted model.

Fig. 2 presents the response surface showing the combined effect of two variables with the third one maintained at its middle value. The effect of ethanol concentration in the solvent can be analyzed through Fig. 2A and B and Eq. 4. The positive coefficient for this variable indicated that DRSA was directly proportional to the concentration of ethanol in the extraction solvent. Additionally, Fig. 2A and B show that DRSA of lettuce extracts increased with increasing ethanol concentration whatever the temperature and time of extraction used. This behavior was associated with the absence of interactions $X_1{}^*X_2$ or $X_1{}^*X_3$ in the model. It is well known that the efficiency of antioxidants extraction depends largely on both the polarity of the solvent and the nature of antioxidant compounds to be extracted [15]. Controversial results are found in the literature in relation to this issue. Some authors reported higher antioxidant activity when using ethanol as extractor solvent on different food matrices [16–18] while others have found the opposite [14,19] and this could be associated with the nature of the antioxidants pool in each food matrix.

Temperature variable positively affects the DRSA (positive coefficient for the linear term X_2), but up to a certain extent. Then, DRSA remained constant for a certain range and finally decreased (Fig. 2A and C). This behavior is associated with the significance of quadratic term for temperature (Table 2) which produces a maximum antiradical activity between 22 and 38 °C, irrespective of the values taken by other tested variables (Fig. 2A and C). High temperature favors extraction by increasing the molecular mobility which has an effect on both the solute solubility and the diffusion coefficient. Reductions in the viscosity and surface tension of the solvent also occur [20,21]. In addition, heating

Table 2
Estimated regression coefficients for RSM analysis of DPPH radical scavenging activity (DRSA) and total phenolics content (TPC).

	DRSA				TPC			
Term	Coded coefficient	SE coefficient	*t*-value	*p*-value[a]	Coded coefficient	SE coefficient	*t*-value	*p*-value[a]
Intercept	61.26	2.80	21.86	<0.001***	39.40	1.39	28.42	<0.001***
X_1	8.12	1.72	4.73	0.005**	2.41	0.85	2.84	0.036*
X_2	4.93	1.72	2.88	0.035*	5.11	0.85	6.01	0.002**
X_3	−3.10	1.72	−1.80	0.131	0.97	0.85	1.14	0.305
X_1^2	0.20	2.53	0.08	0.941	0.36	1.25	0.28	0.787
$X_1 X_2$	−1.35	2.43	−0.56	0.603	−1.43	1.20	−1.19	0.287
X_2^2	−8.88	2.53	−3.52	0.017*	−1.30	1.25	−1.04	0.346
$X_1 X_3$	2.79	2.43	1.15	0.303	1.02	1.20	0.85	0.434
$X_2 X_3$	2.24	2.43	0.92	0.398	0.21	1.20	0.17	0.868
X_3^2	−5.19	2.53	−2.06	0.095	−4.66	1.25	−3.73	0.014*

[a] Coefficients with *p*-value lower than 0.05 were retained in the models.
* Significant with $p < 0.05$.
** Significant with $p < 0.01$.
*** Significant with $p < 0.001$.

might soften the plant tissues, thus more antioxidants would distribute to the solvent [22]. However, beyond a certain temperature, some antioxidants like some phenolic compounds or vitamins can be denatured by chemical or enzymatic reactions [23,24]. Additionally, membranes denaturation could also occur at high temperatures affecting mobility of both solvent and solutes [20]. These kinds of reactions might be responsible for the reduction in DRSA of the lettuce extracts observed beyond 38 °C.

The DRSA was not influenced by the extraction time. This fact could be observed from Fig. 2B and C as DRSA did not change with time for a given solvent concentration and temperature, respectively. It seems that prolonged extraction times are not required to extract compounds that could effectively scavenge free radicals.

The ridge analysis indicated that the maximum DRSA resulted from high ethanol concentration, relatively middle temperature and middle time: $X_1 = 1, X_2 = 0.5, X_3 = 0.5$ in coded values, corresponding to the actual values of 70% ethanol (v/v), 32 °C and 2.5 h, respectively. The model (Eq. 4) predicted that the maximum DRSA obtained with an extraction carried out under these conditions should be 69.62 mg AAE/100 g FW, while TPC (calculated with the model obtained in the following analysis, Eq. 5) should be 43.20 mg GAE/100 g FW.

3.2. Influence of extraction parameters on total phenolics content

Table 2 presents the estimated regression coefficients for TPC obtained with RSM methodology. A simplified model of the polynomial equation that explains total phenolics content of lettuce extracts, considering only the significant terms, is expressed as follows:

$$Y_2 = 39.40 + 2.41 \cdot X_1 + 5.11 \cdot X_2 - 4.66 \cdot {X_3}^2 \quad (5)$$

where Y_2 is the TPC (mg GAE/100 g FW), X_1 is the codified variable for ethanol concentration, X_2 is the codified variable for temperature and X_3 is the codified variable for time of extraction. As it can be seen, only linear terms of solvent and temperature and quadratic term of time resulted significant for this response variable. Other authors, optimizing the same three parameters during polyphenols extraction in different matrices, found various behaviors. Vázquez et al. [14] found that only the linear terms resulted significant for polyphenols extraction from chestnut; Jerez et al. [20] found that time was not significant for pine bark polyphenols; and Saha et al. [25] established that in addition to the linear terms, quadratic and some interaction terms resulted significant for kinema extracts. These discrepancies in the significance of the quadratic model coefficients may be attributed to the particular structure and composition of the vegetable matrices which contains different phenolic compounds as well as other constituents that could affect their extraction. Besides, each biological system can react differently to the processing and extraction conditions and there lies the importance of

Table 3
Parameters of models performance evaluation.

Parameter	Y_1 (DRSA)	Y_2 (TPC)
F-value	5.80	6.95
p-value	0.034	0.023
R^2	0.91	0.93
Coefficient of variation	9.01	6.60
p-value for Lack of Fit test	0.19	0.46

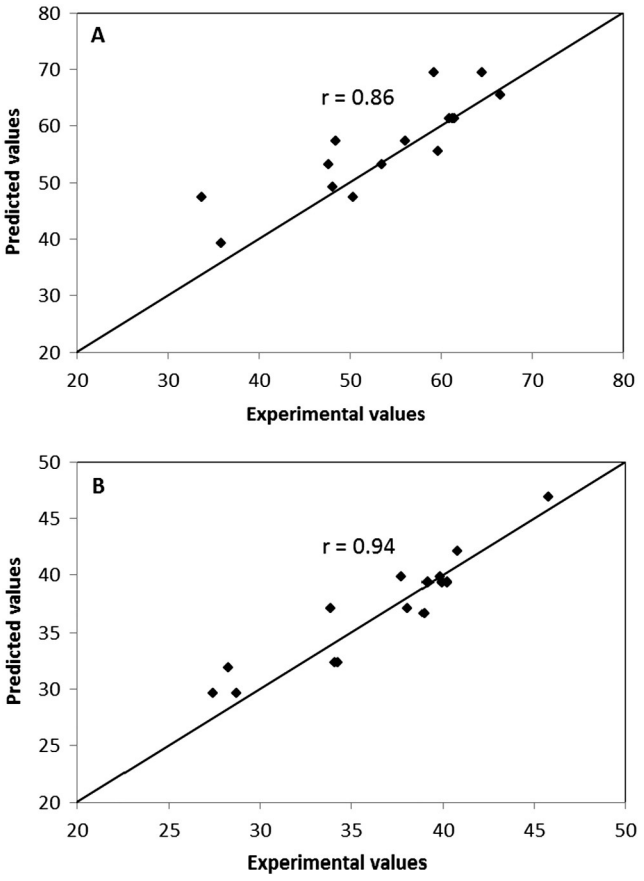

Fig. 1. Experimental versus predicted values for DPPH radical scavenging activity (A) and total phenolics content (B) of lettuce extracts.

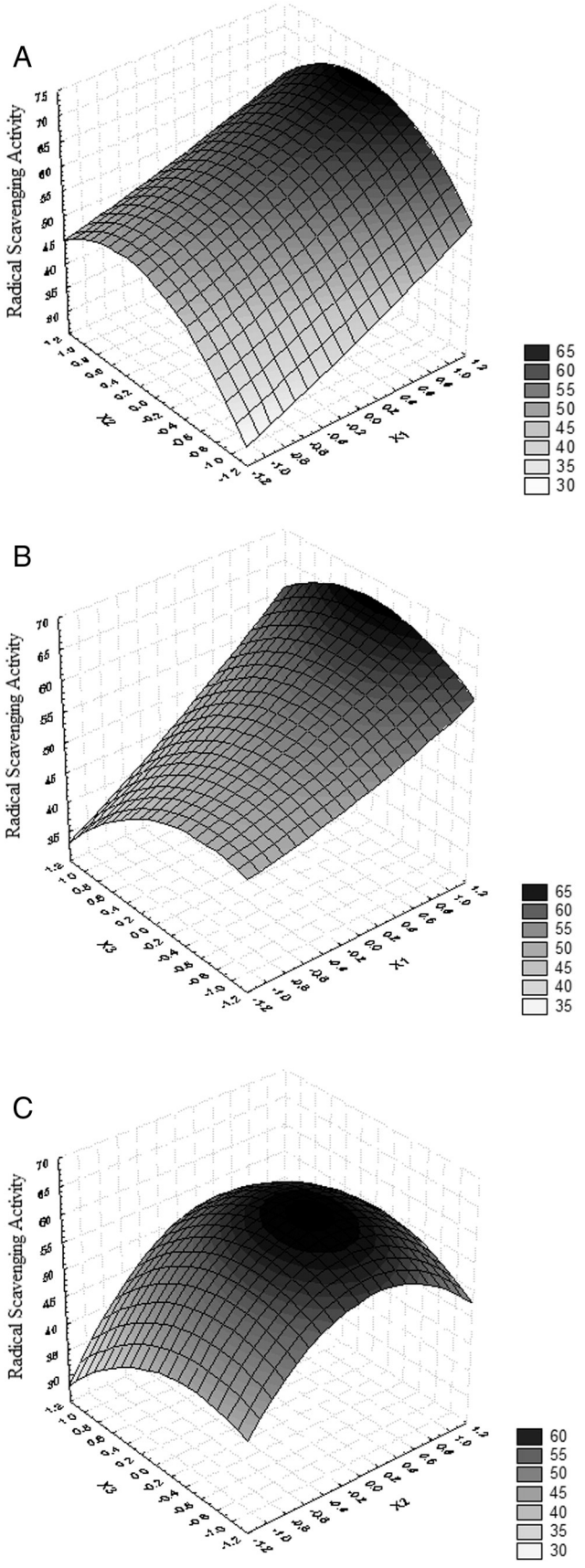

Fig. 2. Response surface plots showing the combined effect of ethanol concentration and temperature (A), ethanol concentration and time (B), and temperature and time (C) on the DPPH radical scavenging activity of lettuce extracts with other variables constant at middle level.

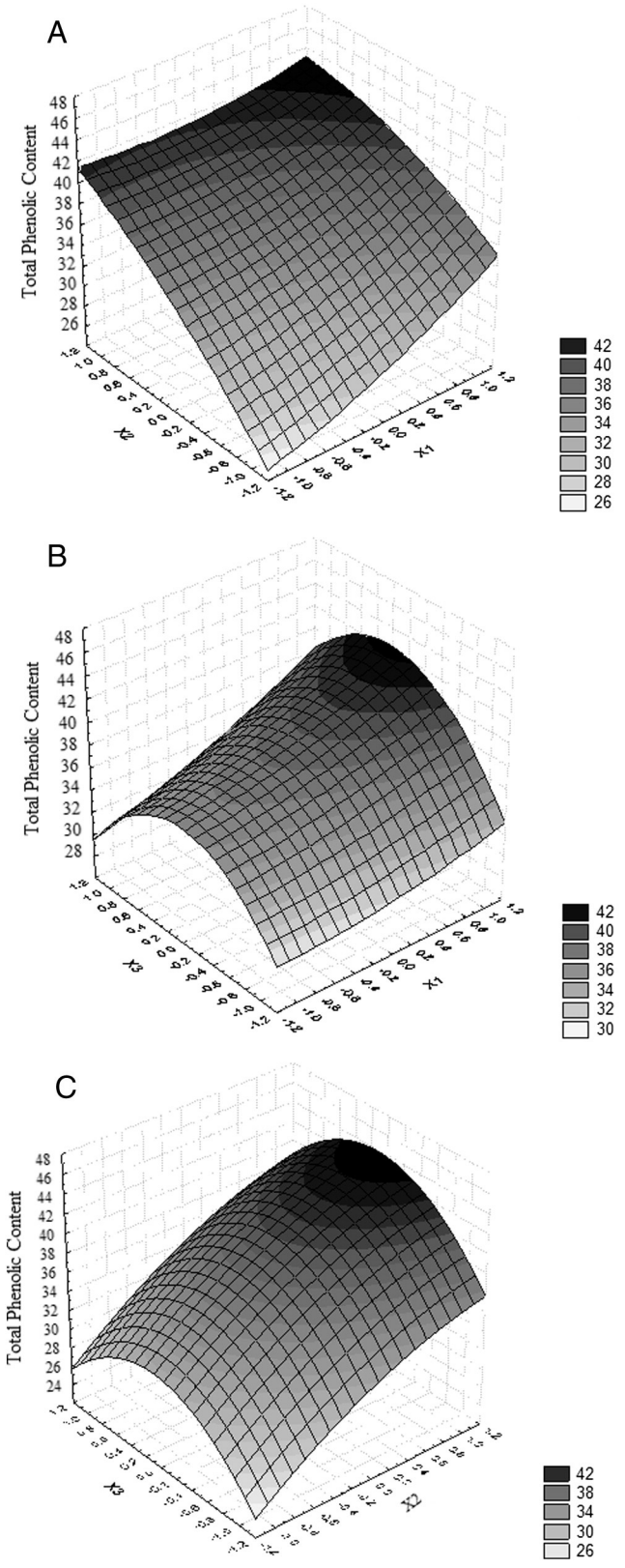

Fig. 3. Response surface plots showing the combined effect of ethanol concentration and temperature (A), ethanol concentration and time (B), and temperature and time (C) on the total phenolics content of lettuce extracts with other variables constant at middle level.

evaluating the effect of extraction factors in each food matrix using multivariable experimental design techniques.

As for the previous response variable, ANOVA showed that TPC model was significant ($p = 0.023$) and adjusted well to experimental data with non-significant Lack of Fit (Table 3). Moreover, the predicted values for TPC, calculated from the simplified model (Eq. 5), presented a high correlation coefficient with experimental results (Fig. 1B) confirming the capacity of the model to describe the TPC of lettuce extracts by the fitted model.

Temperature during extraction resulted in the most significant variable and TPC linearly increased with increasing temperature, independently of both the ethanol concentration and the extraction time (Fig. 3A and C). Besides high temperatures can favor phenolics extraction due to an enhancement of physical factors like solubility, diffusion coefficient, solvent viscosity and surface tension, as mentioned earlier, elevated extraction temperature might also weaken the phenolic–protein and phenolic–polysaccharide linkages, resulting in migration of the phenolic compounds into the extraction solvent [26]. This migration process favors phenolic extraction and thus the TPC values. It is noteworthy that extraction temperatures up to 38 °C did not cause degradation of phenolics as it was suggested when DRSA was used as a measure of antioxidant capacity. This discrepancy in the temperature effect would indicate that antioxidants with antiradical activity other than phenolics (vitamins, for example) are the compounds susceptible to suffer denaturation due to high temperature. Other authors have also informed in different food systems an increase in phenolics extraction with increasing temperature up to 60 °C [8,25], 75 °C [14] and 80 °C [27].

A positive impact of ethanol concentration in the solvent was observed, similar to that found for DRSA, indicating improvements in TPC extraction as ethanol concentration increases (Fig. 3A and B). However, this behavior was more pronounced at low temperatures and middle time. When time and temperature of extraction get closer to their optimum, this is around 2 h and 42 °C, respectively, these variables become more important and the solvent influence get diluted.

The quadratic term of extraction time turned out to be significant, indicating a curvature and nonlinear relationship between time and total phenolics content (Fig. 3B and C). In this sense, TPC increased with increasing extraction time up to 2 hours ($X_3 = 0$), which was the optimum time for obtaining maximum TPC of lettuce extracts. Beyond this value, a decrease in TPC with time was observed whatever the temperature and ethanol concentration assayed. It is known that extended times are expected to favor the extraction of polyphenolic compounds, since it takes time enough to solvent penetration into the plant tissue, dissolving the solute and subsequently diffusing out to the extraction medium [28]. However, large extraction times might also provoke degradation of phenolic compounds due to light or oxygen exposure or enzymatic reactions. It is known that a range of phenolics may be degraded by the enzyme polyphenol oxidase (PPO) [29] and several authors have studied the implication of this enzyme in lettuce [30]. Therefore, losses of phenolics can be derived from enzymatic reactions when high extraction times were used. Several other studies also reported a similar time effect on polyphenol extraction from plant materials [17,24]. However, Vázquez et al. [14] and Saha et al. [25] found that TPC linearly increased with increasing time in chestnut bur and kinema, respectively, and Gomes et al. [8] informed that time variable had no significant effect on the TPC of lettuce by-product extracts.

The optimal conditions for the TPC obtained using ridge analysis were high ethanol concentration and temperature and middle time: $X_1 = 1, X_2 = 1, X_3 = 0$ in coded values, corresponding to the actual values of 70% ethanol (v/v), 42.0 °C and 2 h, respectively. The model (Eq. 5) predicted that the maximum TPC obtained with an extraction carried out under these conditions should be 46.92 mg GAE/100 g FW, while DRSA (calculated with Eq. 4) should be 65.43 mg AAE/100 g FW.

3.3. Simultaneous optimization of antioxidants and phenolic compounds extraction

Total phenolics content showed a similar trend to DRSA. However, it is not surprising to find out that the optimum range of extraction conditions was slightly different for the two responses. Despite both analytical techniques measure the reducing capacity of the sample [31], not all phenolic compounds exhibit antioxidant activity through DPPH assay as each phenolic presents different antioxidant properties, which depends on the chemical structure and –OH position [32]. Moreover, DPPH radical scavenging activity is due not only to phenolic compounds but also to other antioxidants that could be present in the extract [21].

Simultaneous optimization, using the Desirability function (Fig. 4) indicated that the optimum conditions for extraction of antioxidants and phenolics resulted in $X_1 = 1$ (70% ethanol in water), $X_2 = 0.5$ (32 °C), and $X_3 = 0.0$ (2 h), with a desirability value of 0.94. At this point, the investigated responses were theoretically calculated (with Eqs. 2 and 3) as DRSA: 69.62 mg AAE/100 g FW, and TPC: 44.37 mg GAE/100 g FW. These results evidence the advantage of applying simultaneous optimizations because when optimizing only DRSA, a high value was found for this variable but the value obtained under these conditions for TPC was not as good as when optimizing only TPC. In the same way, when optimizing only TPC, a high value was found for this variable but the value obtained under these conditions for DRSA was lower than that found in the previous analysis. In this regard, the simultaneous optimization achieves a compromise finding good values for both variables that are being optimized.

3.4. Validation of the simultaneous optimization model

The new set of lettuce extracts obtained experimentally using optimal extraction conditions predicted by the simultaneous optimization yielded a DPPH radical scavenging activity of 65.33 ± 0.85 mg AAE/100 g FW and a total phenolics content of 42.52 ± 0.87 mg GAE/100 g FW. Relative errors of 6.16 % of the maximum DRSA value and of 4.17 % of the maximum TPC value, predicted by the Desirability function, were determined. These results demonstrated that predicted DRSA and TPC by the simultaneous optimization at optimal extraction conditions agreed with experimental data, confirming the model's validity and robustness.

4. Conclusion

Adequate assessment of antioxidant properties of plant sources is very important because of their potential uses in medicine, food and cosmetics. Thus, standardization of sample preparation, antioxidants extraction and measurement is of essential importance.

In our study, RSM was successfully used to study and model the influence of critical factors (ethanol concentration in the solvent, temperature and extraction time) on the antioxidant extraction from lettuce and simultaneously optimize these factors to yield high total phenolics content and radical scavenging activity in these extracts. ANOVA showed that the solvent concentration and temperature were significant factors to DRSA, whereas also the extraction time was significant for TPC. Optimal conditions for extraction, found with single optimization of DRSA, were 70% ethanol, 32 °C and 2.5 h. Under these conditions the predicted values for DRSA and TPC were 69.62 mg AAE/100 g FW and 43.20 mg GAE/100 g FW, respectively. Meanwhile, when optimizing only TPC, extraction conditions changed (70% ethanol, 42 °C and 2 h) obtaining values of 46.92 mg GAE/100 g FW for TPC and 65.43 mg AAE/100 g FW for DRSA. Simultaneous optimization allowed to find a compromise solution among these values proposing as optimal extraction conditions 70 % (v/v) ethanol, 32 °C and 2 h, which allowed obtaining lettuce extracts with predicted DRSA and TPC equal to 69.62 mg AAE/100 g FW and 44.37 mg GAE/100 g FW, respectively. The validation experiments demonstrated that experimentally

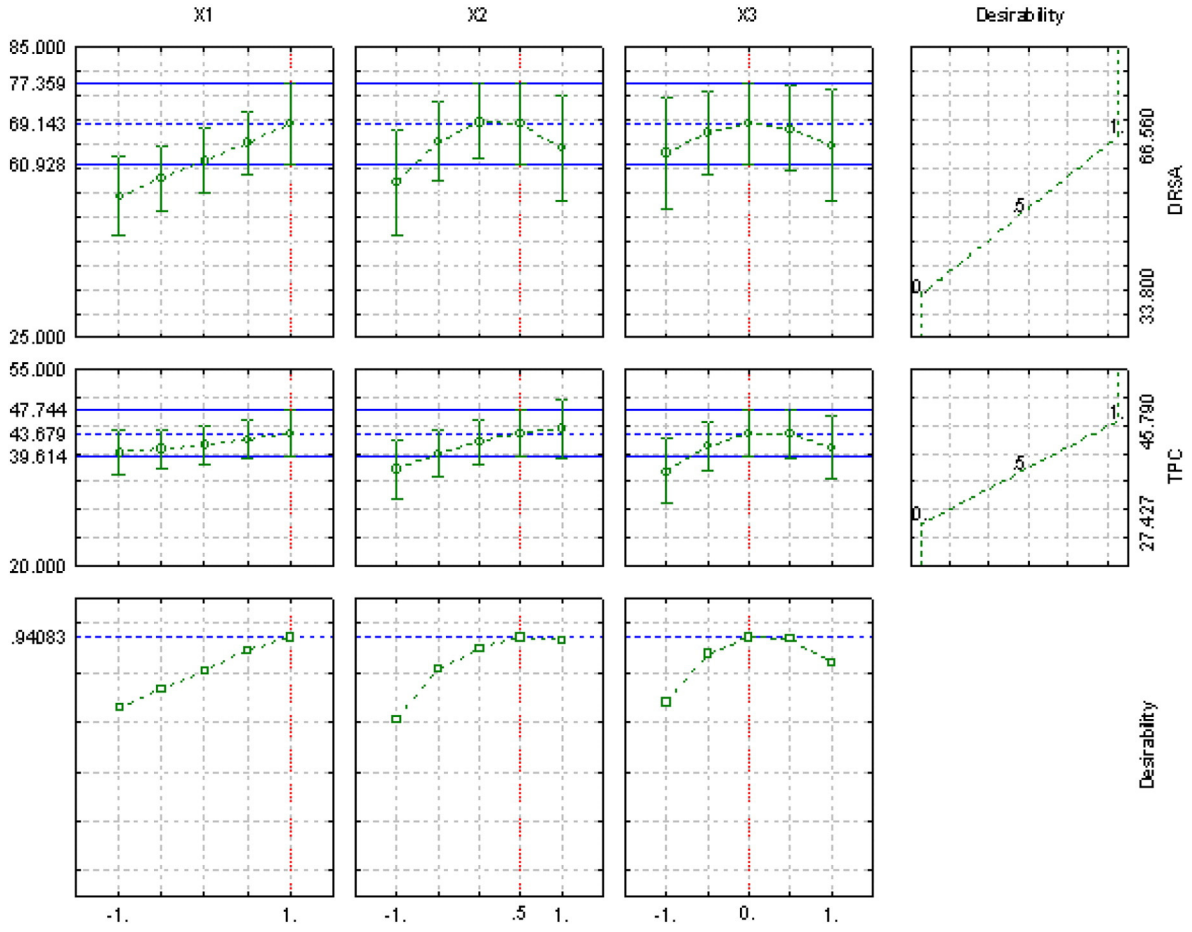

Fig. 4. Profiles for predicted values and Desirability function.

determined values were in close agreement with statistically predicted ones, attesting to the model's robustness.

RSM turns out to be a very useful statistical technique to determine the optimum extraction conditions of lettuce antioxidant compounds. This methodology maximizes amount of information that can be obtained, while limiting the number of individual experiments with consequent economic benefits. When more than one response variable must be optimized, a complementary tool, as the Desirability function, must be applied to simultaneously optimize these variables successfully with the advantage of finding a compromise solution without harming any response as could happen when considering the optimal conditions for only one of them.

Acknowledgements

This work was financially supported by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), the Agencia Nacional de Promoción Científica y Tecnológica (PICT-Foncyt) and the Universidad Nacional de Mar del Plata (UNMDP) of Argentina.

The authors declare that there is no conflict of interest.

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