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Optimization of the enzymatic treatment during aqueous oil extraction from sunflower seeds

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Partially dehulled sunflower seeds were subjected to a hydrolytic treatment with cellulases during aqueous processing for oil and protein extraction. Sub-optimal extraction conditions (particle size and separation technology) were established in order to appreciate the potential improvement caused by the enzymatic treatment and to select the best operational conditions. The effects of three operational variables (extraction–treatment time, water/seeds ratio and enzyme/seed ratio) were studied on three objective functions (the extent of hydrolysis reaction, the oil extraction yield and the percent polyphenolics removal). After 2 h of enzymatic treatment–extraction a practical optimum in the range 7.5–8 g water g^{-1} seeds and 1.25–1.4 g enzyme 100 g^{-1} seeds could be defined. Under these conditions the oil extractability and the polyphenolics removal are improved by more than 30 and 80%, respectively. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Aqueous processing is an alternative technology for the extraction of oil and protein from oilseeds (Rhee *et al.*, 1972; Hagenmaier, 1974; Staron and Guillaumin, 1979; Lawhon *et al.*, 1981; Aguilera *et al.*, 1983; Kim, 1989; Che Man *et al.*, 1996). Like other biorenewable solvents (alcohols, supercritical fluids), the use of water as the most economical extracting agent is gaining interest, especially with the aim of replacing the use of toxic solvents (Shoemaker, 1981; Johnson and Lusas, 1983).

Aqueous extraction is advantageous over conventional pressing and hexane extraction methods since the solvent is neither toxic nor presents any risk of fires and explosion. The operation is more flexible with safer start-up and shutdown in the absence of flammable solvents, favouring less initial capital investment and operation costs and also offering the possibility of processing different temporal crops. The mild operational conditions favour production of high quality products such as oil, that need no further refining, and detoxified meal (Lanzani *et al.*, 1991; Ohlson, 1992). The sequence of steps includes milling of the seeds, mixing with water to extract the oil and centrifugal separation of the liquid and solid phases.

As counterpart, aqueous processing presents lower efficiency of oil extraction than conventional extraction. The oil extraction yields can be improved if an enzymatic treatment is applied during the mixing step (Fullbrook, 1983; Marek *et al.*, 1990; Tano-Debrah and Ohta, 1995*a,b*; Sengupta and Battacharyya, 1996; Tano-Debrah *et al.*, 1996). The cell wall degradation caused by the enzymes increases the permeability to the oil through the seed membranes. The use of several enzymes as cellulases, hemicellulases, pectinases, amylases, proteases has been reported (Lanzani *et al.*, 1975; Bhatnagar and Johari, 1987; Badr and Sitohy, 1992), the multiple activity complexes and enzyme mixtures being especially effective, due to their synergistic action on the demolition of cell walls (Düsterhöft *et al.*, 1993).

Sunflower meal is an important source of good quality protein, although deficient in lysine. It contains few antinutritive compounds; its polyphenolics, through interaction with proteins, can reduce the digestibility of the meal, so their removal is necessary. At the same time, the extraction of polyphenolics and sugars would avoid the dark colour in the processed meal, which limits its use for food purposes. Phenolics content in sunflower defatted meal ranges from 3 to 4% (w/w), 79% of which is soluble and 21% bound to protein (Bau *et al.*, 1983). Chlorogenic and caffeic acids account for 70% of polyphenols present (Mikolajczak *et al.*, 1970; Leung *et al.*, 1981).

Polyphenolics–protein interaction can reduce (besides the protein digestibility) amino acid availability, alter organoleptic and functional properties, shelf-life and stability (Lin *et al.*, 1974; Shamanthaka and Sastry,

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1990). Among several methods proposed to remove polyphenolics are different organic solvents and acidified solvents (Sosulski *et al.*, 1973; Fan and Sosulski, 1976; Sodini and Canella, 1977; Saeed and Cheryan, 1988; Olsen, 1988; Jensen *et al.*, 1990).

The aim of this work was to determine how operational variables affect the efficiency of the enzymatic action during aqueous processing of sunflower into oil and protein, and to develop empirical models for quantitative interpretation of the interrelationships among the variables.

MATERIALS AND METHODS

Partially dehulled sunflower seeds, supplied by Alco (Maia, Portugal), were wrapped in plastic bags and stored at 4°C until use. Seeds were ground and screened to select the fraction size. The seeds contained 53.4% oil (dry basis), 2.04% (dry defatted basis) ethanol-soluble sugars and 2.55% (d.d.b.) chlorogenic acid. The enzyme Celluclast 1.5 L, kindly supplied by Novo Nordisk A/S, was employed.

Enzymatic treatment simultaneous with the aqueous extraction

Particle size was selected between 0.75 and 1 mm. This range, being nearly sub-optimal for extraction (since a reduction in particle size allows the extraction of almost 60% of the total oil) was suitable to appreciate the improvement caused by the enzymatic treatment. Ground seeds were suspended in 0.05 M citrate buffer, pH 4.8. At this pH value, sunflower proteins are insoluble and can be recovered as a concentrate in the solid phase, removing oil and phenolic compounds in the liquid one. The hydrolytic enzymatic treatment to enhance oil extractability was performed during the mixing stage, which was carried out at 150 rpm (in a New Brunswick Innova 4000 orbital shaker) and 50°C (the optimum to preserve the quality of products, and to favour both the activity and stability of enzymes). Optimum pH for these enzymes is in the range 4.5–5.0. Other experimental conditions (water/seeds ratio, enzyme/seeds ratio and extraction–treatment time) depended on the experiment and were varied in the range 5–10 g g^{-1}, 0.5–2 g 100 g^{-1} seeds and 1–3 h, respectively. For this range of times, the observable effect on the oil extraction yield will be ascribed only to the treatment time since, for extraction times higher than 1 h in absence of enzymes, no significant effect on this variable was found (Domínguez *et al.*, 1995).

For the experimental design, sub-optimal conditions for oil recovery were used with only one centrifugation step (11 000 g; 20 min) in a laboratory centrifuge (Beckman J2-20) for separation of solid and liquid phases. The solid residue from the centrifugation was dried and analyzed for residual oil content.

Analytical methods

Oil extraction yield in the liquid phase was calculated as difference between the total oil content of the seeds and the residual in the solid product (measured by Soxhlet with hexane). Reducing sugars content in the liquid phase was determined with the Somogyi-Nelson method (Somogyi, 1952), with D-glucose (Merck) as standard. Hereafter phenolic compounds will be referred to as chlorogenic acid equivalents. The chlorogenic acid content in the aqueous phase was determined by diluting the whey 1:100 with 0.1 M citrate buffer at pH 4.8 to precipitate the soluble proteins. Fifteen ml of this extract were centrifugated at 4200 g for 10 min. The absorbance in the collected supernatant was read at 330 nm in a Hitachi U-2000 spectrophotometer with 0.05 M citrate buffer as blank, using chlorogenic acid (Sigma Chem. Co.) as a standard.

RESULTS AND DISCUSSION

Response surface methodology was applied, using a Box-Behnken factorial design (Box *et al.*, 1988). Three independent and three dependent variables for objective functions were used in order to obtain reliable information on several factors involved in the aqueous processing of sunflower, with enzymes as a processing aid.

The independent variables were **t** (extraction–treatment time, h), **w/s** (water/seeds ratio, g g^{-1}) and **e/s** (enzyme/seeds ratio, g 100 g^{-1}). The objective functions (F_i) were $\mathbf{F_1}$ (reducing sugars, mg g^{-1} seed) as a measure of the extent of the degradation in the cell wall structure caused by the enzyme, $\mathbf{F_2}$ (oil extraction yield, % of the total extractable oil determined by Soxhlet) and $\mathbf{F_3}$ (polyphenolics removal, % of total).

Table 1 lists the set of independent variables corresponding to each experiment, the values of the coded variables being defined by eqns (1)–(3):

$$T = \frac{t - 2}{1} \tag{1}$$

$$W = \frac{w/s - 7.5}{2.5} \tag{2}$$

$$E = \frac{e/s - 1.25}{0.75} \tag{3}$$

The ranges for e/s and t selected were based on those used in the literature (Lanzani *et al.*, 1975; Bhatnagar and Johari, 1987; Badr and Sitohy, 1992) and on previous results (Domínguez *et al.*, 1995). The lowest value of the range of water: seeds ratio was chosen to allow a good mixing in the shaking quipment. A control

Table 1. Operational conditions and experimental results obtained in the study of the enzymatic treatment during the aqueous extraction of sunflower oil

Experiment	T	W	E	t (h)	w/s ($g\,g^{-1}$)	e/s ($g\,100\,g^{-1}$)	Reducing sugars ($mg\,g^{-1}$ seed) F_1	Extracted oil (% total oil) F_2	Polyphenolics (%, total) F_3
1	−1	−1	0	1	5	1.25	0.518	4.34	78.2
2	−1	0	−1	1	7.5	0.5	0.428	4.00	84.7
3	−1	0	1	1	7.5	2	0.684	12.64	82.2
4	−1	1	0	1	10	1.25	0.321	5.094	87.7
5	0	−1	−1	2	5	0.5	0.803	13.01	78.2
6	0	−1	1	2	5	2	0.851	35.65	79.9
7	0	0	0	2	7.5	1.25	0.850	33.07	76.7
8	0	0	0	2	7.5	1.25	0.523	29.83	83.6
9	0	0	0	2	7.5	1.25	1.08	31.65	77.4
10	0	1	−1	2	10	0.5	0.595	7.21	92.4
11	0	1	1	2	10	2	0.875	9.307	91.4
12	1	−1	0	3	5	1.25	1.41	8.183	69.2
13	1	0	−1	3	7.5	0.5	1.09	9.906	85.8
14	1	0	1	3	7.5	2	1.24	4.101	78.1
15	1	1	0	3	10	1.25	1.14	5.281	86.1
16	0	1	0	2	10	1.25	0.847	21.53	89.2
17	0	0	—	2	7.5	0	0.769	≈0	74.2

t,T: extraction-treatment time, w/s, W/S: water/seeds ratio, e/s, E/S: enzyme/seeds ratio. In capital letters when expressed as coded variables.

experiment was performed at the conditions of the central point of the design, but without enzyme (exp. 17).

Experimental results for F_i are also shown in Table 1. These results were used to develop models, according to the generalized eqn (4):

$$F_i = a_O + a_T \cdot T + a_w \cdot W + a_E \cdot E + a_{TW} \cdot T \cdot W + a_{TE} \cdot T \cdot E + a_{WE} \cdot W \cdot E + a_{TT} \cdot T^2 + a_{WW} \cdot W^2 + a_{EE} \cdot E^2 \quad (4)$$

which shows individual and crossed effects of each variable, with three first-order effects (a_T, a_W, a_E) three second-order effects (a_{TT}, a_{WW}, a_{EE}), and the interaction terms (a_{TW}, a_{TE}, a_{WE}).

It can be observed from Table 1 that the production of reducing sugars (F_1) was mainly influenced by the time, being favoured at high values of this variable, as can be seen by comparing experiments 4 and 15, which only differ by T value (−1, 1 respectively), and which offer minimum and nearly maximum values for F_1. Analyzing several pairs of experiments (2,3; 5,6; 10,11) reveals that the greater the E, the greater the concentration of reducing sugars, this effect being more exalted at high W values.

In the control sample a significant effect of extraction time on reducing sugars from 0.049 $mg\,g^{-1}$ seed at 1 h to 0.769 at 2 h and 0.879 at 3 h was noticed (data not shown).

The fact that reducing sugars produced are significantly lower than the reducing sugars concentration registered during the enzyme-aided aqueous extraction of ground sunflower kernels (Domínguez *et al.*, 1995) could be ascribed to the nature of the substrate, containing 15% weight of hulls, a fibrous material composed of 25% cellulose, 28% hemicellulose and 30 % lignin. The high lignification degree of cellulose fibres in the hulls prevents the contact between the enzyme and the substrate, cellulose and hemicellulose being inaccessible to the enzymes. The lignin–cellulose complex in vegetables not only constitutes a resistant material, but also an inactivating agent (Chernoglazov *et al.*, 1988). Another factor responsible for the low production of reducing sugars could be the relatively high particle size of the sunflower ground seeds used in this experiment.

A broad variation in extracted oil (F_2), 4.00–35.65% (of the total extractable oil as measured by Soxhlet), can be obtained depending on the operational conditions during the enzyme-assisted aqueous extraction. The maximum values were those obtained for middle-low water/seeds and middle-high enzyme/seeds ratios (exps. 6 to 9). Under selected separation conditions, hardly any oil could be extracted from the ground seeds, the residual oil in the solid phase being 52.89% (w/w), not significantly different from the initial oil content of the seeds. Oil extraction yields around 30–40% of the total extractable oil are usually reported for the aqueous extraction of ground sunflower (< 1 mm) followed by batch centrifugal separation of the solid and liquid phases (Hagenmaier, 1974; Lanzani *et al.*, 1975; Domínguez *et al.*, 1995). Similar values for control samples were attained with the seeds used in this work with smaller particles (unpublished data). Particle size reduction, incorporation of several centrifugation stages or the use of more efficient separation equipment would provide profitable yields (Rhee *et al.*, 1972; Lanzani *et al.*, 1983; Kim, 1989). However the interest of this work was to obtain a wider range of variation under the different conditions tested, with the aim of better discerning the effects of both the enzymatic and operational conditions.

Values for polyphenolics removal (F_3) varied in a narrow range (78.2–92.4%). For control samples, the polyphenolics removal was 74.2%. The improvement observed in the enzyme-treated samples was probably due to cell wall degradation. That could favour the internal diffusion of polyphenolics in the solid matrix, although the external resistance seems to be more restrictive, because the higher W/S ratio provides the highest polyphenolics removal (see exp. 10 and 11).

Table 2 shows calculated values for the coefficients, as well as their statistical significance (based on a Student t-test). Coefficients with significance of less than 90% (not present in the table) were rejected. The statistical parameters measuring the correlation and the statistical significance of the models, determined by a Fischer test, are also included in this table.

It can be inferred from values in Table 2 that reducing sugars production, F_1, was mainly affected by treatment time, T, the influence of enzyme/seeds ratio, E, being more than three times lower. Only these two linear terms were found to be statistically significant at the 90% level. The linear relationship between reducing sugars concentration in the aqueous extract and treatment time can be considered as a representative measurement of the enzymatic action at this relatively short time.

Water/seeds ratios in the range of those studied in this work did not affect the extent of the enzymatic action. Soluble reducing sugars production was considerably lowered at water/seeds ratios under 1 (w/w) (Domínguez *et al.*, 1993) compared to that achieved when the enzymatic treatment was carried out in aqueous medium (Szakács-Dobozi *et al.*, 1988; Marek *et al.*, 1990; Domínguez *et al.*, 1995; Tano-Debrah *et al.*, 1996).

Figure 1(a) shows the response surface of F_1, reducing sugars, with enzyme/seeds ratio and treatment-extraction time after decoding variables according to eqns (2) and (3), when the water/seeds ratio was kept at 5 (w/w). A continuous increase in reducing sugars production can be noticed when the time increased from 1 to 3 h. A weaker, but similar effect was observed with an increase in the enzyme/seeds ratio from 0.5 to 2 g 100 g^{-1} seeds. Deviation between experimental and predicted values is presented in Fig. 1(b), where the performed experiments have been plotted, indicating their respective numbers.

As can be seen in Table 2, the interrelationship of F_2, oil yield, with the studied variables was characterized by a broad variation range and complex influence of the considered effects. Both the linear terms involving E (significant at the 90% level) and W (significant at the 95% level) influenced the yield of oil extraction. The interaction term including E and W as well as the second-order terms, especially the one depending on the coded treatment time T, were found to be significant.

The response surface in Fig. 2(a), obtained by regression of experimental data, shows the dependence of the oil extraction yield on water/seeds ratio and enzyme/seeds ratio, when using an enzymatic treatment–extraction time of 2 h. A considerable increase in oil extraction yield occurred when the enzyme/seeds ratio increased from 0.5 to 1.25 or when water/seeds ratio decreased from 10 to 7.0. Further increases in E or decreases in W caused limited variations in F_2.

So a maximum oil extraction yield at the central point of the design was located, towards the lower water/seeds ratio and the higher enzyme/seeds ratios. By optimizing the regression equation (obtained when the non-significant terms were neglected) values of W = −0.356 (6.61 g liquid g^{-1} seeds) and E = 0.38 (1.53 g enzyme 100 g^{-1} seeds) were obtained.

An optimum enzyme/seeds ratio of 2 g 100 g^{-1} dehulled seeds have been reported by Badr and Sitohy (1992) at lower water/kernel ratios (1:1 to 1:2.3) with different enzymatic activities and also by Domínguez *et al.* (1995) with 10 g water g^{-1} kernels and a mixture of cellulase and pectinase. Two or three percent appears to be the optimum for enhancing the yield of oil extraction as observed by Lanzani *et al.* (1975) with a similar process and these values were also the most favourable as observed by Bhatnagar and Johari (1987) during hexane-assisted aqueous extraction process. However, for the extraction of shea fat at water/kernel ratio of 2, an optimum enzyme concentration of 1% was found (Tano-Debrah and Ohta, 1995*a*).

A good agreement between experimental and predicted values for the oil extraction yields is presented in

Table 2. Regression coefficients and statistical parameters for the objective functions

F_1		F_2		F_3	
Coefficients	Significance level	Coefficients	Significance level	Coefficients	Significance level
$a_O = 0.8273$	< 0.0001	$a_O = 31.86$	< 0.0001	$a_O = 80.16$	< 0.000
$a_T = 0.3646$	< 0.0001	$a_W = -4.025$	0.0479	$a_W = 6.809$	< 0.000
$a_E = 0.0926$	0.0908	$a_E = 3.446$	0.0945	$a_{EE} = 3.937$	0.0285
		$a_{WE} = -5.136$	0.0805		
		$a_{TT} = -17.51$	0.0001		
		$a_{WW} = -8.365$	0.0122		
		$a_{EE} = -6.943$	0.0272		
Model	< 0.0001	Model	0.0011	Model	< 0.0001
r^2	0.8016	r^2	0.8783	r^2	0.7730
Corrected r^2	0.7710	Corrected r^2	0.7971	Corrected r^2	0.7381
F-ratio	26.2579	F-ratio	10.8220	F-ratio	22.139

F_1:reducing sugars, mg g^{-1} seed, F_2:oil extraction yield and F_3:polyphenolics, % of total.

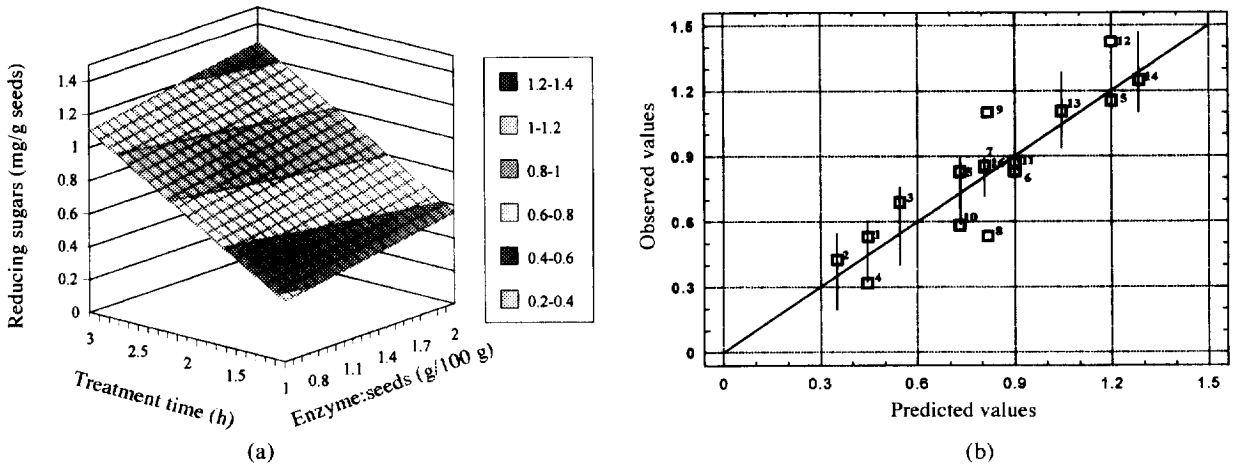

Fig. 1. Response surface (a) and predicted values (b) for reducing sugars production (F_1) as function of T and E, during aqueous extraction and enzymatic treatment performed at 5 g liquid g^{-1} seed.

Fig. 2(b) for a significance level of 95%, the higher deviations being those of experiments 6 and 10.

From data listed in Table 2 it can be deduced that only the term involving the linear effect of the water/seeds ratio exerted a strong influence on the polyphenolics removal, the coefficient being statistically significant at the 99% level. Also the coefficient corresponding to a second order effect of enzyme/seeds ratio was found to be significant (at the 95% limit). In the studied interval, the mathematical model predicted maximum removal of polyphenolics at the experimental conditions defined by high liquid/solid ratios.

Figure 3(a). shows the predictions of the empirical model for dependence of percentage of polyphenolics removal on W and E, relative to experiments lasting 2 h. A continuous increase occurred when the water/seeds ratio was varied from 5 to 10. Despite the presence of hulls, up to 92% removal can be achieved under the best conditions, similar to that achieved during aqueous processing of same-size sunflower kernels (more than 87%) (Domínguez *et al.*, 1994, 1995). Lanzani *et al.* (1983) reported 63% removal of polyphenolics from whole sunflower seeds during the oil extraction with water as extracting agent and 77% from dehulled seeds. Free polyphenolic compounds account for 80% of the total compounds present in sunflower kernels, whereas only 3% of the phenolics contained in the hulls are free (Bau *et al.*, 1983). Particle size slightly influences the extraction of phenolics; so, Sosulski *et al.* (1972, 1973) reported 68% extraction of phenolics from intact kernels whereas, from halves, only 87% was extracted, the testa removal being the more critical factor affecting polyphenolics extraction. Experimental data are, in general, well predicted by the mathematical model for 95% level, as shown in Fig. 3(b).

In any solid–liquid extraction process, the extraction of polyphenolics by acidified water occurs in several steps and the overall rate will be determined by the slowest step. The particle size, to a low extent, and especially the surrounding kernel membrane removal

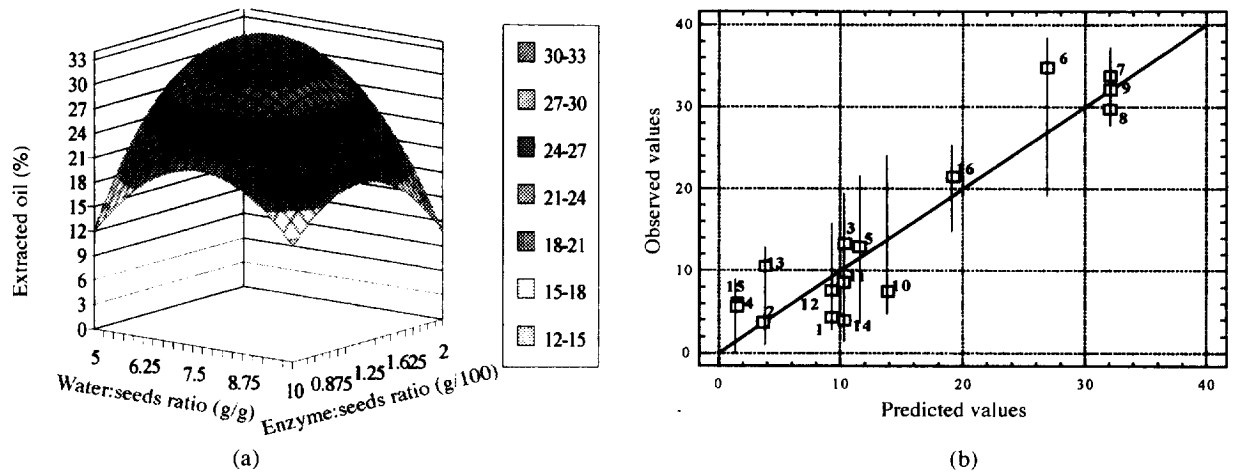

Fig. 2. Response surface (a) and predicted values (b) for the oil extraction yield (F_2) as function of W and E, during aqueous extraction and enzymatic treatment performed after 2 h extraction–treatment.

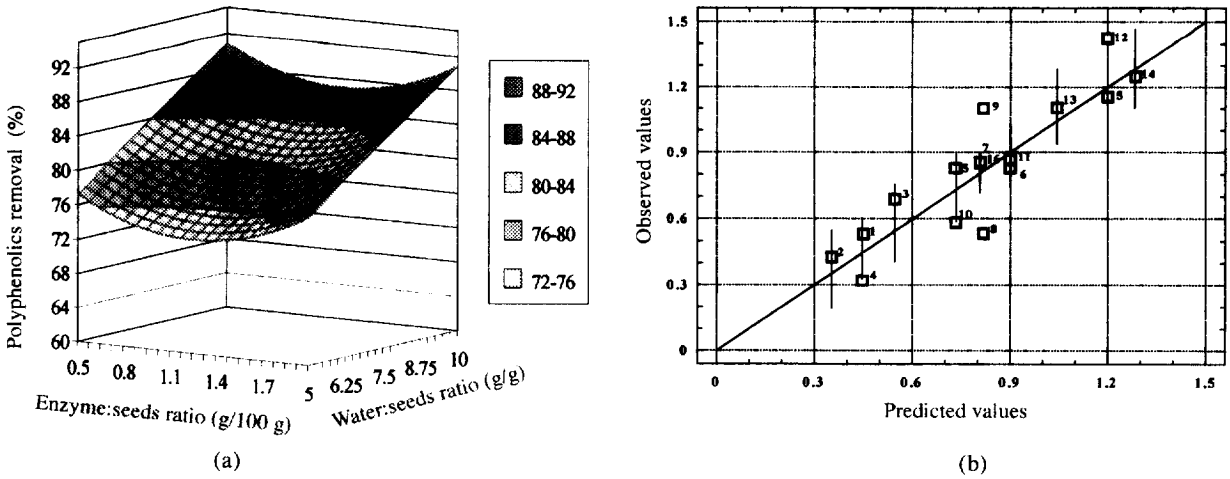

Fig. 3. Response surface (a) and predicted values (b) for polyphenolics compounds removal % (F_3) as function of W and E, during aqueous extraction and enzymatic treatment performed at 2 h.

(Sosulski *et al.*, 1973) are determinants of both extraction yield and rate. If the solvent/seed ratio is high, several stages are used or a continuous process under agitation is performed; the extraction of phenolics from vegetable materials is a diffusion-controlled process (Dibert *et al.*, 1989), unaffected by the flow pattern. However, when the liquid/solid ratio becomes limiting, their concentration in the liquid medium increases and the driving force between surface and the liquid is reduced. This fact was observed in the present experiments, since low liquid/solid ratios were used, and the process consisted of only one mixing-centrifugation stage.

Optimization based on F_2 and F_3

The increase in the oil extraction yield is the objective function most representative of the efficiency of the aqueous process and the main objective when the enzymatic treatment is employed. As a high quality meal is desired, a reduction in polyphenolics must be attained. Therefore the optimization of the treatment should be based on both objective functions. On the basis of the above discussion, the best conditions for oil extraction are found to be low water/seeds ratio and intermediate enzyme/seeds ratios, whereas for polyphenolics removal, only water/seed ratio is crucial to achieve the highest value. It is clear that a 'compromise' solution is needed, for which the following criteria could be used: $F_2 \geq 30\%$ and $F_3 \geq 80\%$ For an enzymatic treatment time of 2 h, water/seeds ratios for which oil yield extraction is $\geq 30\%$ are in the range 7.5–8 and, with these values, polyphenolics removal is $\geq 80\%$ only if the enzyme/seeds ratio is 1.25–1.4 g 100 g^{-1}. The area satisfying both conditions has been plotted in Fig. 4. Estimated values of $F_1 = 0.83$, $F_2 = 33.74$ and $F_3 = 85\%$ were obtained.

The main purpose of this study was to maximize the efficiency of the enzymatic treatment and the oil recovery. Although the degree of polyphenolics removal under these conditions is not the highest attainable in the experimental conditions assayed, it is expected that the use of more centrifugation stages will offer better results than the use of W values even higher than those used in this work.

Effect of the enzyme/seeds ratio on F_2/F_1 ratio

Since the extent of the enzymatic action can be measured by the soluble reducing sugars production, and the oil yield improvement is dependent on cell wall rupture, a relationship between the percentage of oil extracted (F_1) and the reducing sugars (F_2), could be used as an indication of the oil extracted from the ruptured cells. The F_2/F_1 ratio, as a function of enzyme/seeds ratio and process duration is shown in Fig. 5.

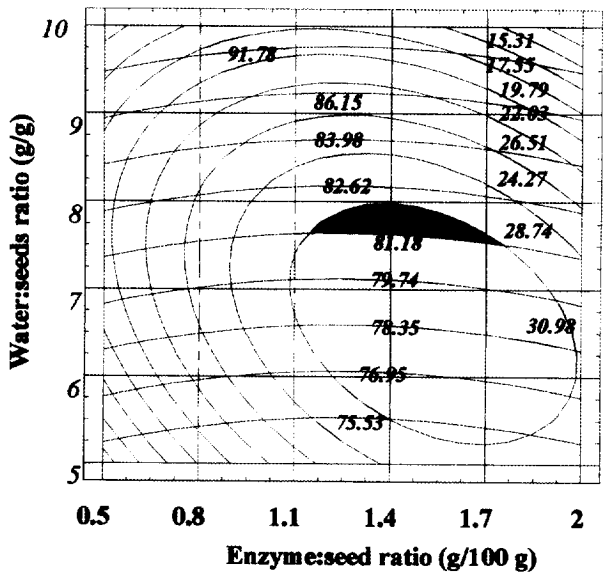

Fig. 4. Practical optimum for oil extraction and polyphenolics removal as a function of water/seeds ratio and enzyme/seeds ratio.

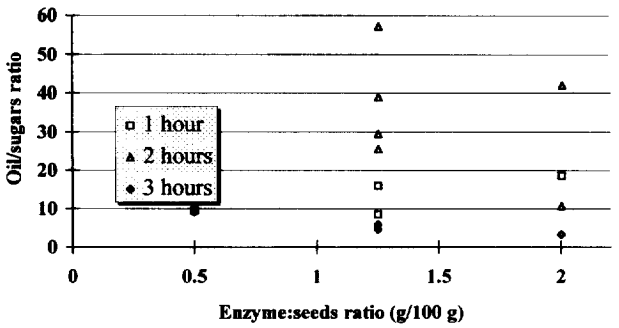

Fig. 5. Oil extraction yield/reducing sugars extraction for different enzyme/seeds ratios during this work.

Maximum efficiency of oil extraction was obtained for 1.25 g enzyme $100\,g^{-1}$ seeds and for 2 h.

CONCLUSIONS

The influence of some operational variables (treatment–extraction time (T), water/seeds ratio (W) and enzyme/seeds ratio (E)) on aqueous extraction of sunflower seeds was assessed by using second-order empirical models derived from experimental data. The degree of cell walls enzymatic attack was found to be dependent on E and T, the oil extractability on W and E and the polyphenolics removal only on W. Since sub-optimal conditions for oil extraction and recovery (particle size and separation conditions, respectively) were used, the presented results are useful for selecting more efficient enzymatic action.

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