Optimization of accelerated solvent extraction of antioxidants from *Spirulina platensis* microalga.

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ABSTRACT

An experimental design has been used to optimize the extraction of antioxidants from the microalga *Spirulina platensis* using Accelerated Solvent Extraction (ASE) with four different solvents (hexane, petroleum ether, ethanol and water). The optimization of the main variables involved in the ASE process (extraction temperature and extraction time) has been performed by means of a Full Factorial (3 levels) design using as responses the extraction yield and the antioxidant activity of the extracts (determined as EC_{50}, i.e. efficient concentration, using an *in-vitro* assay based on a free radical method). The parameters of the model, for each response variable, were estimated by multiple linear regression (MLR). The statistical analysis of the results allowed obtaining mathematical models able to predict the behavior of the different responses selected as a function of the main variables involved in the process. It could be observed that the optimum conditions that maximize yield and minimize EC_{50} depend on the polarity of the solvent used to perform the extractions being the optimum temperature higher when using higher polarity solvents. The effect of extraction time was not as important as the effect of temperature but still had some influence mainly in its interaction with temperature.

Ethanol was finally selected as the extracting solvent for its GRAS (generally recognized as safe) nature and because it provides the higher yields with medium antioxidant activities. The results presented in this work show the possibility of using a fast and easy process to recover natural antioxidants from natural sources such as microalgae.

KEYWORDS: ASE, antioxidant compounds, alga, subcritical water, experimental design, optimization.

INTRODUCTION

In recent years, there has been a growing interest in functional foods, that is, foods able to provide additional physiological benefits for human health others than the basic nutritional and energetic requirements (Goldberg, 1996). Often, functional foods are traditional foods enriched with an ingredient able to provide or promote a beneficial action for human health. These are called functional ingredients. These ingredients are preferred to have a natural origin being commonly extracted from natural sources such as plants or, more recently, algae and microalgae. These type of marine sources are receiving much attention mainly for their content in, for example, polyunsaturated fatty acids (Mahajan & Kamat, 1995; Cohen & Vonshak, 1991), beta-carotene and other pigments (antioxidants) (Madhava, Bhat, Kiranmai, Reddy, Reddanna & Madyastha, 2000; Bhat & Madyastha, 2000), sulphated polysaccharides (anti-virals), sterols (antimicrobials), etc (Richmond, 1988; Ötles & Pire, 2001; Xue et al, 2002). In this work, the microalga *Spirulina platensis* is investigated as natural source of antioxidants, an important kind of compounds for the food industry because of their usefulness as a preservation method and their known beneficial effects for health.

The traditional extraction methods used to obtain these type of products have several drawbacks. They are time consuming, laborious, have low selectivity and/or low extraction yields; moreover, they usually employ large amount of toxic solvents. At present, there is a renewed interest in developing new processes based on the use of sub- and supercritical fluids; that is, environmentally friendly processes that use GRAS solvents (ethanol, water) or small amounts of toxic solvents. These sub- and supercritical processes provide some additional benefits such as higher selectivity and shorter extraction times. Among them, Supercritical Fluid Extraction (SFE) and Accelerated Solvent Extraction (ASE) are two of the most promising processes (King, 2000).

ASE has been used with ethanol as a solvent to study the carotenoids extraction from microalgae *Haematococcus pluvialis* and *Dunaliella salina* (Denery, Dragull, Tang & Li, 2004). The authors demonstrated that similar extraction yields could be obtained with this technique compared to traditional extraction techniques.

In a previous work, we demonstrated the great possibilities of the combined use of ASE, *in vitro* assays and micellar electrokinetic chromatography with diode array detection (MEKC-DAD) to investigate natural sources of antioxidants from microalga *Spirulina platensis* (Herrero, Ibáñez, Señoráns & Cifuentes, 2004). In that paper, we presented the development of a new MEKC-DAD method able to provide a fast profile of the different ASE-extracts that, at the same time, were functionally characterized by an *in-vitro* assay and correlated with the MEKC-DAD profile obtained.

The objective of this work is to optimize, by means of an experimental design using a quadratic mathematical model, the process of extraction of antioxidant compounds from the microalgae *Spirulina platensis.* Different extracting solvents (with different dielectric constant) have been tested in order to evaluate the solvent polarity influence on the ability of extracting natural ingredients with antioxidant activity. The optimization will provide, not only the optimum conditions (in terms of extraction yield and EC_{50} values, that is, the concentration of antioxidant needed to reduce by 50% the initial concentration of a free radical, DPPH) but also mathematical models able to properly predict the behavior of the system considering the factors (extraction temperature and extraction time) that influence the extraction process.

MATERIAL AND METHODS

Samples and chemicals

Microalgae samples (*Spirulina platensis*) consisted of air dried microalgae with 6% moisture weight, from Algamar S.A. (Pontevedra, Spain), stored under dry and dark conditions.

2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH, 95% purity) was obtained from Sigma-Aldrich (Madrid, Spain). Methanol and ethanol were obtained from Scharlau Chemie S.A. (Barcelona, Spain). Hexane, HPLC grade, provided from Lab Scan (Dublin, Ireland) and petroleum ether was purchased from Panreac Quimica S.A. (Barcelona, Spain). The water used was Milli-Q Water (Millipore Corporation, Billerica, MA, USA).

Experimental design.

The effects of two factors, extraction temperature (Tem) and extraction time (tim), on the antioxidant activity and extraction yield of *Spirulina platensis,* were studied using a Full Factorial (3 levels) design. A total of 12 experiments: 9 points of the factorial design, and 3 center points to estate the experimental errors, were carried out in randomized run order. By using this design, the two variables were tested at 3 different levels: extraction temperature at 60, 115 and 175 ºC, and extraction time at 3, 9 and 15 minutes. The response variables selected were EC_{50} (i.e. efficient concentration, as an antioxidant activity measure) and extraction yield (Yield). Table 1 shows the experimental matrix design, with the experimental levels of the independent variables (factors), along with the results obtained for the response analyzed variables for each solvent (Hexane, Petroleum Ether, Ethanol and Water). The quadratic model proposed for each response variable (Y_i) was:

$$Y_i = ß_0 + ß_1\, Tem + ß_2\, tim + ß_{1,1}\, Tem^2 + ß_{2,2}\, tim^2 + ß_{1,2}\, Tem.tim + \text{error} \qquad (1)$$

where $ß_0$ is the intercept; $ß_1$ and $ß_2$ the linear coefficients; $ß_{1,1}$ and $ß_{2,2}$ the quadratic coefficients; $ß_{1,2}$ the interaction coefficient; and error is the error variable. The parameters of the model were estimated by multiple linear regression (MLR) using the program MODDE 5.0, a Software for Design of Experiments and Optimization (Umetrics AB, Umeå, Sweden). This program permits the creation and analysis of experimental designs. The goodness of fit of the model was evaluated by the coefficient of determination (R^2), the residual standard deviation (RSD) and the lack of fit test for the model from the ANOVA table. From the fitted model, the optimum conditions, that maximize the Yield and minimize the EC_{50} response variables, are also provided by the program. Contour plots were developed using the fitted quadratic polynomial equation obtained.

Accelerated Solvent Extraction (ASE)

To perform the extractions with the four different solvents (i.e. Hexane, Petroleum Ether, Ethanol, and Water), an Accelerated Solvent Extraction system ASE 200 from Dionex Corporation (Sunnyvale, CA, USA) was used equipped with a solvent controller unit. Extractions were performed at three different extraction temperatures (60, 115 and 170ºC) and different extraction times (3, 9 and 15 minutes) according to the experimental design employed. Previous to each experiment an extraction cell heat-up was carried out for a given time, which changed according to extraction temperature. Namely, 5 minutes heat-up was used when extraction temperature was set at 60ºC, 6 minutes at 115 ºC and 8 minutes at 170ºC. Likewise, all extractions were performed in 11 mL extraction cells, containing 2.5 g of sample.

Extraction procedure was as follows: i) sample is loaded into cell, ii) cell is filled with solvent up to a pressure of 1500 psi, iii) heat-up time is applied, iv) static extraction takes place (i.e. at 3, 9 or 15 minutes) in which all system valves are closed, v) cell is rinsed (with 60 % cell volume using extraction solvent), vi) solvent is purged from cell with N_2 gas and

vii) depressurization takes place. Between extractions a rinse of the complete system was made in order to overcome any carry-over. The extracts obtained were protected from light and stored under refrigeration until dried. For solvent evaporation a Rotavapor R-200 (from Büchi Labortechnik AG, Flawil, Switzerland) was used when the extracts were obtained with organic solvents and in case of water extracts a Freeze Dryer Unitop 400 SL (from Virtis, Gardiner, NY, USA) was used. Afterwards, different extract solutions were prepared using the same solvent that during extraction, at a known concentration. In the same way, the solutions were stored at 4 ºC and protected from light. When water was used as extracting solvent, care must be taken with the clogging of the extractor lines by the extracted material. To avoid clogging, the microalgae were placed inside a filter paper and the extraction procedure was performed as mentioned.

Antioxidant Activity Determination (In-Vitro Assay)

Antioxidant activity of all extracts was measured using a method based on a procedure described by Brand-Williams et al. (Brand-Williams, Cuvelier & Berset, 1995). The method consists on the neutralization of free radicals of DPPH by the extract antioxidants. The procedure followed was: 23.5 mg of DPPH were weighed and dissolved on 100 ml methanol. This solution was stored at 4ºC. To do the measurements, this stock solution was diluted 1:10 on methanol. Different concentrations of the extracts solutions were used. Then, 0.1 ml of these solutions were added to 3.9 ml diluted DPPH solution to complete the final reaction medium (4 ml). Due to the colored extracts it was necessary to prepare a control (i.e. blank) which consisted of 0.1 ml of each solution added to 3.9 ml of methanol. The reaction was complete after 4 hours at room temperature, and the absorbance was measured at 516 nm in a UV/VIS Lambda 2 spectrophotometer from Perkin Elmer Inc. (Wellesley, MA, USA). Methanol was used to adjust the zero. The absorbance value was obtained by subtract the blank absorbance measurement to the value given by the extracts

solution. The method was calibrated using DPPH solutions of different concentration that allowed to know the DPPH concentration remaining when the reaction was finished. The calibration (n=7; r=0.999) gave the following equation: [DPPH] = (Abs + 0.0029) / 0.0247. For each extract, a known concentration solutions was prepared in order to obtain the remaining DPPH concentration when reaction was finished. The use of these values allowed the estimation of the extract concentration necessary to achieve a 50% reduction of the initial DPPH concentration. This value is known as EC_{50} (Efficient concentration, also called oxidation index) and was utilized to describe the antioxidant activity.

RESULTS AND DISCUSSION

In this study, different extracting solvents (covering a wide range of dielectric constants, that is, 1.9 for hexane, 4.3 for petroleum ether, 24.3 for ethanol and 78.5 for water) have been tested in order to evaluate the influence of the solvent polarity in the ability of extracting natural antioxidants from the microalga *Spirulina platensis.* Accelerated solvent extraction was used since it provides fast extractions with minimum solvent consumption while providing high recoveries. For each of the solvents studied, an experimental design was performed considering the most important factors involved in an ASE process, that is, extraction time and temperature.

Effects of the factors

As mentioned in the Material and Methods section, Table 1 reports the values of EC_{50} and Yield (response variables Y1 to Y8), obtained for all the experiments corresponding to the matrix design. MLR was applied to estimate the parameters of the proposed model in the Equation 1 for each of the eight response variables. A summary of these results is show in Figure 1, where the regression coefficient values for centered and scaled factors are shown as bar graphs for all the responses considered. In the plot, to make possible the

comparison of the coefficients between responses, the corresponding values are normalized by dividing them by the standard deviation of their corresponding responses. Figure 1 shows the importance of the different terms in the model for each of the responses evaluated. As can be seen, temperature (Tem) and its quadratic term (Tem*Tem) have the strongest influence in all response variables, with a positive influence in all of them except for Y7 (EC_{50} values using water as extracting solvent); the extraction time (tim) and its quadratic term (tim*tim) have a lower influence and the temperature-time interaction term (Tem*tim) only shows some effect for more polar extraction solvents (response variables from Y5 to Y8). As can be seen, the factors that mostly influence extraction yields (responses Y2, Y4, Y6 and Y8) have a similar pattern showing an increase on the response by increasing the extraction temperature. This fact can be explained for the increase in the diffusion coefficient of the liquid solvent into the solid matrix when increasing the extraction temperature that favors the kinetics of desorption of the compounds from the matrix. On the other hand, stronger differences among the factors that influence the EC_{50} values (for the different solvents studied and considering that the highest antioxidant activity corresponds to the lowest EC_{50} values) are found, mainly when comparing organic solvents and water. This is probably due to the effect of the change in the dielectric constant of water with the temperature that favors the extraction of less polar compounds, that is, compounds similar to those extracted with medium-low polarity solvents and that seemingly are those who mostly contribute to the antioxidant activity of the microalgae extracts (thus decreasing the EC_{50} value when increasing the amount of non-polar compounds extracted) (Herrero et al, 2004).

The statistical significance of the estimated regression coefficients was analyzed from the table of analysis of variance, and the interaction and quadratic terms of the model not significantly different from zero ($P>0.10$) were excluded from the Equation 1 and the mathematical model was refitted by MLR. The new results are listed in Table 2, and they

include the following information: the regression coefficients obtained, for unscaled factors, the determination coefficient (R^2), the residual standard deviation (RSD) and the P-values from the lack of fit test for the model. From these results, the following conclusions can be drawn: the eight estimated models were found adequate enough to describe the data (P-values of lack of fit test > 0.05); the fraction of variation of the response variable explained by the model (R^2) was superior a 0.80 for the EC_{50} response and higher than 0.96 for the Yield response; the residual standard deviation of the fit for the EC_{50} was lower than 7.5 for three of the four solvents used and slightly higher than 20 for water extraction; when considering the Yield, the RSD values were lower than 0.55 for all the solvents considered. The RSD values, expressed as a percentage of the mean value of the response ($RRSD = RSD/\bar{Y}$), provides a measure of the relative error of the fit; values obtained are also shown in Table 2 being all of them lower than 10%, except the one corresponding to the Y4 response (Ether-Yield) that was slightly higher.

Figure 2 shows the contour plots for the response variables, as a function of temperature and time, the contour plots are used for visually predicting future responses, and for determining factor values that optimize the response function. By analyzing the plots for the EC_{50} responses, and considering that to optimize the antioxidant activity of the extracts the response EC_{50} has to decrease, it can be seen that an increase of temperature produces an increase in EC_{50}, that means a decrease in antioxidant activity, showing, when organic solvents are considered (responses Y1, Y3, Y5), an optimum at intermediate temperatures of the experimental region studied (that is, from 90°C to 120°C); it is also shown that the time factor has a very low influence in the final response. Thus, although similar antioxidant capacity seems to be obtained for the microalgae extracts obtained using hexane, petroleum ether and ethanol, it can be deduced that in general a slightly better antioxidant activity was obtained for hexane-microalgae extracts, that is with lower polarity solvents. This difference can be correlated to the higher amount of non-polar compounds

(carotenoids, among others) that can be extracted using hexane and that contribute to the antioxidant activity of the extracts, as it was suggested in a previous publication (Herrero et al., 2004). When using water as extracting agent, the behavior is completely different decreasing the EC_{50} values when increasing the extraction temperature. As mentioned before, heating water at high temperatures (while keeping it in the liquid state) produces a decrease in its dielectric constant, approaching its behavior to less polar solvent (an increase of temperature from 25°C to 170°C reduces the dielectric constant of water by half, from 80 to 40).

The analysis of the surface plots for the Yield (responses Y2, Y4, Y6 and Y8) shows a typical behavior, increasing the response by raising the extraction temperature; this is also true when increasing the extraction time for all the solvents except for ethanol although the effect of time in ethanol is less important. The predicted Yield values are higher for the more polar solvents, ethanol and water, reaching, when ethanol is considered, values up to 18% at the highest temperature (170°C). This result can be explained through the composition of Spirulina (Richmond, 1998); thus, this microalgae is composed of 50-70% of protein and about 15% of carbohydrates (Richmond, 1998). Therefore, it is expected that by using more polar solvents these polar compounds can be extracted in a higher extent, increasing in this way the yield of extract obtained.

As can be inferred from the comments about the extraction conditions to optimize both, EC_{50} and yield, it will be very difficult to make compatible obtaining large amount of extracts (high yield) together with high antioxidant activity, because the highest yields were obtained at the highest temperatures. Therefore, from the point of view of the whole process, it will be necessary to reach a compromise between the two responses. Table 3 shows the optimum conditions of the extraction process, provided by the statistical program, and the predicted value for the response variables, using the fitted model in Table 2. As can be seen, when minimizing EC_{50}, the optimum temperature depends on the solvent

polarity being the highest when water was considered (T= 170°C). When trying to maximize the extraction yield, we can observe that the optimum temperature was always the highest experimental value, that is, 170°C. As for the extraction time, the optimum was the highest experimental value tested (that is, 15 min) for 5 of the 8 responses analyzed. Nevertheless, we must consider that the effect of the extraction time was not as important as the extraction temperature on the final response (see Table 2).

Interestingly, from the optimum values given in Table 3, it can be also deduced that ethanol extracts posses a good antioxidant activity slightly worse than that obtained with hexane and petroleum ether. This property can be used as an additional advantage taking into account that ethanol, unlike hexane or petroleum ether, is generally considered as GRAS and therefore, can be used as safe solvent for the food industry. Moreover, the yields obtained with ethanol are the highest ones providing a good efficiency of the extraction process.

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FIGURE LEGENDS.

Figure 1. Plot of normalized regression coefficient values, for centered and scaled factors, obtained from MLR, for the eight response variables (Y1 to Y8) studied.

Figure 2. Contour plot for the EC_{50} and Yield, as a function of temperature and time, for each of four studied conditions.

Table 1. Experimental matrix design and results obtained for each of the response variables studied.

			Hexane		Petroleum Ether		Ethanol		Water	
Exp.	Temperature (ºC)	time (min)	EC_{50}(Y1)[a]	YIELD(Y2)[b]	EC_{50}(Y3)	YIELD(Y4)	EC_{50}(Y5)	YIELD(Y6)	EC_{50}(Y7)	YIELD(Y8)
1	60	3	70.5	0.25	108.0	0.34	100.5	6.88	303.9	1.55
2	60	9	82.7	0.5	82.7	0.43	98.8	7.28	350.7	1.62
3	60	15	72.6	0.58	80.5	0.44	84.2	7.21	353.8	1.81
4	115	3	74.7	1.43	76.8	1.32	84.8	12.33	354.6	3.28
5	115	9	74.6	1.73	82.5	1.7	85.8	11.81	333.9	5.0
6	115	9	71.1	1.74	77.5	1.51	87.1	11.26	370.3	4.61
7	115	9	74.8	1.82	70.7	1.64	89.9	11.4	335.5	4.2
8	115	9	72.0	1.74	67.9	1.46	83.2	11.71	348.1	4.41
9	115	15	72.9	1.77	74.9	1.66	89.2	11.94	317.5	4.19
10	170	3	103.2	3.85	117.9	3.28	91.1	19.62	257.2	7.16
11	170	9	110.3	4.28	109.0	2.94	100.1	19.7	257.2	8.22
12	170	15	107.8	4.3	110.3	4.01	98.6	17.14	247.2	10.12

[a]: efficient concentration (µg/ml)

[b]: extraction yield obtained from dry weight/total weight expressed in %.

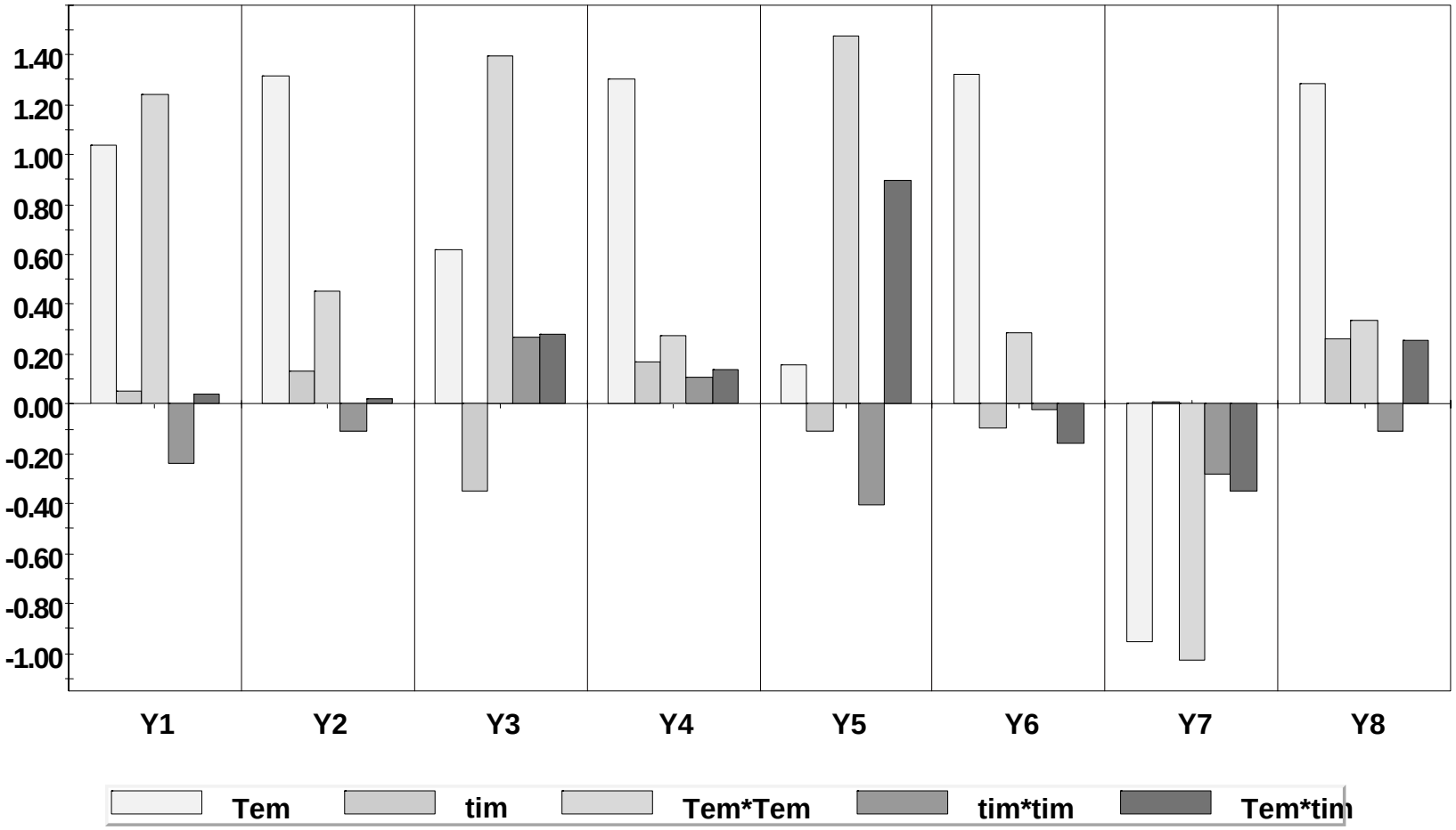
Table 2. Regression coefficients, for unscaled factors, and statistics for the fit, obtained from MLR.

Terms of the model	Regression coefficients:							
	Hexane		Petroleum ether		Ethanol		Water	
	EC_{50}(Y1)	Yield(Y2)	EC_{50}(Y3)	Yield(Y4)	EC_{50}(Y5)	Yield(Y6)	EC_{50}(Y7)	Yield(Y8)
Constant	116.81	0.05948	176.5	-0.32090	143.07	3.32247	217.387	1.70554
Tem	-1.0665^{***}	-0.01493^{*}	-1.8035^{**}	$8.814910^{-5\ ***}$	-0.81861	0.03197^{***}	2.92579^{***}	-0.01693^{***}
Tim	0.1361	0.10674^{*}	-1.0278	0.0325	-2.19570	0.17426	0.07778	-0.12050^{**}
Tem*Tem	0.00589^{***}	0.00021^{*}	0.00871^{***}	0.00012^{*}	0.00294^{**}	0.00041^{**}	-0.01597^{**}	0.00026^{*}
tim*tim		-0.00420^{*}						
Tem*tim					0.01803^{*}	-0.00213^{*}		0.00204^{*}
Statistics for goodness of fit of the model:								
R^2	0.954	0.999	0.879	0.967	0.802	0.991	0.838	0.980
RSD	3.866	0.046	7.243	0.245	3.695	0.537	20.381	0.467
P	0.080	0.425	0.435	0.068	0.257	0.073	0.342	0.235
RRSD (%)	4.70	2.30	8.21	14.16	4.06	4.34	6.38	9.98

R^2, determination coefficient; RSD, Residual Standard Deviation; P, P-value of the lack of fit test for the model; RRSD, the Residual Standard Deviation expressed as a percentage of the mean value of the response; * regression coefficient significant different from zero $p<0.05$; ** regression coefficient significant different from zero ($P<0.01$); *** regression coefficient significant different from zero $p<0.001$

Table 3. Optimum conditions (min EC50 and max Yield), provided by the statistical program, and the predicted value for the response variables, using the fitted model in Table 2.

		Optimum conditions		Predicted		Predicted value for the other response of the process for these ideal conditions.
		Temperature (ºC)	time (min)	value	95% Confidence Interval	
Hexane	EC_{50}	90	3	68.98	63.99 , 73.97	Yield(%) =0.71 (0.64 , 0.78)
	Yield(%)	170	13	4.30	4.23 , 4.37	EC_{50} = 107.64 (101.95 , 113.34)
Petroleum	EC_{50}	103	15	67.74	58.20 , 77.28	Yield(%) =1.43 (1.11 , 1.76)
	Yield(%)	170	15	3.60	3.20 , 4.00	EC_{50} = 106.23 (94.42 , 118.04)
Ethanol	EC_{50}	111	15	85.52	80.42 , 90.52	Yield(%) =10.95 (10.22 , 11.68)
	Yield(%)	170	3	19.94	18.85 , 21.05	EC_{50} = 91.38 (83.82 , 98.95)
Water	EC_{50}	170	3	253.40	220.17 , 286.63	Yield(%) =7.14 (6.18 , 8.09)
	Yield(%)	170	15	9.86	8.91 , 10.82	EC_{50} = 254.33 (221.10 , 287.57)


1.40
1.20
1.00
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-1.00
Y1
Y2
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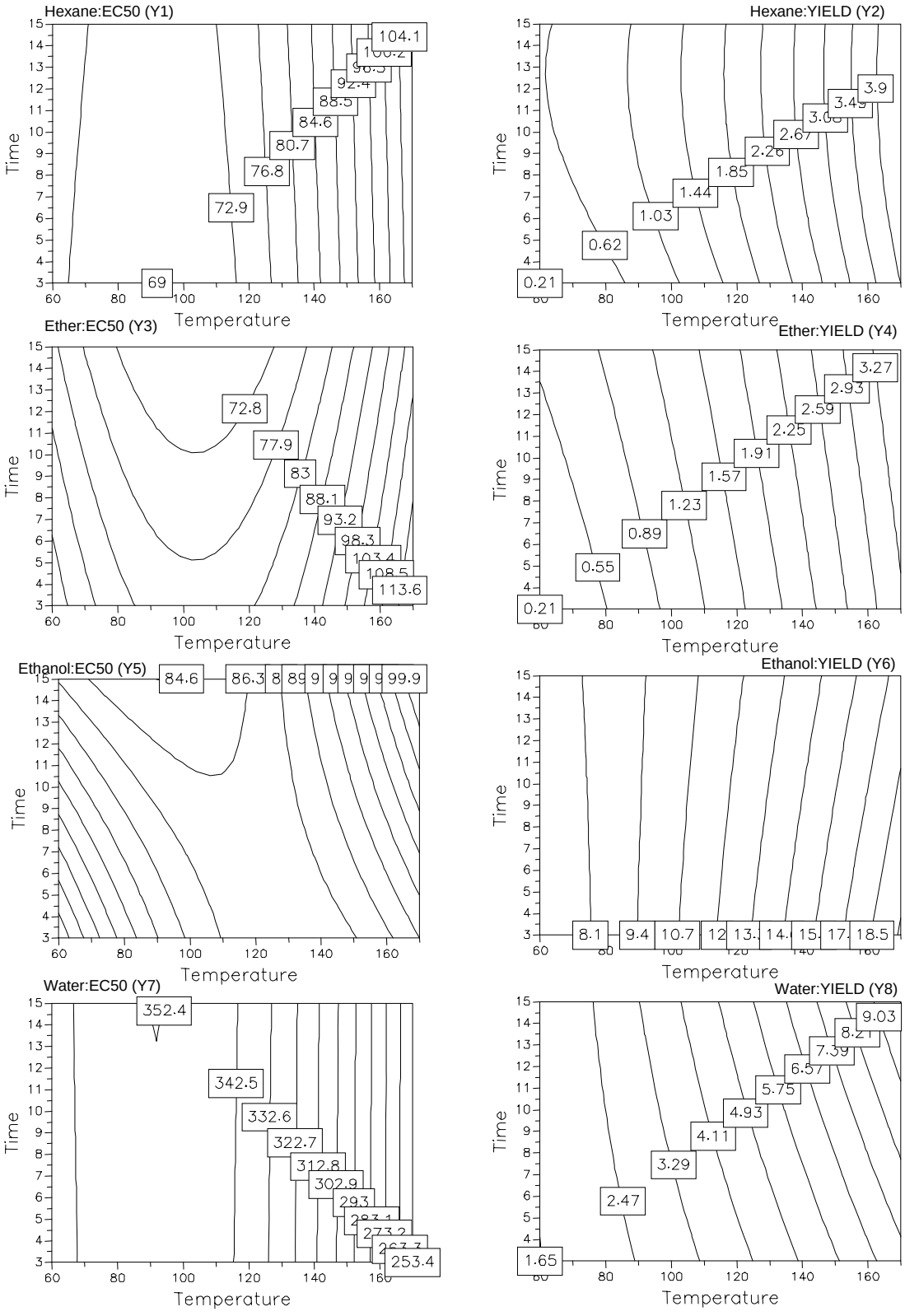
Figure 1.


Hexane:EC50 (Y1)
104.1
88.5
84.6
80.7
76.8
72.9
69
Time
Temperature
Hexane:YIELD (Y2)
3.9
2.67
2.26
1.85
1.44
1.03
0.62
0.21
Time
Temperature
Ether:EC50 (Y3)
72.8
77.9
83
88.1
93.2
98.3
113.6
Time
Temperature
Ether:YIELD (Y4)
3.27
2.93
2.59
2.25
1.91
1.57
1.23
0.89
0.55
0.21
Time
Temperature
Ethanol:EC50 (Y5)
84.6
Time
Temperature
Ethanol:YIELD (Y6)
8.1
9.4
10.7
Time
Temperature
Water:EC50 (Y7)
352.4
342.5
332.6
322.7
312.8
302.9
293.3
253.4
Time
Temperature
Water:YIELD (Y8)
9.03
7.39
6.57
5.75
4.93
4.11
3.29
2.47
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Time
Temperature

Figure 2.