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Journal of Chromatography A, 912 (2001) 61–71

JOURNAL OF CHROMATOGRAPHY A

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Supercritical fluid extraction for liquid chromatographic determination of carotenoids in Spirulina Pacifica algae: a chemometric approach

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Received 16 October 2000; received in revised form 4 January 2001; accepted 8 January 2001

Abstract

An experimental design procedure was used to investigate the effects of some operating parameters on the supercritical fluid extraction of carotenoids β-carotene, β-cryptoxanthin and zeaxanthin from Spirulina Pacifica algae, a carotenoid-rich dietary product. Variables tested were temperature and pressure of the supercritical fluid, dynamic extraction time and percentage of ethanol added as the modifier. Each variable was tested at three levels; 31 experiments were performed in random order. Analyses of the extracts were performed by high-performance liquid chromatography with UV–Vis photodiode array detection. Analytical responses (chromatographic peak areas) were processed by using a stepwise multiple regression analysis, in order to find polynomial functions describing the relationships between variables and responses. For all the analytes the experimental conditions providing the highest extraction yield inside the experimental domain considered were found. Supercritical fluid extraction results obtained in these conditions were compared with those obtained by performing solvent extraction in order to evaluate the effectiveness of the supercritical fluid extraction procedure.

Keywords: Experimental design; Optimization; Carotenoids; Carotenes; Cryptoxanthin; Zeaxanthin

1. Introduction

Carotenoids are highly conjugated polyprenoid compounds which are essential nutrients in the human diet, because of their antioxidant and anti-cancer activity [1]. They can be taken from a variety of natural sources including fruits, vegetables and sea products [2].

Official and conventional methods based on solvent extraction of carotenoids from natural matrices are time-consuming since they require multiple extraction steps and need large amounts of organic solvents, which are often expensive and potentially harmful [3,4]. Owing to problems related to traditional solvent extraction techniques, interest is growing in the development of simpler, faster and more

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0021-9673/01/$ – see front matter
PII: S0021-9673(01)00545-3

efficient methods for carotenoid extraction from food and natural products [5–8].

In the last few years, supercritical fluid extraction (SFE) has proved to be one of the most interesting techniques for solid sample treatment; in fact, a supercritical fluid shows a greater ability to diffuse into the matrix than the corresponding liquid, so improving extraction yield of analyte from complex matrices [9–13]. SFE is a highly desirable alternative to solvent extraction of some classes of natural substances from food: in fact, it provides high speed and efficiency of extraction, eliminating concentration steps and simplifying the analytical method [14]. Supercritical CO_2 is the most commonly used extraction agent because of its non-toxicity, chemical inertia, low cost and easy availability. In addition, carbon dioxide presents a low critical temperature value (T_c=31°C), making it ideal for extraction of thermally labile compounds. For these reasons, SFE using CO_2 has been successfully used for isolation of carotenoids from various vegetables [5–8,15,16].

A limitation of supercritical CO_2 is that it often fails in quantitative extraction of polar analytes from solid matrices, because of the poor solvating power of this fluid and the insufficient interaction between supercritical CO_2 and the matrix [17]. The addition of an organic modifier can greatly improve the extraction efficiency by increasing solubility of the analytes, by reducing their interaction with sample matrix or by inducing matrix modification; in this way, release of the analytes from the matrix can be advantageously enhanced [18–21]. In a research program dealing with the development of sampling treatment procedures and of new methods for the determination of carotenoids [22,23], SFE was evaluated as a new extraction method for carotenoids from a complex natural product.

The aim of this study was to investigate the effect of some experimental parameters on the supercritical fluid extraction of carotenoids β-carotene, β-cryptoxanthin and zeaxanthin (Fig. 1) from Spirulina Pacifica algae (*Spirulina platensis* strain pacifica microalgae) and to find the best experimental conditions for their recovery. An experimental design procedure was used to investigate the effects of four parameters on the SFE performance: temperature and pressure of the supercritical fluid, dynamic extraction time and percentage of ethanol added as the modifier

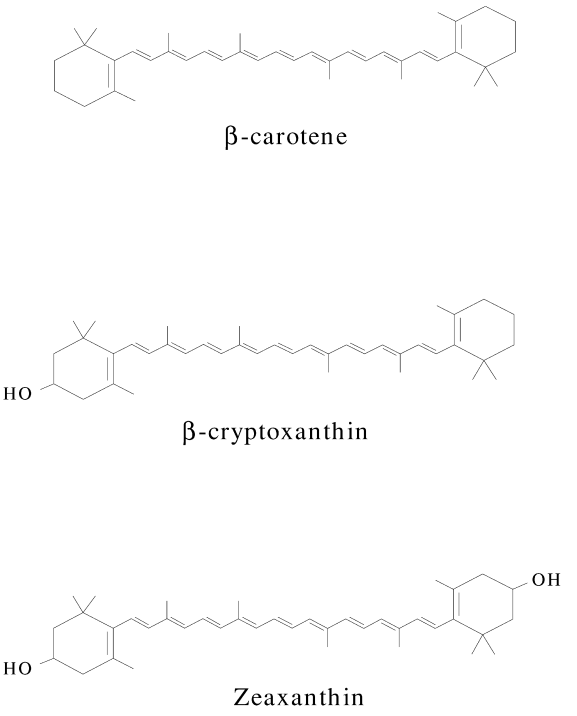

Fig. 1. Structures of carotenoids investigated.

to the CO_2. A similar multivariate approach was previously applied to the optimization of supercritical fluid extraction of carotenes from carrot [15]. In previous studies we successfully used this approach for the optimization of extraction and hydrolysis procedures of natural compounds from food [24,25]. This method makes possible the study of the interactions among the variables and the modelling of multifactorial response surfaces, thus providing a great deal of information about the behaviour of the system; moreover, it requires only a rather small number of experiments [26–28].

Analyses of the extracts were performed by high-performance liquid chromatography (HPLC) with UV–Vis photodiode array detection (DAD).

2. Experimental

2.1. Chemicals

β-Carotene standard (>97% purity) was obtained from Fluka (Buchs, Switzerland); β-cryptoxanthin

and zeaxanthin standards were gifts from Hoffmann–La Roche (Basel, Switzerland). Standard solutions were obtained by dissolving pure compounds in tetrahydrofuran containing butylated hydroxytoluene (BHT) (1%, w/v) as the antioxidant and the solutions were stored in brown flasks at 4°C.

Extractions were carried out with high purity (99.998%) carbon dioxide purchased from Air Liquid (Alphagaz, Milan, Italy).

All organic solvents were HPLC grade; acetonitrile, ethanol, methanol, dichloromethane and tetrahydrofuran were obtained from Carlo Erba (Milan, Italy). Analytical reagent-grade ammonium acetate was supplied by Sigma (Milan, Italy).

The Spirulina Pacifica (*Spirulina platensis* strain pacifica microalgae) powder samples were kindly provided by Cyanotech (Kailua-Kona, HI, USA); they were stored at 4°C until analysis.

2.2. Supercritical fluid extraction

A Hewlett-Packard (Palo Alto, CA, USA) Model 7680T supercritical fluid extractor equipped with a Hewlett-Packard external modifier pump series 1100, was used to extract carotenoids from uniform size powdered Spirulina Pacifica algae having a particle size below 50 μm. Extractions were carried out on 0.5 g of dry powdered algae by using high-purity carbon dioxide (99.998%) with ethanol as the organic modifier. A 7-ml stainless steel extraction cell was used; the components extracted were collected on an octadecyl functionalized silica (ODS) trap.

SFE extractions were carried out by using a combination of a 5-min static extraction and a subsequent dynamic extraction step. Nozzle and trap temperatures were held, respectively, at 85 and 80°C during the extraction step and at 45°C and 30°C, at the end of the extraction. The flow-rate of the supercritical fluid in the dynamic extraction step was fixed to 2 ml min^{-1}.

In order to optimize the SFE procedure, an experimental design approach was followed. The variables assessed were as follows: temperature (40, 60 and 80°C) and pressure (150, 250 and 350 bar) of the supercritical fluid, dynamic extraction time (40, 70 and 100 min) and percentage of ethanol added as the modifier (5, 10 and 15%).

The final volume was made up to 10 ml with tetrahydrofuran containing BHT (1%, w/v) as the antioxidant. The extracts were analysed by HPLC with UV–Vis detection.

2.3. Solvent extraction

Carotenoids were extracted from Spirulina Pacifica powder following a procedure reported in the literature for vegetables [29]. After filtration on 0.2-μm membranes (Lida, Kenosha, WI, USA), the extracts were injected into the HPLC system without any other treatment except for appropriate dilutions.

2.4. Chromatographic conditions

Analyses were carried out by using a high-performance liquid chromatographic system (Waters-Millipore, Bedford, MA, USA) equipped with a Model 625 LC system pump, a Gilson (Villiers-le-Bel, France) Model 231 autosampling injector with Dilutor Model 401 and a Waters Model 996 photodiode array detector. Signal acquisition and integration were performed by using the software Millennium v. 2.0 (Waters).

HPLC separation was performed on a Spherisorb ODS2 column (150×4.6 mm, 3 μm) (Alltech, Milan, Italy) using an isocratic mobile phase [acetonitrile–methanol (ammonium acetate 0.1 *M*)–dichloromethane (71:22:7, v/v/v)] at a flow-rate of 1 ml/min.

After scanning in the 350–600-nm range, analytes were detected at 450 nm for quantitative determination.

2.5. Statistical analysis

Statistical analyses were performed by using the statistical package SPSS version 8.0 (SPSS Italia, Bologna, Italy).

3. Results and discussion

3.1. Supercritical fluid extraction and HPLC separation

The aims of this work were to find the conditions providing the highest SFE recoveries of carotenoids inside the experimental domain explored and to

compare the quantitative results obtained for carotenoids by using SFE and liquid–liquid extraction methods. HPLC–DAD separation and the identification of carotenoids was also carried out for characterization of these extracts. Fig. 2 shows the HPLC–DAD separation of carotenoids extracted by SFE from the algae sample; identification of the analytes extracted from the matrix was performed by injecting standard solutions and was confirmed by the corresponding on-line acquired electronic spectra.

Since various parameters potentially affect the SFE process, the optimization of the experimental conditions represents a critical step in the development of a SFE method. In fact, solubility of the analytes can be controlled by the composition, density and temperature of the extraction fluid; however, the extraction recovery is dependent not only on the operating parameters, but also on sample characteristics (water content, type of matrix, particle size, etc.), making difficult the selection of optimum conditions required for subsequent reliable quantification.

On the basis of preliminary experiments and literature data, some experimental parameters were not varied, i.e., the kind of the collection trap (octadecyl functionalized silica) [16], the static extraction time (5 min) and the kind of modifier (ethanol) [15].

Different organic solvents (i.e., acetonitrile, hexane, tetrahydrofuran) were tested to elute carotenoids from the trap. Among these, tetrahydrofuran was chosen because of its greater ability to quantitatively

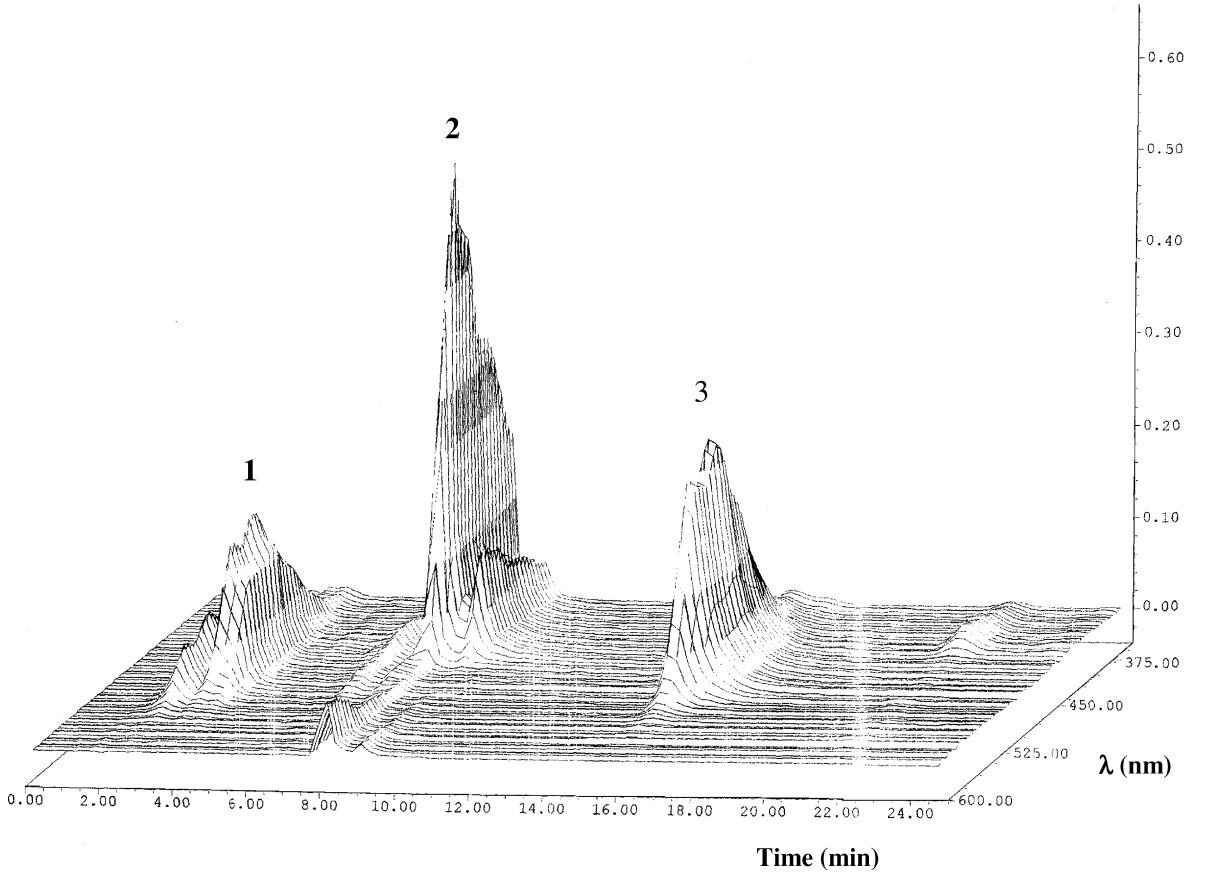

Fig. 2. HPLC–DAD separation of carotenoids extracted by SFE from Spirulina Pacifica algae. Peaks: (1) zeaxanthin; (2) β-cryptoxanthin; (3) β-carotene.

recover carotenoids from the cartridge; elution of analytes from the trap was carried out with three aliquots of 1-ml each of tetrahydrofuran.

In this work, the effect of four parameters (temperature and pressure of the supercritical fluid, dynamic extraction time and percentage of the modifier) on the extraction of some selected carotenoids (β-carotene, β-cryptoxanthin and zeaxanthin) from a powdered sample of Spirulina Pacifica algae was investigated by using an experimental design.

In previous studies, these compounds were the only carotenoids identified in Spirulina Pacifica algae by HPLC coupled with mass spectrometry [22,23].

3.2. Experimental design and data analysis

Preliminarily a k factor ($k=4$) two-level factorial design was used to evaluate the main and interaction effects of the following factors on the SFE efficiency: temperature (X_1) and pressure (X_2) of the supercritical fluid, dynamic extraction time (X_3) and percentage of ethanol added as the modifier (X_4). The experimental domain was defined taking into account both instrumental constraints (pressure range 77–385 bar; temperature range 25–150°C) and experimental limits, with the aim of exploring a wide range of values for each factor considering at the same time symmetrical values. Temperature values higher than 80°C, i.e., the maximum level, were not tested in order to avoid analyte degradation. The maximum dynamic extraction time was set at 100 min to enable the extraction to be performed within a reasonable time. The ethanol percentage range was chosen taking into account analyte polarity and literature data on SFE of carotenoids [15]. Sixteen experiments were performed; in addition, seven experiments were replicated at the center of the experimental domain, in order to estimate the repeatability of the experimental measurements. Factors and levels tested are reported in Table 1.

Table 1
Factors and levels tested (coded values in parentheses) for SFE experiments

Factor	Low level (−1)	Medium level (0)	High level (+1)
Temperature (X_1, °C)	40	60	80
Pressure (X_2, bar)	150	250	350
Dynamic extraction time (X_3, min)	40	70	100
Ethanol percentage (X_4, %, v/v)	5	10	15

Data obtained by performing experiments of the factorial design (Table 2) were analyzed by means of the Yates algorithm, in order to calculate main and interaction effects for each variable. Normal probability plots show for each analyte the significance of the estimated effects (Fig. 3).

As it can be seen from these plots, main effects of the variable temperature (X_1) are not significant for all the analytes, whereas main effects of the variables pressure (X_2), extraction time (X_3) and percentage of the modifier (X_4) resulted to be significant. In addition, interaction effects of the variables X_1 and X_2 are significant for all the analytes except that for β-carotene. On the basis of the results of the factorial design obtained for β-carotene, the variable X_1 should have been excluded from subsequent experiments, because it was not significant either as main and interaction effect.

In a further step, an F-test was performed to evaluate the presence of curvature effects. For this purpose, F was determined applying the following equation:

$$F = (y_0 - y_F)^2/(1/N_0 + 1/N_F)(s_0)^2$$

where y_0 is mean of replicated measurements at the center point, i.e., the experimental response in the center of the experimental domain; y_F the mean of estimated values in the factorial experiments, which estimates the system response in the center of the experimental domain; N_0 the number of experiments at the center point; N_F the number of factorial experiments; and s_0 the experimental error, estimated through the replicated measurements in the center point.

If F resulted to be higher than the tabulated $F_{\mathrm{tab}(1,N_0-1)}$ at the chosen confidence level, a curvature effect occurred. The results of the curvature test (Table 3) show the presence for all the analytes of a curvature effect at a confidence level of 95%, thus indicating that all variables, even if their main and interaction effects were not significant (i.e., variable temperature for β-carotene), had to be included in the subsequent experiments and that a quadratic

Table 2
Experimental matrix and mean values ($n=3$) of observed responses (HPLC peak areas)

No.	Factor				Peak areas (μV s)		
	X_1	X_2	X_3	X_4	Zeaxanthin	β-Cryptoxanthin	β-Carotene
1	1	1	1	1	33 708 302	5 911 231	95 578 668
2	−1	−1	−1	−1	8 775 839	1 788 222	33 638 595
3	1	−1	−1	−1	1 140 350	607 472	23 319 293
4	−1	1	−1	−1	7 803 310	2 556 279	51 460 423
5	−1	−1	1	−1	13 372 012	2 467 817	54 110 253
6	−1	−1	−1	1	23 767 395	3 502 906	77 819 419
7	1	1	−1	−1	15 205 086	3 147 685	69 888 170
8	1	−1	1	−1	2 851 354	1 080 746	40 859 107
9	1	−1	−1	1	13 681 339	2 935 227	64 967 953
10	−1	1	1	−1	12 438 312	2 939 542	76 848 143
11	−1	1	−1	1	26 451 304	4 283 048	87 720 257
12	−1	−1	1	1	27 385 325	3 861 477	62 110 029
13	1	1	1	−1	21 183 944	3 910 252	85 154 746
14	1	1	−1	1	32 527 744	5 286 996	98 187 988
15	1	−1	1	1	18 654 258	3 906 889	78 131 229
16	−1	1	1	1	26 288 005	4 527 979	94 509 406
17	0	0	0	1	29 929 297	4 375 772	67 729 750
18	0	0	0	−1	19 379 736	3 114 551	75 856 039
19	0	0	1	0	30 611 559	4 264 664	103 101 758
20	0	0	−1	0	15 858 909	2 490 928	47 053 288
21	0	1	0	0	39 243 803	4 150 949	94 184 729
22	0	−1	0	0	49 171 339	2 104 672	48 275 791
23	1	0	0	0	24 259 353	3 191 473	58 178 454
24	−1	0	0	0	22 481 469	3 037 032	60 649 624
25	0	0	0	0	26 516 588	3 862 407	78 359 331
26	0	0	0	0	27 812 720	3 692 244	83 022 046
27	0	0	0	0	27 865 825	4 067 031	89 937 524
28	0	0	0	0	25 509 100	3 676 201	87 694 732
29	0	0	0	0	25 166 015	3 703 993	86 403 280
30	0	0	0	0	27 897 439	3 783 734	83 466 346
31	0	0	0	0	27 909 575	4 097 237	88 215 613

model has to be used. On the basis of these findings, additional experiments were performed at the center of each face of the hypercube representing the experimental domain, in order to evaluate which variables are responsible for these curvature effects. A total of 2k star points were then added so that a total of 31 experiments was performed. Experimental data (Table 2) were processed by using the multiple regression analysis, in order to create a mathematical model representing the relationship between factors and responses and to determine the best experimental conditions for the extraction recovery of β-carotene, β-cryptoxanthin and zeaxanthin.

For each compound the following polynomial model was fitted:

$$\hat{Y} = b_0 + \sum_{i=1}^{4} b_i X_i + \sum_{i=1}^{4} \sum_{j=1}^{4} b_{ij} X_i X_j$$

In this equation Y is the predicted response and the X_i variables are the coded values of the factors. The b values are the estimated polynomial coefficients: b_0 is the intercept term; b_i coefficients represent the main effects for each variable; b_{ii} coefficients, present in the quadratic terms, are responsible for the curvature effects and $b_{ij(i\neq j)}$ coefficients describe the interaction effects.

In a further step, statistically significant coefficients were estimated by using the stepwise method. This procedure allows to re-estimate the equations entering the variables into the model step by step; the

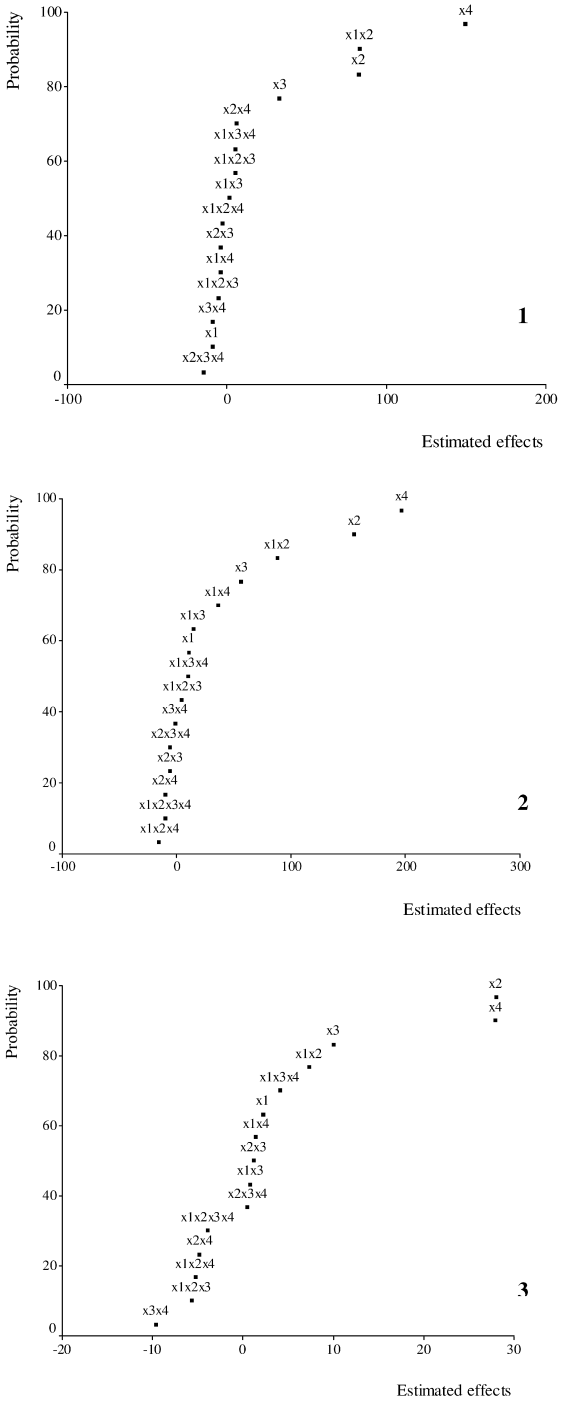

Fig. 3. Normal probability plots of estimated effects for: (1) zeaxanthin; (2) β-cryptoxanthin; (3) β-carotene.

Table 3
Curvature test results[a]

β-Carotene	$F_{calc}=89$
β-Cryptoxanthin	$F_{calc}=46$
Zeaxanthin	$F_{calc}=275$

[a] $F_{tab(1,6)}$ $(\alpha=0.05)=5.99$.

first to be entered is that which shows the strongest correlation with the dependent variable. At each subsequent step, the variable with the strongest partial correlation was entered and variables already included in the model were tested for removal: the maximum probability of F to include a variable in the model was set at 0.1.

The estimated coefficients of the polynomial functions calculated for each analyte by using the stepwise method [30] are reported in Table 4.

Table 5 shows ANOVA results for calculated models. Fig. 4 shows predicted versus experimental values.

Table 4
Regression coefficients of the polynomial functions calculated with the stepwise method (standard errors in parentheses)

	Coefficients ($\times 10^{-6}$)	t values	P
Zeaxanthin			
Constant	28.7 (1.7)	16.82	0.000
X_2	3.1 (1.4)	2.15	0.041
X_4	7.2 (1.4)	4.99	0.000
X_3^2	−10 (2)	−4.59	0.000
X_1X_2	4.2 (1.5)	2.71	0.012
$R^2=0.690$			
β-Cryptoxanthin			
Constant	36 (9)	40.02	0.000
X_2	80 (8)	10.38	0.000
X_3	35 (8)	4.50	0.000
X_4	94 (8)	12.19	0.000
X_1^2	−37 (12)	3.1	0.005
X_1X_2	44 (8)	5.36	0.000
X_1X_4	18 (8)	2.18	0.039
$R^2=0.930$			
β-Carotene			
Constant	79 (3)	24.654	0.000
X_2	15 (3)	5.481	0.000
X_3	8 (3)	2.765	0.010
X_4	12 (3)	4.37	0.000
X_1^2	−12 (4)	−2.857	0.008
$R^2=714$			

Table 5
ANOVA table for refined models

	Sum of squares	df	Mean squares	*F*	*P*
Zeaxanthin					
Regression	2.2×10^{15}	4	5.478×10^{14}	14.465	0.000
Residual	9.8×10^{14}	26	3.787×10^{13}		
Total	3.2×10^{15}	30			
β-Cryptoxanthin					
Regression	3.4×10^{13}	6	5.744×10^{12}	53.263	0.000
Residual	2.6×10^{12}	24	1.078×10^{11}		
Total	3.7×10^{13}	30			
β-Carotene					
Regression	8.8×10^{15}	4	2.194×10^{15}	16.239	0.000
Residual	3.5×10^{15}	26	1.351×10^{14}		
Total	1.2×10^{16}	30			

By analysing the response surfaces and by mathematically calculating the highest values of the variables inside the experimental domain, it was possible to find the best extraction conditions inside the experimental domain (Table 6). Fig. 5 shows three-dimensional plots of the response surfaces for the analytes; in these plots, X_2 and X_4 variables were fixed at their optimum values previously calculated.

3.2.1. Effect of temperature (X_1) and pressure of the supercritical fluid (X_2)

Analyte solubility depends on a complex balance between supercritical fluid density and solute vapour pressure, both controlled by temperature and pressure of the supercritical fluid.

For all the analytes, the temperature of the supercritical fluid was found not to be significant as the main effect; in fact, the linear b_1X_1 term does not appear in the refined models. In the polynomial function obtained for β-cryptoxanthin and for β-carotene, the square term turned out to be significant. A temperature increase, although causing a decrease of the fluid density, could be responsible for an increase in the solvating power because of the increase in the solute vapour pressure.

For zeaxanthin and β-cryptoxanthin, the relationships between temperature and the other variables were more complex than for β-carotene.

As can be inferred from the results, the pressure of the supercritical fluid plays an important role in the SFE of carotenoids from Spirulina Pacifica algae; in fact, it appeared to be significant for all the analytes. This means that extraction recovery is enhanced as the pressure increases. The pressure increase causes an increase of the fluid density and thus it could have a double effect: an increase of the solvating power of the supercritical fluid, responsible for quantitative recoveries, and a reduced interaction between the fluid and the matrix as a consequence of the decreasing of the diffusion coefficient at higher density. No square term $b_{22}X_2^2$ appears in the polynomial functions.

Table 6
Best experimental conditions for SFE recovery of zeaxanthin, β-cryptoxanthin and β-carotene from Spirulina Pacifica algae

Compound	Supercritical fluid temperature (°C)	Supercritical fluid pressure (bar)	Dynamic extraction time (min)	Ethanol percentage (%, v/v)
Zeaxanthin	80	350	70	15
β-Cryptoxanthin	76	350	100	15
β-Carotene	60	350	100	15

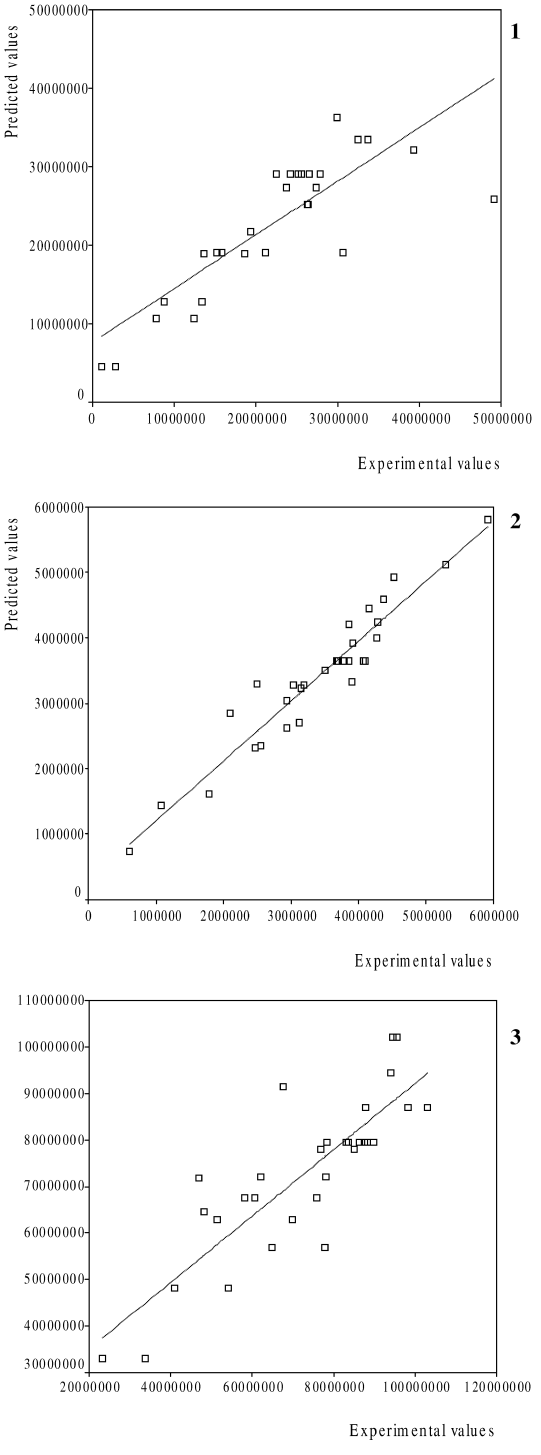

Fig. 4. Predicted versus experimental values for: (1) zeaxanthin; (2) β-cryptoxanthin; (3) β-carotene.

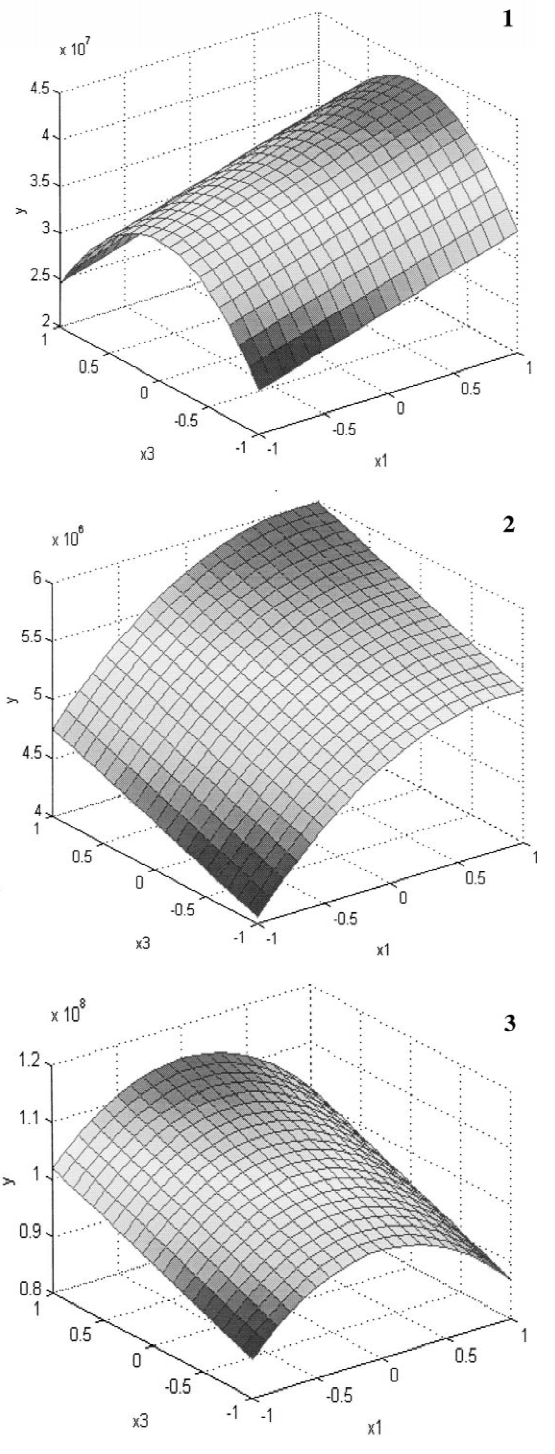

Fig. 5. Response surfaces for: (1) zeaxanthin; (2) β-cryptoxanthin; (3) β-carotene ($X_2 = 1$; $X_4 = 1$).

3.2.2. Effect of the dynamic extraction time (X_3)

In order to achieve high recoveries, a first extraction step in static mode was performed; this step should make possible a better penetration of the fluid in the matrix than the dynamic mode. This step was followed by a dynamic extraction to enhance analyte solubility in the supercritical fluid [31].

In the refined model the main effect of X_3 was found to be significant for β-carotene and β-cryptoxanthin, whereas polynomial function referred to zeaxanthin shows a significant coefficient for the square term (X_3^2). The extraction time does not show significant interactions with the other variables.

3.2.3. Effect of modifier addition (X_4)

An essential drawback in the use of supercritical CO_2 is its low polarity, making the extraction of polar analytes difficult. Nevertheless, this limitation may be overcome by adding to the supercritical CO_2 small amounts of polar modifiers, such as methanol or ethanol, in order to increase its solvation power. A previous work describes ethanol as the most effective modifier in the extraction of β-carotene from vegetables [16], thus it was chosen as the modifier in this study.

For all the analytes the main effect of the variable X_4 is significant with positive coefficients, i.e., the increase of the percentage of ethanol added as the modifier leads to an increase of extraction yield. Further, since analytes with different polarity show a better recovery in the fluid added with ethanol, the effect of the modifier results to be related not only to the change in polarity of the extraction fluid, but also to its interaction with the matrix. No square terms X_4^2 are present in the polynomial functions.

3.3. Comparison between SFE and solvent extraction

In order to compare SFE and liquid extraction performances, data obtained by carrying out SFE in the experimental conditions providing the highest extraction yield inside the experimental domain (Table 6) were compared with those obtained by performing a traditional solvent extraction on the same sample. This extraction procedure [29], which requires multiple extraction and purification steps and the use of a large amount of organic solvents, is very expensive and time-consuming. Carotenoids were determined by constructing calibration curves, the concentrations bracketing those of the algae samples. A one-way analysis of variance (ANOVA) was carried out on data obtained by performing carotenoid extraction with solvent and with supercritical CO_2; ANOVA results did not evidence significant differences between sets of data. The agreement between the results obtained by applying the two different sample treatments confirms the effectiveness of the SFE technique for the extraction of carotenoids from Spirulina Pacifica algae (Table 7). In addition, SFE proved to be easier and faster than solvent extraction and thus more effective and advantageous.

Table 7
Comparison between different extraction techniques of zeaxanthin, β-cryptoxanthin and β-carotene from Spirulina Pacifica algae

Compound	Concentration (mg/100 g)[a]	
	Solvent extraction	Supercritical fluid extraction
Zeaxanthin	50±4	48±3
β-Cryptoxanthin	8±1	7.5±0.1
β-Carotene	120±7	118±7

[a] Mean values ($n=3$)±standard deviation.

4. Conclusions

In the investigation of the effects of the four tested parameters on the SFE recoveries of zeaxanthin, β-cryptoxanthin and β-carotene from algae samples, the use of an experimental design approach allowed to find polynomial functions describing the relationships between variables and responses and to find, for each analyte, the best experimental conditions for the supercritical fluid extraction of these substances inside the experimental domain considered.

The results prove the significance not only of the main effects of pressure and of the percentage of modifier for all the analytes and of the dynamic extraction time for β-cryptoxanthin and β-carotene, but also of the interaction and curvature effects that would have been lost if the traditional one-variable at a time procedure had been used. SFE proved to be a more effective extraction procedure, if compared with the traditional solvent extraction method.

Acknowledgements

The work was financially supported by the Ministero dell'Università e della Ricerca Scientifica e Tecnologica and by Consiglio Nazionale delle Ricerche (Italy). The authors acknowledge Dr. C. Fizet and Dr. W. Schüep of Hoffmann-La Roche (Basel, Switzerland) for generously supplying xanthophyll standards. The authors also thank Dr. R. Todd Lorenz of Cyanotech Corporation (Kailua-Kona, HI, USA) for providing the Spirulina Pacifica algae samples.

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