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Comparison of supercritical fluid and ultrasound-assisted extraction of carotenoids and chlorophyll a from *Dunaliella salina*

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ABSTRACT

In the work described here the extraction processes of carotenoids and chlorophylls were analysed using two extraction techniques, namely ultrasound-assisted extraction and supercritical fluid extraction, and the results are compared. The solvents used for the ultrasound-assisted extraction were *N*,*N*′-dimethylformamide and methanol and for the supercritical fluid extraction, carbon dioxide. The raw material studied was *Dunaliella salina*, a microalgae characterized by the high levels of carotenoids present in its cellular structure. The results indicate that the supercritical fluid extraction process is comparable to the ultrasound-assisted extraction when methanol is used as solvent. In addition, the supercritical extraction process is more selective for the recovery of carotenoids than the conventional technique since it leads to higher values for the ratio carotenoids/chlorophylls. Finally, the effects of pressure and temperature on the extraction yields of the supercritical fluid extraction process were studied.

1. Introduction

At present, microalgae offer great possibilities for the isolation of natural substances of significant commercial interest in industries such as pharmaceuticals, alimentary or cosmetic products. This fact makes microalgae raw materials with a great deal of added value.

Within the wide variety of microalgae types, the marine species in particular are able to produce a variety of substances with a range of properties. These include polyunsaturated fatty acids (PUFAs) [1–3] that protect from cardiovascular illnesses, carotenoids, which are precursors of vitamins and also show antioxidant activity [4,5] and prevent illnesses like cancer and cellular aging [6–8], toxins and bioactive substances with a high antitumor capacity, and micosporines that protect from UV radiation [9–11]. At present, society demands products made with additives that are natural in origin and, wherever possible, are beneficial to human health. In this sense, marine microalgae offer great possibilities as sources of these substances and have attracted close attention from the aforementioned industries due to the economic and social repercussions that the use of this type of additive has in the production of their products. Many of these products are designed for direct human consumption and the extraction technique is extremely important in terms of the appropriate technology to apply.

In this sense, the application of new techniques such as ultrasound-assisted extraction (UAE) and extraction with fluids at high pressure or under supercritical conditions (SFE) constitute extraction methods that reduce the volume of solvent and the extraction time. In addition, in the case of SFE it is possible to minimize the environmental impact of the use of volatile organic compounds (VOCs).

UAE is a good extraction method in comparison with the more traditional approaches due to its high efficiency, low energy requirements and low solvent consumption. This technique has been used systematically in the extraction of substances with low molecular weights [12] and bioactive compounds from plants [13,14]. The improvement in the extraction process on using ultrasound is related to the destruction of the cellular walls, reduction of the particle size, and enhancement of the mass-transfer through the cell wall due to the collapse of bubbles produced by cavitation [15,16].

Carotenoid extraction by SFE represents an alternative to the conventional extraction technique due to the fact that the purification stage is minimized and the extraction time is reduced [17]. The application of this technology in the recovery of pigments from marine microalgae has been widely studied in recent years. Mendes et al. analysed the supercritical extraction process on substances

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of pharmacological interest from different marine microalgae such as *Botryococcus braunii* [18], *Chlorella vulgaris* [19] and *Asthorospira (Spirulina) maxima* [20]. Careri et al. [21], Macías-Sánchez et al. [22,23], Montero et al. [24], Mendiola et al. [25], Gouveira et al. [26] and Canela et al. [27] have applied supercritical extraction to obtain carotenoids from *Spirulina platensis*, *Nannochloropsis gaditana*, *Synechococcus* sp., *Chlorella vulgaris* and *Spirulina maxima*, respectively, with satisfactory results.

Dunaliella salina is a unicellular *Chlorophyta* alga of the *Chlorophyceae* class and *Volvocales* order. The main morphological characteristic that distinguishes this alga from the rest of the *Volvocales* is the absence of a polysaccharide cell wall. For this reason, *D. salina* can be easily digested by humans and animals [28]. It is a microalgae with a high efficiency for the conversion of light energy into biomass and it is also able to accumulate high levels of β-carotene when cultivated under stress conditions [29,30].

When *D. salina* is under appropriate cultivation conditions, it can contain more than 10% of its dry weight as β-carotene (other microalgae and superior plants usually have a content of around 0.3% in β-carotene). This massive accumulation of β-carotene seems to be related to a protection mechanism to counteract the effects of solar radiation. In addition to β-carotene, this microalgae contains other pigments such as chlorophyll a and b, luteine and violaxantine. Depending on the light conditions it can also produce anteraxantine and zeaxantine [31].

There are numerous reports in the literature that describe the study of carotenoid recovery from *D. salina* using a range of extraction techniques [32]. The application of high pressure fluid extraction to this microalgae has been studied by several authors in recent years [33,34]. In relation to the use of supercritical carbon dioxide in the extraction of *D. salina*, only one study has been developed, by Mendes et al. [20], and this involved an analysis of the solubility of the *cis*- and *trans*-β-carotene isomers from this microalgae compared with the solubility of the synthetic *trans*-isomer. The results indicate that the latter compound has a lower solubility in supercritical carbon dioxide than the natural ones.

The work described here involved a comparison of the UAE process and the SFE process with carbon dioxide on carotenoids and chlorophyll. In addition, the program STATGRAPHICS plus 5.1 (1994–2001, Statistical Graphics Corp.) was used to develop empirical equations that have the capacity to predict the extraction yields in the SFE of carotenoids and chlorophyll. The same program also provides useful information concerning the influence of variables on the extraction yields of the process. In this sense, the effects of temperature and pressure in the SFE of carotenoids and chlorophyll were analysed using a multilevel factorial experimental design.

2. Experimental

2.1. Raw material

The algal matter used in this study was provided by the Microalgae Culture Collection of the Institute of Marine Sciences of Andalusia (ICMAN-CSIC, Spain). The biomass was grown in sea water enriched with f/2 medium [35], at temperature in the range 20–35 °C and with atmospheric aeration. After growth was complete, the biomass was lyophilized and stored in a refrigerator in the absence of light until the extraction process was carried out.

2.2. SFE

The experimental work was carried out in SFE equipment (micro-scale) from ISCO (Nebraska), model SFX 220. This equipment has a 0.5 mL extraction cell and the solvent is supplied by a syringe pump with a capacity of 250 mL. The flow rate was controlled manually at the exit of the installation by a thermostatic micrometric valve [22].

The experimental procedure was as follows: firstly, the extraction cartridge was loaded with approximately 0.1 g of the microalgae sample, which was homogenized in order to maintain a constant apparent density in all the experiments; the cartridge was then introduced into the extractor for 15 min to reach the operating temperature; the extractor was pressurized with the CO_2 pump; a static extraction was carried out under working conditions during 15 min; after this time, the micrometric valve was opened and kept at 60 °C. The solvent flow-rate for all experiments was 4.5 mmol/min and the extraction time was 180 min.

The extracted samples were collected in glass tubes containing methanol. After the extraction process, the solvent was removed with a flow of nitrogen at 40 °C. The extracted product was dissolved in 5 mL of methanol and was stored at 4 °C in the absence of light until the measurements were carried out. All the experiments were repeated two times.

2.3. UAE

Methanol and *N*,*N*′-dimethylformamide (DMF) were the solvents selected for the UAE of pigments from *D. salina*. A sample of 0.105 g of lyophilized microalgae was suspended in 5 mL of the solvent. The suspension was sonicated for 3 min in an ultrasound apparatus from Selecta (Spain) and stored for 24 h at 4 °C. After this time, the extract was separated from the pellet and recovered by centrifugation, immediately filtered through a 0.22 μm filter, and finally stored at 4 °C in the absence of light until analysis was carried out. The extraction process was repeated until the liquid extract did not have any coloration (approximately 6 extraction cycles for methanol and 4 extraction cycles for DMF).

2.4. Analytical methods

The total concentrations of carotenoids and chlorophyll were determined by measuring the absorbance of the samples using a U-2010 Spectrophotometer from Hitachi (Japan). The equation proposed by Wellburn [36] was used for the determination of carotenoid and chlorophyll concentrations in the samples of *D. salina*. This equation has more parameters than other equations presented in the literature and allows the determination of the chlorophyll b contained in the samples.

The concentration of total carotenoids was calculated using the following equation:

$$C_{\text{total carotenoids }(x+c)} = \frac{1000A_{470} - 1.63C_a - 104.96C_b}{221} \quad (1)$$

where A_{470} is the absorbance at 470 nm, and C_a and C_b are the concentrations of chlorophyll a and b calculated by:

$$C_a(\mu g/mL) = 16.72A_{665.2} - 9.16A_{652.4} \quad (2)$$

$$C_b(\mu g/mL) = 34.09A_{652.4} - 15.28A_{665.2} \quad (3)$$

where $A_{665.2}$ and $A_{652.4}$ are the absorbance values at 665.2 nm and 652.4 nm, respectively.

3. Experimental results

The experimental results for the extraction yields obtained in the extraction of chlorophylls and carotenoids from lyophilized *D. salina* by SFE with carbon dioxide and UAE using DMF and methanol, along with the carotenoids/chlorophylls ratios, are presented in Table 1 with the confidence limits considering a 95% of confidence

Table 1
Extraction yields of carotenoids and chlorophylls

	Pressure (bar)	Extraction yields		Ratio Car/Chlo
		μg carotenoids/mg dry microalgae	μg chlorophylls/mg dry microalgae	
Temperature (°C)				
SFE-CO_2				
40		0.207 ± 0.004	0.197 ± 0.008	1.05
50	100	0	0	–
60		0	0	–
40		6.43 ± 0.26	0.086 ± 0.003	74.78
50	200	7.03 ± 0.14	0.071 ± 0.002	99.03
60		5.75 ± 0.06	0	–
40		6.30 ± 0.50	0.033 ± 0.003	191
50	300	6.31 ± 0.31	0.184 ± 0.013	34.28
60		14.92 ± 0.89	0.268 ± 0.011	55.66
40		7.67 ± 0.69	0.061 ± 0.004	125.77
50	400	7.28 ± 0.29	0.235 ± 0.009	30.99
60		12.17 ± 0.24	0.227 ± 0.005	53.63
40		4.06 ± 0.24	0.026 ± 0.002	156.31
50	500	1.08 ± 0.03	0.161 ± 0.006	6.72
60		9.30 ± 0.37	0.376 ± 0.019	24.74
UAE-methanol		14.1 ± 1.0	2.5 ± 0.1	5.64
UAE-DMF		27.7 ± 1.4	3.1 ± 0.1	8.93

Car/Chlo: carotenoids/chlorophylls.

level. The yields are expressed in μg of pigment per mg of dry weight of microalgae.

4. Discussion of results

4.1. UAE

From the results presented in Table 1, it can be concluded that the extraction yields obtained for carotenoids and chlorophylls on using DMF as the solvent in the ultrasound-assisted extraction are higher than those obtained with methanol. These results indicate that the use of ultrasound facilitates the penetration of the DMF through the microalgae cell membrane, thus increasing the recovery of the pigments present in the raw material. This finding can be corroborated if we consider that the resulting pellet from the methanol extraction still retains coloration, which is indicative that this process is unable to extract all of the pigments present in the sample.

As far as the carotenoids/chlorophylls ratio is concerned, the extraction with DMF gives higher values than that with methanol. These results show that DMF is more selective than methanol in the recovery of carotenoids from chlorophylls. This behaviour can be attributed to the fact that chlorophyll is heterogenically bound to other compounds in the chloroplast and at least two or even three fractions of chlorophyll exist in the chloroplast. Therefore, the different polarities of methanol and DMF lead to the extraction of different types of chlorophyll and this gives rise to variations in the extraction yield of this substance [37,38].

4.2. SFE

The results of the experimental design analysis are shown in Table 2. Estimates of the effects and interactions between the range of variables under investigation are shown along with the analysis of variance of the extraction process. The sign associated with each of the effects indicates a positive or negative influence on the yield of the process. The analysis of variance with the degree of significance of each factor is represented in Table 2 by the *p*-value; when a factor has a *p*-value below 0.05 this factor influences the process in a significant way.

The results obtained show that temperature and pressure both have a significant positive influence on the extraction process of carotenoids and chlorophylls (*p* value <0.05). On the other hand, the combined interaction of these two variables only has a significant positive influence on the extraction process of chlorophylls.

4.2.1. Effect of pressure

Analysis of the data presented in Table 1 shows that the maximum extraction yield of carotenoids depends on the temperature. At temperatures of 40 and 50 °C, the maximum extraction yield is achieved at 400 bar, while at 60 °C the best value it is obtained at a pressure of 300 bar. On the other hand, in the chlorophyll extraction, it can be seen that at 40 °C the values of the extraction yields are similar and significant differences are not found. At 50 °C of temperature, the highest extraction yield is obtained when the extraction is carried out at a pressure of 400 bar, while at 60 °C the highest chlorophyll yield is obtained at 500 bar.

For each temperature, an increase in the operating pressure leads to two opposing effects: an increase in the solvent power of the carbon dioxide and a decrease in its diffusivity. At relatively low temperatures (40 and 50 °C) the data obtained indicate that the dominant effect is the solvent power of the carbon dioxide until a pressure of 400 bar is reached. Above this value, this effect is unable to counteract the decrease in the solvent diffu-

Table 2
Estimated effect and analysis of variance for the supercritical extraction process for carotenoids and chlorophylls

Variable	Carotenoids		Chlorophylls	
	Effects	*p*-Value	Effects	*p*-Value
Temperature (*T*)	3.494	0.028	0.124	0.009
Pressure (*P*)	5.561	0.011	0.179	0.005
TT	5.747	0.5168	−0.047	0.517
PP	−14.742	0.3796	−0.071	0.380
TP	3.214	0.120	0.210	0.003

sivity and a decrease in the extraction yield of the process is observed.

On the other hand, at 60 °C the effect that prevails up to 300 bar in the SFE of carotenoids is the solvent power of the carbon dioxide, and above this value an increase in pressure does not compensate for the decrease in the diffusivity at 400 and 500 bar, a situation that leads to lower extraction yields. In the SFE of chlorophyll, the increase in the dissolving capacity of the solvent with pressure enables a larger amount of the solute to be recovered up to a pressure of 500 bar.

These results are consistent with those obtained in previous studies in which the microalgae *Nannochlorpsis gaditana* [22] and *Synechococcus* sp. [23] were used as raw materials, and also those obtained by Mendes et al. [20] in the extraction of carotenoids from *Clhorela vulgaris* working in the range 200–350 bar and 40–55 °C.

4.2.2. Effect of the temperature

It can be seen from Table 1 that at a pressure of 200 bar the highest extraction yields are reached when the temperature is 50 °C. This behaviour is similar to that observed in a previous study in which two different microalgae were used: *Nannochloropsis gaditana* [22] and *Synechococcus* sp. [23]. At a pressure of 200 bar, the slight increase in the extraction yield observed on increasing the temperature from 40 to 50 °C it is attributed to an increase in the vapour pressure of the solutes and the increase in the diffusivity of the carbon dioxide. On the other hand, when the temperature is increased from 50 to 60 °C, the effect that prevails is the decrease in the density of the solvent and this is not compensated by the increase in the diffusivity and the vapour pressure of the solutes to be extracted.

The best extraction yields are obtained at a temperature of 60 °C when the operation is carried out at 300, 400 and 500 bar. This behaviour is attributed to the fact that at these pressures the density of the carbon dioxide is greater and, at the same time, the increase in temperature causes increases in the solvent diffusivity and the vapour pressures of the pigments being extracted, thus favouring their dissolution and giving better extraction yields.

4.2.3. Carotenoids/chlorophylls (Car/Chlo) ratio

In order to define the best selective operating conditions for the extraction of carotenoids with respect to chlorophylls, the Car/Chlo ratios presented in Table 1 were analysed. Analysis of these data leads to two conclusions:

The first consideration is that at operating pressures of 300, 400 and 500 bar, the ratio between carotenoids and chlorophylls (Car/Chlo) shows the same variation with temperature. In other words, this ratio decreases on increasing the temperature from 40 to 50 °C, and increases to the highest values when a temperature of 60 °C is reached.

The second conclusion is that the best pressure and temperature conditions to obtain the highest ratio between carotenoids and chlorophyll are 300 bar and 40 °C. These conditions are the most appropriate to carry out the separation and purification of these two types of pigments.

4.3. Empirical correlations

The program STATGRAPHICS was used to analyse the experimental data and this enabled two empirical correlations to be obtained. These correlations are able to relate the variables that influence the SFE extraction process of carotenoids and chlorophylls with carbon dioxide.

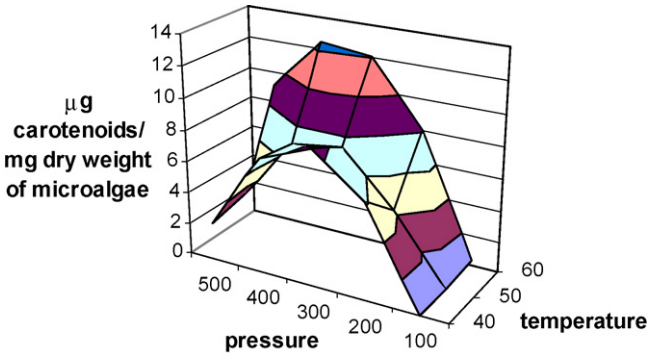

Fig. 1. Estimated values for the extraction yields of carotenoids using the empirical correlation proposed.

In the case of carotenoid extraction, the expression proposed is as follows:

$$R = 61.890 + 8.430 \times 10^{-2}P - 2.940T - 1.843 \times 10^{-4}P^2 + 8.035 \times 10^{-4}PT + 2.873 \times 10^{-2}T^2 \quad (4)$$

where R is the yield of the extraction of carotenoids in μg per mg of dry weight of microalgae, T the temperature [°C] and P is the pressure [bar]. The correlation coefficient obtained is 0.92.

Eq. (4) is represented in Fig. 1 for the different operating conditions. A detailed analysis of the figure indicates that the estimated values are consistent with those obtained experimentally. The highest yield is obtained between 300 and 400 bar at 60 °C.

In the case of chlorophyll extraction, the empirical correlation proposed is as follows:

$$R = -0.170 - 1.644 \times 10^{-3}P + 1.394 \times 10^{-2}T - 8.817 \times 10^{-7}P^2 + 5.240P \times 10^{-5}T - 2.347 \times 10^{-4}T^2 \quad (5)$$

where R is the extraction yield of chlorophylls in g per mg of dry weight of microalgae, T the temperature [°C] and P is the pressure [bar]. The correlation coefficient obtained is 0.92, which is identical to that obtained in the correlation for the carotenoids.

Eq. (5) is represented in Fig. 2 for the different operating conditions. When *D. salina* was used as the raw material it can be seen that the graph leads to a similar conclusion to that described in Section 4.2, i.e., the highest yield is obtained at a pressure of 500 bar and a temperature of 60 °C.

4.4. Comparison of SFE and UAE

A bar chart showing the comparison of the best results obtained by SFE and those obtained by UAE using methanol and DMF as sol-

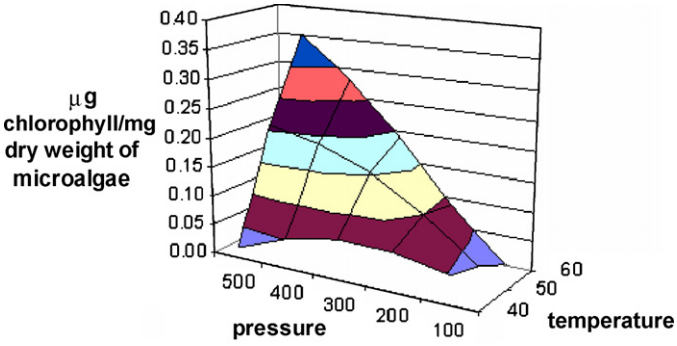

Fig. 2. Estimated values for the extraction yields of chlorophylls using the empirical correlation proposed.

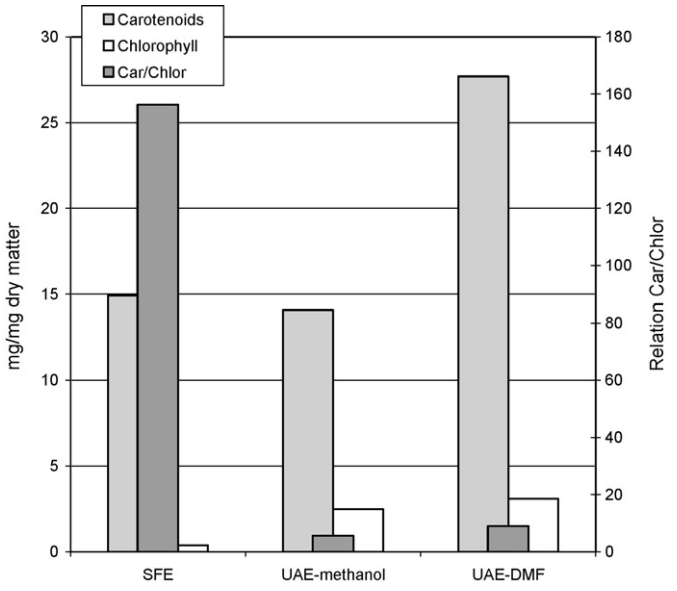

Fig. 3. Comparison of the extraction yields obtained by SFE and UAE on carotenoids and chlorophylls.

vent is shown in Fig. 3 along with the Car/Chlo ratios. Analysis of this figure shows that, for the carotenoid and chlorophyll extraction, the best extraction yields are obtained when the UAE is carried out using DMF as the solvent. On the other hand, the SFE is a more efficient method for the recovery of carotenoids than UAE using methanol as solvent. As far as the Car/Chlo ratio obtained in the SFE process using carbon dioxide is concerned, it is possible to conclude that, in all cases, this ratio is higher than that obtained in the UAE process using methanol or DMF. This suggests that the supercritical extraction process is more selective than the conventional one.

5. Conclusions

The best extraction yields of carotenoids on using *D. salina* as the raw material are obtained at the maximum operating temperature (60 °C) and at a pressure of approximately 400 bar. The best yields in the extraction of chlorophyll are achieved at 60 °C and 500 bar.

Supercritical carbon dioxide is a suitable solvent for the extraction of carotenoids due to the low polarity of these compounds. The SFE process is more selective than the UAE techniques when polar pigments, e.g. chlorophylls, are present in the raw material.

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References

[1] F. García, E. Molina, M.E. Martínez, S. Sánchez, Grasas y aceites 41 (1990) 13–18.
[2] A.P. Scott, C. Middleton, Aquaculture 18 (1979) 227–240.
[3] S. Fujita, T. Watanabe, C. Kitajiwa, Aquaculture 34 (1983) 115–143.
[4] G.W. Burton, J. Nutr. 119 (1989) 109–111.
[5] P. Palozza, N.I. Krinsky, Methods Enzymol. 213 (1992) 403–420.
[6] A. Bendich, J.A. Olson, FASEB J. 3 (1989) 1927–1932.
[7] A. Bendich, in: N.I. Krinsky, M.M. Mathews-Roth, R.F. Taylor (Eds.), Carotenoids: Chemistry and Biology, Plenum Press, New York, 1990, pp. 323–335.
[8] N.I. Krinsky, Pure Appl. Chem. 66 (1994) 1003–1010.
[9] M.A. Borowitzka, L.J. Borowitzka, Microalgal Biotechnology, Cambridge University Press, Cambridge, 1988, p. 477.
[10] R.C. Cresswell, T.A.V. Rees, N. Shah, Algal and Cyanobacterial Biotechnology, Longman Scientific & Technical, 1981, p. 341.
[11] Z. Cohen, Chemicals from Microalgae, Taylor & Francis, 2002, p. 444.
[12] M. Wettasinghe, F. Shahidi, J. Agric. Food Chem. 47 (1999) 1801–1805.
[13] S. Rodrigues, G.A.S. Pinto, F.A.N. Fernandes, Ultrason. Sonochem 80 (2007) 869–872.
[14] V. Flores Péres, J. Saffi, M.I.S. Melecchi, F.C. Abad, R. Jacques, M.M. Martinez, E.C. Oliveira, E.B. Caramão, J. Chromatogr. A (2006) 115–118.
[15] L. Paniwnyk, E. Beaufoy, J.P. Lorimer, T.J. Mason, Ultrason. Sonochem. 8 (2001) 299–305.
[16] M.H. Entazani, S.H. Nazary, M.H.H. Khodaparast, Ultrason. Sonochem. 11 (2004) 379–384.
[17] L. Mathiasson, C. Turner, H. Berg, L. Dahlberg, A. Theobald, E. Anklam, R. Ginn, M. Sharman, F. Ulberth, R. Gaberning, Food Add. Cont. 19 (2002) 632–646.
[18] R.L. Mendes, H.L. Fernandes, J.P. Coelho, J.M.S. Cabral, A.M.F. Palavra, J.M. Novais, J. Appl. Phycol. 6 (3) (1994) 289–293.
[19] R.L. Mendes, H.L. Fernandes, J.P. Coelho, E.C. Reis, J.M.S. Cabral, J.M. Novais, A.F. Palavra, Food Chem. 53 (1995) 99–103.
[20] R.L. Mendes, B.P. Nobre, M.T. Cardoso, A.P. Pereira, A.F. Palavra, Inorg. Chim. Acta 356 (2003) 328–334.
[21] M. Careri, L. Furlattini, A. Mangia, M. Musci, E. Anklam, A. Theobald, J. Chromatogr. A 912 (2001) 61–71.
[22] M.D. Macías-Sánchez, C. Mantell, M. Rodríguez, E. Martínez de la Ossa, L.M. Lubián, O. Montero, J. Food Eng. 66 (2005) 245–251.
[23] M.D. Macías-Sánchez, C. Mantell, M. Rodríguez, E. Martínez de la Ossa, L.M. Lubián, O. Montero, J. Supercrit. Fluids 39 (2007) 323–329.
[24] O. Montero, M.D. Macías-Sánchez, C.M. Lama, L.M. Lubián, C. Mantell, M. Rodríguez, E. Martínez de la Ossa, J. Agric. Food Chem. 53 (2005) 9701–9707.
[25] J.A. Mendiola, L. Jaime, S. Santoyo, G. Reglero, A. Cifuentes, E. Ibañez, F.J. Señoráns, Food Chem. 102 (2007) 1357–1367.
[26] L. Gouveira, B.P. Nobre, F.M. Marcelo, S. Mrejen, M.T. Cardoso, A.F. Palabra, R.L. Mendes, Food Chem. 101 (2007) 717–723.
[27] A.P. Canela, P.T.V. Rosa, M.O.M. Marques, M.A.A. Meireles, Ind. Eng. Chem. Res. 41 (2002) 3012–3018.
[28] K.N. Chidambara Murthy, A. Vanitha, J. Rajesha, M. Mahadeva Swamy, P.R. Sowmya, G.A. Ravishankar, Life Sci. 76 (2005) 1381–1390.
[29] J. Navalho, Biotecnología de Dunaliellsa salina para produçao de betacaroteno Universidade do Algarbe. Unidade de Ciencias e Tecnología dos Recursos Acuáticos, 1997.
[30] A. Ben-Amotz, in: Z. Cohen (Ed.), Chemicals from Microalgae, Taylor & Francis Ltd., 1999.
[31] M. García-González, J. Moreno, J.C. Manzano, F.J. Florencio, M.G. Guerrero, J. Biotechnol. 115 (2005) 81–90.
[32] M.A. Hejazi, C. de Lamarliere, J.M.S. Rocha, M. Vermuë, J. Tamper, R.H. Wijffels, Biotechnol. Bioeng. 79 (1) (2002) 29–36.
[33] J.R. Denery, K. Dragull, C.S. Tang, Q.X. Li, Anal. Chim. Acta 501 (2004) 175–181.
[34] M. Herrero, L. Jaime, P.J. Martín-Álvarez, A. Cifuentes, E. Ibañez, J. Agric. Food Chem. 54 (2006) 5597–5603.
[35] R.R.L. Guillard, J.H. Ryther, J. Microb. 8 (1962) 229–239.
[36] A.R. Wellburn, J. Plant Physiol. 144 (1994) 307–313.
[37] M.E. Deroche, J.M. Briantais, Photochem. Photobiol. 19 (1974) 233–240.
[38] G. Oquist, G. Samuelsson, Physiol. Plantarum 50 (1980) 57–62.