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Supercritical CO_2 extraction of γ-linolenic acid and other lipids from *Arthrospira* (*Spirulina*)*maxima*: Comparison with organic solvent extraction

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Abstract

Freeze-dried biomass of *Arthrospira maxima* Setchell & Gardner was submitted to supercritical CO_2 extraction using a flow type apparatus at a temperature of 50 °C and a pressure of 250 bar.

To increase the yield in either lipids or GLA (γ-linolenic acid), which is mostly contained in the glycolipid fractions, a polar co-solvent (ethanol) was added to the CO_2 and the corresponding supercritical fluid extraction was carried out at temperatures of 50 and 60 °C and at 250 and 350 bar. The use of the co-solvent increased both lipid and GLA yields relative to the extraction with pure CO_2. On the other hand, the increase of pressure and temperature also had a positive effect on the extraction of GLA.

Supercritical extraction was compared with organic solvent extraction, regarding lipid yields, fatty acid composition of total lipids and lipid classes, as well as the distribution of lipids by their classes (neutral lipids, glycolipids and phospholipids).

Keywords: γ-Linolenic; GLA; Supercritical fluid extraction; *Arthrospira maxima*; *Spirulina maxima*; Carbon dioxide

1. Introduction

The cyanobacterium *Arthrospira* (*Spirulina*) can produce large amounts of the valuable γ-linolenic acid (GLA), C18:3 ω6 (Cohen & Heimer, 1992). Other important sources of this polyunsaturated fatty acid are evening primrose, black currant and borage, as well as the fungi *Mortierella* and *Aspergillus* (Gill & Valivety, 1997). On the other hand, up to 25% of the dry *Spirulina* is composed of proteic pigments (the phycobiliproteins), which can be used in human food, animal feed and cosmetic applications.

GLA has an important role in human metabolism, particularly as a precursor of one kind of prostaglandin. Therefore, it has been used in several medical applications, such as the treatment of schizophrenia, multiple sclerosis, dermatitis, pre-menstrual syndrome, atopic eczema, diabetes and rheumatoid arthritis (Kennedy, Reader, & Davies, 1993; Nakahara, Yokocki, Kamisaka, & Suzuki, 1992).

The need for rapid, efficient and safe methods for GLA extraction from natural sources has been emphasized, having in view its human consumption (Reis, Fernandes, Empis, & Novais, 1998). Supercritical fluid extraction is one of the most promising techniques for obtaining this kind of compound, because it is possible to produce solvent-free extracts and to avoid the degradation of thermally labile components (Bruno, Castro, Hamel, & Palavra, 1993). Therefore, several studies of supercritical fluid extraction of oils containing GLA have been reported: from fungi (*Mortierella ramanniana*)

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and using CO_2, N_2O, CHF_3 and SF_6 (Sakaki, Yokochi, Suzuki, & Hakuta, 1990), evening primrose seeds (Favati, King, & Mazzanti, 1991) and *Spirulina platensis* (Santos, Lu, King, & Empis, 1997; Quihui, 1999) using CO_2, borage seeds with CO_2 and a mixture of CO_2 and propane (Illes, Szalai, Szebenyi, Grosz, & Hethelyi, 1994). In the Quihui's (1999) work, the objective was also the removal of the stench of the *Spirulina*, bearing in mind its use for food purposes. Supercritical CO_2 extraction of other compounds from similar organisms (microalgae) has also been reported: hydrocarbons from *Botryococcus braunii* (Mendes et al., 1994) and carotenoids from *Chlorella vulgaris* (Mendes et al., 1995a).

The aim of the present work is to carry out the supercritical CO_2 extraction of the lipids, particularly GLA, from *Arthrospira maxima*, to assess the influence of several operating conditions on the extraction yield and selectivity and to compare the supercritical fluid extraction with conventional extraction using organic solvents.

2. Materials and methods

2.1. Cyanobacterial culture

The *Arthrospira* (*Spirulina*) *maxima* Setchell & Gardner LB 2342 is from the University of Texas culture collection-UTEX (Austin, USA). The growth was carried out with a *Spirulina* medium (Vonshak, 1986). The *Arthrospira* was harvested using a nylon plankton mesh (33 μm) and was then freeze-dried and later stored, for further use, at −20 °C under N_2.

2.2. Supercritical fluid extraction

For the supercritical fluid extraction studies, a flow apparatus was used (Fig. 1), which is already described in detail elsewhere (Mendes et al., 1995b). In this apparatus, the fluid is pumped, under the desired conditions of pressure and temperature, at a flow rate of about 2 g/min, to the extraction vessel (length 25.4 cm and internal diameter 1.27 cm), 11, which contains the dry cyanobacterium. The extracted lipids were collected, at regular time intervals, in a glass U-tube, 13, cooled with an ice bath, after the fluid expansion to atmospheric pressure, and the amount was assessed by gravimetry. At the end of each experiment, the lipids remaining in the tubing before the glass U-tubes were recovered by organic solvent washing, and taken into account in the final yield calculation. The total volume of CO_2 was quantified with a wet test meter, 15. For further analysis, the lipids were stored in acetone under nitrogen at −20 °C. In these studies, a mixture of CO_2 and ethanol (10 mol%) was also used as a supercritical fluid.

For each supercritical experiment, 5 g of ground freeze-dried material, corresponding to 3.6 g of ash-free *Arthrospira maxima*, sieved for particles below 0.2 mm diameter were used. The CO_2 (99.998% purity) and the mixture of CO_2 (90 mol%) plus ethanol (99.998% and 99.5% purity, respectively) were purchased from Air Liquide (Portugal).

2.3. Organic solvent extraction

Dry *Arthrospira* (500 mg) was used to carry out several lipid extractions with organic solvents at 25 °C, for 2 h, with magnetic stirring (100 rpm) in light-protected vessels under nitrogen. The solvents chosen were ethanol, acetone, hexane and a mixture of chloroform, methanol and water (Bligh & Dyer, 1959). All extractions were carried out in duplicate.

The organic and supercritical extracts were loaded into glass columns filled with silicic acid. Fractions eluted with 10 volumes of chloroform, 50 volumes of acetone and 10 volumes of methanol were recovered as neutral lipids, glycolipids and phospholipids, respectively (Kates, 1986). The lipids were quantified gravimetrically prior the fatty acid derivatization and analysis.

2.4. Fatty acids analysis

To determine the content in fatty acids of the freeze-dried biomass, it was transesterified according to the modified Lepage and Roy (1986) method, modified by Cohen, Vonshak, and Richmond (1988).

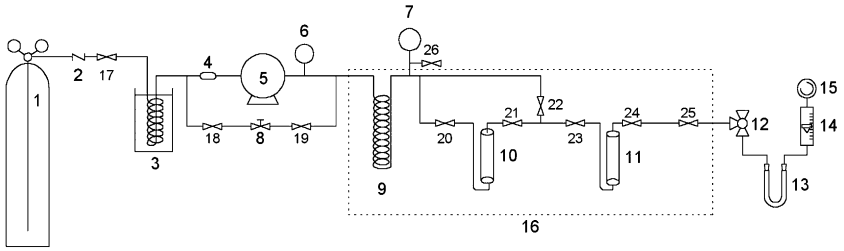

Fig. 1. Schematic diagram of the apparatus for supercritical fluid extraction: 1. gas cylinder, 2. check valve, 3. ice cooler, 4. filter, 5. pump, 6,7. manometers, 8. back pressure regulator, 9. heat exchanger, 10, 11. high pressure vessels, 12. micrometering valve, 13. glass U-tube, 14. rotameter, 15. wet test meter, 16. water bath, 17–26. valves.

Freeze-dried biomass samples (100 mg) or lipidic extracts (at least 10 mg) were transmethylated with 5 ml of methanol/acetyl chloride (95:5 v/v). The mixture was sealed in a light-protected Teflon-lined vial under nitrogen atmosphere and heated at 80 °C for 1 h. The vial contents were then cooled, diluted with 1 ml water and extracted with 2 ml of *n*-heptane. The heptane layer was dried over Na_2SO_4, evaporated to dryness under nitrogen atmosphere and redissolved in heptane, which contained the methyl esters. From each duplicate sample, two independent derivatizations were prepared. Two injections were made for each derivate, thus each fatty acid analysis was the peak area average of eight injections.

All the fatty acid analyses were carried out in a Varian 3300 gas chromatograph equipped with a flame ionization detector. The column was a 0.32 mm × 30 m fused silica capillary one (film: 0.32 μm) Supelcowax 10 (Supelco) with He as carrier at a flow rate of 1.5 ml/min, and its temperature was programmed to 200 °C for 11.5 min; then it was increased to 225 °C at 10 °C/min and maintained at this temperature for 16 min. The injector and detector temperatures and split ratio were 250, 260 °C and 100:1, respectively. Heptadecanoic acid (Merck) was used as internal standard. Peak identification and response factor calculation were carried out using known standards (Sigma and Nu-Chek-prep). From each duplicate sample, two independent derivatizations were prepared. Two injections were made for each derivate, thus each fatty acid analysis was the peak area average of eight injections.

3. Results and discussion

The initial composition of fatty acids of the biomass (assessed by direct transesterification on the freeze dried material), placed in the extractor, was: palmitic (31.7%), palmitoleic (12.4%), stearic (0.6%), oleic (1.2%), linoleic (18.6%), γ-linolenic (35.5%). The content of lipids, as determined by the Bligh and Dyer method, was 7.8 wt% of the dried biomass.

Besides the extractions with organic solvents (hexane, ethanol and acetone) and the mixture of solvents (Bligh and Dyer), the cyanobacterium was submitted to supercritical CO_2 and to a mixture of CO_2 + ethanol (10 mol%). at a pressure of 250 bar and a temperature of 50 °C. The extraction was also carried out with the co-solvent at 60 °C at the same pressure. The residue of the latter extraction was afterwards submitted to a pressure of 350 bar and a temperature of 60 °C. For each condition of pressure and temperature, the lipids were collected after expansion at regular time intervals. The cumulative evolution of the extraction, as a function of the CO_2/biomass weight ratio, is shown in Fig. 2.

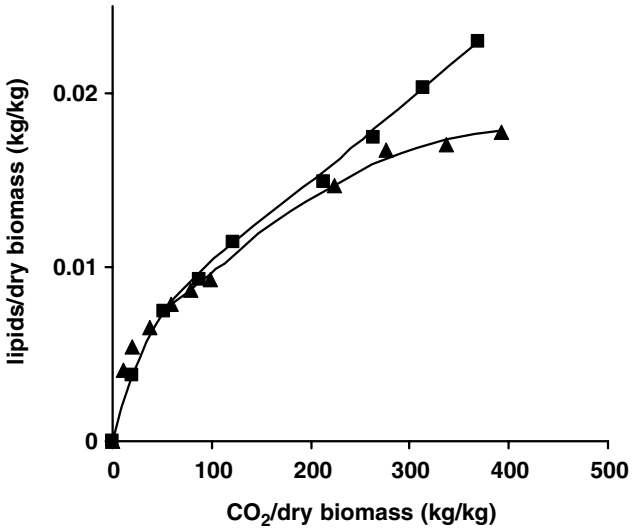

Fig. 2. Extraction of lipids from *Arthrospira maxima* as a function of supercritical solvent biomass ratio at 250 bar and 50 °C. (▲) CO_2 and (■) CO_2 + 10 mol% ethanol.

The curves for pure CO_2 and for CO_2 modified with ethanol overlap up to an amount of CO_2 of about 200 kg/kg of *Arthrospira*. Beyond this point, the yield increases almost linearly. The ethanol can have an entrainment effect on the extraction of the lipids of the *Arthrospira*, which are mainly polar (Reis et al., 1998), increasing its solubility, and can counterbalance the hydrogen bonds and ionic forces between the membrane-associated lipids and proteins (Certik, Andrasi, & Sajbidor, 1996), allowing the lipids to be available for extraction by the supercritical fluid. On the other hand, with pure CO_2, the curve tends rapidly to a plateau, implying a low yield of the extraction but, with the supercritical CO_2 plus ethanol, it is possible to increase the yield steadily, at least up to about 400 kg/kg (CO_2/biomass ratio).

The yields in both lipid and GLA for several conditions of extraction (supercritical and organic solvents) are shown in Table 1. The lipids extracted with the solvent mixture (chloroform, methanol and water), according to the Bligh and Dyer (1959) method, were considered to be 100% of the total lipids existing in the *Arthrospira*, while 100% GLA was obtained by direct transesterification on the freeze-dried biomass (Lepage and Roy method).

The supercritical CO_2 compares well with hexane in the lipid extraction yield, but allows a higher yield of GLA. Among the authorized solvents used in the food industry (ethanol, acetone and CO_2), the highest yield both in lipids and GLA was obtained with ethanol. By supercritical extraction, the highest yield of GLA was obtained at 350 bar and 60 °C with the mixture (CO_2 + 10 mol% ethanol). Although the yields obtained are lower than those obtained by ethanol and acetone, it

Table 1
Lipid and GLA yields for several conditions of extraction

Solvent	Lipids recovery (%)	Lipids/dry biomass (wt%)	GLA recovery (%)	GLA/dry biomass (wt%)
Methanol:acetyl chloride (95/5% v/v)[a]	–	–	100	1.23
Bligh and Dyer mixture	100	7.8	75	0.98
Hexane	33	2.6	1	0.01
Ethanol	73	5.7	72	0.68
Acetone	60	4.7	48	0.63
CO_2[b]	32	2.5	4	0.05
CO_2 + ethanol[c]	40	3.1	13	0.17
CO_2 + ethanol[d]	28	2.2	18	0.24
CO_2 + ethanol[e]	–	–	45	0.44

[a] Lepage and Roy method.
[b] 250 bar/50 °C (1.4 kg CO_2).
[c] 250 bar/50 °C (1.3 kg CO_2).
[d] 250 bar/60 °C (1.2 kg CO_2).
[e] 350 bar/60 °C (0.08 kg CO_2).

is possible to increase them, increasing the supercritical solvent/biomass ratio (Fig. 2).

Table 2 presents the distribution of lipid classes (namely neutral lipids, glycolipids and phospholipids), fatty acid, GLA distribution and profile of the different fatty acids by class of lipids for three conditions of extraction: the Bligh and Dyer (1959) method (which extracts all the lipids), supercritical extraction with pure CO_2 and CO_2 + ethanol. It can be seen, for instance, that when pure CO_2 was used, about 70% of the extracted GLA was in the neutral lipids and only 14% in the glycolipids, but the situation was reversed (73% was in glycolipid fraction) when using CO_2 doped with ethanol. Moreover, of the extracted lipid classes, those extracted with CO_2 were mainly neutral (71%), while those extracted with CO_2 + ethanol were predominantly glycolipidic. The values obtained with the latter mixture are similar to those obtained with the Bligh and Dyer (1959) method. This can be explained in terms of polarity of the solvents involved.

Fig. 3 shows the profile of fatty acids for several conditions and several solvents used for the extraction of the lipids from *Arthrospira*. Among organic solvent systems, acetone yielded the greatest GLA fraction, similar to supercritical extraction with CO_2 and ethanol (250 bar/50 °C), in spite of low lipid and GLA yields. With the Bligh and Dyer solvent mixture, palmitic acid (C16:0) is predominant.

Fig. 4 shows the distributions of the main fatty acids in the glycolipid fraction, for several conditions of extraction. For this fraction, the selectivity in GLA is low when using pure CO_2, but is much higher when CO_2 with ethanol is used. The temperature also has a positive effect on the GLA selectivity. With pure CO_2, on the other hand, in the same fraction, the highest selectivities are for oleic and linoleic acids.

Fig. 5 shows the distribution of the fatty acids in the neutral fraction and it is verified that, again, pure CO_2 is more convenient for extracting linoleic acid while the CO_2 with ethanol co-solvent shows the highest selectivity for GLA.

Table 2
Classes of lipids and distribution of fatty acids by these classes, for several extraction conditions (wt%)

	Neutral lipids			Glycolipids			Phospholipids		
	a	b	c	a	b	c	a	b	c
C16:0	34.2	13.4	23.9	35.7	12.8	14.3	46.8	21.3	18.4
C16:1	7.7	4.7	9.3	15.7	14.4	11.4	4.5	19.3	4.3
C18:0	10.0	8.2	2.1	1.1	4.1	1.8	0.7	6.6	3.3
C18:1	0.5	12.8	12.4	0.1	23.4	2.6	1.9	14.4	12.4
C18:2	26.0	40.5	24.0	12.5	24.5	3.7	38.0	16.7	58.4
C18:3	21.3	19.3	26.8	34.6	19.4	65.3	7.8	20.0	1.7
GLA distribution	21.7	70.2	26.0	70.7	14.0	73.5	7.6	15.8	0.5
FA distribution	25.4	56.9	35.8	50.9	32.7	53.7	23.7	10.4	10.5
Fraction distribution	24.5	70.9	35.3	52.0	12.8	55.6	27.5	16.3	9.1

[a] Bligh and Dyer method.
[b] Supercritical CO_2 extraction (250 bar/50 °C).
[c] Supercritical CO_2 (+10% ethanol) extraction (250 bar/50 °C).

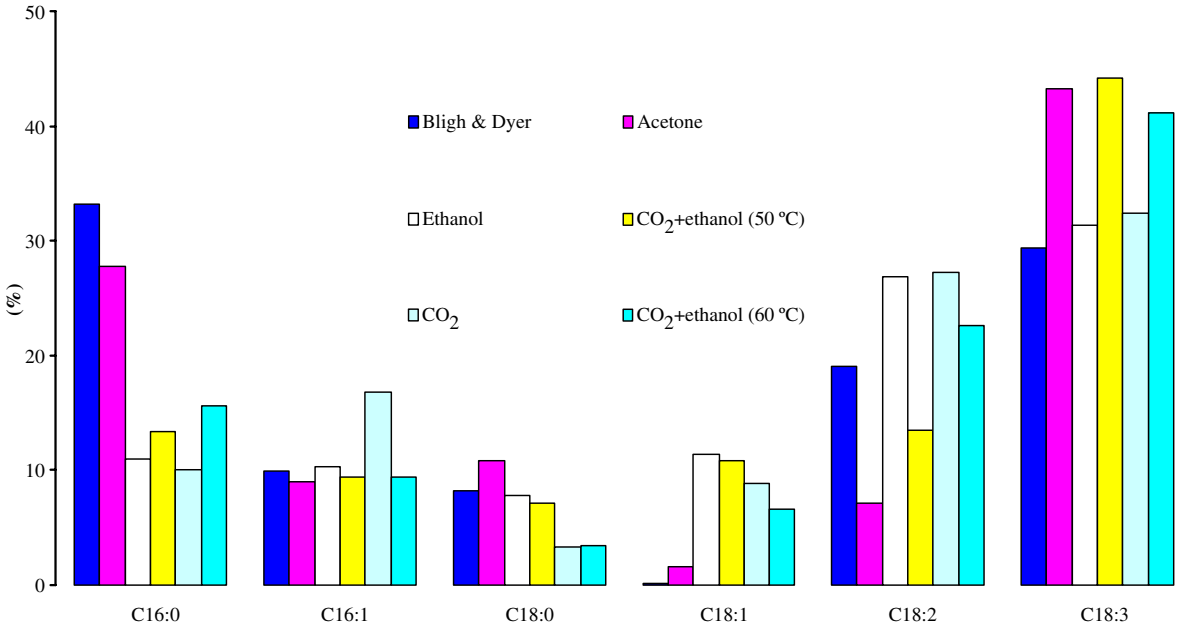

Fig. 3. Profile of fatty acids in the extracts obtained from *Arthrospira maxima*, with several solvents and conditions. Supercritical fluid extraction pressure: 250 bar. Palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and γ-linolenic acid (C18:3).

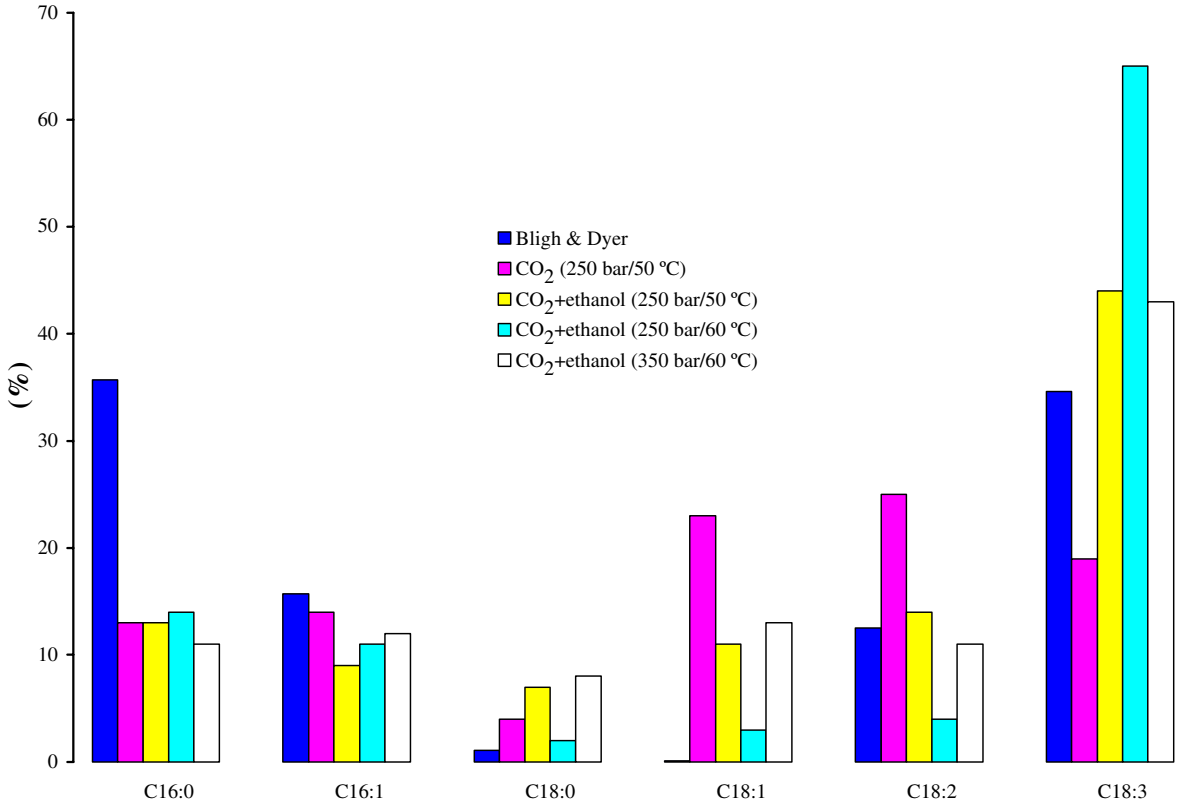

Fig. 4. Profile of fatty acids in the glycolipid fraction of the extracts obtained from *Arthrospira maxima*, with several solvents and conditions. Palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), γ-linolenic acid (C18:3).

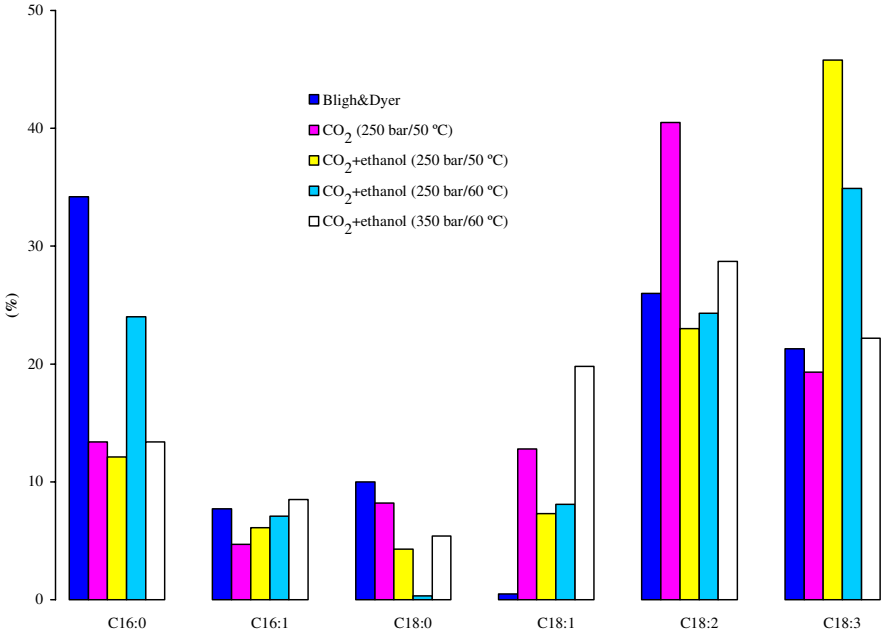

Fig. 5. Profile of fatty acids in the neutral fraction of the extracts obtained from *Arthrospira maxima*, with several solvents and conditions. Palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), γ-linolenic acid (C18:3).

4. Conclusion

Supercritical extraction of lipids from *Arthrospira maxima* with pure supercritical carbon dioxide showed a low yield, when compared with those obtained with a mixture of chloroform, methanol and water (Bligh and Dyer method), acetone and ethanol. The GLA content in the supercritical CO_2 extracts was also lower. On the other hand, it compared well with the yield obtained with hexane.

The use of ethanol mixed with CO_2 increased both lipid and GLA yields and led to greatest GLA fraction, slightly higher than that obtained with acetone. Increasing the temperature of the supercritical extraction, using the modified CO_2 from 50 to 60 °C, led to a higher yield of GLA, but not of the lipids. On the other hand, on increasing the pressure, under these conditions, an improvement in GLA yield was achieved.

Among organic solvents, hexane showed the poorest GLA fraction as well as the lowest yields of lipids and GLA. On the other hand, acetone yielded the greatest GLA fraction, though with a low yield of lipids. Furthermore, among the authorized organic solvents in food industry, ethanol led to the highest yield of lipids and GLA.

References

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology, 37*, 911–917.

Bruno, T., Castro, C. A. N., Hamel, J. F. P., & Palavra, A. M. F. (1993). Supercritical fluid extraction of biological products. In J. F. Kennedy & J. M. S. Cabral (Eds.), *Recovery processes for biological materials* (pp. 303–354). Chichester: John Wiley Sons.

Certik, M., Andrasi, P., & Sajbidor, J. (1996). Effect of extraction methods on lipid yield and fatty acid composition of lipid classes containing γ-linolenic acid extracted from fungi. *Journal of American Oil Chemists Society, 73*, 357–365.

Cohen, Z., Vonshak, A., & Richmond, A. (1988). Effect of environmental conditions on fatty acid composition of the red alga *Porphyridium cruentum*: correlation to growth rate. *Journal of Phycology, 24*, 328–332.

Cohen, Z., & Heimer, Y. M. (1992). Production of polyunsaturated fatty Acids (EPA, ARA and GLA) by the microalgae *Porphyridium* and *Spirulina*. In D. J. Kyle & C. Ratledge (Eds.), *Industrial applications of single cell oils* (pp. 243–273). Champaign: AOCS.

Favati, F., King, J. W., & Mazzanti, M. (1991). Supercritical carbon dioxide extraction of Primrose oil. *Journal of American Oil Chemists Society, 68*, 422–427.

Gill, I., & Valivety, R. (1997). Polyunsaturated fatty acids, part 1: ocurrence, biological activities and applications. *TIBTECH, 15*, 401–409.

Illes, V., Szalai, O., Szebenyi, N. I., Grosz, M., & Hethelyi, I. (1994). Oil recovery from Borage seed with carbon dioxide and propane solvents. In *Proceedings of the 3rd international symposium on supercritical fluids* (pp. 511–516). Nancy: institut National Polytechnique de Lorraine.

Kates, M. (1986). *Techniques of lipidology: isolation, analysis and identification of lipids*. Amsterdam: Elsevier.

Kennedy, M. J., Reader, S. L., & Davies, R. J. (1993). Fatty acid production characteristics of fungi with particular emphasis on gamma linolenic acid production. *Biotechnology and Bioenergy, 42*, 625–634.

Lepage, G., & Roy, C. R. (1986). Direct transesterification of all classes of lipids in one-step reaction. *Journal of Lipid Research, 27*, 114–119.

Mendes, R. L., Fernandes, H. L., Coelho, J. A. P., Cabral, J. M. S., Palavra, A. F., & Novais, J. M. (1994). Supercritical carbon

dioxide extraction of hydrocarbons from the Microalga *Botryococcus braunii*. *Journal of Applied Phycology, 6*, 289–293.

Mendes, R. L., Fernandes, H. L., Coelho, J. P., Reis, E. C., Cabral, J. M. S., Novais, J. M., & Palavra, A. F. (1995a). Supercritical CO_2 extraction of carotenoids and other lipids from *Chlorella vulgaris*. *Food Chemistry, 53*, 99–103.

Mendes, R. L., Coelho, J. P., Fernandes, H. L., Marrucho, I. J., Cabral, J. M. S., Novais, J. M., & Palavra, A. F. (1995b). Applications of supercritical CO_2 extraction to microalgae and plants. *Journal of Chemical Technology and Biotechnology, 62*, 53–59.

Nakahara, T., Yokocki, T., Kamisaka, Y., & Suzuki, O. (1992). Gamma-linolenic acid from genus *Mortierella*. In D. J. Kyle & C. Ratledge (Eds.), *Industrial applications of single cell oils* (pp. 61–97). Champaign: AOCS.

Quihui, H. (1999). Supercritical carbon dioxide extraction of *Spirulina platensis* component and removing the stench. *Journal of Agriculture and Food Chemistry, 47*, 2705–2706.

Reis, A., Fernandes, H. M., Empis, A., & Novais, J. M. (1998). Effect of extraction and purification methods on fatty acid composition and gamma-linolenic acid yield and purity from *Arthrospira* (*Spirulina*) *maxima* biomass. In Y. Le gal & A. Muller (Eds.), *Feuga marine microorganisms for industry* (pp. 34–38). Plouzané(France): Editions IFREMER.

Sakaki, K., Yokochi, T., Suzuki, O., & Hakuta, T. (1990). Supercritical fluid extraction of fungal oil using CO_2, N_2O, CHF_3 and SF_6. *Journal of American Oil Chemists Society, 67*, 553–557.

Santos, R., Lu, T., King, M. B., & Empis, J. A. (1997). Extraction of valuable components from micro-algae *Spirulina platensis* with compressed CO_2. In E. Reverchon (Ed.), *Proceedings of the 4th Italian conference on supercritical fluids and their applications* (pp. 217–224). Salerno (Italy): University of Salerno.

Vonshak, A. (1986). Laboratory techniques for the cultivation of microalgae. In A. Richmond (Ed.), *Handbook of microalgal mass culture* (pp. 117–145). Boca Raton: CRC Press.