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An Evaluation of the Antioxidant Properties of *Arthrospira maxima* Extracts obtained using non-conventional Techniques

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Abstract

In last decade *Arthrospira* has been considered an important source of high quality nutrients and therefore it is suggested as a suitable functional food. In this work *Arthrospira maxima* extracts were obtained in different conditions, varying solvents, time, temperature and extraction techniques, such as microwave- and ultrasound-assisted extraction (MAE and UAE respectively). Total carotenoids and polyphenols were measured using UV determination tests, while HPLC analyses were carried out to identify the main compounds. Antioxidant activity of *A. maxima* extracts was evaluated *in vitro* by Trolox Equivalent Antioxidant Capacity (TEAC) and Oxygen Radical Absorbance Capacity (ORAC). Acetone/hexane and acetone extracts were in general the richest in β-carotene and total carotenoids (20÷33 and 40÷70 mg/g extract, respectively). The best results were obtained under MAE at 90°C for 10 min with the highest yields in term of

total carotenoids and β-carotene (> 2.6 and > 1.0 mg/g algae, respectively). MAE with ethanol (90°C, 30 min) allowed maximization of total carotenoids and β-carotene amount (3.12 and 1.37 mg/g algae, respectively) thanks to the very good extraction yields. Moreover this technique gave an extract with the highest antioxidant activity (812 and 519 μmol Trolox/g extract for TEAC and ORAC respectively). Surprisingly, polyphenols were not detected in *A. maxima* extracts analyzed by HPLC.

Keywords: *Arthrospira maxima*; Microalgae; Antioxidant activity; Carotenoids; Ultrasound; Microwaves

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Introduction

Free radical reactions have been associated with damage to lipids, proteins, cell membranes and nucleic acids and can ultimately result in a variety of chronic diseases [1]. A number of studies have proven that the intake of some vitamins, minerals and other food constituents may help to protect the body against disease [2, 3]. Interest in the use of antioxidant nutritional supplements has boomed in recent decades, while the search for new and alternative sources of antioxidants has led to extraction from microalgae gaining importance [4]. Microalgae, like plants, have developed protective mechanisms against reactive oxygen species (ROS) which involve the production of antioxidant compounds [5, 6]. Their biodiversity, ease of cultivation and modulation of growth conditions are all important factors which mean that microalgae can be counted amongst the top natural resources with high antioxidant potential. *Arthrospira* is a blue green microalga usually cultivated in shallow raceways, that has traditionally been used in Africa and Mexico as an important nutrient source for humans and animals due to its high-protein (55–65%), and its well balanced essential amino acid content [7]. *Arthrospira* also contains a whole spectrum of compounds, including mixed carotene and xanthophyll phytopigments which, together with phycocyanin and polyphenols, seem to be related to its antioxidant activity [8-10]. Since microalgae are a complex matrix, it is important to find optimum extraction conditions. In literature, several solvents were cited for conventional extraction of carotenoids such as hexane, acetone, toluene, diethyl ether, dichloromethane, methanol, ethanol, tetrahydrofuran, the first two being the most commonly used [11-13]. Ethanol, methanol and their mixtures with water, ethyl acetate or acetone have been widely used for polyphenols extraction [14].

Traditional extraction methods usually require a large amount of solvents and long extraction times, giving low yields and moderate selectivity. Several non-conventional

extraction methods have been successfully used for the extraction of bioactive compounds in food and pharmaceutical applications and have provided beneficial results in terms of time, cost and yield [15, 16]. In particular, pressurized liquid extraction (PLE), supercritical fluid extraction with CO_2 and ethane (SFE), MW- (MAE) and US-assisted extractions (UAE) were evaluated in the recovery of carotenoids and polyphenols [17, 18].

The extraction of intracellular bioactive compounds requires the cell wall destruction, which may be enhanced by MAE and UAE [19]. The mechanical effect of US promotes the release of soluble compounds from the plant body, enhancing mass transfer and facilitating solvent access to cell content. US accelerates rehydration and swelling if the matrix has been previously dried [20, 21]. MW heats the sample volume homogeneously by promoting molecular dipole rotation, which increases solvent penetration into the matrix and thus facilitates analyte solvation.

In this work, we report a study of antioxidant activity and an evaluation of carotenoids and polyphenols content for several extracts of *A. maxima*, an *Arthrospira* species that has received so far less study than others. The microalga analysed was cultivated in photobioreactors, an alternative cultivation system that allows a better control of the cultivation parameters and minimizes health risks compared to open ponds cultivation (pollutants, contaminants).

Materials and methods

Microalgae Material

Arthrospira maxima was kindly provided by FiTolife (Turin, Italy) and was cultivated in two meter high plexiglass photobioreactors. Each culture was illuminated with three lamps 24 hours a day (70W, colour temperature 3000°K). Aeration was achieved using an air pump that mixed Zarrouk's medium containing normal concentrations of NaCl (12 mM)

and $NaNO_3$ (14 mM). The culture temperature was maintained between 30 and 33°C, while pH was adjusted to 10 ± 0.5. Conductivity and salinity were measured daily in all photobioreactors using an ADWA (AD 31) conductivity meter. The purity of cultures was periodically checked by microscopic observation according to taxonomy guidelines.

Instrumentation

UV spectra and UV determinations were obtained on a Varian Cary 50BIO UV/Vis spectrophotometer. HPLC analyses were performed on a Waters binary pump 1525 linked to a 2998 PDA detector. Fluorescence measurements were obtained on a PerkinElmer 2030 Multilabel Reader.

Solvents and reagents

Hexane, acetone and methanol (ACS grade, ≥ 99%) (Sigma Aldrich) were used for extractions. Methanol CHROMASOLV® (gradient grade, for HPLC, ≥ 99.9%), acetonitrile CHROMASOLV® (gradient grade, for HPLC, ≥ 99.9%) and pure acetone (ACS grade, ≥ 99.5%) were purchased from Sigma Aldrich for HPLC analyses, while Milli-Q H_2O was obtained in the laboratory from a Milli-Q Reference A+ System (Merck Millipore). Glacial acetic acid (≥ 96%) was purchased from Merck. β-carotene (≥97%) and licopene standards (analytical standard Fluka®) were purchased from Sigma Aldrich. Standards of benzoic, caffeic, ellagic, gentisic, *o*/*m*/*p*-coumaric, ferulic, chlorogenic, vanillic, syringic, protocatechuic, gallic, cinnamic (*trans*), 4-hydroxycinnamic (*trans*), 3,4-dihydroxycinnamic (*trans*), 3-hydroxy-4-methoxycinnamic (*trans*) and 3,4-dimethoxybenzoic acids, as well as coumarin, catechol, (+)-catechin (Fluka®) and quercetin-3-glucoside (Fluka®) were purchased from Sigma Aldrich. ABTS (2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid), diammonium salt, potassium persulfate, Trolox (α-tocopherol-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid), AAPH

(2,2′-azobis(2-amidinopropane) dihydrochloride) and ethanol (ACS grade, ≥ 99%) were purchased from Sigma-Aldrich. Fluorescein (3′,6′-dihydroxyspiro[isobenzofuran-1[3*H*],9′[9*H*]-xanthen]-3-one) was purchased from Riedel-de Haën.

Extraction

Conventional conditions

Finely milled *A. maxima* was placed into a three-necked round bottom flask (250 ml) with the appropriate solvent (ratio of solvent to microalgae was 30:1 ml/g) and the suspension was stirred under nitrogen atmosphere for 24 h. The extraction of the apolar fraction was carried out with hexane (Hex), acetone (Acet) or acetone/hexane 1:1 (Acet/Hex), under stirring at room temperature (rt). The extraction of the polar fraction was carried out using ethanol (EtOH) or ethanol/water 8:2 (EtOH/H_2O) as solvent under stirring at 50°C (see Table 1). Extractions using Acet, Acet/Hex 1:1 and EtOH at 90°C under magnetical stirring were performed in a Pyrex® pressure-resistant tube, using a ratio of solvent to microalgae of 15:1 ml/g in order to compare results with MAE. Suspensions were filtered on a Celite® pad and green extracts were concentrated under vacuum and stored at -20°C.

UAE

A high-power ultrasonic probe system with a titanium horn (frequency 21.1 kHz, max power 250 W, Ø = 2.92 cm) is a commercially available device from Danacamerini s.a.s. (Turin, Italy) [22]. Finely milled *A. maxima* was placed into a three-necked pear-shaped 250 ml flask with the appropriate solvent (ratio of solvent to microalgae was 30:1 ml/g). The suspension was irradiated under nitrogen atmosphere with US at constant power (120 W). The extraction of the apolar fraction was carried out with Hex, Acet or Acet/Hex 1:1, under irradiation for 30 min (15 cycles 1 min on/1 min off) or 1 h (continuous treatment)

(see Table 1). The extraction temperature was kept near rt by means of a thermostated cooling bath. The extraction of the polar fraction was carried out using EtOH or EtOH/H_2O 8:2 as the solvent under irradiation for 30 min (15 cycles 1 min on/1 min off) or 1 h (continuous treatment) near 50°C (see Table 1). Suspensions were filtered on a Celite® pad and the green extracts were concentrated under vacuum and stored at -20°C.

MAE

MW protocols were carried out under pressure in a SynthWave by Milestone (Bergamo, Italy) [23]. Finely milled *A. maxima* was placed into a Pyrex® test tube (50 ml) with Acet, Acet/Hex 1:1, EtOH or EtOH/H_2O 8:2 as the solvent (ratio of solvent to microalgae was 15:1 ml/g). The suspension in the presence of Acet and Acet/Hex 1:1 was irradiated with MW for 10 or 30 min at 90°C, while in the presence of EtOH or EtOH/H_2O 8:2 three different temperatures (90°C, 120°C and 150°C) were tested for 10 and/or 30 min (see Table 1). Extractions were carried out under nitrogen pressure (10 bar for EtOH or EtOH/H_2O 8:2, 40 bar for Acet and Acet/Hex). Suspensions were filtered on a Celite® pad, and the green extracts were concentrated under vacuum and stored at -20°C.

Detection and measurement of carotenoids

The extracts were saponified according to the Cyanotech Corporation method [24]; the diethyl ether solutions were treated with saturated KOH aqueous solutions for 30 min in the dark at room temperature under vortexing every 10 min. The hydrophilic material was then extracted with water and the mixture was centrifuged at 1500 x*g* for 10 min. UV-visible spectra of obtained ether solutions were registered from 200 to 800 nm, while absorbances at 470, 662 and 643 nm were used to calculate carotenoid concentration which

was expressed as μg/ml extract solution; the following equation is specific for the diethyl ether water saturated solution [25].

c_a (μg/mL) = 10.36 A_{662} – 1.28 A_{643}

c_b (μg/mL) = 17.149 A_{643} – 2.72 A_{662}

$_{Cc}$ (μg/mL) = (1000 A_{470} – 1.38 c_a – 48.05 c_b)/211

c_a: Chlorophyll a, c_b: Chlorophyll b, c_c: carotenoid

HPLC analyses of carotenoids

Analyses were performed on a XTerra C18 column (150 mm, 4.6 mm, 5 μm; Waters) using H_2O/MeOH 2:8 (A) and a MeOH/Acet 1:1 mixtures (B) as the mobile phases. Monitoring wavelengths were 430 and 600 nm (PDA range 360-700 nm). The gradient program started from 25% B and moved to 75% B over a 0-8 min period (curve 6, linear), rested at 75% over 8-10 min (curve 6), then moving from 75% to 90% B occurred from 10-18 min (curve 4), from 90% to 100% B over 18-23 min (curve 10), followed by a 100% B step lasting 23-28 min. Linear regression of β-carotene was calculated using solutions at 0.024, 0.066, 0.2, 0.4 and 0.6 mg/ml concentrations in Acet (injection of 20.0 μl). Extracts obtained with Acet, Acet/Hex 1:1 and Hex were dissolved in Acet at concentrations of between 1 and 7 mg/ml (injection of 20.0 μl). Extracts obtained with EtOH and the EtOH/H_2O 8:2 mixture were dissolved in MeOH at concentrations between 10 and 20 mg/ml (injection of 20.0 μl).

HPLC analyses of polyphenols

The analyses were performed on a Synergi Hydro RP C18 (250 mm, 4.6 mm, 5 μm; Phenomenex) using 2% acetic acid (A) and acetonitrile (B) as the mobile phases. Monitoring wavelengths were 280 and 365 nm (PDA range 300-600 nm). The gradient

program started from 0% B where it stayed for 6.5 min. It then rose to 50% B over a 6.5-30 min period, from 50% to 100% B over 30-36 min, followed by a 100% B step over 36-42 min. Extracts obtained with EtOH and the EtOH/H_2O 8:2 mixture were dissolved in MeOH at concentrations between 10 and 20 mg/ml (injection of 20.0 µl).

Antioxidant Activity

Trolox Equivalent Antioxidant Capacity (TEAC)

The antioxidant capacity of the extracts was evaluated using the improved $ABTS^{\cdot+}$ method, as described by Re et al. [26], with slight modifications. Briefly, the $ABTS^{\cdot+}$ radical cation was generated via the reaction of 7 mM ABTS with 2.45 mM potassium persulfate. The reaction mixture was left to stand in the dark for 16 h at room temperature and used within two days. The $ABTS^{\cdot+}$ solution was diluted with a potassium phosphate buffer 50 mM to give an absorbance of around 0.700 at 734 nm. The sample solutions were prepared by dissolving the extracts in EtOH and diluting with the same solvent. 100 µl of the diluted samples were mixed with 1.9 ml of the diluted $ABTS^{\cdot+}$ solution. The absorbance was recorded at 734 nm for 30 min. EtOH did not interfere with the detection. A Trolox solution (final concentration 0-50 µM) was used as a reference standard. The results were expressed as µmol Trolox/g of extract and calculated as mean value ± standard error (SE) of at least three experiments.

Oxygen Radical Absorbance Capacity (ORAC)

The ORAC test was performed on a 96-well black plate. 75 mM potassium phosphate buffer (pH 7.4) was used as a blank and a number of Trolox solutions, ranging from 0.25 to 6 µM, were used as standards [27]. The sample solutions were prepared by dissolving the extracts in an EtOH/buffer mixture (50/50 v/v) and by diluting with a phosphate buffer.

Aliquots of fluorescein solution (150 μl of 48 nM in potassium phosphate buffer) were dispensed into all the wells in order to start incubation. This was followed by 20 μl of either the buffer, standard or sample solution added in duplicate. The plate was covered and incubated in the preheated (37°C) microplate reader for 10 min which included 3 min of shaking. 30 μl of an AAPH solution (133 mM in phosphate buffer) was added and the reaction started when the plate was inserted in the reader at 37°C. All fluorescence measurements are relative to the initial reading of the fluorescence signal and were repeated every min for 35 min at an emission wavelength of 535 nm, while excitation was at 485 nm. The net area under the curve (AUC) was calculated by subtracting the AUC of the blank from the AUC of either the standard or the sample. The Trolox equivalent molar concentrations were calculated using a linear regression equation between Trolox concentration and net AUC. It was decided to calculate the ORAC *relative values* as Trolox micromoles present in 1 g of dried extract to compare the antioxidant activity of the extracts.

Results and discussion

The first step of this work was the extraction of carotenoids and polyphenols exploiting different solvents and techniques. Solvents with differing polarities, such as Hex, Acet, Acet/Hex 1:1, EtOH and EtOH/H_2O 8:2 were used to obtain extracts with carotenoids and/or polyphenols and different antioxidant properties. Conventional extraction procedures were compared to MAE and UAE.

Among the less polar solvents used, Acet and Acet/Hex 1:1 mixture afforded lower extraction yields than Hex in maceration at rt (3.20, 2.44 and 5.85%, respectively). Higher yields were generally observed for extractions with polar solvents (EtOH and EtOH/H_2O 8:2 mixture). Between the different extraction techniques used, UAE (30 min, 1 min

on/off) gave lower yields than the conventional method (24 h). However, these data are still interesting when compared at the same maceration time (see Table 1). In fact, extraction with Hex under magnetic stirring for a time of 30 min gave a 0.024% yield (instead of 5.85% after 24 h), which is much lower than the UAE value at the same time (1.35%), as confirmed in literature [28, 29]. UAE with Acet (1 h) evidenced a slight yield increase compared to maceration (2.35% instead of 1.85%), while with EtOH extraction yield raised from 5.65 up to 9.85%.

MAE (30 min, at 90, 120 and 150°C) provided significant yield improvements in correlation with temperature increases. When applied to less polar solvents, MW gave very interesting results; in fact treatments at 90°C showed higher yields than maceration at rt for 24 h. In general, with Acet and Acet/Hex 1:1 at 90°C better results were obtained with lower extraction times (10 min instead of 30 min), as described also by Pasquet *et al.* [30], while with EtOH this did not happen [31].

According to the composition of *Arthrospira* reported in literature, the apolar extracts should be rich in carotenoids [8]. Moreover it is well known that carotenoids and chlorophylls are thermolabile; several articles described their losses under both conventional and MW heating [32, 33], occurring at a higher temperature for the latter [34]. Therefore, qualitative and quantitative analyses of the carotenoids present in the *A. maxima* extracts were performed. Total carotenoids were determined using a UV method. Since *Arthrospira* extracts are rich in chlorophyll a and other pigments [12] that interfere with the absorbance readings of carotenoids, the saponification and extraction of all the samples tested were performed before spectrophotometric analyses. Results, expressed both as mg of carotenoids per g of extract and mg of carotenoids per g of algae, are reported in Table 1.

HPLC analyses were performed in order to better characterize the carotenoids in *A. maxima* extracts [35]. β-Carotene and lycopene were identified using pure analytical

standards, while the hypothesis of identification for the other compounds was based on a comparison of UV spectra obtained with data reported in the literature (see Supplementary Material) [36]. β-Carotene, the main carotenoid present in all the extracts, was quantified using a standard calibration curve. Results are reported in Table 1, both as mg of β-carotene per g of extract and mg of β-carotene per g of algae.

The Acet/Hex extract (Fig. 1) is the richest in β-carotene (32.6 mg/g extract) among the traditional extracts (maceration, 24 h), while the Acet extract is the richest in total carotenoids (69.1 mg/g extract) (see Table 1). The β-carotene percentage over total carotenoids corresponds to 77.8%, 70.4% and 42.8% for Acet/Hex, Hex and Acet extracts, respectively. Hex gave greater β-carotene selectivity, while Acet extracted even more polar carotenoids, such as xanthophylls [37] (see chromatograms in Fig. 1). Similar selectivity to traditional Acet extract in terms of total carotenoids (around 70 mg/g extract) was obtained with Acet in UAE (1 h) and Acet/Hex 1:1 under conventional and MW heating (90°C, 10-30 min). Acet/Hex 1:1 (10 min at 90°C) and Acet (MAE, 30 min at 90°C) afforded extracts with comparable selectivity to traditional Acet/Hex 1:1 in terms of β-carotene (around 30 mg/g extract).

Extractions with Acet (maceration for 24 h and MAE, 10 min at 90°C) and Acet/Hex 1:1 (MAE, 10 min at 90°C) afforded the best yields in terms of total carotenoids and β-carotene (>2.2 and around 1 mg/g algae, respectively). These results were similar to those obtained from *A. maxima* by Canela *et al.* under SFE conditions [38]. In our experimental conditions, UAE afforded lower contents of total carotenoids and β-carotene than the traditional method. UAE of β-carotene from *A. platensis* gave better results than in our case, but the electrical acoustic intensity used was higher [39].

The best extractions with less polar solvents were those carried out with Acet or Acet/Hex 1:1 under MW irradiation at 90°C for 10 min (see Table 1), considering the highest yield obtained in term of total carotenoids and β-carotene (> 2.6 and > 1.0 mg/g algae,

respectively) and an even high selectivity for these compounds (> 65 and > 23 mg/g extract, respectively).

EtOH also extracted carotenoids fairly well (19.3 mg/g extract) in the conventional maceration at 50°C (Table 1, Fig. 2). However, selectivity for β-carotene over total carotenoids is around 30%, demonstrating that EtOH extracted more xanthophylls than other solvents previously used. Conventional or MW heating at 90°C gave selectivity in term of total carotenoids near 15 mg/g extract in lower extraction times. β-Carotene content was around 6 mg/g extract in the conventional method at 50°C and in MAE at 90°C, while it decreased to 4.5 mg/g extract when temperatures of 120°C or 150°C were reached. Considering UAE, the time increase from 30 min (1 on/1 off cycle) to 60 min (continuous) improved significantly extraction of carotenoids (from 7.84 to 12.4 mg/g extract), but results were still worse than those obtained with the conventional method (24 h, 50°C).

Also with EtOH, MAE provided a real advantage as the carotenoid concentration was almost as good as the traditional method (15.0 *vs* 19.3 mg/g extract) and total carotenoids content in mg/g algae was slightly greater (3.12 *vs* 3.05 mg/g algae), even when compared to the richest apolar extracts (2.81 mg/g algae). MAE at 90°C for 30 min (closed environment, under N_2) promoted the solubilisation and extraction of β-carotene in EtOH more than conventional heating in the same conditions, affording the highest amounts of β-carotene (1.37 *vs* 1.00 mg/g algae) (Table 1). At temperatures of 120 to 150°C, total carotenoid content (mg/g algae) decreased slightly, probably due to partial degradation, despite extraction yields increasing by 5 and 10%.

The addition of H_2O to EtOH caused extraction selectivity for carotenoids to drop further. A comparison of the EtOH/H_2O extract chromatograms, obtained by MAE at different temperatures, showed that the extract at 90°C and 120°C were very rich in carotenoids (3.20 and 3.06 mg/g algae, respectively). When the temperature was increased to 150°C,

the peaks of some compounds disappeared, because the oxidative degradation of carotenoids increased (see Supplementary Material). The data obtained indicate that MW heating in the presence of polar solvents in a closed environment (in the dark) and under N_2 pressure preserved carotenoids from degradation or *cis-trans* isomerization up to 120°C. When the temperature was raised to 150°C, the solvent used was crucial. Indeed, degradation was higher in the presence of H_2O.

A full evaluation of the antioxidant activity of microalgae intracellular metabolites that belong to a variety of chemical families [40] would require a wider range of methods with which to highlight the different mechanisms [41].

The extracts were first evaluated using the improved $ABTS^{\cdot+}$ radical decolourisation assay (TEAC Test), whose use is recommended for plant extracts because the long wavelength absorption maximum at 734 nm eliminates most of the colour interference [42, 43]. Table 2 shows the results expressed as μmol Trolox/g of extract. Fig. 3 reports the time-dependent decrease of $ABTS^{\cdot+}$ absorbance at 734 nm in the presence of different concentrations of EtOH extract that was obtained with MAE at 150°C. The equilibria between $ABTS^{\cdot+}$ and scavengers were reached within 30 min for all fractions.

More significant results in terms of antioxidant capacity were provided by the ORAC test, which is based on the inhibition of the peroxyl radical-induced oxidation of fluorescein, itself initiated by the thermal decomposition of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). This is the only method that combines the evaluation of the inhibition degree and inhibition time in a single quantity (the ORAC value). The ORAC value has been widely used for the standardization of the antioxidant activity of herbal extracts and foods. However, the ORAC value for *A. maxima* has not yet been reported in the literature. The ORAC values obtained for the extracts are reported in Table 2 and are expressed as μmol Trolox/g of extract, like the TEAC test. The fluorescence decay curves

induced by AAPH in the presence of different concentrations of the EtOH extract obtained using UAE (30 min) are reported in Fig. 4.

Different results were obtained for the same extract when the ORAC and TEAC methods were compared. This reflects the differences in the antioxidants' ability to quench the peroxyl radical and reduce $ABTS^{\cdot+}$ *in vitro*. The highest antioxidant activity was found in the TEAC test data. This is probably due to the fact that the compounds present in *A. maxima* may act as stronger electron donors to the ABTS radical cation than as scavengers of peroxyl radicals.

There were no significant differences in antioxidant activity between traditional extracts and UAE 30 min extracts, either they were polar or apolar fractions (Table 2). The prolongation of the treatment time to 1 h increased only TEAC values for the Acet extract. Despite its lower extraction yields, it has to be highlighted that UAE provided extracts with an antioxidant quality that is comparable to fractions obtained with traditional methods, but in a shorter time. This was further supported by the fact that traditional extract (Hex, 30 min, rt) demonstrated very low antioxidant activity (TEAC: not detectable, ORAC: 90 µmol Trolox/g extract).

TEAC tests on the apolar fractions showed that Acet was the best extraction solvent for antioxidants: both UAE (1 h) and MAE gave extracts with a greater antioxidant activity compared to traditional methods (629÷859 *vs* 519÷595 µmol Trolox/g extract, respectively). Indeed the extract obtained with only 10 min of MW irradiation showed a high value of TEAC units. In the ORAC test, no great differences between less polar solvents were observed considering maceration (24 h, rt). Between non conventional techniques, Hex in UAE (30 min) seemed to give better results than Acet (460 and 276 µmol Trolox/g extract, respectively).

Polar extracts were more active than apolar fractions and showed greater antioxidant activity especially in the ORAC test, when EtOH was used alone as the extraction solvent.

EtOH/H_2O 8:2 resulted in less active extracts, even if the extraction yields were greater. This result suggests that EtOH/H_2O 8:2 mixture extracted families of compounds which were not solubilised by less polar solvents and EtOH alone. The amino acids and sugars as well as the derivatives and salts of the alga cultivation itself, which remained on the surface, were possibly extracted as *A. maxima* was not subjected to any washing before extraction. Both the TEAC and ORAC assays showed a significant increase in the antioxidant power of the extracts obtained under MW and US irradiation when the different extraction techniques were compared in the polar fraction. Indeed EtOH extracts, obtained under magnetic stirring for 24 h and US irradiation for only 30 min, gave similar values, 735 and 625 µmol Trolox/g extract (TEAC) respectively, and 442 and 417 µmol Trolox/g extract (ORAC) respectively. Prolongation of UAE to 1 h did not increase the extract antioxidant activity. For MAE, antioxidant power ranged from 747 to 812 µmol Trolox/g extract (TEAC) and from 519 to 605 µmol Trolox/g extract (ORAC) (Table 2). The temperature increase from 90° to 150°C did not highly influence the antioxidant activity of the extracts, while treatment time increase from 10 to 30 min was useful to obtain a greater antioxidant power.

Since polar extracts showed greater antioxidant activity than apolar fractions and according to the composition of *Arthrospira* reported in literature [44-46], the extracts were analyzed to obtain quantitative determination and identification of present polyphenols.

The UV quantification of total phenols using the Folin Ciocalteu method was tried without success because of spectrophotometric interference combined with the excessively low phenolic concentration in the extracts.

The HPLC analyses on *A. maxima* polar extracts were first performed according to the literature [44]. The same operating conditions, such as an RP 18 column (250 mm, 4.6 mm, 5 µm) and mobile phases (ammonium acetate 0.1 N and methanol), were used, while the

same standards were analyzed by monitoring UV signals at 280 and 365 nm. Results were very poor, since neither retention nor separation were observed.

A second method was therefore used, in acidic conditions (2% acetic acid and acetonitrile), which was based on the polyphenol analysis of *A. platensis* [45, 46]. A column with a wide range of analysable compounds (Synergi Hydro RP C18) was chosen in combination with several standards of benzoic, cinnamic acid derivatives and other polyphenols, according to literature data (see Experimental Section). We could not find any correspondence with available standards (see Supplementary Material).

Phycocyanine is another pigment present in *Arthrospira* and probably involved in its antioxidant activity [10, 47]. Phycocyanine has not been extracted in any of our experimental conditions (see Supplementary Material).

Conclusion

The present work has confirmed the antioxidant properties of *A. maxima*. These data prove that this microalgae is an important source of carotenoids and nutrients, no need to mention its high protein quality for diet supplements. ORAC assay, a reference test in food analysis was used as first time for the characterization of *Arthrospira* extracts antioxidant activity.

Acet/Hex and Acet extracts were in general the richest in β-carotene and total carotenoids (20÷33 and 40÷70 mg/g extract, respectively). The best results were obtained under MAE at 90°C for 10 min with the highest yields in term of total carotenoids and β-carotene (> 2.6 and > 1.0 mg/g algae, respectively) and a still high selectivity for these compounds (> 65 and > 23 mg/g extract, respectively).

MAE and UAE have proven to be particularly efficient: not only they provided some extracts with the best antioxidant activity, but they also reduced extraction time, maximizing extraction yields. MAE with EtOH (90°C, 30 min) afforded the highest extraction yield in term of total carotenoids and β-carotene (3.12 and 1.37 mg/g algae,

respectively) with a good selectivity for these compounds (15.0 and 6.62 mg/g extract, respectively). The antioxidant activity of this extract (812 and 519 μmol Trolox/g extract for TEAC and ORAC, respectively) was one the best observed.

Finally, the EtOH extracts, characterized by a good antioxidant activity, are free from the residues of harmful solvents. This could make them interesting components of nutraceutical preparations that may be useful in combating and preventing the damage caused by oxidative stress.

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Figure Captions

Fig. 1 Comparison of HPLC chromatograms of Acet/Hex 1:1, Hex and Acet extracts in traditional conditions

Fig. 2 HPLC chromatogram of EtOH extract in traditional conditions

Fig. 3 $ABTS^{\cdot+}$ radical decolourisation assay in the presence of different concentrations of EtOH extract obtained using MAE at 150°C

Fig. 4 Fluorescence decay curves induced by AAPH in the presence of different concentrations of the EtOH extract obtained with UAE 30 min

Table 1. Total carotenoids and β-carotene content in *Arthrospira maxima* extracts.

Solvent	Method	Time (min)	Temp. (°C)	Yield (%)	Total carotenoids[a]		β-carotene[b]	
					mg/g extract ± SEM	mg/g algae	mg/g extract ± SEM	mg/g algae
Acet	Stirring	24 h	rt	3.20	69.1 ± 6.8	2.21	29.6 ± 1.0	0.946
		30	90	1.81	54.6 ± 2.7	0.988	24.9 ± 0.9	0.451
		10	90	2.79	39.5 ± 2.2	1.10	20.2 ± 0.4	0.563
	UAE	30[c]	rt	1.85	20.8 ± 1.5	0.385	9.61 ± 0.55	0.178
		60	rt	2.35	72.4 ± 7.2	1.70	26.3 ± 0.6	0.616
	MAE	30	90	2.06	63.7 ± 1.3	1.31	33.2 ± 0.9	0.684
		10	90	3.98	65.9 ± 3.3	2.62	25.5 ± 0.8	1.02
Hex	Stirring	24 h	rt	5.85	26.4 ± 1.1	1.55	18.6 ± 0.8	1.09
		30	rt	0.024	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]
	UAE	30[c]	rt	1.35	6.49 ± 0.59	0.0876	4.67 ± 0.38	0.063
Acet/ Hex	Stirring	24 h	rt	2.44	41.9 ± 1.3	1.02	32.6 ± 0.9	0.795
		30	90	2.90	68.8 ± 1.1	2.00	23.9 ± 0.5	0.694
		10	90	1.80	71.6 ± 2.1	1.29	29.7 ± 0.7	0.535
	UAE	30[c]	rt	2.19	21.0 ± 1.0	0.461	6.67 ± 0.67	0.146
	MAE	30	90	2.19	73.4 ± 4.5	1.61	28.5 ± 0.5	0.624
		10	90	4.30	65.4 ± 2.3	2.81	23.9 ± 0.6	1.03
EtOH	Stirring	24 h	50	9.39	19.3 ± 2.0	1.82	5.96 ± 0.36	0.560
		30	50	1.78	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]
		30	90	20.4	15.0 ± 1.0	3.05	4.93 ± 0.19	1.00
	UAE	30[c]	50	5.65	7.84 ± 0.43	0.443	2.44 ± 0.14	0.138
		60	50	9.85	12.4 ± 0.6	1.22	4.27 ± 0.24	0.421
	MAE	30	90	20.8	15.0 ± 0.8	3.12	6.62 ± 0.33	1.37
		10	90	17.0	14.7 ± 0.8	2.50	5.59± 0.27	0.951
		30	120	23.3	11.5 ± 1.8	2.68	4.56 ± 0.22	1.06
		30	150	26.0	9.72 ± 0.98	2.53	4.48 ± 0.18	1.17
EtOH/ H_2O	Stirring	24 h	50	29.7	4.17 ± 0.20	1.22	0.674 ± 0.041	0.200
	UAE	30[c]	50	36.9	3.88 ± 0.44	1.43	0.543 ± 0.032	0.201
	MAE	30	90	31.0	10.3 ± 0.8	3.20	4.28 ± 0.14	1.33
		10	90	31.1	8.35 ± 0.78	2.59	3.21 ± 0.10	0.997
		30	120	35.5	8.61 ± 0.92	3.06	3.05 ± 0.10	1.08
		30	150	42.4	2.64 ± 0.24	1.12	0.321 ± 0.023	0.136

[a] Determined by UV measurements. [b] Determined by HPLC analyses. [c] Real treatment time of 15 min (total time 30 min, 1 on/1 off cycle). [d] Not detectable.

Table 2. Antioxidant activity of *Arthrospira maxima* extracts.

Solvent	Method	Time (min)	Temp. (°C)	TEAC (µmol Trolox/g extract ± SEM)	ORAC (µmol Trolox/g extract ± SEM)
Acet	Stirring	24 h	rt	519 ± 14	353 ± 17
		30	90	523 ± 44	166 ± 13
		10	90	595 ± 47	138 ± 11
	UAE	30[a]	rt	629 ± 16	276 ± 8
		60	rt	859 ± 53	202 ± 22
	MAE	30	90	687 ± 47	254 ± 9
		10	90	711 ± 57	183 ± 12
Hex	Stirring	24 h	rt	594 ± 12	387 ± 27
		30	rt	n.d.[b]	90 ± 7
	UAE	30[a]	rt	556 ± 17	460 ± 28
Acet/ Hex	Stirring	24 h	rt	681 ± 21	356 ± 12
		30	90	478 ± 36	221 ± 24
		10	90	573 ± 40	141 ± 61
	UAE	30[a]	rt	567 ± 12	372 ± 15
	MAE	30	90	528 ± 27	238 ± 13
		10	90	456 ± 47	164 ± 16
EtOH	Stirring	24 h	50	735 ± 24	442 ± 40
		30	50	249 ± 16	291 ± 10
		30	90	529 ± 22	444 ± 19
	UAE	30[a]	50	625 ± 7	417 ± 32
		60	50	413 ± 11	287 ± 11
	MAE	30	90	812 ± 32	519 ± 53
		10	90	445 ± 13	260 ± 30
		30	120	775 ± 30	532 ± 40
		30	150	747 ± 19	605 ± 41
EtOH/ H_2O	Stirring	24 h	50	453 ± 9	396 ± 27
	UAE	30[a]	50	574 ± 16	387 ± 6
	MAE	30	90	748 ± 17	495 ± 19
		10	90	359 ± 7	370 ± 22
		30	120	716 ± 16	533 ± 16
		30	150	693 ± 10	488 ± 18

[a] Real treatment time of 15 min (total time 30 min, 1 on/1 off cycle). [b] Not detectable