Improved extraction of bioactive compounds from biomass of the marine

dinoflagellate microalga *Amphidinium carterae*

M. López-Rodríguez[1], M.C. Cerón-García[1], L. López-Rosales[1], E. Navarro-López[1], A.

Sánchez Mirón[1], A. Molina-Miras[1], A. C. Abreu[2], Ignacio Fernández [2], F. García-

Camacho[1]

[1] Department of Chemical Engineering, Research Centre CIAIMBITAL, University of

Almería, 04120 Almería, Spain

[2] Department of Chemistry and Physics, Research Centre CIAIMBITAL, University of

Almería, Ctra. Sacramento, s/n, 04120 Almería, Spain.

*Corresponding author: mcceron@ual.es

Address: Department of Chemical Engineering, University of Almería. Carretera

Sacramento s/n. E04120, Almería, Spain.

Telephone number: +34 950015981 Fax: +34 950 015484; e-mail address:

mcceron@ual.es

1

Abstract

The extraction of three families of compounds (carotenoids, fatty acids and amphidinols) from the biomass of two strains of *Amphidinium carterae* (ACRN03 and Dn241EHU) was improved by tuning cell disruption and solvent extraction operations. The extraction of carotenoids was evaluated using alkaline saponification (0%-60% KOH d.w.) at different temperatures (25-80°C). High levels of carotenoids were obtained at 60°C using freeze-dried biomass, not subjected to cell disruption methods. The ACRN03 strain required 20% KOH whereas the Dn241EHU strain did not require saponification since carotenoid degradation was observed. The extraction efficiencies were determined with a wide range of pure solvents and mixtures thereof. Two empirical non-linear equations were used to correlate extraction percentages for each family of compounds with the Hildebrand solubility parameter (δ_T) and the polarity index of the solvents (*PI*). Thresholds of δ_T and *PI* of around 20 MPa$^{1/2}$ and 6, respectively, were determined for the extraction of amphidinols, consistent with antiproliferative activity measurements.

Keywords: Extraction process, polyunsaturated fatty acids; carotenoids, polarity index, solubility parameters, peridinin; amphidinols

1. Introduction

The biotechnological production of microalgae biomass has been developing rapidly over recent years due to the high value of the secondary metabolites (SMs) that they can produce, such as carotenoids, polyunsaturated fatty acids, complex polyketides, antioxidants, phenolics, phytosterols, isoprenoids, non-ribosomal peptides, oxylipins and alkaloids, etc. Species selection is considered a crucial matter for this biotechnology's success. In this respect, marine dinoflagellate microalgae stand out given that they produce a wide range of bioactive compounds that are of increasing commercial, biomedical and therapeutic interest (Gallardo-Rodríguez et al., 2012). *Amphidinium carterae* is notable because it synthetizes an important group of polyketide compounds, namely amphidinolides and amphidinols (indistinctly referred to APDs). APDs promote potent anticancer, antifungal and hemolytic activity; thus, they are potentially useful in rational drug design studies (Kobayashi and Kubota, 2010). Recent works have successfully proven the viability of recovering APDs from pilot-plant cultures of *A. carterae* using a scalable process (Molina-Miras et al., 2018b). However, *A carterae*, and marine dinoflagellates in general, also synthesize other high value compounds (e.g. polyunsaturated fatty acids (PUFAs) and carotenoids), which need to be recovered within a specifically-devised bioprocess (Molina-Miras et al., 2018a). As far as is known, only one biorefining approach has been reported recently (López-Rodríguez et al., 2019) for obtaining APDs from dinoflagellates (in particular, *A. carterae*) that includes the recovery of different high-value co-bioproducts.

Nonetheless, there is no single process to completely recover the compound families of interest (carotenoids, polyunsaturated fatty acids and APDs) contained in the *A. carterae* biomass (López-Rodríguez et al., 2019). Carotenoids present very restrictive

3

properties that largely determine any sustainable dinoflagellate-based bioprocess

constructed to produce SMs using a biorefining approach. For example, carotenoids are

more thermolabile, photosensitive and acid-sensitive than the rest of the compounds,

thus limiting their potential exposure to excess heat, light and acids, respectively (Saini

and Keum, 2018). The efficiency of cell disruption methods to facilitate the release of

intracellular carotenoids (and other metabolites) is species-dependent (Cerón-García et

al., 2018). As a result, minimizing carotenoid degradation during analysis and extraction

procedures should be balanced with maximizing the recovery of carotenoids,

polyunsaturated fatty acids and APDs in *A. carterae*. This compromise can be regulated

by the appropriate choice of a single solvent, or solvent mixture, as reported for other

microalgae (Cerón-García et al., 2018). Nevertheless, the diversity and profile of

carotenoids with varied polarity indexes in the biomass complicates their simultaneous

solvent extraction (Saini and Keum, 2018). Despite the numerous studies on carotenoid

extraction using solvents reported in the literature, the generalization of their

recommendations is very limited. Many of them are based on the simple rule "like

dissolves like" applied only in terms of polarity, which has been found to be insufficient

in complex samples. Lately, solvent selection following the Hildebrand solubility

parameter approach has been reported to improve the efficiency of bioactives extraction

from plants (Saha et al., 2015).

The aim of the present study was to characterize the biomass of two *A. carterae*

strains and improve the extraction of the three families of bioactive compounds

(mentioned above) that they contain. A first step aimed to optimize the cell disruption

and biomass alkaline saponification processes; this has not been attempted before for

this strain. The second step was carried out to improve the recovery yields of the three

4

families of compounds using a wide range of single solvents, and mixtures thereof, with different polarity indexes and Hildebrand solubility parameters.

2. Materials and methods

2.1. Microalgae biomass

Biomass from two strains of the marine dinoflagellate microalga *Amphidinium carterae* was used. The Dn241EHU strain was obtained from the Microalgae Culture Collection of the Plant Biology and Ecology Department at the University of the Basque Country (Seoane et al., 2018). The ACRN03 strain was obtained from the Culture Collection of Harmful Microalgae at the IEO (Vigo, Spain). The *A. carterae* Dn241EHU biomass comes from a long-term pilot-scale semicontinuous culture (Molina-Miras et al., 2018a). The *A. carterae* ACRNO3 biomass was obtained from a pilot-scale fed-batch culture (Molina-Miras et al., 2018b). Culture suspensions of the two strains were centrifuged at 700 g in 50 mL falcon tubes. The pellets were softly washed with distilled water. The supernatants were carefully removed, and the tubes containing pellets with cells were immediately frozen. Afterwards, the tubes were freeze-dried. The lyophilized biomass was stored at −22°C to be processed for different purposes.

2.2. Determination of the proximate chemical composition of the biomass

The protein and ash content in the biomass was determined by a method described elsewhere (López et al., 2010). The total lipid content (TLs) in the biomass was ascertained by the method described by (Kochert, 1978). The carbohydrate content

was determined by the difference between 100 and the total of the other fractions, such as proteins, total lipids and ash (Camacho-Rodríguez et al., 2014).

2.3. Determination of the saponifiable lipid fractions and the fatty acid profile in the biomass

The fatty acid (FA) profile and content in the dry biomass were determined by gas chromatography following direct transesterification to obtain the fatty acid methyl esters (FAMEs), as described previously (Rodríguez-Ruiz et al., 1998). The FAMEs were used to calculate the amount of saponifiable (SLs) and transesterifiable (TLs) lipids in the biomass. The percentage of unsaponifiable lipids in the biomass was determined using the difference between the TLs (d.w. %) and SLs (d.w. %).

The SLs were fractionated in neutral lipids (NSLs) and polar lipids, specifically glycolipids (GLs) and phospholipids (PLs), as described elsewhere (Callejón et al., 2014). Briefly, the dry TL extract obtained (as detailed above; Section 2.2) was resuspended in 0.5 mL of chloroform and then fractionated using a single-use silica gel cartridge. Sequential elution with chloroform (30 mL), acetone (30 mL) with chloroform: methanol 85:15 v/v (20m mL), and methanol (30 mL), allowed the NL, GL and PL fractions, respectively, to be collected separately. All the eluents were recuperated in a rotary evaporator and the fractions were converted into FAMEs, as described above (Section 2.2).

2.4 Cell disruption methods

Freeze-drying (FD) is the preferred process for drying microalgae in the value-added biocompounds industry (Lee et al., 2017). It is also considered to be a heat-based

6

disruption method because the cells are damaged during the freeze-thaw cycle (Lee et al., 2017), making subsequent recovery of the intracellular products easier. In this context, all the methods tested in this section were performed with freeze-dried biomass (5 mg samples), in such a way that their effects on cell breakage were also associated with freeze-drying. The control method (CTRL) was carried out using only the freeze-dried biomass. Hence, a total of four cell disruption methods were assayed: (i) ultrasonication (UT) (Selecta, model 3000683, 110 W, frequency 50/60 Hz); (ii) bead milling (BM); (iii) mortar-and-pestle without alumina (MP); and (iv) mortar-and-pestle with alumina present as an abrasive (MPA) at a 1:1 w/w biomass/alumina ratio. The BM-based cell disruption method used a 2.5 L bead mill rotating at a speed of 120 rpm, with 27 mm-diameter ceramic beads. In the MP-assisted procedure, a laboratory mortar-and-pestle with a 250 mL volume was used to ground the lyophilized biomass sample. MP provides good compound recovery but cannot be scaled up for industrial use (Rajesh et al., 2017); consequently, it was tested for comparison purposes only. The alumina in the MPA method was obtained from Sigma-Aldrich (Type WN-3, 013K3422Inc St. Louis, MO, USA). The experiments were operated at room temperature (22-25 °C) for 2 min. The samples from the treatments were saponified and their carotenoid content and profile were determined, as explained elsewhere (Cerón-García et al., 2018). Briefly, the biomass saponification was performed in a tricomponent solution in Pyrex glass tubes at 25ºC with a KOH weight to biomass weight ratio of 0.4 (i.e. 40% d.w.). The carotenoids were analyzed by HPLC using a photodiode array detector. The efficiency of each method was discussed in terms of carotenoid recovery compared to the control. The measurements for each disruption method were carried out in duplicate.

7

2.5. Biomass alkaline saponification

The alkaline treatment used for biomass saponification, as described previously for other microalgae (Cerón-García et al., 2018), was optimized for *A. carterae* by varying the temperature (*T*) and KOH concentration (C_{KOH}). Briefly, 5 mg samples of freeze-dried biomass were placed in Pyrex glass tubes. Then, 1 ml of monophasic tricomponent solution was added to each tube and shaken in a vortex for 20 s over a 2-min period. The tricomponent solution was composed of ethanol, hexane and water in a ratio of 76:18:6 v/v/v while the specific percentage of KOH was calculated on the basis of the sample's dry biomass weight. The temperature of the mixture was controlled by submerging the tubes in a shaking water bath (Julabo® SW22, Julabo USA Inc.). The shaking frequency was set at 3 cycles per minute. After the reaction was completed (2 min), the tubes were left to cool at room temperature. Next, the samples were centrifuged at 12000 rpm for 3 minutes (Eppendorf® MiniSpin Plus® microcentrifuge, Hamburg, Germany). The resulting supernatants were transferred into HPLC vials to determine the carotenoid content and profile as a means of assessing the efficiency of each *T*-C_{KOH} combination. The temperature and KOH concentration were varied from 25ºC to 80°C and from 0% to 60%, respectively. All the experiments were conducted in duplicate and each sample was analyzed in duplicate.

2.6. Solvent extraction of carotenoids

As microalgae biomass contains diverse carotenoids with varying polarity levels, selecting the appropriate solvents is critical for optimizing their extraction. Table 1 displays the range of solvents and mixtures tested as extractants of the carotenoids, fatty

8

acids and amphidinols from *A. carterae* biomass. They cover a wide range of polarity indexes (*PI*). The *PI* values for the solvent mixtures (PI_{mix}) were calculated as follows:

$$PI_{mix} = \sum_{i=1...p} X_i \cdot PI_i \tag{1}$$

where the subscript *i* represents the pure solvent *i*, and *X* is the volumetric fraction of the pure solvent in the mixture (Poole and Poole, 1991). The solubility parameter, originally introduced by Hildebrand, was divided into three partial components by Hansen: a dispersion force component (δ_d), a hydrogen-bonding component (δ_h) and a polar component (δ_p). The total solubility parameter (δ_T) for the pure solvent is given by the equation:

$$\delta_T = \sqrt{\delta_d{}^2 + \delta_p{}^2 + \delta_h{}^2} \tag{2}$$

In the case of solvent mixtures, each partial solubility parameter is calculated as in eq. (1):

$$\delta_k = \sum_{i=1...p} X_i \cdot \delta_{k,i} \tag{3}$$

where *i* represents the solute in the mixture and *k* each partial solubility parameter (i.e., *d*, *p* and *h*). The temperature adjustment for the solubility parameter was carried out as proposed by Barton (1983):

$$\left(\frac{\delta_1}{\delta_2}\right)^2 = \frac{T_2}{T_1} \tag{4}$$

where T_1 is the reference temperature (25 ºC) and T_2 the extraction temperature (60 ºC). The procedure consisted of adding 1 ml of each solvent to 5 mg of dry biomass deposited in a Pyrex glass tube. Samples were subjected to a temperature of 60°C in a

shaking water bath for 2 minutes and energetically mixed every 20 seconds over the 2 minutes. Next, the samples were centrifuged at 12000 rpm for 3 minutes (Eppendorf® MiniSpin Plus® microcentrifuge, Hamburg, Germany). The carotenoids were determined in the resulting supernatants by HPLC, as reported previously (Cerón-García et al., 2018). The experiments were carried out in duplicate. Recovery yields of carotenoids (T-CRs Recovery, %), shown below in Table 4, were calculated with a reference value (1.1% d.w.) that was the maximum obtained using the modified version of the traditional analytical method reported earlier (Cerón et al., 2018), and described briefly in Section 2.5.

2.7. Solvent extraction of fatty acids

To cover the extraction of both polar and non-polar lipids, a selection of the pure solvent and solvent mixtures shown in Table 1 were selected to cover a wide range of *PI* values. The procedure consisted of adding 2 ml of each solvent to 10 mg of dry biomass placed in a Pyrex glass tube. Samples were subjected to a temperature of 60°C in a shaking water bath for 2 minutes and energetically mixed every 20 seconds over the 2 minutes. Next, the samples were centrifuged at 3500 rpm (Heraeus Labofuge 200 Thermo Fisher Scientific, Osterode, Germany) for 4 minutes and the extracts separated for drying in a N_2 stream at 45°C. After this, the fatty acid profile in the extracts was determined by GC, as described in Section 2.2. To calculate the recovery yields of fatty acids (T-FAs Recovery, %), displayed in Table 4 below, the reference value used (18.2% d.w.) was that of the saponifiable fatty acids content in the biomass, measured as described briefly in Section 2.3 (as cited by Rodríguez-Ruiz et al., 1998).

2.8 Determination of amphidinols

The *A. carterae* Dn241EHU strain has recently been shown to produce amphidinols A and B (Abreu et al., 2019). The presence of the A and B amphidinols in the different extracts obtained was preliminarily confirmed by assaying the hemolytic and antitumoral activity (López-Rosales et al., 2015; Molina-Miras et al., 2018b). The antitumoral assays were performed as described elsewhere (Abreu et al., 2019).

Extracts with pure solvent and solvent mixtures obtained from 5 mg of *A. carterae* were processed for the acquisition of the NMR spectra and the quantification of the APDs as previously described (Abreu et al., 2019). Analyses were carried out on a Bruker Avance III HD 600 spectrometer. The relative peak integral at δ_H 5.07 ppm was used in all of the acquired ^{1}H NMR spectra for the quantification of the APDs, as amphidinol A (MW 1338 g/mol), the value of which was based on the TSP signal integral of known concentration. Regarding the recovery yields of amphidinols (T-APDs Recovery, %), shown below in Table 4, these were calculated using the reference value (0.37 d.w.) that was obtained with methanol:water (80:20), according to a previous study ((Rodríguez-Ruiz et al., 1998)).

2.9. Non-lineal fittings

Two non-lineal equations were proposed for fitting the cell content of three groups of compounds (fatty acids, carotenoids and amphidinols) with the polarity index (*PI*) and the total solubility parameter (δ_T) (see below in Figure 3). Equation (1), based on the four-parameter Weibull-type distribution, was used to fit the total carotenoid (T-CRs) and total fatty acid (T-FAs) contents:

$$\text{T-CRs, FAs} = a \cdot \left(\frac{c-1}{c}\right)^{\frac{1-c}{c}} \cdot \left[\frac{x-x_0}{b} + \left(\frac{c-1}{c}\right)^{\frac{1}{c}}\right]^{c-1} \cdot e^{-\left[\frac{x-x_0}{b} + \left(\frac{c-1}{c}\right)^{\frac{1}{c}}\right]^{c}} + \frac{c-1}{c} \quad (1)$$

and the following five-parameter sigmoidal equation was used to fit the total amphidinol content (T-APDs):

$$\text{T}APDs = y_0 + \frac{a}{\left[1 + e^{-\left(\frac{x-x_0}{b}\right)}\right]^{c}} \quad (2)$$

where x represents PI or δ_T. The parameters y_0, x_0, a, b and c were estimated using non-linear regression (Sigma Plot 6.0 software, Systat Software Inc., San Jose, US). Equations (1) and (2) were provided by the SigmaPlot equations library. These do not have direct physico-chemical and biological meaning; both equations are simply mathematical tools for describing the data and, thus, avoiding subjective errors.

2.10. Statistical analysis

All experiments were performed in duplicate. The experimental results were shown as the mean values of the two independent experiments and their standard deviation. Statistical data analyses were performed using the Statgraphics Centurion XVII (version 17.2.04) statistical software (2014, Statpoint Technologies, Inc., Warrenton, VA). The normality and homogeneity tests were performed using the Kolmogorov-Smirnov and Levene tests, respectively. Statgraphics was used for a significant difference analysis with a one-way and multi-way analysis of variance (ANOVA) test.

3. Results and discussion

3.1. Proximate chemical composition

12

Table 2 displays the proximate compositions of both *A. carterae* strains. The profiles were comparable considering the limited percentage differences between them (below 30 %). The discrepancies observed might be attributable not only to intra-specific differences, but also the type of photobioreactor and the operation mode used to produce the biomass of each strain (see details in Section 2.1). On this point, it is well-known that proximate composition is sensitive to the environmental conditions of the culture and the growth phase when the biomass is harvested. Dramatic changes in the proximate cellular components of non-dinoflagellate microalgae have been reported for the same strain when cultured under conditions of both nitrogen sufficiency and deficiency (Thomas et al., 1984). For example, N-deficient cells of *Tetraselmis suecica* and *Dunaliella primolecta* decreased their protein content by about 130%, the carbohydrate content markedly rose to about 300%, and the total lipid content decreased nearly 50%, compared to cells grown in N-sufficient medium (Thomas et al., 1984). Keeping in mind these wide variations reported within a species, the proximate compositions displayed in Table 2 are consistent with those reported for another strain of *A. carterae* (Parsons et al., 1961).

Regarding the total lipids, the saponifiable fraction was predominant in both strains, with relative values of 59.5 % and 73.2 % of the total lipids for ACRN03 and Dn241EHU, respectively. The total FA contents were similar in both strains, with 18.2±1.74% d.w. for DN241EHU and 18.40±2.47 %d.w. for ACRNO3. The FA profiles in Table 3 are in good agreement with those recently reported for both strains when cultured in photobioreactors (López-Rodríguez et al., 2019; Molina-Miras et al., 2018a).

3.2 Comparison of cell disruption methods

13

Figure 1 compares the effectiveness of the four cell breakage methods tested in terms of the amounts of recovered carotenoids and fatty acids. All the cell disruption methods, including the control, were able to disrupt *A. carterae* cells (with cytolysis visible under an optical microscope). However, the extraction capacity was dependent on both the compound family and the method. Regarding carotenoids, all methods extracted peridinin, peridininol diatoxanthin, diadinochrome, pyrrhoxanthin, dinoxanthin, diadinoxanthin and β-carotene. Figure 1A displays the content of the different types of carotenoids in the samples. The profiles were in line with those reported for *A. carterae* Dn241EHU, and consistent with the Type 1 pattern typical of dinoflagellate with peridinin as the major carotenoid (Jeffrey and Wright, 2006). The maximum carotenoid content extracted, $2.17 \pm 0.05\%$, corresponded to the CTRL. Any of the remaining treatments led to lower carotenoid recovery ($p<0.05$); the MPA method, in particular, involved a dramatic decrease in the total carotenoid yield of about 33%, and 56% for peridinin, compared to the CTRL. The degradation of carotenoids seemed to be the most feasible cause because UT, BM, MP and MPA are mechanical disruption procedures where the shear stresses and temperatures involved can affect the quality and structure of the carotenoids, according to a recent review study (Lee et al., 2017). With respect to the total FAs, the effect was radically different compared to carotenoids (Fig. 1B). Only the UT method extracted FAs in lower amounts than the control ($p<0.05$), but to a limited percentage ($< 16\%$). There were no statistically significant differences between BM, MP and the CTRL ($p<0.05$). Only the MPA method slightly improved FA recovery relative to the control ($<6\%$). The individual fatty acids did not appear to follow the same pattern as their sum (i.e. the total FAs). No specific reasons were found to justify the selective action of each method tested. It was

evident that the FD-based method (CTRL) provided the highest recovery values for

both carotenoids and FAs, making it unnecessary to reinforce this pretreatment step

with an additional cell disruption method. A similar conclusion was obtained in studies

on other microalgae (Kim et al., 2012). However, it is well-known that the selection of a

suitable cell disruption method is dependent on the cell-wall's characteristics and

composition, both of which are specific to the microalga species and the culture's

environmental conditions (Gong and Bassi, 2016; Lee et al., 2017). Consequently, the

FD-based method was used for the rest of study.

3.3 Effect of the saponification conditions on carotenoid recovery

Figure 2 shows the effect of saponification T and C_{KOH} (4x6 levels) on the total

carotenoids (CRs) recovered and the peridinin (Pr) contents in both of the *A. carterae*

strains. Figure 2 collates the results from a multi-factor ANOVA. Thus, for each T and

C_{KOH}, the point represented is the average value from all the different C_{KOH} (0, 5, 10, 20,

40 and 60%) and all the temperatures assayed (25, 40, 60 and 80 °C), respectively. Both

factors (T and C_{KOH}) and their interaction (T-C_{KOH}) had a statistically significant effect

on CRs and Pr at the 95.0% confidence level ($p < 0.05$). However, the contribution of

the T-C_{KOH} interaction was substantially lower than those of T and C_{KOH} individually.

Specifically, the combined T-C_{KOH} interaction had a contribution of 14.77%, with the

weight of T (61.39%) higher than C_{KOH} (23.83%). This pattern observed in the ANOVA

was irrespective of the strain, as reported in a recent study carried out on eight

microalgae species of different genera (Cerón-García et al., 2018).

A closer inspection of Figure 2 reveals intraspecific differences in the

saponification effect on CR recoveries, particularly for Pr, as it is the main carotenoid.

15

Compared to the non-saponified control (C_{KOH} =0), saponification of the Dn241EHU

biomass at any C_{KOH} underestimated the CRs on account of Pr degradation, the higher

the C_{KOH} the higher the losses from degradation (Fig. 2A). This is consistent with the

few previous studies reporting Pr as an alkali-labile carotenoid (Barańska and Kaczor,

2016). In contrast, when biomass of the ACRN03 strain was used, saponification at all

C_{KOH} significantly improved the recovery of Pr ($p<0.05$) relative to the control, reaching

a plateau above 20% C_{KOH} (Fig. 2B). The optimal temperature was the same for both

strains, a value of 60º C. A plausible interpretation might be found by considering the

biomass fat content. It is known from the literature that a general rule for reducing the

risk of carotenoid degradation or loss is to apply milder conditions to low-lipid products

in the saponification step, and to then increase the treatment severity as the lipid content

increases (Rodríguez-Bernaldo de Quirós and Costa, 2006). However, the saponifiable

lipid content was similar in both strains.

Therefore, the reason why peridinin degradation was protected during the

ACRN03 biomass saponification might be due to differences in the cell wall

composition of each strain, caused by the culture mode, whether pneumatic stress exists

or not (Gallardo Rodríguez et al., 2016), or even due to the presence of peridinin in ester

form. Peridinin can often undergo hydrolysis, loosing acetic acid to convert into

peridininol, and then in the presence of fatty acids convert into ester form (Sugawara et

al., 2009). As a result, the extraction of carotenoids from the microalgal biomass will

require an alkaline treatment to break up the ionizable lipids (acyl-glycerols) and to

completely destroy the cell wall in order to release those carotenoids that might appear

in esterified form. These esters would remain in the hydro-alcoholic phase; otherwise,

they would remain ionized in the aqueous phase after the alkaline treatment, together

16

with many other lipids such as fats and waxes, and other non-polar compounds (Cerón et al., 2008). As the Dn241EHU biomass presented the highest carotenoid content, it was selected for the rest of the experimental plan.

3.4. Solvent extraction of product contents

With the goal of improving the overall extraction yield of carotenoids, saponifiable lipids (fatty acids) and amphidinols from the *A. carterae* biomass, different solvent systems were used (Table 1). In general, solvent selection is not a straightforward variable since it depends on the type of microalgal species and the target metabolite (Chan et al., 2013; Cerón et al., 2018) that one would like to extract selectively. For instance, as far as is known, there is no unique process to simultaneously recover carotenoids, polyunsaturated fatty acids and APDs from the same type of biomass (López-Rodríguez et al., 2019).

In the total lipid profile determined in the Dn241EHU biomass, the polar constituents are dominant, with 45.1 % of neutral lipids (NLs), 47.3 % of glucolipids (GLs) and 7.6 % of phospholipids (PLs). It is known that in eukaryotic microalgae cells, NLs associate through van der Waals forces to establish lipid globules in the cytoplasm; as a result, polar solvents such as ethanol or acetone are expected to extract them more efficiently. In contrast, non-polar solvents, such as hexane, will extract free NLs from the cells. However, the possibility exists that some neutral lipids may form complexes with polar lipids, and that these associated architectures, which are already hydrogen bonded to the cell membrane proteins, would only be extracted by polar solvents such as acetone, water or ethanol (Ryckebosch et al., 2014a). Consequently, mixtures of polar and non-polar solvents are capable of increasing the extraction yield as they might

17

extract both the neutral and the polar lipids that form part of these complexes, as well as the neutral lipids that are free in the cytoplasm (Ryckebosch et al., 2014a; Balasubramanian et al., 2013).

Given that the families of cellular compounds (carotenoids, polyunsaturated fatty acids and APDs) present different functional groups with variable overall dipolar moments, the polarity parameters of the solvents need to be considered for their optimum extraction. These were calculated for each solvent using eq (1) and the solubility parameter adjusted with temperature using equation 4 (Table 1). In this study, mixtures of different polarity indexes were prepared using different quantities of pure solvents (hexane, acetone, ethanol and water). Figures 3A and B illustrate the extraction percentages obtained for the different extraction systems tested in this work against the polarity indexes and the solubility parameters adjusted with temperature, respectively, for each family of compounds analyzed. In the following subsections, the results from these three families of compounds are discussed separately.

3.4.1. *Carotenoids*

The optimal extraction of carotenoids was achieved using *PI* solvent values of 3-5.5 (Figure 3A) and δ_T values of 16.5-19.5 [MPa$^{1/2}$] (Figure 3B). The maximum content of total carotenoids (T-CRs), 1.7 % d.w, was obtained using acetone: water (99:1) (polarity index: 5.54). Regarding the effect of the solvent system on the recovery yields of T-CRs, Table 4 shows that the maximum value (entry 17) was 175.5±8.8% higher than the control. In particular, the highest peridinin percentage was obtained with the acetone: water (99:1) solvent system (1.56% d.w.±0.07). This peridinin value was higher than those reported by Molina-Miras et al. (2018b); and as López-Rodríguez et

18

al. (2019) previously described, this might be due to the fact that carotenoids such as peridinin are associated with proteins in the photosynthetic complex (Zigmantas et al., 2003) and can only be disrupted by polar organic solvents that are able to dissociate hydrogen bonding (Rychebosch et al., 2014a). Indeed, the acetone: hexane (70:30) mixture (entry 13, Table 4), with a polarity index of 4.36, was the second most effective solvent for carotenoids, with a recovery yield 171.7±8.6 % greater than the control, achieving a 1.67% d.w. of carotenoids (Figure 3A). This is in agreement with previous reports that always describe acetone and hexane as being frequently selected to extract polar and non-polar carotenoids, respectively (Saini et al., 2018). The hexane:acetone (70:30, entry 8, Table 4) and hexane:ethanol (70:30, entry 9, Table 4) solvent mixtures provided similar results of *ca*. 157% of T-CRs (1.54 % d.w.). Lin & Chen (2003) also found that an ethanol;hexane mixture (4:3 v/v) provided the highest carotenoids extraction yield from tomato juice.

A simple variance test (ANOVA) was used to evaluate the influence of the polarity index on carotenoids extraction. From this, target data were obtained which were fitted into a non-linear regression, adjusted to a 4-parameter Weibull-type distribution.

In Table 5, the different parameters are collected for the correlation given by eqs. (1) and (2) between the average concentration of T-CRs with both the polarity index and the solubility parameter (adjusted with temperature). By inspecting these data, it was possible to conclude that the solubility parameter theory is applicable to carotenoids extraction since it improves the recovery of the most abundant carotenoids, such as peridinin (a polar carotenoid), from *Amphidinium carterae.* Although the solvent selection depends on the target carotenoid to be recovered, based on their

19

polarity, the solvent mixtures reported here have demonstrated good performance when extracting both xanthophylls and carotenes.

3.4.2. *Fatty acids*

Regarding the extraction of fatty acids, as shown in Figure 3A, the maximum saponifiable lipid (T-FAs) contents, of *ca*. 17% d.w., were obtained using solvent systems with polarity indexes ranging between 3.4-5.4, corresponding to values between 17-19 [MPa$^{1/2}$] (Figure 3B). Most are mixtures of polar and non-polar solvents such as acetone: hexane or ethanol:hexane. As an example, a 17.28% d.w. content was obtained using acetone:water (99.5:0.5) (polarity index: 5.47), with a recovery of 95.0 ± 5.7 % (entry 16, Table 4) with respect to the initial saponifiable lipid content. Very similar recoveries were obtained with other extraction mixtures that have a low polarity index, such as hexane:acetone 50:50 (entry 11, Table 4) or acetone:hexane 80:20 (entry 14, Table 4) with values of 93.6±5.6 and 94.2±5.6 %, respectively. Mixtures with higher polarity indexes clearly recover lower amounts of FAs (Figure 3A). For instance, solvent mixtures based on ethanol:water 80:20 (entry 24, Table 4), acetone:water 80:20 (entry 25, Table 4) or methanol:water 80:20 (entry 26, Table 4) achieved recovery yields of 45.0±2.7, 42.2±2.5 and 35.7±2.1 %, respectively, with respect to the initial saponifiable lipids. This could be due to the incapacity of very polar solvents to extract free NLs in the cytoplasm, as mentioned above (Balasubramanian et al., 2013).

On the other hand, the use of non-polar solvents, such as hexane (PI=0), achieved a FAs content of 11.2 % d.w. in the biomass, and a T-FAs recovery of 61.5±3.7 % (entry 1, Table 4). Other authors, such as Navarro-López et al. (2016), described similar behaviour in the extraction of saponifiable lipids from the microalgae *Nannochloropsis gaditana* when using mixtures of polar and non-polar solvents for the

20

lipid extraction, achieving a maximum saponifiable lipids extraction yield of 85% using ethanol and hexane as solvents, and only a 36% yield if hexane was used for the lipid extraction. Fatty acids correlate well with the same Weibull-type distribution fitting, as shown in Table 5.

It is worth mentioning that among the variables analysed here, the performance of a particular solvent system strongly depends on the microalgal species. Thus, the yields obtained using hexane with *A. carterae* are higher than those obtained by Ryckebosck et al. (2014a) using the same solvent but with *Nannochloropsis gaditana*. Under these conditions, the authors reported that only 36% of the initial saponifiable lipids contained in the biomass were extracted. The differences between the extraction yields in the two species (*Nannochloropsis gaditana* vs *Amphidinium carterae)*, which have similar lipid profiles (i.e. 40% NLs and 60% polar lipids for the former and 45.1 % NLs and 54.9% polar lipids for the latter), might be explained by the difference in their cell permeability. The *Nannochloropsis* species possesses a thick rigid cell wall that contains a biopolymer named algaenan, making lipid extraction difficult (Ryckebosck et al., 2014b; Navarro-López et al., 2016), while *Amphidinium Carterae* belongs to a group named athecates, as described by Lindemann (1928), which are fragile and easily disrupted, thus allowing the fatty acids to be extracted without further complication.

3.4.3. Amphidinols

With regard to APD extraction, Figure 3A shows that the maximum extraction for T-APDs occurs at polarities between 6 and 7.5, corresponding to solubility parameter values from 22-31 [$MPa^{1/2}$], adjusted with temperature (Figure 3B). Interestingly, APDs increase in a sigmoidal way at polarity values over 6, with the lowest APD content obtained using solvents that have polarities below 6, such as

acetone or hexane. The maximum values, with no significant differences, corresponded to solvents with polarity indexes close to methanol, such as acetone:water, ethanol:water or methanol:water 80:20. From these, target data were obtained which were fitted into a non-linear regression, adjusted to a 5-parameter Sigmoidal-type distribution, as presented in Table 5. The correlation with the polarity index, and the solubility parameter adjusted with temperature, was higher than 0.99. This is the first time that a correlation of APDs with the solvents' polarity and solubility parameters has been presented. Correlations between extraction percentages of bioactive ingredients and solvent polarity (using model solvents with a broad polarity range) have been previously described for plants (Kim et al., 2007). These correlations were demonstrated to be dependent on the compound type (Kim et al., 2007); thus, antioxidant compounds showed a similar correlation as those results for carotenoids and fatty acids presented here, but different to those of APDs.

In general, it can be appreciated that the representations using the δ_T (Fig. 3A) of the solvent system presented less results spread than those using the polarity index (see Fig. 3A). Although bioproducts solubility is commonly found to be associated with the polarity index, it is also well-known that the solubility in binary solvents mixed in a certain ratio may be greater than that of both pure solvents (Jin et al., 2017). This phenomenon, called cosolvency, is better interpreted using the δ_T parameter (Jin et al., 2017). The existence of cosolvency was a feasible explanation in several of the solvent mixtures used here (see Table 4). The δ_T-based Fig. 3B more clearly determined the maximum and threshold values.

3.5. Antiproliferative activity of the *A. carterae* extracts

22

The antiproliferative properties of molecules in the amphidinol family have already been reported (Kobayashi and Kubote, 2010). To link the presence of APDs, observed in the extracts from the solvents shown in Fig. 3A, to a hypothetical antiproliferative effect, their antitumoral activity was measured against the four tumour cell lines. This was carried out on a selection of the extracts whose PI and δ_T values encompassed the abrupt change in APD recovery observed in Fig. 3A. Table 6 shows these results. The PI and δ_T thresholds, of around 6 and 20 MPa$^{1/2}$, respectively, can be appreciated, similar to those observed in Fig. 3A. When these thresholds are exceeded, the antitumoral bioactivity abruptly increased ($<-75\%$). Extracts with δ_T values below 20 MPa$^{1/2}$ (or 6 for PI) presented no bioactivity. This is consistent with the preliminary results previously reported for the same strain of $A.$ $carterae,$ where the existence of a linear correlation was reported between the APD concentration in methanolic:water (80:20) extracts, and its hemolytic activity in sheep blood erythrocytes (Abreu et al., 2019). These assays confirm the importance of using the APD metabolites produced by $Amphidinium$, a basis for their potential medium to large-scale supply in the biomedical industry.

4. Conclusions

Carotenoid extraction from *Amphidinium carterae* should be performed using a low KOH concentration at 60ºC. An effective solvent for extracting metabolites such as carotenoids, fatty acids and amphidinols from *A. Carterae* was identified by varying the solvent species and composition. By correlating the extraction efficiencies with the solvent polarity and solubility parameter, the optimal solvent conditions could be predicted - a solubility parameter above 17, 17 and 22 [MPa$^{1/2}$] with polarities above 4,

23

4 and 6.5, respectively. Hence, the same solvent can be used to extract the carotenoids

and fatty acids, but not the amphidinols. The biomass extracts exhibited potent

antitumoral activities.

Figure captions

Figure 1. Effect of different cell disruption methods on the extraction of (A) carotenoids

and (B) fatty acids from lyophilized *Amphidinium carterae* biomass (Dn241EHU).

CTRL: control; UTS: ultrasounds; BM: bead mill; MWA: mortar without alumina; MA:

mortar with alumina. Data points are averages, and vertical bars are standard deviations

(SD) for duplicate samples. Points without SD bars indicate that the SD was smaller

than the symbol.

Figure 2. Influence of temperature (°C) and KOH concentration (% d.w) on the total

carotenoid content of the different strains (*Amphidinium carterae* ACRNO3 (A) and

Amphidinium carterae Dn241EHU (B)).

Figure 3. Correlation between the extraction of the different compound families

(carotenoids, polyunsaturated fatty acids and APDs) from *Amphidinium carterae*

Dn241EHU (A) with the solvent polarity (B) and with the solubility parameter adjusted

with temperature (the content of carotenoids (T-CRs), fatty acids (FAs) and T-APDs).

24

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

25
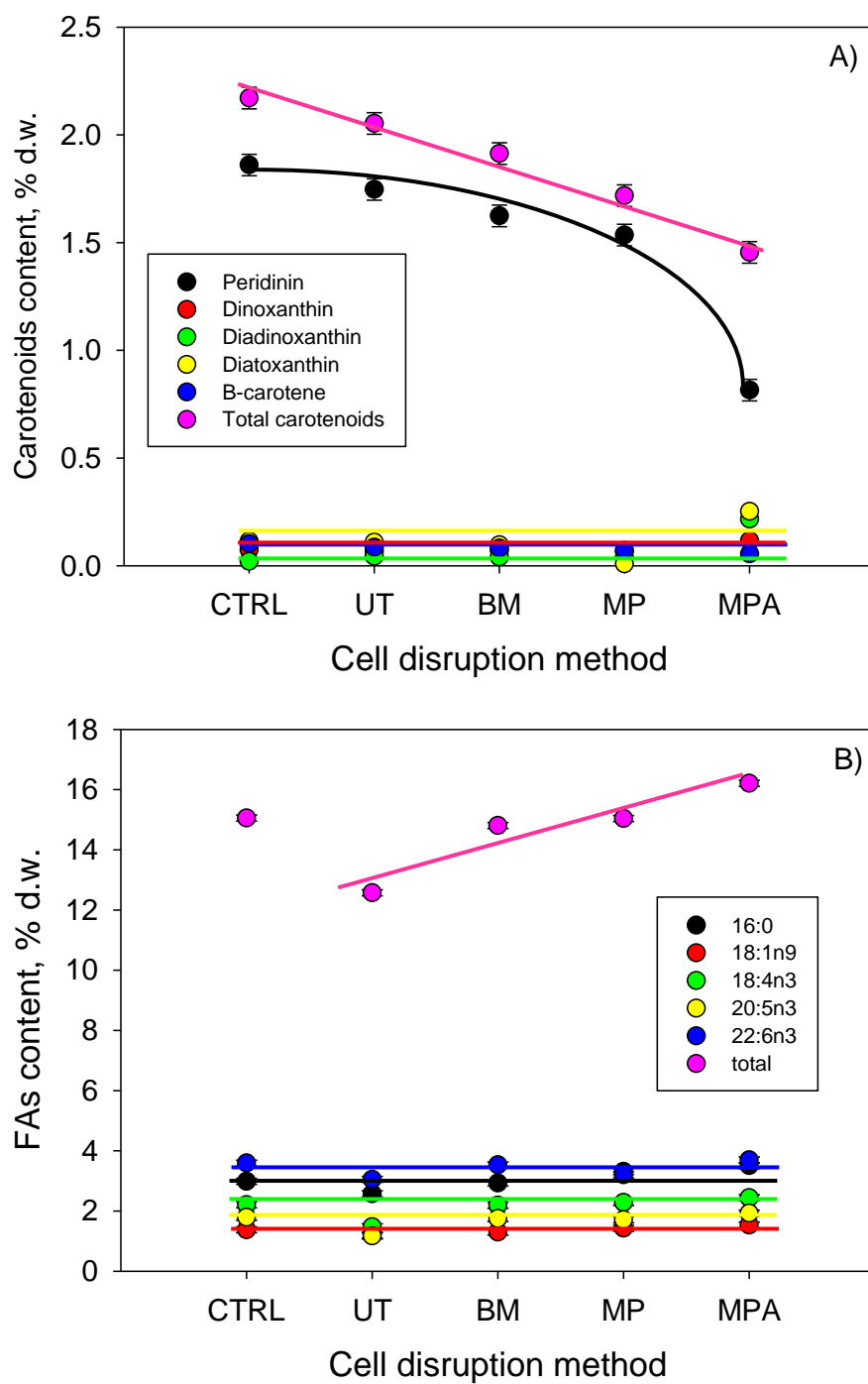
Figure 1

Figure 3

Table 2 revised
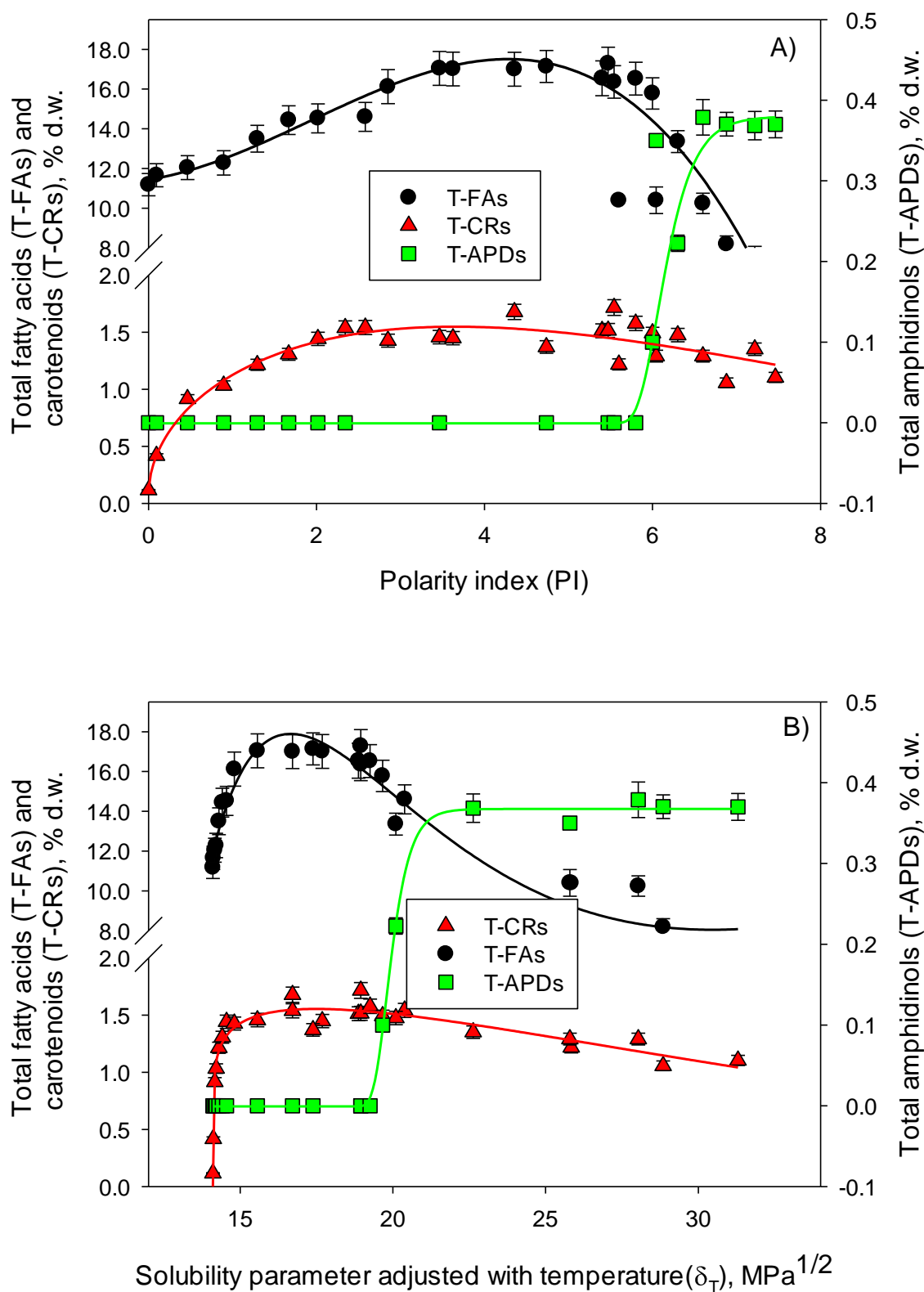
Table 2. Biomass composition (total lipids, saponifiable lipids, proteins, carbohydrates and ashes (% biomass d.w.) identified for the *A. carterae* ACRN03 and *A. carterae* Dn241EHU strains.

Biomass composition	%, Biomass d.w.	
	ACRN03	Dn241EHU
Total lipids	30.9±0.3	23.5±0.5
Saponificable lipids	18.4±2.5	18.2±0.5
Proteins	25.9±0.3	20.9±0.2
Carbohidrates	35.6±0.2	46.3±0.2
Ashes	7.3±0.2	7.8±0.4

Table 3 revised

Table 3. Profile of the percentages of the main fatty acids identified for the *A. carterae* ACRN03 and *A. carterae* Dn241EHU strains.

Fatty acid	ACRN03 (% d.w.)	Dn241EHU (% d.w.)
14:0	0.04±0.0	0.5±0.0
16:0	3.9±0.6	4.4±0.5
18:0	2.2±0.3	2.3±0.1
18:1n9	0.1±0.0	1.9±0.2
18:2n6	0.1±0.0	0.2±0.0
18:3n3	0.02±0.0	-
18:4n3	2.1±0.3	1.8±0.2
20:1n9	0.3±0.0	0.3±0.0
20:4n6	0.4±0.0	
20:5n3	2.7±0.1	2.4±0.1
22:6n3	4.7±0.4	4.4±0.5
Total fatty acids	**18.4±2.5**	**18.2±1.7**

New table4

Table 4. Effect of the extraction solvents on the recovery yields of total carotenoids (T-CRs), fatty acids (T-FAs) and amphidinols (T-APDs). PI is the polarity index; δ_T is the total solubility parameter

Entry	Solvents, v:v	PI	δ_T, MPa$^{1/2}$	T-CRs Recovery, %	T-FAs Recovery, %	T-APDs Recovery, %
1	Hexane HPLC, 100:0	0.00	14.11	11.9 ±0.6	61.4 ±3.6	0.0 ±0.0
2	Acetone:Hexane, 1:99	0.10	14.12	42.7 ±2.1	64.0 ±3.8	0.0 ±0.0
3	Acetone:Hexane, 5:95	0.46	14.17	93.7 ±4.7	66.2 ±3.9	0.0 ±0.0
4	Acetone:Hexane, 10:90	0.90	14.21	105.7 ±5.3	67.5 ±4.0	0.0 ±0.0
5	Acetone:Hexane, 15:85	1.30	14.30	124.2 ±6.2	74.2 ±4.4	0.0 ±0.0
6	Acetone:Hexane, 20:80	1.67	14.42	133.6 ±6.7	79.3 ±4.7	0.0 ±0.0
7	Acetone:Hexane, 25:75	2.02	14.56	147.5 ±7.4	79.8 ±4.7	0.0 ±0.0
8	Hexane:Acetone, 70:30	2.34	16.72	157.3 ±7.8	80.0 ±0.00	0.0 ±0.0
9	Hexane:Ethanol, 70:30	2.58	20.39	157.7 ±7.9	80.2 ±4.7	0.0 ±0.0
10	Diethyl ether, 100:0	2.85	14.81	145.9 ±7.3	88.6 ±5.2	0.0 ±0.0
11	Hexane: Acetone, 50:50	3.46	15.57	149.0 ±7.4	93.6 ±5.5	0.0 ±0.0
12	Hexane:Ethanol, 50:50	3.62	17.69	148.1 ±7.4	93.5 ±5.5	0.0 ±0.0
13	Acetone:Hexane, 70:30	4.36	16.72	171.7 ±8.6	93.4 ±5.5	0.0 ±0.0
14	Acetone:Hexane, 80:20	4.74	17.38	140.2 ±7.0	94.2 ±5.6	0.0 ±0.0
15	Acetone HPLC, 100:0	5.40	18.88	154.7 ±7.7	96.4 ±5.7	0.0 ±0.0
16	Acetone:Water, 99.5:0.5	5.47	18.95	155.0 ±7.8	94.9 ±5.6	0.0 ±0.0
17	Acetone: Water, 99:1	5.54	18.95	175.5 ±8.8	89.9 ±5.3	0.0 ±0.0
18	Ethanol: Water 96:4	5.60	25.84	124.6 ±6.2	57.1 ±3.4	0.0 ±0.0
19	Acetone: Water, 97.2:2.5	5.80	19.25	161.3 ±8.1	90.8 ±5.4	0.0 ±0.0
20	Acetone: Water, 95:5	6.00	19.66	152.6 ±7.6	86.7 ±5.1	27.0 ±1.4
21	Ethanol:Hexane:Water, 80:10:10	6.04	25.79	131.9 ±6.6	57.1 ±3.4	94.6 ±4.7
22	Acetone:Water, 92.5:7.5	6.30	20.10	150.86 ±7.5	73.4 ±4.3	60.2 ±3.0
23	Methanol HPLC, 100:0	6.60	28.03	132.0 ±6.6	56.3 ±3.3	102.4 ±5.1
24	Ethanol:Water, 80:20	6.88	28.85	108.0 ±5.4	45.0 ±2.7	100.0 ±5.0
25	Acetone:Water, 80:20	7.22	22.63	138.2 ±6.9	42.2 ±2.5	99.6 ±4.9
26	Methanol:Water, 80:20	7.46	31.30	112.9 ±5.6	35.7 ±2.1	100.0 ±5.0

Table 5. Values of the parameters that provide the best fitting of the solvent extraction data to eq. (1) and (2), represented in Fig.3A,B. T-FAs: total fatty acids; T-CRs: total carotenoids; T-APDs: total amphidinols; R^2: determination coefficient.

Metabolites	Parameters					R^2
	y_0	a	b	c	x_0	
T-FAs (3A)	11.441	0.645	0.6921	-0.1196		0.911
T-CRs (3A)		1.549	8.189	1.451	3.653	0.891
T-APDs (3A)	-1.144E-9	0.343	0.003	5091.85	5.978	0.964
T-FAs (3B)		23.416	1.953	121.388		0.869
T-CRs (3B)		1.554	0.026	58.489	17.488	0.945
T-APDs (3B)	-0.0006	0.368	0.425	14860.967	15.709	0.998

Table 6. Antiproliferative activity of *A. carterae* biomass extracts with different solvents at polarities from 5.4 to 7.5 for the four human tumor cell lines, as mentioned in Abreu et al. 2019.

Solvent (v/v)	PI	HT-29	A549	MDA-MB-231	PSN-1
Acetone (100)	5.400	145	82	106	53.4
Acetone:water (99:1)	5.542	148.8	81.1	104.3	55.8
Acetone:water (97.2:2.5)	5.770	66.2	52.6	58.5	-21.3
Acetone:water (95:5)	6.030	87.4	25.8	58.5	-40.9
Acetone:water (92.5:7.5)	6.290	-77.1	-76.1	-68.3	-77.5
Methanol (100)	6.600	-86.0	-87.0	-94.0	-88.0
Ethanol:water (80:20)	6.880	-79.5	-82.2	-80.5	-80.1
Acetone:water (80:20)	7.217	-86.8	-82.2	-75.2	-86.1
Methanol:water (80:20)	7.462	-86.8	-75.1	-69.8	-85.2

CRediT author statement

López Rodríguez.: Data in laboratory, Writing- Original draft preparation **Navarro-López and Molina Miras and Abreu** Data in laboratory **López-Rosales:** Conceptualization, Methodology, Software. **Sánchez Mirón**: Visualization, Writing- Reviewing. **Fernández**: Writing- Reviewing and Editing.: **Garcia Camacho and Cerón-García:** Supervision, Writing- Reviewing and Editing