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An integrated approach for the efficient separation of specialty compounds from biomass of the marine microalgae *Amphidinium carterae*

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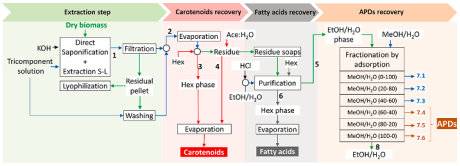
HIGHLIGHTS

- First ever an integrated amphidinol-prioritized fractioning approach.
- The approach readily separates specialty metabolites of the alga Amphidinium carterae.
- Compared to a solvent partitioning method, the integrated approach was more efficient.
- NMR-based metabolomics is a valuable tool for screening solvent extraction methods.

GRAPHICAL ABSTRACT



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ABSTRACT

An amphidinol-prioritized fractioning approach was for the first time developed to isolate multiple specialty metabolites such as amphidinols, carotenoids and fatty acids using the biomass of the marine microalgae *Amphidinium carterae*. The biomass was produced in a raceway photobioreactor and the exhausted culture media were reused, thus fulfilling sustainability criteria employing a circular economy concept. The integrated bioactive compounds-targeted approach presented here consisted of four steps with which recovery percentages of carotenoids, fatty acids and amphidinols of 97%, 82% and 99 %, respectively, were achieved. The proposed process was proved to be a better extraction system for this microalga than another based on a sequential gradient partition with water and four water-immiscible organic solvents (hexane, carbon tetrachloride, dichloromethane and *n*-butanol). The proposed process could be scaled-up as a commercial solid-phase extraction technology well-established for industrial bioprocesses.

1. Introduction

The production of low-value bulk commodities from microalgae is not yet economically feasible (Sarkar et al., 2020; Vermuë et al., 2018). As a result, emphasis has to be given to valorising different biomass fractions for the production of valuable specialty or niche products (e.g., carotenoids, phycobiliproteins or polyunsaturated fatty acids), which are often more expensive than more generic products (Sarkar et al., 2020; Vermuë et al., 2018). In this respect, the interest by compounds derived from marine dinoflagellate microalgae have experienced a

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remarkable increase (Assunção et al., 2017; Chakdar et al., 2021; Cousseau et al., 2020; Karnaouri et al., 2020; Yew et al., 2019).

In the context of biorefining dinoflagellate microalgae, it has recently been shown that the complete recovery of distinct families of important compounds contained in the *A. carterae* biomass within a single process is complex (López-Rodríguez et al., 2019). In that study, different multi-step approaches, originally aimed at separately recovering each family of metabolites from non-dinoflagellate biomass, namely PUFAs, carotenoids, and amphidinols (APDs), were optimized to maximize the recovery of the three families of compounds. Although the results were particularly successful in applying a carotenoid-targeted approach (with recovery percentages of 97% for carotenoids and 80% for fatty acids), all the APDs were unfortunately recovered in a poorly enriched fraction. In contrast, the modified method of Place et al. (2005) accommodates an attractive step based on solid-phase extraction to concentrate the APDs. Consequently, it was hypothesized that integrating the two approaches into one would be a feasible way to improve the viability of the process.

For the purpose of comparison, we employed a solvent-partitioning process that is extensively used to isolate and purify natural marine products. Its usage is indicated in those crude extractions from marine organisms that produce complex matrices of compounds and salts; this is because it allows the successful fractionation of the crude extracts into different classes of natural products with minimal degradation, thus facilitating the subsequent separation stages. A wide variety of biologically active compounds (predominantly medium-to-high polarity compounds) have been isolated utilizing this strategy. Solvent partitioning mainly involves the use of multiple pairs of immiscible solvents in separatory funnels, where the compounds are distributed in each pair of solvents according to their different partition coefficients. In general, the solubility data for interesting compounds are not known so a prior selection of specific solvents is not normally applied. For this reason, the Kupchan solvent-partitioning method (and slight modifications thereof) is usually adopted as a reference method. It has been reported to be highly effective in natural product extracts (Houssen and Jaspars, 2012); however, its application to microalgae has been scarcely studied.

The present work has focused on implementing an APD-prioritized fractionation strategy to isolate important metabolite classes such as APDs, carotenoids and fatty acids, and thus advance the biorefinery concept applied to dinoflagellate microalgae that can provide niche compounds. The biomass used in this study was produced by reusing exhausted culture media based on sustainability criteria encompassing a circular economy concept (Molina-Miras et al., 2020).

2. Material and methods

2.1. The microalga

The marine microalgae used in this work was *Amphidinium carterae* Dn241EHU (Seoane et al., 2018). It is deposited in the microalgae culture collection of the Department of Plant Biology and Ecology at the University of the Basque Country. The inoculum was kept in f/2 medium, as explained elsewhere (Molina-Miras et al., 2018).

2.2. Production of microalgal biomass

The biomass utilised in this study was produced in a long-term (>270 days) culture of *A. carterae* grown in a paddlewheel-driven fiberglass raceway photobioreactor (PBR). The PBR design was previously reported elsewhere (Molina-Miras et al., 2018). Details regarding the operation mode and experimental approach have recently been published (Molina-Miras et al., 2020). Briefly, zenith lighting was distributed across the PBR surface using multicolour LED strips. The environmental conditions were as follows: (a) a sinusoidal diel variation pattern of irradiance with a maximum irradiance of 900 μE m^{-2} s^{-1} at midday in a 24:0h L/D cycle, with a daily mean irradiance of 573 μE m^{-2} s^{-1}; (b) a culture medium composition of f/2 × 3 (N:P = 5); (c) the pH and temperature maintained at 8.5 and 21 ± 1 °C, respectively. The operation mode consisted of repeated semi-continuous cultures in which the exhausted supernatants, obtained by centrifuging each biomass harvesting stage, were completely recycled. The preparation of the reused medium has been described elsewhere (Molina-Miras et al., 2020). Harvesting was carried out once the cultures entered the stationary phase. The cell suspension samples harvested at day 272 were centrifuged at 1000 × g (RINA model 100 U, 200 SM centrifuge). The obtained pellets were gently washed with distilled water. Lastly, the cells were repelleted, lyophilized and stored at –22 °C, ready for use in the different analytical procedures and extraction methods.

2.3. Solvent-partitioning process

Crude extracts from the lyophilized biomass were subjected to a modification of Kupchan's solvent-partitioning process based on a sequential gradient partition with solvents, as shown in Fig. 1 (Riguera, 1997). Compounds contained in the crude extract are distributed into fractions according to their polarity. The process provided five fractions, hexane (Hex), carbon tetrachloride (CCl_4), dichloromethane (DCM), *n*-butanol (BuOH) and water (H_2O) (see Fig. 1). As mentioned above, this partitioning process is thought to facilitate subsequent steps of chemical profiling and biological activity screening in natural marine extracts. The method was carried out in darkness and at a room temperature of 25 °C. The total crude extract was suspended in 80 mL of a 90:10 (v/v) MeOH/water mixture and 80 mL of hexane under a N_2 atmosphere. The mixture was vigorously stirred in a Pyrex glass bottle with a Teflon-coated magnetic stirring bar at 250 rpm for 30 min in a shaking water bath (SW22, JULABO GmbH, Seelbach, Germany), and left to decant overnight in a separating funnel placed in a refrigerated chamber at 5 °C. The hexane upper phase was recovered, and the MeOH/water layer was extracted three more times over three consecutive days with the same hexane volume in each step. Water was added to the remaining MeOH/water layer to reach an 80:20 (v/v) ratio and then 80 mL of CCl_4 were added. After 30 min of stirring, the mixture was left to decant for one night in a separating funnel and again the MeOH/water layer was extracted three more times over three consecutive days with the same CCl_4 volume each time. A similar strategy was applied to the remaining solvents, DCM and *n*-BuOH, using a 60:40 (v/v) MeOH/water ratio and 100% water, respectively. In total, four layers were obtained for each solvent partition. The layers were dried under a 20–100 mBar vacuum in a rotary evaporator (BUCHI Rotavapor™ R-210, Buchi Ibérica, Barcelona, Spain) and stored at − 22 °C until further analysis. The recovery yields for the three families of compounds were determined by adding the amounts obtained in the four extractions performed for each partition (see Fig. 1.)

The solvent-partitioning process described above was applied to three biomass crude extracts obtained with three different solvents: acetone (100%), methanol (100%) and acetone:water (80:20 v/v). This selection was based on a recent work in which we demonstrated that only solvents with polarity indexes (*PI*) and Hildebrand solubility parameters (δ_T) above *ca.* 6 and 20 $MPa^{1/2}$, respectively, allowed amphidinols to be extracted from *A. carterae* biomass (López-Rodríguez et al., 2020), along with different lipid classes, according to their polarity. Thus, methanol (100%; $\delta_T = 28.03$ $MPa^{1/2}$; $PI = 6.6$) and acetone:water (80:20 v/v; $\delta_T = 22.63$ $MPa^{1/2}$; $PI = 7.22$) met the above requirement, but not acetone (100%; $\delta_T = 19.7$ $MPa^{1/2}$; $PI = 5.4$). Preparation of the extracts was carried out in darkness as follows: an 80 mL solvent volume was added to 2.5 g of lyophilized biomass placed in a Pyrex glass bottle. The samples were subjected to a temperature of 40 °C in a shaking water bath at 250 rpm for 60 min under an inert atmosphere with N_2. Then, the mixture was filtered (11 μm, Vidrafoc-Schott; Vidrafoc, Barcelona, España) to separate the spent pellet from the extracting solvent. The resulting spent pellet was re-extracted with 32 mL of fresh solvent (40% of initial volume). After removing the residue by filtration, this volume

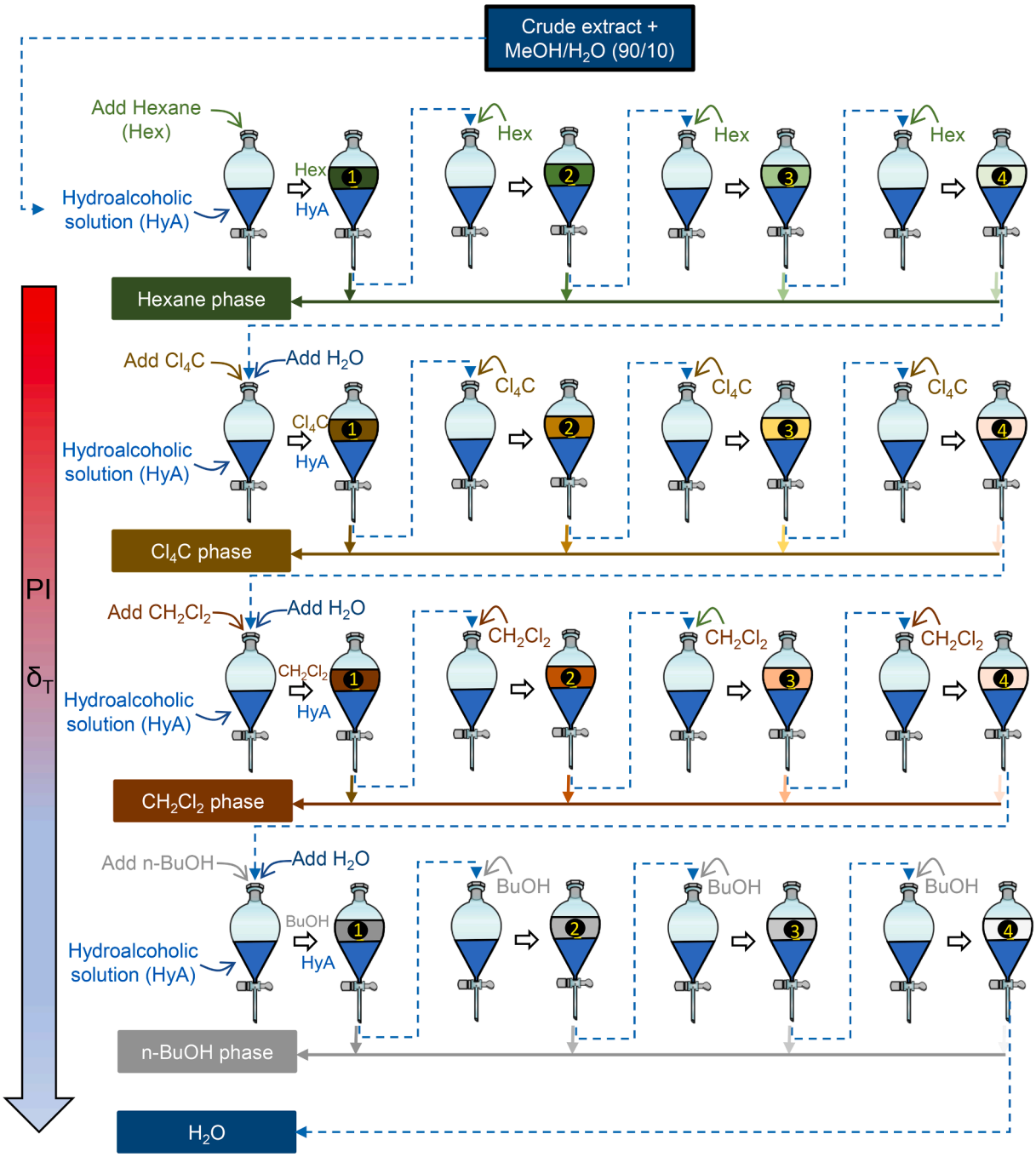

Fig. 1. Solvent-partitioning process of microalgal biomass crude extracts into five fractions (*PI*: polarity indexes; δ_T: Hildebrand solubility parameter).

was mixed with the solvent from the first extraction, dried under a 20–100 mBar vacuum in a rotary evaporator (BUCHI Rotavapor™ R-210, Buchi Ibérica, Barcelona, Spain) at a maximum temperature of 50 °C.

2.4. Integrating extraction approaches targeted at APDs, carotenoid and fatty acids

According to the results obtained in a recent study (López-Rodríguez et al., 2019), a combination of two processes, one targeted at extracting APDs and the other carotenoids, was hypothesized to provide enhanced extraction of the APDs, carotenoids and fatty acids contained in the biomass. Fig. 2 shows the experimental integration of the two processes, assembled in series. In brief, carotenoids are separated first from stream 3 (the hexane phase) and from stream 5 (the acetone:water mixture). Fatty acids are extracted in stream 6. The hydroalcoholic mixture (stream 5) leaves the carotenoid-targeted section to become the input stream for the APD-targeted section. Stream 5 was loaded into a C18 packed bed comprising four disposable C18 cartridges connected in series (Sep-Pak® C18 Plus Light, 130 mg of sorbent per cartridge, 55–105 μm particle size, Waters). This assembly provided a high adsorbent/sample ratio to ensure a high adsorption of organic metabolites from low to moderate polarity. The cartridges were connected to a vacuum collector (Supelco VISIPREP™ DL, 10–15 in-Hg). The conditioning step was carried out as recommended by the manufacturer. Subsequently, elutions were completed with different MeOH/H_2O mixtures ranging from 100% H_2O to 100% MeOH. In this study, the process in Fig. 2 was tested for the first time and 2.5 g samples of dry biomass, not subjected to cell

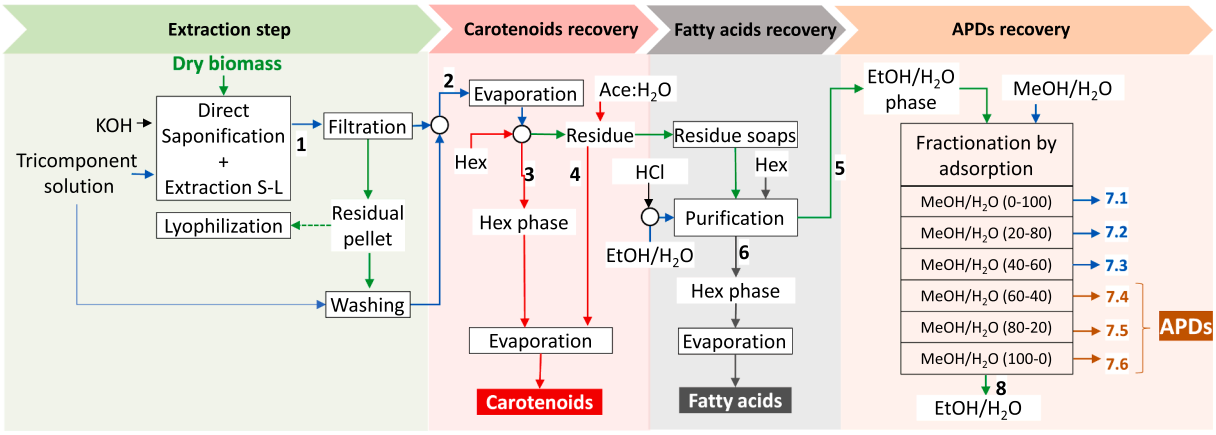

Fig. 2. Flowsheet of the proposed process for optimal extraction of fatty acids, carotenoids and APDs. For the sake of clarity, the different solvent recovery lines have been omitted. The tricomponent solution was composed of ethanol, hexane and water in a ratio of 76:18:6 v/v/v.
Adapted from López-Rodríguez et al., 2019

breakage, were used.

2.5. Analytical procedures

The following techniques were used both in the lyophilized biomass and in the resulting extracts. The carotenoid content and profile were determined using an HPLC photodiode array detector, as previously explained (Cerón-García et al., 2018). The quantified carotenoid pigments comprised diadinochrome, β-carotene, peridininol, diatoxanthin, dinoxanthin, pyrrhoxanthin, diadinoxanthin and peridinin. The total lipid content (TL) in the biomass was ascertained using the method described by (Kochert, 1978). Direct transesterification was used to determine the fatty acid methyl esters (FAMEs) content and profile using gas chromatography coupled to a flame ionization detector (FID) (Agilent Technologies 6890 N Series Gas Chromatograph, Santa Clara, CA, USA) as described earlier (Rodríguez-Ruiz et al., 1998). The quantified FAs comprised tetradecanoic acid (14:0), hexadecanoic acid (16:0), octadecanoic acid (C18:0), oleic acid (18:1n9), 9-eicosenoic acid (20:1n9), stearidonic acid (18:4n3), eicosapentaenoic acid (EPA, 20:5n3) and docosahexaenoic acid (DHA, 22:6n3). Thus, the amount of saponifiable lipids (SLs) were calculated from the FAMEs. The difference between the TLs (d.w. %) and SLs (d.w.) provided the percentage of unsaponifiable lipids in the biomass. The fractionation of the SLs into their neutral lipids (NLs) and polar lipids, specifically glycolipids (GLs) and phospholipids (PLs), was performed as described elsewhere (López-Rodríguez et al., 2020). The measurements were carried out in duplicate.

Localizing the bioactive fractions from the presence of APDs was guided by assays testing the hemolytic and antitumoral activity in the different extracts, as described elsewhere (Abreu et al., 2019; López-Rosales et al., 2015; Molina-Miras et al., 2018). Again, the measurements were carried out in duplicate.

2.6. NMR analysis

The *A. carterae* biomass contains compounds ranging from highly polar zwitterionic metabolites to entirely hydrophobic hydrocarbons, or from small molecules to higher-molecular-weight compounds such as lipids. Thus, the capability of the process described in Fig. 2 for isolating APDs, carotenoids and fatty acids, compared to that displayed in Fig. 1, was addressed using a recent untargeted and fast NMR-based metabolomics approach (Abreu et al., 2019). The high to mid-range polarity compounds were obtained by extracting 5 mg extracts with 600 μL CD_3OD/D_2O KH_2PO_4 buffer (80:20 v/v) at pH 6, containing the sodium salt of 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid (TSP; 0.01% ww) and the enzyme inhibitor sodium azide (NaN_3; 90 μM), with 20 min of sonication followed by 5 min of centrifugation (13500 rpm). The NMR-based metabolic profiles were recorded on a Bruker Avance III HD 600 spectrometer operating at a proton frequency of 600 MHz and using a 5 mm QCI quadruple resonance pulsed field gradient cryoprobe, as explained in detail elsewhere (Abreu et al., 2019). All spectra were acquired without rotation at 293 ± 0.1 K and using a NOESY presaturation pulse sequence (Bruker 1D noesygppr1d). The following metabolites were identified and quantified in the ^{1}H NMR spectra (Abreu et al., 2019): (i) amino acids, **AA** (valine, isoleucine, leucine, threonine, alanine, proline, methionine, glutamate, glutamine, glycine, lysine, aspartate, tryptophan, tyrosine, phenylalanine, histidine); (ii) organic acids, **OA** (lactate, acetate, succinate, fumarate, formate); (iii) sugars, **SA** (β-galactose, β-glucose, α-glucose, α-galactose); (iv) Quaternary ammonium compounds, **QAC** (choline, betaine); (v) Polyhydric alcohols, **PA** (glycerol); (vi) Nitrogenous bases, **NB** (uracil, cytosine); and (vii) APDs. The metabolite assignments in the ^{1}H NMR spectra are given in detail in Abreu et al. (2019). The relative peak integral of diagnostic and isolated signals of each molecule, with respect to the TSP signal integral of known concentration, were used to quantify the above-mentioned metabolites. The measurements were carried out in triplicate. Importantly, as the polar metabolites profile carried all the APDs, the focus was on the polar streams (i.e., 5 and 7 in Fig. 2; fractions *n*-BuOH and H_2O in Fig. 1) because these could potentially carry the APDs.

2.7. Recovery yields of the compounds

The recovery yields of the total carotenoids were calculated with a reference value (0.63 % d.w.), which was the maximum obtained using the modified version of the traditional analytical method reported earlier and cited in Section 2.3 (Cerón-García et al., 2018). To calculate the recovery yields of the total fatty acids, the reference value used (13.53 % d.w.) was that of the saponifiable fatty acid content in the biomass, measured as described earlier (Rodríguez-Ruiz et al., 1998) and cited in Section 2.3. Regarding the recovery yields of the total amphidinols, these were calculated using the reference value (0.69 % d.w.), which was obtained with methanol, following a previously published method (Place and Deeds, 2005).

2.8. Statistical analysis

Two replicates were prepared for each extraction. Each sample was analysed individually, and the data are reported as the mean (n = 2) ± standard deviation. The analysis of variance (ANOVA) was performed to detect any significant differences between the factors at $p < 0.05$. The

software used was Statgraphics Centurion XVI (StatPoint, Herndon, VA, USA).

3. Results and discussion

3.1. Evaluation of the solvent-partitioning process

The solvent-partitioning procedure was applied to three crude biomass extracts obtained with the following solvents, listed in order of the Hildebrand solubility parameter value (δ_T) at 25 °C: acetone (Ace), acetone:water (Ace:H_2O) 80:20 v/v, and methanol (MeOH). In each partition in Fig. 1, the hydroalcoholic phase was extracted four times with the corresponding fresh organic solvent. Table 1 displays the number of extractions needed to ensure the maximum extraction of metabolites for each of the four organic solvents and for the three crude extracts. Overall, the higher the fatty acid content extracted with a particular solvent, the greater the number of extractions needed. This observation was not repeated with the carotenoids. The low carotenoid content in the biomass compared to that of fatty acids (almost ten times lower) was probably the factor responsible for this discrepancy. The hemolytic and antitumoral activity assays performed on all the fractions revealed that only the *n*-BuOH fraction contained APDs. The NMR analysis confirmed the presence of APDs in this fraction.

Fig. 3 displays the yields of the three crude extracts, obtained as described at the end of the section 2.3, for the three target compound families (fatty acids, carotenoids and APDs) (Fig. 3A) and the corresponding recovery yield values after crude extracts were fractionated by the reference method represented in Fig. 1 based on the solvent partitioning (left side in Fig. 3B), and those coming from the process represented in Fig. 2 based on integrating extraction approaches targeting APDs, carotenoid and fatty acids (left side in Fig. 3B). In general terms, the yields of crude extracts in Fig. 3A can be divided into two groups. One consists of acetone and the other of the other two solvents. Acetone extracted a quantity of fatty acids and carotenoids, practically 100% of both, considerably higher than the other two solvents ($p < 0.05$). In contrast, acetone did not extract any amphidinols, while the other solvents did, with similar percentages ($p > 0.05$) well above the 100% corresponding to the analytical reference value. After fractionating the crude extracts (left side in Fig. 3B), this pattern of variation continued to be appreciated, although with significantly decreased recovery yields compared to those of the crude extracts (Fig. 3A). This effect of the solvents is discussed below, based on the Hildebrand solubility parameter (δ_T). The reason for this has been reported in a previous work (López-Rodríguez et al., 2020), the results of which were better interpreted in terms of δ_T than *PI* due to the fact that bioproduct solubility is not only associated with *PI*, but also with phenomena such as cosolvency, dispersive interactions, polar interactions, and hydrogen bonding. Thus, as can be seen in Fig. 3B, the three solvents were able to extract significant amounts of FAs and carotenoids; this is expected for solvents with δ_T values in the 14.5 to 31.3 [$MPa^{1/2}$] range, as previously reported (López-Rodríguez et al., 2020). Acetone provided the maximum extraction percentages ($p < 0.05$), which again is expected based on its δ_T value in the 16.5 to 19.5 [$MPa^{1/2}$] range, considered to be optimum (López-Rodríguez et al., 2020). While the APD extraction was only feasible using the solvents Ace:H_2O and MeOH, both exceeded the δ_T threshold value (around 20 $MPa^{1/2}$), above which APDs can be extracted (López-Rodríguez et al., 2020). The recovery percentages of fatty acids and carotenoids in the three crude biomass extracts were below 100% compared to the analytical reference method (Fig. 3B). However, Ace:H_2O and MeOH recovered over 30% more than that measured in the biomass compared to the analytical reference method. Therefore, it is clear that the solvent-partitioning process is not a reference extraction system and is not sufficiently effective for fatty acids and carotenoids; nonetheless, it performed well for APDs,

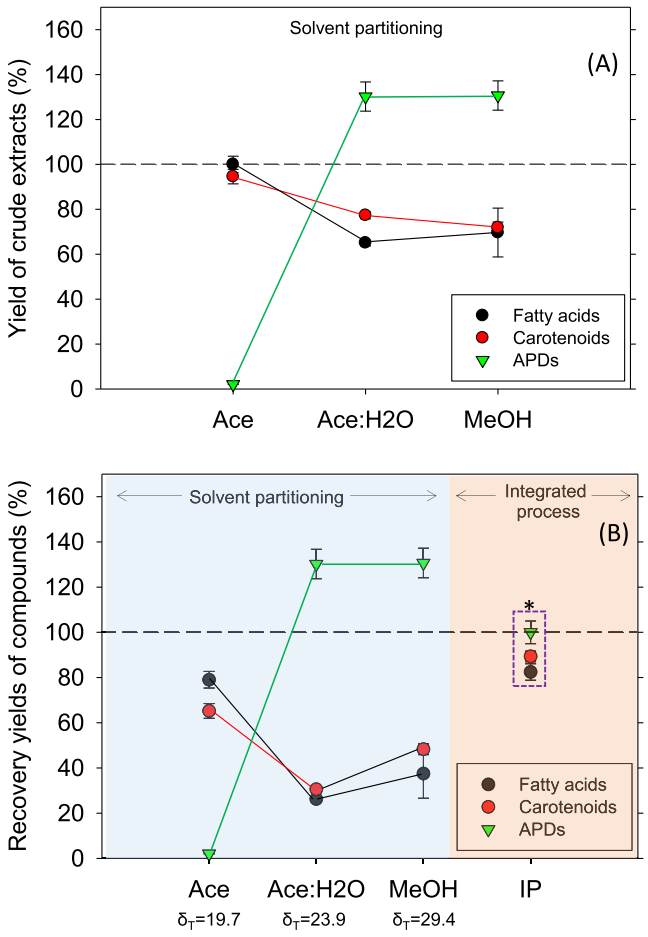

Fig. 3. (**A**) Influence of the solvent type used in the preparation of crude extracts on the extraction yields of the three compound families (fatty acids, carotenoids and amphidinols (APDs). (**B**) Effect of the extraction system for the three compound families on the recovery yields of each of them. (**Left side**) Solvent-partitioning method applied to three crude biomass extracts obtained with acetone (Ace), acetone:water (Ace:H_2O) 80:20 v/v, and methanol (MeOH). δ_T represents the solubility parameter expressed in $MPa^{1/2}$. (**Right side**) Process based on integrating the extraction approaches targeting APDs, carotenoid and fatty acids. The asterisk indicates the goodness of this method. Values are the mean ± standard deviation.

Table 1
Number of extractions needed in the solvent partitioning process to ensure a maximum extraction of metabolites for each of four organic solvents at each of the three crude extracts used. Ace, acetone; Ace:w, acetone:water (80:20); MeOH, methanol; Hexane (hex);carbon tetrachloride (Ctc); dichloromethane; butyl alcohol (BuOH); water (w).

	Fatty acids			Carotenoids			APDs		
Solvent	Ace	Ace:w	MeOH	Ace	Ace:w	MeOH	Ace	Ace:w	MeOH
Hex	3	3	3	1	1	4	nd	nd	nd
Ctc	2	2	2	1	1	4	nd	nd	nd
Dcm	1	1	1	nd	nd	4	nd	nd	nd
BuOH	4	nd	nd	nd	nd	nd	nd	2	2

depending on the solvent used to produce the crude extract. The absence of APDs in the crude acetone extract showed how dramatic the effect can be (of the solvent selected to generate the crude extract) on the recovery yields of the metabolites of interest.

Solvent-partitioning, which consists of simplifying the extract composition into several groups of metabolites sharing similar physicochemical properties, has been roughly used to identify bioactive sterol-rich fractions from non-dinoflagellate microalgae in a few studies (Samarakoon et al., 2013; Sanjeewa et al., 2016). In the study presented herein, a solvent-partitioning procedure was for the first time successfully applied in a systematic way to crude extracts from marine dinoflagellate biomass, as can be seen in Fig. 4. Consequently, when APDs are the main target, separating them by solvent partitioning could be performed in a clear-cut way in the *n*-BuOH fraction (see Fig. 4C) ($p < 0.05$). The desalting capability is inherent in the method as the salts accumulated in the aqueous one.

As mentioned in previous sections, the target lipids were those saponifiable quantified in the form of FAMEs. Saponifiable lipids (SL) accounted for 13.53 ± 0.45 % of the dry biomass. The polar fraction was dominant (47.33 ± 2.37% of glycolipids and 7.63 ± 0.38% of phospholipids), with 45.06 ± 2.25% of non-polar lipids. Therefore, the SL profile in crude extracts is tuned with the polarity of solvents as reported elsewhere (López-Rodríguez et al., 2020). In this sense, acetone, the less polar of the three polar solvents used to produce crude extracts, extracted the highest amounts of fatty acids. Overall, most of the fatty acids migrated to the less polar fraction (*n*-hexane in Fig. 4B) rather than to the remaining fractions of increasing polarity ($p < 0.05$). This suggested a significant defatting capability of *n*-hexane. A small proportion of fatty acids (mainly SDA and EPA) was extracted by the next more polar solvent (CCl4) compared to the n-hexane fraction ($p < 0.05$). Among FAs profile contained in the biomass, only a tiny amount of SFAs were found in DCM fraction.

Conversely, most of the carotenoids were better extracted by CCl_4 ($p < 0.05$) (see Fig. 4A). This result is in line with a previous study that reported the poor capability of hexane to solubilize carotenoids from *A. carterae* biomass, the optimal extraction being achieved using solvents with δ_T values ranging from 16.5 to 19.5 [$MPa^{1/2}$] (López-Rodríguez et al., 2020). However, it is important to mention that some families of metabolites, i.e., fatty acids or carotenoids, are found in successive fractions of the solvent-partitioning process (see below). As a result, tiny amounts of fatty acids and carotenoids were also identified in the solvents with the higher δ_T values (DCM, *n*-BuOH and H_2O).

3.2. Evaluation of the integrated bioactive compounds-targeted approach

Any microalgal strain is a versatile cellular factory for producing value metabolites (Koller et al., 2014). Co-extraction of multiple bioactive compounds commonly found in marine non-dinoflagellate microalgae has been comprehensively reviewed recently (Ma et al., 2020). Co-extraction techniques, taking advantage of similar physicochemical properties of the extracted compounds (e.g. polarity, solubility or molecular weight), are part of microalgae-based integrated biorefinery processes (Gilbert-López et al., 2015; Zhang et al., 2018). However, the case of the dinoflagellates is peculiar within microalgae considering that they can produce unique marker metabolites that are not found in other microalgae groups (Assunção et al., 2017), with physicochemical properties such that specie-specific thorough studies are required for their recovery. *Amphidinium carterae*, species producer of APDs, is an example of that as shown below.

As hypothesized in a previous work (López-Rodríguez et al., 2019), Fig. 2 summarizes the integrated bioactive compounds-targeted approach presented here and which has proven effective for *A. carterae*. Thus, an APD recovery yield close to 100% was feasible in combination with high recovery percentages of the carotenoids and fatty acids present in the biomass, of nearly 100% and 80 %, respectively (see Fig. 3B). Stream 2 in Fig. 2 carried almost 98 ± 5 % of the carotenoid

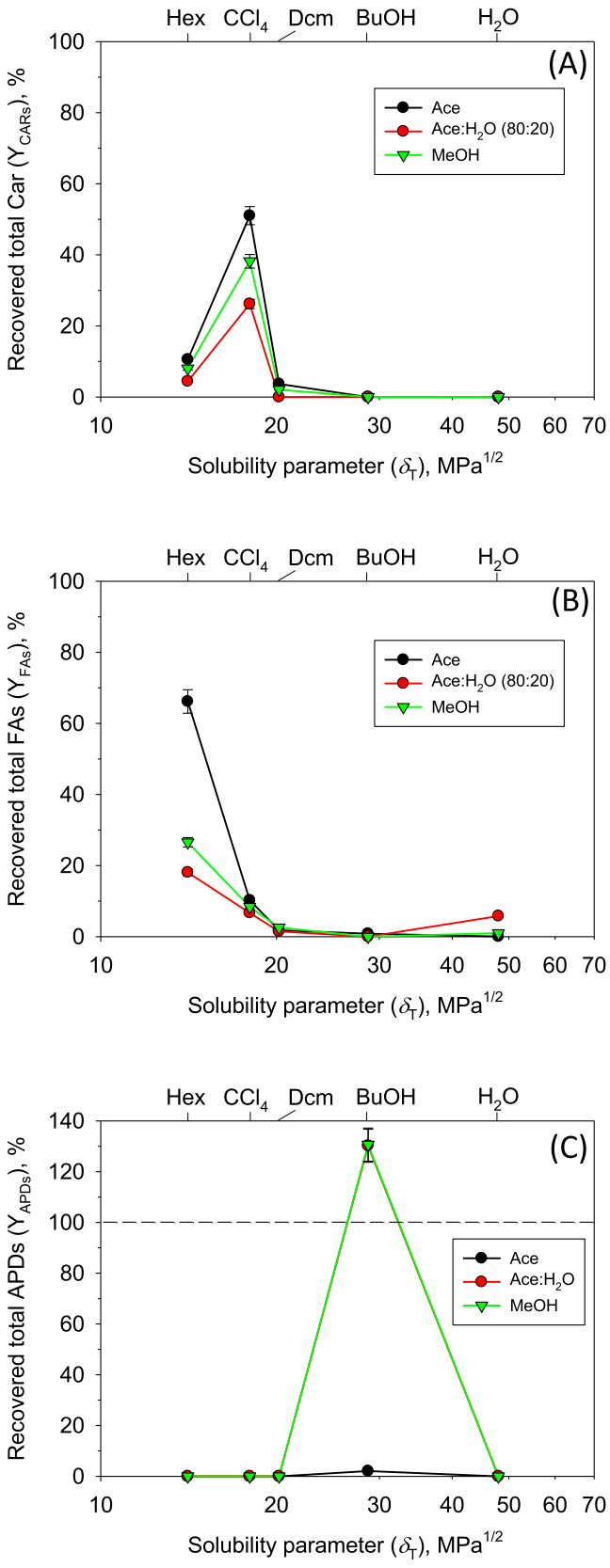

Fig. 4. Distribution of the three metabolite groups throughout the five solvents used in the fractionation of the crude extract. Values are the mean ± standard deviation.

fraction, 98 ± 3% of the fatty acids and 100 ± 5 % of the APDs; the rest of both were found in the residual pellets. The practically complete extraction of carotenoids in stream 2 is indicative of the absence of degradation of them in the saponification step, because of using an optimal KOH-to-biomass ratio (w/w) of 0.4 according to López-Rodríguez et al. (2019). Carotenoids were recovered from the treatments of streams 3 (27.5 ± 1.4 %) and 4 (69.5 ± 3.5%), as shown in Fig. 2. A tiny fraction of fatty acids (1.8 ± 0.1%) was swept along with stream 3 and another 1.0 ± 0.1% with stream 4. Stream 6 allowed us to recover the richest fatty acid fraction (82.2 ± 3.6 %), while it contained an insignificant amount of carotenoids (<1%).

Once again, a bio-guided search for hemolytic activity revealed that only stream 7 was active, with increased performance compared to the crude methanolic extract of the biomass. APDs were recovered in stream 7 with a recovery percentage of 99 ± 5 %. More specifically, the subfractions 7.4 to 7.6 were the most active, with 19.0, 47.9 and 33.1 % of total APDs, respectively. In the present study, fractions were obtained that were more highly enriched in APDs than those obtained in a previous work (López-Rodríguez et al., 2019).

3.3. Metabolomic approach to analyse the solvent extraction systems

Microalgal cultures are an interesting resource for evaluating how a given biosystem responds to changes in abiotic factors (Paliwal et al., 2017). It has been demonstrated that untargeted metabolomics is a valuable approach for increasing our understanding of the metabolic changes appearing in such biological systems (Ding et al., 2019; Hoys et al., 2021), and it has proven particularly useful in evaluating solvent extraction systems applied to plants (Martin et al., 2014), although it has not yet been applied to microalgae. Consequently, as indicated in Section 2.6, an NMR-based metabolomic approach, previously reported for *A. carterae*, was used to assess the processes described in Fig. 1 and Fig. 2. Here, we focus on the NMR metabolomic profiles of the moderately-polar and polar streams containing APDs. As a result, Fig. 5 displays the percentage distribution of the predominantly polar metabolite classes already identified (AA, OA, PA, SA, QAC, NB, and APDs) in both the APD-carotenoid-targeted and solvent-partitioning processes. In the latter, the data correspond to two crude extracts containing APDs: i) MeOH and ii) Ace(80):H_2O(20). The acetone crude extract is not relevant because it did not retrieve any APDs.

For the APD-carotenoid-targeted process (Fig. 5A), the percentages are relative to the compound contents in stream 5, expressed in dry biomass weight (d.w.). In this sense, stream 5 carried 0.49% d.w. of AA, 0.42% d.w. of PA, 0.68% d.w. of APDs, and<0.1% d.w. of OA and QAC; SA and NB were undetected indicating that they were retrieved in previous steps. Fig. 5A shows that the first elution (stream 7.1, MeOH (0%)/H_2O (100%)) swept along>93% of PA, QAC and AA; tiny amounts of these were detected in each of the remaining streams. Organic acids (OA) were eluted in all the streams in significant proportions, without a clear distribution pattern being appreciable. This specific recovery profile, i.e., 23.5, 31.1, 10.5, 12.2, 7.1, and finally 15.2%, is due to the fact that the MeOH/H_2O mixture is not an optimized solvent system, and that the most efficient one is obtained in stream 7.2 (31.1 %). Interestingly, increasing or reducing the water in the above-mentioned ratio does not lead to any improvement. The different pKa of the various organic acids involved, together with their distinct equilibria within the different proportions of water present, could explain why the recovery is maximized in one particular stream but is less efficient in the others.

Based on the data given in Fig. 5A, fractions 7.4 to 7.6 recovered most of the APDs while a small amount of APDs was retrieved from stream 7.1. These results are in agreement with a previous study using the same microalga (López-Rodríguez et al., 2019). A similar distribution pattern was reported for the recovery of karlotoxins from cultures of *Karlodinium*, compounds structurally similar to APDs (López-Rosales et al., 2018). This may be caused by differences in the hydrophobicity of the structural APD analogues, which *A. carterae* DN241EHU is able to synthesize (Wellkamp et al., 2020), due to differences in the functional groups of the molecule (e.g., sulphated APDs). The relatively less hydrophobic APDs would desorb with pure H_2O. Nonetheless, as Fig. 5A shows, the fractions 7.4 to 7.6 are quite enriched in APDs, which facilitates any subsequent purification step if required for a given application. Regarding the hydroalcoholic stream 8, obtained after passing stream 5 through the C18 solid phase (see Fig. 2), no metabolites were detected. The C18 solid phase can retain not only non-polar and moderately polar compounds but also highly polar compounds from a large variety of samples (Andrade-Eiroa et al., 2016). The retention of polar compounds on this non-polar surface (i.e., C18) was further favoured because the amount of organic matter in the mobile phase was small compared to the amount of sorbent (four C18 130 mg cartridges placed in series), and the flow rate through the cartridge below that was 1 mL per minute, as recommended by the manufacturer.

The results from the APD-carotenoid-targeted process contrast with those obtained in the solvent-partitioning procedure (Fig. 5B, C). Even though it could be an advantage for all the APDs to be recovered in one of the fractions (*n*-BuOH), it also swept along significant amounts of AA, OA, PA, SA, QAC and NB.

APDs are polyketide-like secondary metabolites. In principle, the APD-carotenoid-targeted process developed in this work can be extended to prioritized production of other valuable polyketides from other marine dinoflagellate microalgae, such as *Karlodinium veneficum* (López-Rosales et al., 2015). A raceway photobioreactor can be effectively used to grow massive amounts of microalgae such as *A. carterae*. The feasibility of cultivating *A. carterae* in a larger scale has been reported elsewhere for both open pond photobioreactors (Molina-Miras et al., 2018; Molina-Miras et al., 2020) and closed pneumatically agitated bubble column photobioreactors (Fuentes-Grünewald et al., 2016). The pros and cons of both culture systems, widely documented in the literature for other microalgae, are essentially applicable to

Fig. 5. Percentage distribution of the predominant groups of polar metabolome components in the adsorption step of the APD-carotenoid process outlined in Fig. 2 (**A**) and in the solvent-partitioning process (**B, C**) outlined in Fig. 1. Percentages are relative to the content in input stream 5 (**A**) and the initial crude extracts (**B, C**). AA: amino acids; OA: organic acids; SA; sugars; QAC, quaternary ammonium compounds; PA: polyhydric alcohols; NB, nitrogenous bases.

A. carterae. Special attention should be paid to the low tolerance to shear stress of this algae (López-Rosales et al., 2019). The downstream extraction-purification steps of the APDs process allow one to obtain other different families of important compounds such as fatty acids and carotenoids. The process can be scaled-up to a multi-kilogram level as commercial solid-phase extraction technology is well-established in industrial bioprocesses. Given that the materials and solvents used in this process are readily available and relatively inexpensive, it could be technically and economically viable.

4. Conclusions

The feasibility of using a simple solvent-partitioning method to isolate the amphidinols produced by *A. carterae* in a clear-cut way in the *n*-BuOH fraction has been demonstrated. While defatting and desalting was shown to be effective using this approach, the overlapping of metabolites other than amphidinols was inevitably observed. Alternatively, and in the context of biorefining dinoflagellate microalgae, an integrated bioactive compound-targeted approach has proven suitable for readily separating amphidinols, carotenoids and fatty acids. In addition, the NMR-based metabolomics approach was found to be a valuable tool for screening the solvent extraction methods used.

CRediT authorship contribution statement

M. López-Rodríguez: Investigation, Conceptualization, Methodology, Writing - original draft, Data curation. **M.C. Cerón-García:** Conceptualization, Methodology, Data curation, Supervision, Writing - original draft, Project administration, Funding acquisition. **L. López-Rosales:** Investigation, Data curation. **E. Navarro-López:** Investigation, Data curation. **A. Sánchez Mirón:** Investigation, Writing - original draft. **A. Molina-Miras:** Investigation, Data curation. **A.C. Abreu:** Investigation, Methodology, Data curation. **Ignacio Fernández:** Investigation, Methodology, Writing - original draft. **F. García-Camacho:** Conceptualization, Methodology, Formal analysis, Visualization, Supervision, Writing - review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2021.125922.

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