Assessment of multi-step processes for an integral use of the biomass of the marine microalga *Amphidinium carterae*

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Abstract

Sustainable dinoflagellate microalgae-based bioprocess designed to produce secondary metabolites (SMs) with interesting bioactivities are attracting increasing attention. However, dinoflagellates also produce other valuable bioproducts (e.g polyunsaturated fatty acids, carotenoids, etc.) that could be recovered and should therefore be taken into account in the bioprocess. In this study, biomass of the marine dinoflagellate microalga *Amphidinium carterae* was used to assess and optimise three different methods in order to obtain three families of high-value biochemical compounds present in the biomass. The existing processes encompassed a multi-step extraction process for carotenoids, fatty acids and APDs individually and are optimized for the integral valorization of raw *A. carterae* biomass, with SMs being the primary target compounds. Total process recovery yields were 97% for carotenoids, 80% for total fatty acids and 100% for an extract rich in APDs (not purified).

Keywords:

Marine microalgae, *Amphidinium*, Integrated extraction process, Polyunsaturated fatty acids, Carotenoids, Peridinin, Bioactive compounds

1. Introduction

Marine dinoflagellate microalgae have attracted increasing attention in recent years due to their ability to produce high value added bioactive substances (Gallardo-Rodriguez *et al*., 2012; Assunção *et al*., 2017). *Amphidinium carterae* produces an interesting group of polyketide secondary metabolites with potent anticancer, antifungal and hemolytic activities, namely amphidinolides and amphidinols (APDs), which means

that they are considered to be potential sources of new drugs (Kobayashi and Kubota, 2010).

Recent studies have assessed the feasibility of recovering APDs from supernatants of pilot-plant cultures of *A. carterae* using a simple and scalable process (Molina-Miras *et al*., 2018a). The biomass produced in these systems also contains significant quantities of other high-value products, such as carotenoid pigments and fatty acids. The recovery of these products would improve the sustainability and economics of these bioprocesses. In particular, the carotenoid peridinin and the polyunsaturated fatty acids EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) are produced in abundance by *A. carterae* (Molina-Miras *et al*., 2018b; Fuentes-Grunewald *et al*., 2016). Peridinin, a dinoflagellate-associated apocarotenoid with unique photophysical properties, has been reported to have technological applications and to be a potential therapeutic agent against different diseases (Carbonera *et al*., 2014; Onodera *et al*., 2014; Ishikawa *et al*., 2016), whereas EPA and DHA have numerous nutraceutical and pharmaceutical applications (Adarme-Vega *et al*., 2014). Unfortunately, the two methods currently used to obtain carotenoids and fatty acids individually (Fernandez Sevilla et al., 2012; Hita et al., 2015) cannot efficiently extract both families from microalgal biomass.

The concept of biorefining non-dinoflagellate microalgae to produce high value added products (e.g. pigments, proteins, lipids, carbohydrates, vitamins or antioxidants) is well-documented (Chew *et al*., 2017). However, when the priority objective of a marine dinoflagellate-based bioprocess is the production of specific secondary metabolites (SMs), such as APDs from *A. carterae*, the challenge that arises is how to recover the largest amount of the relatively minority metabolites (APDs) while minimising the loss of other valuable by-products. As a result, an efficient extraction

method for APDs and its integration into a multi-product recovery process should be developed. To the best of our knowledge, there is no report in the literature of a biorefining approach that targets SMs from dinoflagellates in which the recovery of multiple high-value co-products is considered.

Procedures for extracting amphidinol-like polyketides contained in dinoflagellate biomass pellets were not originally devised to recover carotenoids and fatty acids (Place *et al*., 2005). Excessively high extraction temperatures, for example, could degrade carotenoids (Araujo *et al*., 2013). In contrast, other methods for extracting lipids are able to preserve carotenoids but are unable to extract all of them (Hita et al., 2015).

With regard to the extraction solvents, studies using organic solvents (acetone, hexane, ethanol, dichloromethane, chloroform etc.) have been carried out to gauge their ability to extract lipids from microalgae cells. Due to toxicity problems (dichloromethane), the use of polar alcohols (less toxic solvents) and solvent mixtures such as hexane/methanol (3:2), hexane/isopropanol (3:2), cyclohexane/1-butanol (9:1) and hexane/ethanol/water (17:77:6), are good candidates amongst non-halogenated solvents. Due to their different polarities, the most suitable options are the use of hexane, acetone and ethanol, or mixtures thereof with different polarities, given that their use in food processing is already accepted (Cerón et al., 2018).

The objective of this work is to assess the impact of three multi-step extraction processes, originally designed to recover a single family of compounds, on the recovery of APDs, carotenoids and polyunsaturated fatty acids (PUFAs) together from *A. carterae* biomass produced in a pilot-scale photobioreactor. Two of the processes have previously been used to extract carotenoids and fatty acids from non-dinoflagellate microalgae individually (Fernández Sevilla *et al* 2012; Hita *et al*., 2015, respectively),

whereas the third has only been used to extract amphidinol-like polyketides (Place *et al.*, 2005). These methods were optimized to maximize the recovery of these three compound families from *A. carterae* biomass.

2. Methods

2.1. Microalgal biomass

Biomass from the marine dinoflagellate microalga *Amphidinium carterae* (strain ACRN03) was produced and used as reported previously (Molina-Miras *et al.*, 2018a). Briefly, a pilot-scale LED-illuminated (80 L) bubble column, similar to that described elsewhere (López-Rosales *et al.*, 2016), was used as photobioreactor (PBR). This PBR was operated in fed-batch mode with a pulse feeding strategy to maintain a stationary growth phase for 10 days (Molina-Miras *et al.*, 2018a). This approach provided a growth pattern strongly limited by the availability of phosphate content in the supernatant, thus stimulating the production of cellular APDs (Molina-Miras *et al.*, 2018a). The biomass was harvested at the end of the culture by centrifugation. Frozen biomass pellet was lyophilized and stored at -22 °C ready for use as a raw material in different analytical techniques and different bioactive extraction methods.

2.2. Analytical procedures

The fatty acid (FA) content and profile in samples were determined by gas chromatography (Agilent Technologies 6890 N Series Gas Chromatograph, Santa Clara, CA, USA) after direct transesterification, as described by Rodríguez-Ruiz *et al.* (1998). The carotenoid content and profile were determined using a photodiode-array HPLC apparatus (Shimadzu SPDM10AV) as explained by Cerón-García *et al.* (2018a). Measurements were carried out in duplicate. The detection of APDs in samples (i.e.

biomass extracts and sub-fractions generated in the methods assayed) was bio-guided by the hemolytic activity thereof, which was determined as reported elsewhere (López-Rosales *et al.*, 2015). A positive control, i.e. the maximum percentage of hemolysis equal to 100%, was obtained using distilled water.

2.3. Multi-step approaches for the extraction of carotenoids, PUFAs and APDs

Three different extraction protocols originally aimed at recovering a family of metabolites, namely PUFAs (Hita *et al.* 2015), carotenoids (Fernández Sevilla *et al.*, 2012), and APDs (Place *et al.*, 2005), were explored and optimized to recover the three families of compounds. Their integration into a single, multistep process was proposed based on the results obtained.

2.3.1. PUFA-targeted approach

The extraction of PUFAs and the remaining fatty acids was based on an earlier method devised for the non-dinoflagellate microalga *Nannochloropsis gaditana* (Hita *et al.*, 2015). The process flowsheet is shown in Fig. 1 and presents some modifications designed to improve the extraction of carotenoids and APDs. The first step is one of these modifications and consists of grinding the lyophilized *A. carterae* biomass to improve the yields of the subsequent extraction steps. Grinding with alumina has been reported to provide high fatty acid recovery percentages for non-dinoflagellate microalgae (Fernández Sevilla et al., 2012). As such, 2.5 g of dry biomass was ground with the same mass of alumina (1:1 w/w) to break the cells.

In a second step, a direct saponification reaction with simultaneous extraction was carried out. This consisted of treating the dry biomass with aqueous ethanol (70 mL 96% EtOH and distilled water, 9.7 mL, PI: 6.8), containing 0.4 g KOH 85% per gram of

dry biomass, instead of the 0.2 g KOH/g biomass reported by Hita *et al*. (2015) due to the higher content of saponifiable lipids in *A. carterae*. The reaction was performed at 60 °C in 250 mL Erlenmeyer flasks under an argon atmosphere. The mixture was stirred magnetically for 30 min instead of 1 hour as reported by Hita *et al*. (2015). The mixture was then centrifuged to separate the ethanolic phase from the residual biomass pellet. The pellet was washed with 32 mL of aqueous ethanol. The two hydroalcoholic phases (streams 1 and 2 in Fig 1) containing the dissolved fatty acid salts were combined for treatment in a third step, which consisted of a multiple liquid/liquid extraction of the unsaponifiable lipids with hexane (PI: 0). Briefly, 32 mL of water was added to the hydroalcoholic phase to increase the water content to 40% w/w, higher than the 30% recommended by Hita *et al*. (2015) in order to enhance the recovery of carotenoids. The extraction of unsaponifiable lipids was carried out at 20 ºC by adding several fractions of hexane to the hydroalcoholic phase in a 1:1 (v/v) proportion. The mixture was stirred magnetically for 10 min at 300 rpm, higher than the value of 250 rpm reported by Hita *et al*. (2015). The two immiscible phases subsequently formed were separated by decantation (hexane phase in stream 3 of Fig. 1) and fatty acids (hydroalcoholic phase in stream 4 of Fig. 1). The extraction process with hexane was repeated until the hydroalcoholic phase was virtually colourless, thus indicating the absence of carotenoids. In a fourth step, the fatty acid salts contained in the hydroalcoholic phase were purified and recovered. Briefly, the pH of the hydroalcoholic phase (stream 4) was adjusted to between 3 and 5 using 37% HCl. Fatty acids were then extracted by adding hexane in a simple liquid/liquid extraction at a 1:1 (v/v) ratio. Extraction was performed under argon atmosphere at 20 ºC, stirring magnetically for 10 min. The hydroalcoholic (stream 5) and hexane phases (stream 6 Fig. 1) were separated by decantation. Streams 1, 3, 4, 5 and 6 were evaluated for carotenoids, fatty acids and hemolytic bioactivity.

2.3.2. Carotenoid-targeted approach

A method previously found to be effective for microalgae was adapted to the carotenoid profile of *A. carterae* (Fernández-Sevilla et al. 2012; Cerón García et al., 2018b) and modified to improve the extraction of carotenoids contained in the biomass and to recover fatty acids and APDs. The process flowsheet is illustrated in Fig. 2. In contrast to Fernandez-Sevilla's method (2012), the procedure starts with a direct saponification and simultaneous extraction using 2.5 g of dry biomass not subjected to cell breakage. Subsequently, 100 mL of a three-component (EtOH/H_2O/Hexane) solvent mixture with a KOH content of 1.177 g (equivalent to 40% (w/w) relative to dry biomass) was used. This KOH proportion ensured both optimal recovery of carotenoids and removal of chlorophylls. The three-component solvent mixture (PI: 5.78) consisted of 87.5 mL 96% EtOH, 6.5 mL distilled water, and 6 mL hexane. The reaction was carried out at 60°C for 30 min instead of 25 ºC and 1 h (Fernandez-Sevilla et al., 2012). The reaction mixture was subsequently separated by filtration using a 12 cm-diameter porous glass plate (60 μm pore diameter, Pobel Madrid, Spain) and the residual pellet retained on the filter was washed with another 40 mL of fresh three-component mixture. Samples from the alkaline and washing treatments were collected for analysis of carotenoids, fatty acids and haemolysis (stream 2 in Fig. 2). The residual pellet was lyophilized for subsequent analysis of fatty acids, carotenoids and hemolytic activity. The hydroalcoholic phase was transferred into a flat-bottomed balloon and evaporated on a rotary evaporator to remove the solvent. This operation started at a temperature of 30 °C and a pressure of 250 mbar, which were varied to a maximum of 55 ºC and a minimum of 80 mbar to remove all solvents. Hexane (a non-polar solvent) was used initially to extract the non-polar carotenoids, followed by acetone:water mixtures (polar solvents) to recover the polar ones. The dry residue was first re-suspended in 50 mL of

hexane at 40 °C then stirred at 150 rpm for 1 h. The hexane phase was saved for further analysis (stream 3 in Fig. 2). The residue obtained was then extracted with acetone and optimised to maximise carotenoid recovery by testing the following four acetone/H_2O mixtures (% v/v): 100:0.00 with a PI: 5.4 (used as control and corresponding to the conditions used by Fernandez-Sevilla *et al*. (2012), 99.5:0.05 (PI: 5.47), 99:1 (PI: 5.54) and 97.5:2.50 (PI: 5.77). The extraction was carried out at 40ºC and 150 rpm for 7 h with all mixtures. The carotenoid-containing acetone extracts (stream 4 in Fig. 2) were then separated from the residue and samples saved for subsequent analysis. Hexane and acetone were recovered using a rotatory evaporator. A section for fatty acid recovery was added to Fernández-Sevilla's original method (2012). Thus, the residue was resuspended in a mixture consisting of 18.2 mL EtOH and 6.8 mL H_2O (ethanol-water phase). Concentrated HCl was then added to the ethanol-water phase with stirring to acidify the mixture (pH 5). This pH guaranteed the integrity of any carotenoids remaining in the ethanol-water mixture, thus allowing them to be recovered from this phase (Hita et al., 2015). The mixture was subsequently acidified to pH 2 to release any fatty acids present in the form of potassium salts. The free fatty acids thus obtained were then extracted with hexane, as previously described in section 2.3.1. Next, 25 mL of hexane was added to the mixture, which was stirred for 5 min at 150 rpm with a magnetic stirrer. The mixture was then poured into a separating funnel and left for 15 min to allow complete separation of the two phases into a lower hydroalcoholic aqueous phase (stream 5 in Fig. 2) and a lighter upper hexane phase (stream 6 in Fig. 2). Both phases were collected separately and an aliquot of each was taken for analysis of the three families of metabolites.

2.3.3. Hemolytic bioactive-targeted approach

The method used to extract amphidinolides was based on the recovery of bioactive compounds from *Karlodinium* cultures described by Place et al. (2005). Thus, the biomass (10 mg dry wt.) was sonicated for 15 min with 1 mL methanol and then centrifuged. The supernatant was injected into a disposable cartridge (Sep-Pak@light C18 Cartridges, 130 mg sorbent per Cartridge, 55-105 µm particle Size, Waters) attached to a vacuum collector (Supelco VISIPREP^{TM} DL, 10-15 in-Hg). This modification of Place's original method (2005) was intended to increase the adsorbent/ extract mass ratio. Thus, the extract was passed through four solid-phase extraction cartridges in series since previous results showed that a single cartridge did not adsorb all of the bioactive compounds.

Cartridges were equilibrated with 20 mL methanol followed by 20 mL distilled water. Once equilibrated, the methanolic biomass extract was diluted with deionized water to a final 20% methanol concentration (Krock et al., 2017) and loaded onto the equilibrated cartridges. The cartridges were eluted with different water-methanol mixtures with different ratios (polarities): 100:0, 80:20, 60:40, 40:60, 20:80, and 0: 100 (H_2O:MeOH), with PI values of between 10.2 and 6.6 The different fractions were dried on a rotary evaporator and the carotenoid and fatty acid contents and hemolytic activity determined.

The polarity index was calculated from the pure components for solvent mixtures as follows:

$$PI_{mix} = \sum_{i=1\ldots p} X_i \,.\, PI_i \qquad (1)$$

where PI_{mix} and PI_i are the polarity indices of the mixture and solvent i, respectively, and X_i is the volumetric fraction of solvent i in the mixture (Poole and Poole, 1991).

3. Results and Discussion

3.1. Optimization of extraction processes

3.1.1. PUFA-targeted approach

The fatty acids present in the biomass were recovered in a higher percentage (94.37%) than the carotenoids (79.5%; see stream 1 in Fig. 1, which corresponds to the crude fatty acid extract produced in the direct saponification). This stream contains fatty acid potassium salts, proteins, APDs, pigments, other unsaponifiable lipids, such as sterols or vitamins, and other lipids. The residual biomass pellet obtained after saponification may still contain some fatty acids, which can be recovered in the ethanol/water washing step to increase the fatty acid yield. Two washes proved sufficient to recover, in total, almost 100% of both fractions (additional 4.33% and 0% in the case of fatty acids and 20.5% and 0% in the case of carotenoids) (stream 2, Fig. 1). As a result, the stream (sum of 1 and 2) that entered the liquid/liquid extraction step with hexane (see Fig. 1) contained essentially 100% of the total fatty acid salts and carotenoids. Table 1a shows the recovery percentages of carotenoids, fatty acids and active metabolites for different steps in our modified version of the process reported by Hita *et al.* (2015). These recovery percentages, namely Y_{carot} $Y_{fatty\ acids}$ and $Y_{bioactive}$, are recovery yields (% d.w.), in other words percentage of carotenoids, fatty acids and bioactive compounds extracted with respect to the total amount of these compounds present in the initial biomass. The fatty acids quantified in stream 2 comprised 0.36% tetradecanoic acid (14:0), 3.64% hexadecanoic acid (16:0), 0.65% octadecanoic acid (C18:0), 1.65% oleic acid (18:1n9), 0.27% 9-eicosenoic acid (20:1n9), 2.59% stearidonic acid (SDA; 18:4n3), 0.32% arachidonic acid (ARA; 20:4n6), 2.15% EPA (20:5n3) and 4.29% DHA (22:6n3) (all percentages based on biomass dry weight). The average total fatty acid content (FA_T) was 15.94±0.8% d.w, which was 22% higher than the content reported by Molina-Miras *et al.* (2018b). These authors concluded that FA_T was not significantly affected by the different environmental culture conditions tested,

therefore there are intraspecies differences in FA_T between the different strains of *A. carterae*. However, the FA profiles were similar, with the exception of ARA content and another unnamed fatty acid, with a content of 0.3% d.w, which could possibly be 18:5n3 (Zhukova and Titlyanov, 2006). The PUFA fraction is higher than 60% FA_T, similar to the value obtained by Molina-Miras *et al*. (2018b). The total pigments content in stream 2 comprised the chlorophylls C2 (0.51% d.w.) and A (0.81% d.w.), and the carotenoid fraction comprised peridinin (0.82% d.w.), dinoxanthin (0.12% d.w.), diadinoxanthin (0.22% d.w.), diatoxanthin (0.25% d.w.) and β-carotene (0.05% d.w.). The pigment profile is similar to that reported by Molina-Miras *et al*. (2018b) for this species. The chlorophylls content is 40% lower because the KOH used hydrolizes these compounds, thereby reducing their content in the extract because of chlorophyll precipitation. With regard to carotenoids, their content (1.45% d.w.) is similar to the value previously reported by the authors (1.34% d.w., Molina-Miras *et al*., 2018b). This demonstrates no influence of the initial saponification step on the recovery of these molecules from the biomass, probably because this strain may contain carotenoids as esters. However, some differences were again found between strains as the ACRN03 strain contains only 0.05% d.w. of β-carotene, which contrasts with the value of 0.30% d.w. for the strain Dn241EHU (Molina-Miras et al., 2018b).

As fatty acid salts are polar, , stream 2 was subjected to repetitive liquid/liquid extractions with hexane to extract the carotenoid fraction (broad range of polarities; see Fig. 1). Emulsions were formed at water contents in the hydroalcoholic phase of more than 50% (w/w), which makes extraction difficult and decreases fatty acid and carotenoid recoveries (Hita *et al*., 2015). No emulsions were observed at 40% (w/w) in our experiments. As the fatty acids were in the form of soaps, they remained in the hydroalcoholic phase (higher solubility), whereas the carotenoids were distributed in

both phases according to their polarity: peridinin is very polar, β-carotene is non-polar and the remaining carotenoids have intermediate polarities. After six extractions, the hexane phase (stream 3 in Fig. 1) contained 62% of total carotenoids (100% β-carotene, 80% diadinoxanthin, and 45% diatoxanthin relative to the amounts determined in the dry biomass), similar to the values reported by Hita (2015). The most polar carotenoids, such as peridinin and dinoxanthin, were mixed with the fatty acid salts and thus completely washed away with the hydroalcoholic phase (stream 4 in Fig. 1). As such, this method is not suitable for the recovery of polar carotenoids.

Prior to recovery of the fatty acids contained in hydroalcoholic stream 4 (purification step), its pH was lowered in order to transform the potassium salts into free fatty acids, thus allowing them to be extracted with hexane (Fig. 1). The fatty acid extraction yield of 78.69% obtained at pH 3 is higher than the value of 67.33% achieved at pH 5. Although an improved yield may be expected upon acidification below pH 2, the risk of emulsion formation when hexane is added increases, as reported for non-dinoflagellate microalgae (González et al., 1998; Hita et al., 2015). EPA and DHA, the most representative fatty acids in *Amphidinium*, were recovered with an extraction yield of 83% and 77%, respectively similar to the total fatty acids yield. In contrast, acidification caused degradation of the carotenoids present in the hydroalcoholic phase (i.e. peridinin and dinoxanthin). As such, this PUFA-targeted approach does not seem to be appropriate for the recovery of two of the most valuable metabolite families from *A. carterae* (or other dinoflagellates containing dinoxanthin or peridinin as principal carotenoids), namely PUFAs and carotenoids.

The presence of APD compounds with hemolytic activity in the different fractions was assayed (Table 1). In the initial extract from the modified process of Hita *et al.* (2015), corresponding to alkaline treatment of the biomass (stream 1, Fig. 1), the

recovery percentage of bioactives was 100%. In contrast, neither of the final hexane phases obtained by decantation, comprising carotenoids and free fatty acids (streams 3 and 5 Fig.1), contained hemolytic APDs. Finally, the percentage recovery for these bioactive compounds was 100% for the hydroalcoholic phase (stream 4, Fig. 1). This means that APDs are successfully extracted by polar solvents due to their high polarity, which is a key issue as they will not contaminate fractions rich in fatty acids and carotenoids.

3.1.2. Carotenoid-targeted approach

A modification of Fernandez-Sevilla's method (2012) was proposed (see section 2.3.2) for extraction of the three families of compounds (see Fig. 2). As expected, the carotenoids present in the biomass were recovered in a high percentage (100%) compared to fatty acids (91.3%). Stream 1 in Fig. 2 corresponds to the crude fatty acid extract produced upon direct saponification (see Table 1b). This stream contains the same compounds as in the previous method (fatty acid potassium salts, proteins, amphidinolides, pigments, such as carotenoids and chlorophylls, other unsaponifiable lipids, such as sterols or vitamins, and other lipids). A simple step of washing the residual pellet allowed 3% d.w. of the carotenoid fraction and 4.65% d.w. of fatty acids to be recovered, with the sum of the two washings reaching almost 97% of the carotenoid fraction and 86.6% of the fatty acids fraction (in stream 2 (Fig. 2).

To recover carotenoids and remove the solvents from stream 3, the extract from the saponification was transferred into a flat-bottomed balloon and evaporated on a rotary evaporator. This procedure differs from that reported by Fernández Sevilla *et al*. (2012) (Figure 2) in that, once the residue had been dried, 50 mL of hexane was added and magnetically stirred at 150 rpm and 40 °C for 1 h. The hexane phase was extracted and saved for further analysis (stream 3). This first extraction with hexane was intended to

effectively recover the non-polar carotenoid β-carotene (96.7% of the total). The residue in the balloon was then extracted with acetone (stream 4, Fig.2), in accordance with previous studies using other strains (Cerón *et al*., 2018b). This extract contained part of the peridinin (40% of the total), dinoxanthin (81% of the total) and diadinoxanthin (30.8% of the total). These results indicate that this non-polar solvent is not suitable for recovering all the carotenoids. Thus, the process was optimized by using different ratios of acetone/water in order to vary the polarity. Data are shown in Table 2. Two such solutions, namely 99:1 and 97.5:2.5 acetone:water (v/v), gave similar results for extraction of these pigments. The total percentage of carotenoids recovered for the first solution was 34% higher than that achieved using the original method (Fernández-Sevilla *et al.* 2012), whereas for the second solution it was 33% higher. Peridinin and diatoxanthin were found to be better extracted by the 99:1 solution, with these two pigments accounting for more than 3.5% d.w. of the biomass, therefore this ratio was chosen as the best alternative as it also minimized dissolution of the fatty acid potassium salts. The acetone/water solution was added to the residual pellet in the balloon and the resulting mixture kept in the bath while stirring at 150 rpm and 40 °C for 7 h. The liquid phase was then removed (stream 4, Fig 2) and the solvents evaporated to recover the carotenoids. The recovery of carotenoids from stream 4 after evaporation was 97% (Table 2). Subsequently, 18.2 mL ethanol and 6.8 mL water were added to the residue left in the balloon (stream 5, Fig 2) in order to solubilise the fatty acid potassium salts and recover the fatty acids in stream 6 (Fig. 2) (Hita *et al.*, 2015). During this purification step, the pH was adjusted to 5 with HCl to free the fatty acids from the salts. This pH value prevented carotenoid degradation in the hydroalcoholic phase (Hita *et al.*, 2015), as reported previously. The fatty acid recovery at this pH was 53.87%, thus meaning that this pH value is too high to efficiently recover free fatty

acids. As such, the hydroalcoholic solution was further acidified to pH 2 and the extraction step repeated in order to determine whether more fatty acids could be recovered from the hydroalcoholic phase. With this treatment, the total recovery of fatty acids increased to 79.83%, which is similar to the value reported by other authors (Hita *et al*., 2015, González *et al*., 1998).

The percentage APD recovery (see Fig 4b and Table 1b) was highest for the initial extract (stream 2, Fig. 2: alkaline treatment of the biomass) and in the hydroalcoholic phases (stream 5, Fig. 2), whereas it was minimal (less than 0.2% of the total) for the acetone/water (stream 4, Fig 2) and hexane phases (streams 3 and 6, Fig. 2). As mentioned above, the hemolytic bioactives are completely extracted by the aqueous phases, in contrast to the non-polar hexane and acetone ones.

3.1.3. *Bioactive compound-targeted approach*

The recovery results obtained for each fraction with the method described in Fig. 3 are shown in Table 1c. It is clear that methanol (stream 1, Fig 3) is not an effective extractant for any of the families of compounds as only around 70% of the total was extracted. In the fractionation step (Fig. 3), carotenoids are distributed between the 100%, 20% and 0% water phases, with the 80:20 MeOH:water mixture (v/v) being the most effective (70% extraction of total carotenoids). This is probably due to the highly polar character of these pigments. The fatty acids were found in the eluate (not adsorbate of C18 cartridges) of the methanolic biomass extract and in the 100:0 MeOH:water solution (37% and 12% of the total, respectively). This could be due to the fact that fatty acids account for a large proportion of the polar lipids (68% of total fatty acids, data not shown), and therefore they do not tend to be absorbed by the non-polar stationary phase. In this process, 51% of the carotenoids and 49% of the total fatty acids were recovered. As such, this method is less effective than the modification of the

method of Fernández-Sevilla *et al*. (2012) and that of Hita *et al*. (2015) for carotenoids and fatty acids (see Table 1b and c).

As expected, the modified method of Place *et al.* (2005) (Fig. 3) allowed 68% of the APDs contained in stream 1 to be recovered (Fig 4c). In the fractionation step, the phases with methanol fractions ranging from 0% to 40% did not contain hemolytic compounds. The 80:20 MeOH:water mixture contained 33% of the APDs, with the 60:40 mixture containing 13% and only a very small percentage in the 100:0 mixture. This means that some water is needed to extract and recover these water-soluble substances. The global recovery was almost 70%, with around 11% not being adsorbed on the column, which could mean that the quantity of biomass used was too high for these cartridges.

As none of the methods assessed was able to recover and purify the three families of compounds in a percentage higher than 90%, further work is needed to solve this problem. In view of the results obtained, a combination of the modified processes of Fernández-Sevilla *et al.* (2012) and Place *et al.* (2005) is proposed in Fig. 5. The two methods are performed in series and linked with the hydroalcoholic mixture (stream 5). In this combined process, carotenoids would be separated first from the hexane phase (stream 3) and then from the acetone:water mixture (stream 4, Fig. 5). Fatty acids would be concentrated in stream 6 and purified by evaporation of the hexane. The residual ethanol/water phase from the lipid purification step (stream 5) is introduced into the adsorption column, which is eluted with different MeOH/H_2O mixtures. The APDs would be recovered for the 60-80% MeOH mixtures and, thus, separated from other compounds such as chlorophylls, proteins or other undesirable lipids.

4. Conclusions

It has been possible to successfully apply and modify two methods originally developed for non-dinoflagellate biomass to recover high percentages of fatty acids and carotenoids from *A. carterae* biomass. The modified methods can be applied to other dinoflagellate species. The modified method of Fernández-Sevilla et al. (2012) allowed 100% of the carotenoids and 98% of the fatty acids to be extracted from the *A. carterae* biomass after saponification. The total process recovery percentages were 97% for carotenoids and 80% for fatty acids after purification, with 100% of APDs also being obtained (not purified). The modified method of Hita *et al.* (2015) was less effective for extraction and purification of the carotenoids family. Finally, the modified method of Place *et al.* (2005), which is not intended for the extraction of carotenoids or fatty acids, performed worse than the modified method of Hita *et al.* (2015). As such, there is no unique method for recovering the three families of compounds from the biomass of *A. carterae* and further work is needed in this respect. The results obtained led us to propose a combination of the methods of Fernández-Sevilla *et al.* (2012) and Place *et al.* (2005), which will be tested in future work.

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Figure Captions

Figure 1. Scheme of the process for recovering mainly fatty acids, as well as bioactive compounds, by modification of the method proposed by Hita *et al.* (2015).

Figure 2. Scheme of the process for obtaining mainly carotenoids, as well as fatty acids and bioactive compounds, by modification of the method proposed by Fernández Sevilla et al. (2012).

Figure 3. Scheme of the process for obtaining mainly bioactive compounds, as well as fatty acids and carotenoids, by modification of the method proposed by Place *et al.* *(*2005).

Figure 4. Percentage hemolytic compounds recovered when using the modified methods of: a) Hita et al. (2015); b) Fernández-Sevilla et al. (2012); and c) Place et al. (2005).

Figure 5. Scheme of the proposed process for obtaining optimal recovery of all compounds (fatty acids, carotenoids and bioactive compounds) involving a combination of the modified methods proposed by Fernández Sevilla *et al.* (2012) and Place *et al.* (2005).

Figure 1

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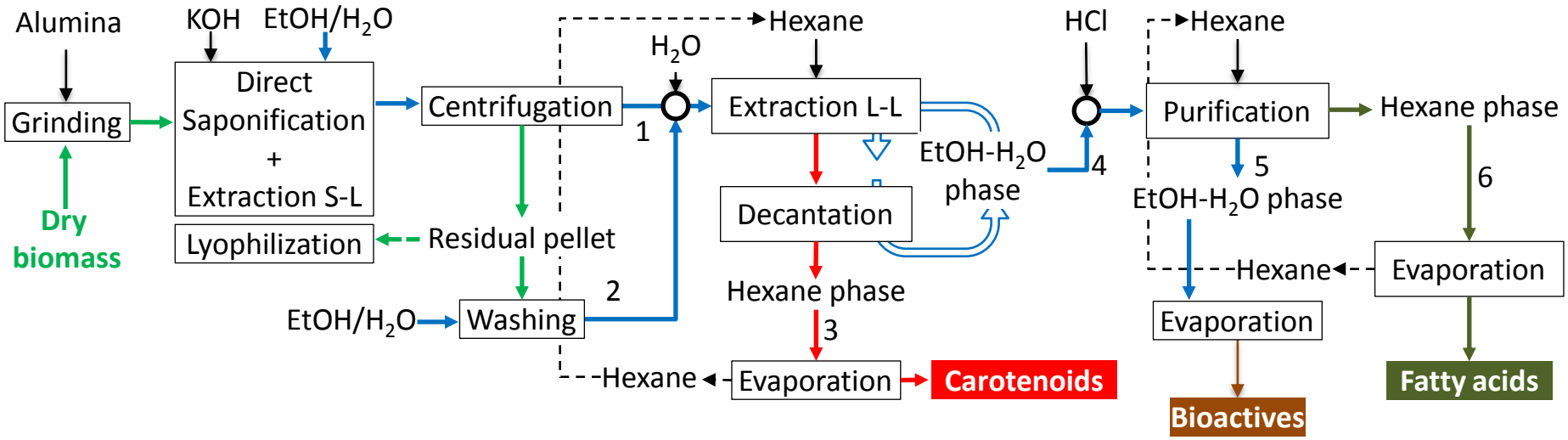

Figure 1

Figure 2

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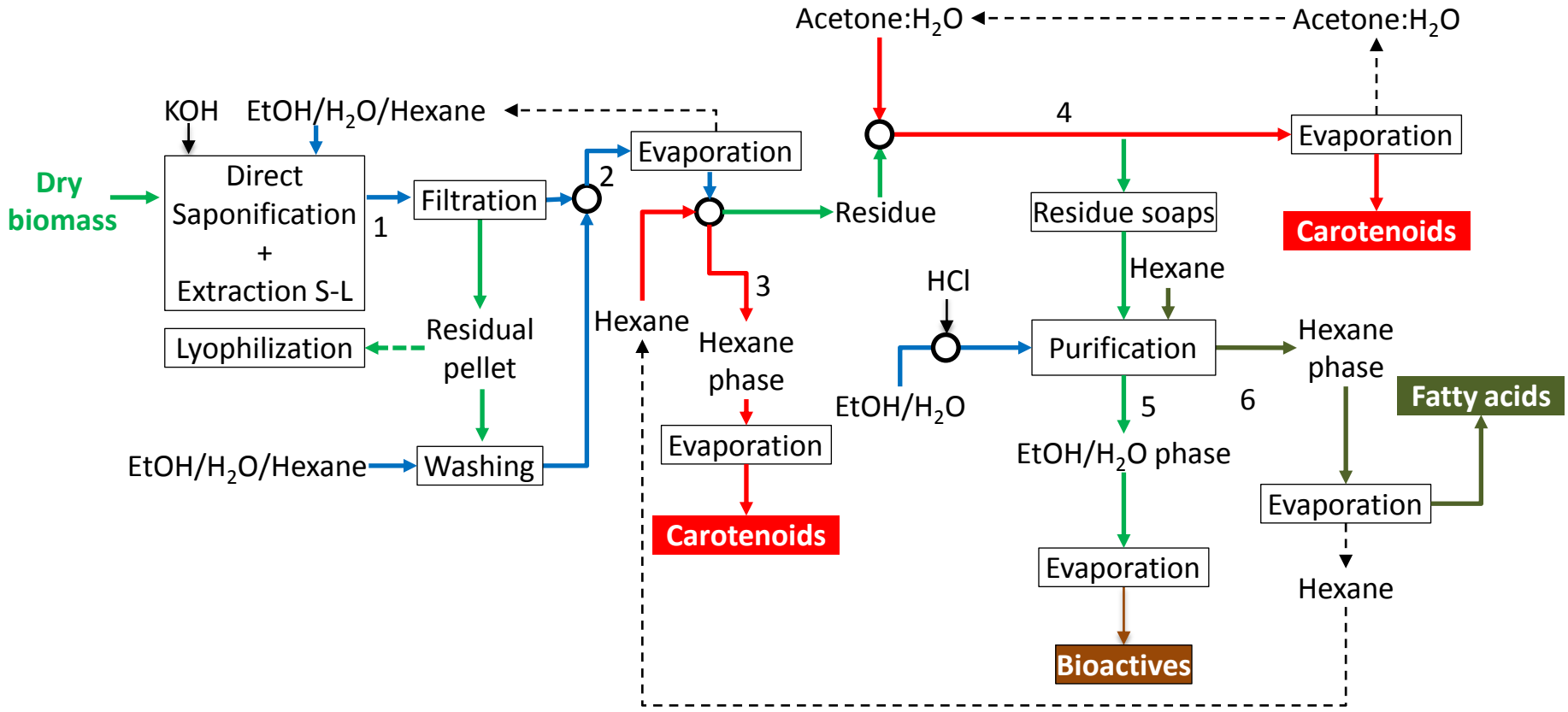

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Figure 3

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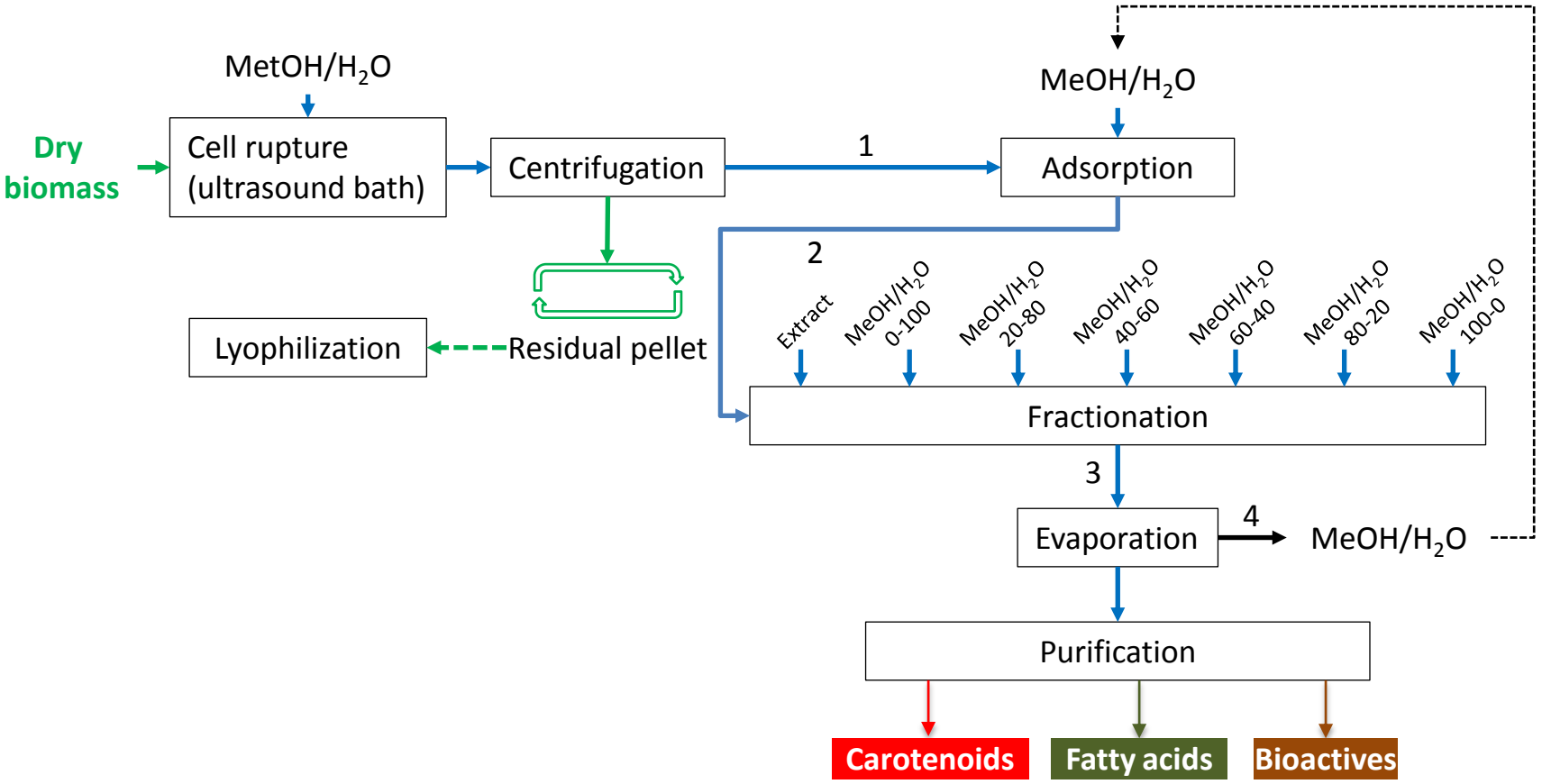

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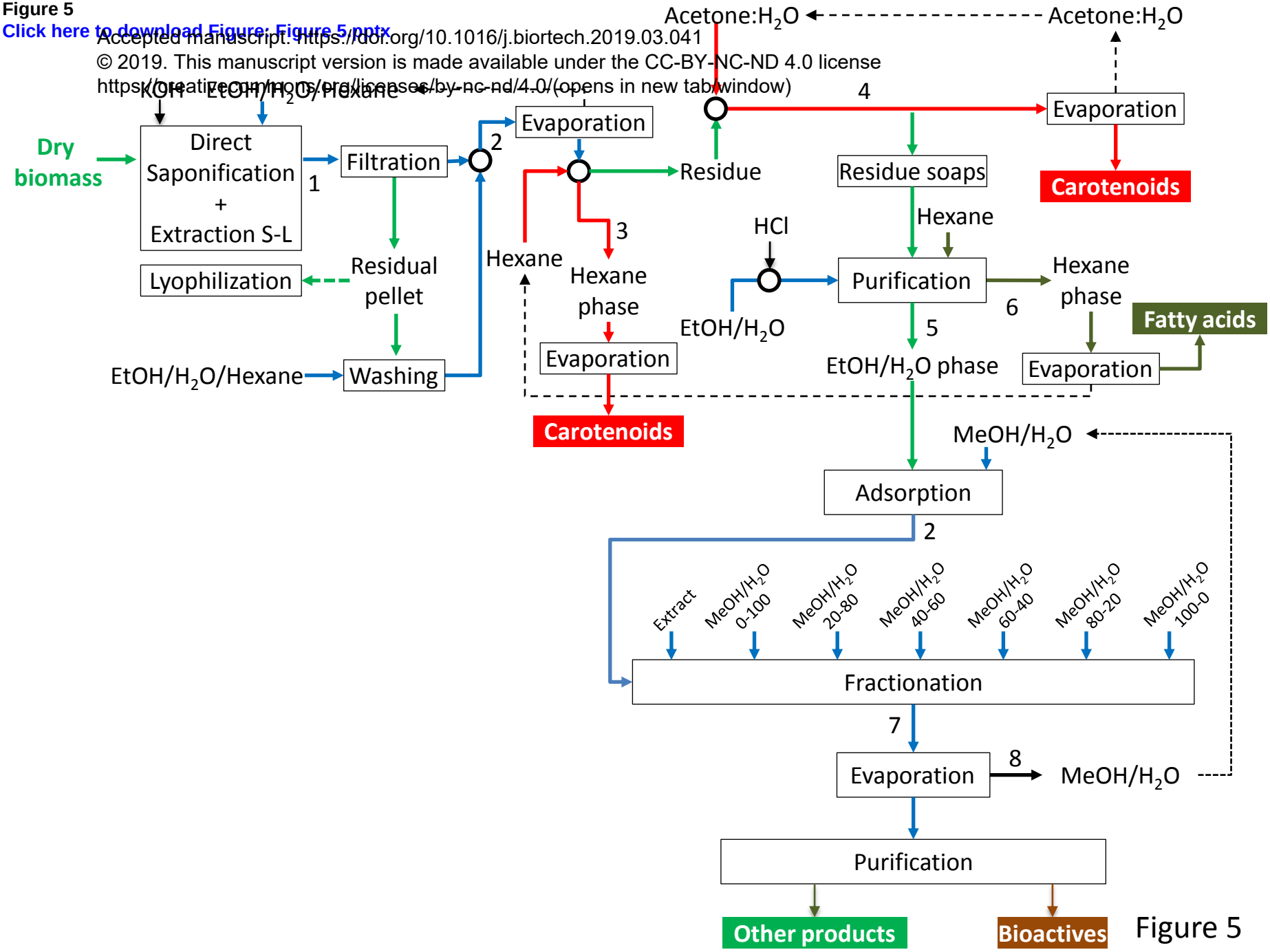

Figure 5

Figure 4

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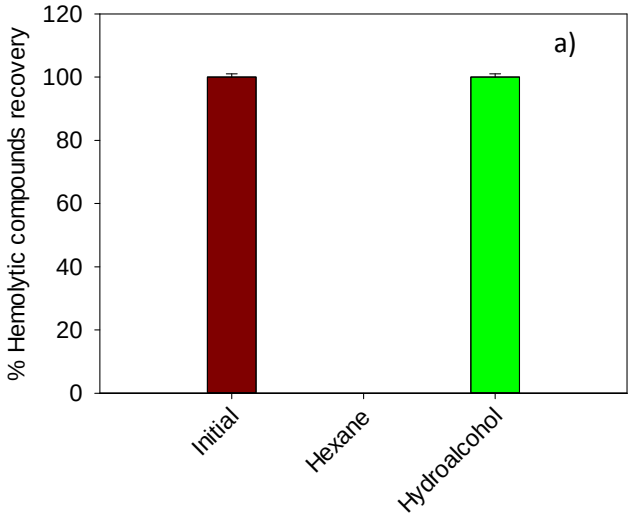

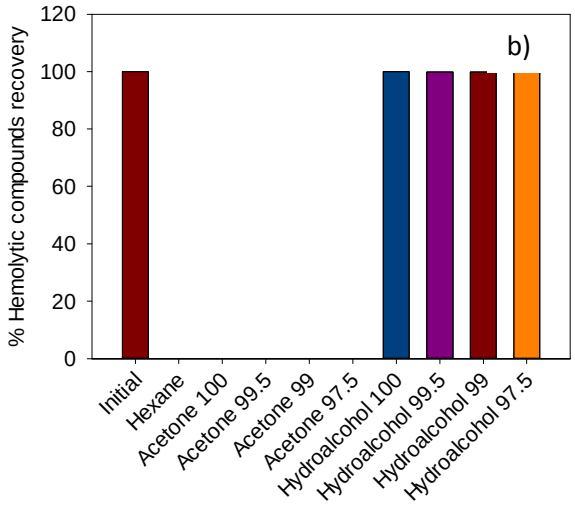

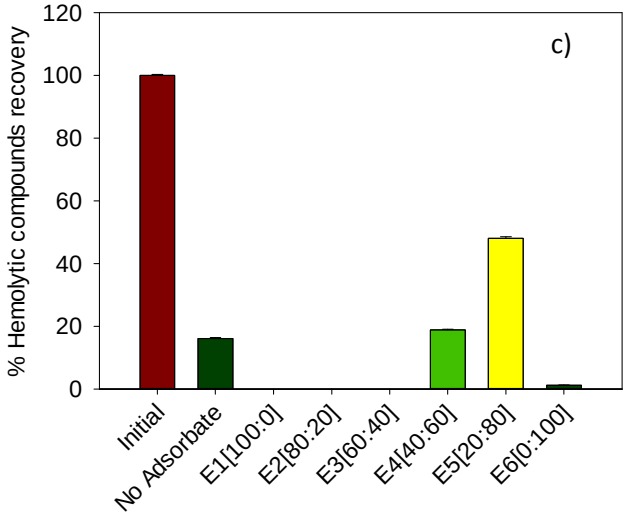

Figure 4

Table 1

Table 1. Recovery percentages of carotenoids, fatty acids and active metabolites for different steps in the modified processes of: (a) Hita *et al.* 2015; b) Fernández Sevilla *et al.* 2012; and c) Place *et al.* 2005).

a) Modification of Hita *et al.* 2015

Step / **Yield**	**Y_{carot}**	**$Y_{fatty\ acids}$**	**$Y_{bioactive}$**
Alkaline extraction	98±2.36	98.89±3.86	100±2.68
Fatty acids recovery (pH 5)	-	67.33±3.37	0.00±0.00
Fatty acids recovery (pH 3)	-	78.69±3.93	0.00±0.00
Final recovery process	**61.90±2.36**	**78.69±3.93**	**100±2.68**

b) Modification of Fernández Sevilla *et al.* 2012

Step / **Yield**	**Y_{carot}**	**$Y_{fatty\ acids}$**	**$Y_{bioactive}$**
Alkaline extraction	100±5	97.81±4.89	100±0.23
Fatty acids recovery (pH 5)	-	53.87±2.15	0.00±0.00
Fatty acids recovery (pH 3)	-	79.83±4.18	0.00±0.00
Final recovery process	**97±+4**	**79.83±4.18**	**100*±2.10**

*The hydroalcoholic phases contained all the bioactive metabolites, which was not the case for the hexane phase in both methods above

c) Modification of Place *et al.* 2005

Step / **Yield**	**Y_{carot}**	**$Y_{fatty\ acids}$**	**$Y_{bioactive}$**
Extraction	68±2.68	61.5±2.89	85.15±0.30
No adsorbed	1.02±0.01	37±2.56	10.93±0.30
Water:methanol (100:0)	9.71±0.53	0.00±0.00	0.00±0.00
Water:methanol (80:20)	0.00±0.00	0.00±0.00	0.00±0.00
Water:methanol (60:40)	0.00±0.00	0.00±0.00	0.00±0.00
Water:methanol (40:60)	0.00±0.00	0.00±0.00	12.86±0.15
Water:methanol (20:80)	36.13±2.63	0.00±0.00	32.71±0.50
Water:methanol (0:100)	4.08±0.08	12±0.36	0.79±0.10
Final recovery process	**51.11±0.93**	**49±0.56**	**68.09±0.25**

Y_{carot} $Y_{fatty\ acids}$ and $Y_{bioactive}$ are recovery yield (% d.w.): Percentage carotenoids, fatty acids and bioactive compounds extracted with respect to the compounds present in the initial biomass.

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Table 2. Carotenoid extraction expressed as dry weight content (% d.w.) using different acetone:water mixtures as solvent.

Acetone:water (%)	Peridinin (% d.w.)	Dinoxanthin (% d.w.)	Diadinoxanthin (% d.w.)	Diatoxanthin (% d.w.)	β-carotene (% d.w.)	Total Carotenoids (% d.w.)
100:0*	0.48±0.02	0.15±0.01	0.09±0.00	0.19±0.01	0.01±0.00	0.92±0.05
99.5:0.05	0.64±0.03	0.16±0.01	0.09±0.00	0.22±0.01	0.01±0.00	1.13±0.06
99:1	0.78±0.04	0.15±0.01	0.10±0.00	0.20±0.01	0.01±0.00	1.24±0.06
97.5:2.5	0.76±0.04	0.16±0.01	0.10±0.00	0.20±0.01	0.01±0.00	1.23±0.06

*This data set corresponds to the method reported by Fernández Sevilla *et al.* (2012).