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A HPLC-DAD method for identifying and estimating the content of fucoxanthin, β-carotene and chlorophyll a in brown algal extracts

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ABSTRACT

Seaweeds are photosynthetic organisms that have high contents of pigments. The coloration of each alga is defined by the content and combination of pigments synthesized, which varies among species and environmental conditions. The most abundant pigments in algae are chlorophylls and carotenoids, lipophilic molecules that can be used as natural colorants and have high acceptance by consumers. In this work, a simple and short hands-on time HPLC-DAD method for identifying and estimating the pigment content of algal extracts, specifically fucoxanthin, β-carotene and chlorophyll a was carried out. Using this optimized method, a pigment screening was performed on the ethanolic extracts obtained by ultrasound-assisted extraction from nine brown algal from the Atlantic coastline: *Ascophyllum nodosum, Bifurcaria bifurcata, Fucus spiralis, Himanthalia elongata, Laminaria saccharina, Laminaria ochroleuca, Pelvetia canaliculata, Sargassum muticum* and *Undaria pinnatifida*. HPLC results permitted to highlight *L. saccharina* and *U. pinnatifida* as promising sources of these three target pigments containing a total amount of 10.5 – 11.5 mg per gram of dry weight. Among them, the most abundant one was fucoxanthin, an added-value compound with a high potential to be commercially exploited by different industries, such as the food, cosmetic, and pharmaceutical sectors.

1. Introduction

The vast number of species belonging to algal contributes to the huge biodiversity present in marine ecosystems (Guiry, 2012). Macroalgae are aquatic macroscopic eukaryotes recognized as primary producers, thus seaweeds have a crucial role in the marine trophic chain. Besides, they also serve as a habitat for other organisms that live on their surface. They can be found at different depths along the water column, from air-water interfaces, attached to solid subtracts, in superficial waters and profundities up to 180 m. Since most of them perform photosynthesis, they are classified as autotrophic beings with high trophic efficiency (Gao & McKinley, 1994). Nevertheless, algae can also live-in deep-sea waters, so they have chemoheterotrophic production as well. Macroalgae can be differently classified according to their life cycle or habitat; however, they are mostly divided in three categories based on the major pigment class they synthesize and the subsequent tissue coloration they acquire: green (Chlorophyceae), brown (Phaeophyceae) and red algae (Rhodophyceae) (Gao & McKinley, 1994; Pepper & Gentry, 2015; Spalding et al., 2019).

Phaeophyceae are considered a major source of macro- and micronutrients but are also an estimated matrix to obtain pigments, fucosterol, sulfated polysaccharides (mainly fucoidans) or phlorotannins which have been attributed with several bioactivities like antioxidant, antimicrobial, immunostimulant, anti-inflammatory, anti-cancer and antidiabetic (Burtin, 2003; MacArtain et al., 2008; Safafar et al., 2015; Silva et al., 2020; Vuong et al., 2018). Regarding their pigment content, the most abundant ones are the carotenoids although others may be present in lower amounts as it is the case of the chlorophylls (Wang et al., 2017). Besides, the pigment production, concentration and distribution in algae may depend on many factors, like light availability and intensity, water temperature, season, weather and depth (Dumay & Morançais, 2016). That is why the analysis and optimization of culture conditions and extraction protocols are key to efficiently recover pigments from a sustainable resource as macroalgae. Chlorophylls and

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carotenoids are lipophilic molecules present in algae that require organic solvents for their extraction. These pigments can be used as natural colorants, which have good acceptance by consumers who show concerns about the effects that the abusive use of synthetic additives may have on human health and the environment. Besides, the biological properties of algal pigments make them a great ingredient to incorporate into different matrixes to improve not only their sensorial quality, but also their nutritional value (Bom et al., 2019). Thus, they result in attractive molecules for cosmetic, food, and pharmacological industries as demonstrated by their market size which shows an increasing tendency, as chlorophyll a represented $279.5 million in 2018 in food nutraceutical and cosmetic industries, fucoxanthin reached $123 million by 2019 and β-carotene more than $520 billion by 2019 due to its applications in food, nutraceutical, pharmaceutical, cosmetics, and animal feed (Global Market Insights, 2020; Market Research Report, 2021; Market Watch, 2021). In addition, biocompounds extracted from algae are natural ingredients that are very valued nowadays as a replacement to synthetic ones with associated side effects.

Chlorophylls are green pigments with non-polar nature. They are characterized for have a magnesium atom bound to a central ring of porphyrin or hydroporphyrin. The most relevant analogs present in algae include chlorophyll a, b, c1, c2 and d. Chlorophylls are the main responsible of the photosynthesis, hence they are the energetic core of the cell (Chen et al., 2017; Osório et al., 2020). Apart for being the key energy suppliers for autotrophic cells *in vivo* they result interesting molecules for their antioxidant properties. However, chlorophylls require determined storage conditions since they become chemically unstable when exposed to light, acidic pH or when stored at high temperatures (Osório et al., 2020). The degradation products generated, mainly phaeophytin or allomerization product, usually occurs after chlorophylls have lost the magnesium ion core (Osório et al., 2020). These unfavorable conditions cause their degradation and further the loss of its color and antioxidant capacity (Chen et al., 2017). Nevertheless, chlorophylls are naturally stabilized by the presence of carotenoids in the cell, therefore if an extract present a mixture of pigments, especially if it is rich in carotenoids, this degradation pathway may get inhibited (Hynninen, 1981). Indeed, the description that the European Commission provides about the chlorophyll as food additive (E140(i)) underlines the presence of carotenoids in the final mixture, among other molecules (European Parliament and Council, 2012). Since chlorophylls are natural molecules, they are well regarded as additive with coloring and antioxidant properties by food, cosmetics, and pharmaceutical sectors.

Carotenoids are classified into two families, carotenes and xanthophylls. Carotenes are non-polar hydrocarbons with a chemical skeleton characterized for the only presence of hydrogens and carbons, as in the case of the main representatives: α-carotene, β-carotene, and lycopene (Lourenço-Lopes, Carreira-Casais, et al., 2021). The β-carotene, a pigment with orange tonality, is the most relevant molecule of this family due to its powerful antioxidant capacity and provitamin A activity. Beside these beneficial properties, the coloring features of naturally obtained β-carotene make them a target molecule for food industry, especially since the 90s when it was approved as food additive by the European Commission under the E160 number (European Parliament and Council, 2012). In addition, this family of molecules has been proved to exert an antioxidant effect *in vivo*. After the excessive exposure to UV radiation, brown macroalgae trigger the synthesis of these molecules. This photoprotective capacity of the carotenoids may mitigate the harmful effects of UV radiation. That is why β-carotene represents a great ingredient not only in the food industry but also as an anti-aging ingredient for cosmetic formulations, among others (Anyanwu et al., 2018).

Fucoxanthin belongs to the xanthophylls group and is the pigment responsible for the color of brown algae. Its structure is characterized by the presence of an allenic bond, and functional carbonyl, hydroxyl and carboxyl moieties that can be responsible for fucoxanthin's diverse properties (Raguraman et al., 2018). In fact, fucoxanthin has gathered much attention lately due to its wide biological properties that include antioxidant, anticancer, anti-inflammatory, anti-angiogenic, cytoprotective, neuroprotective, skin protective and anti-obesity (Wang et al., 2018). The relevance of this carotenoid is also shown by its market size, which is reached US$ 123 million in 2019, reflecting its industrial importance, especially as a food, cosmetic or pharmaceutical ingredient. Indeed, works have proved many of the benefits of fucoxanthin. For instance, this pigment has been described to protect against oxidation processes through a reduction of the UV radiation-induced formation of radical oxygen species (ROS) (Couteau & Coiffard, 2016; Wang et al., 2017). It is a compound of great interest as a food component that may present multiple benefits in the prevention and treatment of chronic diseases, such as obesity (Miyashita & Hosokawa, 2017) and neurological disorders (Hu et al., 2018).

Regarding the chemical characterization of the compounds from complex samples, chromatographic methods are commonly applied to individually separate the molecules to help its detection. Among the currently available techniques in the field of analytical chemistry, high-performance liquid chromatography (HPLC) is one of the most widely used because of its versatility, easy adaptability to the determination of a wide range of compounds, and precision (Gasca-Plou & Torres-Salas, 2003). Concerning the determination of pigments, due to their spectrophotometric properties, the diode-array detector (DAD) has been classically applied for their analytical detection, as it identifies compounds according to their absorbance in the ultraviolet-visible spectrum with good peak resolution and low noise background, acceptable identification and quantification capacity, high reproducibility and easy handling (Swartz, 2010). Regarding the selection of the chromatographic column, we have decided to use an octadecyl-bonded C18 silica column. This type of column is the most common one used in routinary analytical laboratories (Žuvela et al., 2019). It is recommended for general analyzing purposes since it allows the detection of hydrophobic and moderately polar compounds (Žuvela et al., 2019). They permit to use a broad pH range (from 2 to 8), high temperatures (between 50 and 60 °C), and support quite high pressure (variable depending on the column dimensions). Since they are commonly used, they are cost-effective, and the column care is simple and well described.

The main aim of this work is to develop an HPLC-DAD method to identify and quantify relevant pigments extracted from brown algae, namely: fucoxanthin, β-carotene and chlorophyll a. For the purpose, nine brown algae from the Atlantic Coast of the Iberian Peninsula were subjected to pigment extraction: *Ascophyllum nodosum, Bifurcaria bifurcata, Fucus spiralis, Himanthalia elongata, Laminaria saccharina, Laminaria ochroleuca, Pelvetia canaliculata, Sargassum muticum* and *Undaria pinnatifida.* Overall, the optimization of high value-added pigments will contribute to their large-scale exploitation, thus facilitating its applications in different sectors, such as the food, pharmaceutical, and cosmetic industries

2. Materials and methods

2.1. Chemicals and reagents

Fucoxanthin and chlorophyll *a* standards were bought from Sigma (MO, USA), and the β-carotene standard was obtained from TCI (Tokyo, Japan). Ethanol was bought from VWR (PA, USA) and all organic solvents used for the extraction and chromatographic analysis were HPLC-grade. Ammonium acetate was from Carlo Erba (Milan, Italy) (RPE, analytical grade). High purity water was obtained from a Direct-Q 5UV, Millipore equipment (Merck, NJ, USA). Nylon syringe filters (0.22 μm pore size, 25 mm diameter) were from Filter-Lab (Barcelona, Spain).

2.2. Samples collection

Fresh algae were identified and recollected manually at the Galician coastline (NW Spain) and kindly provided by AlgaMar

(www.algamar.com). A total of nine brown algae were used for the development of this work: *Ascophyllum nodosum* (L.) Le Jolis; *Bifurcaria bifurcata* R. Ross; *Fucus spiralis* (L.); *Himanthalia elongata* (L.) S.F.Gray; *Laminaria ochroleuca* de la Pylaie; *Laminaria saccharina* (L.) Lamouroux; *Pelvetia canaliculata* (L.) Decne. & Thur; *Sargassum muricum* (Yendo) Fensholt; and *Undaria pinnatifida* (Harvey) Suringar. Once collected, algae were washed with distilled water and frozen within 24 h after collection at -80°C. Afterwards, samples were lyophilized and pulverized using an automatic grinder to get a fine homogeneous powder and kept at -20°C until analysis. To perform a correct quantification of the target analytes we also determined the humidity content of the lyophilized tissue algae (data not shown).

2.3. Sample extraction method

2.3.1. Heat-assisted extraction (HAE)

Two conditions of time and temperature incubation were tested for analyzing the extractive capacity of the heat-assisted extraction (HAE) using *U. pinnatifida* as representative of the brown algae. It was used with a solid-liquid ratio of 30 g/L (0.6 g of lyophilized alga powder + 20 mL of ethanol). The mixture was vortexed (30 s) and then incubated in an amber glass with direct agitation (450 rpm), for 30 min or 1 h in a water bath at 25 or 45°C, respectively, using an incubator bath to maintain the extraction temperature constant, with individual stirrers (Thermo Scientific™ Cimarec™ i Micro Stirrers). Later, samples were centrifuged (7 min, 8400 rpm, room temperature) and the supernatant filtered by nylon microfilters (pore Ø 0.22 μm) into amber vials. When possible, sample were freshly analyzed otherwise they were stored at -20°C until analysis with the optimized HPLC-DAD method.

2.3.2. Microwave-assisted extraction (MAE)

Two experimental conditions were established to evaluate the efficiency of the microwave-assisted extraction (MAE) to recover pigments from *U. pinnatifida,* used as representative of the brown algae. Same solid-liquid ratio (30 g/L: 0.6 g of lyophilized alga + 20 mL ethanol) was used. Samples were treated at the maximum power of 1400 W, for 3 or 25 min at 2 bars with a microwave instrument (Anton-Paar, Germany). Then, they got centrifuged (7 min, 8400 rpm, room temperature) and the supernatant filtered (0.22 μm pore-filter) into amber vials. Samples were mostly freshly analyzed or frozen (-20°C) until analysis with the optimized HPLC-DAD method.

2.3.3. Ultrasound-assisted extraction (UAE)

All the nine brown algal species were extracted by ultrasound-assisted extraction (UAE). In all cases, 0.6 g of lyophilized samples were mixed with 20 mL of EtOH (solid-liquid ratio of 30 g/L) and vortexed. The UAE conditions were adjusted at 500 W for 55 min, using a CY-500 sonicator (Optic Ivymen System, Spain) and the temperature was maintained using an ice bath for the duration of the ultrasound extraction. Samples were later centrifuged at 8,400 rpm for 7 min at room temperature and the supernatant syringe filtered (0.22 pore size) into amber vials. Samples were freshly analyzed when possible or stored at -20°C until analysis. This same biomass was used for the determination of the dry weight (dw) and extract yield (EY). The optimized HPLC-DAD protocol (M10) was applied to find the pigment content of these algal extracts obtained from UAE.

2.4. Detection of pigments by HPLC-DAD: optimization of the analytical method

HPLC equipment: The method development was performed using a Waters HPLC equipment [including a Waters 600 Controller and Waters 600 Pump, a Waters 2996 Photo Diode-Array detector (1.2 nm optical resolution), a Waters 717 plus Autosampler and a Waters In-Line Degasser AF].

Stationary phase: Analytical separations were performed using a Waters Nova-Pak C18 column (150 × 3.9 mm, 4 μm particle, WAT 086344). The column was thermo-stated at 25°C.

Optimized mobile phases: The mobile phases used for the optimized analytical method were A) 5 mM ammonium acetate in water; B) 5 mM ammonium acetate in MeOH; C) ethyl acetate. The flow rate was fixed at 0.5 mL/min, and the injection volume was 50 μL.

Optimized HPLC gradient: The mobile phase gradient started from 30:70:0 to 5:95:0 of A:B:C in 2 min and was hold for 6 min, then it changed from 5:95:0 to 0:50:50 of A:B:C in 1 min and was hold for 11 min, after it was switched to 50:50:0 of A:B:C, finally it changed from 50:50:0 to 30:70:0 of A:B:C in 15 min and was hold for 5 min to ensure a good HPLC column equilibration (Table 1, conditions for M10).

Method optimization: The optimization of HPLC method for the detection of algal pigments involved the performance of 10 different experimental methods using a total of six mobile phases, differently combined and applied at different flow rates (from 0.35 to 0.8 mL/min), were assessed: 1) 5 mM ammonium acetate in water; 2) 5 mM ammonium acetate in MeOH;H_2O (85:15); 3) 5 mM ammonium acetate in MeOH; 4) 5 mM ammonium acetate in acetonitrile (AcN): H_2O (90:10); 5) isopropanol (IPA); 6) ethyl acetate (Table 1; M1 – M10). Injection volume was 50 μL in all cases.

Pigments detection: Detection was performed by a diode array detector (DAD) between 300 nm and 700 nm (trace analysis). Three analytical standards of fucoxanthin, chlorophyll a and β-carotene were used for creating calibration curves. For the quantification of the chlorophyll a content of the samples it was established a correction factor of 0.96 since after analyzing its chromatogram and checking the purity certify of this analytical standard it demonstrated a purity value of 96%.

2.5. Data analysis

A calibration curve was created for each pigment standard (fucoxanthin, β-carotene, and chlorophyll a). The range of concentrations for each standard was: 1-185 ppm for fucoxanthin, 1-63 ppm for β-carotene and 1-100 ppm for chlorophyll a. Later, results obtained from the samples injections were compared to those obtained from the standards by extrapolation of the calibration curves. All data were expressed as the mean of at least 3 replicates as mg of pig-ment (fucoxanthin, chlorophyll a and β-carotene) or mg of equivalents per g of dw of algal biomass or per g of extract. In the case of the quantification of chlorophyll a data results were corrected using a factor of 0.96 corresponding to its purity. Data obtained with HPLC-DAD were analyzed using the Empower 2 Chromatography Data Software from Waters.

3. Results and discussion

3.1. Optimization of the HPLC detection method

The identification and quantification of compounds through HPLC is one of the most widely used techniques for the analysis of many different compounds. The most employed technique is the reversed phase while regarding the chromatographic column, the C18 alkyl chain ones are the first choice (Žuvela et al., 2019), as demonstrated by the high number of reports in which these conditions were used (Hagerthey et al., 2006; Henriques et al., 2007; Louda et al., 2002; Mantoura & Llewellyn, 1983; Louda et al., 2000; Wright et al., 1991). HPLC methods using C18 columns have been applied for evaluating the pigment profile of brown algae, being the most commonly detected pigments: chlorophylls, including the a, b and c families, and some degradation derivatives like chlorophyllide a and b, and phaeophorbide a and c; xanthophylls, mainly fucoxanthin and some fucoxanthin derivatives, such as fucoxanthinol or 19'-hexanoyfucoxanthin, diadinoxanthin, diatoxanthin, zeaxanthin, neoxanthin, violaxanthin, lutein, alloxanthin, astaxanthin, and canthaxanthin; and carotenes, essentially α- and

Table 1
HPLC conditions assessed for setting up the optimization parameters.

Method[1]	Mobile phases[2]	Flow rate (mL/min)	Time (min)	%A	%B	%C	RT (min)
M1	A: 5mM AA (H_2O)	0.35	0.01	30	70	0	Fx: 3.454
	B: 5mM AA (MeOH)		10	0	100	0	Chl: N.A.
			15	0	100	0	β-car: N. E.
			15.01	30	70	0	
			35	30	70	0	
M2	A: 5mM AA (H_2O)	0.35	0.01	60	40	0	Fx: 29.383
	B: 5mM AA (MeOH)		5	0	100	0	Chl: N.A.
			25	0	100	0	β-car: N. E.
			25.01	60	40	0	
			35	60	40	0	
M3	A: 5mM AA (H_2O)	0.35	0.01	0	100	0	Fx: 4.444
	B: 5mM AA (MeOH)		5	0	100	0	Chl: N.A.
			5.01	30	70	0	β-car: N. E.
			20	30	70	0	
			20.01	0	100	0	
			40	0	100	0	
M4	A: 5mM AA (H_2O)	0.5	2	0	100	0	Fx: 2.219
	B: 5mM AA (MeOH)		7	0	80	20	Chl: N.A.
	C: IPA		17	0	50	50	β-car: N. E.
			21	0	30	70	
			28.5	0	30	70	
			29.5	0	100	0	
			30.5	60	40	0	
M5	A: 5mM AA (H_2O)	0.5	0.01	30	70	0	Fx: 18.616
	B: 5mM AA (MeOH)		2	5	95	0	Chl: N.A.
	C: IPA		8	5	95	0	β-car: N. E.
			9	0	50	50	
			15	0	50	50	
			15.01	50	50	0	
			25	30	70	0	
			30	30	70	0	
M6	A: 5mM AA (MeOH/H_2O)	0.35	0.01	30	70	0	Fx: 11.042
	B: 5mM AA (AcN/H_2O)		2	5	95	0	Chl: N.A.
	C: EtAc		8	5	95	0	β-car: N. E.
			9	0	50	50	
			15	0	50	50	
			15.01	50	50	0	
			25	30	70	0	
			30	30	70	0	
M7	A: 5mM AA (MeOH/H_2O)	0.5	0.01	60	40	0	Fx: 4.116
	B: 5mM AA (AcN/H_2O)		2	0	100	0	Chl: N.A.
	C: EtAc		7	0	80	20	β-car: N. E.
			17	0	50	50	
			21	0	30	70	
			28.5	0	30	70	
			29.5	0	100	0	
			30.5	60	40	0	
M8	A: 5mM AA (MeOH/H_2O)	0.8	0.01	60	40	0	Fx: 9.112
	B: 5mM AA (AcN/H_2O)		5	60	40	0	Chl: N.A.
	C: EtAc		10	0	100	0	β-car: N. E.
			40	0	30	70	
			45	0	30	70	
			46	0	0	100	
			47	0	100	0	
			48	60	40	0	
M9	A: 5mM AA (MeOH/H_2O)	0.5	0.01	30	70	0	Fx: 19.16
	B: 5mM AA (AcN/H_2O)		2	5	95	0	Chl: N.A.
	C: EtAc		8	5	95	0	β-car: N. E.
			9	0	50	50	
			15	0	50	50	
			15.01	50	50	0	
			25	30	70	0	
			30	30	70	0	
M10	A: 5mM AA (H_2O)	0.5	0.01	30	70	0	Fx: 19.068
	B: 5mM AA (MeOH)		2	5	95	0	Chl: 23.754
	C: EtAc		8	5	95	0	β-car: 26.506
			9	0	50	50	
			20	0	50	50	
			20.01	50	50	0	
			35	30	70	0	
			40	30	70	0	

[1] Method 10 (M10) was found as the optimized method applied for the detection of Fx (fucoxanthin), Chl (chlorophyll) and β-car (β-carotene) in algal extracts (N.A.: not analyzed, N.E. not eluted in the run).

[2] Mobile phases: AA (H2O): ammonium acetate dissolved in water; AA (MeOH): ammonium acetate dissolved in methanol; AA (MeOH/H2O): ammonium acetate dissolved in MeOH:H2O (85:15), AA (AcN/H2O): ammonium acetate in AcN(acetonitrile):H2O (90:10), EtAc: ethyl acetate, IPA: isopropanol.

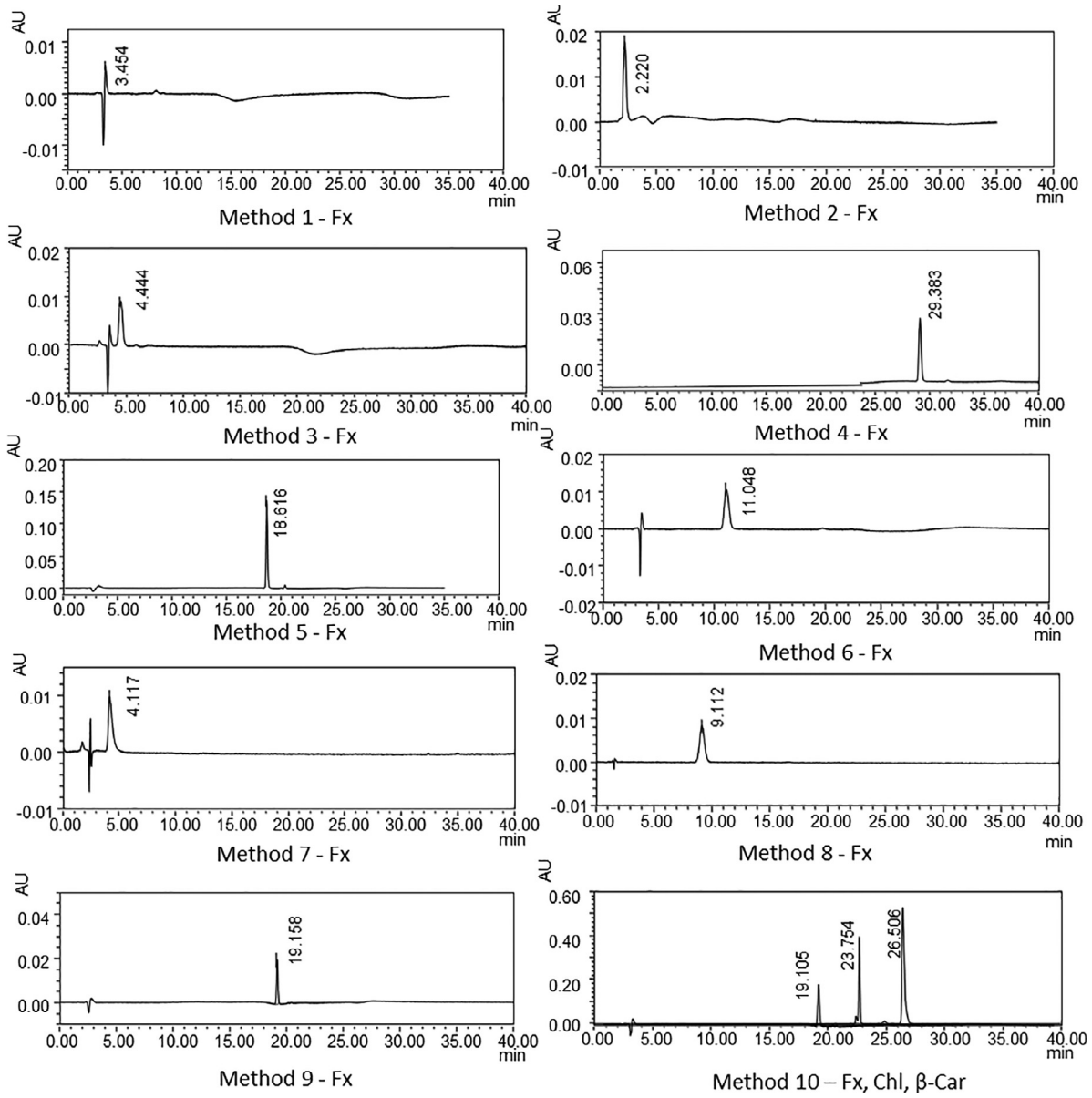

Fig. 1. Chromatograms obtained for standard solutions of pigments applying the HPLC conditions established for methods M1 to M9 and the optimized M10 method. Fx, fucoxanthin; Chl, chlorophyll-a; β-car, β-carotene.

β-carotene (Hagerthey et al., 2006; Henriques et al., 2007; Mantoura & Llewellyn, 1983; Rezanka et al., 2009).

To obtain an optimized method for the detection of the pigments, several HPLC-DAD protocols were evaluated to identify one that provided a good peak resolution and separation. Thus, a total of 10 different methods were evaluated for the simultaneous determination of pigments (Table 1). The corresponding chromatograms are shown in Fig. 1. Among pigment families, the determination of fucoxanthin and β-carotene presented some difficulties. Fucoxanthin exhibited a great variability on its retention times under slight modifications of mobile phases composition. Whereas β-carotene was not detected when the previously proved experimental conditions were applied. For this reason, fucoxanthin and β-carotene were used as key pigments for the optimization of the HPLC-DAD method since chlorophyll eluted in between these two molecules.

The mobile phases of the first three methods (M1-M3) were 5 mM ammonium acetate (AA) in water (A) and 5 mM AA in methanol (B). The run last between 35 and 40 min with a constant flow rate of 0.35 mL/min (Table 1). For M1 the gradient started with a mixture 30/70 of mobile phases A/B and fucoxanthin eluted at 3.454 min (Table 1) while β-carotene was not present in the full run. The peak showed poor resolution and it was too close to the elution front (Fig. 1). For method M2, a more polar gradient was applied (60/40, A/B, Table 1) to try to slightly retard the elution of fucoxanthin and indeed, it appeared later at 29.383 min (Fig. 1) but still no β-carotene peak was seen. In method M3 gradient was 100% of B for 5 min (Table 1) which was enough to make elute fucoxanthin at 4.444 min (Fig. 1), but again β-carotene was not present. For methods M4 and M5 same mobile phases A and B were used but it was incorporated a C mobile phase (IPA 100%) and a flow of 0.5 mL/min was applied, to try to create a higher pressure in the chromatographic column to be elute β-carotene. However, in M4 that presented a similar gradient with M3, fucoxanthin eluted even sooner, at 2.219 min (Fig. 1) and β-carotene was only eluted in the next injection. For M5, gradient showed a less polar profile for the first 15 min (Table 1) which retarded the elution of fucoxanthin to 18.616 min (Fig. 1) but still it did not work for β-carotene. Based on the low multi-detection efficiency of the explained methods, a switch on mobile phases composition was performed, according to the previously reviewed literature

(Hagerthey et al., 2006; Louda et al., 2002; William Louda et al., 2000; Wright et al., 1991)

In this new set of assays the mobile phases selected for methods M6 to M9 were 5 mM AA in methanol:water, 85:15 v/v, (A), 5 mM AA in acetonitrile:water, 90:10 v/v, (B) and ethyl acetate (C), according to some the previously successful published methods [29,32,34,35]. The method M6 had same gradient as M5 but ispopropanol was replaced by ethyl acetate, a more apolar solvent (Table 1), and flow was proved at 0.5 mL/min. Under these conditions, fucoxanthin eluted at 11.042 min with a good resolution but again β-carotene was not detected in the same injection (Fig. 1) it eluted at the end of the running time. Method 7 was similar with M2 but at a higher flow (0.5 mL/min) and with the inclusion of a 7 min window of ethyl acetate (Table 1). These parameters led to fucoxanthin to elute at 4.117 min with a good resolution, but β-carotene was newly not detected (Fig. 1). In the next method, M8, gradient was similar with M7 even though M8 showed a more polar profile and a higher flow rate, at 0.8 mL/min, and achieving a running time of 48 min (Table 1). In M8 fucoxanthin eluted at 9.112 min (Fig. 1), while β-carotene eluted in the following injection by the end of the run with a very weak peak. The method M9, had an identical gradient to M6 but a higher flow, 0.5 mL/min (Table 1). Fucoxanthin showed great resolution with a sharp peak (Fig. 1), but β-carotene was once more not detected. The modifications performed for this previous set of experiments was developed based on the previously published methods. Along these assays we tried to replicate a chromatographic method previously described by (Louda et al., 1998) since the authors used the same column and a similar HPLC equipment as ours to evaluate the concentration of, among others, the three target pigments of our interest: fucoxanthin, β-carotene, and chlorophyll-a. However, as proved by the results displayed (Fig. 1 M1-M9) such conditions did not provide enough peak separation and resolution, probably because despite using the same experimental conditions, different parameters change the results such as columns with different lifetimes.

Hence, we decided to perform another optimization set of experimental conditions. In this case, for M10, a combination of mobile phases from the previous methods was selected: 5 mM ammonium acetate (AA) in water (A), 5 mM AA in methanol (B), as in methods M1 – M5, and ethyl acetate (C), as in methods M6 – M9. The gradient was identical to M6 and M9 that were the ones where fucoxanthin eluted later and, finally, the flow rate was adjusted to 0.5 mL/min, accounting for an experimental time of 40 min (Table 1). The combination of all these adaptations to the method allowed a great separation of the three pigments, fucoxanthin that eluted at 19.068 min, chlorophyll-a at 23.493 min, and β-carotene at 26.260 min, and all peaks showed a good resolution (Fig. 1).

Once M10 was assessed as the optimized method for the simultaneous detection of fucoxanthin, chlorophyll a, and β-carotene, it was applied individually for each compound, preserving the same retention times as seen when they were combined in the same run. Fig. 2 shows the chromatograms obtained for each pigment together with their ultraviolet-visible spectra.

Thus, fucoxanthin exhibited a characteristic wide absorbance peak at ~453 nm, in accordance with the results of other authors (Erdoğan et al., 2021). Chlorophyll-a presented its putative peak band in the red zone of the spectrum at ~662 nm, together with an intense Soret band at ~435 nm, in line with previous studies (Singh et al., 2020). Finally, β-carotene showed a featured two-peak spectra with maximum wavelengths at 454 nm and 480 nm (Rodríguez-Rodríguez et al., 2020). However, the peaks of maximum absorbance may slightly change with respect to those found in the literature because of the influence of solvent on the spectroscopic characteristics of the detected molecules (Lichtenthaler & Buschmann, 2001)

3.2. HPLC-DAD pigment content of brown algal extracts

Classical approaches applied for the extraction of value-added compounds from complex matrices, such as biological samples, have been represented by maceration. It is a conventional extraction method, based on the denaturalization of cell structures through heating delivery, which has been applied to almost any vegetal matrix and any type of sample. Thus, maceration is considered a very versatile technique, simple, cost-effective, and quick. However, its efficiency can be easily improved by using an added source of energy, as it is the case of ultrasounds. In this regard, ultrasound-assisted extraction (UAE) or microwave-assisted extraction (MAE) are based on the exogenous energy application to provoke a further cell-wall breakage that contributes to the release of intracellular biomolecules improving the recovery efficiency. We used *U. pinnatifida* as representative for testing the efficiency of these three methods. Regarding the extraction yield, UAE was the most efficient one by far (151 mg/g dw, Table 2) since the best result with HAE was 44 mg/g dw (1h, 45 °C) and with MAE it was 94 mg/g dw (25 min, 2 bar). Similarly, data showed much better efficiency of pigment extraction for UAE (8.9 mg of carotenoids equivalents/g dw and 0.7 mg of chlorophyll a/g dw, Table 2) against HAE (4.5 mg of carotenoids equivalents/g dw and 0.25 mg of chlorophyll a/g dw) or MAE (3.3 mg of carotenoids equivalents/g dw and none detected c The UAE conditions were adjusted at 500 W for 55 min, using a CY-500 sonicator (Optic Ivymen System, Spain) and the temperature was maintained using an ice bath for the duration of the ultrasound extraction. hlorophylls). Therefore, the use of UAE seemed to provide an improved extraction yield of target compounds in a simple and minimal hands-on time method. Furthermore, it also constitutes a reliable technique, as it has been proven to ensure an efficient extraction of bioactive compounds without interfering with their structural and functional integrity (García-Pérez et al., 2020).

Regarding the extractive capacity of algal pigments using maceration, UAE and microwave-assisted extraction (MAE) scientific literature provide different ranges of values. Fucoxanthin extracted from *Undaria pinnatifida* by maceration (incubated at room temperature for 1 h, using a 1:1:1 mixture of MeOH:H_2O:$CHCl_3$) was quantified in 0.7 mg/g dw (Billakanti et al., 2013). Similar results were obtained by MAE, exhibiting a fucoxanthin content ranging 0.73 – 1.1 mg/g dw in ethanolic extracts obtained under the experimental conditions of 60°C at 300 W for 10 min (Grosso et al., 2015; Xiao et al., 2012). The results of UAE for *Padina tetrastromatica* were in line to those of *U. pinnatifida* reaching values in the range of 0.75 mg/g dw (Raguraman et al., 2018). In the case of other pigments, chlorophyll-a was more efficiently extracted using UAE with respect to maceration, showing an impressive 95% increase in pigment content among the species *Fucus versiculosus* (Bianchi et al., 1997) and *Laminaria japonica* (Lu et al., 2014). In the case of β-carotene, this pigment was usually extracted from microalgae by MAE, showing greater values up to 47 mg/g dw for *Dunaliella bardawil* (Kunjiappan et al., 2018) and 2.04 mg/g dw in *Synechocystis* sp. (Mäki-Arvela et al., 2014), as a result of the induction of the secondary metabolism of these organisms motivated by the application of different inductive signals during their cultivation.

Besides these techniques, the previous literature covers other innovative and alternative techniques for recovering pigments from algae, like supercritical fluid extraction (SFE) or pressurized liquid extraction (PLE). These are both considered green technologies since they present a minimum solvent consumption, and they are able of preserving the integrity of thermo- and light-sensitive compounds. Nevertheless, studies have shown that the pigment concentrations obtained with these techniques were inferior to the yields attributed to UAE and MAE (Grosso et al., 2015; Lu et al., 2014). Moreover, SFE and PLE both need the use of expensive equipment due to their higher complexity and can be more time-consuming, requiring the presence of specialized staff. Overall, UAE constitutes a promising and reliable technique to be applied in the extraction of pigments from brown algae: *Ascophyllum nodosum* (AN), *Bifurcaria bifurcata* (BB), *F. spiralis* (FS), *Himanthalia elongata* (HE), *L. saccharina* (LS), *L. ochroleuca* (LO), *Pelvetia canaliculata* (PC), *Sargassum muticum* (SM), and *U. pinnatifida* (UP).

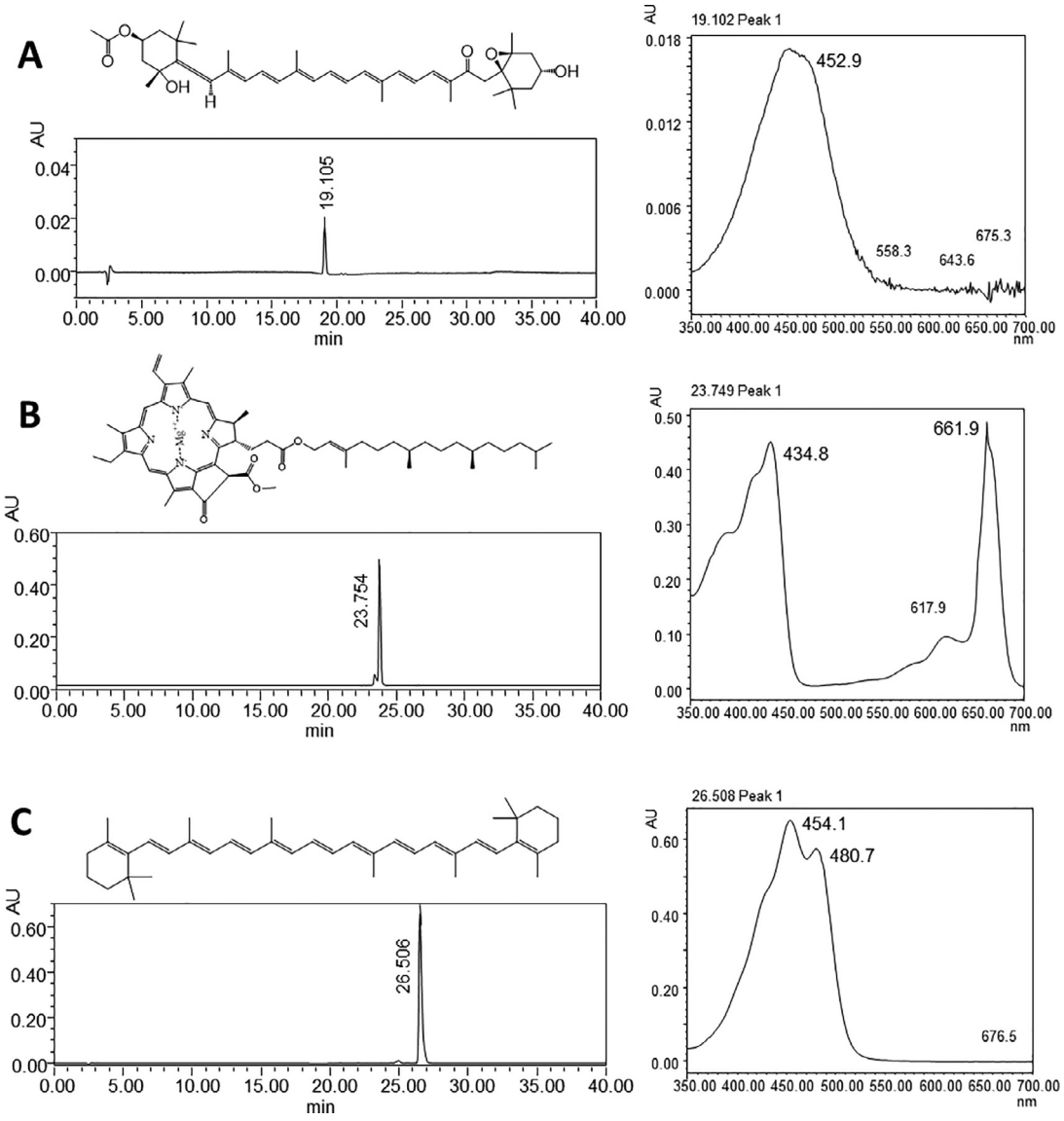

Fig. 2. Chromatograms, absorbance spectra and chemical structure of the standard solutions of (A) fucoxanthin, (B) chlorophyll-a, and (C) β-carotene using the HPLC-DAD optimized method.

Table 2
Extraction yield and HPLC-DAD quantification of pigments from UAE of 9 brown algae. Data were expressed as the mean of 3 injected replicates as mg of pigment or as mg of FX equivalents (eq) per g of dry alga (g dw).

Group	Species	EY (mg/g dw)	FX (mg/g dw)	Chl (mg/g dw)	β-car (mg/g dw)	Other carotenoids (mg of FX eq /g dw)[1]	Total pigment content (mg/g dw)
Group 1	AN	132.6	2.00	0.32	0.05	1.22	3.60
	FS	114.4	2.48	0.39	0.08	1.41	4.39
	HE	105.6	0.67	0.01	0.01	0.33	1.01
	SM	74.8	5.79	0.57	0.06	0.94	7.38
	UP	150.9	6.15	1.58	0.30	2.42	10.5
Group 2	BB	61.6	0.71	0.34	0.08	1.52	2.65
	PC	127.4	2.07	0.25	0.06	0.45	4.52
Group 3	LO	90.5	4.35	0.35	0.03	0.48	5.22
	LS	35.6	9.54	0.67	0.07	0.91	11.2

[1] Quantified according to fucoxanthin calibration curve.Abbreviations: EY, extraction yield; FX, fucoxanthin; Chl, chlorophyll a; β-car, β-carotene; AN, *Ascophyllum nodosum*; FS, *Fucus spiralis*; HE, *Himanthalia elongata*; SM, *Sargassum muticum*; UP, *Undaria pinnatifida*; BB, *Bifurcaria bifurcata*; PC, *Pelvetia canaliculata*; LO, *Laminaria ochroleuca*; LS, *Laminaria saccharina*.

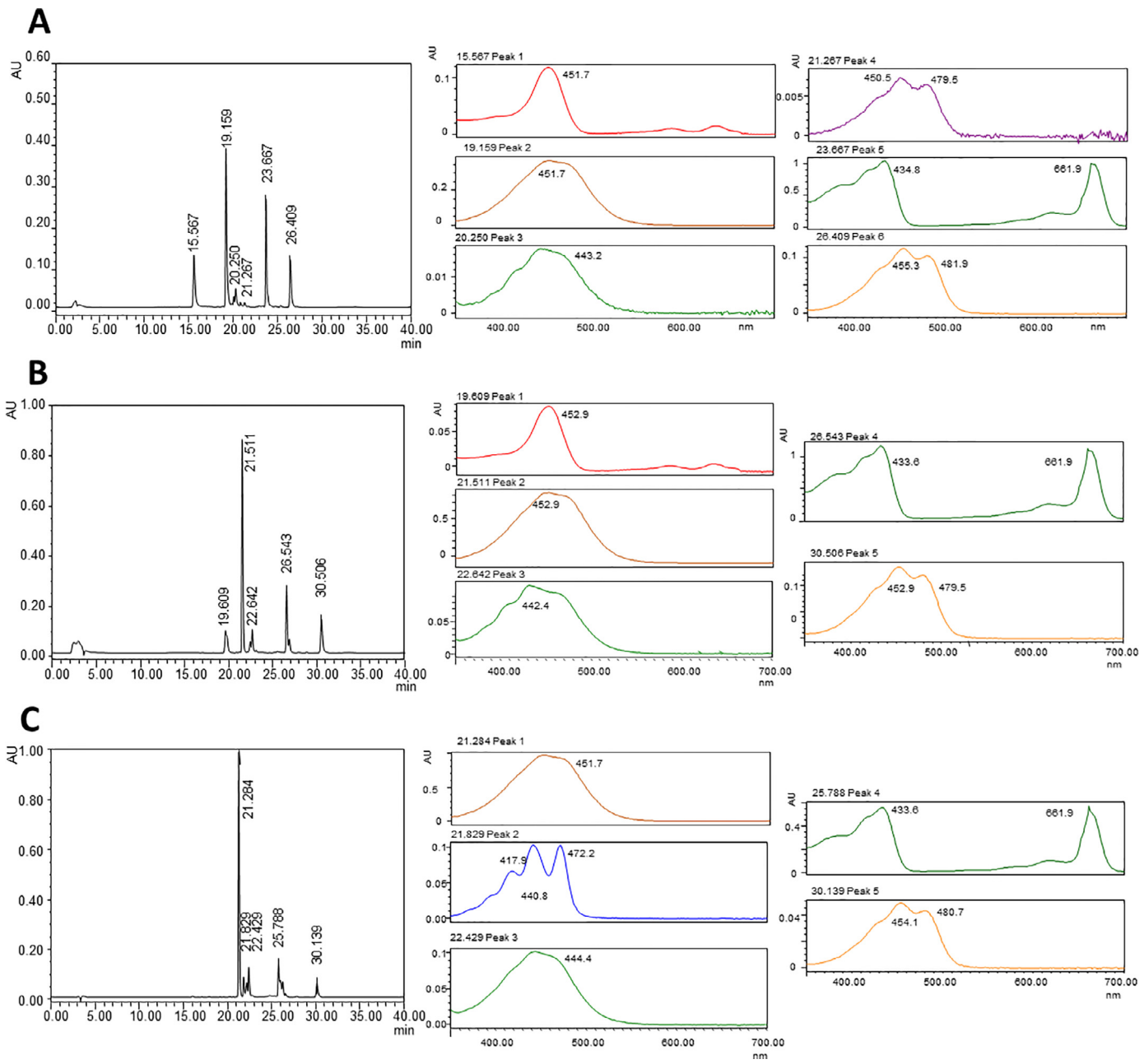

Fig. 3. Chromatograms and UV-Vis absorbance spectra of algal extracts from (A) AN (representing the chromatograms from FS, HE, SM, and UP, as well, Group 1); (B) BB (representing the chromatogram from PC, as well, Group 2); and (C) LO (representing the chromatogram from LS, as well, Group 3).

Results obtained after the HPLC-DAD analysis of these nine brown algae showed the presence of the three target pigments, fucoxanthin, *β*-carotene and chlorophyll, although two different trends in retention times were observed: the extracts of AN, FS, HE, SM, and UP showed similar retention times than those of the analytical standards, ranging 19.1 – 19.2 min for fucoxanthin, 23.6 – 23.8 min for chlorophyll a, and 26.4 – 26.6 min for *β*-carotene (Fig. 3**A**, peaks 2, 5 and 6, respectively), whereas the extracts of BB, LO, LS, and PC exhibited retarded peaks, ranging 21.0 21.5 min for fucoxanthin, 25.7 – 26.5 min for chlorophyll a, 29.6 – 30.5 min for *β*-carotene (Fig. 3**B**, peaks 2, 4, and 5, respectively; and Fig. 3**C**, peaks 1, 4, and 5, respectively). A representative chromatogram of each of these two groups, together with the UV-Vis spectra for each of the detected compounds is shown in Fig. 3. The rest of chromatograms for all the algae analyzed were provided in **Fig. S1**.

Apart from these three target pigments, additional peaks were found across the chromatograms, showing three different groups: AN, FS, HE, SM, and UP showed the same six-peak profile (Group 1; Fig. 3**A**), whereas BB and PC on one hand (Group 2; Fig. 3**B**), and the *Laminaria* species, on the other (Group 3; Fig. 3**C**) exhibited two different five-peak profile. For both Group 1 and Group 2, a sharp peak with a maximum absorbance at ~452 nm was reported at 15.6 min (Fig. 3**A**, peak 1) and 19.6 min (Fig. 3**B**, peak 1), respectively. In all cases this peak eluted before fucoxanthin and, according to previously determined spectra, this compound could be tentatively identified as fucoxanthinol (Airs & Llewellyn, 2006; Louda et al., 2002). Fucoxanthinol is a common fucoxanthin-derived metabolite produced by the deacetylation of the former becoming a non-polar molecule. It can be naturally found as a constituent of algal extracts and it is normally produced during fucoxanthin digestion (Airs & Llewellyn, 2006; Louda et al., 2002; Lourenço-Lopes, Fraga-Corral, et al., 2021).

Another peak, also present in exceptionally low amounts (nearly trace) in the fucoxanthin standard preparation, was also characterized

for presenting a unique absorbance peak with a maximum value around 443 nm. This peak was present in all the analyzed samples, but LS and PC. Its retention time had a very constant value of 20.250 min for Group 1 (Fig. 3**A**, peak 3) and ~22.5 min in BB (Fig. 3**B**, peak 3) and LO (Fig. 3**C**, peak 3). Due to its spectral features and its presence in the fucoxanthin standard, this compound seems to be a fucoxanthin derivative belonging to xanthophyll family.

A third peak eluted at 21.65 ± 0.09 min reported just in *Laminaria* samples (Fig. 3**C**, peak 2) and PC, has an absorbance profile characterized by the presence of three maximum peaks at 418, 441 and 472 nm. This characteristic profile has been previously reported in other extracts and identified as violaxanthin esters, with very similar absorbance peak patterns at ~417, 440 and 470 nm, depending on the esterified moiety (Steingass et al., 2020). Since violaxanthin has been previously described at low amounts in different species of the genus *Laminaria,* these peaks were tentatively identified as violaxanthin (Nielsen et al., 2016). Due to the absence of analytical standard for these compounds, this peak was tentatively quantified with the β-carotene calibration curve achieving values of 12 μg per g of dw for LS and and 21 μg per g of dw for LO.

Finally, a fourth peak with carotenoid-like absorbance profile, presenting two absorbance maxima at 449-455 and 479-484 and eluted at 21.35 ± 0.06 min, was seen only in Group 1 (Fig. 3**A**, peak 4). This last compound has a characteristic absorbance profile, similar with the spectra of 19′-hexanoyloxyfucoxanthin and the 19′-butanoyloxyfucoxanthin-like pigments. Both molecules have been described to have a pronounced minimum between the first and the second maximum absorbance peaks (Wright & Jeffrey, 1987), and it was tentatively identified as a fucoxanthin derivative belonging to xanthophyll family.

All these peaks have been tentatively related to the chemical family of carotenoids, although some of them were identified as fucoxanthin derivatives. Altogether, the content of these molecules in the brown algal extracts were combined and quantified using the calibration curve of fucoxanthin to calculate their content in the extracts.

3.3. Evaluation of pigment content determined for brown algal extracts

Table 2 shows the content of fucoxanthin, chlorophyll a, β-carotene, and other carotenoids of the nine brown algal species reported in the ethanolic extracts obtained by UAE. As stated above, pigments from nine brown seaweed species were extracted by UAE to maximize their recovery. The extraction efficiency was found by the extraction yield (EY), which is the mg of dry extract obtained per gram of dry alga. It is important to note that the algal species subjected to extraction showed a different range of moisture content, ranging 3.6 – 13.1% (data not shown), playing a significant role on EY. Among all analyzed species, the algae from Group 1 showed, in general the highest rates of EY, with UP presenting the maximum values (150.9 mg/g), followed by AN (132.6 mg/g) (Table 2). On the contrary, the lowest yield values were obtained for Group 3, where LS presented the minimum EY value, 35.6 mg/g dw (Table 2).

Regarding the concentration of each of the three pigments analyzed, values showed a huge variability depending on the selected species. Fucoxanthin, as a characteristic pigment of brown algae, was present in all species (Table 2), especially in LS and UP that show the highest contents, 9.54 and 6.15 mg/g dw, respectively. In contrast, HE and PC were the species showing the lowest content ~0.7 mg/g dw. Concerning the other two pigments analyzed, concentrations were negligible in comparison with those of fucoxanthin, ranging 0.01 – 1.58 mg/g dw for chlorophyll a and 0.01 – 0.30 mg/g dw for β-carotene, being UP the species reflecting the highest concentrations and HE the lowest concentrations in both cases (Table 2). Following the same trend, the quantification of other carotenoids, including some fucoxanthin derivatives, ranged 0.33 – 2.42 mg of fucoxanthin equivalents/g dw, with UP showing the highest content (Table 2). Nevertheless, owing to the total pigment content of brown algae, LS shows the highest concentrations, 11.2 mg /g dw, followed by UP, 10.5 mg/g dw, and again HE presents a negligible pigment content of 1.01 mg/g dw (Table 2).

These results show that LS is a rich source of pigments since they are the 32.2% of constituents in the dry extract of this species. In the same way, UP can be considered a potential source of fucoxanthin and fucoxanthin derivatives, in accordance with previous evidence that points at the presence of a wide range of these compounds as the major pigments of this species (Piovan et al., 2013). In this sense, the prevalence of fucoxanthin as the major pigment of brown algae reported in this work is supported by previous findings. In the reviewed literature, the maximum values of the three pigments analyzed reached 0.75 mg/g of fucoxanthin when extracted from *Padina tetrastromatica* by UAE, using 80% ethanol as solvent, a temperature of 50°C and 30 min (Raguraman et al., 2018), 0.5 mg/mL of chlorophyll a in aqueous extracts of UP obtained by UAE at 60°C, 300 W and 30 min (Raguraman et al., 2018; Zhu et al., 2017), and 0.24 mg/g of β-carotene in the methanolic extracts of *Laminaria japonica* obtained by UAE at room temperature for 30 min (Lu et al., 2014). Another study performed with LS extracted up to 0.27 mg/g of chlorophyll a and 0.17 mg/g of fucoxanthin in the acetonic extracts obtained by UAE (Boderskov et al., 2016). Compared with our results, these values are 38.5% and 98.2% lower, motivated by the application of ultrasounds for 60 seconds in this case. Another study, applying UAE through a sonication bath on *L. saccharina*, results were also lower than ours, 0.67 mg/g dw of fucoxanthin, 0.10 mg/g dw of chlorophyll a, and 0.02 mg/g dw of β-carotene, despite using methanol with the antioxidant butylated hydroxytoluene as solvent to prevent pigment degradation (Marinho et al., 2019). Lastly, the application of maceration for the pigment extraction of several brown macroalgae reported much lower fucoxanthin concentrations, < 0.5 mg/g dw, in the acetonic extracts of LS, AN, HE, and PC (Shannon & Abu-Ghannam, 2017). Keeping this in mind, the direct application of the sonication probe into the sample during UAE has proven to be an efficient method for the recovery of pigments from brown algae as it softly hinders the release of the pigments.

Therefore, as observed in this work the selection of the macroalgal species and the extraction techniques are key for obtaining an efficient recovery of the target pigment/s. However, in other to provide a complete scenario regarding the maximal productive species other factors are needed to be studied. The environmental conditions, the presence of stress factors such as excessive radiation or contaminants, need to be further investigated since they can directly affect to the pigment production of macroalgae species. Under this multifactorial analysis it would be possible to better determine the best choice for pigment production at industrial scale

4. Conclusions

In this work, an optimized HPLC-DAD protocol was achieved for the simultaneous detection of three relevant pigments of brown algae: fucoxanthin, chlorophyll-a, and β-carotene. The mobile phases used for the optimized method were 5 mM ammonium acetate in water, 5 mM ammonium acetate in methanol and ethyl acetate for a total run of 40 min with a flow rate of 0.5 mL/min. This protocol allowed a great separation and good peak resolution of the three target pigments, with fucoxanthin eluting at 19.068 min, chlorophyll-a at 23.493 min, and β-carotene at 26.260 min. This optimized method was later applied to the determination of the target pigments in nine brown algae species The samples were subjected to pigment extraction by ultrasound-assisted extraction (UAE), and the obtained extraction yields ranged from 150.9 mg/g for *U. pinnatifida* to 35.6 mg/g dw for *L. saccharina*. The results obtained after the HPLC-DAD analysis of these nine algae displayed the presence of the three target pigments, fucoxanthin, β-carotene and chlorophyll, and some other peaks whose absorbance pattern and retention times were similar with those of fucoxanthin. These peaks were tentatively related to the chemical family of the fucoxanthin and quantified as other carotenoids. As expected, the concentration of the three pigments analyzed in this study when using UAE showed a great vari-

ability depending on the selected species. Among the nine evaluated algal extracts, the most relevant species in terms of pigment concentration were *L. saccharina* and *U. pinnatifida*, especially regarding their fucoxanthin content (9.5 and 6.1 mg/g dw, respectively) but also, for *β*-carotene and chlorophyll, since *U. pinnatifida* was demonstrated to contain 1.6 mg/g dw and 0.3 mg/g dw, respectively while *L. saccharina* reached values of 1.1 mg/g dw and 0.07 mg/g dw, respectively.

Therefore, this paper presents a simple, economic, and minimal hands-on time method to detect and quantify fucoxanthin, *β*-carotene and chlorophyll simultaneously, based on a reliable UAE protocol that requires a short experimental procedure while supplies great extractive rates. Finally, *U. pinnatifida* and *L. saccharina* represent important natural sources of pigments, hence, they can be considered as a sustainable matrix to obtain natural pigments, especially fucoxanthin, a molecule with high demand, for its final commercialization through food, nutraceutical, cosmetic, textile and pharmaceutical industries.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.focha.2022.100095.

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