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Evaluation of fucoxanthin contents in seaweed biomass by vortex-assisted solid-liquid microextraction using high-performance liquid chromatography with photodiode array detection

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ABSTRACT

Fucoxanthin is considered an important marine bioactive compound with biological properties with promising effects, namely on health. A simple and efficient analytical methodology is proposed for its quantification in seaweed biomass by using vortex-assisted solid-liquid microextraction (VASLME) followed by reversed phase high-performance liquid chromatography (RP-HPLC) photodiode array detection (PDA) analysis. This microextraction uses reduced quantities of sample (25 mg) and solvent (300 μL of ethanol) to efficiently extract this high-valued xanthophyll, in a vortex time of 15 min. These extraction parameters were optimized performing a Central Composite Design (CCD) analysis, running 32 individual experiments. In turn, the method validation was assessed. The linearity of the method was confirmed (R^2 = 0.99998) in a concentration range from 12 to 3600 $\mu g \cdot g^{-1}$ dw. Also, good sensitivity and accuracy results were observed through the LOD (3.33 $\mu g \cdot g^{-1}$), LOQ (10.09 $\mu g \cdot g^{-1}$) and recovery (varied from 95 to 97%) assessments. Good precision was also verified, with intra-day variation within 2.0–3.3%, and inter-day within 1.0–3.8%. Matrix effect was also evaluated and an acceptable variation of 3.4% was found. The method applicability was confirmed by the analysis of 22 seaweed biomass samples and fucoxanthin content was found to vary from about 10 to 853 $\mu g \cdot g^{-1}$ dw. This method demonstrated a good performance and can be successfully implemented for a rapid, reliable and accurate screening of fucoxanthin in seaweed biomass.

1. Introduction

Fucoxanthin is a high-value commercial xanthophyll (about 11 €/mg) firstly extracted by Willstätter and Page in 1914 from *Dictyota*, *Fucus* and *Laminaria* brown seaweeds, subsequently also found in other brown seaweeds and diatoms (microalgae). It is one of the most abundant carotenoids, estimated to comprise 10% of the total found in nature [1]. The fucoxanthin content oscillates according to season and life cycle [2]. This carotenoid is linked to chlorophyll *a* and specific proteins of these marine plants, playing an important role on their light harvesting and photoprotection [3]. Its molecular structure includes an unusual allenic bond and oxygenic functional groups, that together constitute a unique arrangement [4] well developed to capture blue and green photons, predominantly present in deeper ocean waters [5]. Fucoxanthin's distinct molecular structure is also responsible for its exceptional biological activity, particularly for its antioxidant properties, that are mainly related with the free radical scavenging and singlet oxygen species quenching [1]. These properties are quite promising on the prevention and treatment of oxidative stress-related diseases [6]. Additionally, fucoxanthin also demonstrates anti-inflammatory, neuroprotective, antiangiogenic, skin protective, anti-obesity, anti-diabetic, anti-cancer, hepatoprotective, cardiovascular and cerebrovascular protective effects [1,7–10]. Recently, other pharmacological activities have been attributed to fucoxanthin, particularly promising for the therapy of the pulmonary fibrosis [9], cerebral ischemic/reperfusion injury [11], hyperglycemia, hyperlipidemia and insulin resistance [12], glycaemic control [13] and liver cancer [14]. These biological properties suggest its high potential for application in human and animal food, health and cosmetics. Thus, there is high interest not also in fucoxanthin high purity extracts but also in fucoxanthin rich supplements to be used as a natural antioxidant for food or beverages preservation.

Due to its high potential, research developments have been improving the industrial potential to purify fucoxanthin from algae biomass. Macroalgae *Saccharina japonica* (formerly *Laminaria japonica*)

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waste parts have been researched to determine their potential as a commercial-scale fucoxanthin resource using conventional extraction methods [15]. Later, new technological approaches such as pressurized liquid methodology [16] and supercritical CO_2/ethanol extraction [17] have been suggested to obtain larger quantities of extracts rich in fucoxanthin from macro and microalgae and to overcome high production costs, namely reducing solvent-usage and time. Other authors proposed microwave [18] and ultrasound [19] assisted techniques as alternatives. Different extraction conditions have also been investigated, varying the solvent type, solvent-to-solid ratio, extraction time, temperature and the extraction technologies, including maceration, ultrasound-assisted extraction, Soxhlet extraction and pressurized liquid extraction [3,20]. Most of these extraction procedures, besides being costly, time-consuming and labour-intensive, often use large amounts of solvent, generating waste and contaminating samples. Also, few studies are devoted to optimize a valid green analytical procedure for the fucoxanthin analysis in algae biomass.

Thus, the main purpose of this work was to develop a simple, fast, cost-effective and environmentally friendly analytical method to rapidly and efficiently assess the fucoxanthin content in seaweed biomass, to determine its potential as a fucoxanthin resource. Therefore, a vortex-assisted solid-liquid microextraction (VASLME) for sample preparation before HPLC-PDA quantification is proposed.

2. Materials and methods

2.1. Chemicals

All chemicals and standards had a purity grade higher than 95%. Methanol UPLC grade and formic acid were supplied by Panreac (Barcelona, Spain), ethanol by Aga (Portugal) and fucoxanthin standard from Sigma (China). Type 1 ultrapure water was obtained with a Simplicity® UV apparatus from Millipore (Milford, MA, USA).

2.2. Sample collection and preparation

Two seaweed biomass sample sets were collected. The first comprises brown macroalgae samples from Madeira archipelago (Portugal) and Galway (Ireland), collected between the intertidal and the subtidal zone up to a 10-meter maximum depth dive. *Dictyopteris polypodioides* (A.P.De Candolle) J.V. Lamouroux 1809: 332, *Dictyota dichotoma* (Hudson) J.V.Lamouroux 1809: 42, *Halopteris filicina* (Grateloup) Kützing 1843: 293, *Halopteris scoparia* (Linnaeus) Sauvageau 1904: 349, *Lobophora variegata* (J.V.Lamouroux) Womersley ex E.C.Oliveira 1977: 217, *Padina pavonica* (Linnaeus) Thivy in W.R.Taylor 1960: 234, *Sargassum vulgare* C. Agardh 1820: 3 and *Zonaria tournefortii* (J.V.Lamouroux) Montagne, 1846 were collected in Madeira archipelago (Porto Santo and Madeira Islands). *Ascophyllum nodosum* (Linnaeus) Le Jolis 1863: 96 and *Fucus vesiculosus* Linnaeus 1753: 1158 were collected in Galway. These samples were transported in seawater and gently rinsed with filtered fresh water except for Galway seaweed, which were air-dried. Afterwards, a primary drying was applied in which seaweed was frozen at −35 °C and freeze-dried under reduced pressure (4×10^{-4} mbar), with a cooling trap set at −56 °C for 5 days. Samples were milled to 200 mesh particle size, vacuum packed and stored at −35 °C until use. These samples were visually identified using the book publications performed by Cabioc'h et al. [21], Braune and Guiry [22], Rodríguez Prieto [23] and Pereira [24]. The second set is composed by 10 samples of beach-cast seaweed collected in the north shore of the island of Gran Canaria (Canary Islands), in “Playa de Las Canteras” from May 29 till October 10, 2017. These were air-dried, milled, packed and sent to our laboratory. The compositional details of these biomasses are presented in Table 1. Seaweed casts are masses of several seaweeds that stay stranded in beaches, affecting tourism, residents, local ecosystems and artisanal fishery. These macroalgae were identified using the book publications of Carrillo and Sansón [25], Haroun et al. [26] and Espino et al. [27].

2.3. Vortex Assisted Solid-Liquid Micro-Extraction optimization

A Central Composite Design (CCD) was implemented to determine the optimum conditions of three parameters of the analytical extraction procedure, namely: sample amount, solvent volume and vortex time. The selected solvent was ethanol at 96% and extraction performed with a Vortex Genie 2, from Scientific Industries. The brown macroalgae *Z. tournefortii* was used for the optimization and validation assessments. For more information, please consult the Supplementary material.

2.4. Chromatographic conditions

A Nexera X2 UHPLC system composed by two binary LC-30AD pumps, a DGU-20 A5 degassing unit, a CTO-20A column oven, a SIL-30AC autosampler and a PDA detector (200–800 nm) SPD-M20A was used for chromatographic analysis. The UV/Vis spectrum of fucoxanthin was used for identification and the 454 nm detection wavelength was used for quantification purposes. A gradient elution with methanol (solution A) and ultra-pure water acidified with 0.1% of formic acid (solution B) was used at 0.3 mL/min flow rate. The gradient started with 6 min of 20% solution A, then, it was gradually set up to 90% in 11 min changed to 100% in 1 min and maintained for 6 min. Finally, solution A was reduced to 5% in 1 min and held for 5 min to prepare the next injection, with a total injection time of 30 min. The mobile phases were previously filtered through a hydrophilic polypropylene 0.2 μm pore size membrane filter (Pall Corporation, Ann Arbor). Sample extracts were separated in a reversed phase Sunshell C18 column (150 × 2.1 mm, 2.6 μm) from ChromaNik Technologies Inc. (Osaka, Japan), thermostated at 30 °C, with an injection volume of 1 μL. All samples were extracted in triplicate and injected twice.

2.5. Method validation

The optimized methodology was validated, assessing linearity, sensitivity, matrix effects, selectivity, precision (repeatability and reproducibility) and accuracy. Linearity was calculated based on linear regression analysis, through correlation coefficient (R^2). Sensitivity was evaluated by the limit of detection (LOD) and limit of quantification (LOQ) according to Eqs. (1) and (2), respectively, where σ is the standard deviation of the y-intercept and *b* the curve slope.

$$LOD = 3.3\frac{\sigma}{b}3\sigma/b \quad (1)$$

$$LOQ = 10\frac{\sigma}{b} \quad (2)$$

For the calibration curve, eight working standard solutions were prepared within 12–3600 $\mu g \cdot g^{-1}$ dw concentration range, by spiking ethanol with the fucoxanthin standard solution.

Matrix effects were also evaluated, based on the ratio between two slope curves: one with the response of the direct injection of fucoxanthin standard working solutions (curve 1) and the other obtained from the extracts of the seaweed biomass (*Z. tournefortii*) spiked with the fucoxanthin standard working solutions (curve 2), as described by Matuszewski [28] (Eq. (3)).

$$\%ME = \left[\frac{(\text{slope of calibration curve 1} - \text{slope of calibration curve 2})}{\text{slope of calibration curve 1}}\right] \quad (3)$$

These analyses intended to determine if the seaweed matrix (ME - matrix effect) had influence on the fucoxanthin extraction.

The method selectivity was verified by checking the absence of interferences at the fucoxanthin retention time of the chromatograms of all samples and standard solutions.

Table 1
Composition of seaweed beach cast samples collected between May 29 and October 10, 2017, in "Playa de Las Canteras", Gran Canaria.

Seaweed code	Prospection date	Seaweed composition
1	29-May-2017	*Dictyota* sp. (32%), *H. incurva* (57%), *L. variegata* (10.7%) and others (0.3%)
2	23-Jun-2017	*A. taxiformis* (16.6%), *C. barbata* (8.4%), *Dictyota* sp. (30.6%), *Jania* sp. (30.5%) and *L. variegata* (13.9%)
3	26-Jun-2017	*A. taxiformis* (50%), *Dictyota* sp. (41.6%) and *H. scoparia* (8.4%)
4	12-July-2017	*A. taxiformis* (45%), *Dictyota* sp. (21%), *L. variegata* (25%) and *H. scoparia* (9%)
5	20-July-2017	*A. taxiformis* (34.8%), *Dictyota* sp. (39.1%), *Jania* sp. (4.3%) and *L. variegata* (21.8%)
6	8-Aug-2017	*A. taxiformis* (30%), *Dictyota* sp. (36%), *L. variegata* (24%) and *H. scoparia* (10%)
7	21-Aug-2017	*A. taxiformis* (28%), *Dictyota* sp. (42%), *L. variegata* (22%) and *H. scoparia* (8%)
8	18-Sep-2017	*A. taxiformis* (33.8%), *C. barbata* (14.5%), *Dictyota* sp. (22.6%), *Laurencia* sp. (0.5%) and *L. variegata* (28.6%)
9	6-Oct-2017	*A. taxiformis* (23.8%), *C. barbata* (22.2%), *Dictyota* sp. (22.2%) and *L. variegata* (31.8%)
10	10-Oct-2017	*C. barbata* (10%), *Dictyota* sp. (20%), *Jania* sp. (25%) and *L. variegata* (45%)

Repeatability and reproducibility were assessed by intra and inter-day analysis, respectively, of *Z. tournefortii* sample and two ethanol standard solutions of fucoxanthin (480 and 2400 $\mu g \cdot g^{-1}$ dw). The assessment of repeatability was obtained through the variation coefficient of ten successive extractions of these samples. The reproducibility was evaluated by the analysis of five extractions of the same samples in three different days, in a time span of 10 days. These results were expressed in percentage to the relative standard deviation (%RSD).

Accuracy was determined through the evaluation of a recovery study, spiking a macroalgae sample (*Lobophora variegata*) at three different fucoxanthin concentrations (24, 480 and 2400 $\mu g \cdot g^{-1}$ dw). Recovery was calculated according to Eq. (4) where SWS is the measured fucoxanthin concentration in a spiked sample, SW is the measured concentration in the sample and S is the concentration of fucoxanthin added to the sample.

$$\text{Recovery}\ (\%) = \frac{SWS - SW}{S} \times 100\ (\%) \tag{4}$$

Finally, the method was applied to 10 different seaweeds (12 samples) and 10 beach-cast seaweeds (10 samples) containing different species of brown seaweed in its composition, in order to confirm the applicability of the proposed methodology for the determination of fucoxanthin in seaweed biomass.

2.6. Extract stability

The extract stability was evaluated at 0, 5 and 10 days after extraction to determine if these extracts could be considered stable. *Z. tournefortii* extracts at two fucoxanthin concentrations, 480 and 2400 $\mu g \cdot g^{-1}$ dw, were used for this assay. These were kept at 10 °C in amber vials. Additionally, two ethanol fucoxanthin standard solutions at same concentration were also tested.

2.7. Statistical analysis

Samples were evaluated using two replicas and three injections, being expressed as mean of six measurements ± standard deviation. Definitive Screening Design (DSD) for design matrix and subsequent data analysis (model estimation and optimization) was achieved using the JMP® ver. 11.1.0 (32-bit) (SAS Institute Inc.).

3. Results and discussion

3.1. Vortex Assisted Solid-Liquid Micro-Extraction optimization

Methanol, acetone and ethanol are the most common solvents used for the extraction of marine pigments (Ragumaran et al. [19]). In this study, methanol was not considered due to its inherent toxicity (class 2 solvent). Kim et al. [3] tested different solvents (water, ethyl acetate, acetone and *n*-hexane) for the extraction of fucoxanthin from the diatom *Phaeodactylum tricornutum* and reported that the best extraction yield was obtained when high purity grade ethanol was used. Although ethanol (class 3 solvent – low toxicity) is not as widely used as acetone (also class 3 solvent) for the extraction of microalgae pigments, it has revealed greater yield for fucoxanthin extraction [3]. For all these reasons ethanol was chosen as the extraction solvent to develop the experimental layout. With the purpose of developing a green extraction procedure, solvent microvolumes were considered. In order to assist the microextraction, ultrasound and vortex were investigated. Tests with ultrasound bath revealed lack of repeatability, therefore, it was decided to proceed with vortex assistance. The use of small quantities of sample was also intended.

The design matrix to determine the best extraction conditions for fucoxanthin was established by a Central Composite Design (CCD), with three factors, volume of ethanol (μL), vortex time (min) and sample amount (mg) at three levels, with center points. For more information about the design, please consult the Supplementary material.

3.2. Method validation

The VASLME followed by HPLC-PDA method was validated for the rapid determination of fucoxanthin content in seaweed biomass. The results are expressed in Table 2. The validation parameters assessed were linearity, sensitivity, matrix effects, selectivity, precision and accuracy, after determining the optimal extraction conditions of fucoxanthin.

No matrix effect was observed, %ME was 3.4%. Therefore, the calibration curve adopted was the one performed by spiking fucoxanthin (Fx) stock solution in ethanol, according to Eq. (5).

$$\text{Fx area} = 2{,}306{,}667 \times \text{Fx concentration}\ \mu g \bullet g^{-1} + 6595 \tag{5}$$

A good correlation coefficient of R2 = 0.99998 was found, supporting the method linearity. Also, excellent sensitivity was obtained, LOD = 3.33 $\mu g \cdot g^{-1}$ and LOQ = 10.09 $\mu g \cdot g^{-1}$. These values are quite lower than the values typically found in macroalgae (about

Table 2
Validation results for VASLME methodology to quantify fucoxanthin in seaweed.

	Parameter	Result
Linearity	Linear regression (y = mx + b)	2,306,667x + 6595
	Linear concentration range	12–3600 $\mu g \cdot g^{-1}$
	R^2	0.99998
Sensitivity	LOD ($\mu g \cdot g^{-1}$)	3.33
	LOQ ($\mu g \cdot g^{-1}$)	10.09
Accuracy	Recovery	%
	SW + 24 $\mu g \cdot g^{-1}$	96
	SW + 480 $\mu g \cdot g^{-1}$	95
	SW + 2400 $\mu g \cdot g^{-1}$	97
Precision	Intra-day (% RSD)	2.0–3.3
	Inter-day (% RSD)	1.0–3.8

All determinations were the result of two replicas each injected three times. LOD – limit of detection; LOQ – limit of quantification; SW – seaweed; RSD – relative standard deviation.

Table 3
Seaweed samples for fucoxanthin yield testing.

Seaweed	Collection site	Prospection date	Fucoxanthin content ($\mu g \cdot g^{-1}$ dw ± SD)
A. nodosum	Galway (Ireland)	Jun/2018	21.6 ± 0.9
D. dichotoma	Porto Santo	Mar/2017	12.2 ± 0.4
D. dichotoma	Madeira	Aug/2018	514 ± 5
D. polypodioides	Madeira	Aug/2018	597 ± 30
F. vesiculosus	Galway (Ireland)	Jun/2018	22 ± 1
H. filicina	Porto Santo	Mar/2017	17.3 ± 0.3
H. scoparia	Madeira	Jan/2016	10.1 ± 0.3
L. variegata	Porto Santo	Mar/2017	40.9 ± 0.8
P. pavonica	Madeira	July/2016	10.2 ± 0.3
S. vulgare	Madeira	Jun/2017	400 ± 14
Z. tournefortii	Madeira	Aug/2016	852 ± 12
Z. tournefortii	Madeira	July/2017	381 ± 3

Data are mean ± standard deviation in micrograms of fucoxanthin per 1 g of algae on a dry weight basis (dw). All determinations were the result of two replicas each injected three times. dw – dry weight; SD – standard deviation.

100–1000 $mg \cdot g^{-1}$ dw) [29]. The method also revealed good precision: 2 to 3.3% of variation in intra-day analyses and 1 to 3.8% for the inter-day analysis. Additionally, the results of the recovery study ranged between 95 and 97%, as summarized in Table 2, demonstrating the accuracy of the method.

After assessing the figures which resulted from the Response Surface Methodology (RSM) assessed in this work and are presented as Supplementary material, fucoxanthin was quantified in 22 samples of algae biomass. The fucoxanthin contents (Tables 3 and 4) were found to vary between 10.1 ± 0.3 and 852 ± 12 $\mu g \cdot g^{-1}$ dw, which are within the calibration range. Fig. 1 shows the typical chromatograms (Fx retention time at 19.9 min) of a standard solution (240 $\mu g \cdot g^{-1}$ dw) and a seaweed biomass sample (brown algae), confirming the method selectivity. It also revealed that the variation between sample replicates never exceeded the 7%, even at levels close to LOQ.

3.3. Extract stability

Sample extracts kept at 10 °C, enclosed in amber vials, were tested for stability and considered suitable due to the low variation detected between initial (0 days), middle (5 days) and the end (10 days) of the assay. For *Z. tournefortii*, the variation of fucoxanthin content was 0.8% and 0.3% for 5 and 10 days, respectively. The fucoxanthin ethanolic solution at 480 $\mu g \cdot g^{-1}$ dw was found to vary 1.3% and 4.3% in 5 and 10 days respectively. For the fucoxanthin solution with 2400 $\mu g \cdot g^{-1}$ concentration, the variation was found to be 0.7% in both dates.

These results indicate that ethanolic extracts of fucoxanthin are stable in the 10 days after sample extraction, regardless of its concentration. Thus, an accurate evaluation of the fucoxanthin concentration in seaweed biomass can be obtained at least for 10-day period at 10 °C and light protected. This result is very important not only for performing the simultaneous extraction of multiple samples and stock before HPLC analysis, but also for eventual industrial applications.

This methodology enables to accurately assess the fucoxanthin content in macroalgae biomass, using small quantities of biomass, allowing sample shipping to dedicated laboratories and perform several extractions at once, reducing extraction time. Stability of the extract is favourable since sample sets could be extracted and analysed with precision within 10 days when kept at 10 °C in amber vials. Table 5 summarizes some fucoxanthin analysis methods. These are compared for the quantity of algal resource needed, solvent and volume used for extraction, health and environmental concerns relating the use of these solvents, extraction methodology and analysis apparatus. The resulting ratio (solvent/algal quantity) presented by other works are usually greater than this work, using higher solvent quantities to extract fucoxanthin from selected samples, producing more waste. Some of these solvents are health concerns or environmentally aggressive, being necessary safer and greener options. Also, simplicity is achieved when smaller quantities of algae, solvent and fewer steps are needed to perform fucoxanthin analysis, resulting in larger number of samples, which can be handled at the same time. Performing the methodology described in this work, it is possible to analyse 50 individual vials in a 3 day period.

Table 4
Seaweed beach casts tested for fucoxanthin concentration.

Seaweed beach cast code	Prospection date	Fucoxanthin concentration ($\mu g \cdot g^{-1}$ dw ± SD)
1	29-May-2017	20.3 ± 1.3
2	23-Jun-2017	14.2 ± 0.6
3	26-Jun-2017	13,2 ± 0,51
4	12-July-2017	33.3 ± 0.8
5	20-July-2017	27.6 ± 1.7
6	8-Aug-2017	32.6 ± 1.2
7	21-Aug-2017	19.6 ± 1.0
8	18-Sep-2017	49.4 ± 2.0
9	6-Oct-2017	22.9 ± 0.6
10	10-Oct-2017	28.4 ± 1.1

Data are mean ± standard deviation in micrograms of fucoxanthin per 1 g of algae on a dry weight basis (dw). All determinations were the result of two replicas each injected three times. dw – dry weight; SD – standard deviation.

3.4. Fucoxanthin content in seaweed biomass

Table 3 reports the evaluation results of 12 samples of 10 different marine brown macroalgae species. The fucoxanthin contents varied significantly between samples, from 10.1 ± 0.3 $\mu g \cdot g^{-1}$ dw to 852 ± 12 $\mu g \cdot g^{-1}$ dw. Samples with high fucoxanthin concentrations, varying from 400 ± 14 to 852 ± 12 $\mu g \cdot g^{-1}$ dw (*Z. toutournefortii*, *D. polypodioides*, *D. dichotoma*, *S. vulgare*), were all collected in Madeira Island seas. These species are comparable to those found by Jaswir et al. [30] in *Sargassum aquifolium* (as *Sargassum binderi*) and *Sargassum ilicifolium* (as *Sargassum duplicatum*), collected in the Straits of Malacca, near Port Dickson (Malaysia), between 730 and 1010 $\mu g \cdot g^{-1}$ dw of fucoxanthin and higher contents than those reported by Kim et al. [3] in *Ecklonia bicyclis* (as *Eisenia bicyclis*) collected in South Korea (260 $\mu g \cdot g^{-1}$ dw). On the other hand, the majority of the brown macroalgae samples evaluated exhibited low fucoxanthin contents (lower than 40.9 ± 0.8 $\mu g \cdot g^{-1}$ dw). It is interesting to notice that this result cannot be attributed to the species, since the same species revealed different concentration levels at different locations (*D. dichotoma*) and collection dates (*Z. tournefortii*). This result might also be related to seasonality, as it has been recently published [6]. Table 4 presents the screening results of 10 beach-cast seaweeds, composed by different brown seaweeds and others (Table 1), sampled in "Playa de Las Canteras", Gran Canaria within the studied collecting period (May 29 and October 10, 2017). The fucoxanthin content was found to vary between 13.2 ± 0.5 $\mu g \cdot g^{-1}$ dw in sample 3 (June 26, 2017) and 49 ± 2 $\mu g \cdot g^{-1}$ dw in sample 8 (September 18, 2017). The fucoxanthin levels of this biomass resource varies along the year and are even comparable to some brown macroalgae samples collected in Madeira Islands and Galway. These algae wastes can be valorised as a relevant bioresource of high-valued bio-compounds for eventual industrial applications, namely through the implementation of biorefinery strategies.

4. Conclusion

A simple and reliable analytical method was successfully optimized and validated to quickly quantify fucoxanthin in seaweed biomass, using reduced amounts of sample and extraction solvent. Simultaneous

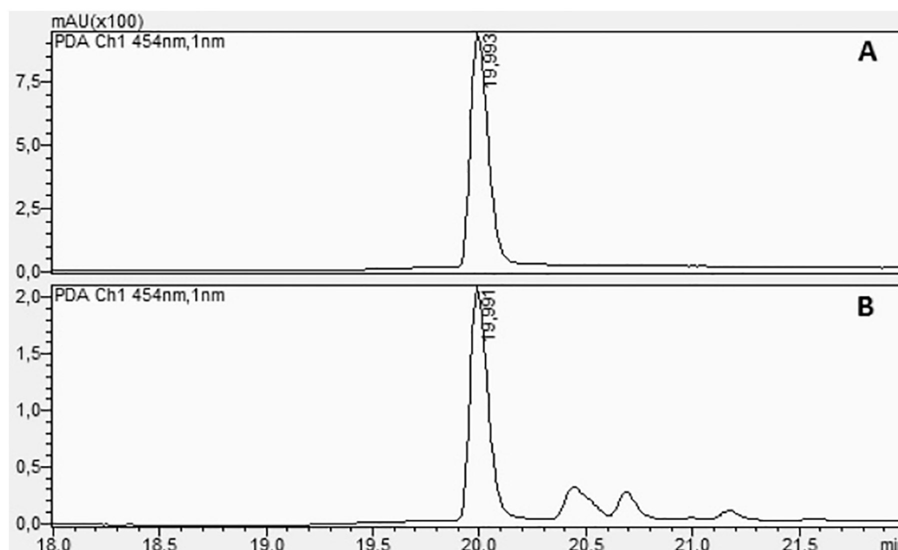

Fig. 1. Chromatograms of a fucoxanthin standard solution of 240 $\mu g \cdot g^{-1}$ dw (A) and a brown seaweed sample (B).

Table 5
Summarized methods for fucoxanthin analysis.

Reference	Quantity of algal resource	Solvent and volume	Health and environmental concerns[a]	Extraction methodology	Analysis apparatus (duration)
Present work	25 mg (dw)	Ethanol (96%), 0.3 mL	9/10–7/10	Vortex assisted extraction (RT, 15 min)	HPLC-PDA (21 min)
Pasquet et al. [18]	50 mg (dw)	Acetone, 30 mL	6/10–7/10	1. Soaking (RT, 120 min); 2. Hot soaking (56 °C, 120 min); 3. UAE (12,2 W, 10 min); 4. VMAE and MAE (50 W, 5 min)	HPLC-PDA (20 min)
Petrushkina et al. [31]	10 mg (dw)	Acetonitrile, 1 mL	2/10–4/10	Shaken (1800 rpm, 20 min)	HPLC-PDA (15 min)
Sun et al. [32]	4 g (dw)	Methanol, 120 mL	4/10–8/10	Vortex assisted extraction (20 min)	UPLC-PDA-TWIMS-QTOF-MS (16 min)

RT – room temperature; UAE – ultrasound assisted extraction; VMAE – vacuum microwave assisted extraction; MAE – microwave assisted extraction; UPLC-PDA-TWIMS-QTOF-MS – ultra performance liquid chromatography coupled to photodiode array detector/travelling wave ion mobility mass spectrometry/quadrupole time-of-flight mass spectrometry.
[a] Retrieved from Curzons et al. [33], higher numbers are preferable.

seaweed biomass VASLME can be performed prior to RP-HPLC-PDA analysis, which takes 30 min. CCD was crucial to optimize the fucoxanthin extraction yield, pointing out that 25 mg of sample with 300 μL of ethanol and vortexed for 15 min are the best experimental combination for the proposed sample preparation procedure. Good results were obtained for all the validation parameters, particularly in terms of sensitivity (LOQ = 10.09 $\mu g \cdot g^{-1}$) and precision (maximum variation of 3.8%). Ethanolic extracts are stable at least for a 10-day period at 10 °C and light protected, allowing the simultaneous extraction of multiple samples and stock them before HPLC analysis. The method proved to be an accurate tool for the evaluation of the fucoxanthin concentration in seaweed biomass, as it was demonstrated by the analysis of 22 samples. Fucoxanthin concentration was found to vary from about 10 to 852 $\mu g \cdot g^{-1}$ dw.

Samples collected in Madeira presented the highest contents (400–852 $\mu g \cdot g^{-1}$ dw), namely those from *Z. tournefortii*, *D. polypodioides*, *D. dichotoma* and *S. vulgare* brown macroalgae. In beach-cast seaweeds from Gran Canaria the fucoxanthin levels (< 49 $\mu g \cdot g^{-1}$ dw) are comparable to some brown macroalgae samples collected in Madeira Islands and Galway. This information can contribute to the development of sustainable strategies to valorise these algae wastes as a relevant bioresource of high-valued bio-compounds for eventual industrial applications.

Declaration of author's contribution

1) Nuno Nunes contribution included analysis and interpretation of the data and wrote the article.
2) João M. Leça also performed the analysis and interpretation of data.
3) Ana C. Pereira contributed with statistical expertise and obtaining of funding.
4) Vanda Pereira contributed with drafting of the article and critical revision of the article for important intellectual content.
5) Sónia Ferraz provided the study materials, obtained funding and revised it critically.
6) Maria do Carmo Barreto performed a critical revision of the article for important intellectual content and obtaining of funding.
7) José C. Marques performed a critical revision of the article for

important intellectual content and Final approval of the article.

8) M.A.A. Pinheiro de Carvalho also performed a critical revision of the article for important intellectual content and final approval of the article.

Also, Nuno Nunes, Vanda Pereira, José C. Marques and M.A.A. Pinheiro de Carvalho take full responsibility for the integrity of the work as a whole.

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

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