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Optimisation of fucoxanthin extraction from Irish seaweeds by response surface methodology

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Abstract Fucoxanthin is a xanthophyll pigment which occurs in marine brown algae (Phaeophyceae). The anti-diabetic, anti-obesity, anti-cancer, and antioxidant properties of fucoxanthin have been widely reported. Macroalgae, particularly brown seaweeds, grow prolifically around Irish coasts, representing a valuable resource of nutraceuticals such as fucoxanthin for functional food applications. The aim of this study was to maximise the solvent extraction yield from three anatomically discrete regions of the seaweed thallus: blade, stipe, and holdfast. Response surface methodology was applied to determine optimum parameters for extraction of fucoxanthin from the seaweed, *Fucus vesiculosus*, as a model species. A central composite design was applied with four extraction variables: time (30–70 min), temperature (30–70 °C), solvent pH (5.0–9.0), and percentage acetone (30–70 %). Fucoxanthin content of extracts was quantified by high-performance liquid chromatography. Percentage acetone was found to have the most significant (P = 0.0002) effect on fucoxanthin yield, followed by pH (P = 0.028) and temperature (P = 0.049). Multiple response optimisation determined that fucoxanthin yield from *F. vesiculosus* may be maximised by incubating at 30.0 °C for 36.5 min, pH 5.7, with 62.2 % acetone. Optimised responses were applied to a further nine brown seaweeds; *Alaria esculenta*, *Ascophyllum nodosum*, *Fucus serratus*, *Himanthalia elongata*, *Laminaria digitata*, *Laminaria hyperborea*, *Pelvetia canaliculata*, *Saccharina latissima*, and *Saccorhiza polyschides*. In all species, the blades contained significantly more fucoxanthin than stipes, while holdfasts contained the least. *Alaria esculenta* blade had the greatest yield (0.870 mg g^{-1} dry mass), followed by *F. vesiculosus* blade (0.699 mg g^{-1}) and *L. digitata* blade (0.650 mg g^{-1}).

Keywords Fucoxanthin · Nutraceutical · Irish brown seaweeds · Yield optimisation by response surface methodology · High-value algal bioactives · Antioxidant

Introduction

Fucoxanthin is a light-harvesting carotenoid that occurs in the chloroplasts of the eukaryotic Chromalveolata (Phylum Ochrophyta), including brown macroalgae, or seaweeds, (Phaeophyceae), and microalgal diatoms (Bacillariophyceae) (Cavalier-Smith and Chao 2006). Fucoxanthin is estimated to account for more than 10 % of the total production of carotenoids in nature, and is responsible for the brown to yellow colour of brown macroalgae (seaweeds) and brown microalgae (diatoms) (Hurd et al. 2014). Fucoxanthin was first isolated in Germany in 1914 from *Dictyota*, *Fucus*, and *Laminaria* (Willstätter and Page 1914). Industrially, Japanese Wakame (*Undaria pinnatifida*) is the most widely utilised seaweed for fucoxanthin extraction due to high concentrations of the pigment in the lipid extract (up to 9.6 % of total lipids) (Maeda et al. 2005). Irish coastlines support the growth of brown seaweeds. The total average annual value of seaweed production in Ireland has been estimated at €23 million. Approximately 36,000 t of seaweed is harvested in Ireland each year, the majority of which is from brown

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species, which is used primarily as fertiliser or incorporated into livestock feed. Only 1 % is processed into higher value nutraceuticals, cosmetics, and foods. However, this small percentage accounts for ~30 % of the total commercial value (Stengel and Dring 1998; Walsh and Watson 2011; Dring et al. 2013; Walsh 2016). The economic justification for this study is the high commercial value of fucoxanthin. In 2015, world fucoxanthin production reached approximately 500 t, with an expected increase of at least 5.3 % per annum between 2016 and 2021 (Joel 2016). The global market for total carotenoids rose in value from US$1.20 billion in 2010 to 1.5 billion in 2014. Although some of these carotenoids are synthetically derived, a substantial section of this market constitutes naturally produced compounds such as β-carotene and astaxanthin a xanthophyll chemically similar to fucoxanthin (Borowitzka 2013; Ulrich 2015). As a functional food, fucoxanthin has already been incorporated as an ingredient in products such as pasta, biscuits, and dips by a number of food companies worldwide (Prabhasankar et al. 2009; Oryza 2011). Fucoxanthin supplements are generally recognised as safe by the European Food Safety Authority, Japan's Food for Specified Health Uses, and the US Food and Drug Administration. The broad health applications of fucoxanthin have only emerged in recent years. It has remained underutilised in food and pharmaceutical applications possibly due to its oxidative instability and the prohibitive costs of inefficient extraction methods. A leaner, efficient extraction methodology has the potential to increase product yield.

Fucoxanthin has been studied clinically for its efficacy against many diseases. It has been shown in vivo to have activity against cancer (Nakazawa et al. 2009; Jaswir et al. 2013), type II diabetes (Oh et al. 2016), obesity (Maeda et al. 2007), cholesterol (Beppu et al. 2012), inflammatory disorders (Shiratori et al. 2005), tumour angiogenesis (Martin 2015), malaria (Briglia et al. 2015), hypertension (Sivagnanam et al. 2015), and as a β-secretase 1 inhibitor in Alzheimer's disease (Jung et al. 2016). Epidemiological data has suggested that the regular consumption of seaweeds can reduce the risk of developing diseases associated with oxidative stress. As a dietary antioxidant, fucoxanthin improves the antioxidant capacity of blood serum levels (Kang et al. 2014), and is capable of quenching reactive oxygen species under hypoxic physiological conditions, unlike the majority of food-derived antioxidants (Kaneko et al. 2013). Reactive oxygen species are known to cause cellular damage, which is implicated in the pathogenesis of disorders such as cardiovascular disease, cancer, metabolic syndrome, and type II diabetes. A study by Zaragozá et al. (2008) on the antioxidant effect of an extract of *Fucus vesiculosus* containing 0.0012 % fucoxanthin, found that antioxidant activity increased in ex vivo assays of erythrocytes and plasma, after 4 weeks of daily oral administration in rats. Significant antioxidant activity was also observed in non-cellular systems and in activated RAW 264.7 mouse leukaemic monocyte macrophage cell lines. Fucoxanthin's anti-diabetic activity has been studied in mice with induced type II diabetes. It has been shown to improve insulin resistance and decrease blood glucose levels via regulation of cytokine secretions from white adipose tissue, and by promoting the recovery of blood glucose uptake to muscle by the up-regulation of GLUT4 mRNA expression. Fucoxanthin has also been shown to affect the peroxisome proliferator-activated receptor γ (PPARγ) and promote gene expression related to lipid metabolism in adipocytes. In cultivated cells, fucoxanthin prevented inflammation and insulin resistance by inhibiting nitric oxide and PGE2 production through the down-regulation of iNOS and COX-2 mRNA expression; as well as adipocytokine production in white adipose tissue (Maeda et al. 2007; Mikami and Hosokawa 2013). A number of human clinical trials with fucoxanthin have been reported. For example, in Japan, a Kombu (*Saccharina japonica*) extract of 3 % fucoxanthin was evaluated for its anti-metabolic syndrome activity in a human clinical trial. A daily dosage of the extract, equivalent to 0.5–1.0 mg pure fucoxanthin day^{-1}, was found to have a significant effect on blood serum parameters related to metabolic syndrome (Oryza 2011). Abidov et al. (2010) conducted a double-blind placebo-controlled study at the Russian Academy of Medical Sciences of 115 non-diabetic, obese, premenopausal women with a liver fat content above 11 %. A daily supplement of 300 mg brown seaweed extract containing 2.4 mg fucoxanthin, combined with 300 mg pomegranate seed oil was administered. An olive oil capsule was administered to the placebo group. The treatment group showed a significant increase in resting energy expenditure and mean weight loss of 4.9 kg after 16 weeks. No toxicity of fucoxanthin extracts has been reported to date, making it an excellent candidate for nutraceutical applications (Maeda et al. 2007; Zaragozá et al. 2008) (Fig. 1).

Fucoxanthin is a xanthophyll and shares some chemical and physical properties with carotenes, such as lipophilicity and antioxidant activity due to their ability to quench reactive oxygen and nitrogen species. However, the presence of oxygen in the hydroxyl and epoxide groups of xanthophylls makes them more polar than carotenes (Landrum 2009). Fucoxanthin can exist in a *trans* or *cis* configuration. The *trans* isomer is the more chemically stable and potent antioxidant of the two, and comprises ~90 % of the fucoxanthin found in nature (Nakazawa et al. 2009). In industry, fucoxanthin is most commonly extracted with solvents such as hexane, methanol, DMSO, ethanol, petroleum ether, diethyl ether, dimethyl ether, acetone, or ethyl acetate, and dried to a powder (Kanda et al. 2014). In algal cells, fucoxanthin is contained in the chloroplasts, within membrane-bound compartments called thylakoids. Fucoxanthin is produced most significantly in the blade of seaweeds, where the majority of photosynthesis occurs due to maximum light exposure at the ocean surface.

(Lobban and Harrison 1994; Kita et al. 2015; Schmid and Stengel 2015). Intra-species fucoxanthin content varies widely. For example, Roh et al. (2008) reported *Undaria pinnatifida* (lyophilised stipe and blade combined) to contain 0.00048 μg g^{-1} (dm) (dry mass) fucoxanthin, while Fung et al. (2013) found 4.96 mg g^{-1} (dm) (lyophilised blade) in the same species. Both intra, and inter, species differences such as this can be attributed to seasonal variations, geographic location, nutrient availability, exposure to sunlight, ontogenetic effects, and extraction methods, (Fung et al. 2013; Gosch et al. 2015; Terasaki et al. 2016).

The aim of this study was to maximise the organic solvent solid-liquid extraction of fucoxanthin from seaweed, using *F. vesiculosus* as a model species, quantify by HPLC, and to apply optimised responses to the holdfast, stipe, and blade of a further nine Irish brown seaweeds.

Materials and methods

Chemicals

Fucoxanthin standard (all-*trans*-fucoxanthin), acetone, hydrochloric acid, ethanol, and methanol were from Sigma-Aldrich (Rep. of Ireland). Ammonium acetate was from BDH Laboratory Supplies (Poole, UK).

Samples

Ten species of Irish brown seaweeds were selected for the study, based upon previously reported fucoxanthin content and commercial availability. Seaweeds were harvested and delivered fresh and whole within 24 h, kept at a constant storage temperature of 4 °C, in darkness. *Fucus vesiculosus*, *Alaria esculenta*, *Ascophyllum nodosum*, *Fucus serratus*, *Himanthalia elongata*, *Laminaria digitata*, *Laminaria hyperborea*, *Pelvetia canaliculata*, *Saccharina latissima*, and *Saccorhiza polyschides* were purchased from Quality Sea Vegetables, Burton Port, Co. Donegal, Rep. of Ireland. Authentication of species was provided by the supplier. Samples were harvested at low tide, in the intertidal area, between the high and low water marks, from the north-western coast of Ireland (54.9823° N, 8.4343° W) in mid July 2015 at mean monthly air and seawater temperatures of 14.5 °C.

Initial solvent trials and RSM range determination for *F. vesiculosus* extraction

Initial trials with ethanol, methanol, DMSO, acetone, hexane, and ethyl acetate determined acetone to have the greatest extraction efficiency for the model species, *F. vesiculosus* holdfast, stipe, and blade. Pre-RSM extraction trials for incubation time, temperature, solvent pH, and percentage acetone were carried out in the following ranges: 30 min–10 h (increments of 30 min), 20–100 °C (increments of 10 °C), solvent pH 5.0–9.0 (increments of 1.0), and 0–100 % acetone (increments of 10 %). pH was adjusted with HCl (0.01 M), based upon previously published extraction protocols. Quantification of fucoxanthin by HPLC determined that there was no statistically significant ($P \geq 0.05$) increase in *F. vesiculosus* fucoxanthin content below or above the following ranges: time (30–70 min), temperature (30–70 °C), solvent pH (5.0–9.0), and acetone (30–70 %). Accordingly then, these ranges were used as the upper and lower limits for the RSM design of experiment.

Response surface methodology design

Response surface methodology was selected as the statistical method as it has been successfully applied in this laboratory for the optimisation of bioactive extraction from a variety of seaweed types. To investigate the effect of factors (incubation time, temperature, pH, and percentage acetone) on the extraction efficiency of fucoxanthin from *F. vesiculosus* (blade), a 2^4 + star central composite design was applied using Statgraphics Centurion XV (StatPoint Technologies Inc., USA). The following equation (Eq. 1) was used to calculate the total number of designed experiments where k is the number of independent variables.

$$N = 2^k + 2k = n_0 \tag{1}$$

Variance analysis was conducted and the following binary quadratic equation (Eq. 2) was constructed where y is the predicted response, B_0 is the intercept term, B_i is the linear coefficient, B_{ij} are the quadratic coefficients, and X_i and X_j are the levels of the independent variables:

$$y = B_0 + \sum_{i=1}^{3} B_i X_i + \sum_{i=1}^{3} B_{ii} X_i^2 + \sum_{i-1}^{3} \sum_{j=i+1}^{3} B_{ij} X_{ij} \tag{2}$$

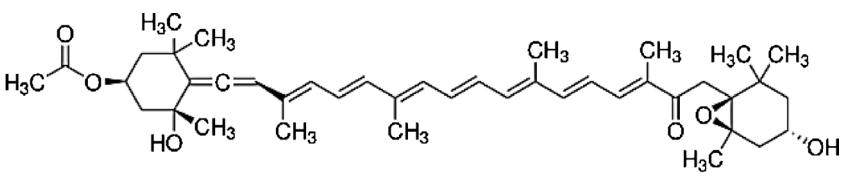

Fig. 1 Molecular structure of fucoxanthin

Four variables were used in this study, therefore values of i and j ranged from 1 to 4. Using a 2^4 + star central composite design with four independent variables, 28 variable combinations in experimental runs were generated by the design. The effects of unexpected variability in the observed responses was minimised by randomisation.

Experimental data generated from the central composite design were fitted to a second-order polynomial regression model (Eq. 3) where Y is the predicted response (here, fucoxanthin), and X_1 (temperature), X_2 (time), X_3 (solvent pH), and X_4 (% acetone) are the coded values of the independent variables.

$$\begin{aligned} Y = {} & B_0 + (B_1X_1) + (B_2X_2) + (B_3X_3) + (B_4X_4) \\ & + \left(B_{12}{X_1}^2\right) + (B_{13}X_1X_2) + (B_{14}X_1X_3) \\ & + (B_{23}X_1X_4) + \left(B_{24}{X_2}^2\right) + (B_{34}X_3X_4) \\ & + (B_{11}X_2X_4) + \left(B_{22}{X_3}^2\right) + (B_{33}X_3X_4) + \left(B_{44}{X_4}^2\right) \end{aligned} \quad (3)$$

RSM statistical analysis Statistical interpretation of experimental data generated by the model was evaluated by analysis of variance (ANOVA) and coefficient of determination, R^2, which measures goodness of fit of the regression model. Regression analysis and response surface plotting was performed to establish optimum parameters for the extraction of fucoxanthin. For each independent variable, quadratic models were represented as response surface optimisation plots. Significance of the model and data was determined at the 95.0 % confidence level ($\alpha = 0.05$).

Sample preparation

Immediately upon delivery, fresh, raw seaweeds were placed in a colander and thoroughly rinsed with cold running tap water (Dublin city mains) to remove epiphytes and debris. With a clean knife, each thallus was divided into its three morphologically discrete sections; holdfast, stipe, and blade; and chopped into 2 cm pieces. Samples were frozen to −80 °C. Aliquots of frozen samples (~10 g) were crushed in a mortar and pestle with liquid nitrogen and stored at −80 °C until extraction.

Extraction procedure using RSM optimised parameters

The extraction procedure was based upon protocols optimised in this laboratory for seaweed (Rajauria et al. 2013). Extraction parameters optimised by RSM for *F. vesiculosus* were used for all samples.

Raw seaweed and solvent were combined in a ratio of 1:10 (*w*/*v*). Nitrogen crushed seaweed (2 g) was incubated in an orbital incubator shaker with acetone (20 mL, 62.2 %, pH 5.7) in a flask covered with Parafilm for 36.5 min at 30 °C, 100 rpm, in the dark. The flask contents were transferred to Nalgene tubes and centrifuged (10 min, 12,000 × *g* 4 °C). The supernatant was retained. The pellet was washed and centrifuged six times with acetone (20 mL, 62.2 %, pH 5.7). The pooled supernatant was filtered (Grade 1 filter paper, 11 μm pore, Whatman, UK) and reduced by evaporation (Laborota 4002 Heidolph rotary evaporator) at 30 °C to 5 mL. Extracts were frozen at −80 °C for HPLC analysis.

Quantification of fucoxanthin

Preparation of seaweed extract stock solutions Frozen seaweed extracts were lyophilised to a powder (0.02 mbar, 50 °C, Labconco Freeze-Drier) for 24 h. Stock solutions of seaweed extracts were prepared for HPLC analysis by dissolving lyophilised seaweed extract (100 mg) in acetone (10 mL, 62.2 %). Samples were syringe-filtered (Sigma-Aldrich Millex Durapore PVDF 0.22 μm pore, 13 mm diam.) into HPLC vials (Waters 2 mL LCGC certified clear glass 12 × 32 mm screw neck vial, with pre-slit PTFE/silicone septa cap).

HPLC-DAD analysis of fucoxanthin Chromatographic analysis of seaweed extracts was carried out according to a modified method developed by Billakanti et al. (2013). Fucoxanthin separation was achieved with HPLC (Alliance-Waters e2695 Separations Module, 400 atm pressure, at 4 °C), equipped with a C_{18} reverse phase column (Waters XSelect, 4.6 mm × 100 mm, 3.5 μm particle size), and a UV photodiode array detector (Waters 2998). Two mobile phases were determined to be optimal for HPLC-DAD analysis of the seaweed extracts. These were solvent A: 20 mM sodium acetate; and solvent B: 100 % methanol. Before use, ddH_2O water was membrane filtered (Merck Millipore Simplicity 185). Mobile phases were filtered (Merck Millipore HVLP 0.45 μm filter) and sonicated (Branson 5510 Ultrasonic Cleaner). Injection volume was 20 μL, with a constant flow rate of 0.15 mL min^{-1}. Detection was performed at 449 nm. A 60-min gradient programme was used, at a constant temperature of 60 °C. Analysis was carried out in triplicate.

HPLC-DAD data analysis Commercial fucoxanthin standard solutions were prepared in concentrations of 5, 10, 20, 30, 40, 50, and 100 μg mL^{-1} in ethanol. The area under the peak (AUP) of those corresponding with retention times for fucoxanthin standards was plotted against concentration (μg mL^{-1}) to make a standard curve. The regression equation was obtained as y = 1,000,000x + 1,000,000. The R^2 value was 0.999. The concentration of fucoxanthin in the seaweed samples was extrapolated from the equation generated.

Statistical analysis

All experiments were conducted in triplicate (n = 3) and replicated at least twice. Results are expressed as mean values ± standard deviation. All statistical analyses and data were fitted to models using Statgraphics Centurion XV. The coefficient of determination (R^2) and mean square error (MSE) were used as criteria for adequacy of fit. Multiple range tests were used to determine least significant differences between samples at the 95.0 % confidence level (α = 0.05).

Results

RSM optimisation of acetone extraction of fucoxanthin from *F. vesiculosus*

Using a 2^4 + star central composite design with four independent variables, 28 experimental runs were generated, with variable combinations of temperature, time, solvent pH, and percentage acetone. Estimation results for fucoxanthin are presented in Table 1, showing the observed, or actual value of fucoxanthin, the predicted value of fucoxanthin using the fitted model, and 95.0 % confidence limits (upper and lower) for the mean response. Experimental run no. 13 showed the highest extraction efficiency of 0.696 ± 0.02 mg g^{-1} (dm) fucoxanthin. The parameters for this run were 40 °C, 40 min, solvent pH 6.0, and 60 % acetone.

The regression equation fitted to the experimental data is shown in Eq. 4, where Y is the predicted response (fucoxanthin), and X_1 (temperature), X_2 (time), X_3 (solvent pH), and X_4 (% acetone) are the coded values of the independent variables.

$$\begin{aligned} Y = {} & -2.15312-(0.0342417*X_1)-(0.00264167*X_2) \\ & +(0.6535*X_3)+(0.05435*X_4)-\left(0.000223333*X_1{}^2\right) \\ & +(0.00044375*X_1*X_2)+(0.0033875*X_1*X_3) \\ & +(0.0000975*X_1*X_4)-\left(0.000395833*X_2{}^2\right) \\ & +(0.0015625*X_3*X_4)+(0.00015*X_2*X_4) \\ & -\left(0.0649583*X_3{}^2\right)-(0.00115*X_3*X_4) \\ & -\left(0.000452083*X_4{}^2\right) \end{aligned} \quad (4)$$

Analysis of variance partitioned the variability in fucoxanthin yield for each of the four independent variables. The statistical significance of each effect, and their interaction amongst each other, was determined by comparing the mean square against an estimate of the experimental error. Three effects, temperature, solvent pH, and percentage acetone were found to be significant. Percentage acetone had the most significant effect on fucoxanthin yield with a P value of 0.0002, followed by solvent pH (P = 0.0284), and temperature (P = 0.0492). Incubation time (P = 0.5599) was not found to be significant at the 95.0 % confidence level. The R-squared statistic indicated that the model as fitted explained 78.58 % of the variability in fucoxanthin yield in *F. vesiculosus*. The standard error of the estimate showed the standard deviation of the residuals to be 0.13, with the average value of the residuals expressed as a mean absolute error of 0.08.

The combination of factor levels required to maximise fucoxanthin yield in *F. vesiculosus* blade were determined to be 30.00 °C, 36.51 min, solvent pH 5.70, and 62.15 % acetone. An optimum value of 0.745 mg g^{-1} (dm) was predicted. The greatest observed value obtained was 0.696 ± 0.02 mg g^{-1} (dm) in experimental run no. 13. This equates to 93.30 % of 0.746 mg g^{-1}, which is in good agreement with the predicted optimum value.

Mathematical modelling

Response surface plots (Fig. 2) were constructed according to the modelled experimental data. In each case, the effects of two variables on fucoxanthin yield were depicted in three dimensional surface plots while the two other variables were kept constant at zero level.

HPLC separation of fucoxanthin

Separation of fucoxanthin was achieved at 449 nm with a C_{18} reverse phase column and a UV photodiode array detector. A 60 min gradient programme at a constant temperature of 60 °C was used with two mobile phases, (A) 20 mM sodium acetate, and (B) 100 % methanol. Figure 3 illustrates the separation of fucoxanthin in an overlay chromatogram at 39.89 min in (A) all-*trans*-fucoxanthin standard peak (10 μg mL^{-1}); (B) *F. vesiculosus* blade; (C) two isomers of cis-fucoxanthin; and (D) an unidentified compound, possibly zeaxanthin.

As discussed in the Introduction, fucoxanthin can exist in a *trans* or *cis* configuration. Peak A in Fig. 3 corresponds with those of the all-*trans*-fucoxanthin standard. It was expected that approximately 10 % of fucoxanthin extracts from each species would contain isomers of *cis*-fucoxanthin. These were detected at retention times of 44.5 and 45.5 min in all extracts. When compared to published chromatograms for similar studies, they are most probably the 13-*cis* and 13′-*cis* isomers of fucoxanthin, ascribed as C in Fig. 3 (Fung et al. 2013; Indrawati et al. 2015). Peak D in Fig. 3 was an unidentified compound detected at a retention time of 49.0 min in all seaweed extracts. It may be zeaxanthin; a xanthophyll that occurs in brown macroalgae with chemical properties similar to fucoxanthin. It is readily soluble in acetone, and absorbs light

Table 1 RSM estimation results for fucoxanthin content in *F. vesiculosus* blade

RSM experiment no.	Observed experimental value mg g^{-1} (dm)	σ (±) mg g^{-1} (dm)	Fitted value mg g^{-1} (dm)	Lower 95 % CL	Upper 95 % CL
1	0.052	0.00	0.136	−0.081	0.352
2	0.380	0.02	0.301	0.085	0.518
3	0.131	0.01	0.304	0.087	0.520
4	0.450	0.03	0.492	0.350	0.633
5	0.090	0.01	0.105	−0.111	0.321
6	0.120	0.02	0.042	−0.175	0.258
7	0.079	0.01	0.140	−0.076	0.356
8	0.054	0.01	0.126	−0.090	0.342
9	0.067	0.00	0.224	0.008	0.440
10	0.079	0.00	0.068	−0.148	0.284
11	0.402	0.01	0.422	0.206	0.638
12	0.481	0.02	0.365	0.149	0.582
13	0.696	0.02	0.675	0.459	0.891
14	0.263	0.03	0.250	0.034	0.466
15	0.507	0.01	0.553	0.337	0.769
16	0.404	0.05	0.364	0.148	0.580
17	0.586	0.03	0.518	0.302	0.735
18	0.413	0.01	0.286	0.070	0.503
19	0.263	0.00	0.362	0.145	0.578
20	0.587	0.02	0.580	0.364	0.796
21	0.445	0.01	0.432	0.216	0.648
22	0.586	0.02	0.492	0.350	0.633
23	0.589	0.01	0.492	0.350	0.633
24	0.475	0.03	0.477	0.261	0.694
25	0.254	0.01	0.100	−0.116	0.316
26	0.342	0.02	0.492	0.350	0.633
27	0.470	0.02	0.421	0.205	0.637
28	0.374	0.01	0.422	0.205	0.638

at wavelengths in the same range as fucoxanthin. It has been reported to have a retention time greater than fucoxanthin, and therefore elute slightly later (Bidigare et al. 2005; Billakanti et al. 2013). Purification of Peak D in Fig. 3 for further identification was not carried out as part of the present study's objective.

The comparative fucoxanthin content of all ten seaweeds and the difference between blade, stipe, and holdfast within each species is presented in Fig. 4. A statistically significant inter-species difference was observed between the means of the ten species, as well as an intra-species difference between blade, stipe, and holdfast.

Dry mass content ranged from the lowest, in *Saccorhiza polyschides* holdfast (0.030 ± 0.001 mg g^{-1}), to the greatest in *Alaria esculenta* blade (0.870 ± 0.030 mg g^{-1}). No fucoxanthin was detected in the holdfasts of *L. digitata*, *A. nodosum*, *L. hyperborea*, *F. serratus*, or *P. canaliculata*. In all ten species, there was a greater fucoxanthin content in the blade compared to the stipe, and least in the holdfast.

Discussion

RSM optimisation Acetone was the most significant RSM extraction variable and has been widely reported in literature as an efficient solvent for fucoxanthin extraction. For example, Schmid and Stengel (2015) successfully extracted fucoxanthin from eight species of Irish brown seaweed using 90 % acetone; and Sudhakar et al. (2013) extracted more fucoxanthin from *Sargassum*, *Padina*, and *Turbinaria* species with 90 % acetone, compared to ethanol. In terms of environmental safety and impact, acetone is also listed as a 'preferred'

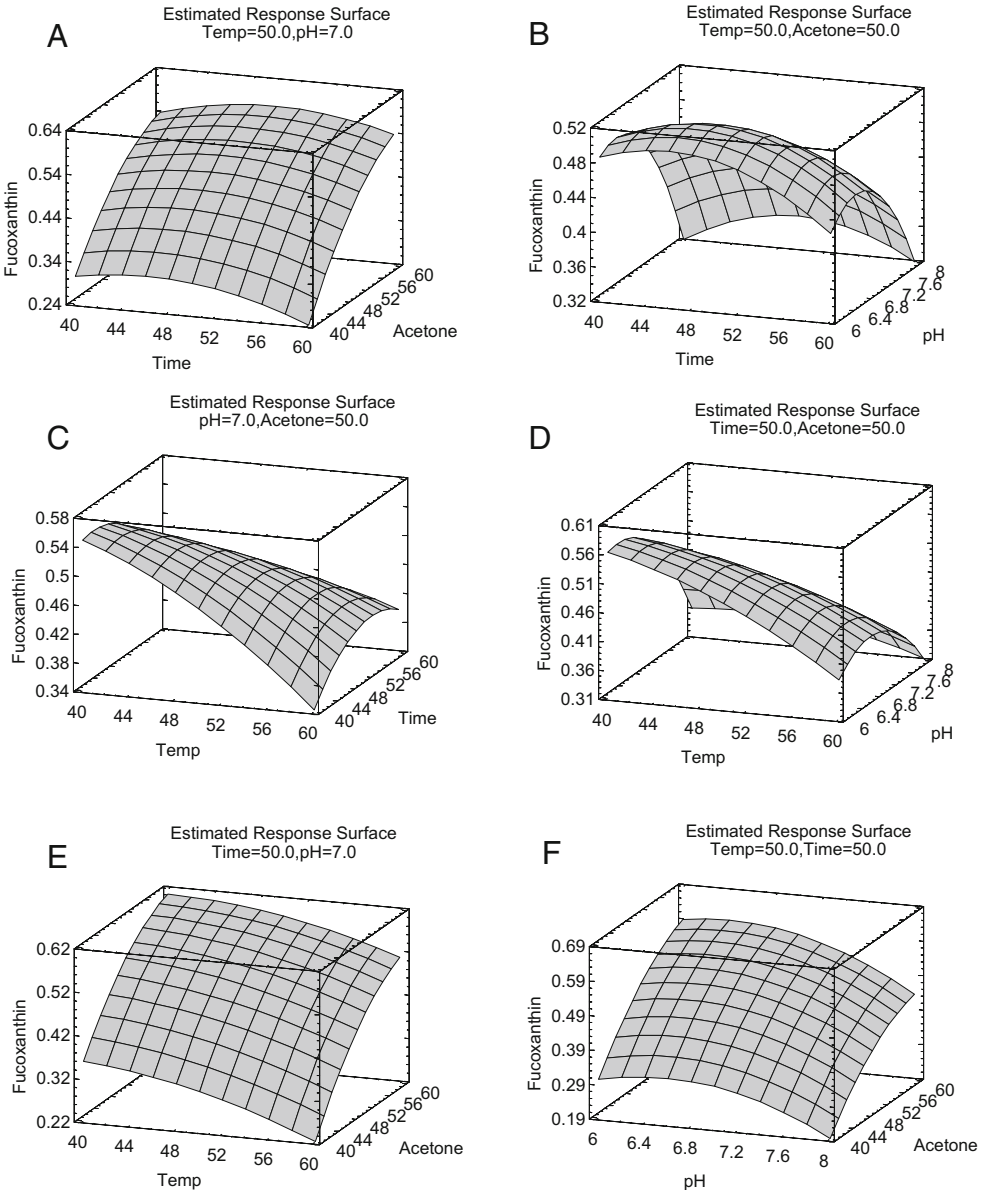

Fig. 2 Multiple response surface optimisation plots for *F. vesiculosus* blade showing the effects of **a** time (min) and acetone (%); **b** time (min) and solvent pH; **c** time (min) and temperature (°C); **d** temperature (°C) and solvent pH; **e** temperature (°C) and acetone (%); and **f** solvent pH and acetone (%) on fucoxanthin yield

solvent in the American Chemical Society's Medicinal Chemistry Solvent Selection Guide (Hargreaves and Manley 2008).

Pigment extraction from macroalgae, particularly the larger kelps and wracks used in the present study, can be challenging due to the tough, polysaccharide-rich nature of the thalli. This may be overcome by maceration with liquid nitrogen. Crushing small aliquots of frozen seaweed in a mortar and pestle with liquid nitrogen ruptures the cell walls of the chloroplasts, releasing pigment-protein complexes from the membrane-bound thylakoids within, exposing them to the acetone solution. In comparison to chlorophyll *a* and *c*, the fucoxanthin pigment-protein complex is less strongly bound to the thylakoid membrane, as it is an accessory pigment synthesised in response to reduced light availability. The second most significant RSM extraction variable was pH, followed by temperature. Time was not found to be significant. Broad ranges have been reported for all three of these parameters, using various protocols. Grosso et al. (2015) reviewed extraction methods ranging from pH 3.8 to 8.5; Bidigare et al. (2005) reported using 0 °C for up to 24 h, Billakanti et al. (2013) 37 °C for 2 h at pH 6.2, Quitain et al. (2013) 40 °C for 3 h, Sivagnanam et al. (2015) 45 °C for 2 h, Roh et al. (2008) 49.85 °C for 50 min, and Shang et al. (2011) 40–

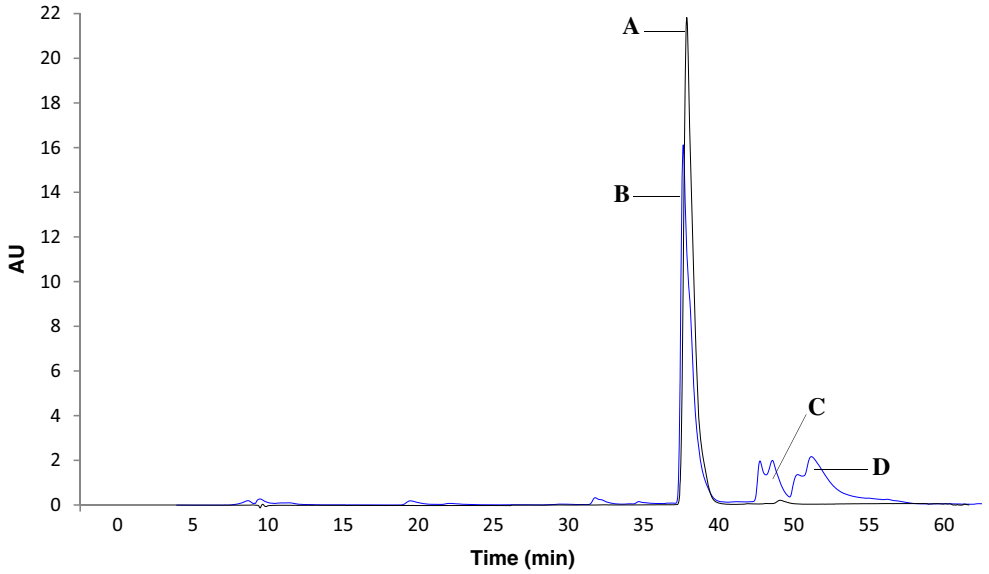

Fig. 3 HPLC separation of fucoxanthin in an overlay chromatogram at 39.89 min in **a** all-trans-fucoxanthin standard peak (10 μg mL^{-1}); **b** *F. vesiculosus* blade; **c** two isomers of *cis*-fucoxanthin; and **d** an unidentified compound, possibly zeaxanthin

100 °C for 5–15 min. Since different algal species, solvents, and extraction methods require different temperatures, pH levels, and incubation times, the optimum parameters calculated in the present study may not be directly comparable to all previously reported methods.

Comparative fucoxanthin content The fucoxanthin results for the ten Irish seaweeds are in line with published values for fucoxanthin content in brown seaweeds from northern European temperate waters. Intra-thallus variations in algal pigment content can be attributed to a greater occurrence of

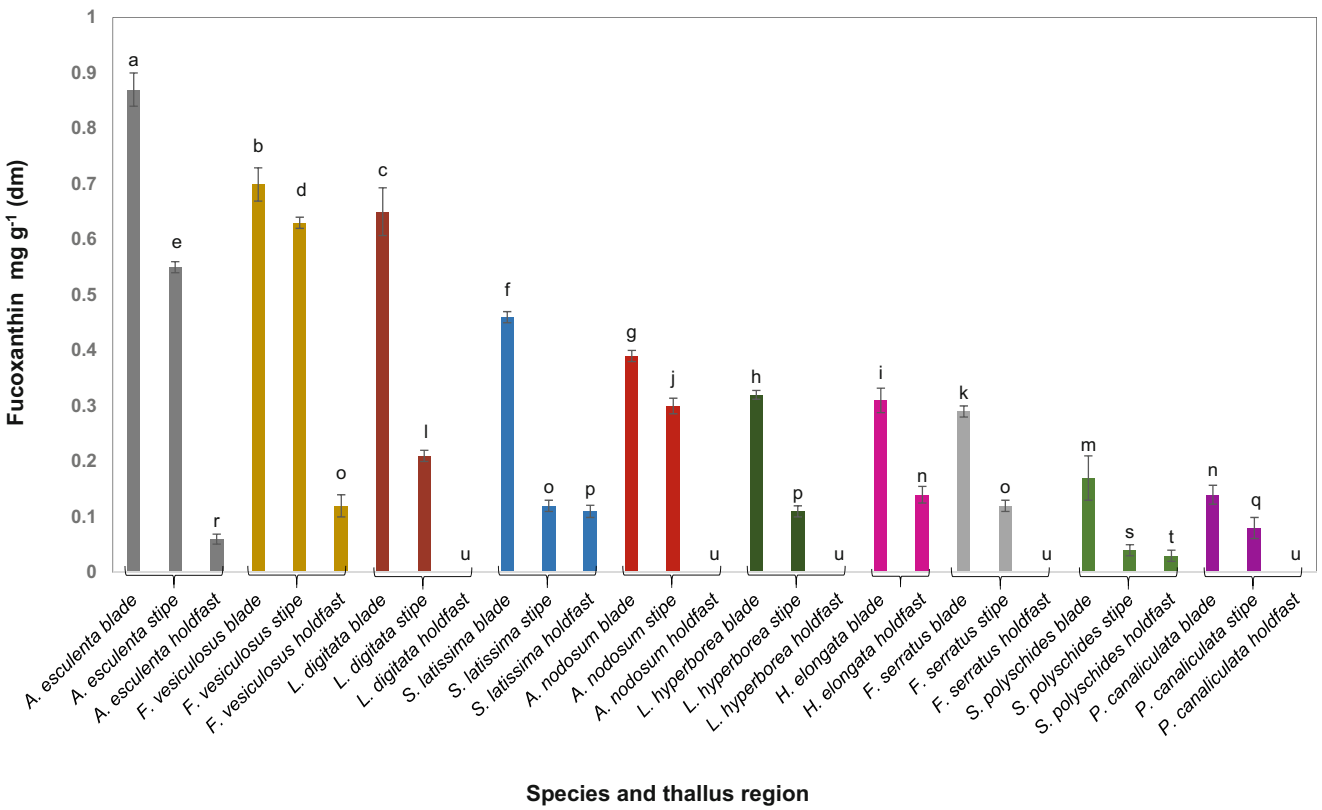

Fig. 4 Comparative fucoxanthin content (mg g^{-1} dry mass) per species and thallus region of ten Irish brown seaweeds. Values are the mean of three replicates ± standard deviation. Letters denote least significant difference between columns ($P \leq 0.05$)

pigment-containing thylakoids in the blades, which function as the photosynthesis engines of macroalgae, compared to the stipe or holdfasts which have structural functions. Photoprotection of thallus regions near the ocean surface against ultraviolet light is another function of fucoxanthin in the blades, where it exerts its powerful antioxidant effect (Lobban and Harrison 1994; Stengel and Dring 1998; Hurd et al. 2014). The majority of observed results were in good agreement with published values for each species. For example, Schmid and Stengel (2015) conducted a study of fucoxanthin, in eight species of Irish brown macroalgae from the west coast of Ireland. Overall, the results of the present study were slightly higher. Schmid and Stengel (2015) reported harvesting of thalli in May 2013, 270 km south of the harvesting region used for the present study. The difference in geographic locations and seasonal variations may account for the difference in fucoxanthin contents, since thalli were harvested for the present study in July 2015. Pigment content variations in algae have been reported due to a number of influences such as seasonal variations, geographic location, sea temperature, nutrient availability, exposure to sunlight, and ontogenetic effects. For example, brown seaweeds harvested from September to March, during the mature phase of the sporophyte, commonly contain higher concentrations of fucoxanthin (Henley and Dunton 1995; Fung et al. 2013; Gosch et al. 2015; Terasaki et al. 2016). This has been attributed to the upregulation of the xanthophyll, or violaxanthin, cycle pathway in reduced levels of sunlight during the winter. Most other published values for fucoxanthin in brown macroalgae are within a range similar to the results of the present study. For example, Ramus et al. (1977) quantified fucoxanthin in the whole thallus of *F. vesiculosus* and *A. nodosum* harvested at Long Island Sound, USA. *Fucus vesiculosus* ranged from 0.202 to 0.751 mg g^{-1}, and *A. nodosum* from 0.178 to 0.304 mg g^{-1}.

Conclusion Fucoxanthin is a bioactive compound found in one of the most prolific and sustainable organisms on the planet, algae. Its efficacy and potential in terms of health applications have been widely reported. The results of this study show the significant distinction between blade, stipe, and holdfast in terms of fucoxanthin content. The findings are in good agreement with international published values for fucoxanthin content. In addition, RSM was shown to be an effective technique for optimising extraction conditions for maximum fucoxanthin yield. These findings may be applied in the development of lean extraction methodologies for value added seaweed products. The Irish government aims to position Ireland as 'The Green Food Island' and develop the annual value of Irish seaweed sector to €30 million by 2020 (Dring et al. 2013). The ten species under study grow prolifically, farmed and wild, around the Irish coast and can be harvested without damage to the base, allowing for continuous regrowth (Taelman et al. 2015). Irish brown seaweeds, particularly *A. esculenta*, *F. vesiculosus*, and *L. digitata* represent a potential source of fucoxanthin for nutraceutical applications.

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