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Phlorotannin Composition of *Laminaria digitata*

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ABSTRACT:

Introduction – Phlorotannins are complex mixtures of phloroglucinol oligomers connected via C-C (fucols) or C-O-C (phlorethols) linkages. Their uniformity in subunits and large molecular weight hamper their structural analysis. Despite its commercial relevance for alginate extraction, phlorotannins in *Laminaria digitata* have not been studied.

Objective – To obtain quantitative and structural information on phlorotannins in a methanolic extract from *L. digitata*.

Methodology – The combined use of ^{13}C and ^{1}H NMR spectroscopy allowed characterisation of linkage types and extract purity. The purity determined was used to calibrate the responses obtained with the colorimetric 2,4-dimethoxybenzaldehyde (DMBA) and Folin–Ciocalteu (FC) assays. Using NP-flash chromatography, phlorotannin fractions separated on oligomer size were obtained and enabled structural and molecular weight characterisation using ESI-MS and MALDI-TOF-MS.

Results – The fucol-to-phlorethol linkage ratio was 1:26 and the extract was 60.1% pure, determined by NMR spectroscopy. For DMBA, the response of the extract was 12 times lower than that of phloroglucinol, whereas there was no difference for FC. By accounting for differences in response, the colorimetric assays were applicable for quantification using phloroglucinol as a standard. The phlorotannin content was around 4.5% DM. Fucol- and phlorethol-linkage types were annotated based on characteristic MS^n fragmentations. Structural isomers of phlorotannins up to a degree of polymerisation of 18 (DP18) were annotated and identification of several isomers hinted at branched phloroglucinol oligomers. With MALDI-TOF-MS phlorotannins up to DP27 were annotated.

Conclusion – By combining several analytical techniques, phlorotannins in *L. digitata* were quantified and characterised with respect to fucol-to-phlorethol linkage ratio, molecular weight (distribution), and occurrence of structural isomers.

Additional Supporting Information may be found online in the supporting information tab for this article.

Keywords: *Laminaria digitata*; phlorotannins; colorimetric assay; RP-UHPLC-UV-MS; MALDI-TOF-MS; NMR spectroscopy

Introduction

Laminariaceae, a family within the brown macroalgae (class Phaeophyceae), is of interest for polysaccharide and protein extraction regarding future needs of feed and food. Usually, brown macroalgae are used for polysaccharide extraction as 38–61% of their dry matter (DM) consists of polysaccharides. Protein contents vary from 3 to 16% DM (Holdt and Kraan, 2011). When the macroalgae are used for polysaccharide extraction, the by-product can be used as animal feed. The protein content of the by-product has increased up to 10–36% DM, comparable to that of rye grass and red clover (Van Soest, 1994), often consumed by cattle. Seaweed proteins are rich in the essential amino acids lysine and methionine. Additionally, brown seaweeds are rich in phlorotannins (Isaza Martínez and Torres Castañeda, 2013).

Phlorotannins are polymers of phloroglucinol (1,3,5-trihydroxybenzene, 126 Da) subunits, which are biosynthesised in the acetate-malonate pathway and polymerised via oxidative coupling (Isaza Martínez and Torres Castañeda, 2013). They resemble terrestrial condensed tannins (also referred to as proanthocyanidins). The subunits can be linked in two ways: aryl-aryl linkages (C-C, fucol type), aryl-ether linkages (C-O-C, phlorethol type). Additionally, two subunits can be linked via double aryl-ether linkages, creating dibenzo-*p*-dioxins (eckol type) (Fig. 1). Within the polymers, combinations of these linkages are possible. Besides, the phlorotannins may contain additional hydroxyl (fuhalols) and halogenic substituents (Isaza Martínez and Torres Castañeda, 2013; Koivikko *et al.*, 2007). Phlorotannins can act as antioxidants, protecting the seaweeds from UV-damage (Shibata *et al.*, 2004) and they can bind proteins, protecting the seaweeds from grazers (Koivikko *et al.*, 2005). The phlorotannin content in seaweeds is highly variable and can vary between 0.5 and 20% DM (Isaza Martínez and Torres Castañeda, 2013), depending on the place of growth, season and presence of grazers (Van Alstyne *et al.*, 1999b).

Phlorotannins are usually extracted in aqueous organic solvents and purified by liquid–liquid partitioning steps, thin layer chromatography or preparative chromatography. The combination of normal phase (NP)-preparative chromatography and reverse phase ultra-high performance liquid chromatography (RP-UHPLC) for phlorotannin characterisation was found to be a successful approach (Montero *et al.*, 2014; Tierney *et al.*, 2014).

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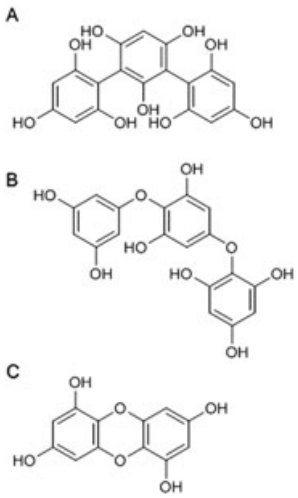

Figure 1. Representative linkage types within phloroglucinol oligomers: fucol type (A), phlorethol type (B) and eckol type (C). Adapted from (Isaza Martínez and Torres Castañeda, 2013).

For terrestrial proanthocyanidins, it has been found that NP-preparative chromatography separated oligomers based on their molecular weight (Appeldoorn *et al.*, 2009).

Structural characterisation of phlorotannins is usually performed using liquid chromatography (LC) coupled to mass spectrometry (MS) or NMR spectroscopy. For chromatography, RP-C18 columns (Koivikko *et al.*, 2007; Shibata *et al.*, 2004) and HILIC columns (Steevensz *et al.*, 2012) have been used. In all cases, chromatographic separation was followed by UV-detection at ranges between 260 and 280 nm and mass detection. The mass detectors attached to currently used chromatographic systems, have a detection limit of approximately 2000 Da and become a limiting factor as phlorotannins are known to be larger than that (Nakai *et al.*, 2006). For the analysis of larger oligomers, above 500 Da, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) has been reported as a suitable technique (Schriemer and Li, 1997).

NMR spectroscopy is generally used for structural characterisation of isolated phlorotannins (Kang *et al.*, 2013), but has also been explored as a way to quantify phlorotannins (Parys *et al.*, 2007), as alternative for colorimetric assays. In analogy to lignin, linkage typologies can be quantified using ^{13}C NMR (Balakshin and Capanema, 2015), and can provide important structural information.

The phlorotannin content of the Laminariacaea family is found to be 0.3–6% DM using the Folin–Ciocalteu (FC) assay (Van Alstyne *et al.*, 1999b). Further information on the structure and molecular weight range of phlorotannins in this family is lacking, despite its commercial relevance. Hence, the present research was performed to determine the molecular diversity of phlorotannins in *Laminaria digitata* by combining quantitative NMR spectroscopy, MS, and chromatographic techniques. The application of NP-chromatography was expected to separate phlorotannins themselves on their degree of polymerisation, creating pools enriched in certain size ranges and improving analysis by RP-UHPLC-UV-MS and MALDI-TOF-MS. The combination of techniques was expected to give an overview of the size and isomeric variation of phlorotannins in *L. digitata*, as complete as possible with currently available technology.

Materials and methods

Macroalgae material

Laminaria digitata was purchased from Bristol Botanicals (Bristol, United Kingdom) in June 2015. Seaweeds were harvested in Scotland in 2015, immediately air dried at 25°C after harvest, and ground into powder before further transportation. It is assumed that these processing steps did not alter the native phlorotannin structure. Final moisture content was 8% *w/w*. In the laboratory the powder was kept in plastic bags in the dark at room temperature.

Chemicals

Methanol, ethyl acetate, hexane, acetone, acetonitrile +0.1% *v/v* formic acid (FA), water +0.1% *v/v* FA, and trifluoro acetic acid (TFA, 99.0% *w/w*) were of analytical grade and obtained from Biosolve (Valkenswaard, The Netherlands). Phloroglucinol (99.0% *w/w*), 2,4-dimethoxybenzaldehyde (DMBA, 98.0% *w/w*), glacial acetic acid (99.0% *v/v*), hydrochloric acid (35.0–37.0% *w/v*), sodium chloride (99.0% *w/w*), dimethylsulphoxide-d_6, acetonitrile-d_3, sodium trimethylsilylpropionate-d_4 (TSP) and chromium(III) acetylacetonate, were obtained from Sigma Aldrich (St Louis, MO, USA). MALDI-TOF-MS grade 2,5-dihydroxybenzoic acid (DHB) was obtained from Bruker Daltonics (Bremen, Germany). Maltodextrin DP20 was purchased from AVEBE (Veendam, The Netherlands).

Sample preparation

Laminaria digitata powder was suspended in 80% (*v/v*) aqueous methanol (25 g/L) and bead-milled using a DYNO®-mill, type MULTI-LAB (Bachhofen AG, Muttenz, Switzerland). The grinding chamber was filled (65% *v/v*) using 0.5 mm silica beads. Macroalgae were ground for 1.5 h at a flowrate of 0.3 L/min. Water (4°C) was circulated in the cooling jacket of the grinding chamber and the algal suspension outside the chamber was kept on ice to avoid heating of the sample. Using these conditions, the suspension leaving the grinding chamber never exceeded 20°C. Afterwards, the suspension was filtered over a cellulose filter (cut off: 8–12 μm Whatman®) and methanol was evaporated by rotary-evaporation. The concentrated extract was diluted with 0.1 M sodium chloride (1:1 *v/v*) and partitioned with ethyl acetate (1:1 *v/v*). After separation, both layers were collected separately. The organic phase was cleaned twice with 100 mL 0.2 M sodium chloride. The aqueous phase was cleaned twice with 200 mL ethyl acetate. The organic phases were pooled and subsequently dried by rotary evaporation to yield the crude extract. The extract was dissolved in methanol at 10 mg/mL and stored at -20°C until further analyses.

NP-flash chromatography

The crude extract was subjected to NP-flash chromatography (Grace, Deerfield, IL, USA), equipped with an evaporative light scattering (ELS)- and UV-detector (400 nm; due to absorbance of acetone at 280 nm, this wavelength could not be used), using a 12 g silica column (18 mL column volume, particle size 40 μm, Grace). The extract (250 mg) was mixed with silica (2.5 g) and injected via dry loading. The compounds were eluted using a combination of hexane (A), acetone (B) and methanol (C) at a flow

rate of 30 mL/min. The elution profile was as follows: 0–1 min, isocratic at 100% A; 1–4 min, 0–80% B; 4–9 min, 80–100% B; 9–12 min, isocratic at 100% B; 12–19 min, 0–20% C; 19–23 min, 20–100% C; 23–25 min, isocratic at 100% C. Fractions (10 mL) were collected in pre-weighed tubes. In total, 51 fractions were collected of which fractions 6–44 contained sufficient material for further analyses.

RP-UHPLC-UV-MS analysis

Flash fractions 6–44 were analysed using an Accela UHPLC system (Thermo Scientific, San Jose, CA, USA) equipped with a pump, autosampler (cooled to 15°C) and photodiode array (PDA) detector (200–700 nm). Samples (2 μL, 3 mg/mL in methanol) were injected onto a UHPLC-BEH-C18 column (2.1 mm × 150 mm i.d., particle size 1.7 μm, Waters, Milford, MA, USA) at 30°C. Water containing 0.1% (*v/v*) formic acid (A) and acetonitrile containing 0.1% (*v/v*) formic acid (B) were used as eluents. The elution profile was as follows: 0–1 min, 1% B; 1–1.5 min, 1–8% B; 1.5–24 min, 8–55% B; 24–25 min, 55–100% B; 25–28 min, 100% B; 28–29 min, 100–1% B; 29–33 min, 1% B. The flow rate was 400 μL/min. The elution was monitored at 266 nm.

MS^n analysis was performed on a Thermo Scientific LTQ-XL using electrospray ionisation (ESI) in negative mode, over a mass range from 300 to 2000 Da. The instrument was tuned using phloroglucinol and the following operation parameters were applied: capillary temperature 350°C, source heater temperature 230°C; for recording MS^2 spectra, the source voltage was 3.5 kV. Compound annotation was based on the MS^n data and UV–vis spectra.

MALDI-TOF-MS analysis

The crude extract and purified phlorotannin fractions were dissolved in methanol (2 mg/mL). DHB was used as matrix (10 mg/mL in 50% (*v/v*) aqueous acetonitrile acidified with 0.3% (*v/v*) TFA). Before spotting on the stainless steel target plate (Bruker Daltonics), sample and matrix were premixed in a 1:1 (*v/v*) ratio. Spotting was performed by pipetting 0.5 μL sample matrix mixture and drying under an air flow. Subsequently, this procedure was repeated with another 0.5 μL and finally with 1.0 μL 1 mM sodium acetate. MALDI-TOF-MS spectra were recorded using an Ultraflextreme workstation controlled by Flexcontrol 3.3 software (Bruker Daltonics) equipped with a N_2 laser of 337 nm and operated in positive mode. The system was calibrated using maltodextrin DP20. Spectra were recorded by automated operation, using positive mode detection over a mass range from 700 to 4000 Da.

Determination of total phlorotannin content

NMR spectroscopy. The crude extract was dissolved (35–60 mg/mL) in a mixture of dimethylsulphoxide-d_6/acetonitrile-d_3 3:1 (*v/v*) containing a relaxation reagent, chromium(III) acetylacetonate (6 mg/mL), and TSP (2.55 mg/mL) as internal standard. Both ^{1}H and ^{13}C NMR spectra were recorded at 303 K in quantitative conditions on a Bruker Avance III 400 MHz NMR Spectrometer (Bruker BioSpin, Rheinstetten, Karlsruhe, Germany) operating at a magnetic field-strength of 9.4 T. ^{13}C NMR spectra were acquired with Inverse Gate Detection, 90° pulse width and 4000 scans. ^{1}H NMR spectra were acquired with a 90° pulse width, 32 scans, 1.4 s acquisition time and 5 s relaxation delay. In order to apply ^{1}H NMR spectroscopy for quantification, the average number of H-atoms on the benzene ring should be known. This was determined using ^{13}C NMR spectroscopy. The value obtained from this technique was then used in ^{1}H NMR spectroscopy for quantification purposes. For quantification, the entire phlorotannin zone was integrated and compared to the area of the internal standard TSP and converted to a mass value according to calculation methodology previously reported (Müller-Maatsch *et al.*, 2014).

The phlorotannin content was also determined by two different colorimetric methods: the DMBA assay (Montero *et al.*, 2014) and the FC assay (Slinkard and Singleton, 1977). For both assays, a calibration curve of phloroglucinol was prepared (0–0.25 mg/mL in methanol). The crude extract was dissolved at the same concentration of phenolics, after accounting for the purity of phlorotannins as obtained by NMR spectroscopy. This allowed comparison of the response of the phloroglucinol calibration to that of the crude extract.

DMBA assay. The DMBA assay was performed as described previously (Montero *et al.*, 2014), with the adaptation that absorbance after 1 h was read at 510 nm using a SpectraMax® M2^e plate-reader (Molecular devices, Sunnyvale, CA, USA).

FC assay. The FC assay was performed as described previously (Slinkard and Singleton, 1977), with adaptations. The sample (20 μL) was diluted with 1.58 mL deionised water and incubated with 100 μL FC reagent for 20 min. Subsequently, 300 μL disodium-carbonate (20% *w/v*) was added to the mixture and after 2 h of incubation the absorbance was read at 765 nm using a UV-1800 Shimadzu spectrophotometer (Shimadzu, Kyoto, Japan).

Results and discussion

Characterisation of the crude extract

The presence of phlorotannins in the crude methanolic extract of *L. digitata* after ethyl acetate partitioning was determined using UV–vis spectroscopy and ^{13}C NMR spectroscopy. The UV–vis spectrum (data not shown) showed λ_{max} values at 270 nm, 410 nm and 664 nm. The latter two maxima indicated the presence of xanthophylls and chlorophylls (Egeland, 2011). The maximum around 270–280 nm is indicative for phenolics. The band around 270 nm represented phlorotannins as the absorbance maximum of phloroglucinol is 269 nm (Koivikko *et al.*, 2007).

It is well known that the phloroglucinol subunits within phlorotannins can be connected via fucol and phlorethol type of linkages (Isaza Martínez and Torres Castañeda, 2013). In order to obtain information on the types of linkages, ^{13}C NMR spectroscopy was used. In a ^{13}C NMR spectrum for phlorotannins, the signals for benzene-based motifs appear from 90 to 165 ppm. Within phlorotannins, the benzene rings can contain the following linkages: C-C (fucol type), C-O-C (phlorethol type), C-H, and C-OH. The latter two are referred to as benzene ring substituents. These four linkages resonate at characteristic wavelengths and are based on the ratios between these signals, the technique can be used to determine the proportion of each linkage present (Kang *et al.*, 2013). Resonances of C-H and C-OH substituents of the phloroglucinol standard were registered at 94.41 and 159.14 ppm, respectively (data not shown). The ^{13}C NMR spectra were interpreted based on phloroglucinol chemical shifts and literature data (Kang *et al.*, 2013; Parys *et al.*, 2010). Within the

spectrum [Fig. 2(A)], the chemical shifts of C-C linkages (99–103 ppm) could be distinguished from those of C-O-C linkages (121–128 ppm and 156–158 ppm) and from C-H substituents (92–97 ppm). As resonances of C-OH (151–160 ppm) signals overlapped with those of C-O-C (156–158 ppm), the proportion of the C-O-C linkages was estimated by the signals at 121–128 ppm. Based on the observed signals, the presence of eckol-linkage types could be excluded as these would resonate at 140 ppm. The proportion of C-OH subunits was estimated by the difference between the overall intensities at 151–160 ppm and those at 121–128 ppm. The relative abundancies of C-H, C-OH, C-C and C-O-C linkages were 22.0%, 32.2%, 1.7% and 44.1%, respectively, corresponding to a molar fucol-to-phlorethol ratio of 1:26. To our knowledge, the ratio between the two subunit linkage types within a mixture has not been reported elsewhere.

Phlorotannin quantification

The purity of the crude extract and therewith the phlorotannin content of *L. digitata* was assayed using several techniques. Colorimetric assays, such as the DMBA-assay (Stern *et al.*, 1996) and the FC-assay (Slinkard and Singleton, 1977), are often used for phlorotannin quantification. NMR spectroscopy is a technique less frequently applied for this purpose (Parys *et al.*, 2007). From the relative abundancies of different carbon typologies within the phlorotannins obtained by ^{13}C NMR spectroscopy, the average number of hydrogens on the benzene ring could be determined. This number was determined to be 1.5 and applied in ^{1}H NMR spectroscopy for quantification (Parys *et al.*, 2007). As most of the linkages were of the phlorethol-type, represented by the 44.1% C-O-C linkages present in our sample, the value of 1.5 was lower than expected. Theoretically, this number would be around two, as only one terminal unit within phlorehol-type chains contains three hydrogens on the benzene ring, whereas all other units contain two hydrogens [Fig. 1(B)]. For fucol types, both terminal units contain two hydrogens, whereas the internal units contain one hydrogen [Fig. 1(A)]. When additional OH substituents or branched chains (one phloroglucinol subunit connected to ≥3 subunits) are present, the average number of hydrogens per benzene ring decreases. The proportion of additional OH groups could not be calculated as the proportion of each oligomeric degree of polymerisation (DP) could not be verified. As the average number of H atoms on the ring was <2, it is likely that branched phlorotannins were present.

In ^{1}H NMR spectroscopy, phlorotannins gave signals at 5.70–6.24 ppm [Fig. 2(B)], which are characteristic for the hydrogens on phenolic rings. For quantification, the entire phlorotannin zone was integrated and related to the area of the internal standard TSP (Parys *et al.*, 2007). This area was then converted to a mass value. Our results were in line with Parys *et al.* (2007), who performed similar assays for different types of brown macroalgae. The purity of the *L. digitata* crude extract was determined to be 60.1% ± 0.3 (*w*/*w*), corresponding to a phlorotannin content of 4.3% DM in the seaweed.

The extract purity, as determined by ^{1}H NMR spectroscopy, was used to evaluate the accuracy and therewith applicability of colorimetric quantification assays for phlorotannins in *L. digitata*. The DMBA reagent reacts specifically with *m*-diphenolics. The DMBA reagent is therefore more specific than the commonly used FC-reagent, as the latter reagent reacts additionally with both mono- and *o*-diphenolics (Stern *et al.*, 1996). Due to lack of polymeric standards, phloroglucinol is mostly used as standard for the calibration curve in both assays. In the DMBA assay, monomeric phloroglucinol is known to react stronger to the

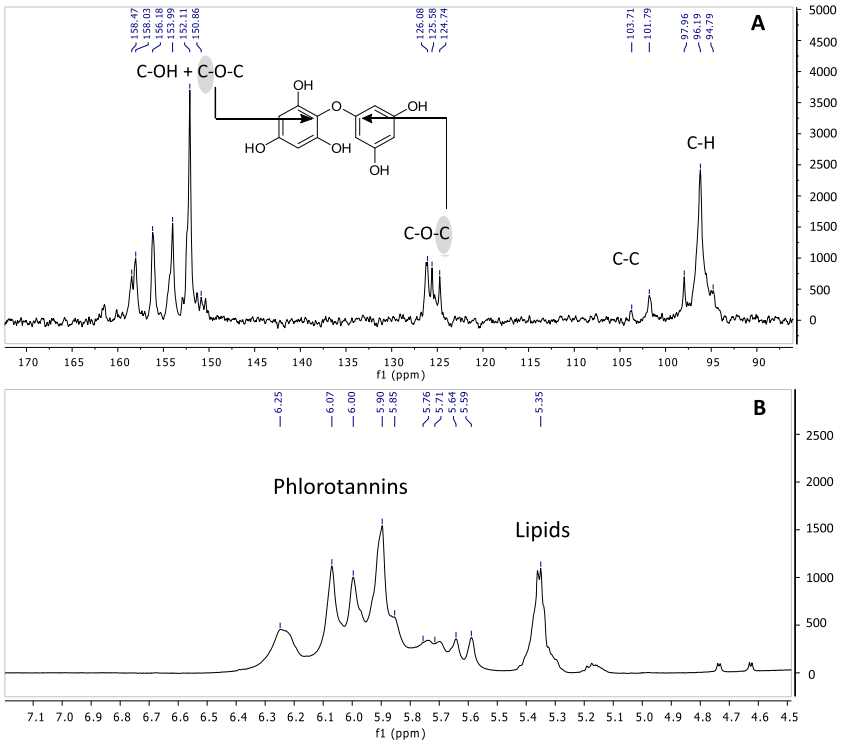

Figure 2. Full ^{13}C NMR (A) and ^{1}H NMR (B) spectra (400 MHz) of the crude extract (30 mg in dimethylsulphoxide-d_6/acetonitrile-d_3 3:1). [Colour figure can be viewed at wileyonlinelibrary.com]

reagent than phlorotannin oligomers, resulting in underestimation of the purity (Parys *et al.*, 2007; Stern *et al.*, 1996). A reaction between phloroglucinol and the reagent yielded a λ_{max} at 495 nm, whereas the crude extract yielded a λ_{max} at 515 nm [Supporting Information Fig. S1(A)]. In the literature, 510 nm is used for colorimetric determination, to compromise for the difference and be close to the λ_{max} of the extract (Stern *et al.*, 1996). To be able to compare our data to the literature, 510 nm was used in this research as well. The difference in response between phloroglucinol and the crude extract was calculated by the ratio between the slope of the phloroglucinol calibration curve and the slope of the crude extract curve. The phloroglucinol curve was 12 times steeper than that of the crude extract (data not shown), despite the fact that the phlorotannin purity in the crude extract was corrected for. This difference in response was applied as correction factor for further quantifications. A similar result was obtained by the developers of the assay, who found phlorotannin reactivities in extracts of 3 to 11 times lower than that of monomeric phloroglucinol, depending on the algal species (Stern *et al.*, 1996). The crude extract contained 63.2 ± 4.2% (*w*/*w*) phlorotannins, and content in *L. digitata* was estimated to be 4.5% DM.

For the FC assay, there was no shift in λ_{max} [Fig. S1(B)] between the monomeric standard and the extract. Quantifying the crude extract based on the FC assay, resulted in 81.5 ± 2.4% (*w*/*w*) phenolics, the content in *L. digitata* corresponded to 5.7% DM.

Comparing all quantification assays, the purities found with the FC assay were the highest, indicating the presence of mono- and *o*-diphenolics. For plorotannin quantification in Laminariaceae, the FC assay is mostly used and content determined in our assay were similar to those reported previously (Stern *et al.*, 1996; Van Alstyne *et al.*, 1999a). The difference in phlorotannin content between the FC and DMBA assay gives an estimation of the amount of non-phloroglucinol phenolics or fuhalol type of tannins. Due to their extra OH group, fuhalol units within the phloroglucinol oligomers do not bear a *m*-diphenolic group and do therefore not react with the DMBA reagent.

In reports using colorimetric quantification, the differences in response between monomeric phloroglucinol and extracts is only limitedly highlighted (Stern *et al.*, 1996). In this study, the methodology for determining a correction factor for quantification of oligomeric phlorotannins using the DMBA assay with a phloroglucinol calibration curve was further refined. Nevertheless, it remains necessary to determine purities by ^{1}H NMR spectroscopy as the phlorotannin composition is species dependent, which makes the response factor species dependent as well.

Characterisation of flash-fractions

To obtain structural and oligomer size information additional to ^{13}C NMR spectroscopy, the crude extract was fractionated by NP-flash chromatography [Fig. 3(A)].

Fractions 6–44 contained sufficient material for analysis by RP-UHPLC-UV-MS. Fractions 6–15, which had yellow and green colours, contained chlorophylls, xanthophylls and lipid components (Supporting Information Table S1) and were not included in further analyses. Within the UV_{266} chromatograms of fractions 16–27 [Fig. 3(B)], two main regions were distinguished. The compounds in the first region, from 0 to 12 min, eluted in separate peaks. The peaks were annotated as phlorotannins based on their UV–vis spectra with absorbance around 270 nm (Koivikko

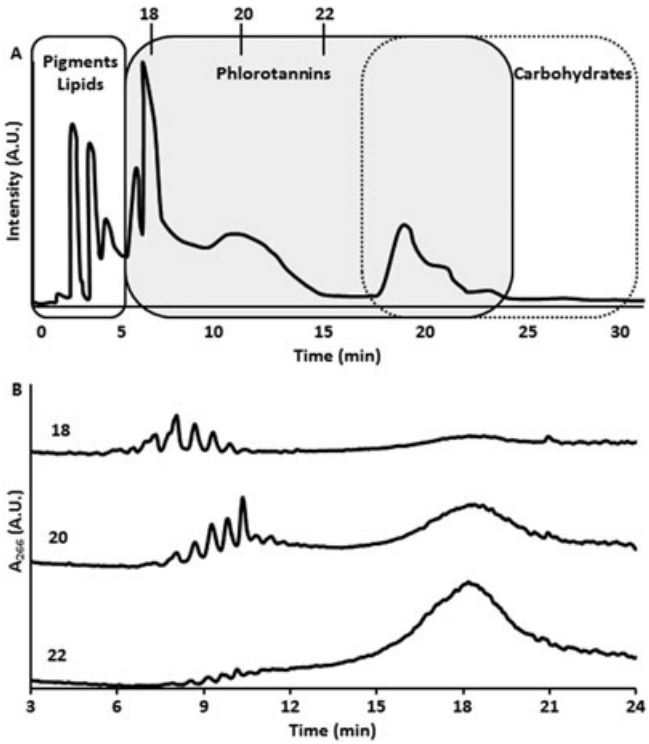

Figure 3. NP-flash separation of pigments and phlorotannins in the crude extract of *Laminaria digitata* (A) and RP-UHPLC-UV chromatograms of fractions 18, 20, 22 at 266 nm (B).

et al., 2007) and ESI-MS/MS spectra in negative mode characterised by losses of 126 Da upon fragmentation, corresponding to the monomeric phloroglucinol subunits (Montero *et al.*, 2014). The second region, from 12 to 24 min, was an unseparated hump containing compounds with low mass signal intensity, albeit with similar absorbance maxima (270 nm). The presence of such an unseparated hump is common for phlorotannin mixtures (Koivikko *et al.*, 2007; Tierney *et al.*, 2014). A shift in proportion between separated peaks and hump of unseparated compounds with increasing fraction numbers was observed. To determine the proportions of separated peaks and the unseparated hump in each fraction, the total area under the A_{266} chromatogram from 2 to 12 min was taken as percentage of the total A_{266} area from 2 to 24 min. In fractions 16–18, the area from 2 to 12 min made up 35% of the total area, which increased to 40% in fraction 19. After this fraction, the proportion decreased to be constant around 5% for fractions 22–27. The hump was not present in fractions 28–44 and these fractions contained carbohydrates as determined by MALDI-TOF-MS analysis (data not shown).

Variability in phlorotannin size and structure

The full-MS and MS^2 fragmentation spectra were used to obtain in depth information on the phlorotannin structures present. Within fucol- and phlorethol-types of phlorotannins, the molecular weight of the phloroglucinol oligomers follows Equation (1):

$$MW = 126 \cdot DP - (2 \cdot (DP - 1)) \quad (1)$$

in which the value 126 represents the molecular weight of the phloroglucinol monomer and DP represents the degree of polymerisation of the oligomer. This results in $[M\text{-}H]^-$ ions with *m/z* values 373, 497, 621, 745, 869, 993, 1117, 1241 for DP3 to

Table 1. Molecular ions and mass fragments of phlorotannins annotated NP-flash chromatography fractions in a *Laminaria digitata* extract using RP-UHPLC–MS.

No	$[M-H]^-$	MS^2	Type	Fraction
DP3				
2[a]	373	**305**[b], 247, 229[c]	Fucol	16
3	373	**305**, *231*[d]	Phlorethol	16
6	373	**247**, *233*, 229, 125	Fucophlorethol	16
DP4				
1	497	**461**, 435, 371, 353, 231	Fucol	16
4	497	371, **353**, 339, 249, 229	Fucol	16–18
22	509	441, **384**, 373, 305, 261	Fuhalol	16
DP5				
7	621	495, *479*, *461*, **373**, 355, 229	Fucophlorethol	16–19
8	621	373, *357*, 447, ***339***, 229	Fucophlorethol	16
15	621	**495**, 477, 371, 229	Fucol	16
27	651	607, 582, ***509***, *465*, 413, 339	Fuhalol	16
DP6				
5	745	**709,** 601, 579, 455, 437, 289	Fucol	16–19
10	745	619, 601, **497**, 479, 353, 335, 229	Fucol	16–22
11	745	*603*, 497, *478*, **371**, *355*, 229	Fucophlorethol	16, 17
19	745	619, **601**, *479*, 461 *355*	Fucophlorethol	16
33	775	731, 633, **586**, 537, 463	Di–fuhalol	16, 17
DP7				
9	869	**833**, 708, 579, 455	Fucol	16–20
13	869	833, 743, **725**, 707, 619, 601, 495, *477*, 371, *355*, 335	Phlorethol	16
16	869	743, ***727***, 621, 603, 479, 353	Phlorethol	20
24	869	**743**, 725, *477*, *355*	Phlorethol	16
38	899	**589**, 663, 855, 537, 463, 373	Di-fuhalol	16
DP8				
12	993	**957**, 849, 831, 709, *603*, 353	Fucophlorethol	17
14[e]	993	**957**, 832, 371		17–21
DP9				
17	1117	**1081**, 973, *849*, 833, 707, 353	Fucophlorethol	18–22
18	1117	**1081**, 956, 745, *727*, 621, *603*, 582, 497, *477* 371, 351	Phlorethol	17–22
26	1117	1081, **973**, 993, 745, 727, 709, 621, *603,* 583, 495, 459, 247, 353	Fucophlorethol	17
DP10				
20	1241	**1205**, 1097, 1079, 975, 745, *727,* 601, 495	Fucophlorethol	17–23
21	1241 $[M-2H]^-$	1205, 1097, 1079, **745**, *727,601, 477*	Phlorethol	17, 18
DP11				
30	682	**610**, 601, 495, *469,* 229	Fucophlorethol	17
DP12				
23	744	*673*, *663*, 621, *601,* 495, 477, **371**, 229	Phlorethol	19–25
24	744	***673***, 663, *601*, 495, 477, 229	Phlorethol	17
DP13				
28	806	745, ***734***, 725, *662*, 601, 477, 371, 353	Phlorethol	18–27
33	806	725, *734*, 724, 672, *663*, 601, **477**, 229	Phlorethol	18
DP14				
31	868	***796***, 787, 745, 477, 495, 353	Phlorethol	18–27
35	868	*796*, 744, 619, **477**	Phlorethol	
DP15				
25	930	*859*, **849**, 745, 734, 725, 495, 477, 371	Fucphlorethol	20–25
37	930	*859*, ***796***, 495, 477	Fucophlorethol	18
39	930	**867**, 805, *726*, 495, 477, 371, *354*	Phlorethol	18
DP16				
29	992	**911**, *849*, 725, 477, 353	Fucophlorethol	19–27

(*Continues*)

Table 1. *(Continued)*

No	[M-H]$^-$	MS^2	Type	Fraction
36	992	957, 911, 867, 849, *816*, 743, 727, 707, 619, 603, **477**, 495	Phlorethol	18
39 *DP17*	992	911, **858**, *851*, 477, 353	Phlorethol	18
32 *DP18*	1054	**973**, 955, 913, 725, 477	Phlorethol	19–27
34	1116	**1035**, *975*, 477	Phlorethol	19–27

[a]Numbering is according to elution order in time.
[b]Fragments in bold represent the main fragment ion.
[c]Fragments underlined represent diagnostic ions for phlorotannins.
[d]Fragments in italic represent diagnostic ions for phlorethols.
[e]Poor fragmentation.

DP10, respectively. These masses were annotated in the different fractions (Table 1). In case of eckol types, the molar mass of the subunit decreases 2 Da with each extra linkage, as two H atoms per monomer are lost during oxidative coupling of subunits (Isaza Martínez and Torres Castañeda, 2013). Eckol types, however, were not annotated by LC–MS, confirming results of ^{13}C NMR spectroscopy.

Oligomers containing more than 10 subunits appeared as double charged ions; [M-2H]$^{2-}$ at *m/z* values 682, 774, 806, 868, 930, 992, and 1054 corresponding to DP11–DP18 (Table 1), respectively (Montero *et al.*, 2014; Steevensz *et al.*, 2012). The composition of each fraction varied, changing from low DP towards higher DP when flash elution progressed. The increased DP with increasing fraction showed that NP-flash chromatography indeed separated phlorotannins based on their DP, similar to proanthocyanidins.

The maximum DP detected by ESI-MS in our samples was 20. Oligomers of DP19 and DP20 had very low signal-to-noise ratio and, therefore, their fragmentation could not be further studied in MS^2. Due to decreasing ionisation potential with increasing molecular weight (Mouls *et al.*, 2011), polymers > DP20 were possibly not detected.

Phlorotannin isomers. The oligomeric fucol- and phlorethol-linkage types differed in fragmentation behaviour. Fucol types [Fig. 4(A)] fragmented at the aryl linkage, visible in the MS/MS spectrum by -125 for a deprotonated monomer or -126 for an uncharged monomer. Additionally, losses of 124*n* (with *n* the number of monomers) for oligomeric fragments (Ferreres *et al.*, 2012; Montero *et al.*, 2014) were identified. These fragmentation patterns are indicative for phlorotannins in general (Ferreres *et al.*, 2012; Heffernan *et al.*, 2015; Montero *et al.*, 2014). With phlorethol-type fragmentation [Fig. 4(B)], the pattern becomes more ambiguous than with fucol types as fragmentation can occur on either site of the ether oxygen. There were the losses of 124*n* resulting from cleavage of phloroglucinol units, not including the ether oxygen. In addition, there were fragments at -142 (phloroglucinol + OH) for the uncharged and -141 for the deprotonated ion, resulting from cleavage including the ether oxygen. Both types of linkages were annotated in the fractions.

Single charged ions. Single charged phlorotannin oligomers from DP3 to DP10 were annotated in fractions 16–23 (Table 1). Monomers and dimers were not annotated during initial screening

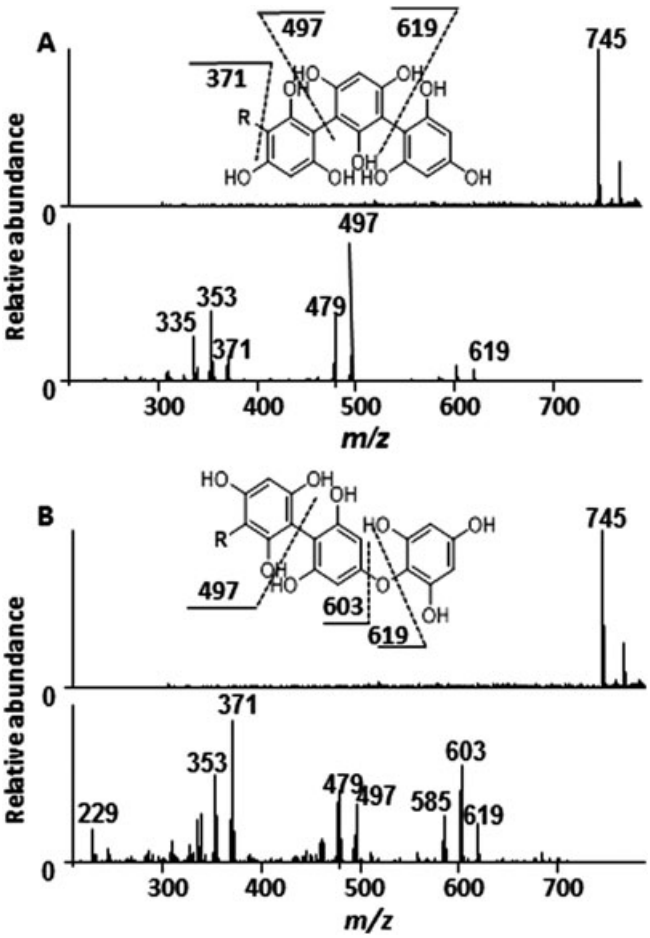

Figure 4. Mass fragmentation patterns and spectra [M-H]$^-$ for a DP6 fucol type (A) and fucophlorethol type (B). Structures are hypothetical, R = additional attachment of three monomers.

and, therefore, full mass spectra from 300 to 2000 Da were recorded during further experimentation. DP3 was found in fraction 16 only, and consisted of one fucol type (**2**) and two phlorethol type (**3**, **6**) oligomers. With increasing fraction number, the lowest DP annotated increased and for each DP, several isomers were annotated. The oligomers **1, 4** (both DP4), **9, 15**

(both DP5), **5** and **10** (both DP6), were fucols. All other single charged oligomers contained either phlorethol type of linkages, or were fucophlorethol type of oligomers. Fucophlorethol types contain both fucol and phlorethol linkages in their structure. Additionally, two fuhalol type of oligomers (**22**, DP4 and **29**, DP5) were annotated.

Within fucol type oligomers, the 2-, 4-, and 6-positions of phloroglucinol are available for extension of the molecule. Linking via these positions creates chains in which subunits are connected at *meta*-positions. Within phlorethol type oligomers the 1-, 3- or 5-hydroxyl positions generally couple to the 2-, 4- or 6-carbon position of the next ring. By using all six available linkage positions, two subunits flanking a central phloroglucinol subunit can extend this central subunit in *ortho*-, *meta*-, or *para*-fashion. In addition, if a phloroglucinol subunit is linked to ≥3 other subunits, branched oligomers are formed. The formation of branched oligomers can be expected as these are created by radical coupling which follows random patterns (Isaza Martínez and Torres Castañeda, 2013). Taken together, the various possibilities for connecting subunits can create a plethora of isomers. Our mass fragmentation data gave information on the type of linkages present within the oligomer, but were not conclusive on linkage positions. The isomers differed in their retention times. It is expected that oligomers with linkages in *ortho*-positions or with branches had lower hydrodynamic volumes than those with *meta*- or *para*-connected subunits, and consequently eluted earlier. This would be in accordance with the elution of branched and linear carbohydrates in which branched ones elute before linear ones on RP-C18 columns (El Rassi, 1996).

Doubly charged ions. The doubly charged ions of DP ≥ 11 oligomers were present from fraction 17 onwards. In fractions 19–27, DP18 was the largest oligomer annotated. All compounds annotated contained phlorethol type of linkages. The annotation of isomers correlated to the data obtained by ^{13}C NMR spectroscopy, which also showed higher abundance of phlorethol type of linkages. With increasing DP, the intensity of the peaks observed dropped, and only trace amounts of higher DPs were visible in the chromatograms. The intensity was too low for fragmentation and confirmation of the phlorotannin structure. In fractions 28 and beyond, no ionisable phlorotannins were detected.

Phlorotannin degree of polymerisation. The use of MALDI-TOF-MS generally allows annotation of molecular weights beyond the detection limit of the ESI-MS (Mouls *et al.*, 2011). The poor ionisation of components under the UV_{266}-hump hinted at presence of phlorotannins > DP18. MALDI-TOF-MS analysis on the crude extract (Fig. 5) and individual fractions showed presence of oligomers from DP7 to DP27, $[M + Na]^+$ 890–3370. The mass differences between peaks resembled 124, the monomeric mass of phloroglucinol minus two H atoms to create a covalent bond. With increasing fraction number, larger DPs were detected, but with low intensities [Supporting Information Fig. S2(A)]. No *m/z* values above 3500 were visible. In fraction 42, the co-elution of carbohydrates from the Flash column might have hindered ionisation of phlorotannins due to a high carbohydrate proportion in the fraction [Fig. S2(B)]. Due to the decrease in ionisation potential with increasing molecular weight, it cannot be excluded that phlorotannins ≥ DP27 were present. To our knowledge, this is the first time that phlorotannins up to DP27 were annotated. MALDI-TOF-MS analysis on phlorotannins has been reported once before, on *Sargassum ringgoldianum*, in which tannins built from

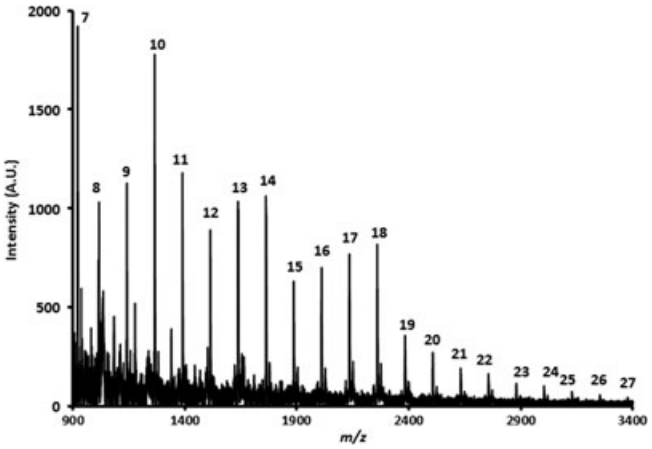

Figure 5. MALDI-TOF mass spectrum $[M + Na]^+$ of phlorotannins DP7–DP27 in the crude extract.

bifuhalol (264 Da) oligomers up to DP8 $[M + Na]^+$ 2136 were visualised (Nakai *et al.*, 2006).

To conclude, the phlorotannins in *L. digitata* were successfully separated on DP using NP-Flash chromatography, and further characterised. Usually, the dominant linkage types of monomers are not reported. In this case it was demonstrated by ^{13}C NMR spectroscopy and MS that phlorotannins in *L. digitata* consist mostly of phlorethol linkage types. Additionally, the oligomeric size of DP27 is the highest annotated so far.

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Supporting information

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