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ORIGINAL ARTICLE

UPLC-MS profiling of low molecular weight phlorotannin polymers in *Ascophyllum nodosum*, *Pelvetia canaliculata* and *Fucus spiralis*

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Abstract Phlorotannins are a group of complex polymers, found in particular brown macroalgae, composed solely of the monomer phloroglucinol (1,3,5-trihydroxybenzene). Their structural complexity arises from the number of possible linkage positions between each monomer unit. This study aimed to profile the phlorotannin metabolite composition and the complexity of isomerisation present in brown macroalgae *Ascophyllum nodosum*, *Pelvetia canaliculata* and *Fucus spiralis* using UPLC-MS utilising a tandem quadrupole mass spectrometer. Phlorotannin-enriched fractions from water and aqueous ethanol extracts were analysed by UPLC-MS performed in multiple reaction monitoring mode to detect molecular ions consistent with the molecular weights of phlorotannins. *Ascophyllum nodosum* and *P. canaliculata* appeared to contain predominantly larger phlorotannins (degree of polymerisation (DP) of 6–13 monomers) compared to *F. spiralis* (DP of 4–6 monomers). This is the first report observing the complex chromatographic separation and metabolomic profiling of low molecular weight phlorotannins consisting of more than ten monomers. Extracted ion chromatograms, for each of the MRM transitions, for each species were analysed to profile the level of isomerisation for specific molecular weights of phlorotannins between 3 and 16 monomers. The level of phlorotannin isomerisation within the extracts of the individual macroalgal species differed to some degree, resulting in substantially different numbers of phlorotannin isomers for particular molecular weights. A similar UPLC-MS/MS separation procedure, as outlined in this study, may be used in the future as a means of screening the metabolite profile of macroalgal extracts, therefore, allowing extract consistency to be monitored for standardisation purposes.

Keywords Phlorotannins · UPLC-MS/MS profiling · Brown macroalgae · Polymers

1 Introduction

Brown algae are a well-known source of phenolic compounds and, in particular, a source of condensed tannin-like dehydro-polymers of phloroglucinol (1,3,5-trihydroxybenzene ($C_6H_60_3$), with a molecular weight of 126.11 Daltons.

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Phlorotannins, which are contained within storage vesicles known as physodes in macroalgae, are becoming the focus of a concerted research effort, due to their potential uses in a range of therapeutics (Tierney et al. 2010). These particular tannin derivatives can constitute of up to 25–30 % of the dry weight of brown macroalgae (Targett et al. 1995), adding to their attractiveness as an extraction target. Many of the potentially useful properties of these compounds can be linked to their antioxidant properties, which are in turn derived from multiple sites at which free radicals can be resonance stabilised (Shibata et al. 2008). In addition to their reported potent antioxidant activity (Kang et al. 2012), phlorotannins have also exhibited anti-diabetic properties (Eom et al. 2012), hyaluronidase inhibition (Ferreres et al. 2012), antimicrobial activity (Nagayama et al. 2002), cytotoxic activity (Li et al. 2011 and antihypertensive activity (Wijesinghe et al. 2011). Phlorotannins are categorised according to the type of bonding between the monomeric units; fuhalols and phlorethols contain only ether linkages, fucols have only phenyl bonds, fucophlorethols consist of both ether and phenyl bonds, and eckols contain dibenzodioxin linkages (Ferreres et al. 2012). Within each phlorotannin class it is possible to have various positions at which a bond can occur between monomers, leading to many structural isomers in addition to conformational isomers. Phlorotannins are known to protect macroalgae against UV irradiation and are likely to act as a chemical defence against herbivores (Boettcher and Targett 1993; Pavia et al. 1997). It is therefore likely that their levels and structural diversity, along with bioactivity, are likely to be affected by seasonality and other factors such as habitat and nutrient availability (Ragan and Glombitza 1986). Generally, phlorotannins have been characterised using spectrophotometric methods such as the Folin–Ciocalteu assays; however, these methods provide no information about the actual phlorotannin composition either in terms of molecular weight and isomer production. For macroalgae phlorotannin polymers to be exploited further it is necessary to develop analytical methods capable of analysing their metabolite profile, both in terms of their molecular weights and the level of isomerisation. With the exception of *Fucus vesiculosus* (Wang et al. 2012) and *Ecklonia cava* species (Kang et al. 2012), the complexity of the phlorotannin composition has meant that relatively few of these molecules have had their structures successfully elucidated. Furthermore, characterisation of these species has been very sporadic with only a relatively small number of tannins identified. Due to the similar chemical properties of phlorotannins and the polymeric nature of their production, only a small number of low molecular weight (degree of polymerisation (DP) of 3–8 monomers) phlorotannin structures have been isolated to date. Recent studies using mass spectrometry have demonstrated that phlorotannins polymers can be detected up to 6 kDa (Steevensz et al. 2012), while size exclusion (Tierney et al. 2013b; Wang et al. 2012) and other studies (Glombitza and Klapperich 1985) suggest that these polymers can reach up to 100 kDa in size.

Recently, ultra performance liquid chromatography-mass spectrometry (UPLC-MS), using high resolution mass spectrometry, was employed to analyse phlorotannins from various species of brown algae (Steevensz et al. 2012). UPLC has been developed to endure much higher system back-pressures than in conventional HPLC, allowing the use of columns with much smaller particle sizes (sub 2 μm) and, therefore, improving the chromatographic speed, sensitivity and resolution (Novakova et al. 2006). Recently we demonstrated that the antioxidant activity of brown macroalgal extracts could be enriched in fractions with molecular weights less than 3.5 kDa from both cold water and aqueous ethanolic extracts (Tierney et al. 2013b). Since it is likely that one of the predominant species responsible for this enrichment are phlorotannins, the principal objective of the present study was to employ UPLC-MS, utilising tandem mass spectrometry (multiple reaction monitoring (MRM) mode) to overcome the problems with chromatographic resolution of different molecular weight phlorotannins and their isomers, observed in other studies (Wang et al. 2012), in conjunction with scanning speeds and sensitivity of a tandem quadrupole mass spectrometer. This method was applied to investigate and profile the relative ratios of low molecular weight (less than 2 kDa) phlorotannins metabolites and provide new information on the numbers of structural isomers in extracts from three species of brown macroalgae common to Ireland; *Ascophyllum nodosum* (*A. nodosum*) (Linnaeus) Le Jolis, *Pelvetia canaliculata* (*P. canaliculata*) (Linnaeus) Decaisne & Thuret and *Fucus spiralis* (*F. spiralis*) Linnaeus.

2 Materials and methods

2.1 Materials and macroalgal material

All chemicals used were reagent-grade. All solvents used were HPLC-grade. BioDesignDialysis Tubing™ with 3.5 kDa cut-off was acquired from Fisher Scientific. Agilent SuperFlash™ SF40-300 g C18 columns and SF10-8 g Si 50 columns were obtained from Apex scientific (Maynooth, Ireland). Varian Bond Elut® Si (500 mg) SPE columns were acquired from JVA Analytical Ltd. (Dublin, Ireland). An Acquity HSS PFP column (100 Å, 1.8 μm, 2.1 mm × 100 mm) was obtained from Waters (Dublin, Ireland).

Brown macroalgal samples used were identified and harvested off the West coast of Ireland in 2010.