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BROWN ALGAL PHLOROTANNINS
Improving and Applying Chemical Methods

by

Riitta Koivikko

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From: Department of Chemistry
University of Turku
Finland

Supervisors: Docent Veijo Jormalainen
Department of Biology
University of Turku
Finland

Docent Jyrki Loponen
Department of Chemistry
University of Turku
Finland

Docent Vladimir Ossipov
Department of Chemistry
University of Turku
Finland

Custos: Professor Kalevi Pihlaja
Department of Chemistry
University of Turku
Finland

Reviewers: Professor Riitta Julkunen-Tiitto
Natural Product Research Laboratory, Faculty of Biosciences
University of Joensuu
Finland

Associate Professor Julia Kubanek
School of Biology & School of Chemistry and Biochemistry
Georgia Institute of Technology
USA

Opponent: Professor Ann E. Hagerman
Department of Chemistry and Biochemistry
Miami University, Oxford Ohio
USA

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PREFACE AND ACKNOWLEDGEMENTS

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ABSTRACT

Phlorotannins are the least studied group of tannins and are found only in brown algae. Hitherto the roles of phlorotannins, e.g. in plant-herbivore interactions, have been studied by quantifying the total contents of the soluble phlorotannins with a variety of methods. Little attention has been given to either quantitative variation in cell-wall-bound and exuded phlorotannins or to qualitative variation in individual compounds.

A quantification procedure was developed to measure the amount of cell-wall-bound phlorotannins. The quantification of soluble phlorotannins was adjusted for both large- and small-scale samples and used to estimate the amounts of exuded phlorotannins using bladder wrack (*Fucus vesiculosus*) as a model species. In addition, separation of individual soluble phlorotannins to produce a phlorotannin profile from the phenolic crude extract was achieved by high-performance liquid chromatography (HPLC).

Along with these methodological studies, attention was focused on the factors in the procedure which generated variation in the yield of phlorotannins. The objective was to enhance the efficiency of the sample preparation procedure. To resolve the problem of rapid oxidation of phlorotannins in HPLC analyses, ascorbic acid was added to the extractant. The widely used colourimetric method was found to produce a variation in the yield that was dependent upon the pH and concentration of the sample.

Using these developed, adjusted and modified methods, the phenotypic plasticity of phlorotannins was studied with respect to nutrient availability and herbivory. An increase in nutrients decreased the total amount of soluble phlorotannins but did not affect the cell-wall-bound phlorotannins, the exudation of phlorotannins or the phlorotannin profile achieved with HPLC. The presence of the snail *Thedoxus fluviatilis* on the thallus induced production of soluble phlorotannins, and grazing by the herbivorous isopod *Idotea baltica* increased the exudation of phlorotannins.

To study whether the among-population variations in phlorotannin contents arise from the genetic divergence or from the plastic response of algae, or both, algae from separate populations were reared in a common garden. Genetic variation among local populations was found in both the phlorotannin profile and the content of total phlorotannins. Phlorotannins were also genetically variable within populations. This suggests that local algal populations have diverged in their contents of phlorotannins, and that they may respond to natural selection and evolve both quantitatively and qualitatively.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications that are referred to in the text by their Roman numerals. Unpublished material is also included in the text.

I Jormalainen, V., Honkanen, T., Koivikko, R., Eränen, J. 2003. Induction of phlorotannin production in a brown alga: defense or resource dynamics? *Oikos* 103, 640-650.

II Koivikko, R., Loponen, J., Honkanen, T., Jormalainen, V. 2005. Contents of soluble, cell-wall-bound and exuded phlorotannins in the brown alga *Fucus vesiculosus,* with implications on their ecological aspects. *Journal of Chemical Ecology* 31, 195-212.

III Koivikko, R., Loponen, J., Jormalainen, V. Colorimetric phlorotannin quantification: Factors generating variation. *Manuscript*

IV Koivikko, R., Loponen, J., Pihlaja, K., Jormalainen, V. 2007 High-performance liquid chromatographic analysis of phlorotannins from the brown alga *Fucus vesiculosus*. *Phytochemical Analysis* 18, 326-332.

V Koivikko, R., Eränen, J. K., Loponen, J., Jormalainen, V. 2008 Variation of phlorotannins among three populations of *Fucus vesiculosus* as revealed by HPLC and colorimetric quantification. *Journal of Chemical Ecology* 34, 57-64.

ABBREVIATIONS

CNB	carbon-nutrient balance
CWB	cell-wall-bound
DAD	diode array detector
DMBA	2,4-dimethoxybenzaldehyde
DW	dry weight
ESI	electrospray ionisation
FC	Folin-Ciocalteu
GC	gas chromatography
GDB	growth-differentiation balance
HPLC	high-performance liquid chromatography
HSQC	heteronuclear single quantum correlation
I.D.	internal diameter
LC	liquid chromatography
MS	mass spectrometry
NMR	nuclear magnetic resonance
NP	normal-phase
PAD	photodiode array detector
PCA	principal component analysis
PTFE	polytetrafluoroethylene (Teflon)
PVPP	polyvinylpolypyrrolidone
r	correlation value
RP	reversed-phase
RSD	relative standard deviation
SD	standard deviation
SE	standard error
TMS	tetramethylsilane
UV	ultra violet
VIS	visible

1. INTRODUCTION

Polyphenolic secondary metabolites are a large and diverse group of chemical compounds which exist both in terrestrial plants and in aquatic macrophytes (Waterman and Mole, 1994). Tannins, a widespread family of phenolic metabolites present in many plants, have received much attention from ecologists during recent decades (Karban and Baldwin, 1997). The word "tannin" comes from the ancient Celtic word for oak, which is a typical source for tannins. Tannins have been traditionally used for millennia to tan animal hides to leather (Hagerman, 2002). Nowadays the term is widely used for large, water-soluble polyphenolic molecules having protein precipitation ability (Strack, 1997).

Tannins are commonly divided into three chemically distinct groups based on their structures. Hydrolysable tannins are characterized by a central polyhydroxyl moiety which is esterified with gallic or hexahydroxydiphenic acid (reviewed in Gross, 1999) and they occur in some green algae and are widely distributed in angiosperms (Waterman and Mole, 1994). Flavonoid-based condensed tannins are found mainly in woody plants and from e.g. red wine, tea and cocoa beans (Santos-Buelga and Scalbert, 2000). The third, less familiar group is the phlorotannins, which consist of polymers of phloroglucinol units and is restricted to the brown algae (Ragan and Glombitza, 1986). During the last two decades, the roles and functions of phlorotannins have been the subject of many studies, particularly those related to plant-herbivore interactions and anti-fouling (Hay *et al.*, 1987; Cronin, 2001; Targett and Arnold, 2001; Amsler and Fairhead, 2006). It has been suggested that tannins and related phenolic substances have primary roles in marine plants as they might serve as osmoregulatory substances in seagrasses and cell wall components in both marine vascular plants and brown algae (Arnold and Targett, 2002). Tannins may affect palatability due to their astringent taste and may act as potential antioxidant agents. It is also possible that they interact strongly in redox reactions of plants (Luck *et al.*, 1994; Larson, 1997).

The few chemical methods available for analysis of phlorotannins were reviewed two decades ago (Ragan and Glombitza, 1986). Thereafter, additional chemical methods for working with phlorotannins are rarely reported in the literature. Most commonly, phlorotannins are quantified in ecological studies with colourimetric methods intended for measurement of total phenolic contents (Amsler and Fairhead, 2006). The lack of more sophisticated analysis methods is largely due to phlorotannins being reactive and polar, and being large and structurally related to each other (reviewed by Amsler and Fairhead, 2006).

Brown algae, which comprise the Class Phaeophyceae, are unique in being very far removed phylogenetically from all other eukaryotic macrophytes (Van den Hoeck *et al.*, 1995). A great deal of the conceptual framework of defensive chemical

ecology is based on studies of vascular plants. The phylogenetic distinctiveness of the Phaeophyceae makes them ideal subjects for testing and extending ideas developed based on studies of vascular plants in trophically analogous but phylogenetically distinct organisms. Brown algae are also very important members of many marine communities ranging from the tropics to polar regions and often dominate these communities in terms of structure and biomass, particularly in temperate and polar waters (Amsler and Fairhead, 2006).

The most important habitat-forming brown alga in the brackish Baltic Sea is the perennial brown alga *Fucus vesiculosus*. Perennial macroalgae have suffered diverse consequences of eutrophication (increased levels of nutrients) in general and, for instance, reduced growing depths have been observed for *F. vesiculosus* (Kautsky *et al.*, 1986) and it has even vanished completely from large areas (Kangas *et al.*, 1982). The harmful effects of eutrophication for perennial macroalgae include decreased light availability due to water turbidity and shading by epiphytic filamentous algae (Kangas *et al.*, 1982). Nutrient availability is an environmental factor commonly found to influence resource allocation of plants (e.g. Koricheva *et al.*, 1998) and thus, the content of total phlorotannins as well (reviewed by Amsler and Fairhead, 2006). The discharge of nutrients from anthropogenic sources during recent decades has promoted eutrophication in coastal areas of the Baltic Sea. The negative effects of eutrophication include increased planktonic algal blooms, increased sedimentation of organic material, enhanced growth of annual filamentous algal species and increased water turbidity (Kangas *et al.*, 1982). Eventually, eutrophication may cause changes in plant-herbivore interactions in littoral communities (e.g. Hemmi and Jormalainen, 2002).

In addition to changes in phlorotannin content caused by nutrients, levels of phlorotannins are also found to vary plastically as a response to other environmental factors such as salinity, light availability, ultraviolet radiation and the intensity of herbivory (reviewed by Targett and Arnold, 1998; Amsler and Fairhead, 2006; Jormalainen and Honkanen, 2008). Among local populations of algae, variation in phlorotannin contents may arise from plastic responses to environmental variation or from genetic divergence among populations, or both. Although the role of genetic differentiation in the among-population variation of phlorotannins has not been directly tested, phlorotannin content in *F. vesiculosus* is found to be genetically variable (Jormalainen and Honkanen, 2004).

2. LITERATURE REVIEW

2.1. Phlorotannins

In the brown algae (Phaeophyceae), the only group of tannins present is the phlorotannins. They are polymers of phloroglucinols (1,3,5-trihydroxybenzene, Fig. 1, i) and may constitute up to 15% of the dry weight of brown algae (Ragan and Glombitza, 1986; Targett and Arnold, 1998). The relationship of phenolic substances to phloroglucinol in brown algae was first mentioned by Crato (1893) and subsequently confirmed numerous times (review by Ragan, 1976). The molecular weights of phlorotannins vary from 126 Da to 650 kDa, but are most commonly found in the 10 to 100 kDa range (Boettcher and Targett, 1993; McClintock and Baker, 2001).

Structural diversity and biosynthesis of phlorotannins
Phlorotannins are dehydro-oligomers or dehydropolymers of phloroglucinols and intensive investigations resulted in the structural elucidation of more than 150 compounds (Ragan and Glombitza, 1986). The monomeric units are linked through aryl-aryl bonds and diaryl ether bonds forming different subgroups of phlorotannins (Glombitza and Pauli, 2003). When aromatic rings are connected purely by aryl-aryl bonds, a group of fucols is formed (Fig. 1, ii). Phlorethols are formed solely by arylether bonds (Fig. 1, iii). Fuhalols are constructed of phloroglucinol units that are connected with para- and ortho-arranged ether bridges containing one additional OH-group in every third ring (Fig. 1, v). When there exists at least one three-ring moiety with a dibenzodioxin element substituted by a phenoxyl group at C-4, the group is named eckols (Fig. 1, vii). They are usually of low molecular size and thus far have only been found in the Alarieae. Carmalols are further derivatives of phlorethols containing a dibenzodioxin moiety. Endofucophlorethols (Fig. 1, iv) and isofuhalols (Fig. 1, vi) are small, distinct, specialized groups.

Phlorotannins are formed biosynthetically via the acetate-malonate pathway, also known as the polyketide pathway, in a process which may involve a polyketide synthase-type enzyme complex (Arnold and Targett, 2002). The exact biosynthetic pathway for phlorotannins is unknown and so are methodologies to monitor phlorotannin synthesis at the genetic or enzymatic levels, which could potentially help to resolve some of the uncertainties regarding phlorotannin biosynthesis (Amsler and Fairhead, 2006).

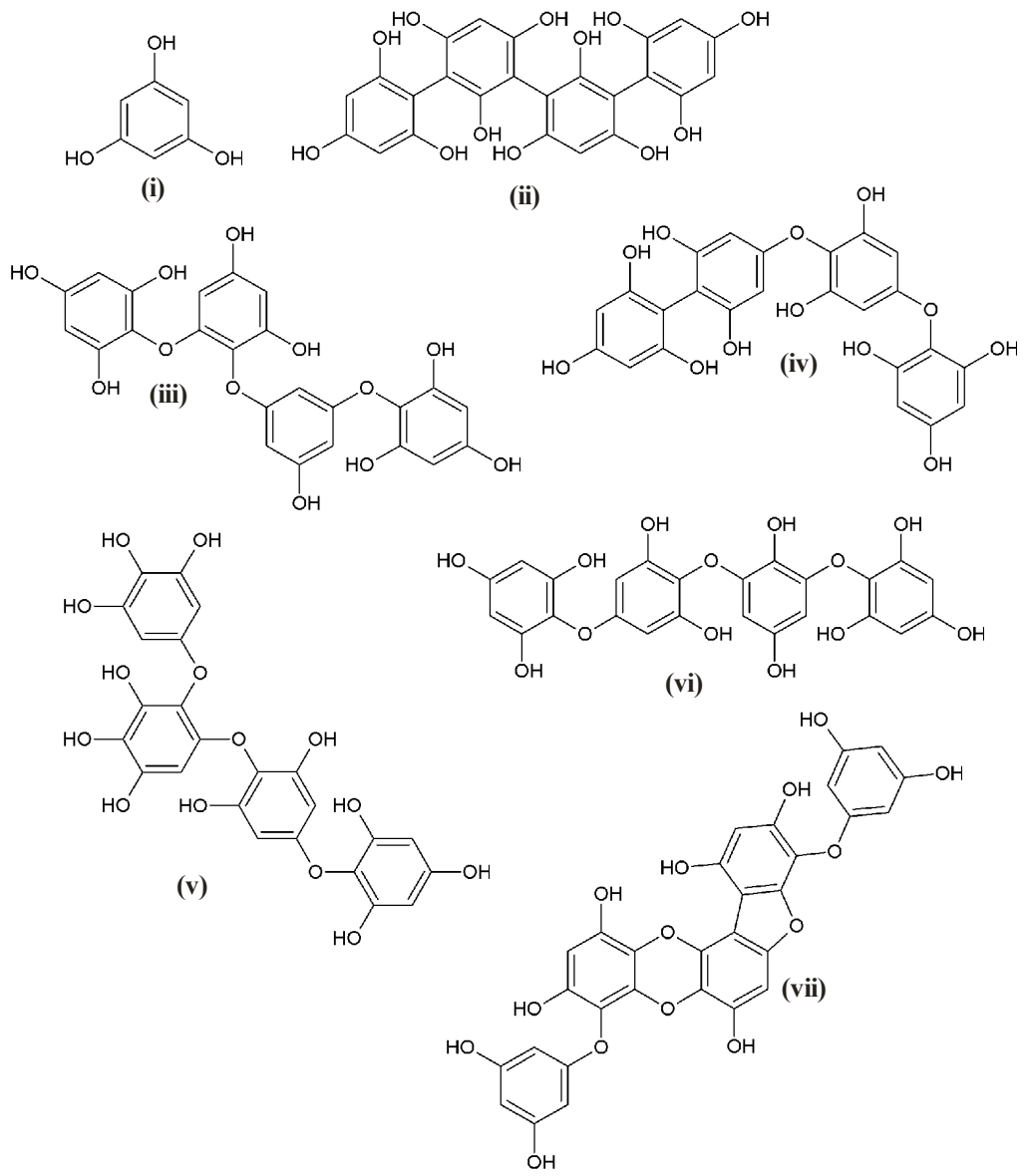

Figure 1. Structures of phloroglucinol (i) and phlorotannins [tetrafucol A (ii), tetraphlorethol B (iii), fucodiphlorethol A (iv), tetrafuhalol A (v), tetraisofuhalol (vi), phlorofucofuroeckol (vii)] (modified after Ragan and Glombitza, 1986).

In the early steps of phlorotannin biosynthesis, two molecules of acetyl co-enzyme A are converted into malonyl co-enzyme A through the addition of carbon dioxide. This changes the acetyl methyl group into a highly reactive methylene and so assists the process of polymerization to occur without the need for a high investment of energy. During further synthesis steps, the carbon dioxide, which was added as an activator, is lost (Figure 2).

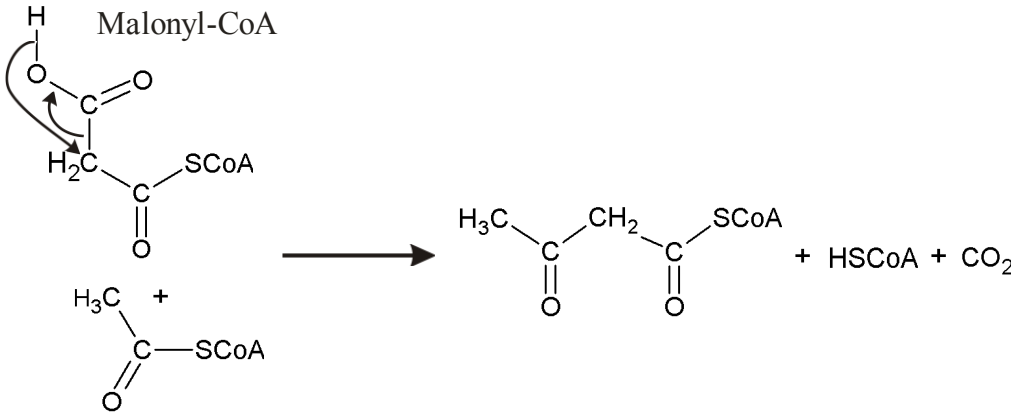

Figure 2. Combination of acetate groups (modified from Waterman and Mole, 1994).

The result of polymerization is a polyketide chain consisting of an acid moiety, and the co-enzyme is lost. The polyketide chain is transformed by intermolecular ring closure and elimination of water to produce hexacyclic ring systems (Figure 3). Triketide, the cyclization product, is not stable and thus undergoes transformation (tautomerisation) into the thermodynamically more stable aromatic form, phloroglucinol, consisting of three phenolic hydroxyl groups (Waterman and Mole, 1994). Further elements of the biosynthetic pathway are unknown.

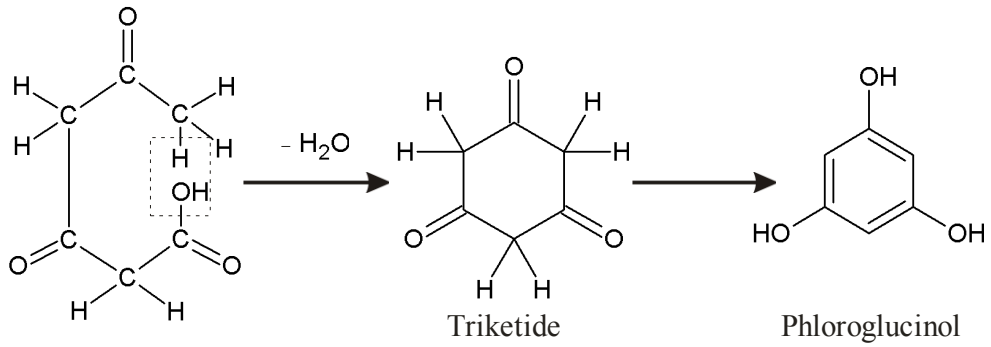

Figure 3. Cyclization of a triketide chain to form phloroglucinol (adapted from Waterman and Mole, 1994).

2.2. Isolation of phlorotannins from algae

Since there is no single protocol for extracting tannins from all plant material, the procedures used for tannins are widely variable (Hagerman, 1988). It may be that acetone in the extraction solvent increases the total yield by inhibiting interactions between tannins and proteins during extraction (Hagerman, 1988) or even by breaking hydrogen bonds between tannin-protein complexes (Porter, 1989). The

solvents most commonly used to extract phlorotannins from *F. vesiculosus* have been aqueous mixtures of ethanol and acetone (e.g. Ragan and Glombitza, 1986).

The well-known feature of free phlorotannins is that they are prone to rapid oxidation (Ragan and Glombitza, 1986). In many studies where individual phlorotannins are isolated, extracted phlorotannins are acetylated with acetic anhydride-pyridine to protect them from oxidation (Li and Glombitza, 1991b; Glombitza and Keusgen, 1995; Glombitza and Schmidt, 1999a; Glombitza and Schmidt, 1999b; Sailler and Glombitza, 1999; Glombitza and Pauli, 2003). As free phenols are needed for bioactivity testing, an efficient method for conversion of peracetylated polyphenolics into free phenolics *via* reductive ester cleavage is also needed. Ester cleavage using lithium aluminium hydride with tetrahydrofuran as solvent seems to be suitable. In the presence of potassium disulphite, rapid oxidation of free phenols can be avoided during hydrolysis by using a surplus of lithium aluminium hydride (Keusgen and Glombitza, 1997).

The addition of ascorbic acid has been effectively used in studies of other tannin groups to prevent oxidation of the extract (Peng and Jay-Allemand, 1991; Santos-Buelga and Williamson, 2003). Ascorbic acid may act as both an antioxidant and a prooxidant (Santos-Buelga and Williamson, 2003). It is thought that the addition of an antioxidant agent increases the stability of tannins.

The ability of tannins to form strong complexes with proteins, either reversibly by hydrogen bonding through peptide or amide linkages, or irreversibly by covalent condensations, is well known (Appel, 1993; Waterman and Mole, 1994; Stern *et al.*, 1996a). This ability can be used to remove tannins from samples by adding substances containing groups similar to peptide bonds e.g. polyvinylpoly-pyrrolidone (PVPP) (Andersen and Sowers, 1968). During analytical quantification of total plant phenolic compounds, tannin removal from the sample using PVPP has been used to quantify the content of non-phenolics as it is reported to bind specifically to polyphenols (Yates and Peckol, 1993; Targett *et al.*, 1995; Van Alstyne, 1995; Cronin and Hay, 1996; Peckol *et al.*, 1996; Stern *et al.*, 1996b; Pavia *et al.*, 1997; Pavia and Toth, 2000a; Toth and Pavia, 2000; Henry and Van Alstyne, 2004). A limited amount of phlorotannins can be bound to a given amount of PVPP and repeated treatment with small quantities of PVPP is recommended (Toth and Pavia, 2001).

2.3. Quantitative chemical analysis of phlorotannins

Colourimetric or biochemical methods for total quantification are very useful and reliable when quantitative information on the whole compound group is needed (Waterman and Mole, 1994). A selective method to degrade phlorotannins into phloroglucinol units by bond-breakage, similar to the acid-butanol assay for condensed tannins (reviewed by Schofield *et al.*, 2001), does not exist (Ragan and

Glombitza, 1986; Waterman and Mole, 1994; Amsler and Fairhead, 2006). Common colourimetric methods for total phenolics are used to quantify brown algal phlorotannins (reviewed by Amsler and Fairhead, 2006 and in Table 1); such assays are a natural choice for the quantification of phenolics in brown algae, using the phloroglucinol monomer as a standard, since they are known to contain no phenolics other than phlorotannins (Stern *et al.*, 1996b). The conceptual basis of the measurement is to quantify the total concentration of phenolic hydroxyl groups present in the extract being assayed, irrespective of the particular molecules in which they occur (Waterman and Mole, 1994). The assay estimating tannin content should give an identical response per mass unit with any of the compounds present in the mixture – otherwise the method will fail to give an accurate estimate for the tannin content in the sample but will only measure a property of the extracts (Scalbert, 1992). The chemical reactions in colourimetric assays do not always follow the stoichiometry; thus, the yield obtained represents an approximation of the compound group under investigation (Rohr, 2002).

The Folin-Ciocalteu method for total phenolics

The most commonly used method for measuring total phenolics is based on phosphotungstic-phosphomolybdic compounds as colour reagents, which can be used in the determination of tyrosine in proteins (Folin and Denis, 1912a; Folin and Denis, 1912b). This Folin-Denis method is capable of detecting tyrosine-containing proteins but is rarely used as such today.

The Folin-Denis reagent was improved by Otto Folin and Vintila Ciocalteu by making it more sensitive to reduction by phenolics and less prone to precipitate (Folin and Ciocalteu, 1927). The first of these improvements was achieved by increasing the ratio of molybdenum to tungsten and using liquid bromine to oxidize any traces of molybdenum-tungsten blue in the freshly prepared reagent. The precipitation problem was solved by addition of lithium sulphate. This Folin-Ciocalteu (FC) assay is also less prone to interference by non-phenolics, and for all the above reasons it has been recommended over the Folin-Denis method (Waterman and Mole, 1994).

Table 1. Different colourimetric methods and extractants used in quantifying brown algal phlorotannins with the range of values measured (Paper III).

Species	Method	Extractant	Content of phlorotannins [mg g^{-1}]
Laminariales	Folin-Ciocalteu	80% aq. methanol[1, 2]	12-66[1], 3-48[2]
	Folin-Denis	70% aq. methanol[3,4]	3-20[3], 4-41[4]
		aq. methanol[5]	54-104[5]
Fucales	Folin-Ciocalteu	80% aq. methanol[1, 2]	3-85[1], 11-60[2]
		aq. methanol[6]	6-77[6]
	Folin-Denis	aq. methanol[5]	9-101[5]
		70% aq. methanol[3,4,7,8]	54-111[3], 8-128[4], 36-56[7],13-47[8]
		85% aq. methanol[9]	19-99[9]
	DMBA	85% aq. methanol[9]	-4-43[9]
Ascophyllum nodosum	Folin-Ciocalteu	60% aq. acetone[10, 11, 12]	25-60[10]; 36-56[11]
		aq. acetone[13]	26-64[13]
		aq. methanol[14]	38-135[14]
	Folin-Denis	aq. methanol[15, 16]	57-114[15]; 48-62[16]
Fucus vesiculosus	Folin-Ciocalteu	aq. acetone[13]	39-73[13]; 29-61[18]
		60%[10] / 70%[17] aq.acetone	26-66[10]; 55-115[17]
		50% aq. methanol:water[19]	27-35[19]
	Folin-Denis	aq. methanol[20, 21]	7-9[20]; 8-28[21]
		70% aq. methanol:water[19]	not reported[19]
	DMBA	85% aq. methanol[22]	6-10[22]
Dictyotales	Folin-Denis	aq. methanol[5]	13-39[5]
		70% aq. methanol[4,7,8]	2-149[4], 29-49[7], 86-158[8]
		85% aq. methanol[9]	3-68[9]
	DMBA	85% aq. methanol[9]	-0.6-95[9]

1 (Van Alstyne *et al.*, 1999b), 2 (Van Alstyne *et al.*, 1999a), 3 (Van Alstyne and Paul, 1990), 4 (Targett *et al.*, 1992), 5 (Steinberg and Vanaltena, 1992), 6 (Connan *et al.*, 2004), 7 (Arnold and Targett, 1998), 8 (Boettcher and Targett, 1993), 9 (Stern *et al.*, 1996b), 10 (Pavia and Toth, 2000b), 11 (Toth and Pavia, 2000), 12 (Toth and Pavia, 2001), 13 (Wikström *et al.*, 2006), 14 (Pavia *et al.*, 1999), 15 (Pavia and Åberg, 1996), 16 (Pavia *et al.*, 1997), 17 (Paper II), 18 (Jormalainen *et al.*, 2003), 19 (Kubanek *et al.*, 2004), 20 (Deal *et al.*, 2003), 21 (Yates and Peckol, 1993), 22 (Arnold *et al.*, 2001)

As with the Folin-Denis method, the FC assay is also based on reduction-oxidation (redox) reactions, which are usually considered to be relatively stoichiometric, and on the redox potential of the phenolic hydroxyl group (Hagerman and Butler, 1989; Waterman and Mole, 1994). The selectivity of the FC assay is based on the oxidation of the phenolic compound. According to the description by Waterman and Mole (Waterman and Mole, 1994), a phenolic compound is oxidized in a base-catalysed redox reaction and a blue-coloured mixture containing the chromophore is formed by reduction of the phosphotungstic-phosphomolybdic complex. The mechanisms of the redox reactions and the structure of the inorganic complex formed in the FC assay are, however, incompletely understood (Waterman and Mole, 1994; Schofield *et al.*, 2001).

DMBA

The DMBA assay is specialised to quantify 1,3- and 1,3,5-substituted phenolics with 2,4-dimethoxybenzaldehyde by forming a pink coloured product. This assay is insensitive to interference, e.g. it does not react with tannic acid containing only ortho- and parahydroxyl-substituted phenolics (Stern *et al.*, 1996b). The DMBA assay requires a standard for each species analysed (Stern *et al.*, 1996b; Amsler and Fairhead, 2006). The chemical basis for the reaction between DMBA and activated phenolics such as phloroglucinol is an electrophilic attack by the aldehyde, and it must be carried out in a strongly acidic solution. The intensity of the colour produced by the reaction is dependent in part on the structure of the aldehyde (Butler *et al.*, 1982).

The variation in reactivity of different phlorotannin fractions with DMBA is somewhat greater than that for e.g. the Folin-Denis method, which is a disadvantage of the assay. This variation in reactivity presumably reflects differences in the chemical structures of different phlorotannins. A purified fraction of phlorotannins used as a standard should eliminate the differences between the reactivity of the standard and that of the samples. Another potential disadvantage of DMBA is that it can form chromophores with some non-polar metabolites but this does not apply in conditions which are used in DMBA analysis for phenolics (Stern *et al.*, 1996b).

Protein-binding assays

Both chemical and biochemical quantification assays are useful for studies of tannin-like phenolics (Waterman and Mole, 1994). Chemical assays are particularly useful for determining the amount of tannin in a sample and for elucidating the structure of the tannin. Protein-binding assays are more useful for determining the potential activity of tannin in a sample (Hagerman and Butler, 1989). In the case of phlorotannins, owing to their protein-binding abilities, which are characteristic of all tannins, the biochemical assays are worthwhile. However, such assays do not reveal the absolute content of tannins. Protein-binding activity depends mainly on the structure of the protein as well as the length and the structure of the tannin

polymer (Haslam, 1996; Waterman and Mole, 1994). Due to the length dependence, optimal-length tannin polymers, i.e. those most suitable for tannin-protein complex formation, are primarily detected. Variation in reaction conditions also generates variability in protein-tannin complexation (Waterman and Mole, 1994). Phlorotannins are found to form covalent bonds with proteins; further, the protein precipitation of phlorotannins varies in a pH-dependent as well as concentration-dependent fashion (Stern *et al.*, 1996a).

2.4. Chromatographic and spectrometric analysis of phlorotannins

In addition to the total content of phlorotannins, knowledge of variation of individual phlorotannin oligo- and polymers would be useful, since their ecological activities and biochemical functions differ from each other. Traditionally studies of plant phenolics have focused on the total phenolic content of extracts, although it is well known that the group consists of a complex set of different types of individual phenolics. Hydrolysable and condensed tannins are nowadays commonly analysed with modern separation methods, whereas phlorotannins are still analysed mainly by colourimetric methods, as total amounts of the whole compound group using phloroglucinol as a standard agent (Stern *et al.*, 1996b; Amsler and Fairhead, 2006).

Due to the high solubility (in water and/or organic solvents) of phlorotannins, high-performance liquid chromatography (HPLC) can offer a suitable tool for qualitative and quantitative analysis. Modern detection methods, such as mass spectrometry (MS) and nuclear magnetic resonance (NMR), can be combined with HPLC (Miliauskas *et al.*, 2006; Schutz *et al.*, 2006), allowing rapid structural analysis and identification of compounds with minimal manipulation of the sample. Applications of liquid chromatography also allow large-scale isolation and purification of individual compounds. Despite extensive research on the HPLC of plant phenolics, analogous studies for brown algal phlorotannins are rare. The analysis of algal polyphenolics has been suggested to be hampered by the difficulty of separating individual compounds from the polymeric mixture that occurs naturally *in vivo* (Stern *et al.*, 1996b). Phlorotannins are difficult to isolate quantitatively due to their large size, structural similarity and reactivity with other compounds.

In brown algal studies, HPLC has been applied for isolation of compounds from crude extracts. Peracetylated alcoholic extracts are eluted via silica columns with various combinations of chloroform, hexane, methanol and/or ethanol with UV detection varying from 235 nm to 275 nm. Several phlorotannins, e.g. fuhalols, deshydroxyfuhalols, sulphated phlorotannins and carmalols have been separated using HPLC and further identified (Koch *et al.*, 1980; Li and Glombitza, 1991b; Li and Glombitza, 1991a; Glombitza and Knöss, 1992; Glombitza and Keusgen, 1995; Glombitza and Schmidt, 1999a; Glombitza and Pauli, 2003). Phloroglucinol is

reported to have a UV absorbance maximum around 280 nm and the phlorotannin fraction around 260 nm (Swanson and Druehl, 2002; Henry and Van Alstyne, 2004).

The techniques most useful for elucidating structures of phlorotannins include ^{1}H and ^{13}C NMR spectroscopy. With these methods the structures of over 150 individual phlorotannins have been studied (reviewed by Ragan, 1985, also Koch *et al.*, 1980; McInnes *et al.*, 1985). The use of mass spectrometry for phlorotannin analysis has been rare, partly due to low ionisation of acetylated derivatives (Koch *et al.*, 1980; Ragan and Jamieson, 1982; Ragan, 1985; Ragan and Glombitza, 1986).

2.5. Multiple biological roles of phlorotannins

Primary roles of phlorotannins

Phenolic substances in brown algae are found in physodes, membrane-bound vesicles, which are accumulated at the zygote periphery early in development and are secreted into the primary zygote wall (Schoenwaelder and Clayton, 1998). At germination, physodes accumulate at the rhizoid tip. Physodes, together with other wall components, contribute to the development of the cell wall (Schoenwaelder and Wiencke, 2000).

It has been suggested that phlorotannins become components of brown algal cell walls when physodes fuse with the cell membrane and the phlorotannins are secreted from cells, complexing finally with alginic acid (Schoenwaelder and Clayton, 1998; Arnold and Targett, 2003) (Figure 4). The cell walls of brown algae are mainly composed of polysaccharides: alginic acid, alginates (carboxylated polysaccharides, salts of alginic acid), and fucans (sulphated polysaccharides), which comprise up to 40% of the thallus dry weight (Mabeau and Kloareg, 1987; Van den Hoeck *et al.*, 1995). Schoenwaelder and Clayton (1998) have suggested, based on microscopic studies, that phlorotannins are likely to be integral structural components of brown-algal cell walls. There are few quantitative data on cell-wall-bound (CWB) phenolics or their functional roles (but see Strack *et al.*, 1989; Peng *et al.*, 1991; Viriot *et al.*, 1993). One possibility is that phenolic compounds are bound to the cell wall during maturation of the plant (Peng *et al.*, 1991). Phenolic compounds are bound with four major types of bonds – hydrophobic, hydrogen, ionic, and covalent – in order of increasing strength (Appel, 1993). Possible linkages between the cell wall (alginic acid) and phlorotannins are the ester bond and the hemiacetal bond, both of which are covalent bonds, thus requiring strong conditions to degrade.

Phlorotannins have a putative role in brown algal reproduction, appearing on the surface of recently fertilized zygotes (Schoenwaelder and Clayton, 1998) where they may prevent multiple fertilizations by inhibiting spermatozoid movement.

Exuded phlorotannins are also known to help form the zygote wall (Schoenwaelder and Clayton, 1998).

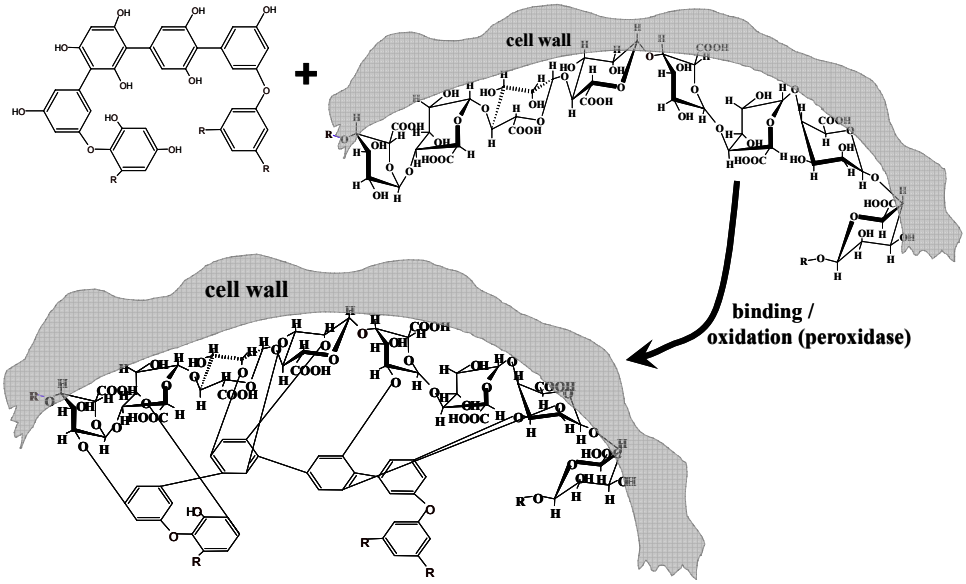

Figure 4. The model of a phlorotannin polymer being bonded covalently to the cell wall via alginic acid.

Secondary roles of phlorotannins

The secondary roles of soluble phlorotannins have been widely studied. They are generally measured as contents of total soluble phlorotannins, which are known to vary due to environmental factors as well as between species and among algal genotypes and populations (Table 2). Phlorotannin content is found to have plastic responses to environmental factors such as light and nutrient availability, ultraviolet radiation and the intensity of herbivory (reviews by Targett and Arnold, 1998; Amsler and Fairhead, 2006; Jormalainen and Honkanen, 2008).

Table 2. Recent examples of functions and responses of phlorotannins studied in different species of brown algae.
Abbreviations of species: A.n. (*Ascophyllum nodosum*), E.b. (*Eisenia bicyclis*), E.c. (*Ecklonia cava*), E.k. (*Ecklonia kurome*), E.r. (*Ecklonia radiata*), F.e. (*Fucus evanescens*), F.se. (*Fucus serratus*), F.sp. (*F. spiralis*), F.v. (*Fucus vesiculosus*), H.e. (*Himanthalia elongata*), L.d. (*Laminaria digitata*), L.h. (*Laminaria hyperborea*), M.i. (*Macrocystis integrifolia*), S.m. (*Sargassum muticum*)
Impact on phlorotannins: decrease/negative (–), increase/positive (+), no impact (o), variable (v).

	Algal species	Direction of impact	Source
THE PLASTICITY OF PHLOROTANNINS TO			
Heavy metals (Cu)	A.n.	**o**	(Toth and Pavia, 2000)
Increasing nutrients	F.v.	–	Paper II
Increasing tissue N	F.v.	–	(Pavia and Toth, 2000b)
Increasing light	F.v., A.n.	+	(Pavia and Toth, 2000b)
Increasing UV-B	M.i.	+	(Swanson and Druehl, 2002)
Wounding	E.r.	+	(Lüder and Clayton, 2004)
THE EFFECT OF PHLOROTANNINS ON			
Antioxidation	8 Phaeophyta	+	(Zubia *et al.*, 2007)
Bacteria	E.k.	–	(Nagayama *et al.*, 2002)
Cell wall construction	F.se., F.sp., H.e.	+	(Schoenwaelder and Wiencke, 2000)
Defence			
Inducible	A.n.	+	(Svensson *et al.*, 2007)
water-borne cues	A.n., F.v.	+	(Toth and Pavia, 2000)
Constitutive			
herbivore performance	F.v.	–	(Jormalainen *et al.*, 2005)
feeding preference	F.v.	–	(Kubanek *et al.*, 2004)
Enzymatic activity	E.b.	–	(Shibata *et al.*, 2003)
Fouling	F.v., F.e.	–	(Wikström and Pavia, 2004)
Human immunodeficiency virus	E.c.	–	(Ahn *et al.*, 2004)
Other algae	E.k.	–	(Nagayama *et al.*, 2003)
VARIATION IN PHLOROTANNIN CONTENTS			
Genetic	F.v.	v	Paper V
Seasonal	S.m.	v	(Plouguerné *et al.*, 2006)
Spatial	A.n.	v	(Hemmi and Jormalainen, 2004)
Within plant	A.n., S.m., L.d., L.h.	v	(Connan *et al.*, 2006)

Resource-based theories, such as the carbon-nutrient balance (CNB) and the growth-differentiation balance (GDB) theories, have been used as the contextual framework to understand allocation between growth and defence (reviewed by Cronin, 2001; Pavia and Toth, 2008). The fundamental aim of these hypotheses is to predict the variability in plant allocation patterns for survival, growth and reproduction (Herms and Mattson, 1992). In GDB, it is hypothesized that resources are allocated between growth and differentiation processes and that differentiation tends to occur after growth (Herms and Mattson, 1992). Further, according to GDB, actively growing tissue should be less defended than differentiated tissue because growth processes precede differentiation processes. The hypothesis behind the CNB suggests that plants allocate all photosynthate to growth processes when light is the limiting factor for growth. On the other hand, under conditions where nutrients are more growth limiting than light, excess photosynthate will be allocated to the production of C-based chemical defences (Bryant *et al.*, 1983).

Phlorotannins can serve in both constitutive and induced defence against herbivory (Amsler and Fairhead, 2006). The inducible defences of plants are phenotypic responses that act to reduce or redistribute the damage inflicted by grazers and therefore decrease the negative fitness consequences of herbivores (Karban and Baldwin, 1997). Induced defences may be more cost effective than constitutive defences in that they allow plants to deploy defences only when they are needed and thus avoid allocating resources to defence when herbivores are absent (Arnold *et al.*, 2001).

The protein-binding ability of tannins has been suggested to be one of the most important factors in reducing the suitability of plants for herbivores (Stern *et al.*, 1996a). On the other hand, depending on the physiological conditions of the gut, formation of protein-tannin complexes might not occur (Martin *et al.*, 1987) or phenolics might be ionized and much of their hydrogen-bonding ability lost (Hagerman and Butler, 1978). As polyphenols are prone to oxidation, one alternative mode of action is the prooxidant activity of phenolic compounds promoting damage to nutrients in the gut lumen and causing oxidative stress in the surrounding gut tissues (Appel, 1993; Barbehenn *et al.*, 2005; Barbehenn *et al.*, 2006).

In addition to soluble phlorotannins in the physodes and bound phlorotannins in the cell wall, brown seaweeds are known to release, i.e. exude, phlorotannins into the surrounding seawater (Carlson and Carlson, 1984; Ragan and Glombitza, 1986; Jennings and Steinberg, 1994; Toth and Pavia, 2000; Swanson and Druehl, 2002). Phlorotannins may also be released indirectly due to grazing or tissue decomposition. Only limited data are available regarding quantities of exuded phlorotannins (see, however, Toth and Pavia, 2000 and Paper II). Such compounds may well be important with respect to natural enemies (Jormalainen and Honkanen, 2004).

Total phlorotannin contents commonly vary among local populations of algae (Pavia *et al.*, 1999; Honkanen *et al.*, 2002; Pavia *et al.*, 2003; Hemmi and Jormalainen, 2004). This variation may arise either from the plastic responses of algae to environmental variation or from the genetic divergence among populations, or both. Although the role of genetic differentiation in the among-population variation of phlorotannins has not been directly tested, such differentiation is possible. Phlorotannin concentration in *Fucus vesiculosus* has been found to vary genetically (Jormalainen and Honkanen, 2004). Based on neutral molecular markers, genetic differentiation has been observed among adjacent *F. vesiculosus* populations in the Baltic Sea (Bergström *et al.*, 2005; Tatarenkov *et al.*, 2007), as well as among populations of *F. serratus*, in which there is substantial genetic sub-structuring at a scale of less than two kilometers along the Atlantic and southern Baltic Sea coastlines (Coyer *et al.*, 2003). While neutral markers indicate differentiation due to genetic drift, spatial variation in natural selection may also lead to among-population differences in the contents of total phlorotannins (Jormalainen and Honkanen, 2004).

3. AIMS OF THE STUDY

The main aim of this study was to develop chemical methods for analysis of brown algal phlorotannins. Using these methods, the contents of phlorotannins as well as their responses to variations in e.g. herbivory and nutrient availability were studied. The specific goals were:

1) To develop and adjust methods to analyse soluble, CWB and exuded phlorotannins (Papers II, III)

2) To develop an HPLC method for more precise phlorotannin studies, to study oxidation prevention and to compare the results achieved using HPLC to those obtained by the method quantifying total phlorotannin contents (Papers IV, V)

3) To analyse the contents of soluble, CWB and exuded phlorotannins (Papers I, II, III, V)

4) To examine the ecological role of phlorotannins with regard to herbivore deterrence; to examine the responses of phlorotannins to nutrient availability; and to examine genetic as well as spatial variation in phlorotannin contents using the adjusted colourimetric method for total phenolics and/or the HPLC method (Papers I, II, V)

4. MATERIALS AND METHODS

More detailed descriptions of the materials and methods used are given in publications I-V.

4.1. Study species

Fucus vesiculosus (L.), bladder wrack, is a brown alga dominating the littoral, non-tidal, rocky shorelines of Finland and the Archipelago Sea in Southwestern Finland. In the littoral community of the brackish Baltic Sea, it is the only canopy-forming seaweed. *F. vesiculosus* is a perennial species, with a life-span of several years for algal individuals. It grows only at the apical tips, where the thallus grows by dichotomous branching. In contrast, the basal thallus is non-growing tissue. During the reproductive period most of the apical tips generate reproductive glands, i.e. generative apices. In the environment of the archipelago, local populations of *F. vesiculosus* are naturally fragmented. The fragmentation is further increased by eutrophication. The isolation of fragmented populations is heightened by the low dispersal ability of fucoid propagules (Chapman, 1995). In this study, the algal material and herbivores were collected between latitudes 59º and 62º. Within these latitudes, the salinity of the brackish Baltic Sea varies from 5 to 6 ‰.

For my studies, either whole individuals or just the parts of the algae that were of interest were collected. The samples were cleaned of epiphytes and placed on ice as soon as possible. In the laboratory, the samples were frozen, freeze-dried, homogenized and stored in the dark at −20°C until analysed.

In the Baltic Sea, the isopod *Idotea baltica* (Pallas) is the most important herbivore of *F. vesiculosus*. *I. baltica* is a generalist feeder, but prefers *F. vesiculosus* over other potential host species (Nicotri, 1980; Jormalainen *et al.*, 2001). For isopods, *F. vesiculosus* provides distinct microhabitats, with the vegetative tips being light green and made of softer tissue, whereas tougher basal parts are darker and often spotted by white epizoites (Tuomi *et al.*, 1989; Merilaita, 1998). *F. vesiculosus* is also host to gastropod grazers. *Theodoxus fluviatilis* and *Physa fontinalis* occur on *F. vesiculosus* but they do not feed on mature thallus (pers. obs. by authors, verified by scanning electron microscopy). Instead, they feed on epibiota and particulate matter on the thallus (Skoog, 1978; Jones *et al.*, 1999; Malm *et al.*, 1999). *T. fluviatilis* is very abundant in the study area, reaching densities of hundreds of individuals per individual alga (Korpinen *et al.*, 2007). *P. fontinalis* is clearly less abundant, occurring in densities of a few individuals per alga (Pettay, 2001).

4.2. Extraction of phlorotannins

Soluble phlorotannins
Various extractants were used to release soluble phlorotannins from the algal matrix (Table 1). Here, the basic procedure for large-scale samples (200 mg dry algal material, Paper I-III, V) was to extract the algal powder four times with 10 ml of 7:3 acetone-water. The acetone was removed in the fume hood, and the resulting aqueous phase was frozen, freeze-dried and dissolved into water.

Minimizing the necessary sample size is beneficial; it reduces the time and reagents needed for each analysis and enables studies on a smaller scale, e.g. of different parts of the algae. A modified method was applied to extract small-scale samples (3 - 20 mg dry algal material) in Eppendorf-tube using 1.5 ml of extractant for each sample (Paper III).

Methods to prevent the oxidation of phlorotannins were studied by visually determining how long the extracted samples containing phlorotannins maintained their initial colour. Oxidation prevention was tested by reducing the temperature, adding different amounts of ascorbic acid (0.0-0.5% (w/v)), reducing pH (with diluted formic acid), adding various other antioxidants (Peterson *et al.*, 1997) and modifying phlorotannins to their acetylated derivatives. The aim was, though, to find a procedure to analyse phlorotannins without modifications since the future goal is to expand the same procedure to a preparative scale. Based on the results of the oxidation prevention tests, the basic extraction procedure for the large samples used for HPLC analyses (Paper IV, V) was enhanced by adding 0.3% (w/v) of ascorbic acid to prevent oxidation of the sample during analysis. Prior to HPLC analysis, all the samples were filtered through 0.45 μm PTFE filters.

Cell-wall-bound phlorotannins
In addition to the studies of the soluble phlorotannins, a method was developed to study the contents of phlorotannins attached to the cell wall (cell-wall-bound (CWB), Paper II). For this, the residues left from the extractions of soluble phlorotannins were first washed. The washing procedure included methanol, water, acetone and diethyl ether, after which the residues were oven dried. To release the CWB phlorotannins, an acidic-degradation method used for wood ellagitannins was tested (Peng *et al.*, 1991). In this acidic treatment the washed insoluble residues were heated with methanol and hydrochloric acid. An alkaline degradation treatment modified from a method applied to spruce needles was also used (Strack *et al.*, 1989). Preliminary tests showed the alkaline degradation to be slightly more effective than the acidic treatment (data not shown), and thus the alkaline treatment was chosen for further analyses. In order to modify the method to suit algal analyses, the following features were studied: (i) the effect of the reaction time, (ii) the strength of the sodium hydroxide (NaOH) solution, (iii) the number of repeated treatments, and (iv) the pH of the final sample. In the modified alkaline treatment,

the total insoluble residue remaining after the extraction of soluble phlorotannins and the washing procedure was suspended in 1 M sodium hydroxide solution. The alkaline treatment was repeated four times to obtain all the CWB phlorotannins. An aliquot from each treatment was neutralized for the FC assay.

Exuded phlorotannins

The aim of paper II was to apply a method to study the quantity of exuded phlorotannins. The water surrounding the growing algae was filtered and stored in dark bottles at 4ºC. An aliquot was taken and evaporated to a smaller, exact volume. As a reference for analyses, artificial seawater containing equal amounts of salt compared to the original samples was prepared. These analyses were carried out using the FC assay in order to quantify the amounts of phlorotannins.

4.3. Quantification of the total phlorotannins

Folin-Ciocalteu assay

To quantify the contents of total phlorotannins in the samples containing soluble, CWB or exuded phlorotannins, a modified FC assay was used. In the standard procedure an aliquot of the diluted sample was mixed in a test tube with 1 N Folin-Ciocalteu reagent (Merck) (1/1, v/v). The mixture was allowed to stand at room temperature, after which aqueous sodium carbonate was added. The samples were incubated in the dark at room temperature then centrifuged, and the absorbances of the supernatants were measured.

For the small-scale samples, different dilutions were used to achieve optimal analysis conditions for the FC assay [dilution ratio = $V_{extract}/(V_{extract} + V_{water})$]. The effect of a catalyst on the FC assay was studied by varying the volume of added Na_2CO_3 solution (1, 2, 4 and 6 ml) and the effect of the reaction time was studied by varying the incubation time (0, 30, 45, 90 and 120 min). The effect of pH, in turn, was tested by adding different amounts of acid to the samples of CWB phlorotannins.

The UV absorbance results were converted into concentrations using a standard curve obtained with phloroglucinol (anhydrous, ICN Biomedicals Inc). The amount of phenolics (P) in the sample was then calculated using a formula modified from Waterman and Mole (1994):

$$P = \frac{V_{solvent}}{V_{extract}} \times \left[\frac{V_{extract} + V_{water}}{V_{fractdil}} \right] \times Z$$

where $V_{solvent}$ = the volume [ml] of solvent into which the W (= the weight of the sub sample [mg]) is diluted
$V_{extract}$ = the sub-volume taken from $V_{solvent}$ for the analysis [ml]
V_{water} = the volume with which $V_{extract}$ is diluted [ml]

$V_{fractdil}$ = the fraction of the diluted sample ($V_{extract}$ + V_{water}) which is used in the assay [ml]
Z = the amount of phenolics in $V_{fractdil}$ calculated using the linear calibration curve [mg]
P = the amount of phenolics in the sample of size W [mg]. The results were converted into % of phlorotannins per dry weight of the sample (= P/W × 100)

In addition to the calibration curve of the phloroglucinol standard, calibration curves were also obtained with two separate phlorotannin-rich *F. vesiculosus* extracts (extract A and B) as standards.

4.4. Qualitative analysis of phlorotannins

Reversed-phase high-performance liquid chromatography
The RP-HPLC system (Merck-Hitachi, Tokyo, Japan) used in paper IV consisted of an L-7100 pump, an L-7200 autosampler, either an L-7455 diode array detector (DAD) or an L-7400 UV-detector, and a D-7000 interface. The column was a Superspher 100 RP-18 (250 × 4 mm i.d.; 4 μm; Merck, Germany). Phenolic compounds were eluted by a gradient of solvents A (1% formic acid in water) and B (acetonitrile). The elution profile used was 0–5 min, 100% A (isocratic); 5–60 min, 0–30% B in A (linear); 60–70 min, 30–60% B in A (linear); 70–80 min, 60% B in A (isocratic); 80–90 min, 60–0% B in A (linear). Phenolic compounds were detected at a wavelength of 280 nm, the flow-rate was 1 mL/min and the injection volume was 20 μL. In addition to formic acid, also acetic acid and 0.05 M phosphoric acid were tested as solvent A since acid is often used to improve chromatographic resolution.

Normal-phase high-performance liquid chromatography
In papers IV and V, the NP-HPLC system used consisted of HP (Hewlett-Packard, Palo Alto, CA, USA) 1090 liquid chromatography with a built-in DAD and HP Chemstations software for LC 3D systems. The column was a LiChrospher Si 60 (250 × 4 mm i.d.; 5 μm; Merck) equipped with a guard cartridge (LiChrospher Si 60; 4 × 4 mm i.d.; 5 μm; Merck). The binary mobile phase consisted of (A) dichloromethane, methanol, water and acetic acid (82:14:2:2, v/v) and (B) methanol, water and acetic acid (96:2:2, v/v). The elution gradient was: 0 min, 100% A (isocratic); 0–30 min 0–17.6% B in A (linear); 30–45 min 17.6–30.7% B in A (linear); 45–50 min 30.7–87.8% B in A (linear); 50–60 min 87.8% B in A (isocratic); 60–80 min 87.8–0% B in A (linear); 80–105 min 100% A (isocratic) (method modified from Adamson *et al.*, 1999). The detection wavelength was 280 nm, the flow-rate 1.0 mL/min and the injection volume 1 μL.

Spectrometric and spectroscopic methods
Preliminary NP-HPLC-ESI/MS studies were conducted in paper IV. The MS (SCIEX API 365, PE Sciex, Foster City, CA, USA) was operated in the negative

ion mode: spray needle voltage, −4200 V; heated nitrogen gas temperature, 310°C; orifice plate voltage, −35 V; ring voltage, −220 V; nebuliser gas (purified air), set at 10; curtain gas (nitrogen) set at 12. Mass data was acquired over a mass range of *m/z* 100–2800 amu in steps of 0.3 amu. The split ratio was 7:3 prior to introduction into the ionisation chamber.

NMR-spectra gave proof of the existence of phlorotannins. The spectra were acquired using a Bruker Avance (Bruker BioSpin Inc., Fällanden, Switzerland) 500 spectrometer (equipped with BBI-5 mm-Zgrad-ATM probe) operating at 500.13 MHz for ^{1}H and 125.77 MHz for ^{13}C. Spectra were recorded at 25°C using acetone-d_6 as a solvent. The ^{1}H spectra were referenced internally to TMS. In addition to the standard ^{1}H spectra, two-dimensional gradient-selected HSQC (heteronuclear single quantum coherence) spectra were also recorded.

4.5. Experimental designs for rearing algae

An experiment to study the induction of phlorotannin production was conducted in paper I. 15 *F. vesiculosus* individuals were collected and cut into 28 pieces per individual to produce clonal material which is commonly used to evaluate genetic effects (Schwaegerle *et al.*, 2000). Twenty-eight flow-through tanks were established with one clone of every algal individual in each and these tanks were assigned to seven treatments, with four replicate aquaria for each treatment. Treatment (1) was the control with no manipulations. Treatment (2) got nutrient enrichment, and in treatment (3) the surface of the thallus was cleaned of the epibiota every second day. Treatment (4) experienced both the nutrient enrichment and removal of epibiota. In treatment (5), *T. fluviatilis* (n=150) grazed on the algae and in treatment (6), *T. fluviatilis* (n=150) was not grazing but was present in an enclosed mesh-net bag. The last treatment (7) had *P. fontinalis* (n=52) grazing on the algae. The algae were reared for two months and the apical thalli that grew during the experiment were used for the phlorotannin analyses. Two replicates per genotype were combined for each treatment. Thus, from each genotype there were 14 replicates, and there were 30 replicates for each treatment.

To study the variation in the contents of soluble and CWB phlorotannins (Paper II), 20 *F. vesiculosus* individuals were collected from one population and placed in 10 outdoor flow-through tanks (60 l) with natural light conditions, with two individuals in each tank. Five microcosms were randomly assigned to a nutrient-enrichment treatment, while the others served as controls. After 40 days, samples were taken for analyses from both the apical parts that had grown during the experiment, and the basal, nongrowing parts of the thallus.

In paper II, variation in the amounts of exuded phlorotannins due to herbivory and nutrients was also studied. Four *F. vesiculosus* individuals were collected and cloned into three pieces each and placed in aquaria under greenhouse lights. The

aquaria were randomly assigned to control, herbivore and nutrient shortage treatments. The nutrient availability of the former two was maintained with added nutrients whereas in the latter, algae faced a nutrient shortage because algae quickly deplete the ambient nutrients in closed aquaria when light is not limited. The herbivore treatment was conducted by adding five *I. baltica* individuals into the aquaria. The experiment was maintained for 14 d, after which the water from each aquarium was collected for analysis of the exuded phlorotannins.

The among-population variation of phlorotannin concentration was studied by colourimetric quantification as well as by HPLC (Paper V). Fifteen *F. vesiculosus* genotypes were collected from three different populations (Aaviikki, Jurmo and Säppi) located 80-185 km apart. Those 45 genotypes were split into four apical pieces which were transferred into a common environment to eliminate the among-population differences due to phenotypic plasticity. Half of the pieces were assigned to the nutrient enrichment treatment while the rest were controls. After three months the apical parts grown during the experiment were collected and analysed for their total phlorotannin content as well as for their phlorotannin profile.

4.6. Statistical analyses

The amount of variance in phlorotannin concentration due to treatments and genotype was compared by using genotypic mean values as the response variables in the statistical analysis (Paper I). To compare the amount of variance in phlorotannins due to the treatments and genotype, the respective variance components were calculated. Correlations between growth and phlorotannin concentration were calculated for each treatment separately by using the genotypic mean values of the traits.

ANOVA was used to test whether the contents of soluble and CWB phlorotannins, and variation in the amount of exudation, differed between nutrient and grazing treatments or thallus type (Paper II). Whether algal growth rates differed between treatments was tested using ANCOVA. Tukey *a posteriori* comparisons were done to compare how each treatment affected the amounts of exuded phlorotannins.

In paper V, the repeatability of the individual peaks in the HPLC profile was studied by examining the consistency of measures within an individual alga (Krebs, 1999). This was done with one-way ANOVA. The Pearson correlation coefficients between individual peaks of the chromatogram and the contents of total phlorotannins were calculated in order to clarify the contribution of the HPLC profile to the total phlorotannin content. Multiple regression analyses were conducted to estimate how well the variation in the phlorotannin profile can explain the variation in total phlorotannin contents. Both population and nutrient enrichment effects (fixed) on the phlorotannin contents were tested by using

mixed-model ANOVAs (genotype as a random effect). To estimate the variation of the individual HPLC peaks among populations, the among-population variance components were calculated. To quantify the effect of nutrient enrichment, the effect sizes of nutrient enrichment for each peak and total phlorotannins were calculated (Gurevitch and Hedges, 2001). MANOVA was applied to test the effects of population, nutrient enrichment and genotype on the phlorotannin profile and to visualize the among-population differences in the phlorotannin profile by conducting a principal component analysis (PCA). In PCA, as well as in multiple regression analyses, only the 7 peaks found to be repeatable were utilized (#3, 7, 8, 11, 13, 16). All statistics were calculated using the SAS statistical package (SAS Institute, 1999)

5. RESULTS AND DISCUSSION

5.1. Extraction of phlorotannins

This section reports on studies of the effects of extractant, extraction repetition and the amount of sample on the phlorotannin yield of samples of *F. vesiculosus*, and the use of this data to optimise extraction conditions.

The polarity of the extractant as well as the solubility of the compounds of interest have a critical effet on the yield of polyphenols. The extraction of different phenolics from different plant material requires extractants with different polarities (Waterman and Mole, 1994; van Beek, 2002). Phenolic compounds are usually easily soluble in extractants less polar than water. The general recommendation for an extractant is a mixture of water and methanol, ethanol, or acetone (Waterman and Mole, 1994). In the extraction of phlorotannins, the efficiency of the solvent generally increased with increasing polarity (Figure 5, Paper II). The 7:3 acetone-water solvent was an exception to this rule since it was the most efficient extractant for brown algae. It may be that acetone increases the total yield by inhibiting interactions between tannins and proteins and other polyols during extraction (Hagerman, 1988) or even by breaking hydrogen bonds (Porter, 1989).

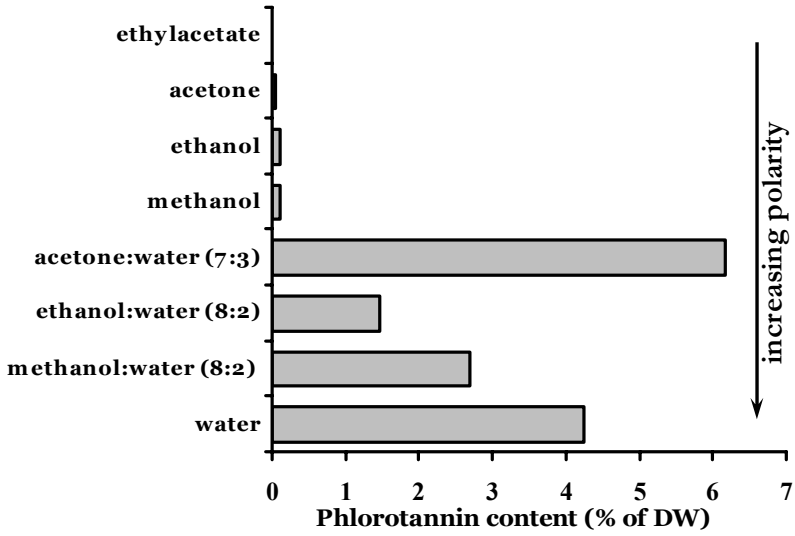

Figure 5. Phlorotannin content when extracting algal material with different solvents. Extractants are arranged from ethyl acetate to water in increasing order of polarity (polarity δ (SI) 18.6; 26.0; 29.6; 20.2; 28.5; 41.1; 42.4 and 47.9 $MPa^{1/2}$ respectively) according to Hildebrand solubility parameters (Barton, 1983).

Reducing the size of the original sample (varying from 3 to 200 mgs) caused variations in the final results (Figure 6, Paper III). Variation among replicates increased with reduction of the sample size below 15 mg. The increase in variation with small samples may indicate that replicate samples may differ in mixing with the extractant as sample is e.g. stucked onto the wall of the mixing tube.

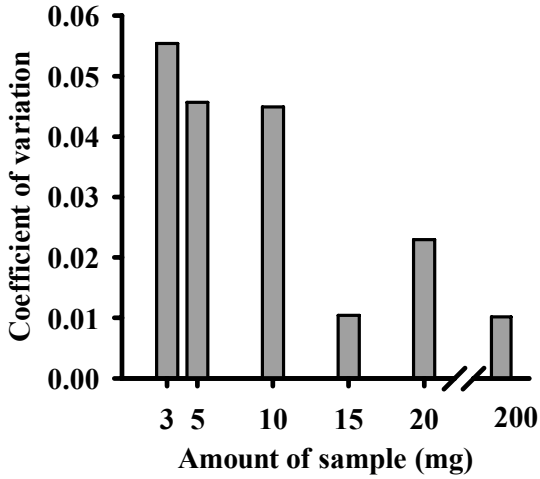

Figure 6. Coefficient of variation (= SD / average) in content of phloroglucinol (percentage of dry weight in phloroglucinol units). Five replicates for each sample size were performed.

The effect of the number of repeated extractions/treatments on the soluble phlorotannins with large (200 mg) as well as small scale samples (3-20 mg) and with CWB phlorotannins were studied (Paper II, Figure 7). For the large scale samples, most of the soluble phlorotannins (80% of the total in 13 consecutive extractions) were released during the first extraction. Four extractions yielded ca. 94% of the total in 13 successive extractions; therefore four repeats were used in further studies. In the extractions of soluble phlorotannins with small scale samples, the first two extractions yielded ca. 95% of the soluble phlorotannins released with 5 successive extractions, thus two repeats were used in further studies.

As with soluble phlorotannins, most of the CWB phlorotannins (69%) were released by the first alkaline treatment. The alkaline degradation was found to be effective in releasing CWB phlorotannins since three treatments released all the detectable phlorotannins. The results were obtained by combining the yields of three treatments. The three parallel treatments showed good repeatability for the treatment.

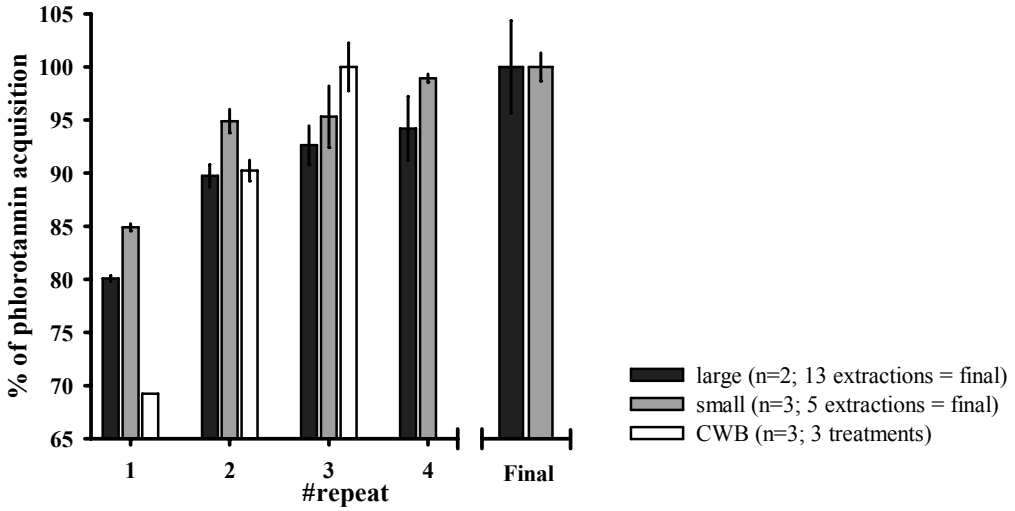

Figure 7. Acquisition of soluble (large and small scale) and CWB phlorotannins when extracted with 7:3 acetone-water (soluble) or treated with alkali (CWB), in consecutive extractions/treatments (mean ± SD).

To prepare the samples for treatment to obtain the CWB phlorotannins, the insoluble residues left from the extractions of soluble phlorotannins were first washed in order to remove all the remaining soluble phlorotannins. The pooled results of soluble phlorotannins from 34 samples represented 8.0% (±0.2) of the dry weight of the sample. The washings of residues were combined and the pooled mean content of the remaining soluble phlorotannins from those 34 samples was 0.36% (±0.03) of dry weight. It can be suggested that the washing procedure prior to alkaline treatment (degradation) minimizes the amount of soluble phlorotannins remained in the fraction of insoluble phlorotannins (see also Salminen, 2003).

5.2. Quantification of phlorotannins

Folin-Ciocalteu –assay for total phlorotannin content

We also examined how changes in pH and dilution of the sample as well as the reaction time and the amount of catalyst used in the assay affect the yield when samples are analysed with the FC assay.

The alkaline degradation of CWB phlorotannins included the reduction of the pH of the CWB phlorotannin liquor aliquot from 13 to 2. The effect of pH within this range on total phlorotannin content measured with the Folin-Ciocalteu method was almost linear; the variation between the high and low ends of the range is about 2% in phloroglucinol units (Figure 8, Paper II). Thus, the fractions were neutralized to make the pH conditions equal in different samples for quantitation with the FC assay.

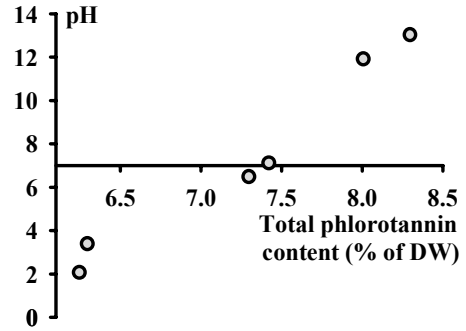

Figure 8. The effect of sample pH on the yield in terms of the total phlorotannin content measured with the FC assay.

The adjustments made to the FC assay conditions for small-sized samples revealed a significant positive correlation between the absorbance and the dilution ratio of a sample; the more diluted the sample, the less intense the absorbance (Figure 9a, Paper III). The absorbance values were converted into phlorotannin content (as percentage of dry weight) by taking into account computationally the factors of sample preparation and dilution and the standard curve of phloroglucinol. The resulting relationship with the dilution ratio was not horizontal, but rather was negative (Figure 9b). Thus a more concentrated sample yielded less phlorotannins. The decrease in yields between the most and least diluted samples (Figure 9b) was considerable, at over 30%. Similarly, colour formation in the acid butanol assay is also found to be affected by the amount of sample (Waterman and Mole, 1994; Schofield *et al.*, 2001).

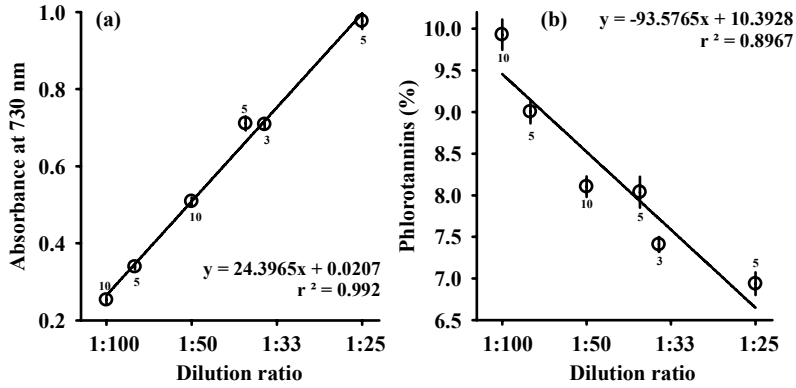

Figure 9. The effect of dilution ratio on the absorbance (a) and content of phlorotannins (b). Each dot represents mean (± SD) of several replicate determinations (m = 20 mg; n = next to the dot).

Possible reasons why different dilutions of the same sample do not give the same results might be the reaction time, binding during extraction or the amount of the catalyst. The yield was not affected by varying the reaction time from zero to 120

minutes. When considering binding during extraction, a more concentrated sample offers more possibilities for reactions between proteins and phenolics, which may cause quenching of absorbance. The relative amount of protein binding to tannins should remain approximately constant since a more concentrated sample with more proteins present should also contain more tannins in the same ratio. It is possible that the reagents, particularly the tungsten-molybdate complex may react differently when the dilution ratio changes: redox reactions may occur more easily at low concentrations than at high ones. The amount of the catalyst, sodium carbonate, clearly affected the phlorotannin yield, which decreased as the catalyst concentration increased; the effect was clearer with the less diluted samples (Figure 10, Paper III). The decrease in the yield was steepest between the addition of one and two ml of catalyst; increasing the amount of the catalyst beyond two ml had no effect on the yield. This implies that reaction conditions in the FC assay should be sufficiently alkaline to allow proton donation from a weakly acidic phenolic compound so that the phenolate ion can be formed. Thus, the dependency of the yield on the dilution ratio may follow from the properties of the sample, the reaction or the reaction conditions.

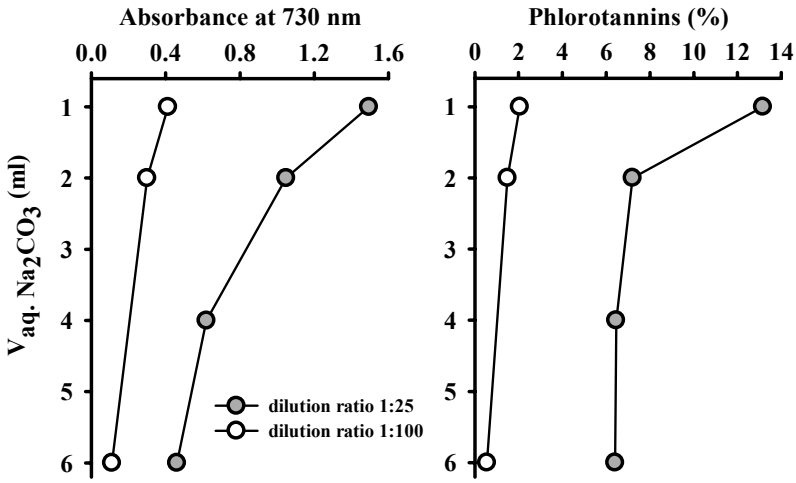

Figure 10. The effect of catalyst (Na_2CO_3) on the yield of phlorotannins measured with the FC assay (modified from paper III).

Several factors during the procedure were found to cause variation in the yield of total phlorotannins. In order to obtain comparable yields within a range of sample sizes, the size of the algal sample, the volume of the extractant, the number of extraction repeats and the dilution ratios were adjusted (Table 3, Paper III).

Table 3. The amounts of samples and extractants and the number of extraction repeats (extraction), dilution ratios (dr) and the amounts of extract and water in determining total soluble phlorotannins for *Fucus vesiculosus*.

Sample [mg]	extraction	dr	$V_{extract}$ [ml]	V_{water} [ml]
200	10 ml × 4	1:100	0.05	4.95
20	0.75 ml × 2	1:35	0.09	3.06
15	0.75 ml × 2	1:28	0.11	2.97
10	0.75 ml × 2	1:19	0.16	2.88
5	0.75 ml × 2	1:10	0.27	2.43
3	0.75 ml × 2	1:6	0.5	2.5

A common problem in quantitative analysis is the lack of proper and reliable standards. To minimize problems arising from the use of inappropriate standards, the use of "self-standards" derived from the plant material itself, has been proposed (Hagerman and Butler, 1989; Waterman and Mole, 1994; Appel *et al.*, 2001). Nonetheless, phloroglucinol is still commonly used as a standard in the quantification of phlorotannins. None of the 22 reviewed studies (Table 1) reported the use of a self-standard, while phloroglucinol was used in twenty studies out of the twenty-two. When a polymeric mixture is analysed using a monomer as a standard, short and long polymers cannot be discriminated, and quantitatively similar determination may contain qualitative differences. If there were a method to break down the phlorotannin polymers into monomers, the use of phloroglucinol would be better justified.

In order to study the effect of the standard, calibration curves were derived for manufactured phloroglucinol and two different kinds of extract from *F. vesiculosus* (Figure 11, Paper III). The latter two acted as self standards. The extraction method of the standard was found to have a profound influence on the results, possibly at least partly due to differences in the contents or composition of compounds between the self-standard and the sample. The more purified self-standard (phlorotannin extract B) resulted in a curve closer to that of phloroglucinol than the less purified extract A. With an increase in the proportion of byproduct compounds in the standard, the amount of the standard needs to be increased to obtain the same substance level as in the more highly purified standard fraction. Consequently, the less purified standard tends to give an overestimation of the phlorotannin contents. Furthermore, the composition of longer versus shorter polymers in the standard versus the sample may differ and potentially invalidate the results.

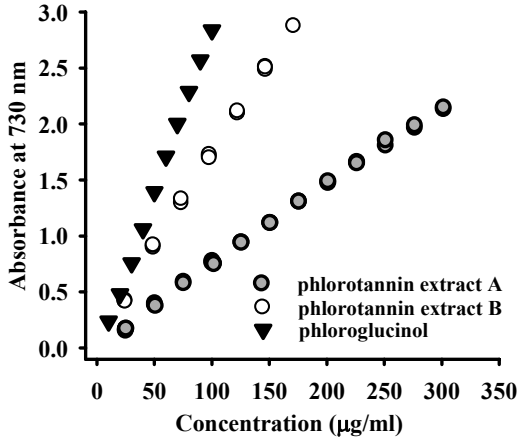

Figure 11. Calibration curves for two self standards (extracts A and B) and for phloroglucinol.

Many factors caused variability in the quantitation of phlorotannins, starting with the sample size and selected extractant. From the perspective of chemical methodology on brown algal phlorotannin quantification for Laminariales, Fucales and Dictyotales, 22 studies were reviewed (Table 1). Both the quantification methods and the extractants were found to be highly variable. Comparison between studies is not possible due to the variation in methods of analysis. In general, the precise analysis conditions, such as the amount of sample or extractant, and dilution ratios, are rarely described in the chemical methodology. In many ecological studies, determination of total phlorotannin appears to be sufficient. Most important is to understand what the method does measure and what kind of methodological artefacts are involved.

5.3. Separation and determination of phlorotannins

With total quantification methods, the possible synergistic as well as antagonist interactions of individual compounds remain undetected, which may be deceptive when chemical data are used to interpret ecological impacts. Liquid chromatographic conditions were studied in order to obtain proper separation of the phlorotannin fraction. Proper separation also benefits MS analysis, as better separation means more purified compounds, which results in better ionisation and identification.

Phlorotannins are able to form net-like structures in addition to chains, and may also occur as halogenated derivatives where phloroglucinol units are combined together via aryl–aryl or diaryl–ether bonds (Ragan and Glombitza, 1986; Figure 1). These characteristics may cause difficulties both in HPLC and NMR analyses.

The present MS analysis yielded no evidence for other tannins or known phenolics. This finding supports the suggestion made by Ragan and Glombitza (1986), that phlorotannins are the only phenolic group in brown algae. Also as the extraction procedure is for phenolic compounds, it was concluded that the detected peaks represent either individual phlorotannins or consist of several, possibly similarly sized phlorotannin polymers.

In order to achieve liquid chromatographic separation of phlorotannins eluent- and gradient-related tests were conducted. The optimization of phlorotannin analysis using RP-HPLC did not produce sufficient separation of peaks at the beginning of the chromatogram under any conditions (Figure 12, Paper IV). Instead of separation, the sample seemed to elute directly with the solvent. The same situation occurred with Sephadex LH 20 fractionation, where the crude extract containing phlorotannins emerged from the column without retention (data not shown). Different phlorotannins are structurally relatively similar to each other (Figure 1), and phlorotannins in general are more compact than other tannin polymers. This may be one reason why they are eluted very rapidly through the RP column, although elution solvents containing phosphoric acid slightly increased the retention of phlorotannins in the column. The separation of the longer condensed tannins is complicated by the increase in the number of isomers with increasing polymer length and the possibility of irreversible adsorption for polymers interacting strongly with the adsorbent material of the column (Labarbe *et al.*, 1999; Lazarus *et al.*, 1999). The same factors may also play a part in complicating the RP-HPLC analysis of phlorotannins.

In NP-HPLC, eluents containing dichloromethane and methanol were applied with different elution gradients. Although modification of the elution profile did not improve the separation of peaks 11-13, NP-HPLC provided a better separation of phlorotannins since they were more clearly retained than in the RP system (Figure 13, Paper IV).

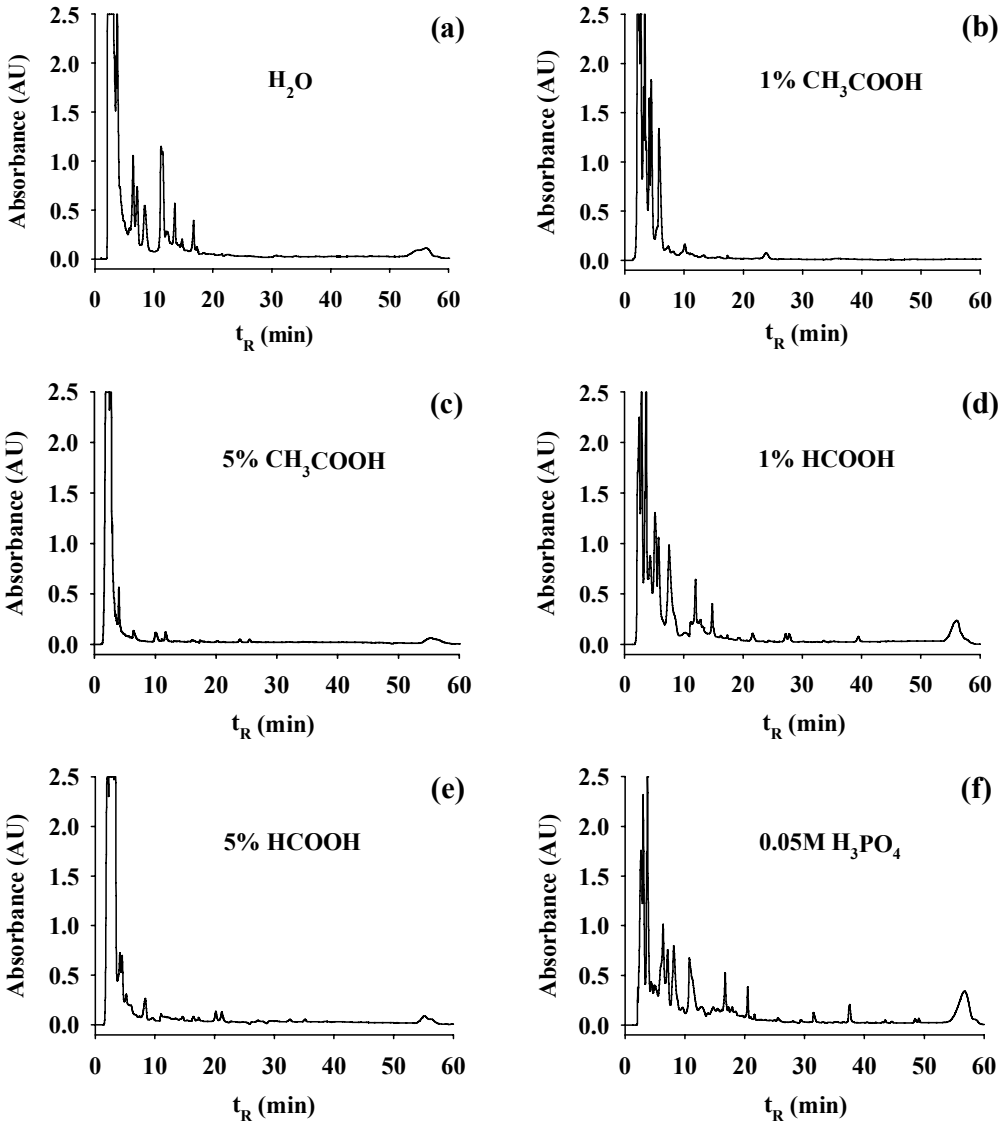

Figure 12. Separation of phlorotannins by RP-HPLC with different mobile phases A (mentioned in the figure). Mobile phase B is acetonitrile in each.

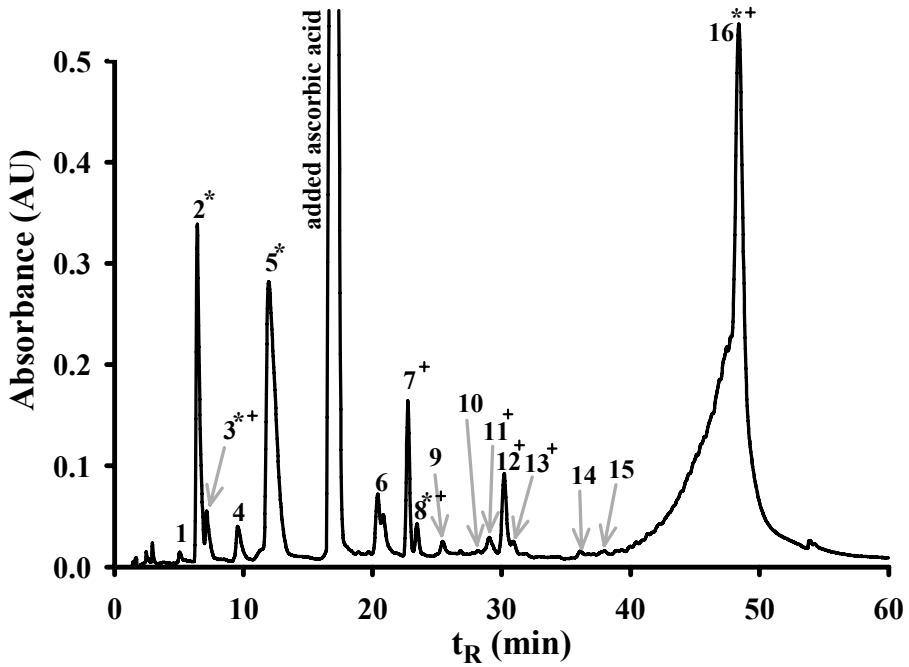

Figure 13. The NP-HPLC chromatogram of phlorotannins. Numbers indicate the peaks which are referred to in the text, the asterisks (*) mark out the peaks with RSD-values below 10%, the crosses (+) mark out the repeatable peaks (modified from paper IV).

When comparing RP and NP column materials, and taking into account the high polarity of phlorotannins, the disadvantage of the non-polar RP column material is that it does not form any proper linkages with phlorotannins. In RP, elution normally starts with a polar eluent, with which in this case the whole polar sample elutes through the column with poor separation. In contrast, NP chromatography has the advantage that the polar functional groups of phlorotannins are prone to be adsorbed to the silica material, resulting in better retention than with the RP material. In addition to being structurally bulkier, which already causes some retention, longer phlorotannin polymers also have more hydroxyl groups; they may therefore attach more tightly to the column material than the shorter oligo- and polymers. Elution starts in NP with a relatively non-polar solvent to release only shorter compounds, and solvent polarity is increased linearly to release longer polymers.

The UV spectra of phlorotannins were recorded directly during HPLC analysis using a diode array detector. These spectra were used for preliminary identification of the type of phlorotannins (Figure 14, Paper IV). The UV spectra of the peaks at short retention times (1-6) clearly differed from those acquired at longer retention times (peaks 7-16). While the UV spectra of the compounds with relatively short retention times had clear maxima between 293 and 297 nm, the UV spectra of the

compounds with longer retention times had a descending slope with a hump around 280 nm. The UV absorption maximum of phloroglucinol occurs at 270 nm. For peak 16 there was a hump at the same place, but the intensity was higher than that of phloroglucinol. All the compounds in the phlorotannin fraction studied do absorb and many of them also have their absorption maxima in the wavelength range of 270-280 nm.

It seems reasonable that phlorotannins emerge in order of the degree of polymerization in NP-HPLC, the shorter oligomers appearing first followed by the longer polymers, as is the situation with, for example, condensed tannins (Hammerstone *et al.*, 1999). Therefore, peak 16 was postulated to contain longer polymers than the early peaks in the chromatogram.

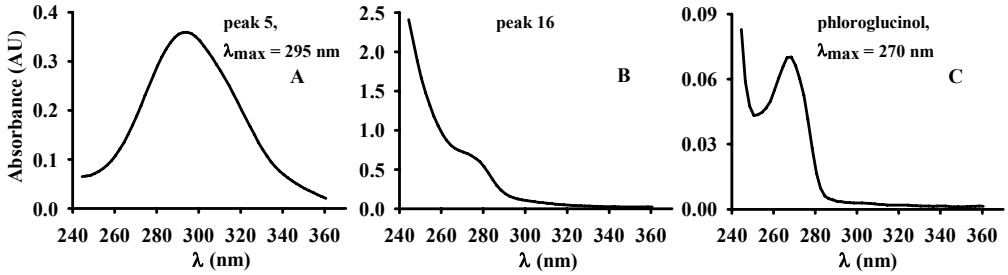

Figure 14. Spectra from UV-DAD for peak numbers 5 (A) and 16 (B) of the phlorotannin extract and phloroglucinol (C). A is an example of the shape of the UV spectra for peaks 1-6, B is an example for peaks 7-16.

The lack of commercial polymeric standards for phlorotannins did not allow identification based on the use of such standards. Hence it was not possible to compare either retention times or UV spectra to identify the compounds. In order to use individual compounds as standards, they must be either prepared synthetically from the monomer, or separated from natural sources with preparative-scale isolation and purification. The latter needs a proper chromatographic separation method that could be applied on a semi-preparative or preparative scale.

Phlorotannins, like other phenolic antioxidants, are prone to rapid oxidation (Ragan and Glombitza, 1986). According to visual tests, both lowering the temperature and the addition of ascorbic acid seemed to prevent oxidation during analysis (Paper IV). In the NP-HPLC chromatograms, samples with ascorbic acid exhibited additional peaks (Figure 15). A concentration of 0.3% (m/v) of ascorbic acid was more effective than lower concentrations, but no further improvement in antioxidant activity occurred when the concentration was increased up to 0.5% (m/v). Thus, on the basis of these results, it can therefore be concluded that the addition of an antioxidant agent increases the stability of phlorotannins.

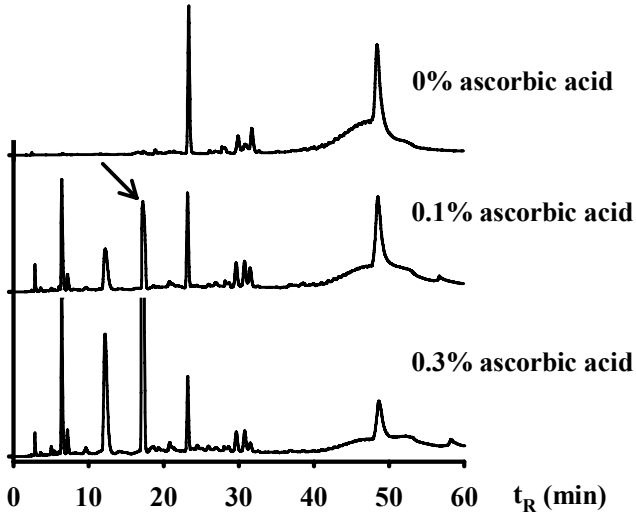

Figure 15. The effect of added ascorbic acid as an oxidation deterrent. The arrow shows the peak from the added ascorbic acid (modified from paper IV).

In addition to the UV detector, the NP separation method was also used with a mass detector. Sufficient chromatographic separation was obtained to allow for primary ionisation of individual peaks. A phlorotannin tetramer with $[2M–1]^-$ = 995.5; $[M–1]^-$ = 497.2; $[M–250–1]^-$ = 247 (Figure 13, peak no. 7) was detected. The $[M–H]^-$ ion of the phloroglucinol molecule was not detected in the crude extract samples, perhaps because in plants phloroglucinol is the starting compound from which the longer phlorotannin chains are generated and it may react very rapidly. This finding supports the belief that the amount of free phloroglucinol is usually considered to be low (Ragan and Glombitza, 1986). A single peak in a mass chromatogram may also contain many relatively similar-sized compounds; it can therefore reduce the selectivity of the mass spectrometric procedure and/or the ionisation efficiency of the mass spectrometer. On the other hand, the preliminary MS results did not provide evidence for the benefits of acetylation (Ragan and Glombitza, 1986). In the preliminary trials with acetylated phlorotannins it was found that they gave at best only weak molecular ions in the negative ion mode (data not shown).

The NMR analysis of peak 16 gave proof of the existence of phlorotannins. The ^{1}H-NMR spectrum displayed (in addition to TMS, solvent and water signals) only two groups of signals lying at regions 5.9-6.4 and 7.7-8.3 ppm. The latter constituted a broad band consisting of exchangeable hydroxyl protons. The former contained the CH protons, which are all in a very similar chemical environment in phlorotannins. The one-bond HSQC spectrum revealed that the carbons bound to protons in the first region resonate at 93-98 ppm. No other correlations were found

in the HSQC spectrum. Thus, the NMR results suggest that the peak contains mainly phlorotannins; no traces from other types of compounds were found.

Stability of the HPLC method and consistency with FC assay
In order to determine the reproducibility of the NP-HPLC analysis, five injections of the same sample on the same instrument were made (Paper IV). The relative standard deviation (RSD %) revealed the reproducibility of the analysis procedure which, calculated on the basis of the area of each peak, varied from 4 to 26% (Figure 13).

To explore the repeatability of the individual peaks in the HPLC profile, 18 samples were analysed twice, replicating the whole process from sample preparation to the calculation of the results (Paper V). As a criterion of repeatability we used the 95% confidence interval of the intra-class correlation coefficient; the measurement was considered non-repeatable when the confidence interval included zero. The consistency measurements of parallel measures within an alga revealed that 7 individual peaks of the NP-HPLC profile were repeatable (Figure 13).

The correlation of the HPLC profile and the total phlorotannin content was studied by calculating the Pearson correlation coefficients. Six of the fourteen analysed peaks from the HPLC chromatogram correlated positively and significantly with the total phlorotannin content (Paper V). It was further estimated how well the variation in the phlorotannin profile can explain the variation in the content of total tannins. Multiple regression analysis revealed that the best-fitting model, which explained 22.9% of the variation in total phlorotannin content, included two peaks from the phlorotannin profile, peaks #13 and #16. The amount of variation explained by within-population analyses differed among populations; in one it increased to 45%. Such variability and the increase in the amount of variance explained at the within-population level were expected because the phlorotannin profiles of the populations differed.

At least two factors contribute to the relatively poor consistency of the total phlorotannins and phlorotannin profile, i.e. the HPLC method may leave some phenolic compounds undetected, while the FC assay might measure some compounds not classified as phlorotannins. It is possible that liquid-chromatographic methods may exclude from quantification individual compounds that occur in low concentrations relative to their UV-response or that have a large molecular structure or an unsuitable polarity for chromatographic separation (Adamson *et al.*, 1999; Lazarus *et al.*, 1999).

5.4. Variation in phlorotannin content and composition in algae

On the basis of the chemical analyses described above, it was found that the amount of phlorotannins in the physodes [8.0 ± 0.2% (mean ± SE)] was

significantly higher, approximately an order-of-magnitude more, than in the cell wall (0.84 ± 0.03%) (Figure 16, Paper II). This is probably due to the fact that they are either bound to the cell walls only in very small amounts or they are rapidly chemically transformed into compounds other than phenolics. Some compounds might also be tightly attached to the cell wall and not be released in alkaline treatment. Although phlorotannins, like other tannins, are terminal products of their synthetic pathway, they can further degrade, for instance, via hydration, or oxidation (Strack, 1997). They can be bound temporarily to the cell wall, and then undergo turnover or degradation, e.g., into cell wall building compounds, complexing with alginic acid. Based on the small amount of phlorotannins found in the cell wall, they seem to play a minor role in cell wall construction as such; if, however, degradation regularly occurs in the cell wall, phlorotannins may function as important transitional compounds, as suggested by Arnold and Targett (2003). Phlorotannins, due to their structure, may become involved in the cell wall via esterification or a hemiacetal reaction. One suggested function may be cell wall hardening (Schoenwaelder and Wiencke, 2000).

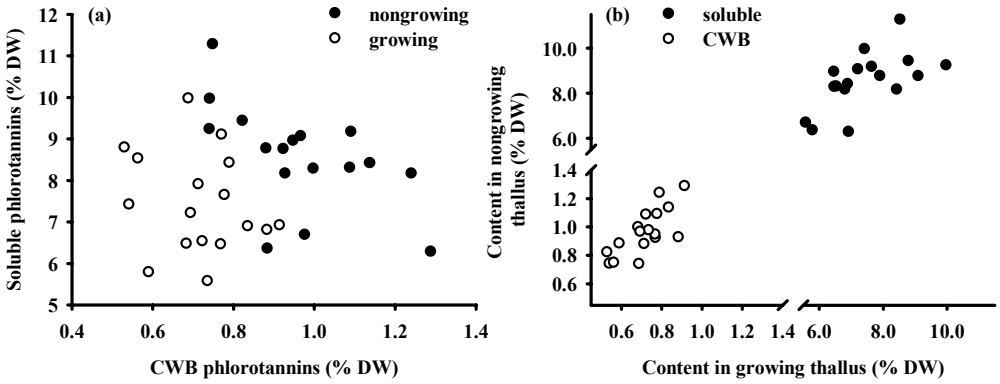

Figure 16. Correlation (a) between soluble and CWB phlorotannins in growing and nongrowing parts of *F. vesiculosus*, and (b) between growing and nongrowing parts of the thallus in soluble and CWB phlorotannins (modified from paper II).

In nongrowing thalli, a significant negative correlation was found between soluble and CWB phlorotannins whereas in growing thalli no such correlation existed (Figure 16a). This may be an indication of active phlorotannin production in the growing thalli. We assume that phlorotannin production decreases as the thallus gets older because of the thickening of the thallus and the consequent decrease in photosynthesis, which may result in a trade-off developing between soluble and CWB phlorotannins. The levels of both remain higher in nongrowing thalli than in actively growing parts, which may be an indication of accumulation over time (Figure 16a). Phlorotannins may also polymerize into longer and more complex forms which are more difficult to degrade or exude than rather short oligomers which may occur in young thallus.

A positive correlation was found between nongrowing and growing thallus in the contents of soluble and CWB phlorotannins; the concentrations of both were lower in growing tissue than in nongrowing tissue (Figure 16b). There was considerable variability among individuals in the contents of both soluble and CWB phlorotannins, with some individuals having a concentration only half that of others. However, within a single individual, when there was a large amount of phlorotannins – soluble or CWB – in growing parts, the amount in nongrowing parts was also large. Variation in the contents of soluble phlorotannins is known to be in a large extent genetic (Jormalainen and Honkanen, 2004) whereas variation in CWB phlorotannins has never been measured before.

5.5. Among-population differences in the contents of phlorotannins

After rearing in a common environment for three months, the three *F. vesiculosus* populations were examined using PCA analysis and it was found that they had diverged with respect to both their total phlorotannin contents and phlorotannin profiles (Paper V). The among-population tests of the individual peaks from the phlorotannin profile revealed a high variability in divergence among the separate peaks; some showed none, while others showed a higher degree of divergence than found in the total phlorotannin content. Although some individuals were mixed with those from other populations, the PCA analysis separated the Aaviikki population quite well (Figure 17), the first two principal components explaining 79% of the total variance in the phlorotannin profile.

The average areas of individual peaks showed similar among-population trends to those of the total phlorotannin contents: the population from Aaviikki had the highest (7.47 ± 0.22, % of DW ± SE), with intermediate and lowest total phlorotannin contents from Jurmo (6.92 ± 0.23) and Säppi (6.01 ± 0.18), respectively (Paper V). The area of the peak did not correlate with the magnitude of the among-population response: a rather small peak had an equally high among-population divergence as one of 10 times higher in intensity. However, the among-population differences in phlorotannin profile are also qualitative because the magnitude of divergence varied between the separate peaks.

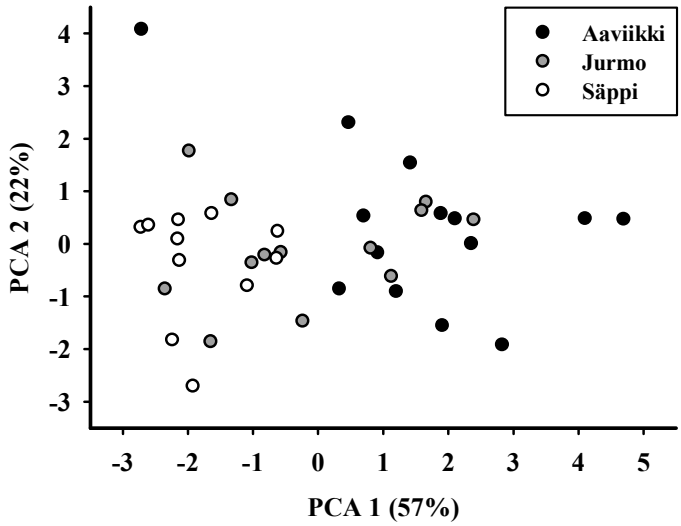

Figure 17. A scatter plot of algal individuals from three populations according to the values of the 1st and 2nd principal components. The PCA axes represents linear combinations of the 7 variables (#3, 7, 8, 11-13, 16) from the phlorotannin profile. Each dot represents an algal genotype (n=38).

The formation of phlorotannin oligo- and polymers in plants from their precursors most likely requires enzyme activity (Waterman and Mole, 1994). Those enzymes involved are transcription products of the plant genome (Waterman and Mole, 1994); thus the genetic variability in the profile and amount of phenolic compounds in a plant must reflect evolutionary differentiation among populations. Such a micro-evolutionary change may be caused either by natural selection or random changes in gene frequencies. The role of genetic drift in the differentiation of phlorotannins cannot be excluded, but they have been shown not to be selectively neutral; the form and intensity of natural selection for total phlorotannins varies among different environments (Jormalainen and Honkanen, 2004). Therefore, the geographic variation in environmental conditions among local populations, i.e. in abiotic and biotic selective agents, may explain the observed differentiation.

These results show that the among-population differences in phlorotannins are genetic in origin, not solely plastic responses to environmental differences. Further support for the genetic variation of phlorotannins came from the marginally significant variation among the cloned genotypes within populations. Thus, not only the total contents of phlorotannins but also the amounts of separate phlorotannins may respond to natural selection and evolve. This implies that the major phenolic group in brown algae may diverge qualitatively among local populations. It might be suspected that peaks which clearly differed among the population could be under differential selection, i.e. having ecological functions whose roles vary among populations.

5.6. Nutrient effects on phlorotannins

Content of total soluble phlorotannins decreased due to nutrient enrichment treatment in three separate studies (Papers I, II and V). Increasing nutrients decreased the contents of soluble phlorotannins in both growing and nongrowing thalli (Figure 18, Paper II). While such nutrient effects are often explained by an increase in growth at the expense of allocation to the production of soluble phlorotannins, this was not the case in paper II, since growth did not increase. In flow-through mesocosms, nutrient enrichment commonly leads to increased growth of epibiota (Paper I); this, in turn, causes shading and may thereby explain the decrease in phlorotannins' production. However, nutrient enrichment did not have a significant effect on the content of CWB phlorotannins in either type of thallus (Figure 18, Paper II), indicating that phlorotannins bound to the cell wall may be relatively stable with respect to environmental variation. Nutrient availability did not affect the exudation of phlorotannins (Paper II). The plastic behaviour of soluble phlorotannins together with the relative stability of CWB phlorotannins supports an approach that uses soluble phlorotannins as the basis for studying phenotypic plasticity in phlorotannin contents.

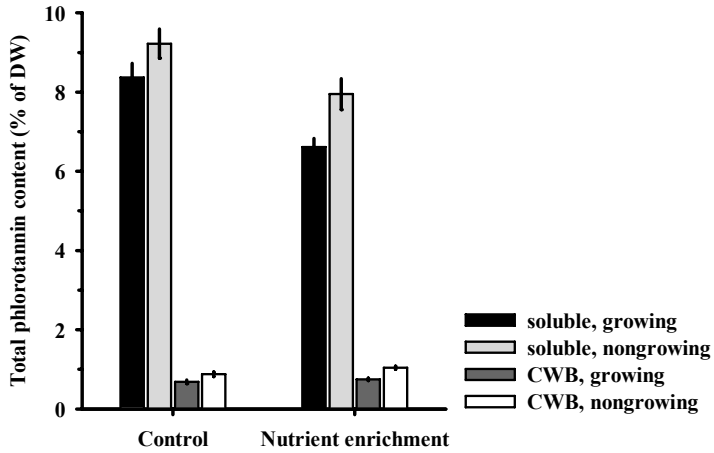

Figure 18. Mean (± SE) contents of soluble and CWB phlorotannins from growing and nongrowing parts of thallus grown in control (n = 8) or nutrient enrichment (n = 9) treatment.

The HPLC profile showed no significant differences between nutrient enrichment and control treatment, only the poorly repeatable peak #6 was higher in the nutrient enrichment treatment than in the control (Figure 19, Paper V). The lack of differences in the phlorotannin profiles between the nutrient levels may indicate that the response to nutrient enrichment includes neither qualitative phlorotannin changes nor quantitative changes in individual phlorotannin components. However, the possibility remains that the profile does not include those phlorotannins that

increase along with the nutrient level. Some compounds might remain undetected or under-represented in liquid chromatography, as earlier discussed, due to their low UV-response relative to concentration, large molecular structure, overlapping of isomers, or an unsuitable polarity for chromatographic determination (Adamson *et al.*, 1999; Lazarus *et al.*, 1999).

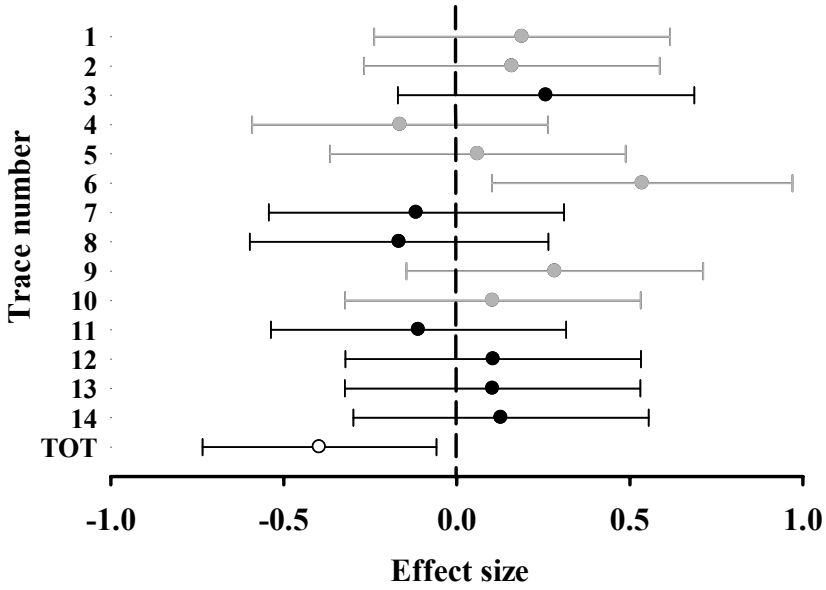

Figure 19. The effect size of nutrient enrichment separately for 14 peaks of the phlorotannin profile, and the total content of phlorotannins (TOT). Grey bars indicate peaks with poor repeatability.

Although nutrient enrichment did not influence phlorotannin composition, since neither individual peaks nor all peaks together from the phlorotannin profile showed any directional response to nutrient enhancement, it did influence total phlorotannin contents (Paper V). Nutrient enrichment may rather have induced a plastic response in compounds other than phlorotannins. As the FC-method gives the total contents of all compounds having a similar functional group, it also measures some compounds not classified as phenolic but which contain an oxidizeable group, e.g. amino acids and proteins, ascorbic acid, urea and diethyl ether, which all are known to cause inaccuracy (Ragan and Glombitza, 1986; Steinberg, 1989; Waterman and Mole, 1994; Van Alstyne, 1995; Stern *et al.*, 1996b). However, it has been suggested that, in algae, the concentrations of compounds other than phlorotannins in the Folin-Ciocalteu assay range from 3 to 5% (Van Alstyne, 1995). An increase of about 10% in the value of the FC assay as a response to nutrient enrichment is unlikely to result from non-phlorotannin compounds alone. But the lack of any response in the phlorotannin profile may

indicate that interference by non-phlorotannin compounds in the determination of total phlorotannins could be stronger than has been conventionally assumed.

The negative influence of nutrient enrichment on the total phlorotannin content supports both the CNB and GDB-hypotheses, which state that nutrient enrichment affects allocation of resources between growth and secondary metabolism. The nutrient responses of total phlorotannins of brown algae have often been successfully explained using these hypotheses (reviewed by Cronin, 2001; Pavia and Toth, 2008) (Papers II and V). In paper I, the washing treatment increased growth both alone and when combined with nutrient addition. The phlorotannin production was decreased when nutrient addition was combined with washing of the algae, which is consistent with the predictions of the resource-based hypotheses, which assume prioritization of growth when nutrient conditions are not limiting. In contrast, the resource allocation hypotheses were not supported in the case of the exuded phlorotannins, nor by the phlorotannin profile (Papers II and V).

5.7. Defensive role of phlorotannins

To study the induced changes in phlorotannin contents a meta-analysis covering the past two decades was recently conducted. The meta-analysis consisted of 23 papers with 48 tests exploring phlorotannin induction after natural or artificial wounding. Effect sizes showed out to be heterogeneous among the studies but positive mean effect sizes and their tendency to stabilize over the time proved that phlorotannins may act as inducible defence chemicals (Jormalainen and Honkanen, 2008).

In the present study, exudation of phlorotannins into surrounding water was observed in all cases, regardless of the nutrient or grazing treatment (Figure 20, Paper II). When algae were grazed by *Idotea*, the amount of exuded phlorotannins was over twice that of the other treatments. (Tukey *a posteriori* comparisons with other groups; $P < 0.05$) (Figure 20). For this there are a few possible explanations. The feeding process may induce defence in algae, consequently i.e. increased excretion of phlorotannin into the water. Alternatively, phlorotannins end up in the water without any induction in the alga as a consequence of the breaking of the surface, chewing action, or through digestion of *Idotea*. In both cases, the consequence of herbivory is an increase in exudation, and it is possible that predators or other plants use exuded phlorotannin as a signal of the presence of herbivores (reviewed by Jormalainen and Honkanen, 2008). If the defence was induced, increases in phlorotannin production and level of soluble phlorotannins would be expected. The content of soluble phlorotannins found in both growing and non-growing parts of the alga showed no change, supporting the idea of exudation due to breakage of the surface.

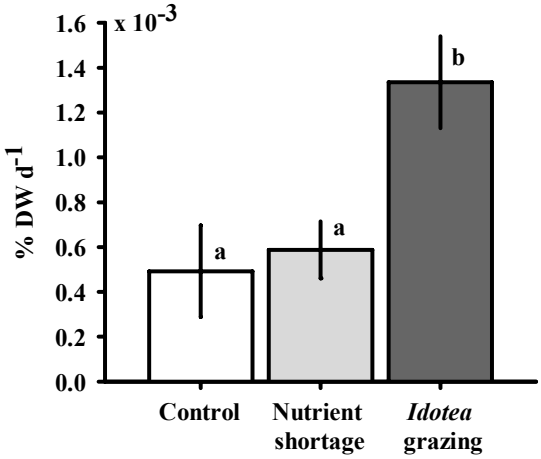

Figure 20. Amount (mean ± SE) of exuded phlorotannins from *F. vesiculosus* in three different treatments (n = 4).

There was variation in the contents of total phlorotannins between treatments in the experiment reported in paper I (Figure 21). Simulation of snail grazing activity by nutrient addition and removal of epibiota and particulate matter from the thallus, effectively decreased the production of phlorotannins in *F. vesiculosus*. This shows that manipulations of resource availability alone are capable of generating significant changes in the production of secondary metabolites.

The grazing action of *T. fluviatilis* on the thallus induced an increase in phlorotannin production. This response clearly differed from the effects of all the other treatments, which either did not affect or else decreased phlorotannin production. The response presupposed physical contact of *T. fluviatilis* with the thallus, as the presence of caged snails in the aquarium had no effect on either growth or phlorotannin content. This response could be interpreted as an induced defence by *F. vesiculosus* against snail grazing. *T. fluviatilis* does not feed on or damage the surface of the photosynthetic thallus of *F. vesiculosus*. Instead, the grazing action of this snail may simultaneously both increase light availability by removal of epibiota and decrease nutrient uptake through removal of the hyaline hairs that have been shown to function in phosphate uptake (Hurd *et al.*, 1993). Subsequently, nutrients may limit growth and the increase of secondary metabolites could be interpreted as a consequence of increased photosynthesis.

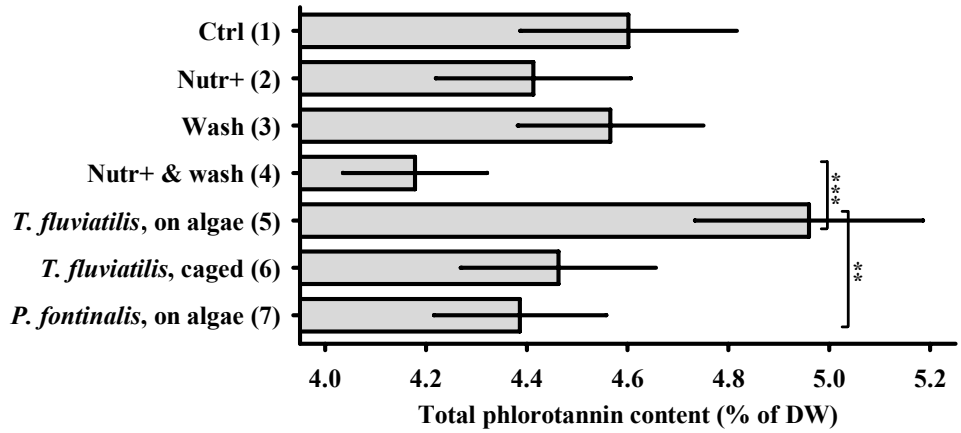

Figure 21. Effect of the treatment on the total phlorotannin content (mean ± SE).
** = P < 0.01, *** = P < 0.001

The presence of *P. fontinalis* on algae had no effect on phlorotannins. The different responses of *F. vesiculosus* to *P. fontinalis* and *T. fluviatilis* show that there is no such general cue that the grazing by snail could trigger phlorotannin production, which could easily be interpreted as an anti-herbivore adaptation.

There was a positive relationship between growth and phlorotannins among genotypes indicating that some genotypes were better able to both grow and produce phlorotannins. We are not aware of any previous reports on genetic trade-offs between growth and secondary metabolism in brown algae, but phenotypic trade-offs have been found to be variable between populations (Yates and Peckol, 1993; Pavia *et al.*, 1999) and in time (Steinberg, 1995). Almost 70% of the variation was genotypic. This clearly emphasizes the importance of genetic variation in secondary metabolism.

6. CONCLUSIONS

Methods to analyse brown algal phlorotannins have been developed in the studies summarized in this thesis. The NP-HPLC method was applied to phlorotannin analysis and, used in conjuction with UV-DAD, at least 16 individual peaks of soluble phlorotannins from the phenolic crude extract of *F. vesiculosus* were found. Preliminary NMR studies verified the existence of phlorotannin structures. While understanding the functions and variability of separate phlorotannins remains a challenge, the HPLC assay of the phenolic fraction provides a method to explore changes in composition and concentration in response to both abiotic and biotic factors. To better understand the suggested multiple roles of phlorotannins, the phlorotannin profile can give more detailed information than the total phlorotannin content.

The FC assay to quantify total phlorotannin content was adjusted as several factors were found to generate variation in yield, demonstrating the necessity for a detailed description of the procedure. The quantification of total phlorotannins from *F. vesiculosus* showed that only a small amount was bound to the cell wall, while the bulk was found in soluble form. This suggests that the chemical role of phlorotannins is mainly secondary, not the construction of the cell wall. The increase in phlorotannin exudation due to herbivory was found to be entirely a consequence of the chewing action of the grazers, as no indication of induced defence was found. Clarification of the biosynthetic pathway of phlorotannins, the enzyme systems involved, and the turnover and degradation processes is needed before our understanding of the functional role of phlorotannins is complete.

An plastic response of phlorotannins to nutrient availability was found as nutrient enrichment decreased the total phlorotannin content, which indicates resource allocation to growth at the expense of phlorotannin production. In addition to plastic responses to environmental factors, genetic variation in phlorotannins among local populations of *F. vesiculosus* was found. Not only the total contents of phlorotannins but also the amounts of individual phlorotannins may respond to natural selection and evolve. This implies that local populations of brown algae may become qualitatively different with respect to their major group of secondary metabolites, which might cause distinct plant-herbivore interactions among populations. No such general cue as e.g. snail grazing triggering phlorotannin production was found. Thus, rather than being caused by induced defence, variation in the phlorotannin contents is more likely due to manipulation of resource availability by *T. fluviatilis*.

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