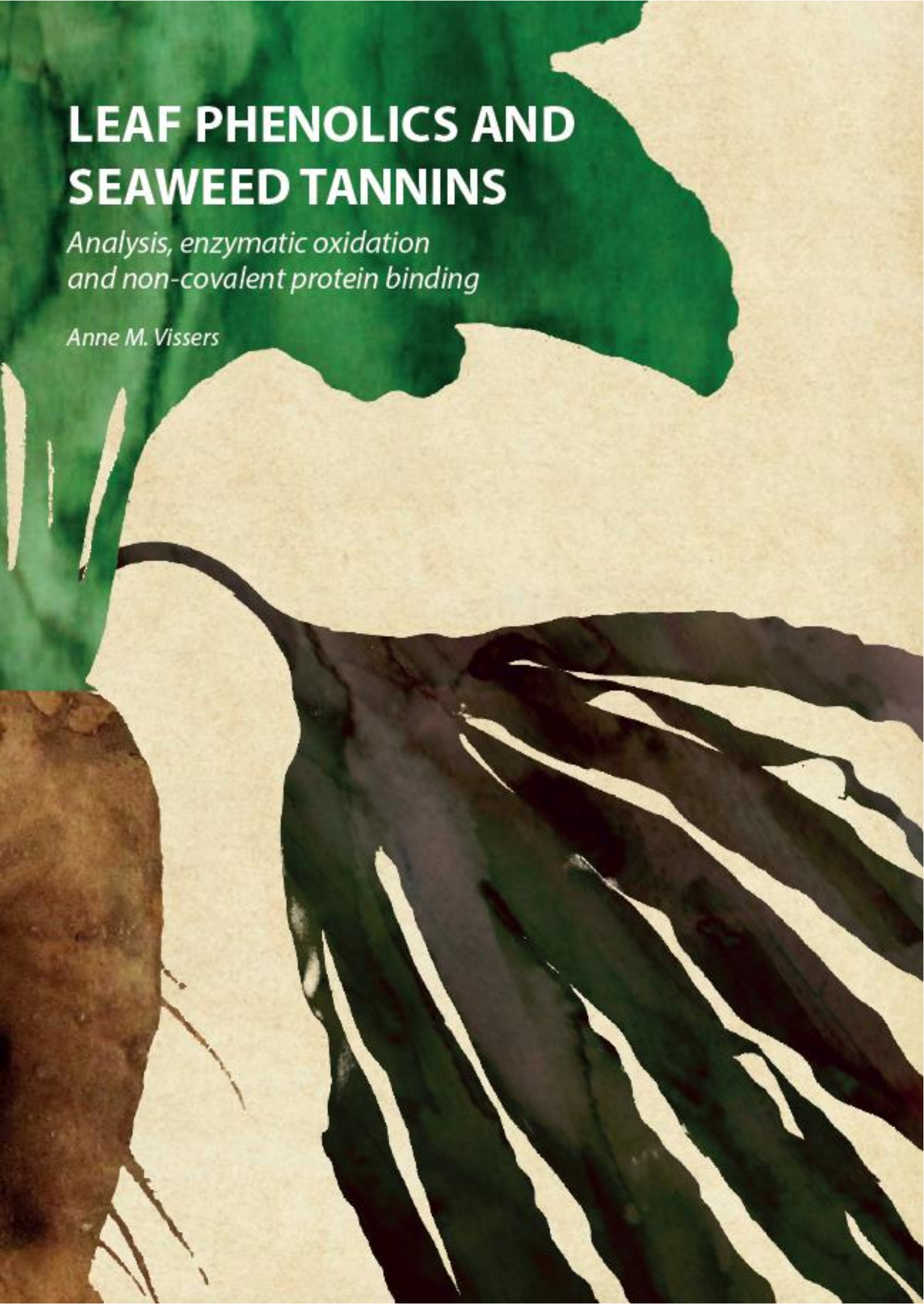
LEAF PHENOLICS AND SEAWEED TANNINS

Analysis, enzymatic oxidation and non-covalent protein binding

Anne M. Vissers



Propositions

1. Total phenolics content and polyphenol oxidase activity in leaves do no predict enzymatic browning
   (this thesis)

2. Due to the high phlorotannin molecular weight and their abundance of isomers, complete mapping of their structures is currently limited by resolution of analytical equipment
   (this thesis)

3. As the best way of learning is by playing games (C. Miller, 2013, *Developments in Business Simulation and Experimental Learning*, 40), gamification requires more attention in higher education

4. Language is an inseparable part of culture (Y. Wang, 2016, *International Journal of Applied Linguistics and English Literature*, 5(1), 215-223) and the increased use of English into other languages threatens cultural diversity

5. The contemporary society emphasises the value of labels given to people and items too much

6. Contact with volunteers enhances rehabilitation of forensic psychiatric inpatients

Propositions belonging to the thesis, entitled

'Leaf phenolics and seaweed tannins
Analysis, enzymatic oxidation and non-covalent protein binding'

Anne M. Vissers
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Leaf phenolics and seaweed tannins

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Anne M. Vissers

Thesis

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Abstract

Upon extraction of proteins from sugar beet leaves (*Beta vulgaris* L.) and oarweed (*Laminaria digitata*) for animal food and feed purposes, endogenous phenolics and proteins can interact with each other, which might affect the protein's applicability. Sugar beet leaf proteins might become covalently modified by phenolics through polyphenol oxidase (PPO) activity. Oligomeric phenolics from seaweed (so-called phlorotannins (PhT)) might bind non-covalently to protein. The first aim of this thesis was to study factors involved in protein modification by phenolics. The second aim was to investigate the effect of PhT supplementation to feed on *in vitro* ruminal fermentation.

Besides PPO activity and the amount of low molecular weight phenolic substrates present, brown colour formation in sugar beet leaves was dependent on the amount of phenolics, which do not serve as a substrate of PPO. These non-substrate phenolics can engage in browning reactions by oxidative coupling and subsequent coupled oxidation of the products formed. Similar reactions might also be involved in covalent protein modification by phenolics, and therewith protein properties.

High molecular weight PhT from *L. digitata* could potentially modify protein properties by non-covalent interactions. *L. digitata* contained PhT with subunits mainly connected via C-O-C linkages, as determined using NMR spectroscopy. Further mass spectrometric analysis revealed the presence of a wide range of oligomers with degrees of polymerisation between 3 and 27. The interaction between PhT and proteins (β-casein and bovine serum albumin) was studied using model systems with different pH values, representing the various environments throughout the ruminants digestive tract. Phlorotannins bound to protein independent of pH, and broadened the pH range of protein precipitation from 0.5 to ~1.5 pH unit around the protein's pI. At the pH of the abomasum of 2-3, the proteins re-solubilised again, presumably by increase in their net charge. Due to their ability to form water insoluble complexes, PhT could improve ruminal fermentation *in vitro* in a dose dependent manner, resulting in lower methane production and ammonia (NH_3) concentration. The decreased NH_3 concentration reflected decreased dietary protein breakdown in the rumen, which is considered a nutritional and environmental benefit.

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Chapter 1

General introduction

The expected increase in world population as well as increase in affluence in many countries comes with increased needs for feed and food proteins. This increasing demand has been a driver for research into alternative protein resources [1]. The alternative resources can consist of novel resources primarily used for proteins or can consist of side streams after industrial processing. Over the years, proteins from terrestrial plant and marine micro- and macro- algal resources have gained more attention as so-called 'green proteins'.

In order to increase the application potential of green protein resources, one or more processing steps are often required. If proteins or other components (e.g. carbohydrates) from green sources are to be extracted, their cells are to be ruptured and their contents released. Cell rupture brings otherwise separated proteins and secondary metabolites together, initiating reaction cascades which can result in modification of proteins. These modified proteins have altered techno-functional and nutritional properties which can be either non-desired or desired. Within both terrestrial and marine resources, phenolics are an important part of these secondary metabolites which can interact with proteins either covalently via the action of polyphenol oxidase (PPO), or non-covalently in case of oligomeric phenolics such as tannins. In this thesis, two green protein resources, PPO containing sugar beet leaves (*Beta vulgaris* L., terrestrial), and tannins containing oarweed (*Laminaria digitata*, marine), will be investigated. Both sugar beets and oarweed can be cultivated within the Netherlands

Green protein resources for feed and food

Green protein resources or fractions thereof after processing can be applied in both the food and feed industry. Sugar beet leaf and brown seaweed proteins have the potential to replace some of the conventional protein resources. Ultimately, this can contribute to decreased cultivation or breeding of conventional resources. Especially the alternatives for soy protein are interesting, which are known to have good techno-functional and nutritional properties and, therefore, they are applied widely in both the food and feed industry [2]. One of the reasons for replacement is that soy has to be imported from Asia and South America as these countries are the best for soy cultivation [3]. **Table 1** presents an overview of the gross chemical composition and presence of phenolics of some conventional and potential alternative resources from which proteins might be isolated.

When the proteins are extracted from its source, the protein concentrates or isolates can be used in food applications. Important properties for food applications are related to techno-functional aspects such as ability to stabilise foams and emulsions [4]. Alternatively, extracted proteins can be used to feed pets and infant animals [5] as the extracts are considered as the highest quality proteins. These animals have higher requirements for amino acids or due to their still immature gastrointestinal tract, a need for highly digestible proteins and amino acids [5]. Proteins of lower nutritional value can be used in feeds for more mature animals such as grower or finished pigs or ruminants as rumen microbes are able to synthesise proteins from non-protein nitrogen (NPN) [6].

Of the daily dry matter intake of ruminants, approximately 68% consists of cellulosic and hemi-cellulosic carbohydrates and 16% of nitrogenous material (proteins and NPN) [7]. In many

countries the feeding of ruminants relies for a large part on forages and corn. These feed resources supply mainly fermentable carbohydrates with a small amount of amino acids. Often, in order to increase milk production, rations are supplemented with soybean- or rapeseed meal. These meals are side streams of oil extraction [8].

Table 1 Chemical composition (% DM) of currently used and alternative feed resources, together with an overview of the molecular weight of the respective phenolics and presence of polyphenol oxidase (PPO)

Source	Protein	Carbohydrate	Ash	Phenolics			Reference
				Content	Size[a]	PPO	
Resources currently used in the feed industry							
Rye grass	8-10	79	10	0.07-0.15	L	+	[9, 10]
Red clover	16	72	9	3-4	L	+	[10, 11]
Soy bean	40-46	31	6	3.5-6	L	-	[12]
Fat extracted soy bean	54-55	37	7	3.5-6	L	-	[10, 13, 14]
Rapeseed meal	43	48	8	6-13	L	-	[10, 15]
Potential resources for the feed industry							
Sugar beet leaves	15-18	58-77	5-18	0.2-1.2	L	+	[16]
Laminaria digitata	4-15	59-82	24-35	0-20	H	-	[17-19]
Alginate extracted *L. digitata*	10-36		58-85	0-40	H	-	

[a] L = low molecular weight phenolics, phenolic acids and flavonoids, functioning as potential substrates for PPO; H = high molecular weight polymeric phenolics, tannins

Leafy proteins can be used in both the food and feed industry. These proteins have good foaming and emulsification properties [4] which makes them suitable for applications in food products after extraction. To my knowledge, sugar beet leaf proteins are not industrially produced yet. The presence of PPO and phenolics is one limitation in the application of sugar beet leaf proteins in food products as the enzyme activity can result in formation of reactive phenolics which create brown pigments. In the feed industry, sugar beet leaves are used for their high nutritional value in rations for ruminants. Digestibility of organic matter is approximately 75% and that of nitrogen approximately 64% [16, 20]. Its protein content is within the range of that of currently consumed fodder crops such as rye grass and red clover (**Table 1**). When sugar beet leaf proteins would be applied as extracts, protein contents comparable to those in soybean meal can be reached.

Brown macroalgae protein extracts have, so far, limited applications in food products as these algae are primarily cultivated for carbohydrate extraction [21]. More often, macroalgae are consumed as

such [17]. Macroalgae are often part of ruminant rations in coastal areas where cattle are kept [22]. Additionally, it has been found to be a good resource for minerals (**Table 1**) [22]. *L. digitata* as such has moderate protein levels, but the protein is of a high quality for ruminants [18]. The protein level increases after carbohydrate extraction [21] to reach contents in the range of currently used fodder crops. The macroalgae contain phlorotannins (PhT) which influence their applicability within feed mixtures. Depending on the type of tannin and the dose, the presence of tannins in feed can be desirable or undesirable.

Terrestrial resource - Sugar beet leaves

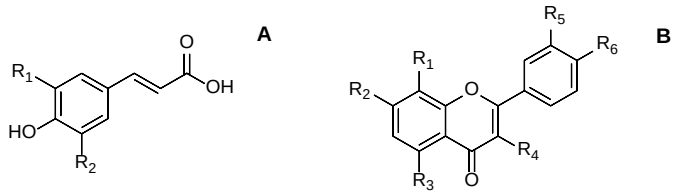
Sugar beets and cultivation

Sugar beets (*Beta vulgaris* ssp *vulgaris*) belong to the family of Amaranthaceae and subfamily of the Betoideae. It includes the cultivar groups of beetroot and leafy vegetables, such as spinach beet and chard [23]. Sugar beet cultivation is mainly directed to the development of beets with high sugar content and is the third most cultivated arable crop in The Netherlands [24]. The Dutch sugar beet production in 2015 equalled $6.9 \cdot 10^6$ tonnes [25]. Sugar beet harvest yields high volumes of beet leaves which can either be left on the field as fertiliser and to improve the soil's structure. On a dry weight basis, the leaves contain approximately 17-35% protein depending on the developmental stage [16, 26]. Due to their relatively high protein content, sugar beet leaf material is attractive for protein extraction and development of protein isolates for food and feed applications. For feed applications, protein isolates have higher value in monogastric than in ruminant species as the former require higher quality proteins [5]. When sugar beet leaf proteins are used in ruminant diets, the leaves are often ensiled [16]. Currently, mild procedures for protein extraction are under development [27] in order to maintain nutritional and techno-functional protein quality.

Sugar beet leaf phenolics

Sugar beet leaves contain mainly phenolic acids, and flavonoids (**Figure 1**). The phenolic acids are mostly present as glycosylated derivatives of hydroxycinnamic acids and important flavonoids are vitexin (8-*C*-glycosyl-apigenin) derivatives [28, 29]. The content and composition of phenolic acids and flavonoids in *B. vulgaris* are dependent on the developmental stage and environmental conditions. Overall, the total phenolics content varies from 2-8 mg/g fresh weight [26, 30, 31].

The quantification of phenolics is often performed by colorimetric or chromatographic methods. In colorimetric assays, the quantification relies on a chemical reaction between the phenolics and the reagent, resulting in colour formation. The Folin-Ciolalteu (FC) assay is most commonly used [32]. A more detailed annotation and quantification can be performed using chromatography combined with UV-detection and mass spectrometry [28, 33]. Mass spectrometric analysis allows characterisation and quantification of individual phenolic compounds. Information on the composition enables prediction of the potential reactivity of multicomponent mixtures of phenolics, which are co-extracted with proteins. Often such information is not available, which makes it challenging to predict whether for instance enzymatic browning [34] can occur during protein extraction.



Coumaric acid: $R_1 = R_2 = H$
Caffeic acid: $R_1 = OH$, $R_2 = H$
Ferulic acid: $R_1 = OCH_3$, $R_2 = H$
Sinapic aicd: $R_1 = OCH_3$, $R_2 = OCH_3$

Vitexin: R_1=Glc, R_2=R_3=R_6=OH, R_4=R_5=H
Quercetin: R_1=H, R_2=R_3=R_4=R_5=R_6= OH

Figure 1 Phenolic acids and flavonoids present in *Beta vulgaris*

Marine resource – *L. digitata*

Macroalgae and cultivation

Macroalgae are multicellular algae which are usually attached to rocks or other substrates. They are classified based on their colour as Chlorophyta (green), Rhodophya (red) and Phaeophyta (brown) macroalgae, depending on their main pigments [35]. Their gross chemical composition is highly dependent on season and growing conditions [17]. Brown macroalgae have generally lower protein contents than green and red macroalgae [21], but the former contain PhT which can potentially modify the properties of (extracted) proteins. Macroalgae have a long use in history. In Asia, they are an important part of the diet while in Europe, macroalgae were used as animal feed or mixed with soil as fertiliser [22].

One of the suitable orders of brown macroalgae is kelp (*Laminariaceae*), of which oarweed is one of the species. As these weeds are more often referred to by its Latin name, *L. digitata*, this term will be used throughout this thesis. The cultivation of *L. digitata* in the Dutch North Sea and the Eastern Scheldt is promising but not yet performed at a sufficiently large scale [36]. Worldwide, *Laminariaceae* contribute to 34% of the dry algal mass harvested and 70% of these are harvested in France, Scotland and Norway [37]. *L. digitata* is used as a resource for extraction of carbohydrates such as laminarin, alginates, cellulose and mannitol [19, 37].

Laminaria phenolics

In *L. digitata*, the phenolics are present in the form of phlorotannins which are comparable to terrestrial condensed tannins (CT). Phlorotannins are composed of phloroglucinol (MW = 126 Da) subunits which are interconnected via C-C (fucol-type) (**Figure 2A**) and C-O-C (phlorethol-type) (**Figure 2B**) linkages. Terrestrial CT consist of catechin and gallocatechin monomers inter-connected via the same linkage types [38]. Some algal species (*Ecklonia cava*) can contain double C-O-C linkages, resulting in dibenzo-*p*-dioxin linkages (eckol-types) (**Figure 2C**) [39]. In this way, oligomers ranging from 250-6,000 Da can be formed, with high variation in degree of polymerisation [39]. Phlorotannin contents in brown macroalgae up to 20% on dry matter basis have been reported [40] (**Table 1**).

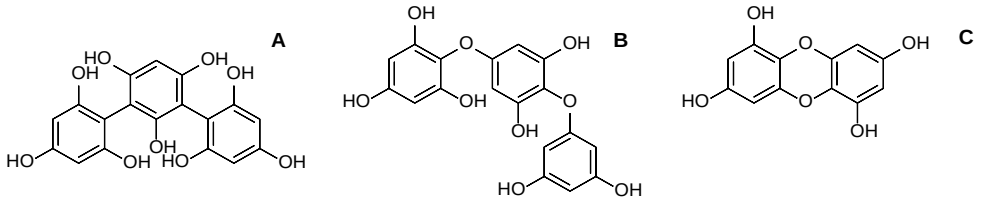


Figure 2 Phloroglucinol trimers of the fucol- (A) and phlorethol- (B) type, and a dimer of the eckol-type (C)

For both PhT and terrestrial CT, structural analysis and quantification is challenging due to the similarities between individual oligomers. These similarities do not allow efficient chromatographic separation based on polarity, as with for instance reversed phase chromatography. For terrestrial hydrolysable ellagitannins (subunit pentagalloyl glucose, MW = 940 Da, consisting of gallic acids connected to a glucose via ester linkages) [41] and condensed proanthocyanidins (subunit catechin, MW = 290 Da, connected via C-C and C-O-C linkages) [38], size based separation was successful and allowed purification of individual oligomeric sizes. Ellagitannins and proanthocyanidins have larger subunit molecular weights than PhT, which makes separation of PhT based on molecular weight more challenging. Efficient isolation of PhT has been obtained by combining NP- and RP-chromatography [42]. Structural information can be obtained by mass spectrometry [42], allowing fragmentation of the molecules. Electrospray mass spectrometric techniques are often limited in their size detection range which results in underestimation of the degree of polymerisation. The use of MALDI-TOF-MS for detection of larger oligomers has only been limitedly applied [43]. Alternatively, NMR spectroscopy can be used. After laborious purification, single molecules can be obtained for detailed structural characterisation [44]. This way provides, however, no general structural or quantitative information on the PhT present. Generic structural and quantitative information can be obtained from the combination of ^{13}C and ^{1}H NMR spectroscopy [45]. The former provides information on the types of linkages and branched oligomers present within the tannins and the latter might be used to determine extract purities.

Similarly to low molecular weight terrestrial phenolics, PhT are mostly quantified by colorimetric assays. Besides the FC-assay, the 2,4-dimethoxybenzaldehyde (DMBA) assay [46] is used. The advantage of the DMBA-assay is the higher specificity towards PhT as the reagent reacts only with *m*-diphenolics. The disadvantage of the use of colorimetric assays for PhT quantification is the lack of appropriate standards. The only available standard is the monomeric phloroglucinol which reacts stronger to the reagent than oligomeric PhT [46], and, therefore, leads to underestimation of the PhT content.

Protein modifying reactions

The main interactions between both low molecular weight phenolics and tannins with proteins and potential effects on food and feed proteins are represented in **Figure 3** [47]. Via low molecular weight phenolics and PPO activity, proteins can become covalently modified. Via tannins, proteins can become non-covalently modified. The PPO-modified proteins become coloured, which is often undesired in food applications [48]. For feed applications, the colour of the proteins is not an important quality parameter, but the modification reactions are associated with decreased solubility [49, 50] and digestibility [51]. A lower digestibility decreases bioavailability of amino acids [52]. The latter aspects have detrimental consequences for both food and feed applications.

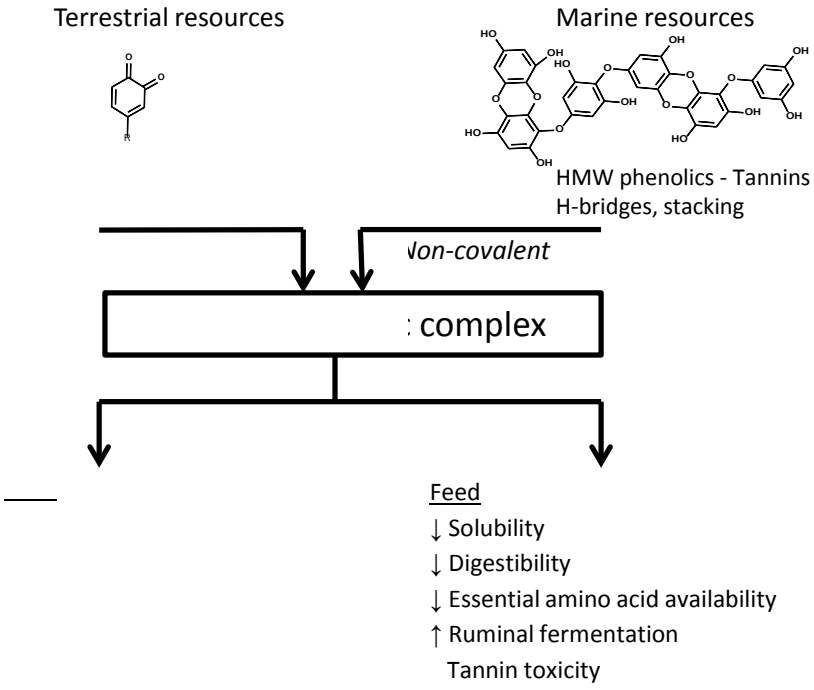


Figure 3 Formation of covalent and non-covalent protein-phenolic complexes and consequences for protein properties

Covalent protein modification by phenolics

The activation of the plant's defence system upon wounding of the plant material [47] can result in covalent modification of proteins. Part of a plant's defence mechanism consists of PPO and phenolics. When these components come together in the presence of oxygen, the phenolics are enzymatically converted into quinones which can react with amino acid residues on proteins [53].

These reactions negatively affect plant protein quality by decreasing protein palatability and nutritional value [47]. With cell rupture during protein extraction [54] and during the wilting period before ensiling grass and leaves [55], PPO can be active.

During enzymatic oxidation, phenolic compounds having a single hydroxyl group or a dihydroxyl function in *ortho*- or *para*-position, can act as substrate for PPO [56, 57]. Both tyrosinases (E.C. 1.14.18.1) and laccases (E.C. 1.10.3.2) are distinguished as PPOs [53]. Tyrosinases can have both cresolase (E.C. 1.14.18.1) and catecholase (E.C. 1.10.3.1) activity (**Figure 4**). Cresolase hydroxylates monophenolics into *o*-diphenolics. These are then substrates for catecholase activity which converts *o*-diphenolics into *o*-quinones.

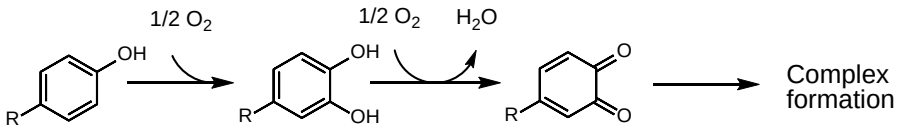


Figure 4 Schematic overview of oxidation of phenolics by tyrosinases which often can perform both cresolase (*o*-hydroxylation) and catecholase (*o*-diphenol oxidation) reactions

Ultimately, the PPO substrates are converted into electron deficient and highly reactive *o*-quinones, which will be part of non-enzymatic continuation reactions. Quinones can be attacked by nucleophiles during oxidative coupling [58] such as amino acids or other phenolics. Within proteins, the side groups of cysteine (sulphuric) and lysine (ε-amino group) are the strongest nucleophiles [59]. Additionally, phenolic acids and flavonoids present in the mixture can serve as nucleophiles, the strength of which depends on the substituents present on the phenolic ring [60]. Within fruits, oxidative coupling is often associated with coupling of substrates to each other [34]. The non-substrate phenolics, however, are also nucleophiles and might participate in the reaction as well. This aspect is not well studied. Oxidative coupling results in formation of phenolic dimers (with the subunits covalently linked to each other) or covalent attachment of phenolics to proteins. All these oxidation and complexation reactions can result in the formation of larger complexes with a brown colour, referred to as 'enzymatic browning'. Due to covalent binding of phenolics to amino acid side chains, the protein structure and accessibility of amide bonds changes, which in turn affects the hydrolysis of proteins [52] by pepsin and trypsin upon ingestion. As a consequence, the nutritional value might be altered.

The activity of PPO in plants and therewith, the extent of covalent protein modification is dependent on the amount of enzyme and the presence of substrates. These parameters are related to the physiological stage of the plant. It has been reported that the total amount of phenolics, as well as their composition, can fluctuate during plant development, and so does PPO activity. The combined increase in PPO activity and total phenolics content during plant development has been reported for the green leafy vegetables kale [61] and lettuce [62]. There are, however, limited literature reports connecting the activity of PPO to the amount and composition of phenolic substrates. In cases

where the relationship has been investigated, total contents of chlorogenic acid or catechin did usually not correlate to PPO activity and browning [63, 64].

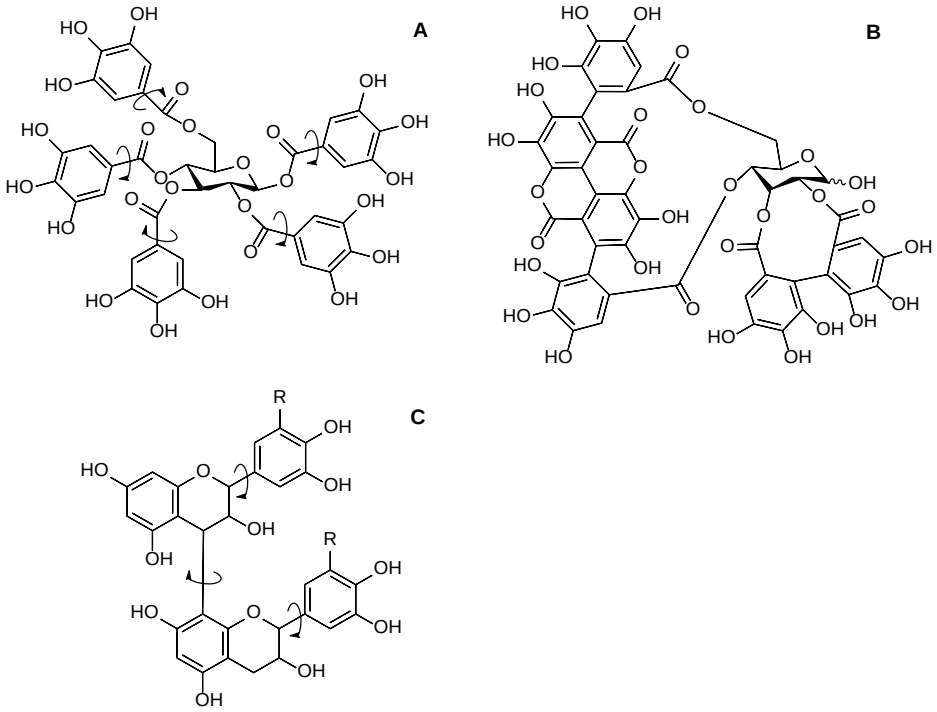


Figure 5 Pentagalloyl glucose (A) and punicalagin (B), representative structures for hydrolysable gallotannins and ellagitannins, respectively. Catechin dimer (C), representative structure for condensed tannins. The arrows indicate bonds around which free rotation is possible

Non-covalent protein modification by phenolics

An alternative defence mechanism of terrestrial plants and marine brown macroalgae is the presence of tannins. These tannins can be hydrolysable or condensed and both are known to interact non-covalently with proteins [65]. By this non-covalent interaction, the solubility of proteins decreases [66] and palatability is reduced due to tannin-induced astringency [67]. The interactions are based on the hydrophobicity of tannins. The tannins bind to proline residues of proteins by hydrophobic CH-π stacking interactions, which are subsequently stabilised by hydrogen bridges induced by the high abundance of hydroxyl groups [65, 68]. There are three important phenolic properties determining their binding affinity to proteins. (i) Molecular weight; tannins of intermediate molecular weight (~1,500 Da) have usually the highest binding affinities [69, 70]. (ii) Molecular flexibility [41]; gallotannins (**Figure 5A**) have usually higher binding affinities than ellagitannins (**Figure 5B**) and CT (**Figure 5C**). The lower binding affinity of ellagitannins than

gallotannins relates to the presence of additional C-C bonds interconnecting gallic acid residues, resulting in decreased rotational freedom or flexibility. Condensed tannins have more bonds with free rotation than ellagitannins, resulting in higher binding affinity for proteins. (iii) Degree of hydroxylation; increased number of hydroxyl groups increases potential for hydrogen bonding and, therewith, stabilisation of the interaction [71]. As a general factor, affecting both phenolics and proteins, the pH is considered important. It is often postulated that the binding between tannins and proteins depends on the pH where binding strength is highest at a pH around the pI of the protein [49]. The binding has been shown to be reversible, and it has been suggested that proteins are released into solution when the pH decreases [72].

Tannin-protein connections formed can result in both soluble and insoluble complexes (**Figure 6**). At a low tannin-to-protein ratio, not all binding sites on the proteins are occupied by single tannin molecules. By increasing the tannin-to-protein molar ratio, the tannins can link proteins to each other, increasing the aggregate size. When the aggregate size becomes sufficiently high, it will cause turbidity of the solution. Increasing the tannin-to-protein ratio even further creates large complexes which are no longer soluble and precipitation occurs [65]. The actual molar ratio at which precipitation occurs is dependent on the tannin molecular weight, the properties of the protein and the pH of the solution. Precipitation is stronger close to the pI of the protein due to decreased electrostatic repulsion [66].

The association between terrestrial tannins and proteins has been widely studied using molecular techniques [68]. Fluorescence quenching is often applied where the decrease in intrinsic fluorescence of tryptophan residues within proteins is measured and can be used to determine binding constants for bimolecular mixtures [70], or to study binding of tannin mixtures to proteins [73].

The direct binding of PhT to proteins has so far not been studied; there is only indirect evidence for their binding to proteins [74]. When tannins are applied in food and feed matrices, it is important to gain information on the binding of these tannins to proteins. The binding behaviour of PhT is expected to be comparable to that of terrestrial tannins.

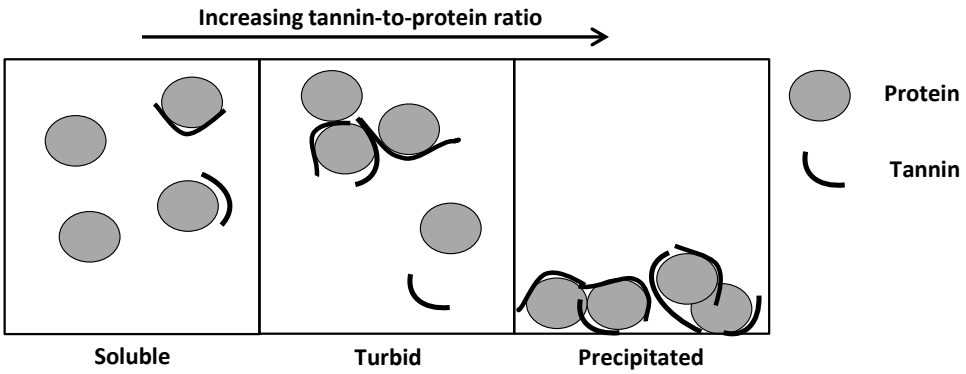


Figure 6 Phlorotannins binding non-covalently to proteins at increasing tannin-to-protein ratio (based on [68])

Digestive processes in ruminants

Ruminal fermentation

When PPO- and tannin-containing resources are incorporated in ruminant feed, their effects on the overall digestion need to be studied. The ruminants' digestive system differs from that of monogastrics in that microbial fermentation occurs prior to the actual digestion and absorption of nutrients in the former, whereas microbial fermentation occurs post digestion and absorption in the latter [75]. This fermentation enables ruminants to utilise large quantities of cellulose-rich materials which, in the case of monogastrics, is, in general, more limited. The majority of the fermentation occurs in the rumen, where pH is around 6-7 (**Figure 7**) [75] and cellulose, hemicellulose and starch are transformed into volatile fatty acids (VFA) by the residing microflora. The VFA are absorbed in the rumen and used by the animal as an energy source for growth, maintenance, reproduction and production. The rumen is combined with the reticulum where indigestible materials such as stones and wood particles are captured. The omasum which follows is physically separated from the rumen, and consists of lamellae absorbing high amounts of water and reducing the volume of the feed material, which enters the abomasum. In the abomasum, the pH is decreased to 2-3 and proteases partially digest the fermented feed material. From the abomasum onwards, the digestion is similar to that in monogastrics [75].

The fermentation efficiency of ruminants is generally low and results in emission of ammonia (NH_3) and methane (CH_4). Of the ingested nitrogen, 55-95% is excreted in the urine or faeces [76]. The excretion of CH_4 decreases feed energy efficiency by 8-12% and, therewith, contributes to 14.5% of the global CH_4 emission [77].

During ruminal fermentation, cellulose and proteins are degraded into CO_2, H_2 and VFA and converted into NADPH (**Figure 7**) [78]. Methanogenic bacteria reduce the H_2 to CH_4 and, therefore, the formation of CH_4 is an essential hydrogen sink [78]. The extent of CH_4 excretion depends on dry matter (DM) intake and diet composition [79].

The important VFA formed upon microbial fermentation are acetic-, propionic, butyric-, and valeric acid, being the primary energy source for the animal [75]. The branched chain VFA iso-valeric- and iso-butyric acid are degradation products of the amino acids valine and leucine, respectively. These branched chain VFA function as growth factors for cellulolytic bacteria and formation of longer branched chain fatty acids [80].

The microbial population is significantly influencing animal performance and, therewith, feed conversion efficiency and excretion. The H_2 released upon cellulose fermentation needs to be removed as it is toxic at high concentrations. The deposition of H_2 can go via formation of CH_4 and propionic acid. There will be, therefore, a competition between methanogenic archaea and propionic acid bacteria. Methanogens are the most sensitive bacteria and their deactivation is reflected by a shift towards propionic acid production [78, 81]. With the increasing pressure on reduction of CH_4 [82] into the environment, strategies to reduce CH_4 excretion by modulation of the feed composition have been investigated. Especially the use of tannins has been highlighted as these components affect ruminal CH_4 synthesis [83].

A way to study the efficiency of fermentation and effects on microbial population is by monitoring gas production *in vitro*. The total gas formation as a result of fatty acid synthesis by ruminal microbiota is a measure of fermentation efficiency [84]. Monitoring techniques for gas production are often combined with determinations of NH_3 (reflecting protein breakdown) and VFAs (reflecting carbohydrate fermentation) providing valuable information on the direction of the fermentation in relation to the substrate used. Alternatively, diets are tested *in situ* in which nylon bags containing test feed are incubated within the rumen [85], or *in vivo* by feeding the test diets [86].

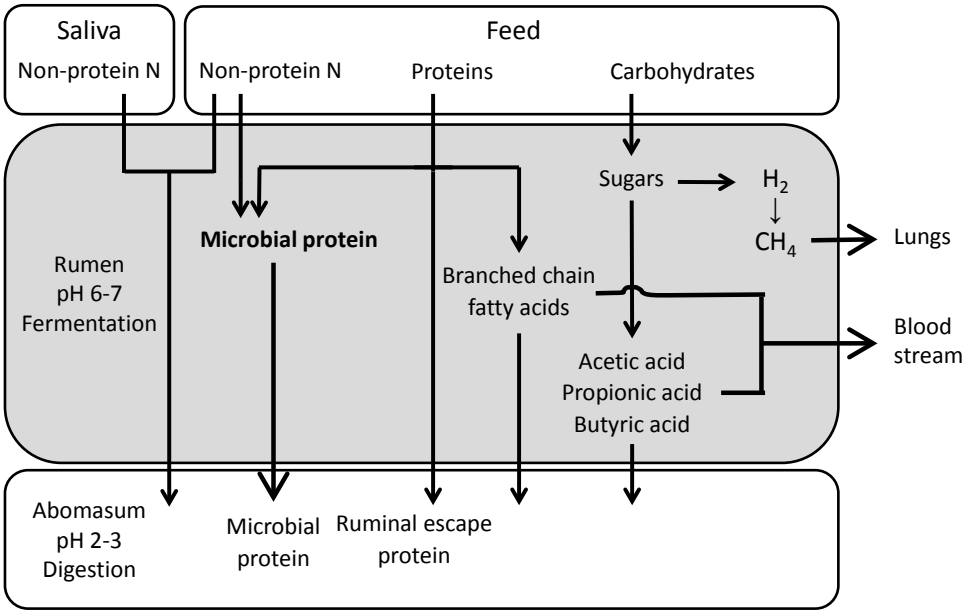


Figure 7 Schematic overview of ruminal fermentation of dietary proteins and carbohydrates

Rumen escape protein

Dietary proteins can be divided into rumen degradable and ungradable protein. The latter is also referred to as 'rumen escape protein' [6, 87]. The microbes can obtain nitrogen for their own metabolism from either the breakdown of dietary proteins or from non-protein nitrogen (NPN) in the form of urea or NH_3 [29]. The NPN in the rumen originates from production of NH_3 from protein breakdown, or from recycling of urea via the blood stream and saliva (19-96% of the nitrogen is recycled [88]). The cow herself is not able to synthesise essential amino acids from NPN and relies on protein hydrolysis in the abomasum. In high protein diets, the excess of protein above the requirement of the animal is excreted in the urine as urea. In low protein diets, NPN is used for the synthesis of microbial mass and as such supplies amino acids to the animal and, therewith, the excretion of urinary nitrogen is decreased [6]. The proportion of rumen escape protein can be modulated in several ways [87] (**Table 2**), which rely on covalent and non-covalent protein modification.

Table 2 Effects and drawbacks of strategies to create ruminal escape protein

Strategy	Effect [a]	Drawback	Reference
Covalent modification			
Heating	Lysine protection Inactivate protease inhibitors	↓ Digestibility and lysine availability at extensive heating Protein-protein crosslinking Difficult to control	[87, 89]
Chemical modification	Lysine protection	↓ Digestibility and lysine availability at high formaldehyde levels Protein-protein crosslinking Chemicals used can be unsafe	[87]
Polyphenol oxidase [b]	Lysine protection	↓ Digestibility and lysine availability at high degree of modification Sufficient PPO levels needed	[55, 90]
Non-covalent modification			
Lipid encapsulation	Physical barrier between dietary protein and microbial proteases	Low release post-rumen	[87]
Encapsulation in pH sensitive polymers	Physical barrier between dietary protein and microbial proteases High release post-rumen Polymers generally not absorbed	High cost	[87]
Tannins [c]	↓ carbohydrate fermentation ↓ CH_4 excretion (inhibit methanogens) Protein release post-rumen	Toxic at high doses Astringent	[84, 85, 90]

[a] The main aim of treatment is to decrease ruminal protein solubility and ruminal protein fermentation

[b] PPO is endogenously present in plant resources, active during ensiling

[c] Tannins are endogenously present in some plant resources and in brown macroalgae

The covalent and non-covalent protein modification approaches have in common that the protein solubility and, therefore, accessibility to microbial enzymes decreases [87]. Within the acidic abomasum, protein unfolding is expected to induce post rumen digestibility as peptide bonds become available for hydrolysis. Soy protein covalently crosslinked by formaldehyde had decreased solubility at rumen pH whereas solubility at abomasum pH was not affected [91]. All covalent treatments are able to protect lysine from ruminal fermentation. For formaldehyde modified proteins, lysine was absorbed in modified form as ε-N-methyl lysine [92]. Lysine is an essential

amino acid, but moderate modification did not significantly influence lysine absorption and total protein digestibility [93]. Too extensive modification, however, leads to losses in bioavailability of amino acids (**Table 2**) [87, 89, 94]. Non-covalent modification strategies target mainly proline and have overall interaction with protein via hydrophobic interactions. Of the strategies to create rumen escape protein, there are two which use endogenous properties of the feed materials, or, more specifically, they use the plant/algal pool of secondary metabolites: (i) The covalent modification by linking polyphenols to proteins by PPO. (ii) The non-covalent binding of tannins to proteins.

By utilising the modified proteins in the feed industry, reactions that are in some food applications considered to be detrimental can become beneficial. Both the resources studied in this thesis appear to be applicable for creation of ruminal escape protein which might be reflected by a decrease in ruminal NH_3 concentration. Additionally, some treatments can modulate CH_4 excretion (**Table 2**). Sugar beet leaves, can provide dietary proteins and contain PPO, therewith creating opportunity for covalent modification of proteins. *L. digitata* applied directly or as industrial side stream after carbohydrate extraction, can provide dietary protein and creates opportunity for non-covalent modification due to the presence of PhT.

Thesis aim and outline

The currently available literature indicates that both covalent and non-covalent modifications by phenolics can have detrimental effects for food applications or for protein digestion, and can have positive effects during ruminal fermentation. The extent of both types of modification and the potential effects on the applicability of the proteins depend on the phenolics present in the resources used and the physiological state of this resource. When sugar beet leaves are used to extract proteins or to be ensiled, there might be significant batch-to-batch variation regarding PPO activity and subsequent browning. This is related to the varying potential for covalent protein modification. Terrestrial tannins have been widely studied as additives in ruminant feed. Information on the effects of PhT in ruminant feed, together with information regarding its protein-complexing and protein release-behaviour is limited. Therefore, this thesis has two main aims:

(i) *To study factors involved in protein modification by phenolics*.
In sugar beet leaves, phenolics contribute to enzymatic browning. It is hypothesised that sugar beet leaves are a good protein resource in terms of amino acid composition and can, therefore, be a valuable protein source in the food and feed industry. The applicability of these proteins is dependent, however, on the extent of enzymatic browning during isolation which increases with plant age. It is hypothesised that the variation in PPO activity combined with the phenolics composition affect enzymatic browning.
In brown macroalgae, PhT modify proteins non-covalently by binding to them. It was aimed to gain information on the complex formation between PhT and proteins together with the re-solubilisation of proteins from these complexes. These interactions might affect fermentation and digestibility in ruminants. Literature reports determining effects of terrestrial tannins in feed *in vivo*, mention limited detrimental effects on total digestive tract protein availability. It is,

therefore, hypothesised that the complex between PhT and proteins can be formed at ruminal pH and that the interactions weaken in the acidic environment of the abomasum.

(ii) *To study the effects of PhT supplementation to feed on ruminal fermentation* in vitro.
Determine the optimal level of PhT addition to feed, a level at which formation of CH_4 and NH_3 decrease without hampering fermentation of carbohydrates and VFA formation.

Regarding PPO mediated modification, the extraction of proteins from sugar beet leaves is taken as example. In **Chapter 2**, the effects of the plant age at harvest on the chemical composition, PPO activity and quality of the extracted proteins in terms of colour is investigated. As the differences observed in these experiments could not provide satisfactory explanations for the difference in colour, a detailed investigation on the phenolic quantities and substrate composition and enzymatic activities is performed in **Chapter 3**.

To study non-covalent modification, the PhT from the brown seaweed *L. digitata* were used. A method for extraction, purification, quantification and determination of the oligomeric composition of these PhT was developed in **Chapter 4**. Information on the potential binding of PhT to proteins and release of proteins throughout the digestive tract is investigated in **Chapter 5**. The relation between pH and binding of PhT to proteins combined with the reversibility of the binding is investigated using a model system consisting of the extracted PhT and animal derived proteins. The purified PhT extract is accordingly tested for its potential to create ruminal escape protein and decrease of CH_4 production *in vitro* in **Chapter 6**. In **Chapter 7***,* the results obtained are discussed in relation to the various techniques to study phenolics extracted from natural materials. Furthermore, the implications of both covalent- and non-covalent protein modification on ruminal fermentation characteristics will be elaborated.

References

1. FAO, Protein sources for the animal feed industry - Expert consultation and workshop Bangkok, 29 April - 3 May 2002. **2004**, FAO: Italy. 24.
2. Fukushima, D., 6 - Soy proteins A2 - Yada, R.Y, *in Proteins in Food Processing*. **2004**, Woodhead Publishing. 123-145.
3. Bouxin, A., Feed & Food - statistical yearbook 2014. **2014**: European Feed Manufacturers Federation.
4. Sheen, S.J., Comparison of chemical and functional properties of soluble leaf proteins from four plant species*. J. Agric. Food Chem.*, **1991**. 39(4): 681-685.
5. McDonald, P., R.A. Edwards, J.F.D. Greenhalgh, C.A. Morgan, L.A. Sinclair, and R.G. Wilkinson, Animal Nutrition. **2010**, Pearson: NJ, USA.
6. Van Soest, P.J., Nitrogen metabolism, *in Nutritional Ecology of the Ruminant*. **1994**, Cornell University Press.
7. Murphy, M.R., Fermentation in the rumen*, in Encyclopedia of Dairy Science*, H. Roginski, Editor. **2011**, Academic Press: NY, USA.
8. Cromwell, G.L., Soybean meal - An exceptional protein source. **2012**, Soybean Meal Infocentre: Ankeny, IA, USA.
9. Rahman, A., C.M. Wallis, and W. Uddin, Silicon-induced systemic defense responses in perennial ryegrass against infection by *Magnaporthe oryzae. Phytopathology*, **2015**. 105(6): 748-757.
10. Van Soest, P.J., Tables of feed composition, *in Nutritional Ecology of the Ruminant*. **1994**, Cornell Univeristy: NY, USA.

11. Kagan, I.A., B.M. Goff, and M.D. Flythe, Soluble phenolic compounds in different cultivars of red clover and alfalfa, and their implication for protection against proteolysis and ammonia production in ruminants. *Nat. Prod. Commun.*, **2015**. 10(7): 1263-1267.
12. Heuzé, V., G. Tran, M. Lessire, and F. Lebas. Soybean seeds. Feedipedia, a programme by INRA, CIRAD, AFZ and FAO **2016**; http://www.feedipedia.org/node/42.
13. Heuzé, V.T., G. and S. Kaushik. Soybean meal. Feedipedia, a programme by INRA, CIRAD, AFZ and FAO **2016**; http://www.feedipedia.org/node/674.
14. Malencic, D., M. Popovic, and J. Miladinovic, Phenolic content and antioxidant properties of soybean (*Glycine max (L.) Merr*.) seeds. *Molecules*, **2007**. 12(3): 576-81.
15. Naczk, M., R. Amarowicz, A. Sullivan, and F. Shahidi, Current research developments on polyphenolics of rapeseed/canola: A review. *Food Chem.*, **1998**. 62(4): 489-502.
16. Tran, G. Sugar beet tops. Feedipedia, a programme by INRA, CIRAD, AFZ and FAO **2012**; http://www.feedipedia.org/node/709.
17. Holdt, S.L. and S. Kraan, Bioactive compounds in seaweed: Functional food applications and legislation. *J. Appl. Phycol.*, **2011**. 23(3): 543-597.
18. Marsham, S., G.W. Scott, and M.L. Tobin, Comparison of nutritive chemistry of a range of temperate seaweeds. *Food Chem.*, **2007**. 100(4): 1331-1336.
19. Schiener, P., K.D. Black, M.S. Stanley, and D.H. Green, The seasonal variation in the chemical composition of the kelp species *Laminaria digitata*, *Laminaria hyperborea*, *Saccharina latissima* and *Alaria esculenta*. *J. Appl. Phycol.*, **2015**. 27(1): 363-373.
20. Aufrere, J. and B. Michalet-Doreau, Comparison of methods for predicting digestibility of feeds. *Anim. Feed Sci. Technol.*, **1988**. 20(3): 203-218.
21. Fleurence, J., 9 - Seaweed proteins A2 - Yada, R.Y, *in Proteins in Food Processing*. **2004**, Woodhead Publishing. 197-213.
22. Makkar, H.P.S., G. Tran, V. Heuzé, S. Giger-Reverdin, M. Lessire, F. Lebas, and P. Ankers, Seaweeds for livestock diets: A review. *Anim. Feed Sci. Technol.*, **2016**. 212: 1-17.
23. ITIS. *Beta vulgaris* L. **2016**; http://www.itis.gov.
24. CBS. Akkerbouwgewassen; productie en regio. **2016**; http://statline.cbs.nl/Statweb/publication/?DM=SLNL&PA=7100OOGS.
25. Suikerunie. Bietenstatistiek Suikerunie - Opbrengst-, kwaliteit- en teeltgegevens. **2015**; http://www.bietenstatistiek.nl/.
26. Biondo, P.B.F., J.S. Boeing, É.O. Barizão, N.E. de Souza, M. Matsushita, C.C. de Oliveira, M. Boroski, and J.V. Visentainer, Evaluation of beetroot (*Beta vulgaris* L.) leaves during its developmental stages: A chemical composition study. *Food Sci. Technol.*, **2014**. 34(1): 94-101.
27. Dijkstra, D.S., A.R. Linnemann, and T.A.J.S. van Boekel, Towards sustainable production of protein-rich foods: Appraisal of eight crops for Western Europe. PART II: Analysis of the technological aspects of the production chain. *Crit. Rev. Food Sci. Nutr.*, **2003**. 43(5): 481-506.
28. Gil, M.I., F. Ferreres, and F.A. Tomás-Barberán, Effect of modified atmosphere packaging on the flavonoids and vitamin C content of minimally processed Swiss chard (*Beta vulgaris* subspecies *cycla*). *J. Agric. Food Chem.*, **1998**. 46(5): 2007-2012.
29. Santos, J.E.P. and J.T. Huber, Feed proteins, *in Encyclopedia of Dairy Science*, H. Roginski, Editor. **2002**, Academic Press.
30. Pyo, Y.H., T.C. Lee, L. Logendra, and R.T. Rosen, Antioxidant activity and phenolic compounds of Swiss chard (*Beta vulgaris* subspecies *cycla*) extracts. *Food Chem.*, **2004**. 85(1): 19-26.
31. Santos, J., M.B.P.P. Oliveira, E. Ibáñez, and M. Herrero, Phenolic profile evolution of different ready-to-eat baby-leaf vegetables during storage. *J. Chromatogr. A*, **2014**. 1327: 118-131.
32. Singleton, V.L., R. Orthofer, and R.M. Lamuela-Raventós, Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent, *in Methods in Enzymology*. **1998**. 152-178.
33. Clifford, M.N., W. Wu, J. Kirkpatrick, and N. Kuhnert, Profiling the chlorogenic acids and other caffeic acid derivatives of herbal chrysanthemum by LC-MS^n. *J. Agric. Food Chem.*, **2007**. 55(3): 929-936.
34. Radi, M., M. Mahrouz, A. Jaouad, M. Tacchini, S. Aubert, M. Hugues, and M.J. Amiot, Phenolic composition, browning susceptibility, and carotenoid content of several apricot cultivars at maturity. *HortScience*, **1997**. 32(6): 1087-1091.
35. Guiry, M.D. What are seaweeds? **2000**; http://www.seaweed.ie/algae/seaweeds.php.

36. Burg, S., M. Stuiver, F. Veenstra, P. Bikker, A. Lopéz Contreras, A. Palstra, J. Broeze, H. Jansen, R. Jak, A. Gerritsen, P. Harmsen, J. Kals, A. Blanco, W. Brandenburg, M. van Krimpen, A.P. Van Duijn, W. Mulder, and L. Van Raamsdonk, A Triple P review of the feasibilty of sustainable offshore seaweed production in the North Sea. **2013**, LEI: Wageningen
37. McHugh, D.J., A guide to the seaweed industry. **2003**, FAO Fisheries Department: Rome, Italy.
38. Appeldoorn, M.M., M. Sanders, J.P. Vincken, V. Cheynier, C. Le Guernevé, P.C.H. Hollman, and H. Gruppen, Efficient isolation of major procyanidin A-type dimers from peanut skins and B-type dimers from grape seeds. *Food Chem.*, **2009**. 117(4): 713-720.
39. Isaza Martínez, J.H. and H.G. Torres Castañeda, Preparation and chromatographic analysis of phlorotannins. *J. Chrom. Sci.*, **2013**. 51(8): 825-838.
40. Koivikko, R., J. Loponen, T. Honkanen, and V. Jormalainen, Contents of soluble, cell-wall-bounds and exuded phlorotannins in the brown alga *Fucus vesiculosus*, with implications on their ecological functions. *J. Chem. Ecol.*, **2005**. 31(1): 195-212.
41. Karonen, M., M. Oraviita, I. Mueller-Harvey, J.P. Salminen, and R.J. Green, Binding of an oligomeric ellagitannin series to bovine serum albumin (BSA): Analysis by isothermal titration calorimetry (ITC). *J. Agric. Food Chem.*, **2015**. 63(49): 10647-10654.
42. Montero, L., M. Herrero, E. Ibanez, and A. Cifuentes, Separation and characterization of phlorotannins from brown algae *Cystoseira abies-marina* by comprehensive two-dimensional liquid chromatography. *Electrophoresis*, **2014**. 35(11): 1644-51.
43. Nakai, M., N. Kageyama, K. Nakahara, and W. Miki, Phlorotannins as radical scavengers from the extract of *Sargassum ringgoldianum. Mar. Biotechnol.*, **2006**. 8(4): 409-414.
44. Kang, H.S., H.Y. Chung, J.H. Jung, B.W. Son, and J.S. Choi, A new phlorotannin from the brown alga *Ecklonia stolonifera. Chem. Pharmaceut. Bull.*, **2003**. 51(8): 1012-1014.
45. Balakshin, M.Y. and E.A. Capanema, Comprehensive structural analysis of biorefinery lignins with a quantitative ^{13}C NMR approach. *RSC Advances*, **2015**. 5(106): 87187-87199.
46. Stern, J.L., A.E. Hagerman, P.D. Steinberg, F.C. Winter, and J.A. Estes, A new assay for quantifying brown algal phlorotannins and comparisons to previous methods. *J. Chem. Ecol.*, **1996**. 22(7): 1273-1293.
47. Sanchez Maldonado, A.F., A. Schieber, and M.G. Gänzle, Plant defence mechanisms and enzymatic transformation products and their potential applications in food preservation: Advantages and limitations. *Trends Food Sci. Technol.*, **2015**. 46(1): 49-59.
48. Kroll, J., H.M. Rawel, and S. Rohn, Reactions of plant phenolics with food proteins and enzymes under special consideration of covalent bonds. *Food Sci. Tech. Res.*, **2003**. 9(3): 205-218.
49. Hagerman, A.E. and K.M. Klucher, Tannin-protein interactions. *Prog. Clin. Biol. Res.*, **1986**. 213: 67-76.
50. Prigent, S.V.E., A.G.J. Voragen, A.J.W.G. Visser, G.A. Van Koningsveld, and H. Gruppen, Covalent interactions between proteins and oxidation products of caffeoylquinic acid (chlorogenic acid). *J. Sci. Food Agric.*, **2007**. 87(13): 2502-2510.
51. Kroll, J., H.M. Rawel, S. Rohn, and D. Czajka, Interactions of glycinin with plant phenols - Influence on chemical properties and proteolytic degradation of the proteins. *Nahrung - Food*, **2001**. 45(6): 388-389.
52. Hurrell, R.F. and P.-A. Finot, Nutritional consequences of the reactions between proteins and oxidized polyphenolic acids, *in Nutritional and Toxicological Aspects of Food Safety*, M. Friedman, Editor. **1984**, Springer US: Boston, MA. 423-435.
53. Kroll, J., H.M. Rawel, and S. Rohn, Reactions of plant phenolics with food proteins and enzymes under special consideration of covalent bonds. *Food Sci. Technol. Res.*, **2003**. 9(3): 205-218.
54. Toivonen, P.M.A. and D.A. Brummell, Biochemical bases of appearance and texture changes in fresh-cut fruit and vegetables. *Postharvest Biol. Technol.*, **2008**. 48(1): 1-14.
55. Lee, M.R.F., Forage polyphenol oxidase and ruminant livestock nutrition. *Front. Plant Sci.*, **2014**. 5.
56. Bittner, S., When quinones meet amino acids: Chemical, physical and biological consequences. *Amino Acids*, **2006**. 30: 205-224.
57. Parveen, I., M.D. Threadgill, J.M. Moorby, and A. Winters, Oxidative phenols in forage crops containing polyphenol oxidase enzymes. *J. Agric. Food Chem.*, **2010**. 58(3): 1371-1382.
58. Kusano, R., T. Tanaka, Y. Matsuo, and I. Kouno, Structures of epicatechin gallate trimer and tetramer produced by enzymatic oxidation. *Chem. Pharm. Bull.*, **2007**. 55(12): 1768-72.
59. Bischoff, R. and H. Schlüter, Amino acids: Chemistry, functionality and selected non-enzymatic post-translational modifications. *J. Proteom.*, **2012**. 75(8): 2275-2296.

60. Teixeira, J., A. Gaspar, E.M. Garrido, J. Garrido, and F. Borges, Hydroxycinnamic acid antioxidants: An electrochemical overview. *BioMed. Res. Int.*, **2013**. 2013: 11.
61. Korus, A., Level of vitamin C, polyphenols, and antioxidant and enzymatic activity in three varieties of kale (*Brassica Oleracea* L. Var. *Acephala*) at different stages of maturity. *Int. J. Food Prop.*, **2011**. 14(5): 1069-1080.
62. Chutichudet, B., P. Chutichudet, and S. Kaewsit, Influence of developmental stage on activities of polyphenol oxidase, internal characteristics and colour of lettuce cv. *Grand Rapids. Am. J. Food Technol.*, **2011**. 6(3): 215-225.
63. Cheng Guiwen, W. and C.H. Crisosto, Browning potential, phenolic composition, and polyphenoloxidase activity of buffer extracts of peach and nectarine skin tissue. *J. Am. Soc. Horticul. Sci.*, **1995**. 120(5): 835-838.
64. Coseteng, M.Y. and C.Y. Lee, Changes in apple polyphenoloxidase and polyphenol concentrations in relation to degree of browning. *J. Food Sci.*, **1987**. 52(4): 985-989.
65. Haslam, E. and T.H. Lilley, Interactions of natural phenols with macromolecules. *Prog. Clin. Biol. Res.*, **1986**. 213: 53-65.
66. Hagerman, A.E. and L.G. Butler, Protein precipitation method for the quantitative determination of tannins. *J. Agric. Food Chem.*, **1978**. 26(4): 809-812.
67. De Freitas, V. and N. Mateus, Nephelometric study of salivary protein-tannin aggregates. *J. Sci. Food Agr.*, **2002**. 82(1): 113-119.
68. Le Bourvellec, C. and C.M.G.C. Renard, Interactions between polyphenols and macromolecules: Quantification methods and mechanisms. *Crit. Rev. Food Sci. Nutr.*, **2012**. 52(3): 213-248.
69. Baert, N., W.F. Pellikaan, M. Karonen, and J.P. Salminen, A study of the structure-activity relationship of oligomeric ellagitannins on ruminal fermentation in vitro. *J. Dairy Sci.*, **2016**. 99(10): 8041-8052.
70. Bohin, M.C., J.P. Vincken, A.H. Westphal, A.M. Tripp, P. Dekker, H.T.W.M. Van Der Hijden, and H. Gruppen, Interaction of flavan-3-ol derivatives and different caseins is determined by more than proline content and number of proline repeats. *Food Chem.*, **2014**. 158: 408-416.
71. Haslam, E., Natural polyphenols (vegetable tannins) as drugs: Possible modes of action. *J. Nat. Prod.*, **1996**. 59(2): 205-215.
72. Jones, W.T. and J.L. Mangan, Complexes of the condensed tannins of sainfoin (O*nobrychis viciifolia scop*.) with fraction 1 leaf protein and with submaxillary mucoprotein, and their reversal by polyethylene glycol and pH. *J. Sci. Food Agr.*, **1977**. 28(2): 126-136.
73. Soares, S., N. Mateus, and V. Freitas, Interaction of different polyphenols with bovine serum albumin (BSA) and human salivary alpha-amylase (HSA) by fluorescence quenching. *J. Agric. Food. Chem.*, **2007**. 55(16): 6726-35.
74. Wang, Y., T.W. Alexander, and T.A. McAllister, In vitro effects of phlorotannins from Ascophyllum nodosum (brown seaweed) on rumen bacterial populations and fermentation. *J. Sci. Food Agr.*, **2009**. 89(13): 2252-2260.
75. Van Soest, P.J., The ruminant forestomach, *in Nutritional Ecology of the Ruminant*. **1994**, Cornell Univeristy: NY, USA.
76. Tedeschi, L.O., C.A. Ramirez-Restrepo, and J.P. Muir, Developing a conceptual model of possible benefits of condensed tannins for ruminant production. *Animal*, **2014**. 8(7): 1095-105.
77. Gerber, P.J., A. Mottet, C.I. Opio, A. Falcucci, and F. Teillard, Environmental impacts of beef production: Review of challenges and perspectives for durability. *Meat Sci.*, **2015**. 109: 2-12.
78. Van Soest, P.J., Microbes in the gut, *in Nutritional Ecology of the Ruminant*. **1994**, Cornell University: NY, USA.
79. Bell, M., R. Eckard, P. Moate, and T. Yan, Modelling the Effect of Diet Composition on Enteric Methane Emissions across Sheep, Beef Cattle and Dairy Cows. *Animals*, **2016**. 6(9): 54.
80. Allison, M.J., M.P. Bryant, I. Katz, and M. Keeney, Metabolic function of branched-chain volatile fatty acids, growth factors for ruminococci. II. Biosynthesis of higher branched-chain fatty acids and aldehydes. *J. Bacteriol.*, **1962**. 83: 1084-93.
81. Ungerfeld, E.M., Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: A meta-analysis. *Front. Microbiol.*, **2015**. 6(FEB).
82. Tamminga, S., A review on environmental impacts of nutritional strategies in ruminants. *J. An. Sci.*, **1996**. 74(12): 3112-3124.
83. Mueller-Harvey, I., Unravelling the conundrum of tannins in animal nutrition and health. *J. Sci. Food Agric.*, **2006**. 86(13): 2010-2037.

84. Hassanat, F. and C. Benchaar, Assessment of the effect of condensed (acacia and quebracho) and hydrolysable (chestnut and valonea) tannins on rumen fermentation and methane production in vitro. *J. Sci. Food Agric.*, **2013**. 93(2): 332-339.
85. Hervás, G., P. Frutos, F.J. Giráldez, Á.R. Mantecón, and M.C. Álvarez Del Pino, Effect of different doses of quebracho tannins extract on rumen fermentation in ewes. *Anim. Feed Sci. Tech.*, **2003**. 109(1-4): 65-78.
86. Hassanat, F., R. Gervais, C. Julien, D.I. Massé, A. Lettat, P.Y. Chouinard, H.V. Petit, and C. Benchaar, Replacing alfalfa silage with corn silage in dairy cow diets: Effects on enteric methane production, ruminal fermentation, digestion, N balance, and milk production. *J. Dairy Sci.*, **2013**. 96(7): 4553-4567.
87. Wu, S.H.W. and A. Papas, Rumen-stable delivery systems. *Adv. Drug Del. Rev.*, **1997**. 28(3): 323-334.
88. Patra, A.K., Urea/ammonia metabolism in the rumen and toxicity in ruminants, *in Rumen Microbiology: From Evolution to Revolution*. **2015**. 329-341.
89. Karlsson, L., M. Ruiz-Moreno, M.D. Stern, and K. Martinsson, Effects of temperature during moist heat treatment on ruminal degradability and intestinal digestibility of protein and amino acids in hempseed cake. *Asian-Austr. J. Anim. Sci.*, **2012**. 25(11): 1559-1567.
90. Copani, G., C. Ginane, A. Le Morvan, and V. Niderkorn, Patterns of in vitro rumen fermentation of silage mixtures including sainfoin and red clover as bioactive legumes. *Anim. Feed Sci. Technol.*, **2015**. 208: 220-224.
91. Chen, L., G. Remondetto, M. Rouabhia, and M. Subirade, Kinetics of the breakdown of cross-linked soy protein films for drug delivery. *Biomaterials*, **2008**. 29(27): 3750-3756.
92. Reis, P.J. and D.A. Tunks, Influence of formaldehyde-treated casein supplements on the concentration of ε-N-methyllysine in sheep plasma. *Aus. J. Biol. Sci.*, **1973**. 26(5): 1127-1136.
93. Oste, R. and P. Sjodin, Effect of Maillard reaction products on protein digestion. In vivo studies on rats. *J. Nutr.*, **1984**. 114(12): 2228-34.
94. Pierpoint, W.S., o-Quinones formed in plant extracts. Their reactions with amino acids and peptides. *Biochem. J.*, **1969**. 112(5): 609-616.

Chapter 2

Effect of plant age on the quantity and quality of the proteins extracted from sugar beet (*Beta vulgaris* L.) leaves

Effects of the developmental stage (e.g. young, mature or senescent) of leaves on their chemical composition have been described in literature. This study focuses on the variation in chemical composition and quantity and quality of proteins extracted from leaves due to variation in plant age (i.e. harvesting time), using leaves from sugar beets grown in field (Rhino, Arrival) and in greenhouse (Isabella). Within the same variety (Rhino-field, Arrival-field, Isabella-greenhouse) the protein content was similar for leaves from young and old plants (22±1, 16±1, and 10±3% w/w DM, respectively). Variation in final protein isolation yield was mostly due to variation in nitrogen extractability (28-56%), although no consistent correlation with plant age was found. A significant effect of the plant age was observed on the quality (colour) of the extracted protein; i.e. brown (indicative of polyphenol oxidase activity) and yellow for extracts from old and young plants, respectively.

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Introduction

Leaves from various plants have long been considered as a source of protein for both food and feed applications [1], due to their nutritional value (e.g. adequate amounts of essential amino acids) [2], good techno-functional properties (e.g. high solubility at a wide pH range) [3] and high abundance in nature. For instance, in the Netherlands in 2011 the sugar beet cultivation area was 72,000 ha [4], which translates to approximately 2.2 Mt leaves per year. The quantity and quality of the proteins extracted from leaves depend on different factors, one of which is the overall chemical composition of the leaves. This composition has been shown to differ among leaves of different age (developmental stages) e.g. young, old or senescent [5-8]. However, when leaves are obtained collectively during harvest of the plants, as for example in the case of sugar beet leaves, an array of leaves of different developmental stages is collected. Also, the harvesting time may vary, since for instance in Northern Europe the harvest of the sugar beets usually takes place from September until December. Therefore, when sugar beet leaves are to be used as a source of protein for food or feed applications it is important to test whether harvesting time; i.e. the plant age, has an effect on the overall chemical composition of the whole foliage, and consequently on the protein quantity and quality of the proteins extracted.

Differences in protein content of whole foliage with varying plant age have been observed. In sugar beet leaves the protein content increased from 26.4% to 31.0% w/w DM in leaves collected 60 and 100 days after sowing, respectively [5]. For alfalfa leaves it was reported that the protein content of the foliage initially increased with plant age by 24% and subsequently decreased by 19% [9].

The protein extractability can be hindered due to the presence of intact cell walls. It was reported that protein extraction from dehulled rapeseed meal was 33% lower when no cell wall degrading enzymes were used [10]. Also, even after the cell wall opening, pectins, that constitute a large part of cell walls of dicotyledon plants, can still hamper the protein isolation due to their interaction with proteins. Overall, it is expected that differences in carbohydrate content will lead to differences in protein extractability. It has been shown that in sugar beet leaves the carbohydrate content decreased from 41% to 31% w/w DM in leaves collected 60 days to 100 days after sowing, respectively [5].

Other compounds present in leaves, like phenolic compounds, are also expected to influence protein extractability [11]. The naturally present phenolic compounds in leaves can, upon cell rupture, be oxidized by endogenous polyphenol oxidases (PPOs), such as catecholase and cresolase [12]. This reaction leads to quinones that can polymerise with phenolic compounds [13] or covalently link to proteins [11]. The latter can potentially hinder protein extractability. The reaction products are dark coloured, thus the colour is an indication of protein modification. Protein modification can negatively affect the quality of the proteins, e.g. decrease their solubility. It has been shown that covalent linkage of enzymatically oxidised phenolic compounds to proteins, like lysozyme or α-lactalbumin, significantly decreased protein solubility at pH values ranging between 3 and 5 [11].

Similar to proteins and carbohydrates, the content of phenolic compounds may also vary with plant age. For example, the total content of phenolic compounds in sugar beet leaves collected 100 days after sowing was approximately 1.5 times higher than that in leaves collected after 60 days [5]. In contrast, in lettuce leaves it was reported that the phenolic compounds content in leaves collected 59 days after sowing was 8.5 times lower than in leaves collected 28 days after sowing, although at 73 days after sowing it was similar to that of 28 days. While the phenolic compounds content showed a fluctuation over time, the PPO activity determined in these leaves showed a constant increase with the lettuce age [14].

From the above, it is evident that the plant age has an effect on the chemical composition of the leaves. The aim of the present research was to determine the effect of plant age on the chemical composition of the sugar beet leaves and thereby its effect on the protein quantity and quality (colour) of the extracted proteins. It was hypothesised that: (i) plant age affects the chemical composition of the sugar beet foliage, (ii) the quantity of protein from sugar beet leaves obtained from old plants is lower than that from leaves from young plants and (iii) in leaves from old plants there is higher PPO activity, which leads to brown colour formation during protein extraction from these leaves.

Materials and Methods

Chemicals and sugar beet leaves

The sugar beet leaves were obtained either from sugar beets grown in a field (*Beta vulgaris* L. var. Rhino, var. Arrival) or grown in a greenhouse (*Beta vulgaris* L. var. Isabella) at Unifarm (Wageningen, The Netherlands). The seeds were provided by Royal Cosun (Breda, The Netherlands; purchased from Kws Saat, Einbeck, Germany). Chlorophyll *a* (purity of 90% w/w, based on HPLC as provided by the supplier) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Gallic acid (purity of 98.5% based on GC as provided by the supplier), (+)-Catechin hydrate (purity of ≥ 98%, based on HPLC as provided by the supplier) and L-tyrosine (purity of ≥ 98% w/w, based on HPLC as provided by the supplier) were purchased from Sigma Aldrich (St. Louis, MO, USA). All other chemicals used were purchased from either Merck (Darmstadt, Germany) or Sigma-Aldrich.

Growth conditions

Field

The sugar beet seeds (pelleted seeds containing fungicides and insecticides) were sown in April 2013 (*Beta vulgaris* L. var. Rhino) and April 2014 (*Beta vulgaris* L. var. Arrival). The plants were protected against weed and fungi. In both years, there was no need for pest control. In 2013, the sugar beet leaves (no stems) were collected 3 and 6 months after sowing and were denoted as LF_{3R} and LF_{6R}, respectively. In 2014, the collection of the leaves took place 3 and 8 months after sowing and the leaves were respectively denoted as LF_{3A} and LF_{8A}. The subscript indicates the age, in months, of the sugar beet and the variety of the sugar beet. The leaves, at the different harvesting times, were collected from different spots in the field to ensure that leaves from regrowth were not used.

Senescent leaves were discarded. The leaves collected in 2013 and 2014 were collectively denoted as field leaves (LF).

Greenhouse

Fifteen seeds of sugar beets (*Beta vulgaris* L. var. Isabella) were sown, each month from December 2012 to July 2013. Seeds were sown in pots (12 x 12 x 20 cm) containing normal soil for flowering plants. The plants were grown in these pots until their first leaf fell off. Next, they were transplanted to trays (40 x 30 x 25 cm) and grown until they had two true leaves. Eight healthy seedlings were then randomly selected and thinned to one per pot (25 x 25 x 25 cm). The plants were grown under a natural photoperiod, with a minimum of 16 h light exposure. In case of shorter natural photoperiod, artificial light was used (SON-T Agro, Philips, Eindhoven, The Netherlands). The humidity in the greenhouse was 65-75% and the temperature was set at 16-18 °C. The plants were watered with tap water twice a day. Pest was biologically controlled using swirski-mite (Koppert biological systems, Berkel en Rodenrijs, The Netherlands). All sugar beets were sown at different time points and harvested at the same time (September 2013) resulting in leaves ranging from 2 (LG_{2I}) to 9 (LG_{9I}) months. In this way, variations due to different storage times before analysis were avoided. The sugar beet leaves (no stems) that were collected from the sugar beets grown in the greenhouse were collectively denoted as greenhouse leaves (LG). Similarly to the collection of leaves from the field, senescent leaves were discarded. The LG_{9I} leaves were mostly senescent leaves, hence, they were excluded from the analyses.

Sugar beet leaves handling

After the sugar beets leaves were collected, the leaves were washed using tap water and stored at 4 ˚C until the excess water was completely drained off (not longer than 36 h). Part of the leaves was stored in vacuum sealed bags at -20 °C until further analysis. The rest of the leaves were freeze-dried. The freeze-dried leaves were ground in an ultra-centrifugal mill (Retsch ZM 200, Haan, Germany) at 6,000 rpm with a 0.5 mm or 0.2 mm sieve, for the leaves grown in the greenhouse and in the field, respectively. The freeze-dried powders were stored at -20 °C until further analyses.

Protein isolation

Sugar beet leaves grown in the field

Frozen sugar beet leaves (13 ± 2.4% w/w DM) were blended for 2 min in a household-type blender with 150 mM sodium phosphate buffer pH 8.0 containing 0.8 M NaCl, using a leaves:buffer ratio of 1:3 (w/w). In another experiment, LF_{6R} was blended under the same conditions in the same buffer supplemented with 0.17% (w/v) $Na_2S_2O_5$ and denoted LF_{6R}, SO_3. The pH of the suspension was readjusted to 8.0 after supplementation of the $Na_2S_2O_5$. The pulp obtained after blending the leaves was left for 1 h at 4 ˚C and subsequently filtered through a Büchner funnel (no filter paper). The filtrate was centrifuged (38,400 × *g*, 4 °C, 30 min) and the supernatant (juice) obtained was subsequently dialysed (MWCO 12,000 - 14,000 Da, Medicell International, London, UK) at 4 °C for 18-24 h against 35 mM sodium phosphate buffer pH 8.0 (LF_{3R}, LF_{3A}, LF_{6R}, LF_{8A}) or against the same buffer containing 0.13% (w/v) $Na_2S_2O_5$ (LF_{6R}, SO_3). Next, the dialysed juices were further dialysed against distilled water at 4 °C for 24 h. The pH of the dialysed juice was lowered to 4.5 using 0.5 M HCl at room temperature. The acidified dialysed juice was kept at 4 °C for at least 1 h and

subsequently centrifuged (5,000 × *g*, 4 °C, 10 min). The pellet obtained was re-dispersed in distilled water and solubilised by adjusting the pH to 8.0 using 0.5 M NaOH at room temperature and subsequently freeze-dried. The obtained leaf soluble protein concentrates obtained were denoted as $LSPC_{LF}^{3F}$, $LSPC_{LF}^{6R}$, $LSPC_{LF}^{6R,SO3}$, $LSPC_{LF}^{3A}$and $LSPC_{LF}^{8A}$. Extractions were performed in duplicate.

Sugar beet leaves grown in the greenhouse

A similar process as for field leaves was followed, albeit with some adaptations; i.e. freeze-dried (96.9 ± 0.4% w/w DM) instead of frozen leaves were used. The leaves were suspended in distilled water at 13 % (w/w). From this point onwards, the same procedure that is described for the protein isolation from the leaves grown in field was followed. The leaf soluble protein concentrates obtained from sugar beet leaves grown in the greenhouse were denoted as $LSPC_{LG}^{2I}$, $LSPC_{LG}^{3I}$. $LSPC_{LG}^{4I}$, $LSPC_{LG}^{5I}$, $LSPC_{LG}^{6I}$, $LSPC_{LG}^{7I}$, $LSPC_{LG}^{8I}$. Extractions were performed twice in two consecutive years.
Dialysed juices obtained from frozen LG were used for the determination of the presence of coloured compounds and PPO activity. The same process as described for the dialysed juices from LF was followed. Extractions in the presence of $Na_2S_2O_5$ were not performed.

Compositional analyses

For each analysis at least two independent samples were taken and each analysis was carried out at least in duplicate. The average and the standard deviation (stdev) obtained from the two independent samples were calculated. All values were expressed on a dry matter basis (DM).

Dry matter content

Dry matter content was determined gravimetrically by drying the samples at 105 °C overnight.

Total nitrogen content

Total nitrogen content (NT) was determined by the Dumas method using a Flash EA 1112 N analyser (Thermo Fisher Scientific, Waltham, MA, USA), according to manufacturer's protocol. Methionine was used as standard for the quantification.

Amino acid analyses

Amino acid composition was determined based on ISO 13903 (2005) [15] method, adjusted for micro-scale. The amide nitrogen from asparagine and glutamine and nitrogen from aspartic acid and glutamic acid were not determined separately. Therefore, nitrogen recovered from these amino acids was calculated assuming that ASX (asparagine/aspartic acid) and GLX (glutamine/glutamic acid) were present as either 100% ASN/GLN or 100% ASP/GLU.

Nitrogen-to-protein (N-prot) conversion factor

Based on the amino acids analysis two N-protein (N-Prot) factors; i.e. k_p and k_a were determined, as described previously [16]. The k_p is the ratio of the sum of amino acid residue weights determined by amino acid analysis to total nitrogen (NT) weight, determined by the Dumas method. The k_a is the ratio of the sum of amino acid residue weights to nitrogen weight from recovered amino acids. Given that the nitrogen recovered from asparagine/aspartic acid and glutamine/glutamic acid was calculated assuming that ASX and GLX were present as either 100% ASN/GLN or 100% ASP/GLU, two k_p and two k_a values were calculated for each sample. The values for k_a are presented as a range

between a lower and an upper limit, calculated with ASX/GLX = 100% ASN/GLN and ASX/GLX = 100% ASP/GLU, respectively. The values for k_p are presented as average between the values calculated with ASX/GLX = 100% ASN/GLN or 100% ASN/GLN because the standard deviations were found to be on average <0.2% of the average values. The k_p/k_a ratio represents the ratio proteinaceous (NAA) over the total nitrogen (NT). The kp calculated for the leaves was used as the N-Prot factor for the respective leaves, whereas the average kp calculated for the $LSPC_{LF}^{3R}$ and $LSPC_{LF}^{6R}$ was used as the N-Prot factor for all LSPCs.

Carbohydrate composition and total uronic acid content

Carbohydrate content was determined as the sum of the uronic acid and the neutral carbohydrate contents. Freeze-dried leaves and LSPC (10-13 mg) were treated with 72% (w/w) H_2SO_4 (1 h, 30 °C) followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100 °C. The suspensions were then centrifuged (16,000 × *g*, 20 °C, 10 min) and the supernatants were used for uronic acid and neutral carbohydrate determinations. Uronic acid content was determined as described previously [17]. A calibration curve with galacturonic acid (12.5-100 mg/L) was used for quantification. Neutral carbohydrates were analysed as free monosaccharides using high performance anion-exchange chromatography on a Dionex Ultimate 3000 system (Thermo Scientific, Sunnyvale, CA, USA) equipped with a CarboPac PA-1 column (2 mm × 250 mm i.d.) in combination with a CarboPac guard column (2 mm × 50 mm i.d.) and a pulsed amperometric detection, as described previously [18] with adaptations. The elution profile that was used for the elution (0.4 mL/min) was as follows: 0-35 min 100% H_2O, 35-50 min from 100% 0.1 M NaOH to 100% 1 M NaOAc in 0.1 M NaOH, 50-55 min 100% 1 M NaOAc in 0.1 M NaOH, 55-63 min 100% 0.1 M NaOH, 63-78 min 100% H_2O. From 0 to 35 min and from 63 to 78 min a post column addition of 0.5 M NaOH at a flow rate of 0.1 mL/min was performed to detect and quantify the monosaccharides eluted. Rhamnose, arabinose, xylose, mannose, galactose, glucose; calibration curves (2-75 mg/L)) were used for the quantification. The total neutral carbohydrate content was calculated as the sum of the weight of the total anhydrous monosaccharides.

Phenolic compounds content

Phenolic compounds were extracted from freeze-dried leaves using 5 consecutive extractions of 30 min at 4 °C in 50% (v/v) aqueous methanol containing 0.5% (v/v) acetic acid, at a leaves to extractant ratio of 1:10 (w/v) [19]. In each extraction, the pellet was separated from the supernatant by centrifugation (8,000 × *g*, 4 °C, 10 min). Fresh solvent was added to the solids after each centrifugation step. The supernatants obtained were combined and filtered (0.45 µm cellulose filter), to yield the final extract. Phenolic compounds content was determined in the final extract using the Folin-Ciocalteu phenol reagent method [20] with adaptations. The extract (20 µL) was diluted with 1.58 mL distilled water and incubated with 100 µL Folin-Ciocalteu reagent for 20 min. Subsequently, 300 µL Na_2CO_3 (20% w/v) were added to the mixture and after 2 h incubation the absorbance at 725 nm was measured. A gallic acid calibration curve (0-100 mg/L) was used for the quantification.

Ash content

Ash content was determined gravimetrically by incinerating the dried samples at 525 °C overnight.

Lipid content

Lipid content was determined gravimetrically using the Folch method [21] with adaptations. In detail, the freeze-dried samples were extracted twice using a methanol/$CHCl_3$ (1:2 v/v) solution at room temperature. The washing of the combined extracts was done by addition of 5 mL of 0.88% (w/v) aqueous KCl solution. The weight of the lipid extract was determined after the solvents were evaporated using a rotary evaporator. From this weight the mass of chlorophyll *a* (described below) was subtracted to obtain the total lipid content.

Lignin content

Acid insoluble lignin was determined gravimetrically, as described previously [22].

Chlorophyll *a* content

Chlorophyll *a* content was determined after dissolving the lipid extract into 1 mL 90% (v/v) aqueous acetone. The absorbance of the solution was measured at 664 nm [23]. For the quantification a calibration curve of chlorophyll *a* in 90% (v/v) aqueous acetone was used.

Nitrogen recovery

To study the plant age effect on nitrogen extraction, the nitrogen extractability; i.e. the proportion of N that was recovered in the juice (nitrogen recovery in the juice%) was calculated as the amount of nitrogen in the juice divided by the amount of nitrogen in the respective pulp*100%. The pulp, rather than the frozen or the freeze-dried leaves, was used as initial reference material for the nitrogen recovery calculation, to avoid any differences caused by leaf to leaf variation. To study the plant age effect on nitrogen isolation, the recovery after dialysis and acid precipitation was calculated as the amount of nitrogen in the dialysed juice or in the LSPC divided by the amount of nitrogen in the juice*100%.

Protein composition

The protein composition was determined using SDS-PAGE under reducing conditions. All samples (freeze dried LG and LF, LF Rhino juice, LF Rhino dialysed juice, LF Rhino protein concentrates; 5.0 g protein/L) were prepared according to the manufacturer's protocol. They were then applied to the gels (any kD, Mini-protean TGX precast protein gels, Bio-Rad Laboratories, Hercules, CA, USA). The proteins were separated on a Mini-protean II system (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol and visualized by staining the gels using coomassie blue stain (InstantBlue, Expedeon, San Diego, CA, USA).

Presence of coloured compounds

The presence of coloured compounds in the dialysed juice was determined by measuring the absorbance from 250 to 750 nm in 1 cm quartz cuvettes using a UV-1800 Shimadzu spectrophotometer and UV Probe 2.00 software (Shimadzu, Kyoto, Japan).

PPO activity assay

PPO-cresolase activity was determined by incubation of the dialysed juice with tyrosine, while PPO-catecholase activity was determined by incubation of the dialysed juice with catechin. The dialysed juice (200 µL) was mixed with 100 µL tyrosine (2 mM in 0.1 M sodium phosphate buffer, pH 6.7) or catechin solution (2 mM in 0.1 M sodium phosphate buffer, pH 6.7) in a 96-well plate. The formation

of oxidation products after the addition of tyrosine was monitored at 520 nm [24] over 1,200 min using a TECAN Infinite F500 spectrophotometer (Männedorf, Switzerland). The formation of oxidation products after the addition of catechin was monitored at 420 nm [25] over 300 min. The formation of oxidation products after the tyrosine addition was monitored over a longer period of time because prior experiments showed that in this case there is a long lag phase before the coloured compounds are formed. Incubation of dialysed juice with buffer was used to verify for autoxidation. Another control was performed by adding tyrosine or catechin to the buffer (0.1 M sodium phosphate, pH 6.7). Reaction rate constants of cresolase and catecholase were calculated from the slopes of the linear segment of the absorbance vs time curves (corrected for the protein content of the sample) and were expressed as $AU \cdot min^{-1} \cdot (mg\ N)^{-1}$.

Results and Discussion

Composition of the sugar beet leaves

The protein content did not considerably vary between the LF (field leaves) from young and old plants (**Table 1**). In the LG (greenhouse leaves), the protein contents for plants of 3-8 months were also quite constant (on average 9.6 ± 2.5% w/w DM) (**Figure 1**). Only the youngest plants (2 months) had a deviating high protein content (22.2 ± 0.2% w/w DM) (**Figure 1**). Overall, the protein content in the LG (LG_{2l} excluded) was 41-59% lower than the protein content in the LF, which shows that the growth conditions have an effect on the protein content in the leaves. That was expected since it is known that environmental conditions; e.g. temperature, rainfall, light etc., can affect the growth response, and thus the chemical composition of the plants [26]. These data suggest that the protein content in the leaves depends on the growth conditions and possibly on the variety, but not on the plant age.

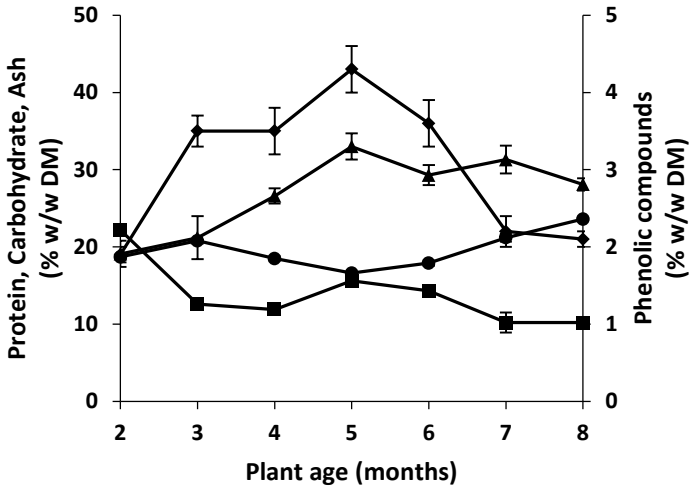

Figure 1 Protein (■), carbohydrate (▲), ash (●) and phenolic compounds contents (◆) in the greenhouse leaves. Error bars indicate stdev

Table 1 Chemical composition (w/w % DM) of field leaves (LF) and corresponding protein concentrates (LSPCs)[a]

Sample	Dry matter [b]	Protein [c]	Total carbohydrates	Lipids [d]	Chlorophyll *a*	Lignin	Ash	Phenolics	Total
LF_{3R}	11.3 (±0.3)	22.8 (±1.7)	19.2 (±0.7)	4.2 (±0.1)	0.3 (±0.1)	6.4 (±0.1)	16.4 (±0.1)	1.5 (±0.3)	70.8
LF_{6R}	14.1 (±0.2)	21.7 (±1.5)	22.9 (±2.2)	8.0 (±1.0)	0.8 (±0.2)	10.1 (±0.6)	15.2 (±0.5)	2.7 (±0.1)	81.4
LF_{3A}	10.6 (± 0.4)	14.6 (±1.0)	31.4 (±0.9)	5.0 (±0.3)	0.3 (±0.1)	13.0 (±0.2)	14.0 (±1.0)	1.0 (±0.1)	79.3
LF_{8A}	15.8 (±0.2)	16.6 (±2.6)	27.3 (±0.4)	7.7 (±0.4)	0.5 (±0.1)	15.8 (±0.3)	11.0 (±2.0)	1.5 (±0.1)	80.4
$LSPC_{LF\ R}^{3}$	93.0 (±0.5)	75.0 (±4.4)	3.1 (±0.5)	4.2 (±0.1)	n.d.[e]	n.d.	1.3 (±0.4)	n.d.	83.6
$LSPC_{LF\ R}^{6}$	91.8 (±1.4)	61.4 (±4.3)	3.8 (±1.2)	3.3 (±0.3)	n.d.	n.d.	2.0 (±0.6)	n.d.	73.2
$LSPC_{LF\ A}^{3}$	95.0 (±0.8)	61.2 (±4.3)	3.7 (±0.2)	4.9 (±0.8)	n.d.	n.d.	1.0 (±0.0)	n.d.	70.8
$LSPC_{LF\ A}^{8}$	94.4 (±1.3)	54.2 (±4.1)	5.1 (±0.4)	7.7 (±0.2)	n.d.	n.d.	2.0 (±0.0)	n.d.	69.0

[a] All data are expressed as average (from at least two independent samples) (±stdev)

[b] In w/w% fresh weight

[c] Determined as $N_T \cdot k_p$

[d] Determined as $ChCl_3$/MeOH soluble material, corrected for chorophyll a concentent

[e] Not determined

Table 2 Amino acid profile (w/w % protein) and amino acid (AA) content (w/w % DM) of filed leaves (LF), greenhouse (GH) and protein concentrates (LSPC) obtained from field Rhino leaves

Sample	ASX [a]	THR	SER	GLX [a]	GLY	ALA	VAL	ILE	LEU	TYR	PHE	HIS	LYS	ARG	CYS	MET	PRO	Total AA
LF_{3R}	10.50 (±0.03)	5.20 (±0.01)	4.90 (±0.00)	12.09 (±0.05)	6.26 (±0.02)	6.56 (±0.02)	6.26 (±0.03)	4.93 (±0.02)	9.47 (±0.01)	4.40 (±0.00)	5.99 (±0.01)	3.09 (±0.01)	5.81 (±0.01)	5.44 (±0.02)	1.72 (±0.00)	2.22 (±0.02)	5.17 (±0.07)	24.68 (±0.15)
LF_{6R}	10.74 (±0.02)	5.20 (±0.01)	4.92 (±0.02)	12.03 (±0.03)	6.43 (±0.02)	6.30 (±0.01)	6.24 (±0.00)	4.85 (±0.01)	9.39 (±0.01)	4.52 (±0.04)	6.02 (±0.02)	3.10 (±0.01)	5.65 (±0.01)	5.44 (±0.00)	1.74 (±0.01)	2.21 (±0.00)	5.24 (±0.05)	23.69 (±0.04)
LF_{3A}	10.61 (±0.05)	5.17 (±0.01)	4.95 (±0.02)	11.49 (±0.07)	6.25 (±0.00)	6.59 (±0.02)	6.31 (±0.01)	4.95 (±0.01)	9.51 (±0.05)	3.96 (±0.03)	5.99 (±0.06)	2.96 (±0.03)	6.72 (±0.03)	5.28 (±0.03)	1.80 (±0.02)	2.14 (±0.05)	5.31 (±0.07)	16.12 (±0.55)
LF_{8A}	10.00 (±0.05)	4.96 (±0.01)	5.32 (±0.03)	11.21 (±0.03)	7.28 (±0.02)	6.39 (±0.02)	5.92 (±0.02)	4.79 (±0.00)	8.71 (±0.00)	4.69 (±0.01)	5.64 (±0.06)	3.49 (±0.02)	6.68 (±0.03)	5.27 (±0.01)	2.28 (±0.02)	2.09 (±0.05)	5.28 (±0.05)	18.07 (±0.69)
LG_{2I}	10.35 (±0.02)	5.08 (±0.03)	4.76 (±0.00)	12.47 (±0.00)	6.02 (±0.02)	6.28 (±0.01)	6.23 (±0.00)	4.95 (±0.05)	9.50 (±0.01)	4.23 (±0.05)	6.05 (±0.01)	2.95 (±0.00)	6.76 (±0.00)	5.37 (±0.01)	1.57 (±0.02)	2.15 (±0.00)	5.28 (±0.04)	26.75 (±0.06)
LG_{3I}	10.67 (±0.06)	4.96 (±0.03)	5.18 (±0.01)	11.64 (±0.06)	6.35 (±0.00)	6.09 (±0.01)	6.13 (±0.01)	5.09 (±0.00)	9.16 (±0.02)	4.17 (±0.02)	5.94 (±0.03)	3.31 (±0.09)	6.66 (±0.03)	4.83 (±0.03)	2.02 (±0.00)	2.00 (±0.01)	5.79 (±0.02)	10.93 (±0.22)
LG_{4I}	10.69 (±0.08)	5.04 (±0.00)	5.24 (±0.04)	11.66 (±0.11)	6.34 (±0.03)	6.10 (±0.02)	6.14 (±0.07)	5.08 (±0.01)	9.15 (±0.07)	4.12 (±0.00)	5.83 (±0.13)	3.26 (±0.02)	6.71 (±0.01)	4.87 (±0.07)	2.04 (±0.03)	2.01 (±0.01)	5.73 (±0.07)	9.92 (±0.42)
LG_{5I}	10.40 (±0.06)	5.01 (±0.01)	5.12 (±0.05)	11.79 (±0.06)	6.32 (±0.04)	6.08 (±0.01)	6.05 (±0.02)	4.97 (±0.00)	9.10 (±0.02)	4.28 (±0.02)	5.95 (±0.07)	3.32 (±0.04)	6.62 (±0.01)	5.39 (±0.10)	1.85 (±0.05)	2.08 (±0.01)	5.63 (±0.02)	16.53 ±0.16)
LG_{6I}	10.51 (±0.07)	4.99 (±0.04)	5.20 (±0.00)	11.85 (±0.14)	6.38 (±0.05)	6.13 (±0.03)	6.04 (±0.06)	4.99 (±0.03)	9.17 (±0.05)	4.11 (±0.04)	5.96 (±0.11)	3.21 (±0.05)	6.66 (±0.01)	4.98 (±0.04)	2.07 (±0.01)	2.04 (±0.01)	5.7 (±0.08)	12.16 (±0.03)
LG_{7I}	10.62 (±0.01)	4.97 (±0.00)	5.30 (±0.02)	12.15 (±0.05)	6.44 (±0.02)	6.03 (±0.03)	5.96 (±0.02)	4.94 (±0.00)	8.94 (±0.00)	4.18 (±0.06)	5.72 (±0.11)	3.32 (±0.03)	6.60 (±0.04)	4.76 (±0.08)	2.28 (±0.00)	1.96 (±0.06)	5.82 (±0.22)	9.37 (±0.09)
LG_{8I}	10.59 (±0.04)	4.94 (±0.01)	5.29 (±0.00)	12.26 (±0.06)	6.47 (±0.02)	6.07 (±0.03)	6.03 (±0.00)	4.92 (±0.03)	9.01 (±0.04)	4.08 (±0.04)	5.77 (±0.02)	3.28 (±0.03)	6.64 (±0.04)	4.69 (±0.12)	2.34 (±0.03)	2.01 (±0.03)	5.62 (±0.15)	9.16 (±0.15)
Average (±stdev)	10.52 (±0.21)	5.05 (±0.10)	5.11 (±0.19)	11.88 (±0.37)	6.41 (±0.31)	6.24 (±0.20)	6.12 (±0.13)	4.95 (±0.09)	9.19 (±0.26)	4.25 (±0.21)	5.90 (±0.14)	3.21 (±0.17)	6.50 (±0.38)	5.12 (±0.30)	1.99 (±0.26)	2.08 (±0.09)	5.51 (±0.25)	
$LSPC_{LF}^{3R}$	10.10 (±0.07)	5.71 (±0.06)	4.14 (±0.03)	11.12 (±0.45)	5.56 (±0.04)	6.25 (±0.00)	6.56 (±0.00)	4.66 (±0.04)	9.50 (±0.05)	5.08 (±0.05)	5.97 (±0.04)	3.02 (±0.02)	6.97 (±0.01)	6.28 (±0.08)	1.58 (±0.02)	2.22 (±0.02)	5.29 (±0.00)	87.34 (±0.16)
$LSPC_{LF}^{6R}$	10.16 (±0.02)	5.50 (±0.00)	4.52 (±0.00)	11.30 (±0.00)	5.79 (±0.00)	6.17 (±0.00)	6.48 (±0.00)	4.77 (±0.00)	9.31 (±0.00)	4.90 (±0.00)	5.82 (±0.00)	2.97 (±0.00)	7.05 (±0.00)	6.21 (±0.00)	1.62 (±0.00)	2.25 (±0.00)	5.2 (±0.00)	74.93 (±0.26)
Average (±stdev)	10.13 (±0.05)	5.60 (±0.15)	4.33 (±0.26)	11.21 (±0.13)	5.67 (±0.16)	6.21 (±0.06)	6.52 (±0.06)	4.72 (±0.07)	9.40 (±0.14)	4.99 (±0.13)	5.90 (±0.11)	2.99 (±0.03)	7.01 (±0.05)	6.24 (±0.04)	1.60 (±0.03)	2.24 (±0.02)	5.25 (±0.07)	

[a] No separate analysis of ASP/ASN and GLU/GLN

The amino acid composition did not differ between the leaves of different plant ages, both for LF and LG samples (**Table 2**). Based on the amino acid analysis the two N-Prot factors k_p and k_a were determined. In the LF, the k_p was 8-13% higher for young than for old plants (**Table 3**). For the LG, the k_p varied between 4.12 and 4.57, while no clear effect of the plant age was identified. Overall, the average k_p calculated for the sugar beet leaves was 4.41 ± 0.26 for all leaves (**Table 3**). This value is similar to the average k_p value (4.46 ± 0.40) reported for other leafy materials [27]. For ka a minimal and maximal value were calculated for each sample, assuming all ASX/GLX to be present as ASP/GLU or as ASN/GLN, respectively (**Table 3**). The k_a factor was similar for all leaves tested (**Table 3**). The average k_a factor (average lower limit and average upper limit) for all leaves (both LF and LG) was 22-43% higher than the average k_p. This indicates the presence of substantial amounts of non-proteinaceous nitrogen (NPN), such as inorganic nitrogen, hormones, chlorophyll and nucleic acids. From the ratio NAA/NT, it was estimated that the leaves from old plants from Rhino and Arrival contain 24-46% and 43-150%, respectively, more NPN than the leaves from young plants (**Table 3**).

Table 3 Proteinaceous nitrogen and nitrogen-to-protein conversion factors k_a and k_p for different materials

Sample	N_{AA}/N_T (%)[b,c]	N-Prot factor k_p[d]	N-Prot factor k_p[e]
LF_{3R}	75 < x < 87	4.72 (*±0.01*)	5.40 < y < 6.31
LF_{6R}	69 < x < 81	4.36 (*±0.01*)	5.39 < y < 6.31
LF_{3A}	79 < x < 92	4.95 (±0.01)	5.39 < y < 6.28
LF_{8A}	70 < x < 80	4.32 (*±0.01*)	5.37 < y < 6.21
LG_{2I}	66 < x < 77	4.15 (*±0.01*)	5.39 < y < 6.31
LG_{3I}	73 < x < 85	4.57 (*±0.01*)	5.41 < y < 6.31
LG_{4I}	66 < x < 77	4.16 (*±0.01*)	5.40 < y < 6.30
LG_{5I}	66 < x < 77	4.12 (*±0.01*)	5.38 < y < 6.26
LG_{6I}	68 < x < 79	4.29 (*±0.01*)	5.40 < y < 6.30
LG_{7I}	68 < x < 80	4.31 (*±0.01*)	5.39 < y < 6.31
LG_{8I}	72 < x < 84	4.53 (*±0.01*)	5.40 < y < 6.32
Average (±stdev)	70 (*±4*) < y < 82 (*±5*)	4.41 (*±0.26*)	5.39 (*±0.01*)< y < 6.29 (*±0.03*)
$LSPC_{LF}^{3R}$	85 < x < 99	5.33 (*±0.01*)	5.39 < y < 6.24
$LSPC_{LF}^{6R}$	82 < x < 95	5.13 l*±0.01*)	5.38 < y < 6.23
Average (±stdev)	84 (*±2*) < x < 97 (*±2*)	5.23 (*±0.14*)	5.39 (*±0.01*)<y<6.23 (*±0.01*)

[a] LF: field leaves, LG: greenhouse leaves, LSPC: protein concentrates
[b] Proteinaceous nitrogen (NAA) as proportion (%) of total nitrogen (NT)
[c] Lower and upper limits represent values calculated with ASX/GLX = 100% ASP/GLU and ASX/GLX = 100% ASN/GLN, respectively
[d] k_p as the average value of k_p calculated with ASX/GLX = 100% ASP/GLU and with ASX/GLX = 100% ASN/GLN
[e] Lower and upper limits represent values calculated with ASX/GLX = 100% ASN/GLN and with ASX/GLX = 100% ASP/GLU, respectively

The total carbohydrate content in LF Arrival was higher in LF_{3A} than in LF_{8A} (31.4 ± 0.9 vs 27.3 ± 0.4% w/w DM), whereas in LF Rhino the carbohydrate content did not differ considerably between the two plant ages (**Table 1**). For the LG an increase from 18.0 ± 1.3% (for 2 months old plants) to 28.2 ± 3.1% w/w DM (for 3-8 months old plants) was observed (**Figure 1**). The main monosaccharides present in all leaves after hydrolysis were glucose and uronic acids (**Table 4**), which are building blocks of cellulose and pectins, respectively.

Table 4 Constituent monosaccharide composition (mol%) of field (LF) and greenhouse (LG) leaves

Sample	Arabinose	Rhamnose	Galactose	Glucose	Xylose	Mannose	Uronic acid
LF_{3R}	17	0	11	38	4	0	29
LF_{6R}	18	0	10	43	3	0	26
LF_{3A}	22	0	5	46	3	0	23
LF_{8A}	22	0	8	44	3	0	23
LG_{2I}	12	0	7	33	4	0	43
LG_{3I}	15	0	4	35	5	0	41
LG_{4I}	15	0	5	36	5	0	39
LG_{5I}	16	0	6	42	5	0	30
LG_{6I}	18	0	6	41	5	0	30
LG_{7I}	19	0	6	36	5	0	34
LG_{8I}	18	0	6	37	5	0	34

The overall protein composition was similar for the leaves of both plant ages (both Rhino and Arrival), as shown by SDS-PAGE under reducing conditions (**Figure 2A**). The most distinctive bands were observed around 50 and 12 kDa, which are indicative of the large and small Rubisco subunits, respectively [28, 29]. In addition two less intense bands were observed at around 37 and 25 kDa, which are indicative of respectively the photosystem II protein D1 (PSII) [30] and to the chlorophyll *a*/*b* binding protein that have been identified in *B. vulgaris* species [31]. In the greenhouse leaves the most distinctive bands were observed around 50 and 25 kDa, whereas no bands were observed at 39 and 12 kDa (with the exception of LG_4) (**Figure 2A**).

For both LF Rhino and LF Arrival, the phenolic compounds content was on average 1.7 times higher in leaves obtained from old than in leaves from young plants (**Table 1**). This is line with previous research [5]. Similar to LF and to literature [5], the phenolic compounds content in leaves from older plants (LG_{3I}-LG_{6I}) was on average 2 times higher than that measured in leaves from younger plants (LG_{2I}) (**Figure 1**). Interestingly, it was observed that with further increase in plant age (leaves from 7-8 months old plants) the phenolic compounds analysed content decreased to the initial value measured, i.e. in leaves from 2 months old plants (**Figure 1**).

The ash content did not differ between the two plant ages, neither for LF Rhino nor for LF Arrival (**Table 1**). For the LG an increase in the ash content from 18.7 ± 0.2% to 23.6 ± 0.2% w/w DM was observed as the plant age increased (**Figure 1**).

For both LF Rhino and LF Arrival, the lipid and lignin contents increased with plant age (with 90 and 54% for lipids and with 58 and 22% for lignin, respectively). The chlorophyll content did not differ

between the two plant ages for LF Arrival, whereas an increase from 0.3 ± 0.1% in LF_{3R} to 0.8 ± 0.2% in LF_{6R} (**Table 1**) was observed, for LF Rhino.

In total, 71-81% of the total dry matter in the LF samples was annotated (**Table 1**). This incomplete mass balance cannot be explained. It should be noted that such incomplete analysis is often observed in literature [1, 32], and it should be taken into account when explaining variations in chemical composition among different samples.

Nitrogen recovery

The nitrogen that was extracted in the juice; i.e. the nitrogen extractability, of LF Rhino was quite constant for both plant ages (53.0 ± 5.0 vs 45.6 ± 2.9% for LF_{3R} and LF_{6R}, respectively, **Table 5**). In contrast, for LF Arrival a 39% decrease in the nitrogen extractability was observed as the plant age increased from 3 to 8 months (**Table 5A**). For the LG samples a decrease of even 50% was observed as the plant age increased from 2 to 5 months (**Table 5B**). It must be noted that the absolute age of the plants grown in the field and in the greenhouse may perhaps not represent exactly the same developmental stage of the plant, given the fact that based on previous observations at Unifarm the growth of the sugar beets; i.e. time until harvest, in a greenhouse was faster than in a field. For the LG samples, it was observed that the nitrogen extractability increased again at higher plant age. Other researchers have also reported that the nitrogen extractability (nitrogen recovered in the juice/nitrogen in the pulp) from bean leaves initially decreased with plant age and subsequently increased [33]. For other species, e.g. lucerne, the nitrogen extractability was shown to be constant (on average 78 ± 5%) with age, whereas for sunflower leaves the nitrogen extractability showed a sharp decrease from 62% (leaves collected in July) to 22% (leaves collected in September) [33]. Overall, it was shown that nitrogen extractability from leaves of different species changes with age, but not always in the same way [33]. In this study, we also show that nitrogen extractability changes with age, even within the same species. Despite the variation in the nitrogen extractability, the nitrogen recovery in the dialysed juice was quite constant with plant age (**Table 5**). This shows that, given the fact that the low molecular N-containing compounds were removed after dialysis, the protein recovery was quite constant with plant age. The nitrogen recovery in the subsequent stage (LSPC) was also quite constant with plant age, with the exception of LF Rhino for which the nitrogen recovery in $LSPC_{LF}^{6R}$ was 13% higher than in $LSPC_{LF}^{3R}$ (**Table 5A**).

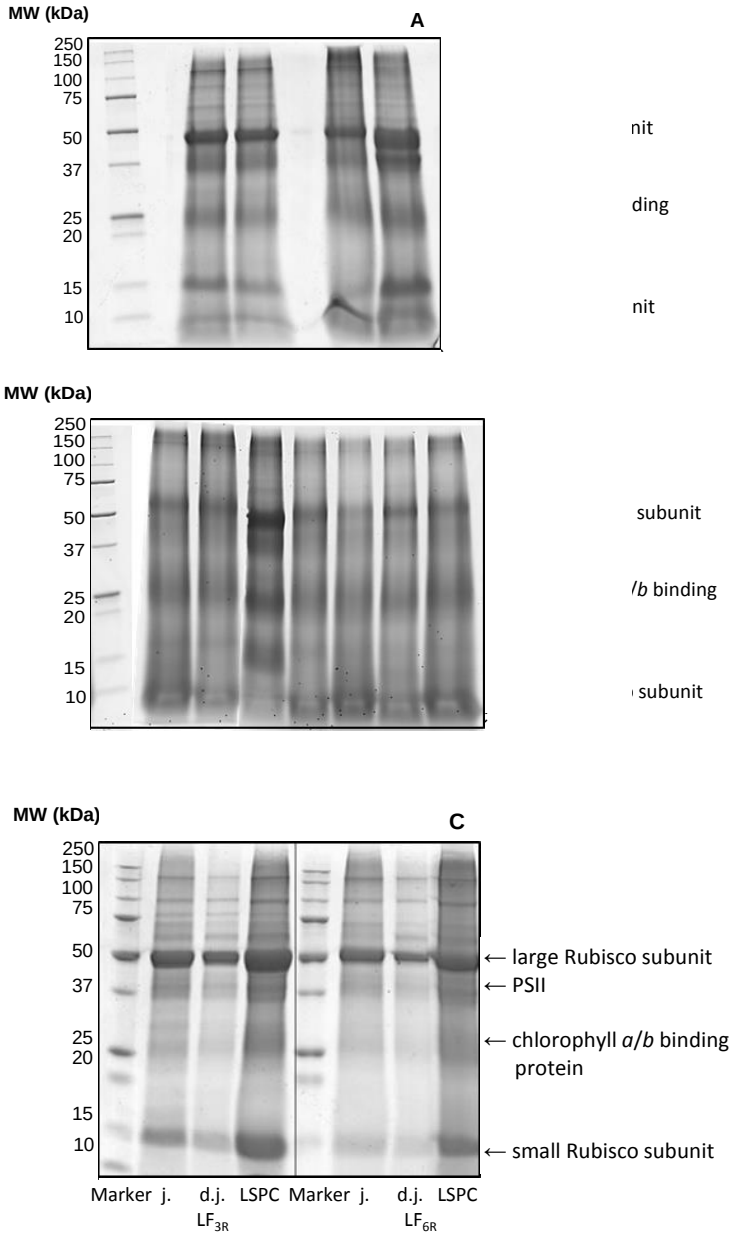

Figure 2 SDS-PAGE gels stained by coomassie blue; (**A**) leaves from field (**B**) leaves from greenhouse (C) j. = juice, d.j. = dialysed juice, LSPC = leaf soluble protein concentrates extracted from the field (Rhino) leaves

Table 5 Nitrogen recovery (%) at each processing step in field (A) and greenhouse (B) leaves

A	Recovered in:	LF_{3R}[a]	LF_{6R}[a,b]	LF_{3A}[a]	LG_{8A}[a]
Extraction	Juice	53.0 (±5.0)	45.6 (±2.9)	48.3 (±1.4)	29.3 (±3.8)
Isolation	Dialysed juice	62.2 (±3.5)	63.9 (±3.2)	59.9 (±6.1)	57.7 (±2.9)
	LSPC	44.9 (±1.7)	50.9 (±0.4)	41.6 (±1.7)	42.8 (±4.3)

B	Recovered in:	LG_{2I}[c]	LC_{3I}[c]	LG_{4I}[c]	LG_{5I}[d]	LG_{6I}[c]	LG_{7I}[c]	LG_{8I}[c]
Extraction	Juice	55.6 (±0.3)	44.4 (±4.2)	41.1 (±5.7)	27.7	40.1 (±7.4)	46.1 (±6.5)	46.1 (±6.0)
Isolation	Dialysed juice	44.8 (±6.0)	55.5 (±6.1)	45.0 (±3.6)	44.4	52.3 (±2.3)	59.5 (±1.4)	62.1 (±5.5)
	LSPC	38.6 (±7.0)	45.2 (±7.1)	34.7 (±9.7)	34.4	43.5 (±1.8)	45.6 (±4.2)	n.d.[e]

[a] Average (±stdev) of two repeated extractions in the same year
[b] Similar values were also obtained when proteins were extracted in the presence of disulfite
[c] Average (±stdev) of two repeated extractions in two consecutive years
[d] Extraction done once
[e] Not determined

Chemical composition of the protein concentrates obtained from field leaves

In protein concentrates obtained from LF of both varieties the protein contents did not differ between the two plant ages (Table 1). The k_p factors calculated for $LSPC_{LF}^{3R}$ and $LSPC_{LF}^{6R}$ were quite similar; on average 5.23 ± 0.14 (**Table 3**). The k_a factors calculated for these samples ranged from 5.39 ± 0.01 to 6.23 ± 0.01 for both $LSPC_{LF}^{3R}$ and $LSPC_{LF}^{6R}$ (**Table 3**). It was observed that the k_a factor for the protein concentrates (LSPC) was 3-19% higher than the respective k_p factor, whereas for the leaves (LF) this difference was higher (22-43%), as discussed previously. This indicates that the isolation method leading to the concentrates indeed led to removal of NPN.

The total carbohydrate content in $LSPC_{LF}^{8A}$ was higher than in $LSPC_{LF}^{3A}$ (5.1 ± 0.4 vs 3.7 ± 0.2% w/w DM), whereas for LF Rhino the carbohydrate content was similar in protein concentrates from both plant ages (on average 3.5 ± 0.5% w/w DM) (**Table 1**).

In protein concentrates obtained from LF Rhino the lipid content was higher for leaves from young than for leaves from old plants (**Table 1**). For protein concentrates obtained from LF Arrival the opposite was the case (**Table 1**). The ash content in $LSPC_{LF}^{8A}$ was higher than in $LSPC_{LF}^{3A}$, whereas in protein concentrates from LF Rhino the ash content was the same for both plant ages (**Table 1**).

Protein composition of the protein concentrates obtained from field leaves

The overall protein composition was similar for the protein concentrates (from LF Rhino) of both plant ages, as shown by SDS-PAGE under reducing conditions (**Figure 2**). For all samples (juice, dialysed juice, protein concentrate) the most distinctive bands were observed around 50 and 12 kDa (large and small subunits of Rubisco, respectively [28, 29]). This suggests that the isolation method

did not lead to loss of Rubisco. The band at around 25 kDa present in leaves was not apparent in this case. This indicates that the isolation method was adequate for the removal of the green colour.

PPO-mediated browning in extracts of field and greenhouse leaves from old plants

A striking observation was that the juice obtained from leaves of old sugar beets (LF_{6R}) was brown, whereas the juice from leaves of young sugar beets (LF_{3R}) was yellow (**Figure 3A**, insert). The same effect was observed for LF Arrival and for LG (**Figures 3A**, **B** inserts).

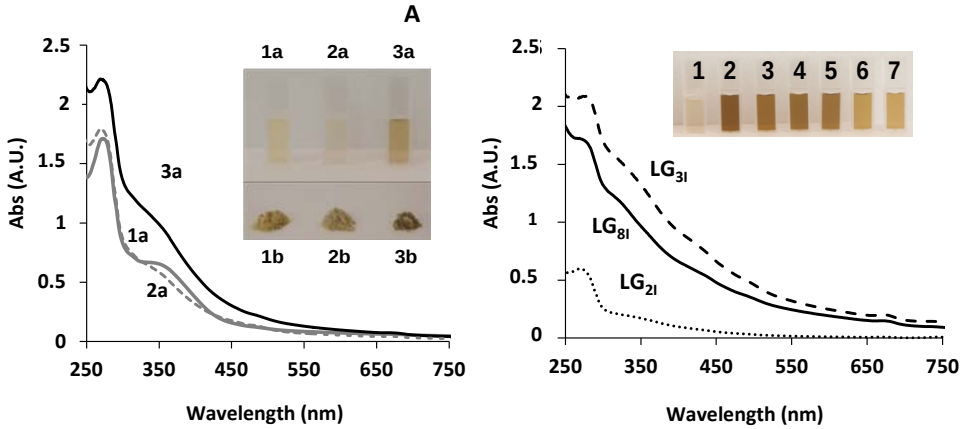

Figure 3 UV-vis spectra of the dialysed juices obtained from the field leaves. Insert: cuvettes containing dialysed juice from: LF_{3R} (1a), $LF_{6R,\ SO3}$ (2a), LF_{6R} (3a) and protein concentrates: $LSPC_{LF}^{3R}$ (1b), $LSPC_{LF}^{6R,SP3}$ (2b), $LSPC_{LF}^{6R}$ (3b) (A), UV-vis spectra of the dialysed juices obtained from the greenhouse leaves. Insert: cuvettes containing dialysed juice from 1-7: LG_{2I} to LG_{8I} (B)

The brown colour observed in these juices was still present in the high molecular mass (> 12 kDa) fraction after dialysis and acid precipitation (**Figure 3A**, insert). Hence, it is stated that most of the brown colour observed was associated with high molecular weight compounds. In another study, it was shown that the brown colour observed during protein extraction from potato tubers was due to oxidised phenolics [34]. In the same study, it also was shown that most of the oxidized phenolics were associated with high molecular weight compounds, which is in line with our observation. Therefore, it was hypothesised that the brown compounds observed in our study were formed by quinones, which were produced via PPO-mediated oxidation of the phenolic compounds. To test whether PPO activity (catecholase/cresolase activity) was indeed the reason for the brown colour formation in the juices from the old plants, juice from LF_{6R} was prepared in the presence of sulfite. Sulfite is known to prevent enzymatic browning, by inactivating the PPO and/or by formation of sulfo-adducts that do not contribute to browning [24]. Indeed, in the presence of sulfite, the juice from the leaves of old plants (LF_{6R}, SO_3) was not brown (**Figure 3**), which indicates that the PPO activity was responsible for the brown colour formation. The PPO activity was determined in the dialysed juice of LF_{6R}, SO_3 and surprisingly, after the removal of sulfite, the PPOs were still active (**Figures 4A**, **C**). It has been shown previously that when chlorogenic acid was incubated with

tyrosinase and sulfite, the enzyme was irreversible inactivated, while no colour development was observed [35]. As discussed before, sulfite can lead to the formation of sulfo-adducts that do not contribute to browning. Hence. the latter is suggested to be the case in the present study.

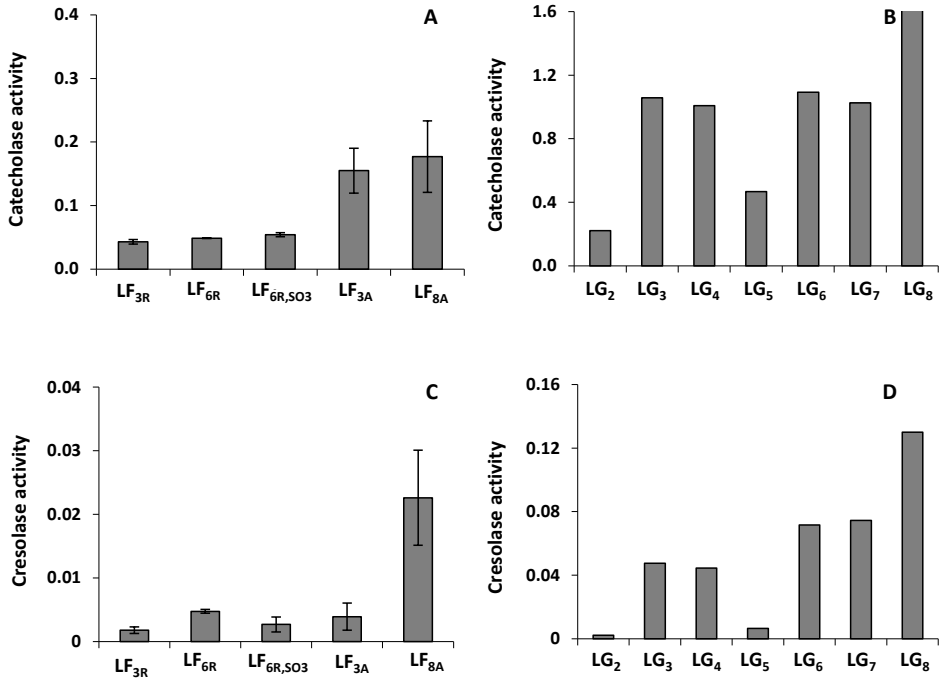

Figure 4 Catecholase (on catechin) (AU·min^{-1}·(mg N)$^{-1}$) (A, B) and cresolase (on tyrosine) (AU·min^{-1}·(mg N)$^{-1}$) (C, D) activity measured in the dialysed juice obtained from the field (A, B) and the greenhouse (B, D) leaves

Given the fact that the brown colour formation in the (dialysed) juices from the leaves of the old plants was attributed to PPO activity, the lack of brown colour formation in the (dialysed) juice of the leaves from young plants was consequently thought to be due to absence of PPO activity. Surprisingly, the catecholase activity measured in the dialysed juice from leaves of young plants was similar to that in the dialysed juice of leaves from old plants (**Figure 4A**). The cresolase activity was higher in the dialysed juice of leaves from old plants than in that of leaves from young plants (0.005 AU·min^{-1}·(mg N)$^{-1}$ in LF_{6R} vs 0.002 AU·min^{-1}·(mg N)$^{-1}$ in LF_{3R} and 0.023 AU·min^{-1}·(mg N)$^{-1}$ in LF_{8A} vs 0.004 AU·min^{-1}·(mg N)$^{-1}$ in LF_{3A}) (**Figure 4C**). In the LG dialysed juices both the catecholase and the cresolase activity were higher in leaves from old plants than in leaves from young plants (**Figures 4B**, **D**). The values determined for LG_{5I} (0.47 AU·min-1·(mg N)$^{-1}$ and 0.007 AU·min^{-1}·(mg N)$^{-1}$, for catecholase and cresolase activity, respectively) deviated very strongly from the values determined for the other samples (LG_{3I}-LG_{4I} and LG_{6I}-LG_{8I}) and were, therefore, not included in further considerations. Interestingly, the cresolase activity measured in LF_{3A} (yellow juice and dialysed juice) was similar to the cresolase activity measured in LF_{6R} (brown juice and dialysed juice) (**Figure 4C**). This indicates that the cresolase activity itself does not explain the colour difference between the

juices of leaves from old and young plants. Furthermore, the catecholase activity in LG_{2I} dialysed juice (yellow juice and dialysed juice) was 1.2-4.4 times higher than the catecholase activity in the brown juices and dialysed juices obtained from the field leaves. Thus, the PPO activity (cresolase and/or catecholase) per se, as determined with the typical methods used throughout literature, cannot explain the striking difference in colour between the (dialysed) juices and the LSPC from young and old plants.

Overall, it is concluded that the age of the sugar beets does not affect the protein content in the whole foliage. The nitrogen extractability varies with plant age, although no consistent correlation with plant age is found. The nitrogen recovery in the subsequent isolation stages is independent of plant age. Thus, the variation in the final yield of protein extraction is mostly due to the variation in nitrogen extractability. A significant effect of the plant age is observed on the quality (colour) of the protein extracted from the sugar beet leaves; i.e. the protein extracted from leaves of older plants is brown, whereas the protein extracted from leaves from young plants is yellow.

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References

1. Edwards, R.H., R.E. Miller, D. De Fremery, B.E. Knuckles, E.M. Bickoff, and G.O. Kohler, Pilot plant production of an edible white fraction leaf protein concentrate from alfalfa. *J. Agric. Food Chem.*, **1975**. 23(4): 620-626.
2. Makkar, H.P.S. and K. Becker, Nutrional value and antinutritional components of whole and ethanol extracted *Moringa oleifera* leaves. *Anim. Feed Sci. Technol.*, **1996**. 63(1): 211-228.
3. Betschart, A. and J.E. Kinsella, Extractability and solubility of leaf protein. *J. Agric. Food Chem.*, **1973**. 21(1): 60-65.
4. SuikerUnie. **2016**; http://www.suikerunie.nl/Sustainability-number-of-beet-growers.aspx.
5. Biondo, P.B.F., J.S. Boeing, É.O. Barizão, N.E. de Souza, M. Matsushita, C.C. de Oliveira, M. Boroski, and J.V. Visentainer, Evaluation of beetroot (*Beta vulgaris L.*) leaves during its developmental stages: A chemical composition study. *Food Sci. Technol.*, **2014**. 34(1): 94-101.
6. Byers, M. and J.W. Sturrock, The yields of leaf protein extracted by large-scale processing of various crops. *J. Sci. Food Agric.*, **1965**. 16(6): 341-355.
7. Nordkvist, E. and P. Åman, Changes during growth in anatomical and chemical composition and *in-vitro* degradability of lucerne. *J. Sci. Food Agric.*, **1986**. 37(1): 1-7.
8. Tambari, U., B. Aliero, S. Muhammad, and L. Hassan, Interaction effect of season, habitat and leaf age on proximate composition of *Senna occidentalis* and *senna obtusifolia* leaves grown in Fadama and Upland locations in Sokoto, Nigeria. *Nig. J. Basic Appl. Sci.*, **2015**. 23(1): 39-44.
9. Addy, T.O., L.F. Whitney, and C.S. Chen, Mechanical parameters in leaf cell rupture for protein production, *in Leaf Protein Concentrates*, L. Telek, H. Graham, and H.D. Graham, Editors. **1983**, AVI Publishing: Westport, CT, USA.
10. Rommi, K., T.K. Hakala, U. Holopainen, E. Nordlund, K. Poutanen, and R. Lantto, Effect of enzyme-aided cell wall disintegration on protein extractability from intact and dehulled rapeseed (*Brassica rapa L.* and *Brassica napus L.*) press cakes. *J. Agric. Food Chem.*, **2014**. 62(32): 7989-7997.

11. Prigent, S.V.E., A.G.J. Voragen, A.J.W.G. Visser, G.A. Van Koningsveld, and H. Gruppen, Covalent interactions between proteins and oxidation products of caffeoylquinic acid (chlorogenic acid). *J. Sci. Food Agric.*, **2007**. 87(13): 2502-2510.
12. Pourcel, L., J.M. Routaboul, V. Cheynier, L. Lepiniec, and I. Debeaujon, Flavonoid oxidation in plants: from biochemical properties to physiological functions. *Trends Plant Sci.*, **2007**. 12(1): 29-36.
13. Cheynier, V., C. Owe, and J. Rigaud, Oxidation of grape juice phenolic compounds in model solutions. *J. Food Sci.*, **1988**. 53(6): 1729-1732.
14. Chutichudet, B., P. Chutichudet, and S. Kaewsit, Influence of developmental stage on activities of polyphenol oxidase, internal characteristics and colour of lettuce cv. *Grand Rapids. Am. J. Food Technol.*, **2011**. 6(3): 215-225.
15. 13903:2005, I., Animal feeding stuff - Determination of amino acids content. International organisation for standardisation: Geneva, Switzerland.
16. Mossé, J., Nitrogen to protein conversion factor for ten cereals and six legumes or oilseeds. A reappraisal of its definition and determination. Variation according to species and to seed protein content. *J. Agric. Food Chem.*, **1990**. 38(1): 18-24.
17. Murciano Martínez, P., R. Bakker, P. Harmsen, H. Gruppen, and M. Kabel, Importance of acid or alkali concentration on the removal of xylan and lignin for enzymatic cellulose hydrolysis. *Ind. Crops Prod.*, **2015**. 64: 88-96.
18. Jurak, E., M.A. Kabel, and H. Gruppen, Carbohydrate composition of compost during composting and mycelium growth of *Agaricus bisporus*. *Carbohydr. Polym.*, **2014**. 101(1): 281-288.
19. Narváez-Cuenca, C.E., J.-P. Vincken, C. Zheng, and H. Gruppen, Diversity of (dihydro) hydroxycinnamic acid conjugates in Colombian potato tubers. *Food Chem.*, **2013**. 139(1-4): 1087-1097.
20. Slinkard, K. and V.L. Singleton, Total phenol analysis: Automation and comparison with manual methods. *Am. J. Enol. Vitic.*, **1977**. 28(1): 49-55.
21. Folch, J., M. Lees, and G.H. Sloane Stanley, A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.*, **1957**. 226(1): 497-509.
22. Kabel, M.A., G. Bos, J. Zeevalking, A.G.J. Voragen, and H.A. Schols, Effect of pretreatment severity on xylan solubility and enzymatic breakdown of the remaining cellulose from wheat straw. *Bioresour. Technol.*, **2007**. 98(10): 2034-2042.
23. Jeffrey, S.W. and G.F. Humphrey, New spectrophotometric equations for determining chlorophylls *a*, *b*, *c1* and *c2* in higher plants, algae and natural phytoplankton. *Biochem. Physiol. Pflanz.*, **1975**. 167: 191-194.
24. Kuijpers, T.F.M., C.-E. Narváez-Cuenca, J.-P. Vincken, A.J.W. Verloop, W.J.H. van Berkel, and H. Gruppen, Inhibition of enzymatic browning of chlorogenic acid by sulfur-containing compounds. *J. Agric. Food Chem.*, **2012**. 60(13): 3507-3514.
25. Gowda, L.R. and B. Paul, Diphenol activation of the monophenolase and diphenolase activities of field bean (*Dolichos lablab*) polyphenol oxidase. *J. Agric. Food Chem.*, **2002**. 50(6): 1608-14.
26. Gupta, A.J., H. Gruppen, D. Maes, J.-W. Boots, and P.A. Wierenga, Factors causing compositional changes in soy protein hydrolysates and effects on cell culture functionality. *J. Agric. Food Chem.*, **2013**. 61(45): 10613-10625.
27. Milton, K. and F.R. Dintzis, Nitrogen-to-protein conversion factors for tropical plant samples. *Biotropica*, **1981**. 13(3): 177-181.
28. Uniprot, Ribulose biphosphate carboxylase large chain - *Beta vulgaris*.
29. Uniprot, Ribulose bisphosphate carboxylase small chain - *Beta vulgaris*.
30. Uniprot, Photosystem II protein D1 - *Beta vulgaris*.
31. Uniprot, Chlorophyll a-b binding protein, chloroplastic - *Beta vulgaris*.
32. Tamayo Tenorio, A., J. Gieteling, G.A. de Jong, R.M. Boom, and A.J. van der Goot, Recovery of protein from green leaves: Overview of crucial steps for utilisation. *Food Chem.*, **2016**. 203: 402-8.
33. Butler, J.B., An investigation into some causes of the differences of protein expressibility from leaf pulps. *J. Sci. Food Agric.*, **1982**. 33(6): 528-536.
34. Narvaez-Cuenca, C.E., J.-P. Vincken, and H. Gruppen, Quantitative fate of chlorogenic acid during enzymatic browning of potato juice. *J. Agric. Food. Chem.*, **2013**. 61(7): 1563-72.
35. Kuijpers, T.F., H. Gruppen, S. Sforza, W.J. van Berkel, and J.-P. Vincken, The antibrowning agent sulfite inactivates *Agaricus bisporus* tyrosinase through covalent modification of the copper-B site. *FEBS J.*, **2013**. 280(23): 6184-95.

Chapter 3

The content of caffeic acid derivatives, oxidative coupling and coupled oxidation drive enzymatic browning in sugar beet (*Beta vulgaris* L.) leaves

Sugar beet (*Beta vulgaris* L.) leaves of 8 month (8_m) plants showed more enzymatic browning than those of 3 month (3_m). Total phenolic content increased from 4.6 to 9.4 mg/g FW in 3_m and 8_m, respectively, quantified by RP-UHPLC-UV-MS. The PPO activity was 6.7 times higher in extracts from 8_m than from 3_m leaves. Substrate content increased from 0.5 to 2.4 mg/g FW in 3_m and 8_m, respectively, of which caffeic acid glycosyl-esters were most important, increasing 10 fold with age. Caffeic acid glycosides and vitexin derivatives were no substrates. In 3_m and 8_m, non-substrate-to-substrate ratios were 8:1 and 3:1, respectively. A model system showed browning at 3:1 ratio due to formation of products with extensive conjugated systems through oxidative coupling and coupled oxidation. The 8:1 ratio did not turn brown as oxidative coupling occurred without much coupled oxidation. We postulate that differences in non-substrate-to-substrate ratio and therewith extent of coupled oxidation explain browning.

Based on: Anne Vissers, Alexandra Kiskini, Roelant Hilgers, Marina Marinea, Peter Alexander Wierenga, Harry Gruppen, Jean-Paul Vincken – *Submitted to J. Agric. Food Chem.*

Introduction

For future protein demand, sugar beet leaves (*Beta vulgaris* L.) seem an attractive new protein source [1]. For the extraction of proteins present, cell rupture is needed [2]. Cell rupture brings polyphenol oxidases (PPOs) stored within chloroplasts and phenolics stored within vacuoles together in the presence of oxygen [2]. In such situations, PPOs oxidise phenolics into highly reactive quinones, which engage in browning reactions. Also, these quinones are prone to nucleophilic attacks by e.g. cysteine and lysine residues, resulting in covalent binding to proteins [3]. These reactions affect the final techno-functional properties of the proteins extracted [4]. Often, PPO activity varies with plant age, environmental influences and activation of latent enzymes in plants [5, 6]. Currently, there is limited information on the PPO activity in *Beta vulgaris* species [1].

Beta vulgaris belongs to the family of *Amaranthaceae*. Phenolics found in this family can be divided into phenolic acids and flavonoids. Phenolic acids (e.g. hydroxybenzoic and hydroxycinnamic acids), together with the flavonoid subclasses flavonols (e.g. quercetins) and flavones (e.g. vitexins) have been reported in *Beta vulgaris* beets and leaves [7, 8]. Similar to PPO activity, the content of total phenolics in leaves has been reported to vary with plant age. In lettuce, the total phenolic content decreased by 88% from 28 to 59 days after planting, followed by an increase to the starting content 73 days after planting. The increase in total phenolic content coincided with increases in PPO activity and browning [5]. To our knowledge, no investigations on the relation between the phenolic composition and extent of brown colouring have been performed.

Plants contain reducing agents that can, in principle, delay enzymatic browning. Of these, ascorbic acid (AA) and glutathione (GSH) are the most abundant [9]. These molecules can reduce the enzymatically formed quinones back into *o*-diphenolics, upon which AA and GSH are converted into dehydro-ascorbic acid (DHA) and glutathione disulfide (GSSG), respectively. During plant growth and development, changes in AA and GSH contents have been reported [5, 10]. The content of AA and GSH may affect the extent of enzymatic browning.

Earlier studies in our laboratory [1] showed that aqueous extracts made from leaves of 3-month old sugar beet plants had a yellow colour, whereas similar aqueous extracts made from leaves of 8-month old plants were brown. The observations did not depend on year of harvest or growing condition (field or greenhouse). Neither the twofold increase in PPO activity, measured as cresolase (hydroxylation of monophenolics into *o*-diphenolics) and catecholase (oxidation of *o*-diphenolics into *o*-quinones) activities, nor the twofold increase in total phenolic content could satisfactorily explain the differences in colour between the two plant ages [1]. Hence, the main aim of this research was to find explanations for the difference in colour formation upon cell rupture in sugar beet (*Beta vulgaris* L.) leaves harvested after 3 and 8 months. It was hypothesised that the composition of individual phenolics changes over time, which might lead to increased quantities of mono- and *o*-diphenolic substrates in leaves of older plants.

In this research, the composition of substrate phenolics was determined in leaves from 3- and 8-month old plants. Subsequently, the oxidative enzyme activity towards various classes of endogenous phenolic compounds was measured. In addition, the reaction products were analysed.

Materials and Methods

Chemicals

UHPLC-grade water and acetonitrile were obtained from Biosolve (Valkenswaard, The Netherlands). Vitexin was obtained from Rotichrom® TLC (Karlsruhe, Germany). All other chemicals and extraction solvents used were of analytical grade and purchased from either Merck (Darmstadt, Germany) or Sigma–Aldrich (St. Louis, MO, USA).

Sugar beet leaves harvesting and storage

The leaves of sugar beets (*Beta vulgaris* L. var. Arrival) harvested in 2014, 3 (3_m) and 8 months (8_m) after sowing the seeds, and used in our previous research [1], were used. After collecting the leaves, one part was stored in vacuum sealed bags at -20 °C. The other part of the leaves was freeze dried, ground into powder in an ultracentrifugal mill (Retch ZM 200, Haan, Germany) at 6,000 rpm. The freeze dried powders were stored at -20 °C. Dry matter contents of the leaves were determined gravimetrically after drying overnight at 105 °C. Dry matter contents were 10.6% (±0.4) and 15.8% (±0.2) w/w fresh weight for 3_m and 8_m, respectively.

Preparation of the phenolics extracts

Phenolics were extracted (w/v ratio 1:10) from freeze dried leaves with 50% (v/v) methanol containing 0.5% (v/v) acetic acid using four consecutive extractions. During each extraction, samples were first sonicated in an Elmasonic S40H sonicator (Elmasonic, Zwolle, The Netherlands) for 5 min and then stirred for 30 min at 4 °C in darkness. After centrifugation (4 °C, 5 min, 5,000 $\times$ *g*), fresh solvent was added to the pellet, after which the procedure was repeated. The four extracts were combined and methanol was removed by evaporation under reduced pressure at 40 °C. The aqueous residue was freeze dried and used for further analyses. For each plant age, samples were extracted in triplicate and analysed separately.

Fractionation of phenolics

One gram of freeze-dried phenolic extract was fractionated using a Flash chromatography system (Grace, Deerfield, IL, USA) equipped with a 40 g Reveleris C18 Flash cartridge (45 mL column volume (CV), particle size 40 µm, Grace). Elution of phenolic compounds (30 mL/min) was performed using milliQ water containing 1% (v/v) acetic acid (eluent A) and with acetonitrile containing 1% (v/v) acetic acid (eluent B). The cartridge was equilibrated with 3 CVs eluent B, followed by 3 CVs eluent A. Elution conditions were as follows: 0-2 min, 0% B; 2-10 min, 0-60 % B; 10-11 min, 60-100% B; 11-13 min, 100% B. Fractions (10 mL) were collected and pooled into 4 pools based on ELS and/or A_{280} nm responses. Pools 1-4 were taken between 0-3.5 min, 3.5-5.5 min, 5.5-8 min and 8-13 min, respectively. Pools 2 and 3 were used for further analyses. Acetonitrile was evaporated from the pools under reduced pressure at 40 °C. The remaining aqueous phase was freeze-dried and stored at -20 °C prior to further analyses. For both 3_m and 8_m, two fractionation runs were performed.

Annotation and quantification of phenolics

The phenolic fractions were analysed using UHPLC-UV-MS on an Accela system (Thermo Scientific, San Jose, CA, USA) equipped with a pump, degasser, auto-sampler (set at 15 °C) and photodiode

array detector. Samples (10 µL, 1 g/L in 50% (v/v) aqueous methanol) were injected onto a Hypersil Gold aQ column (Thermo Scientific; 150 x 2.1 mm i.d.; particle size 1.9 µm). The column oven temperature was set at 30 °C. Elution of phenolic compounds (400 µL/min) was performed using water containing 1% (v/v) acetic acid (eluent A) and acetonitrile containing 1% (v/v) acetic acid (eluent B). The elution profile for the pool enriched in phenolic acids (pool 2) was as follows: 0-4 min, 1% B; 4-16 min, 1-22% B; 16-18 min, 22-100% B; 18-20 min, 100% B; 20-21 min, 100-1% B; 21-24 min, 1% B. The elution profile for the pool enriched in flavonoids (pool 3) was as follows: 0-4 min, 1% B; 4-8 min, 1-17% B; 8-25 min, 17-30% B, 25-27 min, 30-100% B; 27-29 min, 100% B; 29-30 min, 100-1% B; 30-32 min, 1% B. For both pools, throughout elution, PDA spectra (200-600 nm) were recorded.

Mass spectrometric (MS^n) data were obtained by analysing samples on a LTQ-XL Velos mass spectrometer (Thermo Scientific) equipped with a heated electrospray ionisation probe coupled to the UHPLC system. The instrument was tuned with caffeic acid for pool 2 and with apigenin for pool 3, with capillary temperature at 350 °C, source heater temperature at 230 °C, and source voltage at 3.5 kV. A full mass spectrum was recorded over an *m/z* range of 150-1500. The annotation of compounds was performed based on the parent molecular ion, MS^n data and UV-vis spectra. Data acquisition and reprocessing were performed with Xcalibur 2.07 software (Thermo Scientific). For quantification of phenolics, external calibration curves were used. Quantities of phenolic acids were expressed as caffeic acid equivalents as 42% w/w of the phenolic acid derivatives found contained a caffeic acid backbone. Quantities of flavonoids were expressed as vitexin (IUPAC: (1*S*)-1,5-anhydro-1-[5,7-dihydroxy-2(4-hydroxyphenyl)-4-oxo-4H-chromen-8-yl]-D-glucitol) equivalents as 78% w/w of the flavonoids found were vitexin derivatives. For both compounds, 7 concentrations were taken for calibration, based on full MS intensity and measured in duplicate. For caffeic acid: 1.15-115.0 mg/L, R^2 0.91, limit of quantification (LOQ) was 0.3 mg/L. For vitexin: 0.1-13.5 µg/mL, R^2 0.97, LOQ was 0.05 mg/L. For quantification of individual compounds, correction factors were determined by dividing the molecular weight of the analyte by that of the calibrant (**Table 1**). The MS response was multiplied by the correction factor to obtain the amounts present [11]. Amino acid derivatives were quantified using caffeic acid as the standard. The phenolic contents in the leaves were expressed in mg/g fresh weight (FW).

Phenolics identification strategy

Phenolics were identified using UV-vis spectra and mass spectra. Phenolic acids show absorbance around 280 nm and often a shoulder around 320-355 nm [12]. Flavonoids show absorbance around both 265 and 350 nm [12]. Diagnostic ion screening allowed annotation of hydroxycinnamic acid [13, 14] and flavonoid [7, 15] backbones. The presence of phenolic derivatives was determined by the presence of characteristic neutral losses upon MS^2 and MS^3 fragmentation. Phenolic acid-glycosides (**Figure 1A**) and flavonoid *O*-glycosides were annotated by a neutral loss of 162 (*O*-hexose) or 132 (*O*-pentose) [16]. Glycosylation in phenolic acid glycosyl-esters (**Figure 1B**) was annotated by a neutral loss of 162 (*O*-hexose) [14, 16] and additional polyol fragment ions (-30, -60, -90) [13] in MS^2, originating from fragmentation within the glycosyl residue. *C*-glycosylation could be distinguished

from *O*-glycosylation by a neutral loss of 120 (*C*-hexose) [7, 16, 17]. Acetylation and malonylation were annotated by neutral losses of 42 and 86, respectively [16].

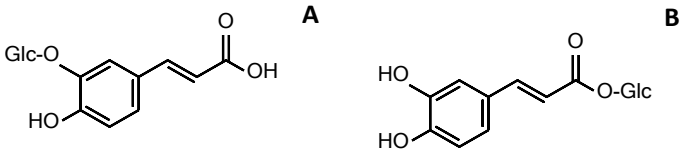


Figure 1 Structures of caffeic acid glycoside (A) and caffeic acid glycosyl-ester (B)

Preparation of the enzyme extracts

For enzyme extraction, 50 g frozen leaves from the 3 and 8 months old plants were used, extracted and described previously [1]. Dialysed enzyme extracts were used immediately for enzyme activity assays.

PPO activity assay and quantification of reacting phenolics

PPO activities in sugar beet leaves were determined by oxygen consumption. The oxygen consumption analyses were performed using an Oxytherm System (Hansatech, Kings Lynn, UK). Aliquots (700 µL) of dialysed enzyme extract were incubated with 200 µL (5 g/L) Flash-fractionated phenolics, 1.2 g/L catechin or 0.9 g/L vitexin. Substrates were dissolved in 150 mM sodium phosphate buffer pH 6.7. Oxygen consumption was monitored over 30 min. The rate of oxygen consumption during the first 10 s was taken as measure for PPO activity, expressed as nmole O_2/(s·g) FW.

Quantification of phenolics before and after reaction with leaf dialysed enzyme extracts was performed using RP-UHPLC-UV-MS, as described above. An aliquot (200 µL) of dialysed enzyme extract was incubated with 100 µL of Flash-fractionated phenolics (5 g/L), dissolved in 150 mM sodium phosphate buffer pH 6.7. After 20 h the reaction was stopped by addition of 300 µL methanol. A sample at t_0 was prepared in the same way to correct for trace amounts of phenolics present in the dialyzed enzyme extract. In this sample, methanol was added directly to prevent enzyme reaction.

Contributions of non-substrate phenolics to browning via oxidative coupling was investigated using a model system containing combinations of chlorogenic acid (ChA) and ferulic acid (FerA), ChA and sinapic acid (SinA), the three phenolic compounds combined, or each phenolic compound individually. Equimolar contents of phenolics ChA:SinA and ChA:FerA:SinA (1 mM) and different molar ratios of ChA:FerA (1:1, 1:3, 1:8) in 10 mM sodium phosphate buffer pH 6.7, were incubated with 15 U [18] mushroom tyrosinase for 30 and 60 min. Reaction products were diluted 10 times in 50% (v/v) aqueous methanol. The reaction mixtures with 1:1 molar ratios were analysed using RP-UHPLC-UV-MS as described above with an altered gradient: 0-1 min, 5% B; 1-9 min, 5-50% B;

9-10 min, 50-100% B; 10-12 min, 100% B, 12-13 min, 100-5% B; 13-15 min, 5% B. Injection volume was 5 µL. The UV-vis spectra of all reaction mixtures were recorded using a UV-1800 Shimadzu spectrophotometer and UV Probe 2.00 software (Shimadzu, Kyoto, Japan).

Ascorbic acid and GSH quantification

The method to quantify ascorbic acid (AA) and dehydro-ascorbic acid (DHA) in leaves was adapted from Davey *et al*. (2003) [9]. Freeze dried leaves (50 mg) ground in liquid nitrogen using a ball mill (Retch MM 4400, Haan, Germany) and 0.5 mm stainless steel beads. Next, 1.5 mL 3.3% (w/v) *meta*-phosphoric acid was added and the mixture was sonicated on ice for 10 min. The mixture was centrifuged (25,000 × *g,* 10 min, 4 °C) and the supernatant was filtered (0.45 µm cellulose filter, Whatman®, Dassel, Germany) prior to AA and GSH analysis. To convert DHA and GSSG into AA and GSH, respectively, 100 µL of the filtered supernatant was mixed with 5 mM DTT in 400 mM Tris base pH 9.0 and incubated at room temperature in the dark for 15 min. The reaction was stopped by addition of 50 µL 8.5% (w/v) *o*-phosphoric acid. Four samples of both ages were injected onto a ProntoSIL 120-3 C18 AQ (Knauer, Berlin, Germany) column and eluted isocratically with water containing 0.04% (v/v) H_3PO_4, 0.25% (v/v) methanol, and 0.1 mM EDTA. Flow rate was 0.35 mL/min and elution was followed by a wash step with 30% (v/v) aqueous acetonitrile. AA and GSH were detected at 243 nm and 197 nm, respectively. For quantification, external AA and GSH calibration curves were used (0-0.1 g/L in 3% (v/v) *m*-phosphoric acid containing 2.5 mM DTT, $R^2 = 0.98$ for both AA and GSH). The DHA and GSSG contents were determined by the differences in AA and GSH contents after and before DHA and GSH reduction, respectively.

Results

Annotation of phenolics

The phenolics composition was analysed in 3_m and $8_{m\ sugar}$ beet leaves. In order to facilitate annotation of phenolics, the crude extract was fractionated into 4 pools using RP-Flash chromatography. Pool 1 contained sugars, as annotated in LC-UV-MS; components eluted in the void of the column, and had no $A_{280\ nm}$ absorbance. During MS/MS fragmentation, characteristic neutral losses of -162 for sugars were observed. Pool 4 contained saponins, which had high column retention and no $A_{280\ nm}$ absorbance.

Pool 2 (**Figure 2A**, **Table 1**) consisted mainly of *O*-glycosylated derivatives of caffeic- (**2**, **4**, **6**), ferulic- (**20**, **23**, **26**-**28**) and sinapic acid (**21**, **24**). Glycosylated coumaric acids were not annotated. Trace amounts of dopamine (**3**, **5**, **8**-**10**, **16**) and tryptophan (**13**) derivatives were annotated. Pool 3 (**Figure 2B**) consisted mainly of iso-rhamnetin, quercetin and vitexin (8-*C*-glycosides of apigenin) derivatives. Vitexins have been previously identified in Swiss chard (*Beta vulgaris* subsp. *cycla*) [7], passiflora [17] and tea leaves [19]. The vitexin derivatives included those which contained an extended *C*-glycosyl residue: vitexin-2"-*O*-glycoside (**33**), vitexin-2"-*O*-pentoside (**34**), glycosyl-malonylvitexin (**38**) and pentosyl-malonylvitexin (**40**).

Based on the phenolic structure, PPO substrates were annotated (**Table 1**). Phenolics containing a free hydroxyl (e.g. coumaric acid) or a free *o*-dihydroxyl (e.g. caffeic acid) motif on the benzene ring could be considered as potential substrates. The presence of mono- or *o*-dihydroxyl motifs in a phenolic molecule does not necessarily mean that it is a PPO substrate, as other substituents may be present. Phenolics containing a single hydroxyl- and additional *o*-methyl substituents on the benzene ring (e.g. ferulic acid) are not considered to be substrates, because an *o*-diphenol, and subsequently an *o*-quinone, cannot be formed. Additionally, phenolic acid glycosides (**Figure 1A**) and phenolic acid glycosyl-esters (**Figure 1B**) were distinguished. In the former, the glycosyl residue is connected via an acetal linkage to an OH group on the phenolic ring, which thereby hinders quinone formation. In the latter, the glycosyl residue is esterified to the carboxyl group, maintaining the *o*-diphenol function. Caffeic acid-glycoside (**2**) and dopamine-glycosides **3** and **5** were tentatively annotated as glycosides (**Table 1**). The derivatives of caffeic- (**4**, **6**), sinapic- (**24**) and ferulic acid (**27**, **28**) were tentatively annotated as glycosyl-esters. Derivatives of ferulic acid, sinapic acid and iso-rhamnetin are not substrates for PPOs, as one or more of the hydroxyl oxygens are methylated. Qualitatively, the phenolics profile obtained (**Table 1**) correlated with those of *Beta vulgaris* species, determined previously [8, 20, 21].

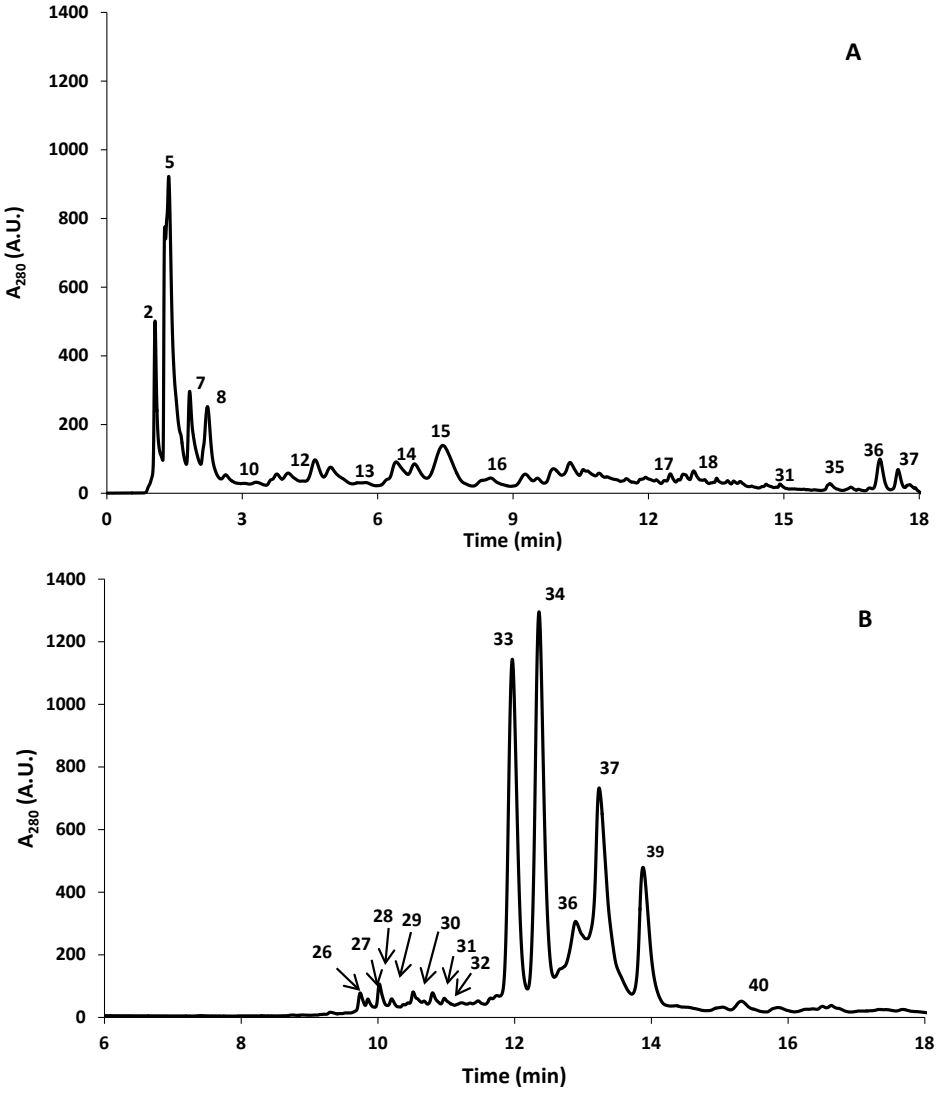


Figure 2 RP-UHPLC-UV chromatograms at 280 nm of pool 2 (phenolic acids) (A) and pool 3 (flavonoids) (B). Numbers represent compounds as listed in Table 1

Table 1 Phenolics annotated and quantified in the aqueous methanolic extract from sugar beet leaves

Comp no.	UV max	$[M-H]^-$	MS^2 fragments	MS^3 fragments	Identification	MWCF	3_m (mg/g FW)	8_m (mg/g FW)
Caffeic acid derivatives								
1[a]	355	215	**179**[b], 161	**161**, 89	Caffeic acid derivative	1.20	0.08	0.17
2	267, 379	341	**281**, 179, 161, 119	**89**, 161, 119	Caffeic acid glycoside [13,14]	1.91	0.94	2.12
4	280	773	**661**, 306	**306**, 593, 272, 481, 179	Caffeoyl-spermine-glycosyl-ester derivative [14]	4.32	0.04	0.02
6	279, 339	383	**341**, 343, 179	**179**, 161, 119	Caffeic acid acetyl-glycosyl-ester derivative	2.14	n.q.[c]	0.18
7	255, 273	297	**179**, 161, 237	**161**, 89, 135	Caffeic acid derivative	1.66	0.01	0.02
11	279, 339	261	**181**, 199, 215	**163**, 135, 119	Dihydrocaffeic acid sulfate-ester	1.46	n.q.	0.03
18	300	487	**307**, 161, 179		Caffeic acid derivative	2.72	n.q.	1.47
Amino derivatives								
3	280	330	**220**, 240, 255	**150**, 135, 109	Dehydro-dopamine derivative	1.84	n.q.	n.q.
5	280	314	**224**, 278, 194	**152**	Dopamine glycoside	1.75	0.12	0.17
8	261	344	**150**, 133	**133**, 107	Dehydro-dopamine derivative	1.92	0.02	0.06
9	255, 277	282	**150**	**133**	Dehydrodopamine-pentose	1.58	n.q.	n.q.
10	279	192	**192**, 150	**150**, 121	Dehydrodopamine acetyl derivative	1.07	n.q.	n.q.
13	280	203	**159**, 131, 203	**131**, 148	Tryptophan [21]	1.13	n.q.	n.q.
16	284	354	**192**, 264, 294	**150**, 174. 192, 232	Dehydrodopamine glycoside	1.98	n.q.	n.q.
Sinapic acid derivatives								
12	310	439	**241**	**97**, 139, 223	Sinapic acid derivative	2.45	n.q.	n.q.
21	320	651	**223**, 489	**208**, 179	Sinapic acid-*O*-glycosyl-derivative	3.64	0.01	0.70
24	252, 322	445 [d]	**385**	**223**, 153, 161, 179	Sinapic acid-glycosyl ester [14]	2.49	0.16	0.34
29	325	489	**223**, 205	**164**, 179	Sinapic acid derivative	2.73	0.24	0.21
5-OH Ferulic acid derivatives								
14	309	355	**191**, 209, 147	**85**, 147	5-OH Ferulic acid derivative	1.98	n.q	n.q
15	311	355	**191**, 209, 111	**85**, 147	5-OH Ferulic acid derivative	1.98	n.q.	n.q.
Coumaric acid derivatives								
17	355	305	**225**, 165, 97	**165**, 181, 147	Dihydro-*p*-coumaric acid derivative	1.70	n.q.	0.01
22	284	337	**191**, 289, 163,	**85**, 147, 173	5-*p*-Coumaroylquinic acid [19]	1.88	0.2 [e]	0.28
25	284	337	**173**, 191, 163, 130	**85**, 57, 111	4-*p*-Coumaroylquinic acid	1.88		

Table 1 continued

Comp no.	UV max	[M-H]$^-$	MS^2 fragments	MS^3 fragments	Identification	MWCF	3_m (mg/g FW)	8_m (mg/g FW)
Ferulic acid derivatives								
20	325	621	**193**, 459, 445, 265	**149**, 178, 134	Ferulic acid-*O*-glycosyl-derivative	3.47	0.04	0.63
23	314	487	**193**, 355, 149	**149**, 178, 193, 134	Ferulic acid-*O*-pento-glycoside	2.72	n.q.	n.q.
26	284, 330	445	**283**, 343, 301, 401	**138**, 165, 193, 286	Ferulic acid-*O*-glycosyl-derivative	2.49	n.q.	0.26
27	325	355	**193**, 175, 217, 134	**134**, 149, 178	Ferulic acid-glycosyl ester	1.98	0.32	0.46
28	287, 323	517	**193**, 355, 337	**134**, 149, 178	Ferulic acid-glycosyl ester	2.89	0.4	0.13
30	323	443	**267**, 193, 134	**249**, 113, 175	Ferulic acid derivative [14]	2.47	0.29	0.12
31	326	473	**267**, 193	**249**, 113, 175	Ferulic acid derivative	2.64	0.39	0.53
Quercetin derivatives								
32	271, 201, 395	625	**301**, 300, 273, 179	179, 257, 151	Quercetin-*O*-diglycoside [8,15]	1.45	0.03	0.05
Vitexin derivatives								
33	269, 331	593	**431**, 293	**293**	Vitexin-2″-*O*-glycoside [17,19]	1.38	0.34	0.41
34	269, 332	563	**413**, 443, 311, 293	**293**	Vitexin-2″-*O*-pentoside [7,17]	1.31	0.34	0.36
35	269, 330	431	**311**, 341, 191	**283**, 311, 191	Vitexin	1.00	n.q	0.02
36	269, 330	635	**473**, 311	**311**, 413	Vitexin-*O*-glycoside	1.47	0.30	0.20
38	269	679	**635**, 593	**455**, 413, 293, 269	Glycosyl-malonylvitexin	1.58	n.q.	n.q.
40	269, 330, 395	649	**605**, 455	**455**, 311, 473, 563, 269	Pentosyl-malonylvitexin [7]	1.51	0.13	0.13
41	268, 331	473	**413**, 293	**293**, 161	Vitexin acetyl derivative	1.10	0.03	n.q.
Iso-rhamnetin derivatives								
37	254, 351	639	**315**, 300, 271, 255	**300**, 315, 287, 272, 107, 151	Isorhamnetin-*O*-diglycoside [7, 15]	1.48	0.14	0.35
39	267, 341	609	**315**, 300	**300**	Isorhamnetin derivative [28]	1.41	0.13	0.09

[a] The numbers correspond to the peak numbers in **Figure 2**. MWCF: molecular weight correction factors used for quantification

[b] Bold numbers represent a relative abundance of 100%. Values underlined represent diagnostic ions for the respective compounds [14, 15, 17]

[c] n.q. = not quantified, concentration was below the limit of quantification

[d] Parent ion for compound **24** is supposed to be an acetic acid adduct (M+60-H]$^-$

[e] For quantification, **22** and **25** were combined

Quantification of phenolics

The majority of the crude methanolic extract consisted of mono- and disaccharides which were collected in pool 1 (49.5 (±2.9) % w/w of the extract) by Flash fractionation. For 3_m and 8_m, pools 2 and 3 represented 8.7 (±1.7) % w/w and 14.8 (±1.5) % w/w of the methanolic extract, respectively. The total contents of phenolics quantified were 4.6 (±0.7) mg/g FW and 9.4 (±0.8) mg/g FW in 3_m and 8_m, respectively (**Table 2**). Contents were comparable to those found in Swiss chard (*Beta vulgaris*, subsp. *cycla*) [8]. Phenolic acid derivatives represented the major part of the phenolics and increased upon aging from 3.1 mg/g FW in 3_m to 7.6 mg/g FW in 8_m. Of the phenolic acids, caffeic acid glycosyl-ester content showed the largest increase from 0.2 mg/g FW in 3_m to 1.9 mg/g FW in 8_m. The overall content of coumaric acid derivatives was 0.3 mg/g FW for both ages. Sinapic acid derivatives content increased, from 0.4 mg/g FW in 3_m to 1.2 mg/g FW in 8_m leaves. Contents of quercetin- (0.04 mg/g FW), vitexin- (1.1 mg/g FW) and iso-rhamnetin (0.4 mg/g FW) derivatives did not change with age. In both plant ages, amino acid derivatives (**3**, **5**, **8**-**10**, **16**) comprised 2% w/w of the total phenolics.

Table 2 Contents of phenolic acids and flavonoids (mg/g fresh weight) in leaves of sugar beets harvested 3 and 8 months after sowing

Phenolic class	3_m	8_m	Substrate for PPO
Amino acids			
Dopamine derivatives	0.1 (± 0.04)	0.2 (± 0.05)	Yes
Tryptophan	n.q. [1]	n.q.	No
Sub-total	*0.1*	*0.2*	
Phenolic acids			
Coumaric acid derivatives	0.2 (± 0.05)	0.3 (± 0.1)	Yes
Caffeic acid glycosides	0.9 (± 0.2)	2.1 (± 0.2)	No
Caffeic acid-esters	0.2 (± 0.03)	1.9 (± 0.1)	Yes
Ferulic acid derivatives	1.4 (± 0.07)	2.1 (± 0.1)	No
Sinapic acid derivatives	0.4 (± 0.03)	1.2 (± 0.06)	No
Sub-total	*3.1*	*7.6*	
Flavonoids			
Vitexin derivatives	1.1 (± 0.3)	1.1 (± 0.1)	No
Quercetin glycoside	0.03 (± 0.00)	0.05 (± 0.0)	Yes
Isorhamnetin derivatives	0.3 (± 0.03)	0.4 (± 0.1)	No
Sub-total	*1.4*	*1.6*	
Total	**4.6**	**9.4**	

[1] n.q. = not quantified, concentration below quantification limit

The overall PPO substrate composition was categorized by taking into account that caffeic acid glycosides, ferulic- and sinapic acid derivatives were not substrates for PPO and were referred to as non-substrates. The 3_m leaves consisted of 1.3 mg/g FW cresolase substrates, 0.3 mg/g FW of catecholase substrates and 3.0 mg/g FW non-substrates. The 8_m leaves consisted of 1.4 mg/g FW cresolase substrates, 2.2 mg/g FW catecholase substrates and 5.8 mg/g FW non-substrates. The non-substrates represented 65% w/w and 62% w/w of the total phenolics in 3_m and 8_m, respectively. Expressing the contents on molar basis gave similar results.

PPO activity

The PPO activity was analysed by incubation of the dialysed enzyme extracts with catechin and Flash-fractionated phenolic pools and monitoring the oxygen consumption (**Figure 3**). The reaction continued until all oxygen was depleted. For catechin, reaction rates (0.03 ± 0.01 nmole O_2/(s·g) FW) were similar for 3_m and 8_m dialysed enzyme extracts. Activity differences observed were comparable to those determined previously, with a colorimetric activity determination [1].

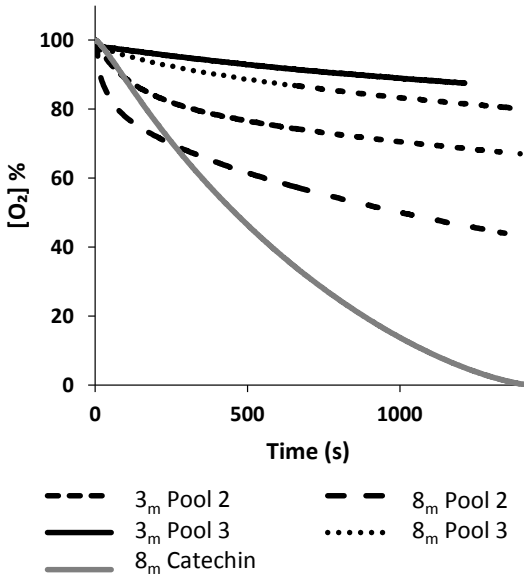


Figure 3 Monitoring of oxygen consumption over time during incubations of dialysed leaf enzyme extract from sugar beet leaves of 3- and 8-month old plants with pool 2 (1.0 g/L), pool 3 (1.0 g/L) and catechin (1.2 g/L) (only 8_m is presented; 3_m leaf extracts gave similar profiles)

The dialysed enzyme extracts had a higher activity towards pool 2 than towards pool 3. The colour changes and reaction rates were high in the beginning, especially for 8_m incubated with pool 2. For this pool, the initial rates were 6.7 ± 0.6 times higher in 8_m than in 3_m, i.e. 0.05 nmole O_2/(s·g) FW and 0.29 nmole O_2/(s·g) FW for 3_m and 8_m, respectively. For both 3_m and 8_m, incubations with pool 2 resulted in a red/brown colour (SI **Figure S1A**). For both plant ages, the dialysed enzyme extracts had low activity towards pool 3 and rates were similar, 0.02 and 0.04 nmole O_2/(s·g) FW for 3_m and 8_m, respectively. During the reaction, the colour did not change (SI **Figure S1A**). The lack of activity towards pool 3 was not expected as vitexin was the main flavonoid present (**Table 2**).

Potentially, vitexin is a cresolase substrate due to the monohydroxylated benzene ring, which might be converted into an *o*-diphenol. The lack of activity raised the question whether vitexin is indeed a cresolase substrate. This was tested by incubating vitexin with dialysed enzyme extract and determination of oxygen consumption (data not shown). No activity was observed and the non-reactivity of vitexin is most likely related to the 8-*C*-glycosyl function, which hinders access to the active site.
As both pools consisted of mono- and *o*-diphenolics, no distinction between cresolase and catecholase activity could be made. In order to distinguish these enzyme activities, UHPLC-UV-MS analysis, determining the contents of individual phenolics upon incubation, was performed.

Quantitative fate of phenolics during incubation with PPO

Regarding the quantification of the incubations with Flash pools, only results obtained with pools 2 and 3 from 3_m plants are shown (**Figure 4**), as those obtained from 8m were comparable. Prior to incubation, pool 2 from 3_m contained 8 mol% mono-, 12 mol% o-diphenolics and 80 mol% non-substrate phenolics. After incubation of this pool with dialysed enzyme extract of both plant ages, the total amount of phenolics originally present decreased (**Figure 4A**, SI **Table S1**) by 42%, for enzyme extract of both plant ages. Amino acid-derivatives were not quantified as the amounts found were below the limit of quantification. No UV- or MS-peaks indicating the formation of particular oxidative condensation products were annotated. The solution gave increased signal intensity above 400 nm after incubation, indicating formation of compounds with large conjugated systems. The caffeic acid glycosyl-esters content in pool 2 was about 12 mol% and these molecules were the main reactants (**Figure 4A**). The total content of caffeic acid esters decreased by 60% and 49% in 3_m and 8_m, respectively. No changes in compound **2** (caffeic acid glycoside) were found (SI **Table S1**), confirming that it is not a catecholase substrate. The total concentration of coumaric acid derivatives did not change in 3_m and decreased by 79% in 8_m. All three components (**17**, **22** and **25**) reacted. Ferulic- and sinapic acid derivatives decreased for both plant ages, except the ferulic-*O*-glycoside (**27**). Overall, sinapic acid diglycosides (**23**, **28**) decreased by 30%, while sinapic- and ferulic acid monoglycosides (**21**, **24**, **20**, **26**, and **27**) decreased by 60%. This demonstrated that non-substrate phenolics also participate in the reaction.

Prior to incubation, pool 3 from 3_m contained 16 mol% mono-, 9 mol% *o*-diphenolics and 75 mol% non-substrate phenolics, taking into account that vitexin was not a substrate. The pool contained 69 mol% vitexin derivatives and about 2 mol% caffeic acid esters (**Figure 4B**, SI **Table S1**). The total decrease (26% for both plant ages) in phenolics quantified after reaction was mainly caused by a decrease in the concentration of the vitexin derivatives, which were not substrates. It is likely that the decrease in vitexin derivatives was caused by coupling to enzymatically formed quinones of caffeic acid esters.

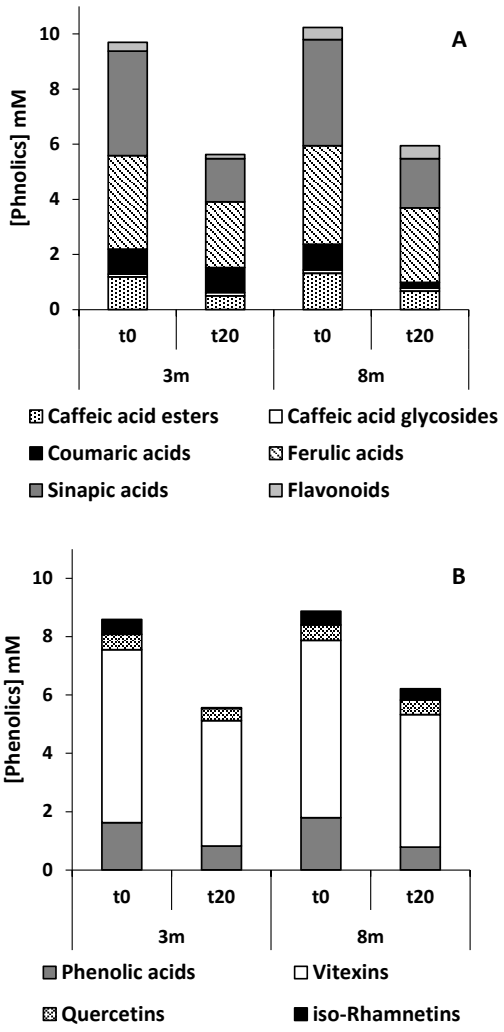


Figure 4 Contents (mM) of phenolics pool 2 (A) and pool 3 (B) before (t_0) and after (t_{20}) incubations with oxidative enzymes extracted from 3 (3_m) and 8 (8_m) month old leaves

Model incubations

As ferulic acid, sinapic acid (pool 2) and vitexin derivatives (pool 3) were not PPO substrates, their decrease might be related to their participation in non-enzymatic continuation reactions forming oxidative coupling products [22]. In this process, nucleophilic phenolics, which are not necessarily PPO substrates, attack the enzymatically formed quinones and form a covalent bond. To test whether this could indeed explain the observed decrease of non-substrates, a model system was used in which chlorogenic acid (ChA) was incubated with tyrosinase either with or without an equimolar amount of ferulic acid (FerA) or sinapic acid (SinA). After 30 min, a ChA-dimer (*m/z* 705) was annotated in all samples. In addition, when a mixture of phenolics was incubated, products with *m/z* values of 545 (ChA-FerA) (**Figure 5A**) and 575 (ChA-SinA) (SI **Figure S2B**) were annotated. This was indicated by MS^n fragmentation spectra showing the loss of ChA and diagnostic ions for ChA, FerA, and SinA (SI **Figure S3**). After 60 min of incubation, $(FerA)_2$-ChA with *m/z* 737 and $(ChA)_2(SinA)_2$ with *m/z* 1153 were tentatively annotated. Sinapic acid was somewhat more reactive than FerA as after 30 min of incubation all SinA and 85% of the initial FerA had reacted. Sinapic acid was also more reactive than FerA when all three compounds were incubated together (data not shown). The final reaction mixtures showed absorbance around 400 nm (**Figures 5B**, SI **Figure S2**), which was expected to relate to formation of coupling products with enlarged conjugated systems. The PDA spectra of the dimers (λ_{max} 320-340 nm) themselves had no absorbance in the visible wavelength range. With increasing FerA-to-ChA molar ratio, the colour formation decreased (**Figure 5C**).

Reducing agent levels

The freeze-dried 3_m leaves contained less AA than 8_m ones, i.e. 5.5 ± 1.5 mg/100 g FW and 17.5 ± 4.1 mg/100 g FW in 3_m and 8_m, respectively. GSH was not detected. After reduction with DTT, the contents of AA and GSH did not increase, indicating that both DHA and GSSG were not initially present.

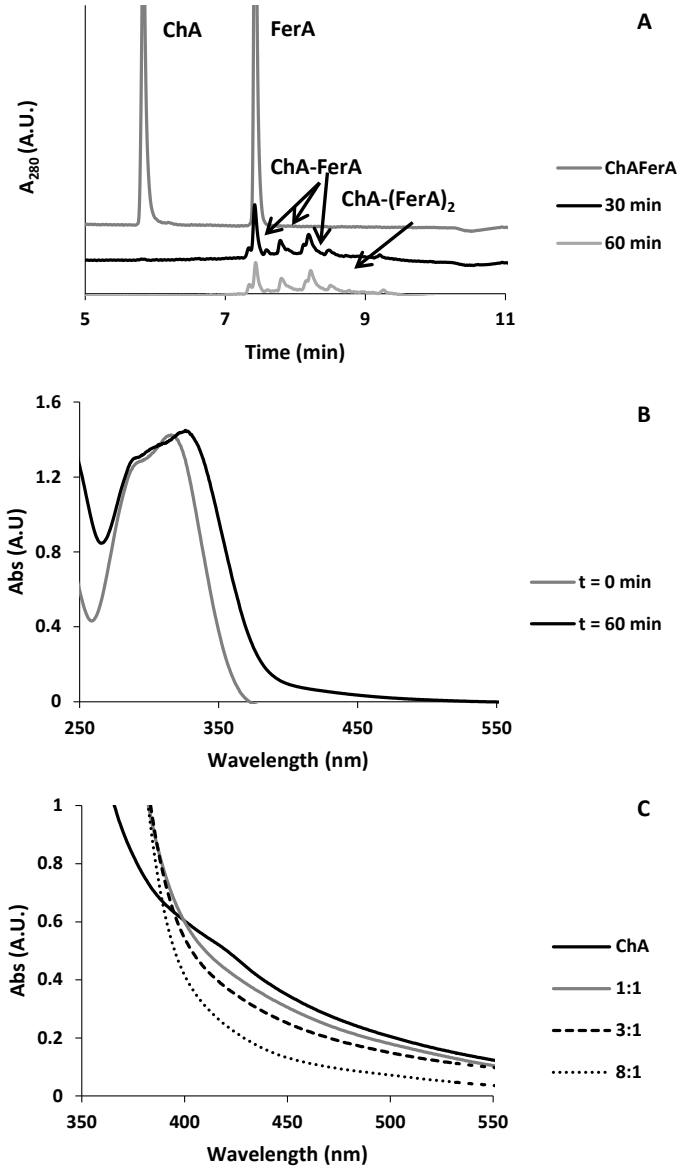


Figure 5 RP-UHPLC-PDA traces of chlorogenic acid (ChA) (0.1 mM) and ferulic acid (FerA) (0.1 mM) at several time points after incubation with mushroom tyrosinase (A) and absorbance spectra of the reaction mixtures before and after 60 min incubation (B). Absorbance spectra of ChA after 60 min incubation with mushroom tyrosinase at increasing molar ratios of FerA-to-ChA (C)

Discussion

In this study, three aspects that can influence the extent of enzymatic browning in leaves from sugar beet plants were considered.

PPO activity and substrates

In our previous study [1], we observed that the cresolase activity increased 6 fold from 3-8 months, whereas the catecholase activity did not change. The cresolase activity in 8_m was still 10 times lower than catecholase activity. Activities were determined using pure catechin and tyrosine of which it is unknown whether these are ideal substrates for PPO from sugar beet leaves, as these substrates are normally not present in high amounts. In the present research, pools of endogenous leaf phenolics were used, which were assumed to be better PPO substrates than catechin and tyrosine. The leaf extracts had highest activity towards pool 2 (**Figure 3A**) and the activity increased 6.7 fold with age. The compositions of residual phenolics after incubation (**Figure 4**) were similar for both plant ages, indicating that the enzymes by themselves had similar substrate specificities. The difference in activity might be caused by activation of latent enzymes in 8_m leaves, resulting in higher absolute amounts of PPO. Similar observations have been made for red clover and wheat leaves [6, 23]. The amounts of PPO, however, were not quantified. The difference in activity might explain part of the differences in browning behaviour of sugar beet leaves. Nevertheless, also an influence of substrate composition is expected.

Of the PPO substrates annotated in the leaves, mainly the caffeic acid esters reacted, independent of the enzyme extract applied (**Figure 4A**). Monophenolic derivatives decreased three times less than *o*-diphenolic derivatives. Tyrosinase-like enzymes possess both the cresolase and catecholase activity within a single enzyme. The use of *o*-diphenolics is preferred and the hydroxylation step (cresolase activity) is known to be rate limiting [24]. Within the 8_m leaves the total amount of diphenolic substrates was 10 fold higher than in 3_m and we propose that this contributed to higher browning potential. There were no changes in total monophenolic content and this represented on average only 3.5% of the total phenolics pool. Therefore, cresolase activity is expected to have minor influence. Additionally, low monophenolic content might explain the low cresolase activity observed previously as the same leaves were used for this research [1].

Contributions of non-substrates

The majority of the leaf phenolics were not substrates for PPO. The decreases observed in these derivatives were caused by combinations of oxidative coupling [22] and coupled oxidation [25] reactions, as evidenced with the model substrate ChA. The mechanisms are proposed in **Figure 6A**, in which ChA is displayed more generically as caffeic acid ester (CafAe). Initially, monomeric CafAe is enzymatically oxidised into a quinone. Subsequently, there are two continuation reactions in which this quinone can participate. The first one is oxidative coupling, in which the quinone is attacked by nucleophiles. Nucleophiles present in the model system were CafAe, FerA and SinA. The reaction of these phenolics towards quinones is expected to follow their nucleophilic strength; i.e. CafAe = SinA > FerA [26]. These differences in nucleophilicity can explain the faster decrease of SinA than of FerA in the leaf phenolic extracts upon incubation with the dialyzed enzyme extract (**Figure 4A**).

The nucleophilic attack on the quinone yields a dimer, such as $(CafAe)_2$ or FerA-CafAe. Thereby, the quinone motif is reduced back into its *o*-diphenol. This *o*-diphenolic dimer has potential to be oxidised. Due to its size, it is unlikely that enzymatic oxidation occurs directly. The re-oxidation of the *o*-diphenolic motif can occur via coupled oxidation [25]. This coupled oxidation is the second continuation reaction for the CafAe quinone (**Figure 6A**). In this reaction, the enzymatically formed quinone of monomeric CafAe can oxidise the dimer and reduce back into a monomeric CafAe again. For CafAe-FerA or CafAe-SinA dimers, only the CafAe motif will be oxidised. As FerA and SinA do not possess an *o*-diphenolic structure, they are not expected to be oxidised by quinones. These oxidised dimers can participate in further oxidative coupling reactions.

The oxidative coupling and coupled oxidation reactions can comprise various cycles, the number of which depends on the ratio of non-substrates-to-substrates present in the mixture. The elongation of phenolics by subsequent cycles of oxidative coupling and coupled oxidation are schematically shown in **Figure 6B**. At high ratio (8:1 in the model system), mainly CafAe-FerA dimers will be formed. Due to the relatively low CafAe concentration, all CafAe is consumed after a few reaction cycles and connected to non-substrate phenolics. This results in small oligomers without an extensive conjugated system. The dimers found during the model incubations did not show absorbance in the visible wavelength range. The formation of smaller oligomers explains the decreased colour formation at increased FerA:CafAe ratio in the model system (**Figure 5C**). At low FerA:CafAe molar ratios (3:1 and 1:1 in the model system), there is increased probability of $(CafAe)_2$ formation (**Figure 6B**). This dimer is prone to further coupled oxidation as there is sufficient CafAe present, which can be oxidised into quinones by PPO. The monomeric CafAe quinones oxidise the dimers into quinones. These dimeric quinones are then further elongated by oxidative coupling. During the oxidative coupling, a dimer can be attacked by a monomeric as well as a dimeric nucleophile, resulting in the formation of many different coupling products, which are not necessarily a linear chain as depicted in **Figure 6B**.

In the same way, the non-substrate-to-substrate ratio is suggested to influence the degree of browning in sugar beet leaves. In 3_m leaves, the molar non-substrate-to-phenolic acid-ester ratio was 8:1, whereas in 8_m leaves this ratio was 3:1. The high ratio in 3_m leaves inhibits extensive polymerisation and browning.

Reducing agents

Taking into account the presence of reducing agents, AA and GSH were potential candidates. It was hypothesised that the AA content was higher in the younger leaves. The hypothesis had to be rejected as the AA content was 3- fold higher (w/w) in 8_m than in 3_m. On a molar basis, the substrate contents were 8 and 3 times higher than AA contents in 3_m and 8_m leaves, respectively. Therefore, the activity of AA as reducing agent cannot be a major factor of importance. Plants possess the capacity to reduce DHA back into AA by the expression of dehydroascorbate reductase. This enzyme reduces DHA back to AA by oxidising GSH into GSSG [27]. This mechanism does not seem to be at play in sugar beet leaves as GSH was not detected.

To conclude, there are several factors influencing the extent of enzymatic browning in plant materials. The increases in enzyme activity and phenolics content have been reported earlier. With the observations in this research, we postulate that besides PPO the ratio between the non-substrate-to-substrate phenolics drive enzymatic browning, rather than the absolute quantities of phenolics.

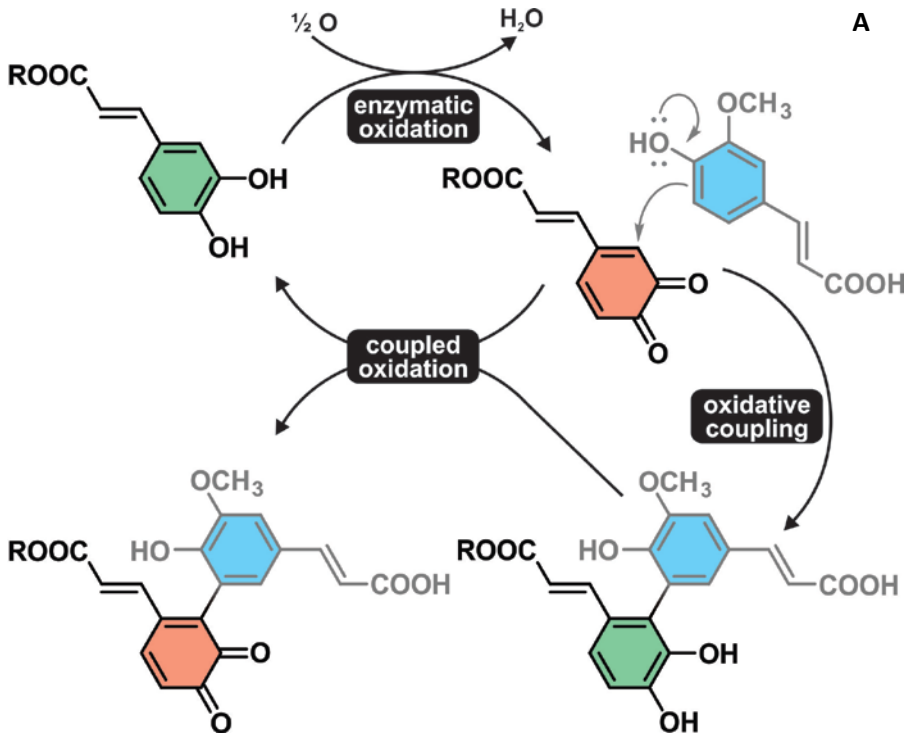
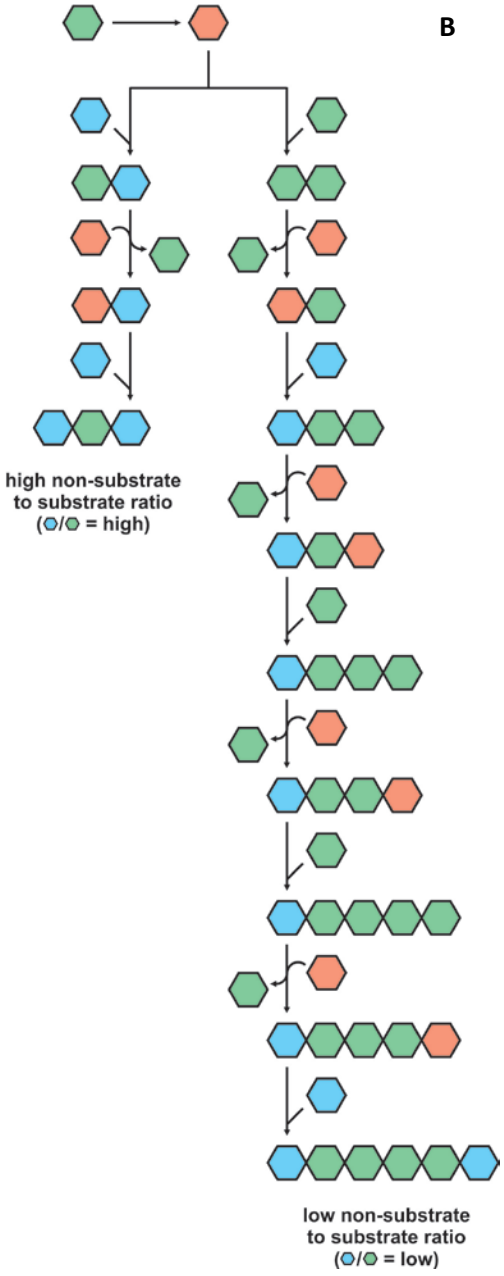


Figure 6 Proposed mechanism of enzymatic oxidation of a caffeic acid ester, followed by oxidative coupling to ferulic acid, and subsequent coupled oxidation (A). Schematic representation of coupled oxidation and oxidative coupling reactions at high and low non-substrate-to-substrate ratio (B). At high ratio, the limited substrate concentration restricts quinone formation and the number of subsequent elongation cycles, resulting in smaller oligomers without an extensive conjugated system. At low ratio, elongation to phenolics with a more extensive conjugated system, and consequently brown colour formation, can occur. For clarity, only linear reaction products are depicted, but it cannot be excluded that also branched products are formed


B
high non-substrate
to substrate ratio
(⬡/⬡ = high)
low non-substrate
to substrate ratio
(⬡/⬡ = low)

Acknowledgements

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References

1. Kiskini, A., A. Vissers, J.P. Vincken, H. Gruppen, and P.A. Wierenga, Effect of plant age on the quantity and quality of proteins extracted from sugar beet (*Beta vulgaris* L.) leaves. *J. Agric. Food Chem.*, **2016**: 8305-8314.
2. Toivonen, P.M.A. and D.A. Brummell, Biochemical bases of appearance and texture changes in fresh-cut fruit and vegetables. *Postharvest Biol. Technol.*, **2008**. 48(1): 1-14.
3. Kroll, J., H.M. Rawel, and S. Rohn, Reactions of plant phenolics with food proteins and enzymes under special consideration of covalent bonds. *Food Sci.Technol. Res.*, **2003**. 9(3): 205-218.
4. Bittner, S., When quinones meet amino acids: Chemical, physical and biological consequences. *Amino Acids*, **2006**. 30(3 SPEC. ISS.): 205-224.
5. Chutichudet, B., P. Chutichudet, and S. Kaewsit, Influence of developmental stage on activities of polyphenol oxidase, internal characteristics and colour of lettuce cv. *Grand Rapids. Am. J. Food Technol.*, **2011**. 6(3): 215-225.
6. Winters, A.L., F.R. Minchin, T.P.T. Michaelson-Yeates, M.R.F. Lee, and P. Morris, Latent and active polyphenol oxidase (PPO) in red clover (*Trifolium pratense*) and use of a low PPO mutant to study the role of PPO in proteolysis reduction. *J. Agric. Food Chem.*, **2008**. 56(8): 2817-2824.
7. Gil, M.I., F. Ferreres, and F.A. Tomás-Barberán, Effect of modified atmosphere packaging on the flavonoids and vitamin C content of minimally processed Swiss chard (*Beta vulgaris* subspecies *cycla*). *J. Agric. Food Chem.*, **1998**. 46(5): 2007-2012.
8. Pyo, Y.H., T.C. Lee, L. Logendra, and R.T. Rosen, Antioxidant activity and phenolic compounds of Swiss chard (*Beta vulgaris* subspecies *cycla*) extracts. *Food Chem.*, **2004**. 85(1): 19-26.
9. Davey, M.W., E. Dekempeneer, and J. Keulemans, Rocket-powered high-performance liquid chromatographic analysis of plant ascorbate and glutathione. *Anal. Biochem.*, **2003**. 316(1): 74-81.
10. Takahama, U., M. Hirotsu, and T. Oniki, Age-dependent changes in levels of ascorbic acid and chlorogenic acid, and activities of peroxidase and superoxide dismutase in the apoplast of tobacco leaves: Mechanism of the oxidation of chlorogenic acid in the apoplast. *Plant Cell Physiol.*, **1999**. 40(7): 716-724.
11. Chandra, A., J. Rana, and Y. Li, Separation, identification, quantification, and method validation of anthocyanins in botanical supplement raw materials by HPLC and HPLC-MS. *J. Agric. Food Chem.*, **2001**. 49(8): 3515-3521.
12. Kontogianni, V.G., Novel techniques towards the identification of different classes of polyphenols, *in Polyphenols in Plants: Isolation, Purification and Extract Preparation*, R.R. Watson, Editor. **2014**, Academic Press: NY, USA. 159-185.
13. Clifford, M.N., W. Wu, J. Kirkpatrick, and N. Kuhnert, Profiling the chlorogenic acids and other caffeic acid derivatives of herbal chrysanthemum by LC-MS^n. *J. Agric. Food Chem.*, **2007**. 55(3): 929-936.
14. Narváez-Cuenca, C.E., J.P. Vincken, and H. Gruppen, Identification and quantification of (dihydro) hydroxycinnamic acids and their conjugates in potato by UHPLC-DAD-ESI-MSn. *Food Chem.*, **2012**. 130(3): 730-738.
15. Justesen, U., Negative atmospheric pressure chemical ionisation low-energy collision activation mass spectrometry for the characterisation of flavonoids in extracts of fresh herbs. *J. Chromatogr. A*, **2000**. 902(2): 369-379.
16. Cuyckens, F. and M. Claeys, Mass spectrometry in the structural analysis of flavonoids. *J. Mass Spectrom.*, **2004**. 39(1): 1-15.
17. Sakalem, M.E., G. Negri, and R. Tabach, Chemical composition of hydroethanolic extracts from five species of the Passiflora genus. *Rev. Bras. farmacog.*, **2012**. 22(6): 1219-1232.

18. Kuijpers, T.F.M., C.E. Narváez-Cuenca, J.P. Vincken, A.J.W. Verloop, W.J.H. Van Berkel, and H. Gruppen, Inhibition of enzymatic browning of chlorogenic acid by sulfur-containing compounds. *J. Agric. Food Chem.*, **2012**. 60(13): 3507-3514.
19. Dou, J., V.S.Y. Lee, J.T.C. Tzen, and M.R. Lee, Identification and comparison of phenolic compounds in the preparation of oolong tea manufactured by semifermentation and drying processes. *J. Agric. Food Chem.*, **2007**. 55(18): 7462-7468.
20. Kujala, T., J. Loponen, and K. Pihlaja, Betalains and phenolics in red beetroot (*Beta vulgaris*) peel extracts: extraction and characterisation. *Z. Naturforsch. C* **2001**. 56(5-6): 343-348.
21. Olsen, B.W., R.F. Madsen, and W.K. Nielsen, Sugar beet phenols - 2. Investigation of phenolic compounds from sugar beet in relation to the formation of colour. *Int. Sugar J.*, **1979**. 81(972): 362-367.
22. Kusano, R., T. Tanaka, Y. Matsuo, and I. Kouno, Structures of epicatechin gallate trimer and tetramer produced by enzymatic oxidation. *Chem. Pharm. Bull.*, **2007**. 55(12): 1768-1772.
23. Jukanti, A.K., P.L. Bruckner, and A.M. Fischer, Molecular and biochemical characterisation of polyphenol oxidases in developing kernels and senescing leaves of wheat (*Triticum aestivum*). *Funct. Plant Biol.*, **2006**. 33(7): 685-696.
24. Aniszewski, T., R. Lieberei, and K. Gulewicz, Research on catecholases, laccases and cresolases in plants. Recent progress and future needs. *Acta. Biol. Cracov. Ser. Bot.*, **2008**. 50(1): 7-18.
25. Tanaka, T., C. Mine, K. Inoue, M. Matsuda, and I. Kouno, Synthesis of theaflavin from epicatechin and epigallocatechin by plant homogenates and role of epicatechin quinone in the synthesis and degradation of theaflavin. *J. Agric. Food Chem.*, **2002**. 50(7): 2142-2148.
26. Teixeira, J., A. Gaspar, E.M. Garrido, J. Garrido, and F. Borges, Hydroxycinnamic acid antioxidants: an electrochemical overview. *BioMed. Res. Int.*, **2013**. 2013: 11.
27. Gallie, D.R., The role of l-ascorbic acid recycling in responding to environmental stress and in promoting plant growth. *J. Exp. Bot.*, **2013**. 64(2): 433-443.

Supporting information

Table S1 Quantification of phenolics (mM) in pools 2 and 3 during incubations with endogenous leaf oxidases from 3_m and 8_m leaves

No.	$[M-H]^-$	Compound	Pool 2 (mM)				Pool 3 (mM)			
			3_m t_0	3_m t_{20}	8_m t_0	8_m t_{20}	3_m t_0	3_m t_{20}	8_m t_0	8_m t_{20}
1	215	Caffeic acid derivative	0.03	0.02	0.02	0.03	0.01	0.064	0	0.02
2	341	Caffeic acid-glycoside	0.10	0.12	0.11	0.11	0.03	0.08	0.05	0.08
4	773	Caffeoyl-spermine-glycosyl-ester	0.12	0.09	0.12	0.10	0.05	0.10	0.04	0.04
6	383	Caffeic acid acetyl-glycosyl-ester	0.01	0.00	0.01	0.01	0.00	0.00	0.00	0.00
7	297	Caffeic acid derivative	0.94	0.38	1.01	0.42	0.11	0.04	0.54	0.18
11	261	Dihydrocaffeic acid sulfate-ester	0.01	0.00	0.04	0.01	0.00	0.00	0.03	0.00
18	487	Caffeic acid derivative	0.07	0.00	0.12	0.08	0.00	0.00	0.05	0.01
12	439	Sinapic acid derivative	0.15	0.07	0.14	0.00	0.00	0.00	0.00	0.05
21	651	Sinapic acid-*O*-glycosyl derivative	0.85	0.50	0.92	0.7	0.26	0.19	0.33	0.25
24	445	Sinapic acid-*O*-glycoside	2.80	1.00	2.79	1.09	0.09	0.00	0.08	0.00
29	489	Sinapic acid derivative	0.67	0.23	0.78	0.33	0.53	0.06	0.64	0.09
17	305	Dihydro-p-coumaric acid derivative	0.06	0.00	0.07	0.00	0.00	0.00	0.01	0.00
22	337	5-*p*-Coumaroylquinic acid	0.62	0.52	0.64	0.10	0.02	0.01	0.04	0.01
25	337	4-*p*-Coumaroylquinic acid	0.22	0.20	0.22	0.07	1.42	0.00	1.42	0.00
20	621	Ferulic acid-*O*-glycosyl derivative	0.26	0.10	0.27	0.09	0.03	0.01	0.03	0.04
23	487	Ferulic acid-*O*-pento-glycoside	0.50	0.36	0.51	0.36	0.02	0.01	0.03	0.01
26	445	Ferulic acid-*O*-glycosyl derivative	0.94	0.38	1.01	0.42	0.11	0.04	0.54	0.18
27	355	Ferulic acid-glycosyl ester	0.60	0.59	0.67	0.66	0.14	0.03	0.20	0.05
28	517	Ferulic acid-glycosyl ester	1.52	0.99	1.56	1.13	0.79	0.62	0.83	0.83
30	443	Ferulic acid derivative	0.63	0.40	0.63	0.50	0.08	0.07	0.08	0.07
31	473	Ferulic acid derivative	0.38	0.30	0.44	0.32	0.03	0.03	0.10	0.01
32	625	Quercetin-*O*-diglycoside	0.03	0.02	0.03	0.04	0.52	0.53	0.53	0.50
33	593	4''-*O*-Glycosylvitexin	0.03	0.00	0.03	0.00	0.35	0.00	0.35	0.00
34	563	Vitexin-*O*-pentosyl derivative	0.02	0.01	0.03	0.01	0.65	0.35	0.65	0.24
35	431	Vitexin	0.02	0.01	0.02	0.01	0.79	0.45	0.78	0.52
36	635	Vitexin-*O*-glycoside	0.04	0.01	0.07	0.02	1.24	1.1	1.27	1.28
38	679	Glyosyl-malonylvitexin	0.06	0.02	0.07	0.03	1.28	1.19	1.29	1.27
41	473	Vitexin acetyl derivative	0.00	0.04	0.12	0.02	1.62	1.21	1.73	1.24
37	639	Isorhamnetin-*O*-diglycoside	0.00	0.00	0.01	0.01	0.03	0.02	0.03	0.03
39	609	Isorhamnetin derivative	0.12	0.03	0.07	0.00	0.48	0.00	0.43	0.35

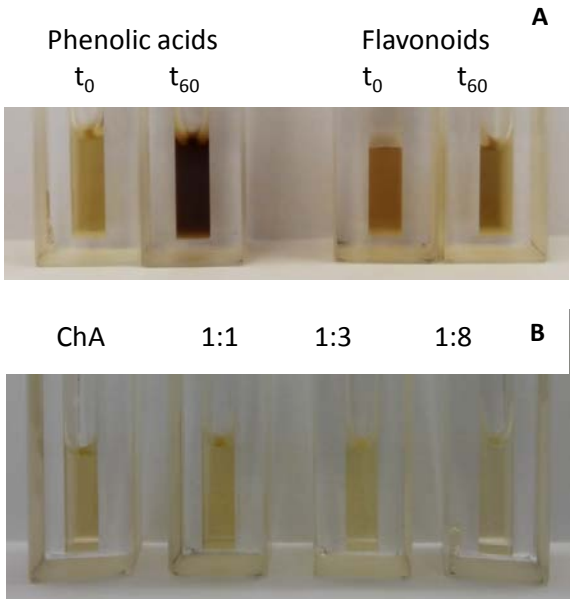

Figure S1 Colours of pool 2 (phenolic acids) and 3 (flavonoids) upon incubation with dialyzed leaf enzyme extract at start (t_0) and after 60 min (t_{60}) (A). Colours of chlorogenic acid (ChA) increasing molar proportion of ferulic acid upon incubation with tyrosinase after 60 min (B)

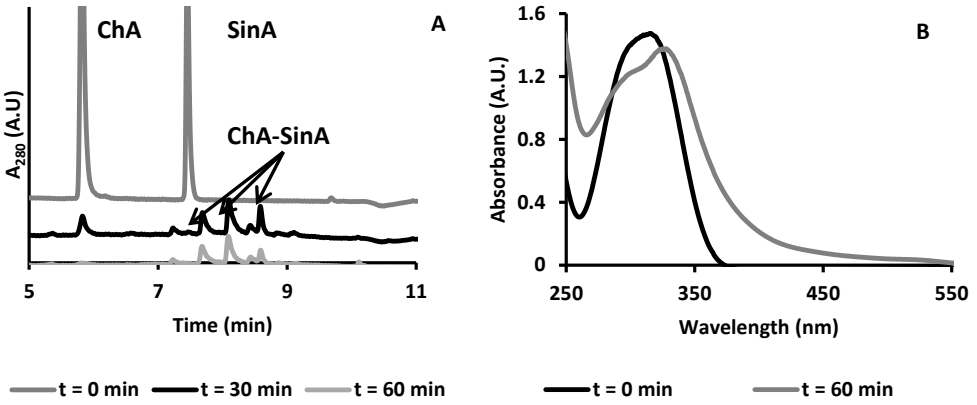

Figure S2 RP-UHPLC-DAD traces of chlorogenic acid (ChA) (0.1 mM) and sinapic acid (SinA) (0.1 mM) incubated with mushroom tyrosinase at several time points (A) and absorbance spectra of the reaction mixtures before and after 60 min incubation (B)

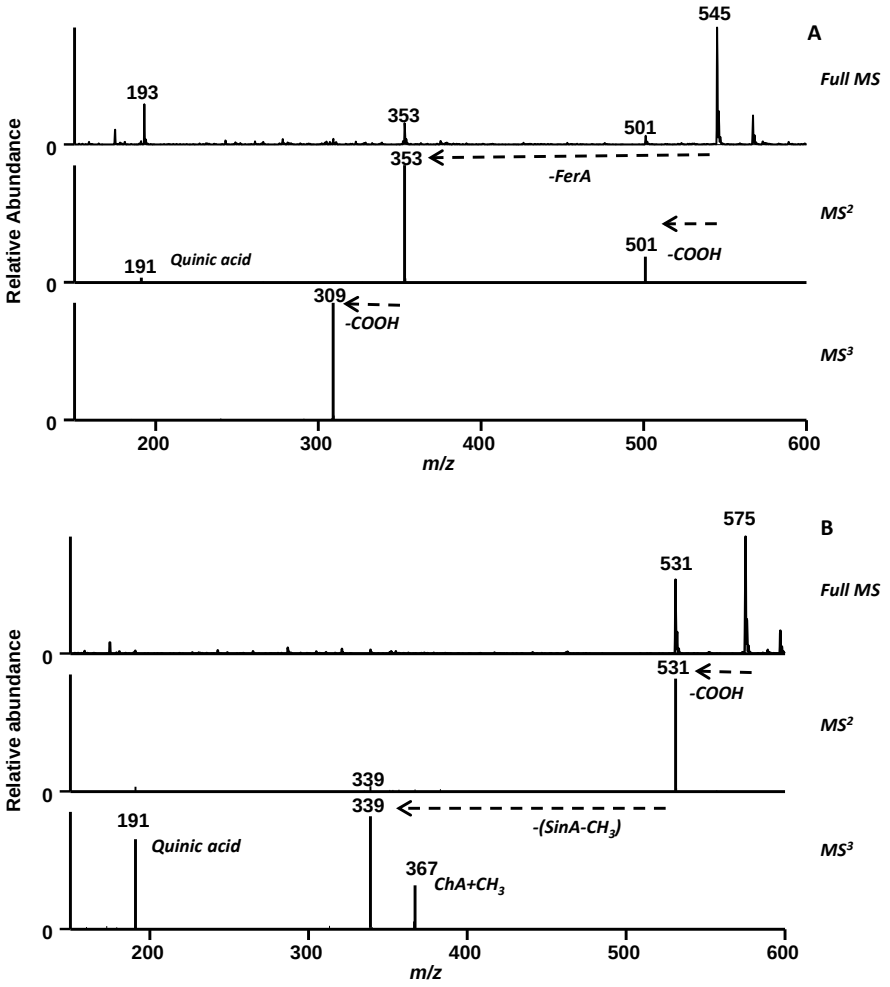

Figure S3 Mass fragmentation spectra of ChA-FerA (A) and ChA-SinA (B)

Chapter 4

Phlorotannin composition of *Laminaria digitata*

Phlorotannins (PhT) are complex mixtures of phloroglucinol oligomers connected via C-C (fucols) or C-O-C (phlorethols) linkages. Their uniformity in subunits and large molecular weight hampers their structural analysis. To obtain quantitative and structural information on PhT in a methanolic extract from *Laminaria digitata*, several techniques were applied. With ^{13}C NMR spectroscopy, a fucol-to-phlorethol linkage ratio of 1:26 was found. The extract purity was determined to be 60% using quantitative ^{1}H NMR spectroscopy, and PhT content in L. digitata was around 5% DM. The extract purity was used to calibrate the responses obtained with the colorimetric 2,4-dimethoxybenzaldehyde (DMBA) and Folin-Ciocalteu (FC) assays. For the DMBA-assay, there was a difference in λ_{max} for the phloroglucinol monomer and crude extract. There were no difference in λ_{max} for the FC-assay. By accounting for the differences in response, the colorimetric assays are applicable for quantification using phloroglucinol as a standard for calibration.

Using NP-Flash chromatography, fractions separated on degree of polymerisation were obtained which enabled detailed structural analysis by ESI-MS. With characteristic fragmentation spectra, fucol and phlorethol type of linkages were annotated. Structural isomers of PhT oligomers up to DP18 were annotated and identification of several isomers hinted at branched phloroglucinol oligomers. This was confirmed by ^{13}C NMR spectroscopy in which the average number of H atoms per benzene ring was determined to be 1.5. With MALDI-TOF-MS larger PhT up to DP27 were annotated.

Based on: Anne Vissers, Augusta Caligiani, Stefano Sforza, Jean-Paul Vincken, Harry Gruppen – *Accepted for publication in Phytochem. Anal.* **2017**

Introduction

Laminariaceae, a family within the brown macroalgae (class Phaeophyceae), is of interest for polysaccharide and protein extraction regarding future needs of feed and food. Usually, brown macroalgae are used for polysaccharide extraction as 38-61% of their dry matter consists of polysaccharides. Protein contents vary from 3-16% DM [1]. When the macroalgae are used for polysaccharide extraction, the by-product can be used as animal feed. The protein content of the by-product has increased up to 10-36% DM, comparable to that of rye grass and red clover [2], often consumed by cattle. Seaweed proteins are rich in the essential amino acids lysine and methionine. Additionally, brown seaweeds are rich in phlorotannins (PhT) [3].

Phlorotannins are polymers of phloroglucinol (1,3,5-trihydroxybenzene, 126 Da) subunits, which are biosynthesised in the acetate-malonate pathway and polymerised via oxidative coupling [3]. They resemble terrestrial condensed tannins (also referred to as proanthocyanidins). The subunits can be linked in two ways: aryl-aryl linkages (C-C, fucol-type), aryl-ether linkages (C-O-C, phlorethol-type). Additionally, two subunits can be linked via double aryl-ether linkages, creating dibenzo-p-dioxins (eckol-type) (**Figure 1**). Within the polymers, combinations of these linkages are possible. Besides, the PhT may contain additional hydroxyl (fuhalols) and halogenic substituents [3, 4]. The PhT content in brown macroalgae varies between 0.5 and 20% DM [3].

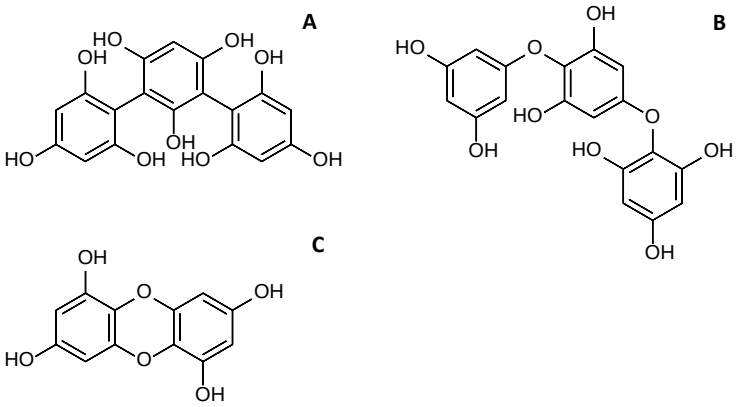

Figure 1 Representative linkage types within phloroglucinol oligomers: fucol- (A), phlorethol- (B) and eckol-type (C). Adapted from [3]

Phlorotannins are usually extracted in aqueous organic solvents and purified by liquid-liquid partitioning steps, thin layer chromatography or preparative chromatography. The combination of NP-preparative chromatography and RP-UHPLC for PhT characterisation was found to be a successful approach [5, 6]. For terrestrial proanthocyanidins, it has been found that NP-preparative chromatography separated oligomers based on their molecular weight [7].

Structural characterisation of PhT is usually performed using liquid chromatography coupled to mass spectrometry or NMR spectroscopy. For chromatography, RP-C18 columns [4, 8] and HILIC columns

[9] have been used. In all cases, chromatographic separation was followed by UV-detection at ranges between 260-280 nm and mass detection. The mass detectors attached to currently used chromatographic systems, have a detection limit of approximately 2,000 Da and become a limiting factor as PhT are known to be larger than that [10]. For the analysis of larger oligomers, above 500 Da, MALDI-TOF-MS has been reported as suitable technique [11].

NMR spectroscopy is generally used for structural characterisation of isolated PhT [12], but has also been explored as a way to quantify PhT [13], as alternative for colorimetric assays. In analogy to lignin, linkage typologies can be quantified using ^{13}C NMR [14], and can provide important structural information.

The PhT content of the Laminariacaea family is found to be 0.3-6% DM using the Folin-Ciocalteu (FC) assay [15]. Further information on the structure and molecular weight range of PhT in this species is lacking. Hence, the present research was performed to characterise the content, molecular weight- and isomer variation of PhT from *Laminaria digitata* by combining information obtained from several analytical techniques. The application of NP-chromatography was expected to separate PhT themselves on their degree of polymerisation, creating pools enriched in certain size ranges and improving analysis by RP-UHPLC-UV-MS and MALDI-TOF-MS. The combination of techniques was expected to give an overview of the size and isomeric variation of PhT in *L. digitata*, as complete as possible with currently available technology.

Materials and Methods

Macroalgae material

L. digitata was obtained from Bristol Botanicals (Bristol, United Kingdom) as air dried powder (moisture content 8% w/w).

Chemicals

Methanol, ethyl acetate, hexane, acetone, acetonitrile + 0.1% v/v formic acid (FA), water + 0.1% v/v FA, and trifluoro acetic acid (TFA, 99.0% w/w) were of analytical grade and obtained from Biosolve (Valkenswaard, The Netherlands). Phloroglucinol (99.0% w/w), 2,4-dimethoxybenzaldehyde (DMBA, 98.0% w/w), glacial acetic acid (99.0% v/v), hydrochloric acid (35.0-37.0% w/v), sodium chloride (99.0% w/w), dimethylsulphoxide-*d6*, acetonitrile-*d3*, sodium trimethylsilylpropionate-*d4* (TSP) and chromium (III) acetylacetonate, were obtained from Sigma Aldrich (St. Louis, MO, USA). MALDI-TOF-MS grade 2,5-dihydroxybenzoic acid was obtained from Bruker Daltonics (Bremen, Germany). Maltodextrin DP20 was purchased from AVEBE (Veendam, The Netherlands).

Sample preparation

L. digitata powder was suspended in 80% (v/v) aqueous methanol (25 g/L) and bead-milled using a DYNO®-mill, type MULTI-LAB (Bachhofen AG, Muttenz, Switzerland). The grinding chamber was filled (65% v/v) using 0.5 mm silica beads and the macroalgal suspension was ground for 1.5 h at a flowrate of 0.3 L/min. Afterwards, the suspension was filtered over a cellulose filter (cut off: 8-12 µm Whatman®) and methanol was evaporated by rotary-evaporation. The concentrated extract was

diluted with 0.1 M sodium chloride (1:1 v/v) and partitioned with ethyl acetate (1:1 v/v). After separation, both layers were collected separately. The organic phase was cleaned twice with 100 mL 0.2 M sodium chloride. The aqueous phase was cleaned twice with 200 mL ethyl acetate. The organic phases were pooled and subsequently dried by rotary evaporation to yield the crude extract. The extract was dissolved in methanol at 10 g/L and stored at -20 °C until further analyses.

NP-Flash chromatography

The crude extract was subjected to NP-Flash chromatography (Grace, Deerfield, IL, USA), equipped with an ELS- and UV detector (400 nm; due to absorbance of acetone at 280 nm, this wavelength could not be used), using a 12 g silica column (18 mL column volume, particle size 40 µm, Grace). The extract (250 mg) was mixed with silica (2.5 g) and injected via dry loading. The compounds were eluted using a combination of hexane (A), acetone (B) and methanol (C) at a flow rate of 30 mL/min. The elution profile was as follows: 0-1 min, isocratic at 100% A; 1-4 min, 0-80% B; 4-9 min, 80-100% B; 9-12 min, isocratic at 100% B; 12-19 min, 0-20% C; 19-23 min, 20-100% C; 23-25 min, isocratic at 100% C. Fractions (10 mL) were collected in pre-weighed tubes. In total, 51 fractions were collected of which fractions 6-44 contained sufficient material for further analyses.

RP-UHPLC-UV-MS analysis

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

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

MALDI-TOF-MS analysis

The crude extract and purified PhT fractions were dissolved in methanol (2 g/L). 2,5-Dihydroxybenzoic acid was used as matrix (10 g/L in 50% (v/v) aqueous acetonitrile acidified with 0.3% (v/v) TFA). Before spotting on the stainless steel target plate (Bruker Daltonics), sample and matrix were premixed in a 1:1 (v/v) ratio. Spotting was performed by pipetting 0.5 µL sample matrix mixture and drying under an air flow. Subsequently, this procedure was repeated with another 0.5 µL and finally with 1.0 µL 1 mM sodium acetate. MALDI-TOF-MS spectra were recorded using an Ultraflextreme workstation controlled by Flexcontrol 3.3 software (Bruker Daltonics) equipped with a N_2 laser of 337 nm and operated in positive mode. The system was calibrated using maltodextrin DP20. Spectra were recorded by automated operation, using positive mode detection over a mass range from 700-4,000 Da.

Determination of total phlorotannin content

NMR spectroscopy

The crude extract was dissolved (35-60 g/L) in a mixture of dimethylsulfoxide-*d6*:acetonitrile-*d3* 3:1 (v/v) containing a relaxation reagent, chromium(III) acetylacetonate (6 g/L), and sodium trimethylsylilpropionate-*d4* (TSP) (2.55 g/L) as internal standard. Both ^{1}H and ^{13}C NMR spectra were recorded at 303 K in quantitative conditions on a Bruker Avance III 400 MHz NMR Spectrometer (Bruker BioSpin, Rheinstetten, Karlsruhe, Germany) operating at a magnetic field-strength of 9.4 T. ^{13}C NMR spectra were acquired with Inverse Gate Detection, 90° pulse width and 4000 scans. ^{1}H NMR spectra were acquired with a 90° pulse width, 32 scans, 1.4 s acquisition time and 5 s relaxation delay. In order to apply ^{1}H NMR spectroscopy for quantification, the average number of H-atoms on the benzene ring should be known. This was determined using ^{13}C NMR spectroscopy. The value obtained from this technique was then used in ^{1}H NMR spectroscopy for quantification purposes. For quantification, the entire PhT zone was integrated and compared to the area of the internal standard TSP and converted to a mass value according to calculation methodology previously reported [16].

The PhT content was also determined by two different colorimetric methods: the DMBA assay [5] and the Folin-Ciocalteu assay [17]. For both assays, a calibration curve of phloroglucinol was prepared (0-0.25 g/L in methanol). The crude extract was dissolved at the same concentration of phenolics, after accounting for the purity of PhT as obtained by NMR spectroscopy. This allowed comparison of the response of the phloroglucinol calibration to that of the crude extract.

DMBA-assay

The DMBA-assay was performed as described previously [5], with the adaptation that absorbance after 1 h was read at 510 nm using a SpectraMax® M2^{e} plate-reader (Molecular devices, Sunnyvale, CA, USA).

FC-assay

The FC-assay was performed as described previously [17], with adaptations. The sample (20 µL) was diluted with 1.58 mL deionised water and incubated with 100 µL FC reagent for 20 min. Subsequently, 300 µL disodium-carbonate (20% w/v) was added to the mixture and after 2 h of incubation the absorbance was read at 765 nm using a UV-1800 Shimadzu spectrophotometer (Shimadzu, Kyoto, Japan).

Results and discussion

Characterisation of the crude extract

The presence of PhTs in the crude methanolic extract of *L. digitata* after ethyl acetate partitioning was determined using UV-vis spectroscopy and 13C NMR spectroscopy. The UV-vis spectrum (data not shown) showed λ_{max}s at 270 nm, 410 nm and 664 nm. The latter two maxima indicated the presence of xanthophylls and chlorophylls [18]. The maximum around 270-280 nm is indicative for phenolics. The band around 270 nm represented PhT as the absorbance maximum of phloroglucinol is 269 nm [4].

It is well known that the phloroglucinol subunits within PhT can be connected via fucol and phlorethol type of linkages [3]. In order to obtain information on the types of linkages, ^{13}C NMR spectroscopy was used. In a ^{13}C NMR spectrum for PhT, the signals for benzene-based motifs appear from 90-165 ppm. Within PhT, the benzene rings can contain the following linkages: C-C (fucol-type), C-O-C (phlorethol-type), C-H, and C-OH. The latter two are referred to as benzene ring substituents. These four linkages resonate at characteristic wavelengths and based on the ratios between these signals, the technique can be used to determine the proportion of each linkage present [12]. Resonances of C-H and C-OH substituents of the phloroglucinol standard were registered at 94.41 and 159.14 ppm, respectively (data not shown). The ^{13}C NMR spectra were interpreted based on phloroglucinol chemical shifts and literature data [12, 19]. Within the spectrum (**Figure 2A**), the chemical shifts of C-C linkages (99-103 ppm) could be distinguished from those of C-O-C linkages (121-128 ppm and 156-158 ppm) and from C-H substituents (92-97 ppm). As resonances of C-OH (151-160 ppm) signals overlapped with those of C-O-C (156-158 ppm), the proportion of the C-O-C linkages was estimated by the signals at 121-128 ppm. Based on the observed signals, the presence of eckol linkage types could be excluded as these would resonate at 140 ppm. The proportion of C-OH subunits was estimated by the difference between the overall intensities at 151-160 ppm and those at 121-128 ppm. The relative abundancies of C-H, C-OH, C-C and C-O-C linkages were 22.0%, 32.2%, 1.7% and 44.1%, respectively, corresponding to a molar fucol-to-phlorethol ratio of 1:26. To our knowledge, the ratio between the two subunit linkage types within a mixture has not been reported elsewhere.

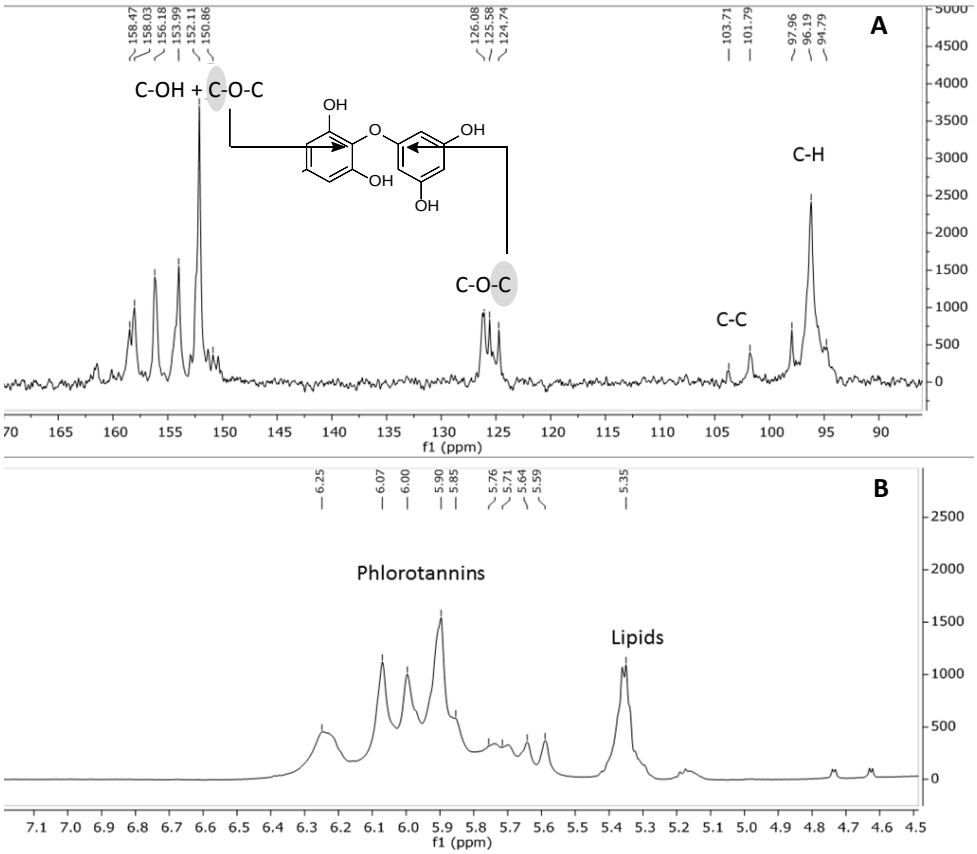

Figure 2 Full ^{13}C (A) and ^{1}H (B) NMR spectra (400 MHz) of the crude extract (30 mg in DMSO/CD_3OD 3:1)

Phlorotannin quantification

The purity of the crude extract and therewith the PhT content of *L. digitata* was assayed using several techniques. Colorimetric assays, such as the DMBA- [20] and the FC-assay [17], are often used for PhT quantification. NMR spectroscopy is a technique less frequently applied for this purpose [13]. From the relative abundancies of different carbon typologies within the PhT obtained by ^{13}C NMR spectroscopy, the average number of hydrogens on the benzene ring could be determined. This number was determined to be 1.5 and applied in ^{1}H NMR spectroscopy for quantification [13]. As most of the linkages were of the phlorethol type, represented by the 44.1% C-O-C linkages present in our sample, the value of 1.5 was lower than expected. Theoretically, this number would be around 2, as only one terminal unit within phlorehol type chains contains 3 hydrogens on the benzene ring, whereas all other units contain 2 hydrogens (**Figure 1B**). For fucol types, both terminal units contain 2 hydrogens, whereas the internal units contain one hydrogen (**Figure 1A**). When additional OH substituents or branched chains (one phloroglucinol subunit

connected to ≥ 3 subunits) are present, the average number of hydrogens per benzene ring decreases. The proportion of additional OH groups could not be calculated as the proportion of each oligomeric DP could not be verified. As the average number of H atoms on the ring was < 2, it is likely that branched PhT were present.

In ^{1}H NMR spectroscopy, PhT gave signals at 5.70-6.24 ppm (**Figure 2B**), which are characteristic for the hydrogens on phenolic rings. For quantification, the entire PhT zone was integrated and related to the area of the internal standard TSP [13]. This area was then converted to a mass value. Our results were in line with Parys *et al*. (2007), who performed similar assays for different types of brown macroalgae. The purity of the *L. digitata* crude extract was determined to be 60.1% ± 0.3 (w/w), corresponding to a PhT content of 4.3% DM in the seaweed.

The extract purity, as determined by ^{1}H NMR spectroscopy, was used to evaluate the accuracy and therewith applicability of colorimetric quantification assays for PhT in *L. digitata*. The DMBA-reagent reacts specifically with *m*-diphenolics. The DMBA-reagent is therefore more specific than the commonly used FC-reagent, as the latter reagent reacts additionally with both mono- and *o*-diphenolics [20]. Due to lack of polymeric standards, phloroglucinol is mostly used as standard for the calibration curve in both assays. In the DMBA-assay, monomeric phloroglucinol is known to react stronger to the reagent than PhT oligomers, resulting in underestimation of the purity [13, 20]. A reaction between phloroglucinol and the reagent yielded a λ_{max} at 495 nm, whereas the crude extract yielded a λ_{max} at 515 nm (SI **Figure S1A**). In literature, 510 nm is used for colorimetric determination, to compromise for the difference and be close to the λ_{max} of the extract [20]. To be able to compare our data to literature, 510 nm was used in this research as well. The difference in response between phloroglucinol and the crude extract was calculated by the ratio between the slope of the phloroglucinol calibration curve and the slope of the crude extract curve. The phloroglucinol curve was 12 times steeper than that of the crude extract (data not shown), despite the fact that the PhT purity in the crude extract was corrected for. This difference in response was applied as correction factor for further quantifications. A similar result was obtained by the developers of the assay, who found PhT reactivities in extracts of 3-11 times lower than that of monomeric phloroglucinol, depending on the algal species [20]. The crude extract contained 63.2 ± 4.2% (w/w) PhT, and content in *L. digitata* was estimated to be 4.5% DM.

For the FC-assay, there was no shift in λ_{max} (SI **Figure S1B**) between the monomeric standard and the extract. Quantifying the crude extract based on the FC-assay, resulted in 81.5 ± 2.4 % (w/w) phenolics, the content in *L. digitata* corresponded to 5.7% DM.

Comparing all quantification assays, the purities found with the FC-assay were the highest, indicating the presence of mono- and *o*-diphenolics. For PhT quantification in Laminariaceae, the FC-assay is mostly used and content determined in our assay were similar to those reported previously [20, 21].

The difference in PhT content between the FC- and DMBA-assay gives an estimation of the amount of non-phloroglucinol phenolics or fuhalol type of tannins. Due to their extra OH group, fuhalol units within the phloroglucinol oligomers do not bear a *m*-diphenolic function and do therefore not react with the DMBA-reagent.

In reports using colorimetric quantification, the differences in response between monomeric phloroglucinol and extracts is only limitedly highlighted [20]. In this study, the methodology for determining a correction factor for quantification of oligomeric PhT using the DMBA-assay with a phloroglucinol calibration curve was further refined. Nevertheless, it remains necessary to determine purities by ^{1}H NMR spectroscopy as the PhT composition is species dependent, which makes the response factor species dependent as well.

Characterisation of the Flash-fractions

To obtain structural and oligomer size information of PhT additional to ^{13}C NMR spectroscopy, the crude extract was fractionated by NP-Flash chromatography (**Figure 3A**).

Fractions 6-44 contained sufficient material for analysis by RP-UHPLC-UV-MS. Fractions 6-15, which had yellow and green colours, contained chlorophylls, xanthophylls and lipid components (SI **Table S1**) and were not included in further analyses. Within the $A_{266\ nm}$ chromatograms of fractions 16-27 (**Figure 3B**), two main regions were distinguished. The compounds in the first region, from 0-12 min, eluted in separate peaks. The peaks were annotated as PhT based on their UV-vis spectra with absorbance around 270 nm [4] and ESI-MS/MS spectra in negative mode characterised by losses of 126 Da upon fragmentation, corresponding to the monomeric phloroglucinol subunits [5]. The second region, from 12-24 min, was an unseparated hump containing compounds with low mass signal intensity, albeit with similar absorbance maxima (270 nm). The presence of such an unseparated hump is common for PhT mixtures [4, 6]. A shift in proportion between separated peaks and hump of unseparated compounds with increasing fraction numbers was observed. To determine the proportions of separated peaks and the unseparated hump in each fraction, the total area under the $A_{266\ nm}$ chromatogram from 2-12 min was taken as percentage of the total $A_{266\ nm}$ area from 2-24 min. In fractions 16-18, the area from 2-12 min made up 35% of the total area, which increased to 40% in fraction 19. After this fraction, the proportion decreased to be constant around 5% for fractions 22-27. The hump was not present in fractions 28-44 and these fractions contained carbohydrates as determined by MALDI-TOF-MS analysis (data not shown).

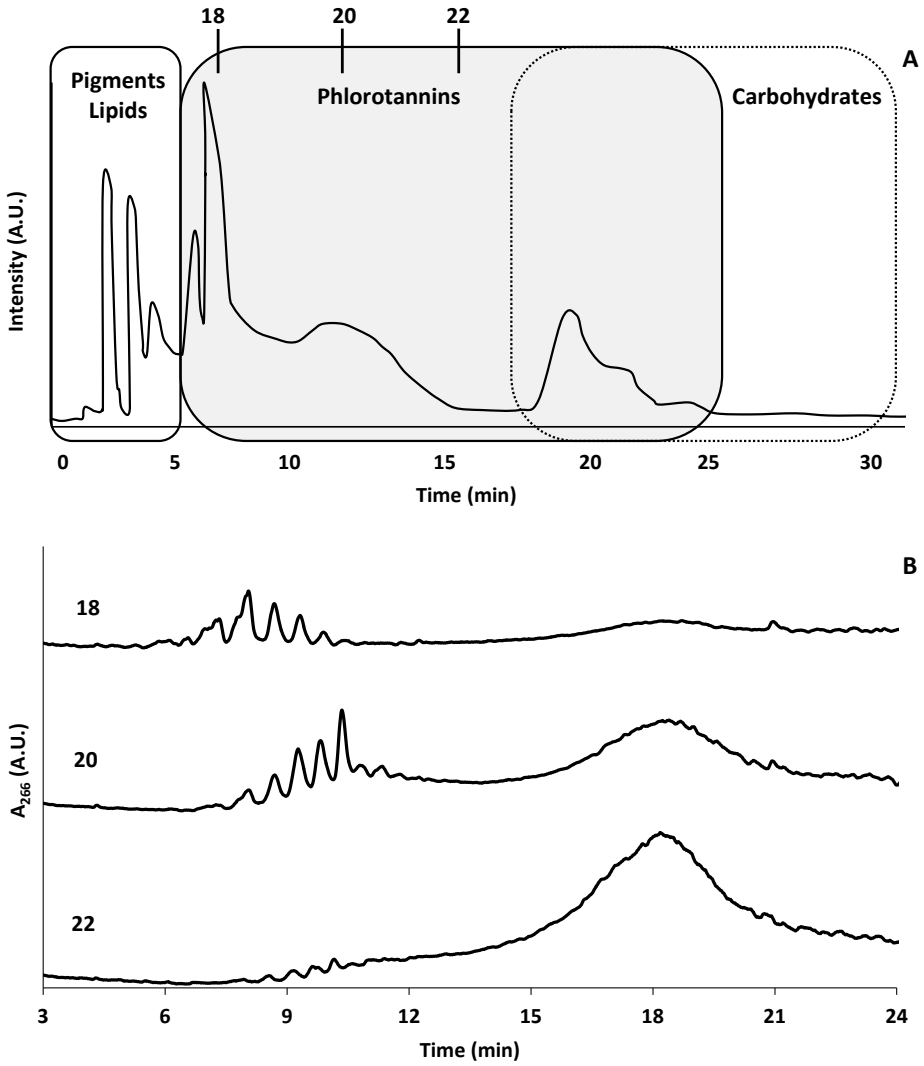

Figure 3 NP-Flash separation of pigments and phlorotannins in the crude extract of *Laminaria digitata* (A) and RP-UHPLC-UV chromatograms of fractions 18, 20, 22 at 266 nm (B)

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

No	$[M-H]^-$	MS^2	Type	Fractions
DP3				
2[a]	373	**305**[b], 247, 229[c]	Fucol	16
3	373	**305**, *231*[d]	Phlorethol	16
6	373	**247**, *233*, 229, 125	Fucophlorethol	16
DP4				
1	497	**461**, 435, 371, 353, 231	Fucol	16
4	497	371, **353**, 339, 249, 229	Fucol	16-18
22	509	441, **384**, 373, 305, 261	Fuhalol	16
DP5				
7	621	495, *479*, *461*, **373**, 355, 229	Fucophlorethol	16-19
8	621	373, *357*, 447, ***339***, 229	Fucophlorethol	16
15	621	**495**, 477, 371, 229	Fucol	16
27	651	607, 582, ***509***, *465*, 413, 339	Fuhalol	16
DP6				
5	745	**709**, 601, 579 , 455, 437, 289	Fucol	16-19
10	745	619, 601, **497**, 479, 353, 335, 229,	Fucol	16-22
11	745	*603*, 497, *478*, **371**, *355*, 229,	Fucophlorethol	16, 17
19	745	619, **601**, *479*, 461 *355*	Fucophlorethol	16
33	775	731, 633, **586**, 537, 463	Di-fuhalol	16, 17
DP7				
9[e]	869	**833**, 708, 579, 455	Fucol	16-20
13	869	833, 743, **725**, 707, 619, 601, 495, *477*, 371, *355*, 335	Phlorethol	16
16	869	743, ***727***, 621, 603, 479, 353	Phlorethol	20
24	869	**743**, 725, 477, *355*	Phlorethol	16
38	899	**589**, 663, 855, 537, 463, 373	Di-fuhalol	16
DP8				
12	993	**957**, 849, 831, 709, *603*, 353	Fucophlorethol	17
14[e]	993	**957**, 832, 371		17-21
DP9				
17	1117	**1081**, 973, *849*, 833, 707, 353	Fucophlorethol	18-22
18	1117	**1081**, 956, 745, *727*, 621, *603*, 582, 497, *477* 371, 351	Phlorethol	17-22
26	1117	1081, **973**, 993, 745, 727, 709, 621, *603*, 583, 495, 459, 247, 353	Fucophlorethol	17
DP10				
20	1241	**1205**, 1097, 1079, 975, 745, *727*, 601, 495,	Fucophlorethol	17-23
21	1241	1205, 1097, 1079, **745**, *727, 601, 477*	Phlorethol	17, 18

Table 1 continued

	[M-2H]$^-$			
DP11				
30	682	**610**, 601, 495, *469,* 229	Fucophlorethol	17
DP12				
23	744	*673*, *663*, 621, *601,* 495, 477, **371**, 229	Phlorethol	19-25
24	744	***673***, 663, *601*, 495, 477, 229	Phlorethol	17
DP13				
28	806	745, ***734***, 725, *662*, 601, 477, 371, 353	Phlorethol	18-27
33	806	725, *734*, 724, 672, *663*, 601, **477**, 229	Phlorethol	18
DP14				
31	868	***796***, 787, 745, 477, 495, 353	Phlorethol	18-27
35	868	*796*, 744, 619, **477**	Phlorethol	18-27
DP15				
25	930	*859*, **849**, 745, 734, 725, 495, 477, 371	Fucphlorethol	20-25
37	930	*859*, ***796***, 495, 477	Fucophlorethol	18
39	930	**867**, 805, *726*, 495, 477, 371, *354*	Phlorethol	18
DP16				
29	992	**911**, *849*, 725, 477, 353	Fucophlorethol	19-27
36	992	957, 911, 867, 849, *816*, 743, 727, 707, 619, 603, **477**, 495,	Phlorethol	18
39	992	911, **858**, *851*, 477, 353	Phlorethol	18
DP17				
32	1054	**973**, 955, 913, 725, 477	Phlorethol	19-27
DP18				
34	1116	**1035**, *975*, 477	Phlorethol	19-27

[a] numbering is according to elution order in time

[b] fragments in bold represent the main fragment ion

[c] fragments underlined represent diagnostic ions for phlorotannins

[d] fragments in italic represent diagnostic ions for phlorethols

[e] poor fragmentation

Variability in phlorotannin size and structure

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

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

in which 126 represents the molecular weight of the phloroglucinol subunit and DP represents the degree of polymerisation of the oligomer. This results in $[M\text{-}H]^-$ ions with *m/z* values 373, 497, 621, 745, 869, 993, 1117, 1241 for DP 3 to 10, respectively. These masses were annotated in the different fractions (**Table 1**). In case of eckol types, the molar mass of the subunit decreases two Da with each extra linkage, as two H atoms per monomer are lost during oxidative coupling of subunits [3]. Eckol types, however, were not annotated by LC-MS, confirming results of ^{13}C NMR spectroscopy.

Oligomers containing more than 10 subunits appeared as doubly charged ions; $[M\text{-}2H]^{2-}$ at *m/z* values 682, 774, 806, 868, 930, 992, and 1054 corresponding to DP11 to 18 (**Table 1**), respectively [5, 9]. The composition of each fraction varied, changing from low polymerisation degrees towards higher degrees when Flash elution progressed. The increased DP with increasing fraction showed that NP-Flash indeed separated PhT based on their DP, similar to proanthocyanidins.

The maximum degree of polymerisation detected by ESI-MS in our samples was 20. Oligomers of DP19 and DP20 had very low signal-to-noise ratio and, therefore, their fragmentation could not be further studied in MS2. Due to decreasing ionisation potential with increasing molecular weight [22], oligomers >DP20 were possibly not detected.

Phlorotannin isomers

The oligomeric fucol- and phlorethol linkage types differed in fragmentation behaviour. Fucol types (**Figure 4A**) fragmented at the aryl linkage, visible in the MS/MS spectrum by -125 for a deprotonated subunit or -126 for an uncharged subunit. Additionally, losses of 124*n* (with *n* the number of subunits) for oligomeric fragments [5, 23] were identified. These fragmentation patterns are indicative for PhT in general [5, 23, 24]. With phlorethol type fragmentation (**Figure 4B**), the pattern becomes more ambiguous than with fucol types as fragmentation can occur on either site of the ether-oxygen. There were the losses of 124*n* resulting from cleavage of phloroglucinol units, not including the ether oxygen. In addition, there were fragments at -142 (phloroglucinol + OH) for the uncharged and -141 for the deprotonated ion, resulting from cleavage including the ether oxygen. Both types of linkages were annotated in the fractions.

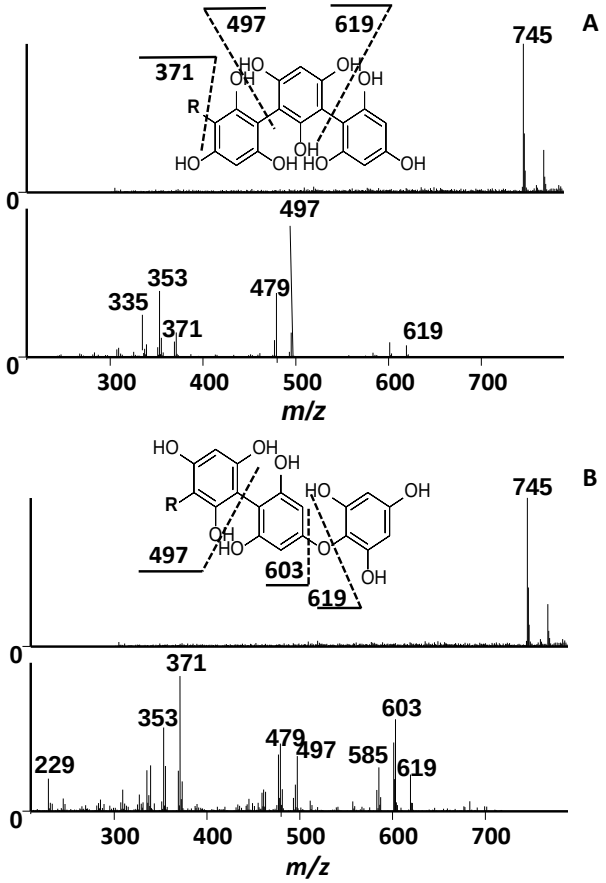

Figure 4 Mass fragmentation patterns and spectra $[M-H]^-$ for a DP6 fucol type (A) and fucophlorethol type (B). Structures are hypothetical, R = additional attachment of three subunits

Single charged ions

Single charged PhT oligomers from DP3 to DP10 were annotated in fractions 16-23 (**Table 1**). Monomers and dimers were not annotated during initial screening and, therefore, full mass spectra from 300-2,000 Da were recorded during further experimentation. DP3 was found in fraction 16 only, and consisted of one fucol type (**2**) and two phlorethol type (**3**, **6**) oligomers. With increasing fraction number, the lowest DP annotated increased and for each DP, several isomers were annotated. The oligomers **1**, **4** (both DP4), **9**, **15** (both DP5), **5** and **10** (both DP6), were fucols. All other single charged oligomers contained either phlorethol type of linkages, or were fucophlorethol type of oligomers. Fucophlorethol types contain both fucol and phlorethol linkages in their structure. Additionally, two fuhalol type of oligomers (**22**, DP4 and **29**, DP5) were annotated.

Within fucol type oligomers, the 2-, 4-, and 6-positions of phloroglucinol are available for extension of the molecule. Linking via these positions creates chains in which subunits are connected at *meta*-positions. Within phlorethol type oligomers the 1-, 3- or 5- hydroxyl positions generally couple to the 2-, 4- or 6-carbon position of the next ring. By using all six available linkage positions, two subunits flanking a central phloroglucinol subunit can extend this central subunit in *ortho*-, *meta*-, or *para*-fashion. In addition, if a phloroglucinol subunit is linked to ≥ 3 other subunits, branched oligomers are formed. The formation of branched oligomers can be expected as these are created by radical coupling which follows random patterns [3]. Taken together, the various possibilities for connecting subunits can create a plethora of isomers. Our mass fragmentation data gave information on the type of linkages present within the oligomer, but were not conclusive on linkage positions. The isomers differed in their retention times. It is expected that oligomers with linkages in *ortho*-positions or with branches had lower hydrodynamic volumes than those with *meta*- or *para*-connected subunits, and consequently eluted earlier. This would be in accordance with the elution of branched and linear carbohydrates in which branched ones elute before linear ones on RP-C18 columns [25].

Doubly charged ions

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

Phlorotannin degree of polymerisation

The use of MALDI-TOF-MS generally allows annotation of molecular weights beyond the detection limit of the ESI-MS [22]. The poor ionisation of components under the A_{266}-hump hinted at presence of PhT > DP18. MALDI-TOF-MS analysis on the crude extract (**Figure 5**) and individual fractions showed presence of oligomers from DP7 to DP27, $[M+Na]^+$ 890-3370. The mass differences between peaks resembled 124, the monomeric mass of phloroglucinol minus two H atoms to create a covalent bond. With increasing fraction number, larger DPs were detected, but with low intensities (SI **Figure S2A**). No *m/z* values above 3500 were visible. In fraction 42, the co-elution of carbohydrates from the Flash column might have hindered ionisation of PhT due to a high carbohydrate proportion in the fraction (SI **Figure S2B**). Due to the decrease in ionisation potential with increasing molecular weight, it cannot be excluded that PhT ≥ DP27 were present. To our knowledge, this is the first time that PhT up to DP27 were annotated. MALDI-TOF-MS analysis on PhT has been reported once before, on *Sargassum ringgoldianum*, in which tannins built from bifuhalol (264 Da) oligomers up to DP8 $[M+Na]^+$ 2136 were visualised [10].

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

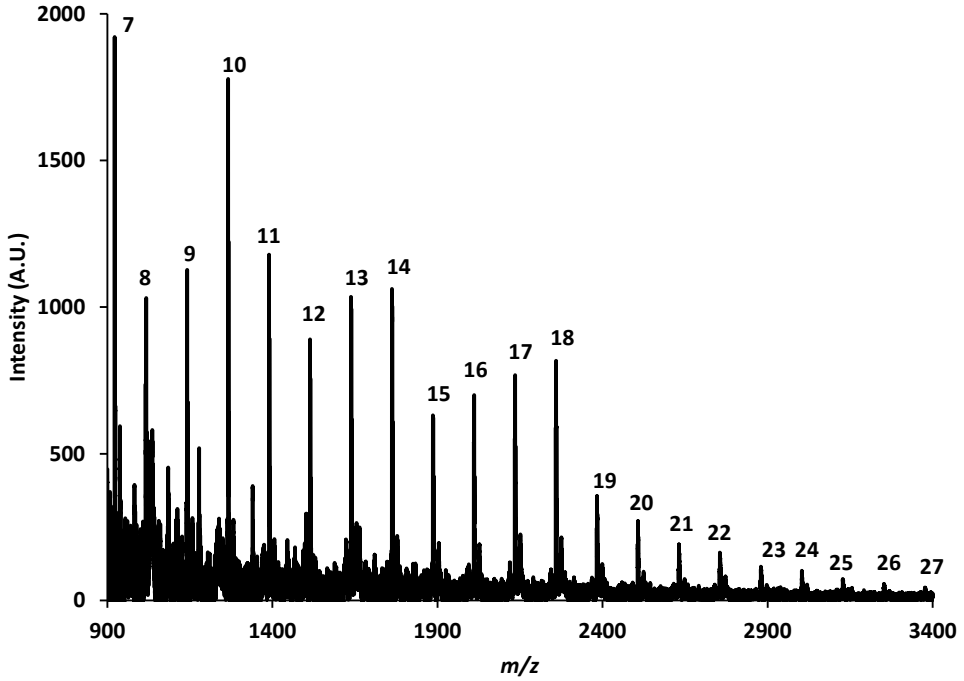

Figure 5 MALDI-TOF mass spectrum $[M+Na]^+$ of phlorotannins DP7-DP27 in the crude extract

Acknowledgements

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References

1. Holdt, S.L. and S. Kraan, Bioactive compounds in seaweed: Functional food applications and legislation. *J. Appl. Phycol.*, **2011**. 23: 543-597.
2. Van Soest, P.J., Tables of feed composition, *in Nutritional Ecology of the Ruminant*. **1994**, Cornell University: NY, USA.

3. Isaza Martínez, J.H. and H.G. Torres Castañeda, Preparation and chromatographic analysis of phlorotannins. *J. Chromatogr. Sci.*, **2013**. 51: 825-838.
4. Koivikko, R., J. Loponen, K. Pihlaja, and V. Jormalainen, High-performance liquid chromatographic analysis of phlorotannins from the brown alga *Fucus vesiculosus. Phytochem. Anal.*, **2007**. 18: 326-332.
5. Montero, L., M. Herrero, E. Ibáñez, and A. Cifuentes, Separation and characterization of phlorotannins from brown algae *Cystoseira abies-marina* by comprehensive two-dimensional liquid chromatography. *Electrophoresis*, **2014**. 35: 1644-1651.
6. Tierney, M.S., A. Soler-Vila, D.K. Rai, A.K. Croft, N.P. Brunton, and T.J. Smyth, UPLC-MS profiling of low molecular weight phlorotannin polymers in *Ascophyllum nodosum*, *Pelvetia canaliculata* and *Fucus spiralis. Metabolomics*, **2014**. 10: 524-535.
7. Appeldoorn, M.M., J.P. Vincken, M. Sanders, P.C.H. Hollman, and H. Gruppen, Combined normal-phase and reversed-phase liquid chromatography/esi-ms as a tool to determine the molecular diversity of a-type procyanidins in peanut skins. *J. Agric. Food Chem.*, **2009**. 57: 6007-6013.
8. Shibata, T., S. Kawaguchi, Y. Hama, M. Inagaki, K. Yamaguchi, and T. Nakamura, Local and chemical distribution of phlorotannins in brown algae. *J. Appl. Phycol.*, **2004**. 16: 291-296.
9. Steevensz, A.J., S.L. MacKinnon, R. Hankinson, C. Craft, S. Connan, D.B. Stengel, and J.E. Melanson, Profiling phlorotannins in brown macroalgae by liquid chromatography-high resolution mass spectrometry. *Phytochem. Anal.*, **2012**. 23: 547-553.
10. Nakai, M., N. Kageyama, K. Nakahara, and W. Miki, Phlorotannins as radical scavengers from the extract of *Sargassum ringgoldianum. Mar. Biotechnol.*, **2006**. 8: 409-414.
11. Schriemer, D.C. and L. Li, Mass discrimination in the analysis of polydisperse polymers by MALDI Time-of-Flight Mass Spectrometry. 2. Instrumental issues. *Anal. Chem.*, **1997**. 69: 4176-4183.
12. Kang, Y.M., S.H. Eom, and Y.M. Kim, Protective effect of phlorotannins from Eisenia bicyclis against lipopolysaccharide-stimulated inflammation in HepG2 cells. *Environ. Toxicol. Phar.*, **2013**. 35: 395-401.
13. Parys, S., A. Rosenbaum, S. Kehraus, G. Reher, K.W. Glombitza, and G.M. König, Evaluation of quantitative methods for the determination of polyphenols in algal extracts. *J. Nat. Prod.*, **2007**. 70: 1865-1870.
14. Balakshin, M.Y. and E.A. Capanema, Comprehensive structural analysis of biorefinery lignins with a quantitative ^{13}C NMR approach. *RSC Advances*, **2015**. 5: 87187-87199.
15. Van Alstyne, K.L., J.J. McCarthy Iii, C.L. Hustead, and L.J. Kearns, Phlorotannin allocation among tissues of northeastern Pacific kelps and rockweeds. *J. Phycol.*, **1999**. 35: 483-492.
16. Müller-Maatsch, J., A. Caligiani, T. Tedeschi, K. Elst, and S. Sforza, Simple and validated quantitative ^{1}H NMR method for the determination of methylation, acetylation, and feruloylation degree of pectin. *J. Agric. Food Chem.*, **2014**. 62: 9081-9087.
17. Slinkard, K. and V.L. Singleton, Total phenol analysis: automation and comparison with manual methods. *Am. J. Enol. Viticult.*, **1977**. 28: 49-55.
18. Egeland, E.S., Data sheets aiding identification of phytoplankton carotenoids and chlorophylls, *in Phytoplankton pigments - characterizaion, chemotaxonomy and applications in oceanography*, S. Roy, C.A. Llewellyn, E.S. Egeland, and G. Johnsen, Editors. **2011**, Cambridge University Press: NY, USA.
19. Parys, S., S. Kehraus, A. Krick, K.W. Glombitza, S. Carmeli, K. Klimo, C. Gerhäuser, and G.M. König, *In vitro* chemopreventive potential of fucophlorethols from the brown alga *Fucus vesiculosus* L. by anti-oxidant activity and inhibition of selected cytochrome P450 enzymes. *Phytochemistry*, **2010**. 71: 221-229.
20. Stern, J.L., A.E. Hagerman, P.D. Steinberg, F.C. Winter, and J.A. Estes, A new assay for quantifying brown algal phlorotannins and comparisons to previous methods. *J. Chem. Ecol.*, **1996**. 22: 1273-1293.
21. Van Alstyne, K.L., J.J. McCarthy Iii, C.L. Hustead, and D.O. Duggins, Geographic variation in polyphenolic levels of northeastern Pacific kelps and rockweeds. *Mar. Biol.*, **1999**. 133: 371-379.
22. Mouls, L., J.-P. Mazauric, N. Sommerer, H. Fulcrand, and G. Mazerolles, Comprehensive study of condensed tannins by ESI mass spectrometry: average degree of polymerisation and polymer distribution determination from mass spectra. *Anal. Bioanal. Chem.*, **2011**. 400: 613-623.
23. Ferreres, F., G. Lopes, A. Gil-Izquierdo, P.B. Andrade, C. Sousa, T. Mouga, and P. Valentão, Phlorotannin extracts from fucales characterized by HPLC-DAD-ESI-MS^{n}: Approaches to hyaluronidase inhibitory capacity and antioxidant properties. *Mar. Drugs*, **2012**. 10: 2766-2781.
24. Heffernan, N., N.P. Brunton, R.J. FitzGerald, and T.J. Smyth, Profiling of the molecular weight and structural isomer abundance of macroalgae-derived phlorotannins. *Mar. Drugs*, **2015**. 13: 509-528.
25. El Rassi, Z., Recent progress in reversed-phase and hydrophobic interaction chromatography of carbohydrate species. *J. Chrom. A*, **1996**. 720: 93-118.

Supporting information

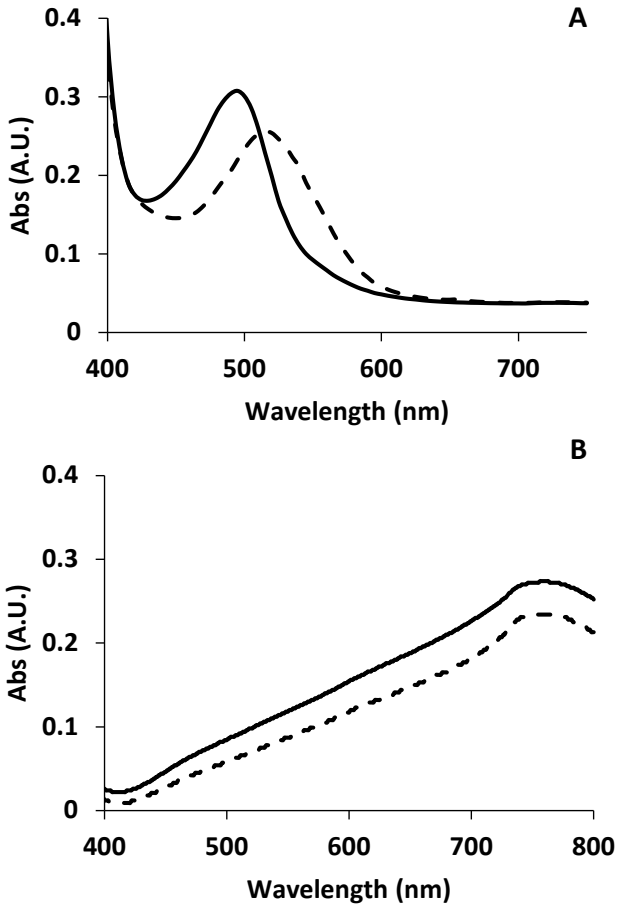


Figure S1 Absorbance spectra of 0.1 g/L phloroglucinol (solid line) and 0.15 g/L crude extract (dotted lines) in methanol, for the DMBA assay (A) and Folin-Ciocalteu assay (B)

Table S1 Molecular ions and fragment ions for compounds annotated in *Laminaria digitata* extract

Ion	UV_{max}	*m/z*	MS^2	Identification	Fraction
$[M-H]^-$	281, 318, 380	587	541, 507,	Peridininol	12-16
$[M-H]^-$		339	339, 295	Dihydroxy-prostaenoic acid	15
$[M-H]^-$		337	337, 319, 301, 275, 191, 145	5,6-Dihydroxy-eicosatrienoic acid	10
$[M-H]^-$		315	271, 253, 315, 203	9-Oxo-prostatetraenoic acid	10
$[M-H]^-$		311	311, 243, 183	Eicosatetaenoic acid	6 - 16
$[M-H]^-$		317	317, 299, 273, 255, 219, 163	Hydroxy-eicosopentaenoic acid	6 - 10
$[M+Na]^+$		409	391, 353, 325, 181	Cholesterol	14, 15
$[M+Na]^+$		435	417, 379, 351, 199, 181	Fucosterol	14, 15
$[M-H]^-$		327	327, 283, 265, 117	Dococahexaenoic acid	11 - 13
$[M+Na]^+$		487	487, 469, 403, 329	β-Sitosterol	14, 15,
$[M-H]^-$		355	355, 311, 293, 117	Trihydroxy-prostaenoic acid	6-11
$[M-H]^-$		381	381, 345, 337, 319, 117	trihydroxy-prostadienoic acid	6-11
$[M+H]^+$	442, 604, 650	593	533	Pheophorbide *a*	14, 15
$[M-H]^-$		319	319, 301, 275, 257, 207	Eicosatetraenoic acid	All
$[M-H]^-$		301	301, 257, 203	Eicosopentaenoic acid	All
$[M-H]^-$		303	303, 259, 205	Arachidonic acid	All

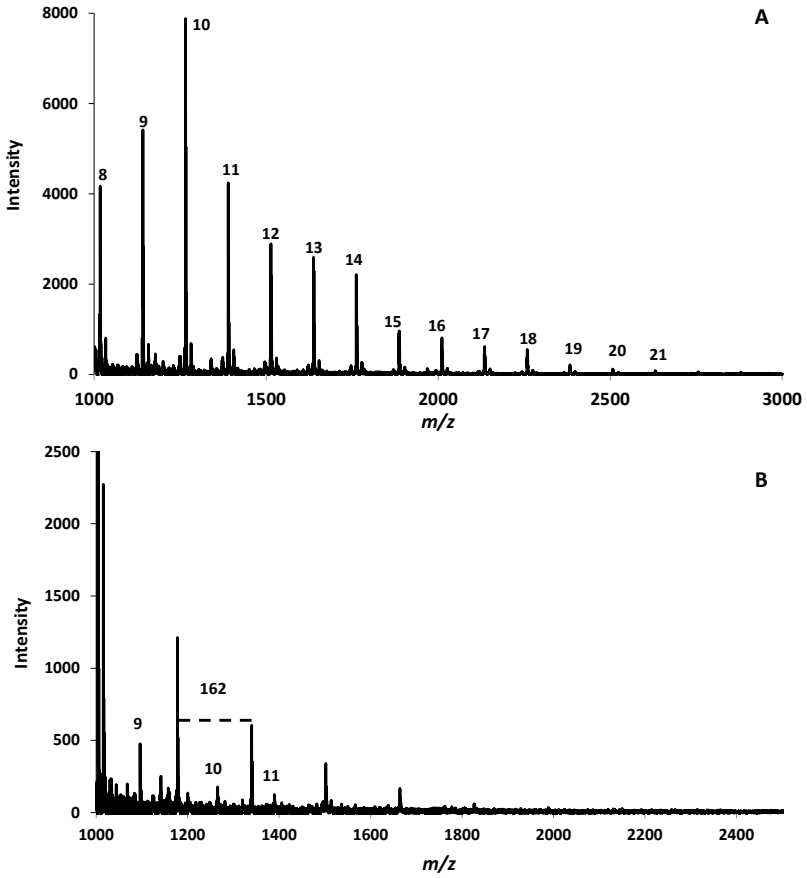


Figure S2. MALDI-TOF mass spectra $[M+Na]^+$ of phlorotannins in fraction 19 (A) and fraction 42, sugars were co-eluting (B)

Chapter 5

Re-solubilisation of protein from water insoluble phlorotannin-protein complexes upon acidification

Marine phlorotannins (PhT) from *Laminaria digitata* might protect feed proteins from ruminal digestion by formation of insoluble non-covalent tannin-protein complexes at rumen pH (pH 6-7). Formation and disintegration of PhT-protein complexes was studied with β-casein (random coil) and bovine serum albumin (BSA, globular) at various pH. Phlorotannins had similar binding affinity for β-casein and BSA as pentagalloyl glucose and higher than ellagic acid, as studied by fluorescence quenching. The affinity of PhT for both proteins was independent of pH (pH 3.0, 6.0 and 8.0). Addition of PhT increased the pH range of protein precipitation from 0.5 to ~1.5 pH unit around the proteins' pI. Complete protein re-solubilisation from insoluble PhT-protein complexes, was achieved at pH 7 and 2 for β-casein and BSA, respectively. It was demonstrated that PhT modulate the solubility of proteins at neutral pH, and that re-solubilisation of PhT-protein complexes at acidic pH is mainly governed by the charge state of the protein.

Based on: Anne Vissers, Annelies Blok, Adrie Westphal, Wouter Hendriks, Harry Gruppen, Jean-Paul Vincken – *Submitted to J. Agric. Food Chem.*

Introduction

Oligomeric phenolics, usually referred to as tannins, occur in both terrestrial and marine resources. In terrestrial resources, hydrolysable (e.g. ellagitannins (**Figure 1A**) and gallotannins like pentagalloyl glucose (**Figure 1B**)) [1] and condensed (CT) (e.g. proanthocyanidins, built from catechin and/or gallocatechin subunits) tannins [2] are distinguished. In marine resources, only brown macro algae contain tannins [3]. These latter tannins phlorotannins (PhT) and differ from terrestrial CT in their building block, i.e. phloroglucinol (**Figure 1C**). The phloroglucinol subunits are interconnected by carbon-carbon (C-C) or ether (C-O-C) bonds (**Figure 1D**) [3]. Phlorotannins with molar mass up to 1,738 Da have been fully characterised [3]. There are, however, indications that PhT can be larger [4]. In contrast to terrestrial tannins, there is little knowledge on the interactions between PhT and proteins. Terrestrial tannins can bind non-covalently to proteins and, in some cases, induce precipitation [5].

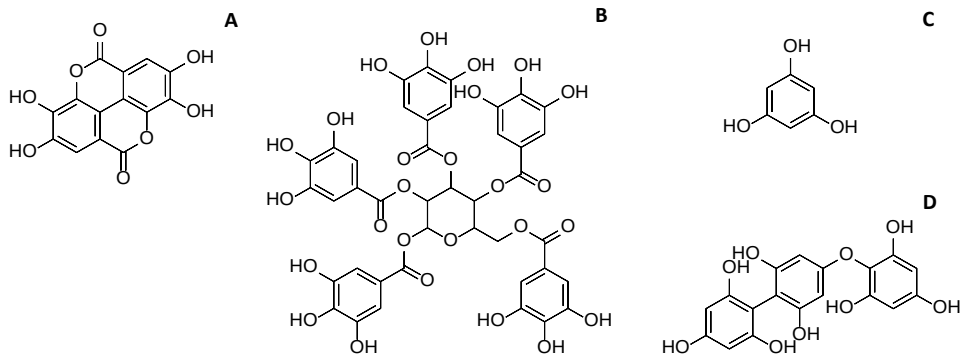


Figure 1 Ellagic acid (A), pentagalloylglucose (building blocks glucose and gallic acids) (B), as representatives of hydrolysable tannins. Phloroglucinol (C), the building block of phlorotannins and a representative phloroglucinol trimer in which phloroglucinol subunits are connected via C-C and C-O-C bonds (D)

The interaction between tannins and proteins is hydrophobically driven [6]. Tannins bind mainly to proline residues [5, 7], via CH-π stacking [8]. The connections are then reinforced by hydrogen bridges between the carbonyl oxygens of the peptide bonds, flanking the proline residue, and the phenolic hydroxyl groups of the tannin [5]. The resulting complexes can be either water soluble or water insoluble. Insoluble complex formation can occur at high tannin-to-protein molar ratios [5]. Additionally, increasing the molar mass of tannin (up to ~3,000 Da) [7], molecular flexibility [9] and a pH close to the protein's pI favor precipitation of complexes [10, 11]. Effects of pH on tannin-protein binding as such have not been reported [12] and are unlikely given the drivers for binding mentioned above. Tannins have higher affinity for polyethylene glycol (PEG) than for proteins due to the abundance of ether oxygens within the linear PEG polymers, which create ample hydrogen bonding opportunities along with hydrophobic interactions [13]. Challenging of tannin-protein complexes with PEG has shown that the interaction between tannin and protein is reversible [14].

Several adverse and beneficial effects of non-covalent tannin-protein complexation have been reported, i.e. reduced protein digestibility [15] and altered sensorial properties [16]. In the ruminants' digestive system, tannins are reported to have beneficial effects. Due to tannin-protein binding and formation of insoluble complexes, protein degradation within the rumen decreases, thereby, enabling delivery of intact protein further down the digestive tract [17]. It is generally assumed that the interactions within tannin-protein complexes leaving the rumen are weakened upon pH changes further down the digestive tract [11, 14]. It has been reported [14] that no protein was released from an insoluble CT-protein complex at pH 4-7, correlating to the rumen pH of 6-7 [18]. At pH 1-3, which is in the range of abomasum pH, proteins were released. This pattern suggests a diminished affinity of tannins to protein at low pH.

The binding of tannin to protein in the presence and absence of PEG at different pH is schematically presented in **Figure 2**. The properties of proteins are influenced by pH. At their pI, proteins have zero net charge and a lack of electrostatic repulsion [19], resulting in low solubility. Away from the pI, the net charge of the proteins increases resulting in enhanced protein solubility due to electrostatic repulsion. Important proteins in ruminant feed originate from grass and soy, which have wide pI ranges from 4.5-7 [20, 21]. The pH conditions, where no protein release from the tannin-protein complex was observed previously [14], correlate to pI values of the feed proteins.

In the absence of tannins, proteins precipitate in a range ~0.5 pH unit around their pI [22]. In the presence of tannins, the pH range for precipitation of tannin-protein complexes widens to 1-1.5 pH unit around the protein's pI [10, 23]. The more distant the pH is from the pI, the more charged the proteins become, as a result of which the tannin-protein complex can become water soluble (**Figure 2A**). In presence of PEG, tannins preferentially bind to PEG instead of protein, and the pH range for protein precipitation is similar to that without tannin, i.e. pI $\pm$ ~0.5 (**Figure 2B**). The wider pH range for protein precipitation by tannins is assumed to explain the protective effect against proteolysis during ruminal fermentation. Little is known about the behaviour of PhTs in relation to protein binding as affected by pH.

The aim of this research was to (i) determine the pH dependency of PhT to protein binding, and (ii) to map the release of protein from PhT-protein complexes as a function of pH. Based on the knowledge of terrestrial tannin-protein binding mechanisms and protein charge effects in relation to pH, it is hypothesised that PhT will bind to proteins in a similar manner as terrestrial tannins and that their binding affinities are independent of pH. The solubility of PhT-protein complexes in relation to pH is hypothesised to be related to the charge state of the protein. Despite the presence of PhT, protein solubility increases when pH << pI or pH >> pI, and protein can re-solubilise.

The experiments were conducted using β-casein, a random coil protein as this has been shown to be a good phenolics-binding protein [24]. In addition, bovine serum albumin (BSA), a model for globular proteins, was chosen because of its known binding of phenolics and its physiological function as transport protein [25]. Both proteins have a similar pI value, i.e. 5.1 [24] and 4.9 [26] for β-casein and BSA, respectively. The effect of tannin molar mass on binding to protein was tested by comparison of a PhT extract to ellagic acid (EA) and pentagalloyl glucose (PGG) (**Figure 1A** and **B**), representing building blocks for ellagitannins and gallotannins.

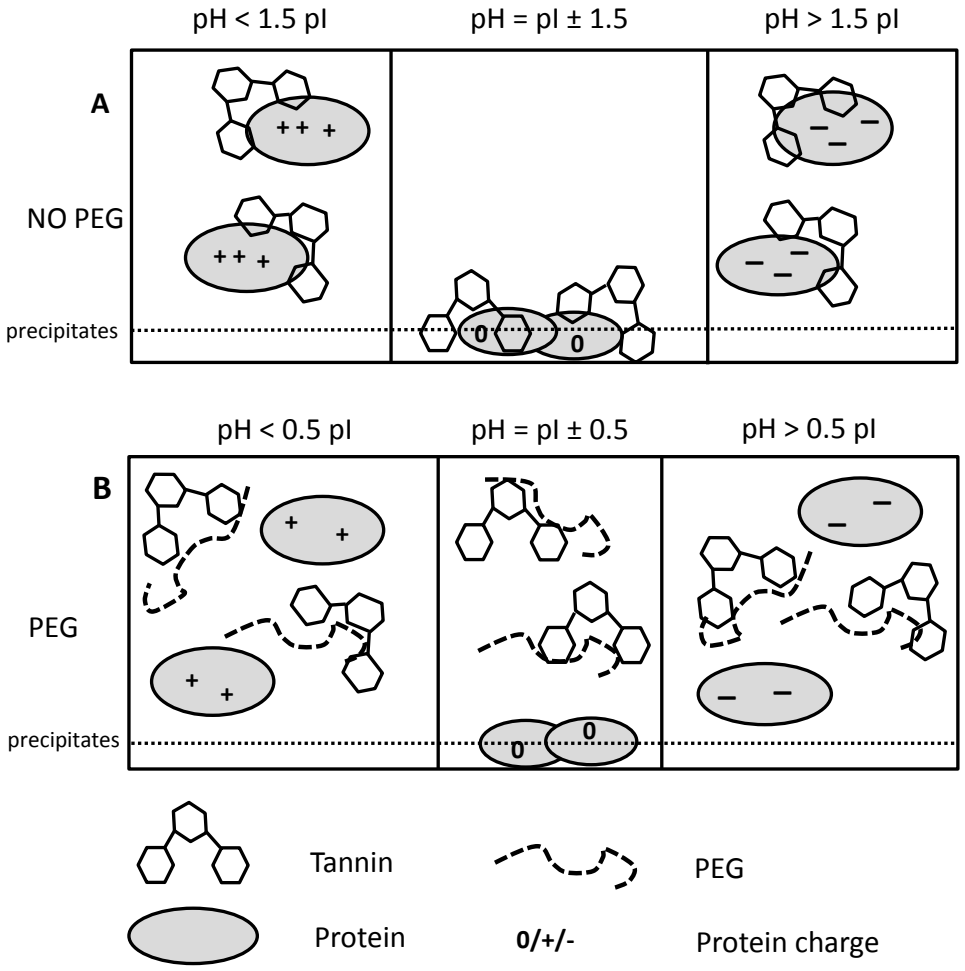


Figure 2 Model for tannin-protein complexation at pH < pI, pH = pI, pH > pI, without polyethylene glycol (PEG) (A) and with PEG (B)

Materials and methods

Materials

Bovine β-casein (98% w/w), BSA (96% w/w), EA (95% w/w) and PGG (96% w/w) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Kelp powder (*Laminaria digitata*) was obtained from Bristol Botanicals (Bristol, UK). Organic solvents used were of UHPLC-MS grade and obtained from Biosolve BV (Valkenswaard, The Netherlands). Water was obtained from a milli-Q system (Millipore, Billerica, MA, USA). All other chemicals were obtained from Sigma-Aldrich or Merck (Darmstadt, Germany).

Phlorotannin extraction

L. digitata powder (100 g) was suspended in 80% (v/v) aqueous methanol (25 g/L) and bead-milled using a DYNO®-mill, type MULTI-LAB (Bachhofen AG, Muttenz, Switzerland). The grinding chamber was filled (65% v/v) using 0.5 mm silica beads and macro algae were ground for 1.5 h using a flow rate of 0.3 L/min. Afterwards, the suspension was filtered over a cellulose filter (cut off: 8-12 µm Whatman®) and methanol was evaporated by rotary-evaporation (R-210/215, Büchi Labortechnik, Flawil, Switzerland). The concentrated extract was diluted with 0.1 M sodium chloride (1:1 v/v). This suspension was partitioned with ethyl acetate (1:1 v/v) and after separation, both layers were collected separately. The aqueous phase was cleaned twice with 200 mL ethyl acetate while the organic phase was cleaned twice with 100 mL 0.2 M sodium chloride. The three organic phases were pooled and dried by rotary evaporation, dissolved in methanol and stored at -20 °C until further analysis. This material was denoted as PhT extract solution.

NP-Flash chromatography

The PhT extract solution was subjected to a NP-Flash chromatography unit (Grace, Deerfield, IL, USA), equipped with an ELS detector and an UV detectors (set at 400 nm), using a 12 g silica column (18 mL column volume, particle size 40 µm, Grace). The extract (250 mg) was mixed with 2.5 g silica and injected via dry loading. The compounds were eluted using a combination of hexane (A), acetone (B) and methanol (C) at a flow rate of 30 mL/min. The elution profile was as follows: 0-1 min isocratic at 100% A, 1-9 min 0-100% B, 9-11 min, isocratic at 100% B, 11-19 min 0-20% C, 19-23 min 20-100% C and 23-25 min isocratic at 100% C. Phlorotannins eluted between 8-25 min. Between 8-11 min, PhT co-eluted with pigments while PhT eluting between 20-25 min co-eluted with sugars (annotated by MALDI-TOF-MS). In order to limit contamination with pigments and sugars, only the PhT fraction eluting between 11-20 min was used for further experimentation.

Fluorescence quenching assay

Non-covalent tannin-protein binding was studied by fluorescence quenching, using the intrinsic fluorescence of tryptophan residues. The tryptophan emission around 350 nm [27] is quenched by binding of ligands. Sodium phosphate buffers (10 mM) of pH 3.0, 6.0, and 8.0 were prepared by mixing 10 mM Na_2HPO_4 and 10 mM NaH_2PO_4 solutions in the correct amounts to obtain the set pH values. Protein stock solutions of either 10 µM β-casein or BSA were prepared these buffers. Protein concentration was determined using molar absorption at 280 nm and molar extinction coefficients of 11,443 $M^{-1}cm^{-1}$ [24] and 43,824 $M^{-1}cm^{-1}$ [28] for β-casein and BSA, respectively were used. Tannin solutions were prepared by dissolving a PhT Flash fraction (1 g/L) in 10 mM sodium phosphate

buffer pH 8.0, 6.0 and 3.0 containing 10% (v/v) methanol. These were further diluted in the same buffers to obtain concentration ranges from 0-0.1 g/L. Ellagic acid and PGG solutions (0-100 µM) were prepared in the three buffers, mentioned above.

Quenching experiments were performed in SterilinTM black microtiter plates (Thermo Scientific, San Jose, CA, USA). For determination of the binding curves, 100 µL protein solutions were mixed with 100 µL tannin solutions, in triplicate. These mixtures were incubated for 10 min in the dark at 25 °C under continuous shaking at 300 rpm (Thermomixer comfort, Eppendorf, Hamburg, Germany). Intrinsic protein fluorescence was measured in a Spectramax M2^e microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 25 °C, with λ_{ex} = 280 nm, λ_{em} = 300-600 nm with 10 nm band width.

When performing fluorescence quenching, inner filter effects related to absorbance of the ligand in both the excitation and emission wavelengths were corrected when necessary [29]. Corrections were performed using equation (1) [30, 31].

$$F_{corr} = F_{obs} \times 10^{(A_{ex} \times d_{ex} + A_{em} \times d_{em})} \quad (1)$$

in which F_{corr} = corrected fluorescence intensity, F_{obs} = observed fluorescence intensity at emission wavelength, A_{ex} and A_{em} are the absorbances measured at excitation and emission wavelength, respectively, d_{ex} and d_{em} refer to the relative path lengths in excitation and emission direction, respectively. The actual path length was determined according to the manual of the supplier [32], using the pH 8.0 buffer. The path length was determined to be 0.05 cm and assumed to be equal for both d_{em} and d_{ex}.
After correction, data were analysed using least square regression analysis with a 1:1 binding model assuming the formation of a non-fluorescent protein-tannin complex using equation (2) [30].

$$[PT] = \frac{([P] + [T] + K_d) - \sqrt{([P] + [T] + K_d)^2 - 4 \times [P] \times [T]}}{2} \quad (2)$$

In which [PT] is the molar concentration of the protein-tannin complex, [P] is the molar protein concentration, [T] is the molar tannin concentration and K_d the dissociation constant. As the exact molar weight of the PhT mixture was not known, an apparent K_d ($K_{d\text{-}app}$) was determined under that assumption of an average PhT MW of 2,000 Da.

Protein precipitation assay

Sodium phosphate buffers (10 mM) of pH 3.0, 4.0, 5.0, 6.0, 6.5, 7.0 and 8.0 were prepared as described above. The PhT Flash pool (10 g/L in methanol) was diluted up to 1 g/L in the different buffers. β-Casein and BSA were each dissolved in water at 2 g/L. To induce complexation, 1 mL buffer, 1 mL protein solution and 1 mL PhT solution were mixed in a glass tube and incubated at room temperature for 15 min. Final pH was measured after complexation and, to avoid dilution, not adapted when changes occurred. In those cases, the actual pH values are provided in the results section. The tubes were centrifuged (4,000 × *g*, 10 min, 20 °C). The PhT content in the supernatants was assayed using a colorimetric 2,4-dimethoxybenzladehyde assay [4]. The PhT concentration was determined using a calibration curve (0-0.8 g/L) of a PhT extract of known purity (60% w/w [33]).

Reversibility of phlorotannin-protein binding

The re-solubilisation of protein from the PhT-protein complex was determined based on a previously described protocol [14], with adaptations. Briefly, 2 g/L BSA or 1 g/L β-casein solution in 10 mM sodium phosphate buffer (pH 6.0) were prepared. Of these solutions, 1 mL was mixed with 0.1 mL (for BSA) or 0.05 mL (for β-casein) PhT Flash pool (10 g/L in methanol). The mixtures were incubated for 24 h at 39 °C and then centrifuged (10,000 × *g*, 5 min, 20 °C). The supernatant was carefully removed with a pipet. For the first series, PEG (10 mg) was added as powder to the pellet and mixed 1 mL 10 mM sodium phosphate buffer of various pH values (pH = 2.0-8.5). For the second series, only buffers were used. The mixtures were incubated for 2 h at 39 °C and 500 rpm (Thermomixer comfort, Eppendorf) and centrifuged (10,000 × *g*, 5 min, 20 °C). Controls using only protein were treated in the same way. The protein content in the supernatant was determined using the DUMAS method with a Flash EA 111 NC analyser (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Nitrogen conversion factors were 6.37 and 6.08 for β-casein and BSA, respectively. The factors were calculated based on the amino acid sequences. As only 75% of the initial protein present precipitated, the re-solubilisation of BSA from the precipitate was corrected for protein loss in the supernatant. For β-casein, all the proteins precipitated and no corrections were required. The percentage of protein re-solubilised into solution was calculated using equation (3).

$$P_r = \frac{P_s}{P_p} \times 100\% \qquad (3)$$

in which P_r, P_s and P_p represent the amounts of protein (mg) re-solubilised, present in solution after re-solubilisation, and present in the initial pellet, respectively. The PhT content in the supernatants was determined colorimetrically as described above. Interactions between proteins and PEG were determined using fluorescence quenching as described above. Determination was done at pH 7.0 for PEG-to-protein weight ratios of 0.5, 1, 2, 4, 8, 16 and 32.

Results and Discussion

For the application of PhT in ruminant feed, their protein binding behaviour was studied. It was hypothesised that the binding behaviour of PhT from *L. digitata* was similar to terrestrial tannins and that binding would not be affected by pH.

Binding of phlorotannin, ellagic acid and pentagalloyl glucose

The binding behaviour of PhT (polydisperse mixture with estimated average MW 2,000 Da) to β-casein and BSA was compared to those of EA of 302 Da and PGG of 904 Da. Binding was studied using fluorescence quenching at pH 8.0. The intensity of the tryptophan fluorescence emission spectra of both β-casein (**Figure 3A**) and BSA (**Figure 3B**) decreased upon addition of tannins. For some tannins, shifts in maximum emission wavelength can occur upon binding to proteins [30], but this was not observed for the tannins tested. For comparison (**Figures 3C** and **3D**), the normalised relative emissions at 350 nm were plotted versus the tannin concentration. The fluorescence decreased steeply at low tannin concentrations, and levelled off at higher concentrations. A similar relationship has been obtained in other model systems using proteins combined with catechins [30], terrestrial condensed [34] and hydrolysable tannins [9].

The binding parameters of the ligands (K_d for PGG and EA, $K_{d\text{-}app}$ for PhT) for both proteins at pH 8.0 are summarised in **Table 1**. A lower K_d value reflects stronger binding. For both proteins, binding affinity for PGG and PhT was in similar order of magnitude. Ellagic acid had a low binding affinity for both proteins. Binding of EA to β-casein was similar to that of epigallocatechin gallate to this protein.[30] For both proteins, an increase in binding affinity correlated with phenolic molecular weight and molecular flexibility. Flexible phenolics, like PGG have high binding affinity to proteins as they can orient themselves towards the protein. Ellagic acid is relatively small and rigid; it does not contain groups with rotational freedom. For EA, there were no differences in binding affinity between proteins. In case the protein is flexible as well, like β-casein, the protein can orient itself towards the phenolic compounds. Bovine serum albumin, however, has usually higher binding affinities than expected for globular proteins as it contains hydrophobic sites with high affinity for phenolics [7, 35]. These factors could have resulted in similar affinities for both proteins.

Within the PhT mixture used, phloroglucinol oligomers DP3-27 (250-3,348 Da) have been annotated [33]. It is likely that medium size PhT (~ 600-1,500 Da, DP 5-12) have the highest affinity to proteins, in analogy to ellagitannin oligomers [9]. Karonen *et al*. (2015) found that purified ellagitannin oligomers from tetramers to octamers had similar binding affinities to BSA, whereas those of dimers and trimers were lower. Ellagitannin flexibility was more determinant for binding than the actual molar mass [9]. Following these relations, it was expected that PhT behaved similarly to flexible terrestrial tannins. Phlorotannins in *L. digitata* all consist of phoroglucinol units linked by C-C and C-O-C linkages [3]. With each additional subunit, one or two additional free rotating bond(s), respectively, are incorporated in the PhT oligomer. For the PhT mixture, results were comparable to those obtained previously [36, 37], based on binding of crude tannin mixtures to salivary α-amylase.

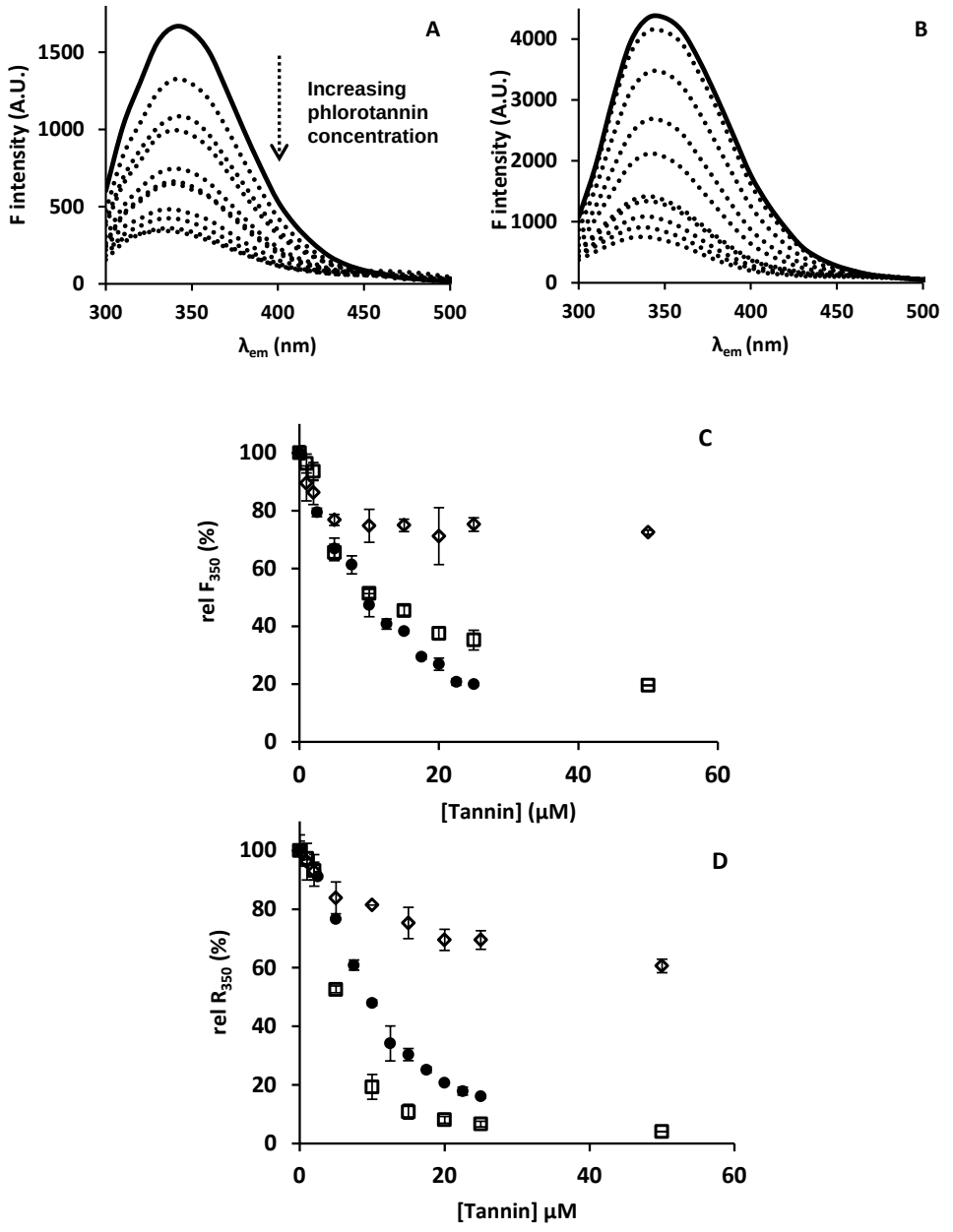


Figure 3 Fluorescence emission spectra (λ_{ex} = 280 nm) of β-casein (A) and BSA (B) quenched by increasing tannin concentration. Normalised fluorescence (λ_{em} = 350 nm) binding curves for binding of phlorotannins (0-25 µM, assuming an average MW = 2,000 Da) (●), pentagalloylglucose (0-50 µM) (□) and ellagic acid (0-50 µM) (◊) to β-casein (B) and BSA (D) at pH 8.0

Effect of pH on phlorotannin-protein binding

With decreasing pH, the emission maxima of BSA (**Figure 4A**) shifted from 345 nm at pH 6.0 and 8.0 to 355 nm at pH 3.0, in accordance with earlier observations [27, 38]. The $K_{d\text{-}app}$ values (**Table 1**) showed no pH-effect for binding of PhT to either β-casein (**Figure 4B**) or BSA (**Figure 4C**). For the occurrence of PhT-protein binding, it did not matter whether the protein had a net negative (pH > pI) or positive (pH < pI) charge, as binding affinities were in similar orders of magnitude and showed no trend. The results indicated that pH as such was not a significant contributor to affinity of PhT for proteins, in analogy to terrestrial tannins [37].

Table 1 Summary of the interactions (K_d and $K_{d\text{-}app}$) of pentagalloyl glucose (PGG), ellagic acid (EA) and phlorotannins (PhT) to β-casein and BSA at various pH conditions, determined using fluorescence quenching

Ligand	pH	K_d (× 10^{-6} M)	
		β-Casein	BSA
PGG	8.0	15.9 (±0.04)	5.6 (±0.6)
EA	8.0	261.8 (±40.7)	290.0 (±33.4)
PhT [1]	8.0	11.4 (±0.5)	9.5 (±0.2)
PhT	6.0	12.9 (±1.8)	6.0 (±0.03)
PhT	3.0	18.1 (±0.9)	10.7 (±0.1)

[1] for PhT, $K_{d\text{-}app}$ was determined assuming an average MW = 2,000 Da

Protein precipitation by phlorotannins

As PhT-protein binding occurred at every pH, the effects of the pH on the type of aggregate formed (soluble or insoluble) was studied. The formation of insoluble aggregates was studied by performing a precipitation assay [23]. Phlorotannins were able to precipitate both β-casein and BSA at pH conditions around the proteins' pIs. Upon PhT addition, the initially transparent solutions turned turbid immediately, indicating rapid complexation.

β-Casein. Around its pI, β-Casein alone precipitated, whereas in the presence of PhT the pH range at which this protein precipitated was broadened, as expected (**Figure 5A**). After complexation, 60-90% of the initial PhT was still detected in the supernatant from pH 3.0-6.4 (**Figure 5B**), consistent with the range of protein precipitation. In all cases, supernatants were turbid. The turbidity of the supernatant indicated the presence of smaller complexes which did not precipitate upon centrifugation [5].

BSA. Without tannins, BSA was soluble over the entire pH range (**Figure 5C**). In presence of PhT, complete protein precipitation occurred around the pI. The PhT co-precipitated with the protein for 78% (**Figure 5D**), resulting in transparent colourless solutions after centrifugation. The samples at pH < 4.8 and > 5.5 were yellow, suggesting that not all tannins had precipitated.

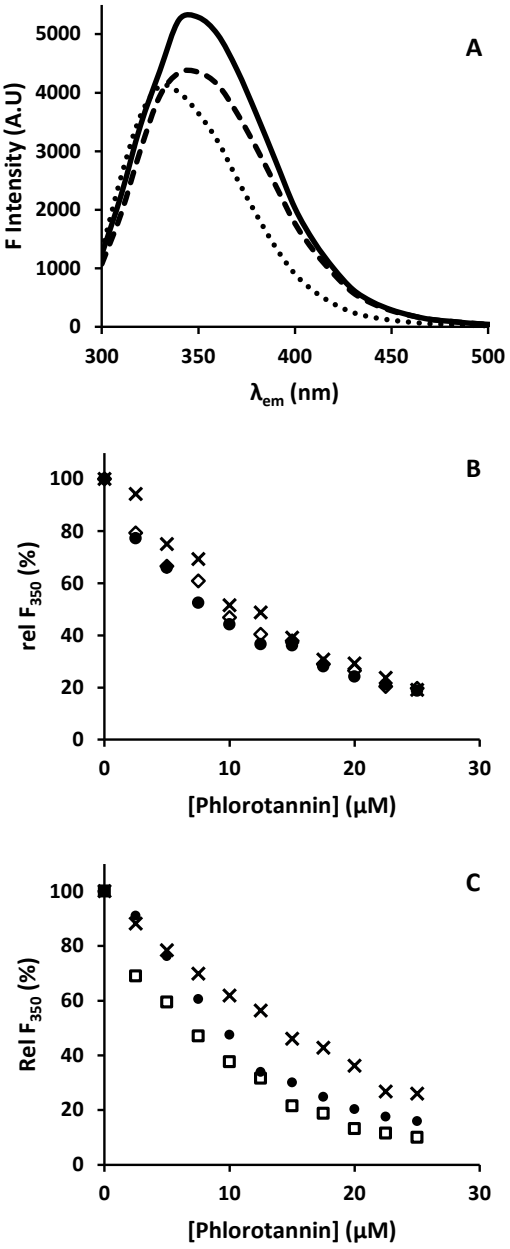
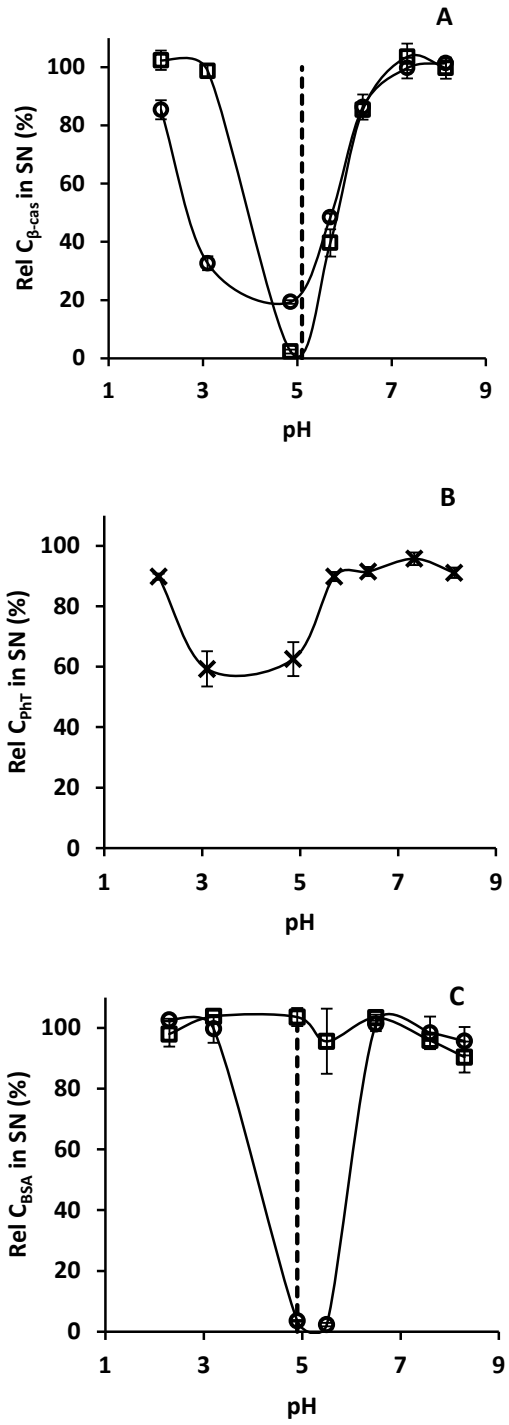


Figure 4 Fluorescence spectra of BSA (λ_{ex} = 280 nm) at pH 8 (—), pH 6.0 (- -) and pH 3.0 (···) (A). Normalised fluorescence intensity, λ_{em} = 350 nm, of 5 µM β-casein (B), 5 µM BSA (C) quenched by increasing phlorotannin (PhT) concentration (0-25 µM, assuming an average MW = 2,000 Da) at pH 8.0 (●), 6.0 (□) and 3.0 (×)


A
Rel $C_{\beta\text{-cas}}$ in SN (%)
100
80
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40
20
0
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B
Rel C_{PhT} in SN (%)
100
80
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20
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1
3
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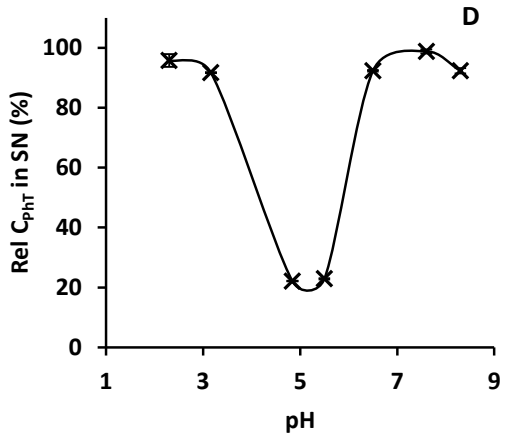

Figure 5 Proportions (%) of soluble protein (A, C) and soluble phlorotannin (PhT) (×, B,D) in supernatant (SN) after precipitation of protein (□) or mixtures of PhT/protein (❍) for β-casein (A, B) and BSA (C, D) as function of pH, (- -) represents proteins' pI.

Protein re-solubilisation

Phlorotannins and proteins were incubated at pH 6.0 and 39 °C, and after 24 h the suspensions were centrifuged. Subsequently, the insoluble PhT-protein complexes (pellets) were exposed to various pH conditions, in presence or absence of PEG, to study re-solubilisation of these insoluble PhT-protein complexes. As it was impossible to determine whether solubilised protein and PhT were still bound to each other, we prefer to speak of protein re-solubilisation rather than of protein release. There were no interactions between PEG and proteins as determined by fluorescence quenching (data not shown). The addition of PEG resulted in formation of soluble PhT-PEG complexes as no pellet was formed upon centrifugation of PhT/PEG combinations. It should be noted that the PhT concentration could no longer be quantified in presence of PEG as reactivity towards the DMBA reagent decreased.

Re-solubilisation in absence of PEG. When PEG was absent in the solution, there was complete re-solubilisation of β-casein at pH 7, with and without PhT (**Figure 6A**). At pH 4 and 5 there was no re-solubilisation, independent of PhT, correlating to the pI range of β-casein. Unexpectedly, even at pH 2 only 4% of the β-casein was re-solubilised, whereas that value was 26% in absence of PhT. At pH 2, the protein has a net positive charge of +22, which should be sufficient for re-solubilisation, considering that a charge of -7.7 at pH 7 yielded only soluble protein (complexes).

For the BSA/PhT combination (**Figure 6B**), there was no re-solubilisation at pH 4 and 5, around BSA's pI. There was complete re-solubilisation in pH 2, whereas 43% BSA released at pH 7. The higher re-solubilisation further away from BSA's pI might be related to the charge of the protein. At pH 3, BSA (mature protein) has a charge of +164 while at pH 7, the charge is only -29 [39]. The higher

positive charge at pH 3 compared to the negative charge at pH 7 might result in higher protein solubility. For both proteins, complete protein re-solubilisation was associated with minor PhT re-solubilisation, 24% for the PhT/β-casein combination and 12% for PhT/BSA**.** The tannins remained insoluble in fully aqueous solutions as opposed to the 10% (v/v) methanolic buffers used to prepare the complexes.

Re-solubilisation in presence of PEG. In presence of PEG, the proteins re-solubilised from the tannins due to the high affinity of tannins for PEG, depending on the pH. In presence of PEG, there was a slightly higher re-solubilisation of β-casein from the PhT-β-casein complexes than in its absence (**Figure 6A**), 20% and 14% for pH 2 and 3, respectively. For BSA, PEG increased the protein re-solubilisation from PhT-BSA complexes with 28-67% from pH 3-7 (**Figure 6B**) without affecting the overall shape of the BSA solubility curve. Even around the pI of BSA, 30% protein re-solubilised.

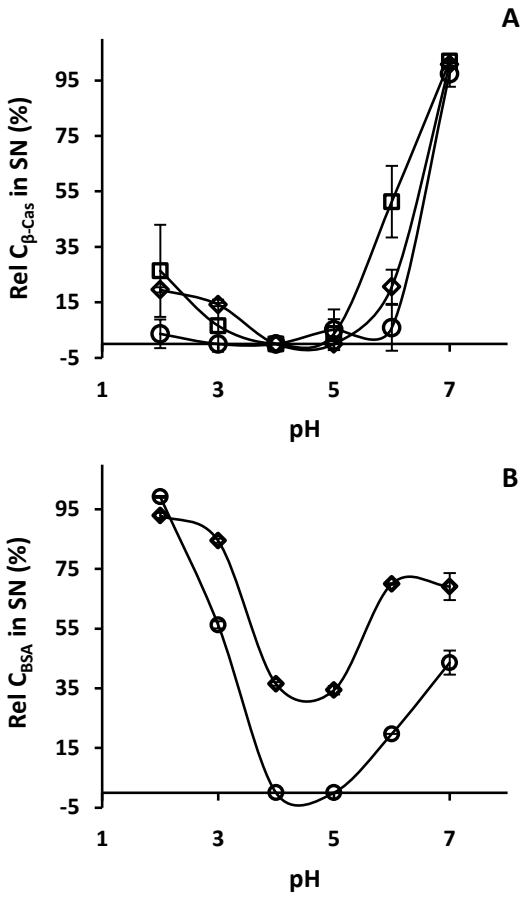

Figure 6 Protein (%) re-solubilisation from precipitated protein (□), PhT/protein combinations (❍) and PhT/protein/PEG combinations (◊) for β-casein (A) and BSA (B) as function of pH

The results of the PEG treatment agree with a previous study [14]. Despite PEG addition, not all proteins re-solubilised and it might be that the insoluble PhT-protein complex consisted of proteins entangled with PhT, thereby, maintaining their insolubility. When no complete protein re-solubilisation was achieved, it was assumed that the strength of electrostatic repulsions within the protein were insufficient to bring large aggregates into solution.

Overall, our results show that the proteins re-solubilise together with minor amounts of PhT, but our experiments are inconclusive about their actual release. In presence of PEG, our results show that proteins indeed re-solubilise more extensively. The addition of PEG is not required to assess protein re-solubilisation behaviour, but its addition confirms that PhT is able to delay the re-solubilisation of proteins, until more extreme pH conditions are reached.

Extrapolation to protein protection and digestion in ruminants

Regarding the application of tannins in ruminal feed to act as protein protecting agents, the various pH regimes along the digestive tract need to be taken into account. Due to the widening of the pH range for protein precipitation by PhT, insoluble PhT-protein complexes are created at rumen pH. When the pH decreases to far below the pI for globular proteins (as in the abomasum), the increased charge of proteins re-solubilises protein again. As there is minor tannin re-solubilisation, minor re-complexation of tannins and proteins can be expected. The re-solubilisation is determined by the charge of the proteins, rather than by their release from tannins as reported previously [14] (**Figure 2**). The re-solubilisation of proteins is expected to facilitate efficient digestion of feed proteins after ruminal fermentation.

References

1. Arapitsas, P., Hydrolyzable tannin analysis in food. *Food Chem.*, **2012**. 135(3): 1708-1717.
2. Quideau, S., D. Deffieux, C. Douat-Casassus, and L. Pouységu, Plant polyphenols: Chemical properties, biological activities, and synthesis. *Angew. Chem. Int. Edit.*, **2011**. 50(3): 586-621.
3. Isaza Martínez, J.H. and H.G. Torres Castañeda, Preparation and chromatographic analysis of phlorotannins. *J. Chromatogr. Sci.*, **2013**. 51(8): 825-838.
4. Montero, L., M. Herrero, E. Ibáñez, and A. Cifuentes, Separation and characterization of phlorotannins from brown algae *Cystoseira abies*-marina by comprehensive two-dimensional liquid chromatography. *Electrophoresis*, **2014**. 35(11): 1644-1651.
5. Haslam, E., Natural polyphenols (vegetable tannins) as drugs: Possible modes of action. *J. Nat. Prod.*, **1996**. 59(2): 205-215.
6. Kilmister, R.L., P. Faulkner, M.O. Downey, S.J. Darby, and R.J. Falconer, The complexity of condensed tannin binding to bovine serum albumin - An isothermal titration calorimetry study. *Food Chem.*, **2016**. 190: 173-178.
7. Deaville, E.R., R.J. Green, I. Mueller-Harvey, I. Willoughby, and R.A. Frazier, Hydrolyzable tannin structures influence relative globular and random coil protein binding strengths. *J. Agric. Food Chem.*, **2007**. 55(11): 4554-61.
8. Hagerman, A.E. and L.G. Butler, The specificity of proanthocyanidin-protein interactions. *J. Biol. Chem.*, **1981**. 256(9): 4494-4497.

9. Karonen, M., M. Oraviita, I. Mueller-Harvey, J.-P. Salminen, and R.J. Green, Binding of an oligomeric ellagitannin series to bovine serum albumin (BSA): analysis by isothermal titration calorimetry (ITC). *J. Agric. Food Chem.*, **2015**. 63(49): 10647-10654.
10. Naczk, M., D. Oickle, D. Pink, and F. Shahidi, Protein precipitating capacity of crude canola tannins: Effect of pH, tannin, and protein concentrations. *J. Agric. Food Chem.*, **1996**. 44(8): 2144-2148.
11. Perez-Maldonado, R.A., B.W. Norton, and G.L. Kerven, Factors affecting in vitro formation of tannin-protein complexes. *J. Sci. Food Agric.*, **1995**. 69(3): 291-298.
12. Rawel, H.M., K. Meidtner, and J. Kroll, Binding of selected phenolic compounds to proteins. *J. Agric. Food Chem.*, **2005**. 53(10): 4228-4235.
13. Jones, D.E., Banana tannin and its reaction with polyethylene glycols. *Nature*, **1965**. 206(4981): 299-300.
14. Jones, W.T. and J.L. Mangan, Complexes of the condensed tannins of sainfoin (*Onobrychis viciifolia* scop.) with fraction 1 leaf protein and with submaxillary mucoprotein, and their reversal by polyethylene glycol and pH. *J. Sci. Food Agric.*, **1977**. 28(2): 126-136.
15. Jansman, A.J., Tannins in feedstuffs for simple-stomached animals. *Nutr. Res. Rev.*, **1993**. 6(1): 209-36.
16. Cala, O., N. Pinaud, C. Simon, E. Fouquet, M. Laguerre, E.J. Dufourc, and I. Pianet, NMR and molecular modeling of wine tannins binding to saliva proteins: Revisiting astringency from molecular and colloidal prospects. *FASEB J.*, **2010**. 24(11): 4281-4290.
17. Tedeschi, L.O., C.A. Ramírez-Restrepo, and J.P. Muir, Developing a conceptual model of possible benefits of condensed tannins for ruminant production. *Animal*, **2014**. 8(7): 1095-1105.
18. Van Soest, P.J., Rumen microbes, *in Nutritional Ecology of the Ruminant*. **1987**, Cornell University: NY, USA. 152-177.
19. Singh, K., M. Shandilya, S. Kundu, and A.M. Kayastha, Heat, acid and chemically induced unfolding pathways, conformational stability and structure-function relationship in wheat α-amylase. *PLoS ONE*, **2015**. 10(6).
20. Pearsall, W.H. and J. Ewing, The isoelectric points of some plant proteins. *Biochem. J.*, **1924**. 18(2): 329-339.
21. Petersen, A., R. Suck, S. Hagen, O. Cromwell, H. Fiebig, and W.M. Becker, Group 13 grass allergens: structural variability between different grass species and analysis of proteolytic stability. *J. Allergy Clin. Immunol.*, **2001**. 107(5): 856-62.
22. Damodaran, S., Amino acids, peptides and proteins, *in Fennema's Food Chemistry*, S. Damodaran, K.L. Parkin, and O.R. Fennema, Editors. **2008**, CRC Press: NY, USA.
23. Hagerman, A.E. and L.G. Butler, Protein precipitation method for the quantitative determination of tannins. *J. Agric. Food Chem.*, **1978**. 26(4): 809-812.
24. Bohin, M.C., J.-P. Vincken, H.T.W.M. Van Der Hijden, and H. Gruppen, Efficacy of food proteins as carriers for flavonoids. *J. Agric. Food Chem.*, **2012**. 60(16): 4136-4143.
25. Kragh-Hansen, U., Structure and ligand binding properties of human serum albumin. *Dan. Med. Bull.*, **1990**. 37(1): 57-84.
26. Vlasova, I.M., V.V. Zhuravleva, and A.M. Saletsky, Denaturation of bovine serum albumin initiated by sodium dodecyl sulfate as monitored via the intrinsic fluorescence of the protein. *Russ. J. Phys. Chem. B*, **2014**. 8(3): 385-390.
27. Lakowicz, J.R., Protein fluorescence, *in Principles of Fluorescence Spectroscopy*. **2006**, Springer: NY, USA.
28. Gill, S.C. and P.H. von Hippel, Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.*, **1989**. 182(2): 319-326.
29. van de Weert, M. and L. Stella, Fluorescence quenching and ligand binding: A critical discussion of a popular methodology. *J. Mol. Struc.*, **2011**. 998(1–3): 144-150.
30. Bohin, M.C., J.-P. Vincken, A.H. Westphal, A.M. Tripp, P. Dekker, H.T.W.M. Van Der Hijden, and H. Gruppen, Interaction of flavan-3-ol derivatives and different caseins is determined by more than proline content and number of proline repeats. *Food Chem.*, **2014**. 158: 408-416.
31. Kubista, M., R. Sjöback, S. Eriksson, and B. Albinsson, Experimental correction for the inner-filter effect in fluorescence spectra. *Analyst*, **1994**. 119(3): 417-419.
32. SpectraMax M2^e - Multimode plate readers user guide. **2006**.
33. Vissers, A.M., A. Caligiani, F. Sforza, H. Gruppen, and J.-P. Vincken, Chapter 4: Phlorotannins of *Laminaria digitata in This thesis*. **2017**.
34. Bose, A., Interaction of tea polyphenols with serum albumins: A fluorescence spectroscopic analysis. *J. Lumin.*, **2016**. 169: 220-226.

35. Dufour, C. and O. Dangles, Flavonoid–serum albumin complexation: determination of binding constants and binding sites by fluorescence spectroscopy. *Biochimica et Biophysica Acta (BBA) - General Subjects*, **2005**. 1721(1–3): 164-173.
36. Rawel, H.M., S.K. Frey, K. Meidtner, J. Kroll, and F.J. Schweigert, Determining the binding affinities of phenolic compounds to proteins by quenching of the intrinsic tryptophan fluorescence. *Mol. Nutr. Food Res.*, **2006**. 50(8): 705-713.
37. Soares, S., N. Mateus, and V. De Freitas, Interaction of different polyphenols with Bovine Serum Albumin (BSA) and Human Salivary α-Amylase (HSA) by fluorescence quenching. *J. Agric. Food Chem.*, **2007**. 55(16): 6726-6735.
38. Bhattacharya, M., N. Jain, K. Bhasne, V. Kumari, and S. Mukhopadhyay, pH-induced conformational isomerization of Bovine Serum Albumin studied by extrinsic and intrinsic protein fluorescence. *J. Fluoresc.*, **2011**. 21(3): 1083-1090.
39. Olsen, M.H.M. and C.R. Sondergard, Propka: A Protein pKa Predictor - Online Software. **2010**: Copenhagen, Denmark.

Chapter 6

Laminaria digitata phlorotannins decrease protein degradation and methanogenesis during *in vitro* ruminal fermentation

Phlorotannins (PhT) are marine condensed tannins consisting of phloroglucinol subunits connected via C-C and C-O-C linkages. They have non-covalent protein binding properties and are, therefore, expected to be beneficial in protecting protein from hydrolysis during ruminal fermentation. In this study, the effectiveness of a methanolic PhT extract from *Laminaria digitata* was investigated in vitro on reduction of CH_4 and protection of dietary protein in ruminal fluid. A 7 × 2 factorial design of treatments was used to test 6 levels of tannin addition (10, 20, 40, 50, 75 and 100 g/kg tannin free grass silage) with or without addition of polyethylene glycol (PEG). Total gas production (GP) was monitored continuously for 72 h and at multiple time points headspace samples were analysed for CH_4. The GP and CH_4 curves were fitted through a modified multiphasic Michaelis-Menten model. Total volatile fatty acids (VFA) compositions and ammonia (NH_3) were measured as end point parameters.

Addition of PhT had a linear (P < 0.0001) and quadratic (P = 0.0003) effect on GP and CH_4, respectively. Optimal dosage of PhT was 40 g/kg grass silage as at this point CH_4 decreased significantly from 24.5 to 15.2 mL/g organic matter (OM) (P < 0.0001), without significantly affecting GP (P = 0.3115), total VFA (P = 1.000), and pH (P = 1.000). A trend (P = 0.0903) was observed for a decrease in NH_3 from 0.49 to 0.39 mmol/g OM indicating protection of protein. Addition of PEG inhibited the effect of tannins and did not change the various parameters compared to the control. Effectiveness of PhT was compared to hydrolysable and terrestrial condensed (CT) tannins on a molar basis using literature data. Phlorotannins behaved similar to CT with respect to CH_4 reduction and protein protection but appeared to be less effective.

Based on Anne Vissers, Wilbert Pellikaan, Anouk Bouwhuis, Wouter Hendriks, Harry Gruppen, Jean-Paul Vincken – *to be submitted*

Introduction

Cattle provide valuable resources for human consumption, such as milk and meat. Keeping cattle has a large environmental impact due to excretion of methane (CH_4) and ammonia (NH_3). Ruminal carbohydrate fermentation releases hydrogen gas (H_2) and methanogenic archaea utilise H_2 to reduce CO_2 to CH_4 and keep low H_2 pressure in the rumen. This CH_4 formation, however, limits overall energy-efficiency by 8-12% and contributes significantly to the environmental CH4 emission as ruminants have been estimated to contribute approximately 14.5% to the global CH4 emission [1]. In addition, ruminal nitrogen (N) use is relatively inefficient as 55-95% of the ingested N is excreted via the urine or faeces [2]. The excreted urea can be hydrolysed and is susceptible to NH_3 volatilisation and emission [3].

The current increase in world population and, therewith, the demand for agricultural raw materials, increases the need to find strategies to improve the efficiency of ruminant feed conversion and decrease CH_4 excretion. One of the strategies is the use of tannins [4]. These phenolic compounds produced by plants and macro algae as secondary defence metabolites, range in molecular weight between 250-3,000 Da [5]. There are several types of tannins of terrestrial and marine origin. Hydrolysable (HT) and condensed (CT) tannins are both present in terrestrial plants. Hydrolysable tannins, e.g. gallotannins (**Figure 1A**) and ellagitannins (**Figure 1B**), consist of phenolic building blocks linked via esters and are sensitive to acid hydrolysis. The CT, e.g. proanthocyanidins (**Figure 1C**), are connected by carbon-carbon (C-C) and sometimes additional ether (C-O-C) bonds. In addition, there are the marine phlorotannins (PhT) (**Figure 1D**) produced solely by brown macro algae. These tannins are built from phloroglucinol subunits (126 Da) connected via either C-C bonds, C-O-C bonds or both [6]. As their building blocks are different from terrestrial proanthocyanidins, but contain similar linkages between subunits, PhT can be considered as the marine version of CT.

Tannins are known to have a non-covalent binding ability to both proteins and fibres, of which affinity towards proteins is the highest [7]. By binding both dietary proteins and microbial digestive enzymes via proline residues, ruminal fermentation of proteins and carbohydrates decreases. The application of terrestrial tannins to improve ruminal fermentation has been studied extensively, both *in vitro* [8-11], and *in vivo* [12-14]. Low dosage addition of tannins to the feed improved the use of non-protein N (NPN) for microbial metabolism in the rumen and decreased N excretion. In addition, it increased the fraction of 'ruminal escape protein' and absorption of dietary amino acids post-rumen [4, 15]. It also resulted in decreased ruminal methanogenesis [2].

In contrast to terrestrial tannins, the effect of PhT on ruminant feed efficiency has received far less attention [16]. In the present study, the effect of PhT dose on *in vitro* rumen fermentation of grass silage was studied. Total gas and CH_4 production, VFA formation and ammonia nitrogen (NH_3) content were taken as indicators for efficient conversion of ruminant feed. The efficiency is known to be influenced by type of tannin, being hydrolysable or condensed [17], tannin size [18], and variation in subunit composition of terrestrial CT [19]. The PhT in *Laminaria digitata* range in degree of polymerisation from 3-27 units, linked by single linkages [20]. The occurrence of C-C and C-O-C linkages between subunits allows rotational freedom and, therewith, oligomer flexibility. As the PhT subunits are smaller than those of terrestrial CT, the rotational freedom within PhT is expected to be

higher. Consequently, it is anticipated that PhT are more effective in CH_4 reduction and protecting protein from hydrolysis than terrestrial CT.

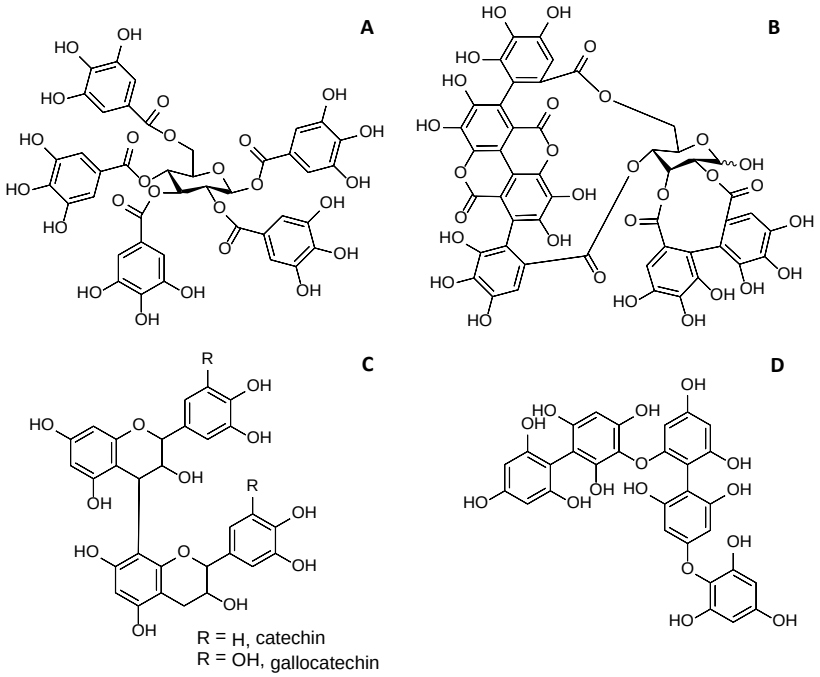

Figure 1 Structure of the hydrolysable tannins, pentagalloyl glucose (A) and punicalagin (B). Structural characteristics of terrestrial condensed tannin dimer (C) and phlorotannin oligomer DP5 (D)

Materials and methods

Phlorotannin extraction

L. digitata was obtained as air dried powder (8% w/w moisture) from Bristol Botanicals Ltd. (Bristol, United Kingdom). The powder was suspended (25 g/L) in 80% (v/v) aqueous methanol and bead-milled using a DYNO®-mill, type MULTI-LAB (Bachofen AG, Mittenz, Switzerland). The grinding chamber (65% v/v) was filled with 0.5 mm silica beads and the macro algae suspension was ground in about 1.5 h at a flow rate of 0.3 L/min. Afterwards, the suspension was filtered over a cellulose filter (cut off: 8-12 µm Whatman®) and methanol was evaporated by rotary-evaporation. The concentrated extract was diluted with 0.1 M sodium chloride (1:1 v/v). This suspension was partitioned with ethyl acetate (1:1 v/v). After phase separation, both layers were collected separately. The organic phase was cleaned twice with 200 mL 0.2 M sodium chloride (1:1 v/v). The aqueous phase was cleaned twice with 200 mL ethyl acetate. The three organic phases were pooled, dried by rotary-evaporation and stored in a desiccator until further use. The aqueous phase was not

used for further experimentation. The extract purity was analysed by the 2,4-dimethoxybenzaldehyde assay as described previously [21]. For the calibration curve, a 60% (w/w) pure PhT extract was used, The extract purity was determined using quantitative ^{1}H NMR spectroscopy [20].

Experimental design

The effect of the PhT dose on fermentation characteristics was tested with tannin-free grass silage as substrate, using a 7 × 2 factorial arrangement of treatments. The chemical composition of the silage was: dry matter (DM) = 890 g/kg; organic matter (OM) = 893 g/kg DM; crude protein (CP) = 197 g/kg DM; neutral detergent fibre (NDF) = 459 g/kg DM, acid detergent fibre (ADF) = 280 g/kg DM; acid detergent lignin (ADL) = 21 g/kg DM. The PhT extract was added to the substrate to reach an effective tannin concentration of 10, 20, 40, 50, 75 or 100 (g/kg substrate), with (+PEG) and without (-PEG) polyethylene glycol (PEG; MW 6000) at PhT:PEG = 1:10 w/w [9]. As the extract could contain impurities influencing in vitro fermentation, PEG was included as tannin binding agent which counteracts the effect of tannins [22]. A control without addition of PhT was included within the same run. Incubations were performed in 250 mL bottles (Schott bottle, GL45, Mainz, Germany) containing 250 mg grass silage. For each treatment, triplicate bottles were used within one run and two separate runs were performed. Each run was performed using the same PhT extract. All components were combined in a dry state just before addition of the rumen buffer, the latter which was prepared as described previously [23].

Each of the two runs was performed using the combined rumen fluids from two different rumen cannulated lactating Holstein-Friesian cows (in total, 4 different animals were used), which were fed a grass and corn silage diet (1:1 ratio based on product), with the following nutrient composition (g/kg DM): CP = 119; NDF = 394; ADF = 235; ADL = 16; starch = 175. In addition, animals received concentrates. Animals were fed in the morning and the diet was provided ad libitum with access to water at all times. The handling of the animals was approved by the Institutional Animal Care and Use Committee of Wageningen University & Research (Wageningen, The Netherlands), and in accordance with the Dutch legislation on use of experimental animals. Rumen fluid was collected before the morning feeding in pre-warmed and CO_2-filled thermos flasks and transported within 15 min to the laboratory. All further handling was performed under a constant flow of CO_2 in order to maintain anaerobic conditions. To monitor anaerobic conditions, resazurin was used as an indicator. For each run, the rumen fluids of the two cows were pooled 1:1 (v/v), filtered through two layers of cheese cloth, mixed 1:15 (v/v) [18] with a freshly prepared buffer as described by Williams *et al*. [23] and Pellikaan *et al*. [24].

Thirty mL of buffered rumen fluid was added to the bottles, which were incubated for 72 h in shaking water baths at 39 °C, with individual bottles connected to an automated gas production (GP) measuring system [24]. Blanks were included. In the rumen buffer without substrate and tannins, gas productions were 3.8 ± 6.5 mL and 4.8 ± 1.7 mL in run 1 and 2, respectively. Gas production with PhT extracts without PEG and substrate were 0.6 mL in run 1 and 4.8 mL in run 2 while GP in PhT extracts with PEG were 0.9 mL in run 1 and 3.0 mL in run 2.

After 72 h incubation, the pH of the mixture was recorded and fermentation fluid from each bottle was analysed for volatile fatty acids (VFA) and ammonia (NH_3) concentration.

Cumulative gas and methane production

For CH_4 production, aliquots of 10 µL were taken during several time points (0, 2, 4, 6, 8, 10, 12, 24, 30, 36, 48, 56, 72 h) from the head space of the fermentation bottles using a gas tight syringe (Hamilton 1701N, point style 5 needles, 51 mm, Hamilton, Bonaduz, Switzerland) as described previously [24]. Samples were directly injected onto a GC, which was equipped with a stainless steel column (PoraPack Q, 50-80 mesh, 6 m × 0.53 mm i.d., 25 µm film thickness, Grace Altech, Breda, The Netherlands) and connected to a flame ionisation detector maintained at 150 °C. Oven temperature was maintained at 60 °C, N_2 was used as carrier gas. Before, during and after analysis, a CH_4 standard from a synthetic air mixture (CH_4 = 7,524 ppm, Linde Gas Benelux, Schiedam, the Netherlands) was injected and used as external calibrant. The cumulative gas and CH_4 data obtained were plotted against time and data points were fitted through a non-linear modified Michaelis-Menten model [25]. Biphasic and monophasic models were used to fit the curves for GP and CH_4 (GP_{CH4}) production, respectively [25]. With this equation, the asymptotic gas concentrations and fermentation half times (GP-$T2_{1/2}$ and CH_4-$T_{1/2}$, respectively), were determined. The $T_{1/2}$ represents the exponential phase of the monophasic model, $T2_{1/2}$ represents the second exponential phase in the biphasic model.

Chemical analyses

The chemical composition of the grass silage was analysed according to the procedures described previously [19]. The OM was calculated as: DM – crude ash. For NH_3 determination, 750 µL reaction fluid was deproteinised by 750 µL trichloroacetic acid (100 g/L of trichloroacetic acid neutralised by NaOH). Samples were centrifuged at 14,000 × *g* at 20 °C for 10 min. Concentration of NH_3 in the supernatant was determined in duplicate as described previously [26]. For VFA analysis, 750 µL reaction fluid was acidified with 750 µL internal standard (isocaproic acid) in a 4% (w/w) *o*-phosphoric acid solution. Gas chromatography was conducted as described previously [9].

Statistical analyses

The effect of PhT dose (T) in combination with PEG (P) treatment was analysed by analysis of variance using the MIXED procedure of SAS (2012). The average of triplicate bottles within one run were taken as statistical units and analysed as a 7 × 2 factorial design including run as random factor. The following statistical model was applied:

$$Y_{ij} = \mu + T_i + P_j + (T \times P)_{ij} + \varepsilon_{ij}$$

in which Y_{ij} = the dependent variable, μ = the overall mean, T_i = the PhT dose (i = 1 to 7), P_j = the effect of PEG (j = 1 or 2), $(T \times P)_{ij}$ = the PhT dose and PEG interaction, and ε_{ij} = the residual error term. Differences in means were analysed by the Tukey-Kramer's test using the LSMEANS statement and values were significant at $P < 0.05$. To test linear (L) and quadratic (Q) effects of tannin dose with or without PEG addition, non-linear polynomial contrasts were used.

Results

Effect on total gas and methane production

The overall fermentation performance upon inclusion of PhT in grass silage was determined by total GP (**Figure 2A**, **Table 1**). Phlorotannin addition changed the characteristics of fermentation. For the control, 10 and 20 g PhT/kg grass silage addition, curves were similar and only one exponential phase was visible. At 40 g PhT/kg and higher additions the shapes of the fermentation curves started to deviate from the control and became biphasic (**Figure 2A**). The GP after 72 h (GP_{72h}) production of the control was 186.7 mL/g OM and was not affected up to 50 g PhT/kg addition (P = 0.1097) (**Table 1**). Afterwards, GP started to decline linearly (P < 0.001), resulting in 42% GP_{72h} reduction at 100 g PhT/kg addition, compared to the control. Patterns were similar for GP_{24h} and GP_{72h} with increasing PhT addition. For the control and 10 g PhT/kg addition, similar GP_{24h} and GP_{72h} values were obtained.

The data for CH_4 production could all be fitted with a monophasic model (**Figure 2B**). There was a similar direction of the effect of PhT on CH_4 (GP_{CH4}) production, with the difference that production decreased significantly from additions of 40 g PhT/kg onwards (**Table 2**). After 24 h, GP_{CH4} decreased (P = 0.0002) from 19.0 g/kg OM in the control to 7.6 ml/g OM at 40 g PhT/kg addition. The decrease (P < 0.001) in GP_{CH4} after 72 h followed a quadratic trend, and decreased with 39% at a 40-50 g/kg addition and with 73% at a 100 g PhT/kg addition. The half time ($T_{1/2}$) is the time-point at which half the asymptotic gas or CH_4 volume is produced (mL/g OM), which provides information on the rate of microbial fermentation for GP (**Table 1**) and GP_{CH4} (**Table 2**). For both GP and GP_{CH4}, fermentation delayed with PhT addition. For total GP, GP-$T2_{1/2}$ increased (P < 0.001) from 11 h for the control to 38 h for 100 g PhT/kg addition. For CH_4 production CH_4-$T_{1/2}$ increased (P = 0.0013) from 15 h for the control to 38 h at 100 g PhT/kg addition. The proportion of CH_4 in the total gas decreased by 45-50% at 75-100 g PhT/kg addition which was significant.

Effect on volatile fatty acid production

There was a linear effect on decrease in total VFA production upon PhT addition (**Table 3**). All proportions of individual VFAs decreased (acetic acid (HAc), butyric acid (HBu), branched chain fatty acids (HBr)) or increased (propionic acid (HPr) and valeric acid (HVa)) quadratically with tannin addition. The HAc proportion stabilised at additions of 40 g PhT/kg and higher around 53 mol%, corresponding to a decrease of 8% compared to the control. The HPr proportion increased (P < 0.0001) from 29 mol% in the control to 35 mol% at 40 g PhT/kg addition and remained stable with further PhT addition. Butyric acid decreased (P < 0.0001) from 8.1 mol% in the control to 6.1 mol% at 40 g PhT/kg and further to 5.0 mol% at 100 g PhT/kg addition. The proportion of HVa was only affected at 100 g PhT/kg addition. HBr represents the sum of iso-butyric and iso-valeric acid which are fatty acids formed by the breakdown of the amino acids valine and leucine. Their relative proportion decreased quadratically (P = 0.0366) from 3.3 mol% in the control to 2.6 mol% at 40 g PhT/kg and stabilised with further tannin addition.

Effect on ammonia nitrogen and pH

Ammonia nitrogen (NH_3) (**Table 3**) decreased (P = 0.0951) from 0.49 to 0.39 mmol/g OM at 50 g PhT/kg addition. The pH was not affected.

Effect of PEG addition

Addition of PEG to the fermentation mixtures counteracted the effect of PhT. Gas production kinetics were identical for all PhT levels and for most parameters studied. The addition of PEG started to deviate significantly from –PEG (**Table 1**-**3**) at 50 g PhT/kg addition. Addition of PEG did not change the values of the parameters compared to the control and for all parameters the interaction term (Dose × PEG) was significant except for total VFA (P = 0.0802) and NH_3 (P = 0.0538) for which a trend was observed.

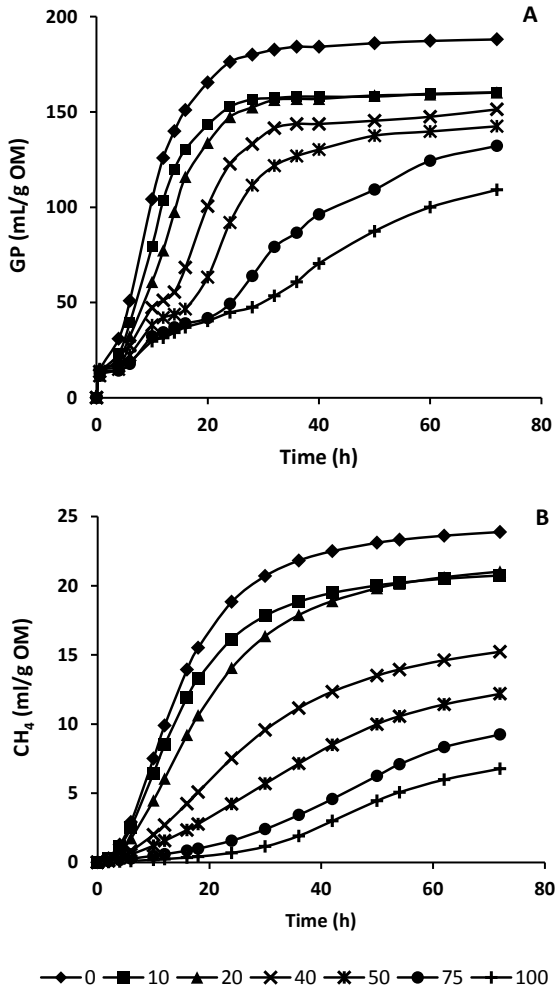

Figure 2 Kinetics of total gas- (A) and methane production (B) of grass silage with increasing phlorotannin concentration (g PhT/kg grass silage)

Table 1 Total gas (GP) production after 24 and 72 h, gas production half time (GP-$T2_{1/2}$) from fermentations containing increasing phlorotannin (PhT) dose without (–) and with (+) polyethylene glycol (PEG) addition

Dose PhT g/kg feed)	$GP_{12\ h}$ (mL/g OM)		$GP_{72\ h}$ (mL/g OM)		GP-$T2_{1/2}$ (h)	
	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG
0	173.4 [a]	137.9	186.7 [a]	177.3	10.8 [a]	10.1
1	160.3 [a]	157.1	160.3 [a]	186.4	11.1 [a]	9.9
2	117.3 [a]	157.3	160.2 [a]	189.3	13.5 [a]	10.2
4	119.6 [a]	157.8	151.4 [a]	190.1	19.1 [b]	10.1
5	90.1 [b]	148.8	142.5 [b]	178.1	23.3 [b]	10.5 *
7.5	45.9 [c]	161.7 *	125.5 [b]	197.2 **	34.7 [c]	10.2 ***
10	43.9 [c]	142.7 *	109.1	172.3 **	37.7 [c]	10.3 ***
S.E.M	41.48		38.32		2.19	
P-values						
Dose L	<0.0001	0.9715	<0.0001	0.7863	<0.0001	0.9065
Dose Q	0.5976	0.3984	0.7020	0.1794	0.7254	0.9328
PEG	0.0003		<0.0001		<0.0001	
Dose × PEG	0.0112		0.0108		<0.0001	

GP_{24h}, GP_{72h} = total gas volume produced per gram OM initial grass silage after 24 h and 72 h of incubation; GP-$T2_{1/2}$ = half time of asymptotic gas production in the second phase of the fermentation; S.E.M. = standard error of the mean; Dose L = linear PhT effect, Dose Q = quadratic PhT effect

[abc] similar superscripts in a column indicate no difference ($P > 0.05$) between PhT doses

* $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$ indicates difference to corresponding PEG values

Table 2 Methane (GP_{CH4}) production after 24 and 72 h, methane production half time (GP_{CH4}-$T_{1/2}$) and calculated CH_4 proportions (CH_4%) from fermentations containing increasing phlorotannin (PhT) dose without (–) and with (+) polyethylene glycol (PEG)

Dose PhT g/kg feed)	$GP_{CH4-24h}$ (mL/g OM)		$GP_{CH4-72h}$ (mL/g OM)		GP_{CH4}-$T_{1/2}$ (h)		CH_4% (% of GP)	
	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG
0	19.0 ^{a}	19.0	24.5 ^{a}	24.4	14.5 ^{a}	14.1	13.5 ^{a}	14.3
1	17.9 ^{a}	20.0	23.4 ^{a}	25.2	15.2 ^{a}	13.9	14.8 ^{a}	13.8
2	14.1 ^{a}	19.3	21.0 ^{a}	26.0 **	18.5 ^{a}	15.3	13.3 ^{a}	13.8
4	7.6 ^{b}	18.7 ***	15.2 ^{b}	24.2 ***	25.9 ^{ab}	14.7	10.2 ^{ab}	13.1
5	4.2 ^{bc}	17.5 ***	12.2 ^{bc}	24.5 ***	41.0 ^{b}	15.9 ***	8.9 ^{bc}	14.1 **
7.5	1.8 ^{bc}	18.6 ***	9.0 ^{cd}	25.0 ***	39.1 ^{b}	15.6 ***	7.4 ^{bc}	12.9 **
10	0.7 ^{c}	18.8 ***	6.8 ^{d}	24.9 ***	38.1 ^{b}	15.0 **	6.8 ^{c}	15.3 ***
S.E.M	1.28		2.35		3.15		1.46	
P-values								
Dose L	<0.001	0.4388	<0.001	0.8799	<0.001	0.6834	<0.001	0.5005
Dose Q	0.0003	0.4191	0.0006	0.7931	0.0046	0.6993	0.0778	0.0368
PEG	<0.001		<0.001		<0.001		<0.001	
Dose × PEG	<0.001		<0.001		0.0005		<0.001	

$GP_{CH4-24h}$, $GP_{CH4-72h}$ = total methane volume produced per gram OM initial grass silage after 24 h and 72 h of incubation; GP_{CH4}-$T_{1/2}$ = half time of asymptotic methane production; CH_4% = methane percentage in total gas after 72 h; S.E.M. = standard error of the mean; Dose L = linear PhT effect, Dose Q = quadratic PhT effect

^{abc} similar superscripts in a column indicate no difference ($P > 0.05$) between PhT doses

$^{**}P < 0.01$; $^{***}P < 0.001$ indicates difference to corresponding PEG value

Table 3 Fatty acids and NH_3-N produced during *in vitro* fermentations containing increasing phlorotannin (PhT) dose without (-) and with (+) polyethylene glycol (PEG)

Dose PhT (g/kg feed)	tVFA (mmol/g OM)		HAc (% of tVFA)		HPr (% of tVFA)		HBu (% of tVFA)		HVa (% of tVFA)		HBc (% of tVFA)		NH_3 (mmol/g OM)	
	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG
0	8.1 ^{a}	8.3	56.8 ^{a}	57.3	29.2 ^{a}	28.5	8.1 ^{a}	8.2	2.6 ^{a}	2.6	3.3 ^{a}	3.3	0.49 ^{a}	0.53
1	8.0 ^{a}	8.4	57.1 ^{a}	57.8	29.5 ^{a}	28.1	7.8 ^{a}	8.3	2.6 ^{a}	2.6	3.1 ^{a}	3.3	0.39 ^{a}	0.50
2	8.1 ^{a}	8.6	55.6 ^{a}	57.4	31.8 ^{a}	28.5 **	7.1	8.2 ***	2.6 ^{a}	2.6	2.9 ^{a}	3.3	0.42 ^{a}	0.50
4	7.9 ^{a}	8.5	53.5 ^{b}	57.1 **	35.3 ^{b}	28.9 ***	6.1 ^{b}	8.1 ***	2.5 ^{a}	2.6	2.6 ^{a}	3.3	0.39 ^{a}	0.54
5	7.4 ^{ab}	8.0	52.7 ^{b}	56.6 ***	36.7 ^{b}	29.3 ***	5.6 ^{b}	8.1 ***	2.6 ^{a}	2.7	2.4 ^{b}	3.3 **	0.27 ^{a}	0.46
7.5	7.6 ^{ab}	8.7	51.8 ^{b}	57.2 ***	37.7 ^{b}	28.9 ***	5.0 ^{c}	8.1 ***	3.2 ^{ab}	2.6	2.2 ^{b}	3.2 **	0.22 ^{b}	0.52 *
10	6.5 ^{b}	8.4 **	52.2 ^{b}	57.3 ***	36.6 ^{b}	28.7 ***	5.0 ^{c}	8.1 ***	3.7 ^{b}	2.6 **	2.5 ^{b}	3.2 **	0.20 ^{b}	0.51 **
S.E.M	0.26		0.39		0.39		0.26		0.17		0.18		0.05	
P-values														
Dose L	<0.001	0.8416	<0.001	0.4921	<0.001	0.2238	<0.001	0.1476	<0.001	0.9873	<0.001	0.5199	<0.001	0.9948
Dose Q	0.1461	0.9632	0.0003	0.2382	<0.001	0.1974	<0.001	0.4142	0.0032	0.0032	0.0015	0.9741	0.4892	0.6868
PEG	<0.001		<0.001		<0.001		<0.001		0.0302		<0.001		<0.001	
Dose × PEG	0.0802		<0.001		<0.001		<0.001		0.0089		0.0065		0.0425	

tVFA = total volatile fatty acid (tVFA = HAc+HPr+HBu+HVa+HBc); HAc = acetic acid; HPr = propionic acid; HBu = butyric acid; HVa = valeric acid; HBc = branched chain fatty acids

(iso-butyric + iso-valeric acid); S.E.M. = standard error of the mean; Dose L = linear PhT effect, Dose Q = quadratic PhT effect

^{abc} similar superscripts in a column indicate no difference ($P > 0.05$) between PhT doses

$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ indicates difference to corresponding PEG value

Discussion

Phlorotannis can reduce methane excretion

For environmental reasons, CH_4 reduction is beneficial [1], and as total GP is a measure of OM digestion, it should preferably not decrease. Within a normal fermentation process, initially the small and easily degradable sugars are fermented in the timespan of several hours. Afterwards a second fermentation phase of larger carbohydrates starts, correlating to the exponential second phase [19], visualised in **Figure 2**. Due to the higher contribution of the second phase to the total gas production, GP data were fitted using a biphasic modified Michaelis-Menten model [25]. Phlorotannin addition at 40 g/kg grass silage reduced the CH_4 production by 40% after 24 h fermentation without hampering OM degradation as reflected by GP, GP-$T2_{1/2}$ (**Table 1**) and total VFA (**Table 2**). The PhT inhibitory effects were not observed when the doses were tested in presence of PEG. Polyethylene glycol is able to quench tannins from the fermentation mixture as tannins have higher binding affinity for PEG than for proteins [22, 27]. Incubating the PhT blanks with rumen buffer did not result in GP. Additionally, PEG addition was able to revert the action of PhT and maintain GP. These two observations indicated that the 40% impurities present in the PhT extract did not influence the fermentation. These results agree with those found previously [16], where addition of 40 g/kg PhT from *Ascophyllum nodosum* improved *in vitro* fermentation and where PEG addition counteracted the dose effect. In their case, however, the optimum could not be verified as no higher dosages were investigated.

The CH_4 reducing effect of tannins can be due to three mechanisms. (i) Tannins could decrease fibre digestibility by either binding to the fibres or by binding to the microbial fibre digesting enzymes [28], resulting in less H_2 release and, therewith, CH_4 formation. (ii) The population of methanogens themselves could be reduced [29]. (iii) A relative increase in HPr synthesis at the expense of HAc and HBu. This results in competition between formation of HPr and CH_4 as both require H_2 [30].
The first effect seemed to play a role in the present study as both GP and VFA decreased linearly with PhT addition. Both parameters are related to carbohydrate digestion [30]. Even though the final VFA level was not affected at 40 g PhT/kg, there were changes in the VFA profile. Acetic acid decreased from 57 to 54 mol%. Usually HAc makes op 50-60% of the fatty acids and is mainly used for gluconeogenesis [30]. The HAc proportion was still within this range (**Table 3**).

No conclusions regarding the second effect could be drawn as in the present study the microbial population was not determined. There are, however, indications that methanogens were negatively affected by PhT addition [31].

Regarding the third effect, there was a 20% increase in HPr proportion (**Table 3**) at 40 g PhT/kg addition. The results correlate with earlier studies which showed that when the activity of methanogens is inhibited, H_2 is directed more into HPr production [18].

Phlorotannins can protect protein from hydrolysis during fermentation

At 40 g PhT/kg addition, the NH_3 was decreased by 21% (**Table 3**), indicating protection of protein from degradation. A decrease in NH_3 indicates a decrease in dietary protein digestion and increase in use of NPN for microbial protein synthesis [30]. Decreased dietary protein breakdown in the rumen results in higher amounts of protein available for digestion in the abomasum. In addition, the

increased use of NPN for microbial protein synthesis increases the flow of microbial protein towards the abomasum. The NH_3 reduction reflects the binding of PhT to dietary proteins and to microbial excreted digestive enzymes [32] and direct inhibition of bacterial growth [33]. Additional evidence of the protein protective effect was provided by the decrease in proportion of HBr, which was lowered by 20% (**Table 3**). The proportion of HBr consisted of the combined proportions of iso-butyrate and iso-valerate, degradation products from valine and leucine, respectively, These fatty acids function as co-factors for cellulolytic bacteria and their synthesis of long chain fatty acids [30, 34]. The HBr content should, therefore, not decrease substantially. Additionally, rumen microbes do not grow optimally on NH_3 alone and fermentation efficiency improves when proteins and peptides can be used as well [35].

Improvement effects of condensed-, hyrolysable- and phlorotannins

In most *in vitro* ruminal fermentation studies, the dosages of CT, HT or PhT were added on a weight bases. Mostly, levels around 40 g PhT/kg were applied when no dose response tests were performed [19, 36]. Dosage of tannins on weight base alone complicates the comparison of effectiveness as within the different types of tannins used there is variation in molecular weight of the tannin building blocks as well as the range of oligomer length (**Table 4**), which are important factors influencing tannin effectivity. Building blocks of HT are usually larger than those of CT, as the former are built from galloyl-glucose units [37].

Table 4 Characteristics of phloro- condensed and hydrolysable tannins

Tannin type	Subunit	Subunit MW (Da)	Linkages	DP range	No. rotating bonds per dimer	Microbial fermentation
Condensed	Catechin	290	C-C	2->10	3	[38, 39]
	Gallocatechin	458				
Hydrolysable	Pentagalloyl glucose [a]	940	Ester	2-10	Variable [b]	[40] [c]
Phlorotannin	Phloroglucinol	126	C-C, ether	3-27	1	Not reported

[a] Pentagalloyl glucose represents the intermediate building block for both gallo- and ellagitannins [37]
[b] The number of free rotating bonds within the dimer depends on the extent of gallic acid crosslinking
[c] Toxicity reported upon *in vivo* ruminal inoculation at 3 mg/kg body weight

The effects of *L. digitata* PhT were compared to other studies in which *A. nodosum* PhT [16], sainfoin (*Onobrychis viciifolia*) CT [41] or both HT and CT from various sources [42] were tested *in vitro*. For all tannin dosages, molar additions were estimated based on monomeric equivalents for which the assumptions are summarised in **Table 4**. The reductions in CH_4 production and NH_3 concentration were calculated as change relative to the control substrate without tannin addition and were plotted versus the molar tannin dose (**Figure 3**). On weight basis, all tannins would have been equally effective. All three types of tannins were effective in reducing GP and CH_4 production as well

as NH_3 concentration, with HT [42] being more effective. Already at 5 mmole additions, 20% CH_4 reductions were achieved while for CT and PhT these reductions were achieved at around 20 mmole. Effects of PhT and CT showed similar trends. The PhT from *A. nodosum* [16] were similar to those of *L. digitata*. The lowest NH_3 reduction was obtained by PhT, followed by CT and then HT. To achieve 50-60% NH_3 reduction, < 10 mmol HT were needed while 60 mmol PhT monomers would be necessary to reach this same reduction. For CT, approximately 15 mmole would be sufficient.

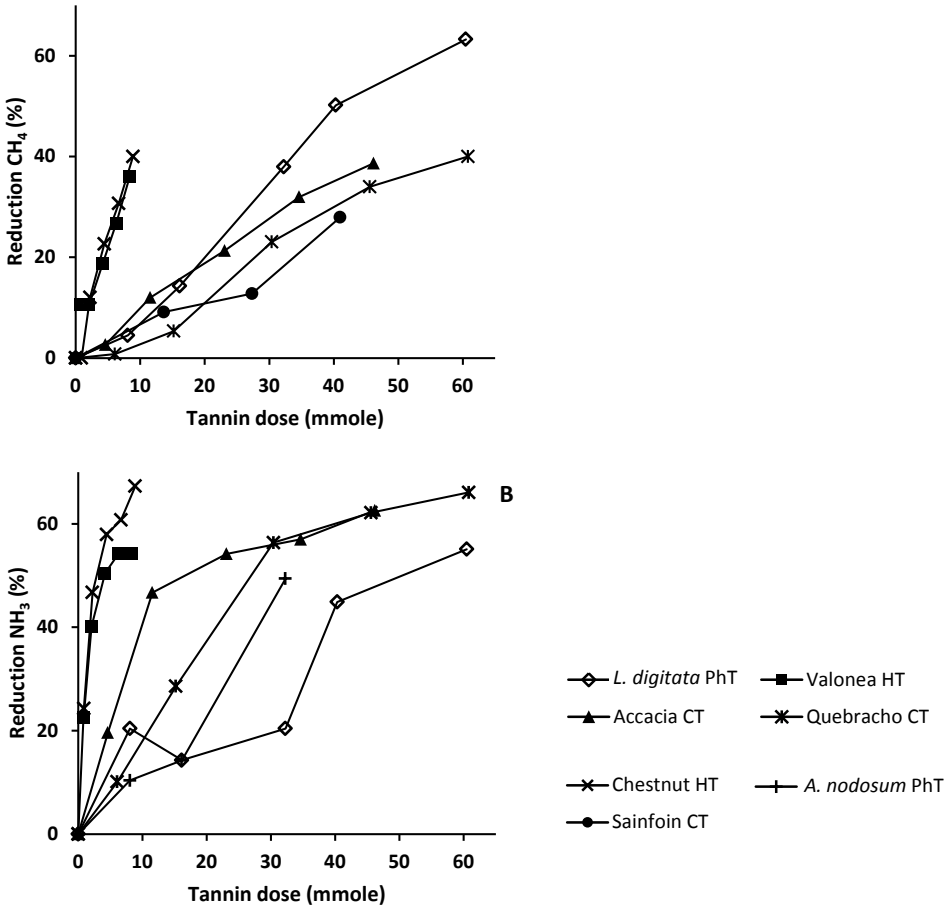

Figure 3 Comparison between effectivity of phlorotannin (PhT), condensed- (CT) and hydrolysable tannin (HT) on reduction of CH_4 (A) and NH_3 (B) when added to feed during *in vitro* fermentation. Data sources for chestnut, valonea, accacia and quebracho: [42]; sainfoin: [41] *A. nodosum*: [16]

There are several molecular properties of tannins which are important in terms of the effects on fermentation. The first is the abundance of hydroxyl groups which are important for protein-tannin and carbohydrate-tannin complexation [7, 43, 44]. For CT, there is a positive effect of the

contribution of gallocatechin units within the tannin oligomer [19], probably related to increased binding by the increase in hydroxyl groups per monomeric unit. The second is the degree of polymerisation (DP). Although it has been reported that tannin oligomers in higher molecular weight ranges bind proteins more effectively, there is an optimum DP. For ellagitannins, trimers to pentamers showed positive effects on fermentation while dimers and hexamers to nonamers were less effective or even decreased fermentation [18]. As all the extracts used in these studies were mixtures, the set-up of the current trial cannot discriminate for potential effects of DP. The third property is molecular flexibility which is related to presence of free rotational bonds. This property is most likely one of the reasons for higher effectivity of HT. Hydrolysable tannins have a higher binding affinity to proteins compared to PhT and CT [20] caused by the presence of galloyl groups with high rotational freedom (**Figure 1**, **Table 4**). These can, therefore, orient themselves to the protein, creating stronger connections [43]. Ellagitannins are expected to have less rotational freedom compared to gallotannins due to the multiple connections between subunits (Figure 1B). The HT used for comparison originated from chestnut and valonea and in both, ellagic acid and gallic acid units were identified [42]. Comparing HT to CT and PhT, the latter two have fewer free rotating bonds within their oligomers (**Table 4**). The resulting lower flexibility of CT and PhT might reflect lower protein binding affinity and, therewith, a smaller reduction in NH_3 can be reached (**Figure 3**).

A last potential parameter of influence can be the potential metabolism of tannins (**Table 4**), which has mainly been reported for HT [39]. Metabolism of HT can occur both in the rumen and further down the GI tract [40]. The possibility of microbial fermentation of HT and release of toxic reaction products also contributes to their toxicity [45]. To our knowledge, microbes able to break down PhT oligomers have not been identified. As the PhT tend to have lower potential to be metabolised by ruminal microbes, there are probably lesser toxic effects compared to HT.

Conclusions

Application of a PhT extract from *L. digitata* at 40 g/kg to grass silage effectively reduced CH_4 production without significantly decreasing organic matter degradation as reflected by total gas production and volatile fatty acid formation. Additionally, PhT decreased protein degradation and additionally, ruminal microbes used NPN for their metabolism, reflected by reduction in NH_3.

References

1. Gerber, P.J., A. Mottet, C.I. Opio, A. Falcucci, and F. Teillard, Environmental impacts of beef production: Review of challenges and perspectives for durability. *Meat Sci.*, **2015**. 109: 2-12.
2. Tedeschi, L.O., C.A. Ramírez-Restrepo, and J.P. Muir, Developing a conceptual model of possible benefits of condensed tannins for ruminant production. *Animal*, **2014**. 8(7): 1095-1105.
3. Oenema, O., D. Oudendag, and G.L. Velthof, Nutrient losses from manure management in the European Union. *Livest. Sci.*, **2007**. 112(3): 261-272.
4. Mueller-Harvey, I., Unravelling the conundrum of tannins in animal nutrition and health. *J. Sci. Food Agric.*, **2006**. 86(13): 2010-2037.
5. Claussen, T.P., P.B. Reichardt, J.P. Bryant, and F. Provenza, Condensed tannins in plant defence: a perspective on classical theories, *in Plant Polyphenols*, R.W. Hemmingway and P.E. Laks, Editors. **1992**, Plenum Press: NY, USA. 639-651.

6. Isaza Martínez, J.H. and H.G. Torres Castañeda, Preparation and chromatographic analysis of phlorotannins. *J. Chrom. Sci.*, **2013**. 51(8): 825-838.
7. Le Bourvellec, C. and C.M.G.C. Renard, Interactions between polyphenols and macromolecules: Quantification methods and mechanisms. *Criti. Rev. Food Sci. Nutr.*, **2012**. 52(3): 213-248.
8. Calabrò, S., A. Guglielmelli, F. Iannaccone, P.P. Danieli, R. Tudisco, C. Ruggiero, G. Piccolo, M.I. Cutrignelli, and F. Infascelli, Fermentation kinetics of sainfoin hay with and without PEG. *J. Anim. Physiol. Anim. Nutr.*, **2012**. 96(5): 842-849.
9. Pellikaan, W.F., E. Stringano, J. Leenaars, D.J.G.M. Bongers, S.V.L.V. Schuppen, J. Plant, and I. Mueller-Harvey, Evaluating effects of tannins on extent and rate of in vitro gas and CH_4 production using an automated pressure evaluation system (APES). *Anim. Feed Sci. Tech.*, **2011**. 166-167: 377-390.
10. Tiemann, T.T., P. Avila, G. Ramírez, C.E. Lascano, M. Kreuzer, and H.D. Hess, *In vitro* ruminal fermentation of tanniniferous tropical plants: Plant-specific tannin effects and counteracting efficiency of PEG. *Anim. Feed Sci.Tech.*, **2008**. 146(3–4): 222-241.
11. Toral, P.G., G. Hervás, H. Missaoui, S. Andrés, F.J. Giráldez, S. Jellali, and P. Frutos, Effects of a tannin-rich legume (*Onobrychis viciifolia*) on *in vitro* ruminal biohydrogenation and fermentation. *Span. J. Agric. Res.*, **2016**. 14(1).
12. Anantasook, N., M. Wanapat, A. Cherdthong, and P. Gunun, Effect of tannins and saponins in *Samanea saman* on rumen environment, milk yield and milk composition in lactating dairy cows. *J. Anim. Physiol. Anim. Nutr.*, **2015**. 99(2): 335-344.
13. Ávila, S.C., G.V. Kozloski, T. Orlandi, M.P. Mezzomo, and S. Stefanello, Impact of a tannin extract on digestibility, ruminal fermentation and duodenal flow of amino acids in steers fed maize silage and concentrate containing soybean meal or canola meal as protein source. *J. Agric. Sci.*, **2015**. 153(5): 943-953.
14. Jolazadeh, A.R., M. Dehghan-banadaky, and K. Rezayazdi, Effects of soybean meal treated with tannins extracted from pistachio hulls on performance, ruminal fermentation, blood metabolites and nutrient digestion of Holstein bulls. *Anim. Feed Sci. Tech.*, **2015**. 203: 33-40.
15. Barahona, R., C.E. Lascano, R. Cochran, J. Morrill, and E.C. Titgemeyer, Intake, digestion, and nitrogen utilization by sheep fed tropical legumes with contrasting tannin concentration and astringency. *J. Anim. Sci.*, **1997**. 75(6): 1633-1640.
16. Wang, Y., Z. Xu, S.J. Bach, and T.A. McAllister, Effects of phlorotannins from *Ascophyllum nodosum* (brown seaweed) on *in vitro* ruminal digestion of mixed forage or barley grain. *Anim. Feed Sci. Tech.*, **2008**. 145(1–4): 375-395.
17. Jayanegara, A., G. Goel, H.P.S. Makkar, and K. Becker, Divergence between purified hydrolysable and condensed tannin effects on methane emission, rumen fermentation and microbial population *in vitro*. *Anim. Feed Sci. Tech.*, **2015**. 209: 60-68.
18. Baert, N., W.F. Pellikaan, M. Karonen, and J.P. Salminen, A study of the structure-activity relationship of oligomeric ellagitannins on ruminal fermentation *in vitro*. *J. Dairy Sci.*, **2016**. 99(10): 8041-8052.
19. Huyen, N.T., C. Fryganas, G. Uittenbogaard, I. Mueller-Harvey, M.W.A. Verstegen, W.H. Hendriks, and W.F. Pellikaan, Structural features of condensed tannins affect *in vitro* ruminal methane production and fermentation characteristics. *J. Agric. Sci.*, **2016**(154): 1474-1487.
20. Vissers, A.M., A. Caligiane, S. Sforza, H. Gruppen, and J.-P. Vincken, Chapter 4 - Phlorotannins of *Laminiaria digitata, in This thesis*. **2017**.
21. Montero, L., M. Herrero, E. Ibáñez, and A. Cifuentes, Separation and characterization of phlorotannins from brown algae *Cystoseira abies-marina* by comprehensive two-dimensional liquid chromatography. *Electrophoresis*, **2014**. 35(11): 1644-1651.
22. Makkar, H.P.S., M. Blümmel, and K. Becker, Formation of complexes between polyvinyl pyrrolidones or polyethylene glycols and tannins, and their implication in gas production and true digestibility in *in vitro* techniques. *Br. J. Nutr.*, **1995**. 73(6): 897-913.
23. Williams, B.A., M.W. Bosch, H. Boer, M.W.A. Verstegen, and S. Tamminga, An *in vitro* batch culture method to assess potential fermentability of feed ingredients for monogastric diets. *Anim. Feed Sci. Tech.*, **2005**. 123-124 Part 1: 445-462.
24. Pellikaan, W.F., W.H. Hendriks, G. Uwimana, L.J.G.M. Bongers, P.M. Becker, and J.W. Cone, A novel method to determine simultaneously methane production during *in vitro* gas production using fully automated equipment. *Anim. Feed Sci. Tech.*, **2011**. 168(3-4): 196-205.
25. Groot, J.C.J., J.W. Cone, B.A. Williams, F.M.A. Debersaques, and E.A. Lantinga, Multiphasic analysis of gas production kinetics for in vitro fermentation of ruminant feeds. *Anim. Feed Sci. Tech.*, **1996**. 64(1): 77-89.

26. Weatherburn, M.W., Phenol-hypochlorite reaction for determination of ammonia. *Anal. Chem.*, **1967**. 39(8): 971-974.
27. Jones, W.T. and J.L. Mangan, Complexes of the condensed tannins of sainfoin (*Onobrychis viciifolia scop*.) with fraction 1 leaf protein and with submaxillary mucoprotein, and their reversal by polyethylene glycol and pH. *J. Sci. Food Agric.*, **1977**. 28(2): 126-136.
28. Barry, T.N., T.R. Manley, and S.J. Duncan, The role of condensed tannins in the nutritional value of *Lotus pedunculatus* for sheep: 4. Sites of carbohydrate and protein digestion as influenced by dietary reactive tannin concentration. *Br. J. Nutr.*, **1986**. 55(1): 123-137.
29. Jayanegara, A., S. Marquardt, E. Wina, M. Kreuzer, and F. Leiber, *In vitro* indications for favourable non-additive effects on ruminal methane mitigation between high-phenolic and high-quality forages. *Br. J. Nutr.*, **2013**. 109(4): 615-622.
30. Van Soest, P.J., Rumen microbes, *in Nutritional Ecology of the Ruminant*. **1987**, Cornell University Press: NY, USA. 152-177.
31. Wang, Y., T.W. Alexander, and T.A. McAllister, *In vitro* effects of phlorotannins from *Ascophyllum nodosum* (brown seaweed) on rumen bacterial populations and fermentation. *J. Sci. Food Agric.*, **2009**. 89(13): 2252-2260.
32. Horigome, T., K. Okamoto, and R. Kumar, Effects of condensed tannins prepared from leaves of fodder plants on digestive enzymes *in vitro* and in the intestine of rats. *Br. J. Nutr.*, **1988**. 60(2): 275-285.
33. Min, B.R., G.T. Attwood, W.C. McNabb, A.L. Molan, and T.N. Barry, The effect of condensed tannins from *Lotus corniculatus* on the proteolytic activities and growth of rumen bacteria. *Anim. Feed Sci. Technol.*, **2005**. 121(1): 45-58.
34. Allison, M.J., M.P. Bryant, I. Katz, and M. Keeney, Metabolic function of branched-chain folatile fatty acids, growth factors for ruminococci II: Biosynthesis of higher branched-chain fatty acids and aldehydes. *J. Bact.*, **1962**. 83(5): 1084-1093.
35. Hoover, W.H. and S.R. Stokes, Balancing carbohydrates and proteins for optimum rumen microbial yield. *J. Dairy Sci.*, **1991**. 74(10): 3630-3644.
36. Carreño, D., G. Hervás, P.G. Toral, A. Belenguer, and P. Frutos, Ability of different types and doses of tannin extracts to modulate *in vitro* ruminal biohydrogenation in sheep. *Anim. Feed Sci. Tech.*, **2015**. 202: 42-51.
37. Niemetz, R. and G.G. Gross, Enzymology of gallotannin and ellagitannin biosynthesis. *Phytochem.*, **2005**. 66(17): 2001-2011.
38. Appeldoorn, M.M., J.-P. Vincken, A.M. Aura, P.C.H. Hollman, and H. Gruppen, Procyanidin dimers are metabolized by human microbiota with 2-(3,4-dihydroxyphenyl)acetic acid and 5-(3,4-dihydroxyphenyl)-γ-valerolactone as the major metabolites. *J. Agric. Food Chem.*, **2009**. 57(3): 1084-1092.
39. Bhat, T.K., B. Singh, and O.P. Sharma, Microbial degradation of tannins - A current perspective. *Biodegrad.*, **1998**. 9(5): 343-357.
40. González-Barrio, R., P. Truchado, R. García-Villalba, G. Hervás, P. Frutos, J.C. Espín, and F.A. Tomás-Barberán, Metabolism of oak leaf ellagitannins and urolithin production in beef cattle. *J. Agric. Food Chem.*, **2012**. 60(12): 3068-3077.
41. Hatew, B., E. Stringano, I. Mueller-Harvey, W.H. Hendriks, C.H. Carbonero, L.M.J. Smith, and W.F. Pellikaan, Impact of variation in structure of condensed tannins from sainfoin (*Onobrychis viciifolia*) on *in vitro* ruminal methane production and fermentation characteristics. *J. Anim. Phys. Anim. Nutr.*, **2016**. 100(2): 348-360.
42. Hassanat, F. and C. Benchaar, Assessment of the effect of condensed (acacia and quebracho) and hydrolysable (chestnut and valonea) tannins on rumen fermentation and methane production *in vitro. J. Sci. Food Agric.*, **2013**. 93(2): 332-339.
43. Bohin, M.C., J.-P. Vincken, A.H. Westphal, A.M. Tripp, P. Dekker, H.T.W.M. Van Der Hijden, and H. Gruppen, Interaction of flavan-3-ol derivatives and different caseins is determined by more than proline content and number of proline repeats. *Food Chem.*, **2014**. 158: 408-416.
44. Stern, J.L., A.E. Hagerman, P.D. Steinberg, and P.K. Mason, Phlorotannin-protein interactions. *J. Chem. Ecol.*, **1996**. 22(10): 1877-1899.
45. Zhu, J., L.J. Filippich, and M.T. Alsalami, Tannic acid intoxication in sheep and mice. *Res. Vet. Sci.*, **1992**. 53(3): 280-292.

Chapter 7

General discussion

In this thesis, sugar beet leaves (*Beta vulgaris L.*) and the brown macroalgae *Laminaria digitata* were investigated as terrestrial and marine resources for protein use, respectively, in relation to their interactions with phenolics. Both resources can provide high quality food and feed material. When the resources are processed for protein extraction, proteins can interact with phenolics, leading to modified proteins with lesser value. Within sugar beet leaves, the modifications take place via polyphenol oxidases (PPO) and endogenous phenolic substrates which make sugar beet leaf proteins prone to covalent modification by attachment of phenolics to proteins. Within *L. digitata,* high molecular weight phlorotannins (PhT) are prone to non-covalent binding to protein. A schematic representation of both types of interactions is shown in **Figure 1**. Covalent and non-covalent protein modifications are reported to have both detrimental and beneficial effects regarding their techno-functional and nutritional properties. In this thesis, the first aim was to study factors involved in protein modification by phenolics. For sugar beet leaves, the composition of phenolics was investigated in relation to brown colour formation. Moreover, the non-covalent binding of PhT to proteins was studied in model systems. The second aim was to investigate the effect of PhT supplementation to feed on *in vitro* ruminal fermentation. In this chapter, the analysis of phenolics and factors involved in phenolic-mediated protein modification will be discussed in relation to consequences for both food and feed applications.

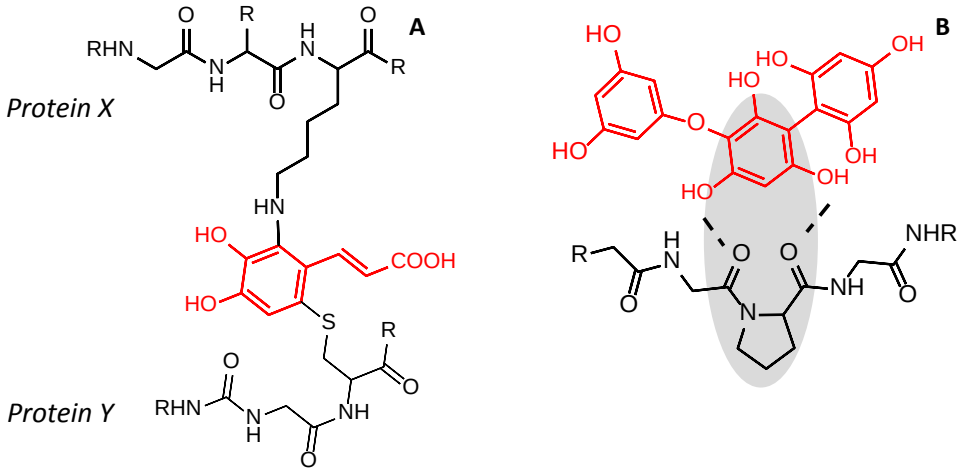

Figure 1 Schematic representation of covalent modification of proteins by phenolics via lysine or cysteine, resulting in crosslinking of proteins (A) or non-covalent binding of phlorotannins to proline residues via CH-π stacking (shaded oval), stabilised by hydrogen bridges (dashed lines) (B). The structures shown are hypothetical, adapted from [1]

Implications for PPO mediated protein modification

The results obtained in **Chapters 2** and **3**, indicated that changes in polyphenol oxidase (PPO) activity and phenolic content and composition influenced the degree of enzymatic browning upon cell damage and subsequent protein extraction. Both the PPO activity and total amount of phenolics in the leaves increased twofold when the age of sugar beet plants increased from 3- to 8-months. In **Chapter 2**, the total phenolic content in sugar beet leaves was quantified in the crude methanolic extract using the Folin Ciocalteu (FC) assay, but the twofold increase measured did not explain the differences in browning observed to a satisfactory extent.

Non-substrate phenolics participate in browning by oxidative coupling and coupled oxidation

The quantification of individual phenolics in **Chapter 3** revealed differences in phenolic composition between the two plant ages, particularly in the ratio of PPO substrates to non-substrates. These differences in composition matched well with the PPO mediated browning observed. The results in this thesis highlight the importance of detailed compositional analysis of phenolics in order to predict or explain reactivity of phenolics in enzymatic browning reactions.

Often, phenolics are quantified as total phenolics using colorimetric assays, such as the FC assay (**Chapter 2**) [2], but this does not provide an accurate prediction of the amount of PPO substrate present. Individual phenolics can be assayed using chromatographic separation and quantification by UV-detection at characteristic wavelengths [3] or mass intensity signals (**Chapter 3**). Within UV-quantification, the molar extinction coefficient of reference compounds is used. The molar extinction coefficient at a certain wavelength is compound specific and can vary between compounds with the same phenolic skeleton. For the caffeic acid derivatives annotated in **Chapter 3**, the molar extinction coefficients have not been reported. It has been reported that when chlorogenic acid is bound to a sulfite group, the extinction coefficient decreases from 18.4 $M^{-1}cm^{-1}$ to 9.4 $M^{-1}{\cdot}cm^{-1}$ [4]. Often, quantities are represented as equivalents of the reference compound but differences in molar extinction coefficient induced by variations in the UV-vis spectrum can result in under- or overestimations of the individual phenolic content. Additionally, structural information cannot be obtained from the UV-vis spectrum as this does not provide information on the type of and position of the substituent. Detailed structural information of isomers can only be obtained by mass fragmentation.

It was demonstrated (**Chapter 3**) that the non-enzymatic continuation reactions were the main drivers for colour formation. The substitution of phenolics determines participation in enzymatic oxidation, oxidative coupling reactions, or coupled oxidation reactions [5]. The phenolics not able to act as a PPO substrate, e.g. ferulic acids and caffeic acid glycosides, participated in oxidative coupling reactions. The PPO substrates, e.g. caffeic acid glycosyl esters, were able to participate in all three reactions due to their *o*-dihydroxyl function on the phenolic ring. Phenolics with monohydroxylated functions, such as coumaric acid- or vitexin derivatives, are potential PPO substrates or participated in oxidative coupling reactions. The reactivity as a PPO substrate was mainly influenced by substituents present. In vitexin, the bulky 8-*C*-glycosyl function induced steric hindrance [6] and

made vitexin effectively a non-substrate, despite the monohydroxylated B-ring. Due to this and their inability to form quinones, vitexins could, therefore, only participate in oxidative coupling reactions. The coupled oxidation reactions were the main drivers for colour formation by formation of large phenolic complexes. During tea leaf fermentation [5] and browning of fruits [7, 8], these oxidative coupling and coupled oxidation reactions in relation to colour formation have already been described. In these studies, however, the focus was mainly on the reactions between PPO substrates [8], and only potential substrate phenolics were quantified. This PhD thesis underlines that the composition of non-substrate phenolics should not be neglected, when trying to understand the browning of leaf materials.

Required conjugate size for browning

In **Chapter 3**, it was demonstrated that high non-substrate-to-substrate ratios inhibited browning by the formation of small oxidative coupling products without significant absorbance in the visible wavelength range (400-800 nm). For instance, the chlorogenic acid-ferulic acid dimers formed during the model incubations did not absorb in the range from 400-500 nm (**Figure 2**). During the reaction, minor amounts of trimers were identified, and insoluble material was formed. The latter could not be analysed using RP-UHPLC-UV-MS. Due to the low trimer quantity, their PDA spectra could not be obtained. It has been demonstrated previously [9] that catechin trimers formed upon oxidation by PPO showed absorbance from 400-550 nm, whereas monomers and dimers did not. At low non-substrate-to-substrate conditions, nascent phenolics can enter multiple cycles of coupled oxidation and oxidative coupling, increasing the size of the oligomeric products and create colour (**Chapter 3**). The fact that dimeric oxidation products do not yet show colour should be taken into account when measuring PPO activity colorimetrically. In both **Chapters 2** and **3**, higher PPO activity correlated to more browning. In **Chapter 2**, the PPO activity determinations were based on colour formation by the oxidised coupling products. The use of colorimetric determination of PPO activity in the visible wavelength (420 nm is often used [10], **Chapter 2**) might result in underestimation of the actual activity. In order to determine enzyme activities as such, oxygen consumption determinations as performed in **Chapter 3** are better, as they do not rely on formation of coloured conjugates.

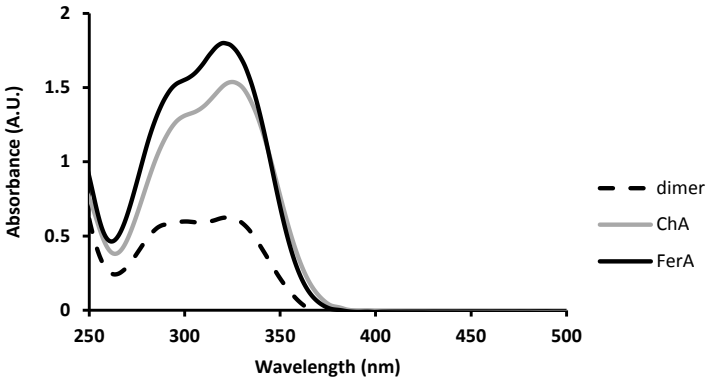

Figure 2 Absorbance spectra of 0.1 mM ChA, 0.1 mM FerA, and a dimer formed from ChA and FerA upon coupled oxidation during incubation with tyrosinase. As the dimer is formed during oxidation, the exact concentration is unknown

Participation of phenolics and amino acids in oxidative coupling

The sugar beet leaves under study contained mixtures of phenolics (phenolic acids, flavonoids), various amino acids, and proteins. An important question is whether the phenolic quinones will react with nucleophilic amino acid residues (free amino acids [11] or amino acids as part of protein) or with other nucleophilic phenolics in solution. For potential covalent protein modifications, free amino acids will not be considered in this discussion. Important factors affecting nucleophilicity of molecules are the presence of electron donating or withdrawing groups in the structure [12], the type of solvent, the solvent pH [12] and presence of bulky groups inducing steric hindrance [13]. With such a complex set of parameters affecting nucleophilicity, it is difficult to accurately predict which compounds in the mixture are most prone to react.

In the model incubations with sugar beet leaf phenolics, the phenolic acid derivatives were more reactive than the flavonoids as 50% of the phenolic acids participated versus only 30% of the flavonoids (**Chapter 3**). Electron donating groups on hydroxycinnamic acids are hydroxyls and *O*-methyls. The expected reactivity, based on nucleophilic substituents directly attached to the phenolic ring and presence of bulky groups within the molecule, decreases: caffeic acid > sinapic acid > ferulic acid > coumaric acid > vitexin. It has been shown previously [13] that the reactivity of hydroxycinnamic acid aglycons and flavonoid aglycons depended more on the position and number of hydroxyl functions than on the phenolic skeleton. The ranking is based on potential reactivity of the phenolic aglycones. Potential steric effects of substituents are omitted. All the phenolics can participate in oxidative coupling to quinones. Caffeic acid ester derivatives are the only phenolic acids present which are able to react with nucleophilic amino acid residues on proteins as these can form *o*-quinones. Nucleophilic amino acids on proteins reported to react with *o*-quinones are cysteine, lysine, tryptophan [13], tyrosine, proline, methionine and histidine [14]. The sulphur containing cysteine is expected to be the most reactive due to its high nucleophilicity followed by lysine, which contains an ε-amino group [15]. Amino acids with carboxylic acid- and aliphatic side chains are the least nucleophilic [15].

When non-substrate phenolics and proteins are present, it is likely that both the non-substrate phenolics and nucleophilic amino acid side chains on proteins participate in oxidative coupling. In cases that caffeic acid quinones react with e.g. cysteine or lysine, the reduced quinone can participate in coupled oxidation. The resulting *o*-quinone can participate in oxidative coupling with another amino acid residue of the same or a second protein. In case of a second protein, the second cycle of coupled oxidation and oxidative coupling can ultimately crosslink proteins (**Figure 1A**) [16], resulting in increased molecular weights and potential for protein precipitation. It should be noted, however, that reactivity of cysteine can be limited when poorly accessible inside proteins [17] and when part of disulfide bridges [18].

Consequences of covalent protein modification in feed applications

As mentioned in the introduction of this thesis, the activity of PPO in forages and grasses can be used for the benefit in ruminant feed. In food applications, the reaction is referred to as enzymatic browning, and often undesired. In that respect, it is associated with decreased solubility [19] and digestibility [20]. The PPO can become active when proteins are extracted (**Chapters 2** and **3**) or when silage [21] is created from leafy resources. When silage is produced from grasses or legumes, it starts with a wilting process. In this step, leaves are cut and aerated. This aeration of damaged cells allows PPO to work. The resulting attachment of phenolics to proteins has been hypothesised to be the reason for limited proteolysis during ensiling [22] and during ruminal fermentation [23]. The decrease in ruminal proteolysis is thought to contribute to the increased amount of rumen escape protein. Consequently, more protein will be available for digestion in the abomasum and further [22]

Besides covalent modification through PPO activity, proteins can become covalently modified upon heating (Maillard reaction) [24] and by treatment with formaldehyde [25]. In all situations, electrophilic groups react with nucleophilic amino acid residues (**Table 1**). These covalent modifications can significantly affect the suitability of proteins for both food and feed applications. Formaldehyde treatment is only applied for feed purposes, and aims to stabilise proteins within the rumen to protect them from ruminal fermentation.

Table 1 Overview of covalent protein modification treatments and reactive groups involved

Source	Reactive group	Reactive amino acids
Maillard reaction [24]	α-Dicarbonyl	*Lys* > N-terminal amino groups
Formaldehyde crosslinking [25, 26]	Formaldehyde	Cys[2] > *Lys* > *His* > *Trp* > N-terminal group
PPO oxidation [27, 28]	o-Quinone (also α-dicarbony)l	Cys[2] > *Lys* > *His* > *Trp* > N-terminal group > Pro > Tyr

[1] Amino acids in *Italic* are essential amino acids
[2] Only Cys which is not part of disulfide bridges can react

Comparing the reactivity's of amino acids in all three reactions, attachment of phenolics to proteins tends to have higher risk for detrimental effects than Maillard reaction, due to the higher participation of essential amino acids. The initial stage of the Maillard reaction involves carbonyl formation and attachment of the reducing sugar to lysine. Upon further heating, formation of α-dicarbonyl Amadori compounds can react with other substituents, mainly amino acids, in the mixture. Cysteine has been reported in the formation of advanced glycation product [29], formed during continuation reactions with Amadori compounds formed after linkage of the sugar to the amino group. During modification of phenolics by PPO, cysteine is expected to react first, when not present in disulfide bridges, followed by lysine. For both food and feed applications, lysine is an essential amino acid and its presence and availability needs to be controlled. By overheating [24] or extensive modification by PPO [30], bioavailability of (essential) amino acids might de decreased.

Factors affecting tannin-mediated protein modification

Structural characterisation of phlorotannins

There is evidence that tannin structure (including size) significantly alters its interactions with proteins. Techniques for detailed characterisation of both terrestrial and marine tannins are, therefore, required. In **Chapter 4**, PhT extracted from *L. digitata* were successfully analysed using several techniques, which all provided information regarding tannin structure (**Table 2**). According to our knowledge, the DP27 annotated is the largest PhT oligomer identified. MALDI-TOF-MS was a good tool to be used as it allows characterisation of larger oligomers.

The use of pyrolysis GC-MS was tested for its suitability to analyse the subunit composition of PhT. Pyrolysis at 650 °C resulted in cleavage of the ether (C-O-C) bonds within phlorethol types of tannins (data not shown). The fucol type (C-C) of linkages were not fragmented as these would need pyrolysis at 1100 °C [31], above the maximum temperature of the pyrolyser used (T_{max} = 950 °C). As not all bonds were cleaved, the technique provided solely information on the presence of phlorotehol types of linkages, and was not conclusive on the presence of C-C linkages. If the desired high temperatures can be reached and C-C bonds would be cleaved, pyrolysis GC-MS might be used to quantify the ratio between fucol and phlorethol type of linkages.

Table 2 Overview and applicability of techniques used to characterise the structure of phlorotannins in *L. digitata*

Technique	Information on	
	Molecular size	Linkage type
^{13}C NMR	+	++
RP-UHPLC-UV-MS	+	++
MALDI-TOF-MS	++	--
Pyrolysis GC-MS	--	+

Taking all the results of **Chapter 4** together, the combination of ^{13}C and ^{1}H NMR spectroscopy was the most suitable analytical technique, applicable for determination of the PhT content of the extract, and to quantify the linkages (C-C, C-O-C) and benzene ring substituents (C-H, C-OH) present. Additionally, the average number of hydrogens on the phenolic ring provided information on the structure and indicated presence of branched PhT, phloroglucinol subunits linked to three other phloroglucinol subunits. The occurrence of these branched PhT has been described before [32] and some structures were characterised by NMR spectroscopy after acetylation and purification [33]. The fact that branched PhT exist within mixtures aids in the understanding of the complexity of LC-MS/MS profiles. Nevertheless, LC-MS/MS analysis is not conclusive on the position of the linkages and the point of branching cannot be identified. Branching of PhT might also influence their affinity to proteins. Branched PhT are more compact than linear ones, and consequently they display less rotational freedom of the subunits, and, therewith, lower affinity for proteins.

Phlorotannin isomers binding to proteins

Different PhT are expected to vary in binding affinities towards proteins. The isomers can vary in their linkage types between subunits, either C-C or C-O-C. Additionally, the subunits can be attached at different positions on the benzene ring. With respect to each other, subunits can be linked via, e.g. *ortho-, meta* or *para* fashion (**Figure 3**). Both aspects might influence protein binding.

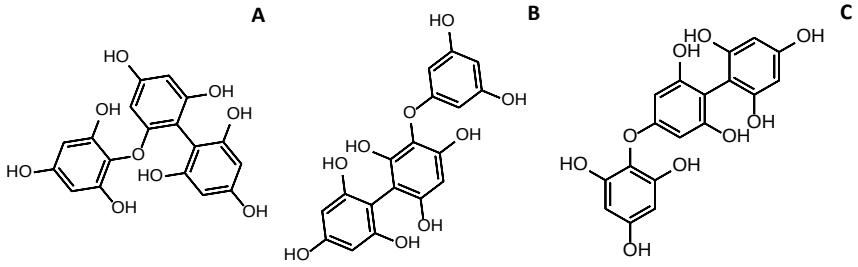

Figure 3 Phloroglucinol trimers with subunits connected via *ortho-* (A), *meta-* (B) and *para-* (C) fashion

The binding of tannins to proteins occurs via hydrophobic interaction (stacking) and subsequent stabilisation by hydrogen bridges between the phenolic rings and the proline residues of the protein (**Figure 1B**). Rotational freedom of tannin subunits and intermediate tannin size are positively correlated to binding affinity to proteins [34]. Both the linkage types and linkage fashions influence the PhT flexibility. Within the linkage types, C-O-C linked subunits are expected to be more flexible than C-C linked subunits. The ether contains two linkages around which free rotation is possible. For the various linkage fashions, the order of flexibility is expected to have the following order: *para* > *meta* > *ortho*. The former two are expected to be less affected by steric hindrance. The *o*-connected isomers have the phenolic rings close to each other, decreasing ability to orient themselves towards the protein molecule. Within individual phloroglucinol oligomers, the subunits are not necessarily linked by a single linkage type or single fashion. Together with branching, the various bonds between subunits (C-C, C-O-C) and the various fashions by which the subunits are connected make it

difficult to predict the binding affinity of PhT mixtures for proteins, not in the least because it is difficult to analyse the precise composition of the PhT mixture.

In **Chapter** 3, the coupling of phenolics to each other via repeated cycles of oxidative coupling and coupled oxidation was described. This process creates large phenolic structures which do resemble tannins to a certain extent. The structures are high in molecular weight with increased hydrophobicity and do contain phenolic groups. These properties make non-covalent interaction between the enlarged phenolic structures and proteins likely (**Figure 1B)**. These aspects should be considered when the application of PPO modified protein extracts is studied

Assay phlorotannin-protein binding affinity

By studying the binding between PhT and proteins using fluorescence quenching and protein precipitation in **Chapter 5**, the first direct evidence for interaction between these molecules was provided. The fluorescence quenching assay was conducted using a microplate set-up, scaling down conventional fluorescence quenching assays [35-37]. It allowed the use of small sample volumes and, therefore, allowed for rapid screening of binding affinities under various conditions. In conventional fluorescence quenching assays, the ligands are often stepwise titrated into the protein solution [35], or mixtures are prepared in large volumes within cuvettes [38]. The former is a time consuming process as only one sample at a time can be measured. The large sample volumes required in the latter method can be a disadvantage when low amounts of phenolic ligand are available. These methodologies are therefore less applicable for screening purposes. A potential complicating factor in the study of tannin-protein interaction by fluorescence quenching are inner filter effects. An important cause for inner filter effects are the absorbance of quenchers in the wavelength range of emission [39]. When substantial absorbance takes place, the fluorescence intensity measured needs to be corrected for ligand absorbance at both excitation and emission wavelength [35]. For PhT (**Chapter 5**), only minor corrections were needed as the tannins showed minor absorbance at the emission wavelength used (λ_{em} = 350 nm).

Besides fluorescence quenching, techniques such as isothermal titration calorimetry (ITC) [34], ultrafiltration [40], mass spectrometry [41] and NMR spectroscopy [42] are used for analysis of tannin-protein interactions. For bimolecular tannin-protein systems, ITC is a suitable technique [34]. Together with conventional fluorescence quenching assays [35], ITC is seen as low throughput method and not suitable for the analysis of PhT mixtures. In this laboratory, ultrafiltration has been applied for bimolecular systems [40]. For phlorotannins it was found to be not suitable as the high hydrodynamic volumes of tannins clogged the membrane pores. Additionally, the tannins interacted with the cellulosic membrane material [43]. LC-MS can be useful when binding of tannin mixtures to proteins is studied, to identify preferential binding. The results obtained in **Chapter 4** highlighted that UHPLC-MS was not sufficient in analysing all the tannins present due to the high numbers of isomers and limiting detection range of the mass detector and, therefore, UHPLC-MS was not applicable for binding studies. NMR spectroscopy can be used to determine the phenolic's binding site within a protein, visualised by specific chemical shifts, but it requires high sample volumes and it is sensitive to precipitations [44]. Overall, fluorescence quenching appears to be the most suitable

method when phlorotannins are to be assayed, given that the protein under study contains at least one tryptophan residue.

Protein precipitation by tannins and effects on post-rumen digestibility

In **Chapter 5**, the re-solubilisation of the protein from the insoluble PhT-protein complexes upon changes in the pH was shown to be mainly dependent on protein solubility. The presence of PhT widened the pH range for precipitation from 0.5 to 1-1.5 pH unit around the proteins' pI. Due to structural similarities between terrestrial and marine tannins (high abundance of hydroxyl groups and high molecular weight [1]), the broadening of the pH range for protein precipitation observed for PhT was similar to that for terrestrial hydrolysable and condensed tannins [45]. When the pH decreased far (≥ 2 pH units) below the pI, electrostatic interactions were sufficient to induce tannin-protein complex re-solubilisation with globular proteins such as BSA. The random coil β-casein showed poor re-solubilisation, probably related to protein-protein interactions rather than tannin presence.

The precipitation and re-solubilisation of proteins and therewith potential influence on post-rumen protein digestibility are probably dependent on the types of proteins present in the feed. Feed proteins are usually a mixture of different proteins with varying pI values. Proteins consumed by ruminants originate from forages and from soy or rapeseed present in the concentrate supplements [46]. Within the forages, approximately 70% of the proteins in forages resemble rubisco which has a pI between 5.1-5.7 [47]. The pI of soybean proteins ranges between 4.5-5.6 [48] and those of rapeseed have a wide pI range between 5.2-8 [49]. At the normal ruminal pH of 6-7, these proteins are predominantly present in an insoluble form when bound to tannins, despite their pI values below the rumen pH. In a previous study [50], pepsin (pI 2.0-2.8 [51]) and lysozyme (pI 11.35 [52]) were used as models for acidic and alkaline proteins, respectively. There was minor protein precipitation by tannins at pH 6-7 for both proteins [50]. It can, therefore, be postulated that potential feed proteins with pI values in the acidic or alkaline range are less likely to be present in an insoluble tannin-protein complex within the rumen as these proteins are still sufficiently charged. In the acidic abomasum, insoluble proteins regain their charge and electrostatic repulsions will bring the proteins and bound tannins back into solution. It might be assumed that soluble tannin-protein complexes are easier to degrade by proteases than insoluble complexes.

Practical applicability of phlorotannin from *L. digitata*

The NH_3 and CH_4 mitigating properties of PhT in **Chapter 6** showed that PhT can be applied as effective strategy in creating ruminal escape protein and decrease CH_4 excretion. The effective dosages required for reduction in CH_4 and protein protection for terrestrial HT [53] were lower and more effective than terrestrial CT and PhT. These hydrolysable tannins, however, can be metabolised within both the rumen [54] and further down the GI tract [55]. Tannases, produced by microorganisms in the gastrointestinal tract, are able to utilise HT and produce volatile fatty acids which can then, when absorbed by the animals, be further used in the citric acid cycle to provide energy [56]. The microbial fermentation also contributes to the higher toxicity level of HT [57], as the metabolites formed can be toxic. Terrestrial CT are only metabolised to a minor extent [58]. For PhT, metabolism has, to our knowledge not been reported in the scientific literature. Due to the

highly condensed structure, metabolism of the oligomers seems unlikely. The terrestrial CT and PhT were equally effective in CH_4 reduction and PhT were slightly less effective in protein protection. Despite the slightly lower effectiveness in protein protection, the applicability of PhT in ruminant feed seems feasible, due to low risks of becoming toxic.

The effects of PhT were assayed *in vitro*, using a 60% pure PhT extract (**Chapter 4**). Even though the PhT were highly effective in protecting dietary protein from ruminal degradation, the application of such a highly purified extract would be challenging in practice. The gross composition of *L. digitata* used in this thesis is provided in **Table 3.** The protein content was determined by DUMAS with a nitrogen-to-protein conversion factor of 5.13 [59]. The fat content was determined gravimetrically by extraction in chloroform/methanol [60]. The ash content was not determined, but in literature it was estimated to be 30% DM [61].

Table 3 Macro and micronutrient composition of *L. digitata* before and after extraction of alginates

Nutrient (g/kg DM)	*L. digitata*	Residue after alginate extraction
protein and non-protein nitrogen	142	266.9
Carbohydrate	468	-
Fat	45	84.6
Phlorotannin	45	84.6
Ash	300 [1]	563.9

[1] Ash content taken from Schiener *et al.* 2015

Assuming that a lactating dairy cow of 680 kg consumes roughly 28 kg dry feed on a daily basis [62], of which 4.5 kg is nitrogenous material and PhT are incorporated at 40 g/kg diet, the daily tannin intake would be roughly 1.1 kg. The PhT content of *L. digitata* is approximately 45 g/kg DM. To extract the required 1.3 kg PhT and creating a 60% pure extract, 24.8 kg dry seaweed would be needed. The DM content of macroalgae is approximately 150 g/kg fresh weight [63]. To reach the daily tannin amount, ~166 kg fresh *L. digitata* would be needed, which is challenging in practice.

When a side stream after extraction of carbohydrates (often performed in hot acid or alkaline solutions [64]) would be used, the picture changes. Cell wall bound PhT are extracted in alkaline or acidic solutions without hampering the tannin structure [65]. After extraction of carbohydrates, the remaining pellet is enriched in PhT and proteins. The contents of all nutrients are roughly doubled (**Table 3**). When this pellet is taken as such or as a starting point for further purification, the macroalgae have increased applicability. Its applicability, however, is still challenging in practice. The application of the residue can theoretically provide substantial amounts of protein and other nutrients (**Table 4**). In order to meet the daily tannin requirements, 13.2 kg of the residue has to be fed. At these levels, however, there is overfeeding of ash (**Table 4**). Additionally, it is likely that the proteins present in the residue have a decreased value due to the hot acidic or alkaline extraction treatment. Proteins have usually limited stability at these conditions [18]. Due to the decreased protein quality and the overfeeding of ash, further purification of the tannins by an additional

extraction step in organic solvent would be beneficial, to decrease the ash content and enrich the extract in PhT.

Despite the effectiveness of PhT in feed, practical applicability of *L. digitata* PhT as purified extract or side stream after carbohydrate extraction is challenging as the PhT content in *L. digitata* is generally low. The use of macroalgae which are higher in PhT content are in that respect preferred. Examples of other species are *Ascophyllum nodosum* and *Ecklonia cava*, containing ~9% [66] and ~20% [67] PhT on a dry matter basis. The resulting residue after carbohydrate extraction would yield a higher tannin content in the residue, decreasing feeding requirements. There is, however, still extensive processing required. These high processing and feeding requirements limit the applicability of brown macroalgal tannins with the purpose PhT addition to the ruminant's diet.

Table 4 Daily intake of nitrogen, carbohydrates, fats, ash and phlorotannins by lactating dairy cow and supply of these by addition of the residue of *L. digitata* after carbohydrate extraction

Nutrient	Total daily intake [1] (kg)	Supply by residue after extraction [2] (kg)
Nitrogen	4.5	3.5
Carbohydrate	18.8	-
Fat	1.2	1.3
Ash	2.2	7.4
Phlorotannin	1.3	1.3

[1] Daily intake is based on a lactating cow of 680 kg, consuming 27.3 kg DM feed [62] (phlorotannins not included)

[2] Supply is based on feeding 13.2 kg of the *L. digitata residue* after carbohydrate extraction

Prospects

Green protein resources as animal feed

The determination of the amino acid composition of sugar beet leaf proteins in **Chapter 2** showed that these have a good amino acid composition to be applied in food and feed. After extraction, the proteins can be applied in pet food or young animals. When applied as silage or just as the unprocessed source, the resources can be utilised in ruminant feed. When ruminants are considered, lysine and methionine are usually the first limiting amino acids [46, 68]. The amino acid composition of sugar beets is comparable to that of soybeans, being slightly higher in lysine and slightly lower in arginine content (**Table 5**). Just like rye grass, sugar beet leaf proteins contain more lysine than red clover and corn. With respect to the high similarity between amino acid composition of sugar beet leaf and soy, sugar beet leaf proteins are a very good source for replacement of soybean proteins. In all cases, the cysteine content is limited [17].

For *L. digitata*, the amino acid composition was not determined and values from literature were used. Macroalgae harvested in colder climates had similar lysine and a higher methionine content

[69-71] compared to soy. Macroalgae collected in warmer climates contain more lysine than soy, but have a similar methionine content [56] (**Table 5**). Despite the lower lysine content in macroalgae than in soy, macroalgal proteins are still potentially valuable for animal feed purposes. They might be used to supplement lysine poor corn.

When the proteins from sugar beet leaves and *L. digitata* can be extracted without modification of proteins by phenolics, either covalently via PPO or non-covalently via PhT, respectively, the extracts might be applied in food for pets or young animals. For these purposes, leafy resources with low PPO activity, low phenolics content and high phenolic non-substrate-to-substrate ratio are useful. These resources do not show extensive browning and polymerisation of phenolics (**Chapter 3**). Regarding macroalgal applications, brown ones are less suitable for protein extraction due to relatively low protein content and presence of PhT. Other macroalgal sources, e.g. green and red, are more suitable as these are higher in protein content and do not contain PhT [63].

Table 5 Composition of essential amino acids and cysteine in agricultural and marine resources

Source	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val	Cys[a]
Soybean [b]	7.3	2.8	4.6	7.8	6.3	1.4	5.3	3.9	1.3	4.6	1.5
Corn silage [b]	1.9	1.8	3.3	8.6	2.5	1.5	3.8	3.2	1.2	4.4	1.3
Rye grass [c]	6.0	2.2	4.7	9.4	5.6	2.2	5.7	5.2	n.d.	6.5	1.4
Red clover [d]	4.4	3.1	4.8	6.9	4.7	1.9	3.9	1.7	1.3	4.1	1.8
Sugar beet leaf [e]	5.4	3.2	4.9	9.3	6.2	2.2	5.9	5.1	n.d.	6.2	1.9
L. digitata (Norway) [f]	5.5	1.9	4.3	8.4	5.9	2.9	5.5	6.1	n.d.	5.8	n.d.
L. digitata (Japan) [g]	5.3	4.3	4.7	8.0	8.6	2.7	5.1	6.1	0.3	10.8	2.0

Composition expressed as % total protein

n.d. = not determined

[a] Cys is not an essential amino acid but can participate in covalent protein modification

[b] NRC 2001, [c] Edmunds (2012) [d] Purwin (2015) [2] **Chapter 2**, [f] Mæhre (2014), [g] Kolb *et al.* (2004)

Applications for covalently and non-covalently modified proteins

When proteins are modified during processing, or when resources are fractionated into fractions enriched in e.g. PhTs, both resources can be applied in ruminant feed. Modification of proteins by phenolics provide opportunity to protect protein from ruminal degradation and can in some cases even mitigate CH_4 excretion.

For sugar beet leaves, their application after ensiling is most promising. Ensiling allows PPO to be active and create covalently modified protein. Additionally, ensiling is an economically valuable process due to limited processing [72]. Especially the leaves with low non-substrate-to-substrate ratio are suitable, as these showed extensive browning, polymerisation of phenolics and potentially attachment of phenolics to proteins. *In vivo*, the use of silage from red clover in which PPO had been

active reduced the ruminal NH_3 content by 20-66% with respect to silages from alfalfa, which is known to contain no PPO [21, 73]. It might even be considered to ensile corn or other forage sources in presence of these leaves. For the co-ensiling of alfalfa and red clover, nitrogen utilisation and rumen NH_3 improved compared to ensiling either alfalfa or red clover alone [21]. Overall, the feeding improvement effects were related to decreased ruminal protein digestibility rather than carbohydrate digestibility. The total dry matter digestibility, VFA production and the acetate-to-propionate ratios were not affected by covalently modified proteins, but the proportions of branched chain fatty acids decreased [74], indicating decreased protein breakdown. Additionally, the polymerisation of phenolics via oxidative coupling and coupled oxidation might create tannin like compounds providing opportunity for non-covalent protein modification. The extent at which this occurs is unknown.

For macroalgae, application of purified PhT or extracts enriched in PhT are useful. *In vivo* and *in situ* trials using proteins non-covalently modified by CT from quebracho (83 g/kg DM feed) showed a 25% reduction in ruminal NH_3 concentration without influencing the overall protein digestibility [75]. Application of condensed tannins from *Lotus* species (22-55 g/kg feed DM) increased duodenal flow of NH_3 by 27-62%. Higher doses (75-100 g/kg DM feed) reduced concentrations even further, but hampered voluntary feed intake and weight gain [76]. In all cases, both carbohydrate and protein fermentation were affected. The former correlated to decrease in CH_4 formation.

In **Table 5**, the consequences of covalent and non-covalent protein modification for application in ruminant feed are compared. Based on the scientific literature and the results of **Chapter 6**, the application of tannins seems to be more beneficial than the use of PPO modified proteins related to the additional decrease in CH_4. Additionally, the ability to control the extent of modification [73] is important. It is likely that high PPO activity and PPO substrate contents combined with long incubation times increase the risk of over-modification of the protein and significant loss of amino acid bioavailability [27].

Table 6 Comparison between applicability of PPO modifications and tannins in protecting protein from ruminal fermentation

	PPO modification	Tannins
Protein protection	Yes	Yes
CH_4 mitigation	Unknown	Yes
Availability essential amino acids	Lys availability ↓	Not affected
Ability to control modification	Challenging due to non-enzymatic continuation reactions	By dose adjustment
Toxicity	No	Potentially
Endogenous enzyme activity	Variable	Not relevant
Phenolic composition and content	Variable	Variable

To summarise, the potential application of sugar beet leaves and macroalgae in food and feed require different strategies regarding control of protein modification. For food and animal feed applications requiring high protein quality, browning and covalent protein modification is undesired as these aspects are attributed to decreased protein quality regarding techno-functionality and nutritional value. For these purposes, leafy resources high in non-substrate-to-substrate ratio or leafy resources in which the activity of PPO is inhibited (e.g. by sulphite [77]), are useful. Alternatively, green and red macroalgae can be considered as these are usually higher in protein content and lack tannins [63]. For feed applications, covalent modification of proteins can be beneficial as the modified proteins are more resistant against ruminal fermentation. Non covalently modified proteins, by PhT from seaweeds are preferred over covalently modified proteins as the non-covalent modification has additional benefits in addition to formation of rumen bypass protein: it hinders CH_4 formation, does not involve losses of amino acid bioavailability and dosage can be better controlled.

References

1. Le Bourvellec, C. and C.M.G.C. Renard, Interactions between polyphenols and macromolecules: Quantification methods and mechanisms. *Crit. Rev. Food Sci. Nutr.*, **2012**. 52(3): 213-248.
2. Singleton, V.L., R. Orthofer, and R.M. Lamuela-Raventós, Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent, *in Meth. Enzymol.* **1998**. 152-178.
3. Radi, M., M. Mahrouz, A. Jaouad, M. Tacchini, S. Aubert, M. Hugues, and M.J. Amiot, Phenolic composition, browning susceptibility, and carotenoid content of several apricot cultivars at maturity. *HortSci.*, **1997**. 32(6): 1087-1091.
4. Narváez-Cuenca, C.E., T.F.M. Kuijpers, J.P. Vincken, P. De Waard, and H. Gruppen, New insights into an ancient antibrowning agent: Formation of sulfophenolics in sodium hydrogen sulfite-treated potato extracts. *J. Agric. Food Chem.*, **2011**. 59(18): 10247-10255.
5. Kusano, R., T. Tanaka, Y. Matsuo, and I. Kouno, Structures of epicatechin gallate trimer and tetramer produced by enzymatic oxidation. *Chem. Pharm. Bull. (Tokyo)*, **2007**. 55(12): 1768-72.
6. Parveen, I., M.D. Threadgill, J.M. Moorby, and A. Winters, Oxidative phenols in forage crops containing polyphenol oxidase enzymes. *J. Agric. Food Chem.*, **2010**. 58(3): 1371-1382.
7. Nicolas, J.J., F.C. Richard-Forget, P.M. Goupy, M.J. Amiot, and S.Y. Aubert, Enzymatic browning reactions in apple and apple products. *Crit. Rev. Food Sci. Nutr.*, **1994**. 34(2): 109-157.
8. Radi, M., M. Mahrouz, A. Jaouad, M. Tacchini, S. Aubert, M. Hugues, and M.J. Amiot, Phenolic composition, browning susceptibility, and carotenoid content of several apricot cultivars at maturity. *HortScience*, **1997**. 32(6): 1087-1091.
9. Uchida, K., K. Ogawa, and E. Yanase, Structure determination of novel oxidation products from epicatechin: thearubigin-like molecules. *Molecules*, **2016**. 21(3): 273.
10. Gowda, L.R. and B. Paul, Diphenol activation of the monophenolase and diphenolase activities of field bean (*Dolichos lablab*) polyphenol oxidase. *J. Agric. Food Chem.*, **2002**. 50(6): 1608-1614.
11. Gzik, A., Accumulation of proline and pattern of α-amino acids in sugar beet plants in response to osmotic, water and salt stress. *Environ. Exp. Bot.*, **1996**. 36(1): 29-38.
12. McMurry, J., Chapter 8: aromatic compouds, *in Organic Chemistry: A Biological Approach*, J. McMurry, Editor. **2007**, Thomson Brooks/Cole: CA, USA.
13. Rawel, H.M., D. Czajka, S. Rohn, and J. Kroll, Interactions of different phenolic acids and flavonoids with soy proteins. *Int. J. Biol. Macromol.*, **2002**. 30(3-4): 137-150.
14. Matheis, G. and J.R. Whitaker, Modification of proteins by polyphenol oxidase and peroxidase and their products. *J. Food Biochem.*, **1984**. 8(3): 137-162.
15. Bischoff, R. and H. Schlüter, Amino acids: Chemistry, functionality and selected non-enzymatic post-translational modifications. *J. Proteom.*, **2012**. 75(8): 2275-2296.

16. Rawel, H.M. and S. Rohn, Nature of hydroxycinnamate-protein interactions. *Phytochem. Rev.*, **2010**. 9(1): 93-109.
17. Miseta, A. and P. Csutora, Relationship between the occurrence of cysteine in proteins and the complexity of organisms. *Mol. Biol. Evol.*, **2000**. 17(8): 1232-1239.
18. Damodaran, S., Amino acids, peptides and proteins, *in Fennema's Food Chemistry*, S. Damodaran, K.L. Parkin, and O.R. Fennema, Editors. **2008**, CRC Press: NY, USA.
19. Prigent, S.V.E., A.G.J. Voragen, A.J.W.G. Visser, G.A. Van Koningsveld, and H. Gruppen, Covalent interactions between proteins and oxidation products of caffeoylquinic acid (chlorogenic acid). *J. Sci. Food Agric.*, **2007**. 87(13): 2502-2510.
20. Hurrell, R.F., P.A. Finot, and J.L. Cuq, Protein-polyphenol reactions: 1. Nutritional and metabolic consequences of the reaction between oxidized caffeic acid and the lysine residues of casein. *Br. J. Nutr.*, **1982**. 47(2): 191-211.
21. Broderick, G.A., R.P. Walgenbach, and S. Maignan, Production of lactating dairy cows fed alfalfa or red clover silage at equal dry matter or crude protein contents in the diet. *J. Dairy Sci.*, **2001**. 84(7): 1728-1737.
22. Lee, M.R.F., Forage polyphenol oxidase and ruminant livestock nutrition. *Front. Plant Sci.*, **2014**. 5(DEC).
23. Copani, G., C. Ginane, A. Le Morvan, and V. Niderkorn, Patterns of *in vitro* rumen fermentation of silage mixtures including sainfoin and red clover as bioactive legumes. *Anim. Feed Sci. Technol.*, **2015**. 208: 220-224.
24. Faldet, M.A., L.D. Satter, and G.A. Broderick, Determining optimal heat treatment of soybeans by measuring available lysine chemically and biologically with rats to maximize protein utilization by ruminants. *J. Nutr.*, **1992**. 122(1): 151-160.
25. Lu, K., W. Ye, L. Zhou, L.B. Collins, X. Chen, A. Gold, L.M. Ball, and J.A. Swenberg, Structural characterization of formaldehyde-induced cross-links between amino acids and deoxynucleosides and their oligomers. *J. Am. Chem. Soc.*, **2010**. 132(10): 3388-3399.
26. Metz, B., G.F.A. Kersten, P. Hoogerhout, H.F. Brugghe, H.A.M. Timmermans, A. De Jong, H. Meiring, J. Ten Hove, W.E. Hennink, D.J.A. Crommelin, and W. Jiskoot, Identification of formaldehyde-induced modifications in proteins: Reactions with model peptides. *J. Biol. Chem.*, **2004**. 279(8): 6235-6243.
27. Hurrell, R.F. and P.A. Finot, Nutritional consequences of the reactions between proteins and oxidized polyphenolic acids. *Adv. Exp. Med. Biol.*, **1984**. 177: 423-435.
28. Kroll, J., H.M. Rawel, and S. Rohn, Reactions of plant phenolics with food proteins and enzymes under special consideration of covalent bonds. *Food Sci. Tech. Res.*, **2003**. 9(3): 205-218.
29. Alt, N., J.A. Carson, N.L. Alderson, Y. Wang, R. Nagai, T. Henle, S.R. Thorpe, and J.W. Baynes, Chemical modification of muscle protein in diabetes. *Arch. Biochem. Biophys.*, **2004**. 425(2): 200-206.
30. Rawel, H.M., K. Meidtner, and J. Kroll, Binding of selected phenolic compounds to proteins. *J. Agric. Food Chem.*, **2005**. 53(10): 4228-4235.
31. Jackson, E.A., X. Xue, H.Y. Cho, and L.T. Scott, Probing mechanisms of aryl-aryl bond cleavages under flash vacuum pyrolysis conditions. *Aust. J. Chem.*, **2014**. 67(8-9): 1279-1287.
32. Martinez, J.H. and H.G. Castaneda, Preparation and chromatographic analysis of phlorotannins. *J. Chromatogr. Sci.*, **2013**. 51(8): 825-38.
33. Glombitza, K.W. and J. Große-Damhues, Antibiotics from Algae XXXIII: Phlorotannins of the brown alga *Himanthalia elongata*. *Planta Med.*, **1985**. 51(01): 42-46.
34. Karonen, M., M. Oraviita, I. Mueller-Harvey, J.P. Salminen, and R.J. Green, Binding of an oligomeric ellagitannin series to bovine serum albumin (BSA): Analysis by isothermal titration calorimetry (ITC). *J Agric. Food Chem.*, **2015**. 63(49): 10647-10654.
35. Bohin, M.C., J.P. Vincken, A.H. Westphal, A.M. Tripp, P. Dekker, H.T.W.M. Van Der Hijden, and H. Gruppen, Interaction of flavan-3-ol derivatives and different caseins is determined by more than proline content and number of proline repeats. *Food Chem.*, **2014**. 158: 408-416.
36. Rawel, H.M., S.K. Frey, K. Meidtner, J. Kroll, and F.J. Schweigert, Determining the binding affinities of phenolic compounds to proteins by quenching of the intrinsic tryptophan fluorescence. *Mol. Nutr. Food Res.*, **2006**. 50(8): 705-713.
37. Soares, S., N. Mateus, and V. De Freitas, Interaction of different polyphenols with Bovine Serum Albumin (BSA) and Human Salivary α-Amylase (HSA) by fluorescence quenching. *J. Agric. Food Chemi.*, **2007**. 55(16): 6726-6735.

38. Papadopoulou, A., R.J. Green, and R.A. Frazier, Interaction of flavonoids with bovine serum albumin: A fluorescence quenching study. *J. Agric. Food Chem.*, **2005**. 53(1): 158-163.
39. Lakowicz, J.R., Quenching of fluorescence, *in Principles of Fluorescence Spectroscopy*, J.R. Lakowicz, Editor. **2006**, Springer NY, USA. 277-327.
40. Bohin, M.C., J.P. Vincken, H.T.W.M. Van Der Hijden, and H. Gruppen, Efficacy of food proteins as carriers for flavonoids. *J. Agric. Food Chem.*, **2012**. 60(16): 4136-4143.
41. Canon, F., A. Giuliani, F. Paté, and P. Sarni-Manchado, Ability of a salivary intrinsically unstructured protein to bind different tannin targets revealed by mass spectrometry. *Anal. Bioanal. Chem.*, **2010**. 398(2): 815-822.
42. Cala, O., N. Pinaud, C. Simon, E. Fouquet, M. Laguerre, E.J. Dufourc, and I. Pianet, NMR and molecular modeling of wine tannins binding to saliva proteins: Revisiting astringency from molecular and colloidal prospects. *FASEB J.*, **2010**. 24(11): 4281-4290.
43. Lopes, G., C. Sousa, L.R. Silva, E. Pinto, P.B. Andrade, J. Bernardo, T. Mouga, and P. Valentão, Can phlorotannins purified extracts constitute a novel pharmacological alternative for microbial infections with associated inflammatory conditions? *PLoS ONE*, **2012**. 7(2).
44. Charlton, A.J., N.J. Baxter, M.L. Khan, A.J.G. Moir, E. Haslam, A.P. Davies, and M.P. Williamson, Polyphenol/peptide binding and precipitation. *J. Agric. Food Chem.*, **2002**. 50(6): 1593-1601.
45. Hagerman, A.E. and K.M. Klucher, Tannin-protein interactions. *Prog. Clin. Biol. Res.*, **1986**. 213: 67-76.
46. Council, N.R., Nutrient requirements of Dairy Cattle: seventh revised edition, 2001. **2001**, Washington, DC: The National Academic Press. 408.
47. Mummenhoff, K. and H. Hurka, Rubisco as a taxonomic tool: Introducing a simple electrophoretic technique for separating the holoenzyme. *Plant Syst. Evol.*, **1986**. 153(3): 281-285.
48. Chove, B.E., A.S. Grandison, and M.J. Lewis, Emulsifying properties of soy protein isolate fractions obtained by isoelectric precipitation. *J. Sci. Food Agric.*, **2001**. 81(8): 759-763.
49. Dong, X.Y., L.L. Guo, F. Wei, J.F. Li, M.L. Jiang, G.M. Li, Y.D. Zhao, and H. Chen, Some characteristics and functional properties of rapeseed protein prepared by ultrasonication, ultrafiltration and isoelectric precipitation. *J. Sci. Food Agric.*, **2011**. 91(8): 1488-98.
50. Hagerman, A.E. and L.G. Butler, Protein precipitation method for the quantitative determination of tannins. *J. Agric. Food Chem.*, **1978**. 26(4): 809-812.
51. Malamud, D. and J.W. Drysdale, Isoelectric points of proteins: A table. *Anal. Biochem.*, **1978**. 86(2): 620-647.
52. Sigma-aldrich, Product information Lysozyme from chicken egg white, Sigma-Aldrich, Editor. **2016**: St. Louis, USA.
53. Hassanat, F. and C. Benchaar, Assessment of the effect of condensed (acacia and quebracho) and hydrolysable (chestnut and valonea) tannins on rumen fermentation and methane production *in vitro*. *J. Sci. Food Agric.*, **2013**. 93(2): 332-9.
54. Singh, B., T.K. Bhat, O.P. Sharma, S.S. Kanwar, P. Rahi, and A. Gulati, Isolation of tannase-producing Enterobacter ludwigii GRT-1 from the rumen of migratory goats. *Small Rum. Res.*, **2012**. 102(2–3): 172-176.
55. González-Barrio, R., P. Truchado, R. García-Villalba, G. Hervás, P. Frutos, J.C. Espín, and F.A. Tomás-Barberán, Metabolism of oak leaf ellagitannins and urolithin production in beef cattle. *J. Agric. Food Chem.*, **2012**. 60(12): 3068-3077.
56. Bhat, T.K., B. Singh, and O.P. Sharma, Microbial degradation of tannins-a current perspective. *Biodegradation*, **1998**. 9(5): 343-57.
57. Zhu, J., L.J. Filippich, and M.T. Alsalami, Tannic acid intoxication in sheep and mice. *Res. Vet. Sci.*, **1992**. 53(3): 280-292.
58. Appeldoorn, M.M., J.P. Vincken, A.M. Aura, P.C. Hollman, and H. Gruppen, Procyanidin dimers are metabolized by human microbiota with 2-(3,4-dihydroxyphenyl)acetic acid and 5-(3,4-dihydroxyphenyl)-gamma-valerolactone as the major metabolites. *J. Agric. Food Chem.*, **2009**. 57(3): 1084-92.
59. Lourenço, S.O., E. Barbarino, J.C. De-Paula, L.O.d.S. Pereira, and U.M.L. Marquez, Amino acid composition, protein content and calculation of nitrogen-to-protein conversion factors for 19 tropical seaweeds. *Phycol. Res.*, **2002**. 50(3): 233-241.
60. Folch, J., M. Lees, and G.H. Sloane Stanley, A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.*, **1957**. 226(1): 497-509.

61. Schiener, P., K.D. Black, M.S. Stanley, and D.H. Green, The seasonal variation in the chemical composition of the kelp species *Laminaria digitata*, *Laminaria hyperborea*, *Saccharina latissima* and *Alaria esculenta. J. Appl. Phycol.*, **2015**. 27(1): 363-373.
62. Murphy, M.R., Nutrients, digestion and absorption*, in Encyclopedia of Dairy Science*. **2011**, Elsevier: NU, USA.
63. Holdt, S.L. and S. Kraan, Bioactive compounds in seaweed: functional food applications and legislation*. J. Appl. Phycol.*, **2011**. 23(3): 543-597.
64. McHugh, D.J., A Guide to the Seaweed Industry. **2003**, FAO: Rome.
65. Koivikko, R., J. Loponen, T. Honkanen, and V. Jormalainen, Contents of soluble, cell-wall-bound and exuded phlorotannins in the brown alga *Fucus vesiculosus*, with implications on their ecological functions*. J. Chem. Ecol.*, **2005**. 31(1): 195-212.
66. Pavia, H., G.B. Toth, A. Lindgren, and P. Åberg, Intraspecific variation in the phlorotannin content of the brown alga *Ascophyllum nodosum. Phycologia* **2003**. 42(4): 378-383.
67. Stern, J.L., A.E. Hagerman, P.D. Steinberg, F.C. Winter, and J.A. Estes, A new assay for quantifying brown algal phlorotannins and comparisons to previous methods*. Journal of Chemical Ecology*, **1996**. 22(7): 1273-1293.
68. Lapierre, H., D. Pacheco, R. Berthiaume, D.R. Ouellet, C.G. Schwab, P. Dubreuil, G. Holtrop, and G.E. Lobley, What is the true supply of amino acids for a dairy cow? *J. Dairy Sci.*, **2006**. 89, Supplement: E1-E14.
69. Bogolitsyn, K.G., P.A. Kaplitsin, and A.S. Pochtovalova, Amino-acid composition of arctic brown algae*. Chem. Nat. Comp.*, **2014**. 49(6): 1110-1113.
70. Mæhre, H.K., M.K. Malde, K.E. Eilertsen, and E.O. Elvevoll, Characterization of protein, lipid and mineral contents in common Norwegian seaweeds and evaluation of their potential as food and feed*. J. Sci. Food Agric.*, **2014**. 94(15): 3281-3290.
71. Manns, D., A.L. Deutschle, B. Saake, and A.S. Meyer, Methodology for quantitative determination of the carbohydrate composition of brown seaweeds (*Laminariaceae*)*. RSC Advances*, **2014**. 4(49): 25736-25746.
72. Pezzuolo, A., D. Boscaro, F. Marinello, and L. Sartori. Alternative maize ensiling techniques for anaerobic digestion process: Economic and energetic comparison. in *Engineering for Rural Development* **2016**.
73. Broderick, G.A., R.P. Walgenbach, and E. Sterrenburg, Performance of lactating dairy cows fed alfalfa or red clover silage as the sole forage*. J. Dairy Sci.*, **2000**. 83(7): 1543-1551.
74. Broderick, G.A., A.F. Brito, and J.J.O. Colmenero, Effects of feeding formate-treated alfalfa silage or red clover silage on the production of lactating dairy cows*. J. Dairy Sci.*, **2007**. 90(3): 1378-1391.
75. Hervás, G., P. Frutos, F. Javier Giráldez, Á.R. Mantecón, and M.a.C. Álvarez Del Pino, Effect of different doses of quebracho tannins extract on rumen fermentation in ewes*. Anim. Feed Sci. Tech.*, **2003**. 109(1–4): 65-78.
76. Barry, T.N. and W.C. McNabb, The implications of condensed tannins on the nutritive value of temperate forages fed to ruminants*. Br. J. Nutr.*, **1999**. 81(4): 263-272.
77. Kuijpers, T.F.M., H. Gruppen, S. Sforza, W.J.H. Van Berkel, and J.P. Vincken, The antibrowning agent sulfite inactivates Agaricus bisporus tyrosinase through covalent modification of the copper-B site*. FEBS J.*, **2013**. 280(23): 6184-6195.

Summary

With the increasing world population, the demand for food and feed proteins increases. The use of novel plant based and marine protein resources has been increasingly investigated. These resources can be fractionated into streams enriched in proteins and the fractions can be applied in food and feed products. Plant and marine resources contain phenolic secondary metabolites (including tannins) which can interact with proteins, altering their functional and nutritional properties. In order to maintain the desired quality of the protein extracts, knowledge on the factors affecting such modification by phenolics need to be unraveled. Hence, the first aim of this thesis was to study factors potentially involved in protein modification (both covalently and non-covalently) by phenolics. The second aim was to investigate the effect of tannin supplementation to feed on *in vitro* ruminal fermentation, and to link this to the altering pH conditions throughout the ruminant's digestive system.

In **Chapter 1**, sugar beet leaves (*Beta vulgaris* ssp *vulgaris*) as terrestrial and the brown seaweed *Laminiaria digitata* as marine resource of proteins were explored for their applicability as ingredient in food and feed, as well as the composition of phenolic compounds of these resources and factors influencing the applicability of these resources. Proteins in sugar beet leaves can become covalently modified by relatively small phenolics in presence of polyphenol oxidase (PPO). In *L. digitata*, the phenolic compounds are represented by oligomeric phlorotannins (PhT), which can alter protein properties by non-covalent binding. The changes in functional and nutritional properties of modified proteins are usually associated with deterioration of protein quality. Nevertheless, the modification of proteins might also be applied for the benefit in ruminal fermentation.

In **Chapter 2**, sugar beet leaves differing in variety, growing environment (field and greenhouse) and plant age (3, 6 or 8 months old) were used. The focus was on the variation in chemical composition of these leaves combined with the quantity and quality of proteins extracted. Within the same variety, the gross composition was not affected by plant age; protein contents were 10-22% w/w DM, depending on the variety. Variation in nitrogen extractability was 28-56%, and did not show a relation to plant age. The plant age significantly affected the enzymatic browning in which proteins extracted from young plants (3 months) were yellow and those from old plants (6 and 8 months) were brown. Both the yellow and brown protein extracts contained active PPO. There were twofold increases in PPO activity and total phenolics content with age, independent of the variety. The occurrence of enzymatic browning could not be explained by variation in gross composition as such.

An in depth analysis on the phenolics composition and their quantities present in 3 month (3_m) and 8 month (8_m) old sugar beet leaves was performed by RP-UHPLC-MS in **Chapter 3**. From 3_m to 8_m, there was 6.7 fold increase in PPO activity towards endogenous substrates; total phenolic content increased twofold. The PPO substrate content increased from 0.53 to 2.45 mg/g FW from 3_m to 8_m, respectively. Caffeic acid glycosyl-esters were the most important, increasing tenfold with age. In addition to substrate phenolics, non-substrate phenolics played an important role in enzymatic browning through participation in oxidative coupling and coupled oxidation reactions. Rather than the absolute amounts of phenolics, it is postulated that enzymatic browning was driven by the non-substrate-to-substrate ratio. These ratios determine the number of consecutive oxidative coupling and coupled oxidation cycles taking place and hence the size of the conjugated system of the coupling products. In 3_m and 8_m the non-substrate-to-substrate ratios were 8:1 and 3:1, respectively. A model system using these ratios showed browning at the 3:1 molar ratio, due to

formation of phenolics with extensive conjugated systems through numerous oxidative coupling and coupled oxidation cycles.

In **Chapter 4**, several analytical techniques were employed to characterise and quantify PhT in *L. digitata*. The combined use of ^{13}C NMR and ^{1}H NMR spectroscopy was suitable for determination of the fucol and phlorethol linkage ratios and sample purity. The fucul:phlorethol linkage ratio was 1:26 and the extract contained 60% (w/w) of PhT. The purity determined by NMR spectroscopy allowed determination of a correction factor to be applied in the colorimetric DMBA assay. The response of the oligomeric mixture was 12 times lower than that of the PhT monomeric subunit phloroglucinol. Further structural information was obtained by ESI-MS. Structural isomers of PhT oligomers up to DP18 were annotated and the presence of several isomers hinted at branched phloroglucinol oligomers. The use of MALDI-TOF-MS allowed annotation of PhT up to DP27.

Tannins are known to modulate protein nutritional properties due to non-covalent binding to proteins. Throughout the ruminant's digestive tract the pH varies. Therefore, the effect of pH on complex formation between PhT and proteins was investigated in **Chapter 5**. The use of fluorescence quenching was employed to compare the binding of PhT to β-casein and bovine serum albumin (BSA) with pentagalloyl glucose (PGG) and ellagic acid (EA). Phlorotannins and PGG showed binding affinities for the two proteins in similar orders of magnitude, whereas EA showed lower binding affinity. The binding of PhT to both β-casein and BSA were independent of pH. Within a precipitation assay, PhT-protein complexes aggregated at high tannin-to-protein ratios and broadened the pH range of protein precipitation from 0.5 pH unit to ~1.5 pH unit around the pI of the proteins. Protein re-solubilisation from complexes versus pH was studied in presence and absence of the tannin binding agent polyethylene glycol (PEG). After preparing the PhT-protein complexes at ruminal pH of 6.0, complete re-solubilisation was observed for BSA at pH 2, and for β-casein at pH 7. Complete protein re-solubilisation coincided with partial tannin re-solubilisation, hinting at potential re-complexation or re-solubilised PhT and proteins. These results suggest that some proteins might escape ruminal fermentation (pH 6-7) by binding to PhT. In the abomasum (pH 2-3), these proteins can become available for further digestion and absorption.

The application of the extracted PhT as agent to improve ruminal fermentation was assayed in **Chapter 6**. The effects were visualised by creating a dose-response curve (10, 20, 40, 50, 75 and 100 g PhT/kg tannin free grass silage) *in vitro*, including and excluding the tannin binding agent PEG. The reduction of CH_4 production and NH_3 concentration were used as parameters to study decrease in methanogenic activity and protection of protein from ruminal fermentation, respectively. The optimal dosage of PhT was 40 g/kg grass silage. At this point. CH_4 decreased significantly from 24.5 to 15.2 mL/g organic matter), without significantly affecting fermentation performance as determined by total gas production and formation of volatile fatty acids. The concentration of NH_3 decreased from 0.49 to 0.39 mmol/g organic matter, indicating protection of dietary protein. Addition of PEG inhibited the effect of tannins and did not change the fermentation parameters compared to the control. Effectiveness of PhT was compared to terrestrial hydrolysable and condensed tannins on a molar basis using literature data. Phlorotannins behaved similar to condensed tannins with respect to CH_4 reduction and protein protection. The hydrolysable tannins, however, seemed more effective.

In **Chapter 7** the methodology applied to characterise and quantify phenolics within mixtures was further discussed, together with a discussion on the potential nutritional effects of both covalent- and non-covalent protein modification. Phenolics appear to have potential with respect to protein modification. In sugar beet leaves, the composition of potential substrates and non-substrates for PPO affected browning. The oxidised phenolics might engage in covalent protein modifications by binding to nucleophilic amino acid side chains in the protein. Within *L. digitata*, the PhT allowed binding to proteins and formation of insoluble PhT-protein complexes at ruminal pH. The insoluble complex formation improved ruminal fermentation.

Samenvatting

Met de toename van het aantal wereldbewoners stijgt ook de vraag naar eiwitten voor gebruik in de levensmiddelen- en diervoedingsindustrie. In dit kader worden plantaardige en mariene eiwitbronnen steeds vaker onderzocht. Via fractionering van deze bronnen kunnen extracten verkregen worden die verrijkt in eiwitten als ingrediënt in andere producten gebruikt kunnen worden. Naast eiwitten bevatten plantaardige en mariene bronnen secundaire metabolieten, waarvan polyfenolen (incl. tannines) een onderdeel zijn. Polyfenolen kunnen aan eiwitten binden en zo de functionele en nutritionele eigenschappen beïnvloeden. Om de gevraagde eiwitkwaliteit te behouden is het van belang kennis op te doen over de factoren die een rol spelen bij deze modificatiereacties. Het eerste doel van dit onderzoek was het bestuderen van factoren die invloed hebben op de eiwitmodificatie door polyfenolen (covalent en niet-covalent). Het tweede doel was het bestuderen van de invloed van het toevoegen van tannines aan diervoeding op de *in vitro* pens-fermentatie, in samenhang met de verschillende pH condities in het verteringssysteem van runderen.

Hoofdstuk 1 bespreekt blad van suikerbiet (*Beta vulgaris* ssp *vulgaris*) als plantaardige eiwitbron en het bruin zeewier *Laminiaria digitata* als mariene eiwitbron in relatie tot hun toepasbaarheid als ingrediënt in levensmiddelen en diervoeding. Factoren die een rol spelen bij de eventuele bruikbaarheid zijn de aanwezigheid en samenstelling van polyfenolen. Suikerbietenblad bevat kleine polyfenolen en polyfenol oxidase (PPO) waardoor de aanwezige eiwitten covalent gemodificeerd kunnen worden. *L. digitata*, bevat polyfenolen in de vorm van oligomere phlorotannines (PhT), welke de eiwit eigenschappen veranderen via niet-covalente binding. De veranderingen van gemodificeerde eiwitten worden over het algemeen geassocieerd met verlies van functionele- en nutritionele eiwitkwaliteit. Desalniettemin zouden gemodificeerde eiwitten bij kunnen dragen aan het verbeteren van de pens-fermentatie in runderen.

In **Hoofdstuk 2**, wordt blad van suikerbiet met variatie in ras, groeiomstandigheden (akker en kas) en leeftijd van de plant (3, 6 of 8 maanden oud) vergeleken. De focus lag op de variatie in chemische samenstelling van het blad in combinatie met de kwantiteit en kwaliteit van de geëxtraheerde eiwitten. Binnen een ras had leeftijd van de plant geen effect op de chemische samenstelling; eiwitgehaltes varieerden van 10-22% w/w droge stof, afhankelijk van het ras. Variatie in extraheerbaarheid van stikstof was 28-56%, en niet gerelateerd aan de leeftijd van de plant. De leeftijd van de plant had een significant effect op enzymatische bruinkleuring; eiwitten geëxtraheerd uit blad van jonge planten (3 maanden) waren geel en die uit oude planten (6 en 8 maanden) waren bruin. De gele en bruine eiwitextracten bevatten beiden actief PPO met een verdubbeling van PPO activiteit en totaal polyfenolgehalte met toename in leeftijd, onafhankelijk van het ras. Het ontstaan van bruine eiwit extracten kon niet verklaard worden door de variatie in chemische samenstelling.

In **Hoofdstuk 3** wordt bestudeerd of de verschillen in bruinkleuring verklaard kunnen worden door variatie in polyfenolsamenstelling en gehalte. Deze parameters zijn bepaald in blad van suikerbiet van 3- (3_m) en 8 maanden (8_m) oude planten, door gebruik te maken van RP-UHPLC-UV-MS. Van 3_m naar 8_m was er 6.7 maal toename in PPO activiteit voor endogene substraten; het totaal polyfenol gehalte verdubbelde. Het PPO substraatgehalte steeg van 0.53 in 3_m naar 2.45 mg/g vers gewicht in 8_m. koffiezuur glycosyl-esters waren de belangrijkste substraat polyfenolen, en het gehalte vertienvoudigde met leeftijd. Naast de substraat polyfenolen spelen de niet-substraat polyfenolen een belangrijke rol in enzymatische bruinkleuring via oxidatieve koppeling en gekoppelde oxidatie

reacties. Hierdoor ontstond het idee dat niet het totaal gehalte aan polyfenolen verantwoordelijk is voor bruinkleuring maar dat deze reactie gestuurd wordt door de niet-substraat-tot-substraat ratio. De ratio bepaalt het aantal opeenvolgende cycli van oxidatieve koppeling en gekoppelde oxidatie en daarmee de grootte van het geconjugeerde systeem van de gevormde koppelingsproducten. In 3_m en 8_m waren de niet-substraat-tot-substraat ratio's op mol-basis respectievelijk 8:1 en 3:1. In een model systeem, gebruikmakend van deze ratio's, was er bruinkleuring bij een 3:1 ratio doordat er complexen met grote geconjugeerde systemen gevormd werden via vele cycli van oxidatieve koppeling en gekoppelde oxidatie.

In **Hoofdstuk 4** worden de PhT in *L. digitata* gekarakteriseerd en gekwantificeerd door middel van verschillende analytische technieken. Door ^{13}C NMR en ^{1}H NMR spectroscopie te combineren konden de fucol:phlorethol verbindingsratio's en de zuiverheid van het extract bepaald worden. De fucul:phlorethol verbindingsratio was 1:26 en het extract bevatte 60% (w/w) PhT. De zuiverheid van het extract, bepaald via NMR spectroscopie werd gebruikt om een correctiefactor voor de colorimetrische DMBA assay te bepalen. De reactiviteit van het mengsel van PhT was twaalf maal lager dan dat van het PhT monomeer phloroglucinol. De PhT structuur werd verder ontrafeld via ESI-MS waarbij structuur isomeren van PhT oligomeren tot DP18 konden worden geannoteerd. De aanwezigheid van verschillende isomeren maakt het aannemelijk dat vertakte phloroglucinol oligomeren aanwezig zijn. Het gebruik van MALDI-TOF-MS maakte het mogelijk oligomeren tot DP27 te annoteren.

Door non-covalente binding aan eiwitten kunnen tannines de nutritionele eigenschappen van eiwitten beïnvloeden. **Hoofdstuk 5** beschrijft de invloed van de pH op deze complex formatie en modelleert de verschillende pH milieus in het verteringskanaal van runderen. De binding van PhT aan β-caseïne en runder serumalbumine (BSA) werd vergeleken met de eiwitbinding van pentagalloyl glucose (PGG) en ellaginezuur (EZ) door gebruik te maken van 'fluorescence quenching'. De bindingsaffiniteiten van PhT en PGG voor beide eiwitten lagen in dezelfde orde van grootte en waren hoger dat die van EZ. De bindingsaffiniteiten van PhT aan β-caseïne en BSA waren onafhankelijk van pH. Met een precipitatie test werd aangetoond dat PhT-eiwit complexen neersloegen bij hoge tannine-tot-eiwit ratio's en dat het pH gebied voor eiwit precipitatie zich verbreedde, van 0.5 pH eenheid tot ~1.5 pH eenheid rondom de pI van de eiwitten. De mate waarin eiwitten terugkwamen in oplossing vanuit de onoplosbare complexen werd bekeken in relatie tot de pH in aan- en afwezigheid van de tannine-binder polyethylene glycol (PEG). Na het vormen van de PhT-eiwit complexen bij pens pH van 6.0 kwam alle BSA bij pH 2 en alle β-caseïne bij pH 7 terug in oplossing, onafhankelijk van de aanwezigheid van PEG. In deze situaties kwam PhT ten dele terug in oplossing. Dit wees op mogelijke complexvorming tussen her-opgeloste eiwitten en PhT. De resultaten suggereren dat sommige eiwitten beschermd kunnen worden tegen pens fermentatie (pH 6-7) door gebonden te worden door PhT. In de lebmaag (pH 2-3) kunnen deze eiwitten weer beschikbaar komen voor verdere vertering en absorptie.

Hoofdstuk 6 onderzoekt hoe een PhT extract ingezet kan worden om de pens fermentatie te verbeteren. De effecten werden zichtbaar via een dosis-respons curve (10, 20, 40, 50, 75 en 100 g PhT/kg tanninevrij kuilgras) *in vitro*, met en zonder toevoeging van PEG. De afname in CH_4 productie werd gebruikt als maat voor afname in methanogene activiteit, de afname in NH_3 concentratie als maat voor het beschermen van eiwitten tegen pens fermentatie. De optimale dosering van PhT was

40 g/kg kuilgras. Bij deze dosering daalde het CH_4 gehalte significant van 24.5 naar 15.2 mL/g organische stof (OS), zonder de kwaliteit van de fermentatie (gemeten als totaal geproduceerd gas en formatie van vluchtige vetzuren), negatief te beïnvloeden. De NH_3 concentratie daalde van 0.49 naar 0.39 mmol/g OS, wat duidde op bescherming van eiwitten. Toevoeging van PEG ging de werking van pHT tegen en er was daardoor geen effect van het toevoegen van PhT op de kwaliteit van fermentatie ten opzichte van de controle. De effectiviteit van PhT is vergeleken met die van plantaardige hydrolyseerbare en gecondenseerde tannines op molaire basis via gegevens uit de literatuur. Phlorotannines en plantaardige gecondenseerde tannines hadden vergelijkbare effecten wat betreft vermindering in CH_4 en het beschermen van eiwitten. De hydrolyseerbare tannines daarentegen, leken meer effect te hebben op de pens-fermentatie.

Hoofdstuk 7 evalueert de methodologie die gebruikt is om polyfenolen in mengsels te karakteriseren en te kwantificeren. De mogelijke nutritionele effecten van covalente en niet-covalente modificatie van eiwitten door polyfenolen worden bediscussieerd. Polyfenolen hebben zeker de mogelijkheid om de nutritionele eigenschappen van eiwitten te beïnvloeden. In blad van suikerbiet was er het effect van de PPO substraat-tot-niet-substraat ratio op het ontstaan van enzymatische bruining. De geoxideerde polyfenolen kunnen eiwitten covalent veranderen door te binden aan nucleofiele zijketens van aminozuren in het eiwit. In *L. digitata*, zorgen de PhT voor het vormen van onoplosbare complexen door te binden aan eiwit bij pH van de pens. De onoplosbare complexen verbeteren pens-fermentatie.

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Anne

About the author

Curriculum Vitae

Anne Maria Vissers was born on February 12 1989 in Hoorn, The Netherlands. After graduation from secondary school (VWO, RSG Enkhuizen) she started the Bachelor Food Technology, Wageningen University, in September 2007. During her BSc she did a minor in Biomedical Sciences at Utrecht University. In 2010 she started the MSc program Food Technology with specialisation in Product Design. She did a thesis at te Food Process Engineering department on the milling and air-classification of peas to create pea protein concentrates with potential for meat replacer development. Afterwards she joined the team at the State Key Laboratory of Food Science and Technology, Nanchang University, China, for her internship where she worked on the development of extruded oat products high in β-glucans. In January 2013 she obtained her MSc degree in Food technology. In February of the same year she started her PhD research within the IPOP project TripleP@Sea, under supervision of Dr. Ir. Jean-Paul Vincken, Prof. Dr. Ir. Harry Gruppen and Prof. Dr. Ir. Wouter Hendriks. The results of her PhD research are presented in this thesis.

Currently, Anne is working as researcher and educational assistant at the Laboratory of Food Chemistry.

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List of publications

P.J.M. Pelgrom, **A.M. Vissers**, R.M. Boom, M.A.I. Schutyser; Dry fractionation for production of functional pea protein concentrates, *Food Res. Int.* 52 (2013) 232-239

A. Kiskini, **A. Vissers**, J.-P. Vincken, H. Gruppen, P. Wierenga; Effect of plant age on the quantity and quality of proteins extracted from sugar beet (*Beta vulgaris* L.) leaves, *J. Agric.Food Chem.* 66 (44), **2016**, 8305-8314

A. Vissers, A. Kiskini, R.J. Hilgers, M. Marinea, P.A. Wierenga, H. Gruppen, J.-P. Vincken; The content of caffeic acid derivatives, oxidative coupling and coupled oxidation drive enzymatic browning in sugar beet (*Beta vulgaris* L.) leaves, *Accepted for publication in J. Agric. Food Chem,* **2017**.

A. Vissers, A. Caligiani, S. Sforza, J.-P. Vincken, H. Gruppen; Phlorotannin composition of *Laminaria digitata*, *Accepted for publication in Phytochem. Anal.,* **2017**

A. Vissers, A.E. Blok, A.H. Westphal, W.H. Hendriks, H. Gruppen, J.-P. Vincken; Re-solubilization of protein from water insoluble phlorotannin-protein complexes upon acidification, *Submitted for publication in J. Agric. Food Chem.*

A. Vissers, W.F. Pellikaan, A. Bouwhuis, W.H. Hendriks, H. Gruppen, J.-P. Vincken; *Laminaria digitata* phlorotannins decrease protein degradation during *in vitro* ruminal fermentation, *to be Submitted*

Overview of completed training activities

Discipline specific activities

Courses

- Advanced Statistics – Design of Experiments (WIAS), Wageningen, The Netherlands 2013
- Applied Biocatalysis (VLAG/ORC), Wageningen, The Netherlands, 2014
- Industrial Food Proteins [1] (VLAG/FCH), Wageningen, The Netherlands, 2015
- Advanced Food Analysis [1] (VLAG/FCH), Wageningen, The Netherlands, 2015

Conferences

- Minisymposium WBox2: Redox Biocatalysis, Wageningen, The Netherlands, 2013
- 3rd Infogest conference, Wageningen, The Netherlands, 2014
- 7th Oxyzymes conference, Vienna, Austria, 2015
- 3rd Seagiculture conference, Terneuzen, The Netherlands, 2014
- 9th European Conference on Marine Natural Products, Glasgow, United Kingdom, 2015 [1]
- 28th International Conference on Polyphenols, Vienna, Austria, 2016 [1]

General courses

- VLAG PhD Introduction week (VLAG), The Netherlands, 2013
- Teaching and supervising thesis students (ESD), 2013
- Data management (WGS), 2013
- Project and time management (WGS), 2014
- Techniques for Scientific Writing (WGS), 2014
- Philosophy and Ethics of Science (VLAG), 2014

Additional activities

- Preparation of the PhD research proposal
- PhD Trip FCH to Germany and Scandinavia, 2014 [1,2]
- PhD trip FCH to Japan, 2016 [1,2]
- BSc/MSc student presentations and colloquia 2013-2017
- PhD Presentations, 2013-2017
- VLAG PhD Council, 2013-2016

[1] Poster presentation, [2] Oral presentation
VLAG: Graduate school for Nutrition, Food Technology, Agrobiotechnology and Health Sciences
ORC: Laboratory of Organic Chemistry
FCH: Laboratory of Food Chemistry
WIAS: Wageningen Institute of Animal Sciences
ESD: Educational Staff Development
WGS: Wageningen Graduate School

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