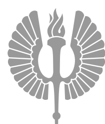

Turun yliopisto
University of Turku

UNDERSTANDING THE BIOACTIVITY OF PLANT TANNINS: DEVELOPMENTS IN ANALYSIS METHODS AND STRUCTURE-ACTIVITY STUDIES

Marica Engström

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ABSTRACT

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ENGSTRÖM, MARICA THERESE: Understanding the bioactivity of plant tannins: developments in analysis methods and structure–activity studies

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Tannins, typically segregated into two major groups, the hydrolyzable tannins (HTs) and the proanthocyanidins (PAs), are plant polyphenolic secondary metabolites found throughout the plant kingdom. On one hand, tannins may cause harmful nutritional effects on herbivores, for example insects, and hence they work as plants' defense against plant-eating animals. On the other hand, they may affect positively some herbivores, such as mammals, for example by their antioxidant, antimicrobial, anti-inflammatory or anticarcinogenic activities. This thesis focuses on understanding the bioactivity of plant tannins, their anthelmintic properties and the tools used for the qualitative and quantitative analysis of this endless source of structural diversity.

The first part of the experimental work focused on the development of ultra-high performance liquid chromatography−tandem mass spectrometry (UHPLC-MS/MS) based methods for the rapid fingerprint analysis of bioactive polyphenols, especially tannins. In the second part of the experimental work the *in vitro* activity of isolated and purified HTs and their hydrolysis product, gallic acid, was tested against egg hatching and larval motility of two larval developmental stages, L1 and L2, of a common ruminant gastrointestinal parasite, *Haemonchus contortus*. The results indicated clear relationships between the HT structure and the anthelmintic activity. The activity of the studied compounds depended on many structural features, including size, functional groups present in the structure, and the structural rigidness. To further understand tannin bioactivity on a molecular level, the interaction between bovine serum albumin (BSA), and seven HTs and epigallocatechin gallate was examined. The objective was to define the effect of pH on the formation on tannin–protein complexes and to evaluate the stability of the formed complexes by gel electrophoresis and MALDI-TOF-MS. The results indicated that more basic pH values had a stabilizing effect on the tannin–protein complexes and that the tannin oxidative activity was directly linked with their tendency to form covalently stabilized complexes with BSA at increased pH.

TIIVISTELMÄ

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ENGSTRÖM, MARICA THERESE: Analyysimenetelmien kehittäminen ja rakenne–aktiivisuusmääritykset kasvitanniinien bioaktiivisuuden ymmärtämiseksi

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Tanniinit, joiden kaksi pääryhmää ovat hydrolysoituvat tanniinit (HT) ja proantosyanidiinit (PA), ovat kasvien yleisesti tuottamia sekundäärimetaboliitteja. Tanniineilla voi olla sekä negatiivisia että positiivisia vaikutuksia niitä syöviin eliöihin. Ne voivat aiheuttaa haitallisia ravitsemuksellisia vaikutuksia esimerkiksi niitä syöville hyönteisille. Toisaalta tanniinit voivat olla hyödyllisiä kasvinsyöjille, kuten nisäkkäille, esimerkiksi niiden hapettumista, mikrobiaalista toimintaa, syöpää sekä tulehduksia ehkäisevien ominaisuuksien takia. Tässä väitöskirjatyössä keskityttiin kasvitanniinien bioaktiivisuden ymmärtämiseen, niiden loisia/loistauteja ehkäiseviin ominaisuuksiin sekä näiden rakenteellisesti monimuotoisten yhdisteiden kvalitatiivisiin ja kvantitatiivisiin analyysimenetelmiin.

Kokeellisen työn ensimmäisessä osassa kehitettiin erittäin korkean erotuskyvyn nestekromatografia–tandemmassaspektrometriaan (UHPLC-MS/MS) perustuvia nopeita ”sormenjälki”-analyysimenetelmiä tanniinien kvalitatiiviseen ja kvantitatiiviseen analyysiin. Toisessa osassa testattiin eristettyjen ja puhdistettujen HT:ien *in vitro* -vaikutusta märehtijöiden ruuansulatuselimistön loisen, *Haemonchus contortuksen,* munien kuoriutumiseen ja loistoukkien liikkuvuuteen. Tulosten perusteella HT:n rakenteen ja antiloisaktiivisuuden välillä havaittiin selvä yhteys: tutkittujen yhdisteiden aktiivisuus riippui monista rakenteellisista ominaisuuksista, kuten HT:n koosta, rakenteen funktionaalisista ryhmistä ja rakenteellisesta jäykkyydestä. Tanniinien bioaktiivisuuden ymmärtämiseksi molekyylitasolla malliproteiinin ja seitsemän HT:n sekä epigallokatekiinigallaatin välistä vuorovaikutusta tutkittiin geelielektroforeesilla ja MALDI-TOF-massaspektrometrialla. Tarkoituksena oli tutkia pH:n vaikutusta tanniini–proteiini-kompleksien muodostukseen sekä tarkastella muodostuvien kompleksien pysyvyyttä. Tulokset osoittivat emäksisen pH:n stabiloivan tanniini–proteiini-komplekseja, ja että tanniinin hapettumisherkkyys oli suoraan verrannollinen sen kykyyn sitoa korkeassa pH:ssa proteiinia kovalenttisesti.

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Working on this thesis has been an amazing journey filled with the excitement of discovering new things, the enjoyment of things working as planned, the frustration when not and the pleasure of seeing the results of the consistent and relentless work. None of this would have been possible without the support of my supervisors, colleagues, friends and family.

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Turku, May 2016

"If we knew what it was we were doing, it would not be called research, would it?"

~ Albert Einstein

"I believe in God, only I spell it Nature."

~ Frank Lloyd Wright

"Going to the woods is going home."

~John Muir

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications and some unpublished results. The publications are referred in the text by their Roman numerals.

I Engström, M.T.; Pälijärvi, M.; Fryganas, F.; Grabber, J.; Mueller-Harvey, I.; Salminen, J.-P. Rapid qualitative and quantitative analysis of proanthocyanidin oligomers and polymers by UPLC-MS/MS. *J. Agric. Food Chem.* 2014, 62, 3390–3399.

II Engström, M.T.; Pälijärvi, M.; Salminen, J.-P. Rapid fingerprint analysis of plant extracts for ellagitannins, gallic acid and quinic acid derivatives, and quercetin-, kaempferol- and myricetin-based flavonol glycosides by UPLC-QqQ-MS/MS. *J. Agric. Food Chem.* 2015, 63, 4068–4079.

III Engström, M.T.; Suber, M.; Li, M.; Salminen, J.-P.; Hagerman, A.E. The oxidative activity of ellagitannins dictates their tendency to form highly stabilized complexes with bovine serum albumin at increased pH. Submitted to *J. Agric. Food Chem*.

IV Engström, M.T.; Karonen, M.; Ahern, J.R.; Baert, N.; Payré, B.; Hoste, H.; Salminen, J.-P. 2016. Chemical structures of plant hydrolyzable tannins reveal their *in vitro* activity against egg hatching and motility of *Haemonchus contortus* nematodes. *J. Agric. Food Chem.* 2016, 64, 840–851.

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ABBREVIATIONS

BSA	Bovine serum albumin
DHHDP	Dehydrohexahydroxydiphenoyl
EHA	Egg hatch assay
ESI	Electrospray ionization
ET	Ellagitannin
GA	Gallic acid
GG	Galloyglucose
GIN	Gastrointestinal nematode
GT	Gallotannin
HHDP	Hexahydroxydiphenoyl
HRF	Heterocyclic ring fission
HT	Hydrolyzable tannin
LC 50	50% lethal concentration
LC-DAD	Liquid chromatography-diode array detector
LC-MS	Liquid chromatography-mass spectrometry
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry
MRM	Multiple reaction monitoring
MS/MS	Tandem mass spectrometry
MW	Molecular weight
NBT	Nitroblue tetrazolium
NHTP	Nonahydroxytriphenoyl
PA	Proanthocyanidin
PC	Procyanidin
PD	Prodelphinidin
PGG	Pentagalloylglucose
QM	Quinone methide fragmentation
QqQ	Triple quadrupole
RDA	Retro-Diels-Alder fragmentation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SRM	Single reaction monitoring
TEM	Transmission electron microscopy
UHPLC	Ultra-high performance liquid chromatography

1. INTRODUCTION

A single plant synthesizes hundreds of secondary metabolites, which are essential for the survival of the plant due to their defensive properties, including resistance against pathogens and herbivores as well as UV radiation (Haslam 1989; Stafford 1991). Polyphenols are the most common class of secondary metabolites and they are found in almost every plant species and in every part of the plant (Haslam 1989). Tannins are the most complex class of polyphenols and compounds found in a wide range of plant species (Bernays et al. 1989; Haslam, 1989) and are conventionally classified into two major groups: hydrolyzable tannins (HTs) and proanthocyanidins (PAs). The biochemical definition of tannins describes them as compounds having a tendency to interact and form insoluble precipitates with proteins and other biological macromolecules in aqueous solutions (Bate-Smith and Swain 1962; Hagerman 2012). Tannin–protein interactions have great importance in numerous plant-related domains and the ability of tannins to bind proteins is one explanation for their defensive properties (Appel 1993; Salminen and Karonen 2011; Constabel et al. 2014) as well as for their nutritional benefits (Mueller-Harvey 2006; Quideau et al. 2011; Li and Hagerman 2013).

Over the last few decades, the use of tannin containing plants as natural anthelmintics in the control of ruminant parasites has received increasing interest. The parasitic infections of the gastrointestinal tract are one major threat associated with the production of various livestock species worldwide (Sykes 1994; Jackson et al. 2009; Hoste et al. 2012). They affect the health and welfare of cattle, sheep and goats by causing e.g. anemia, anorexia, impaired digestion and nutrient absorption, which consequently result in significant production losses (Holmes 1987; Sykes 1994; Hoste et al. 2001; Hoste et al. 2012). For more than 50 years, the solution for this problem has been the repeated use of synthetic, chemical anthelmintic drugs (Hoste et al. 2012). However, this exclusive reliance on the use of synthetic anthelmintic drugs to fight parasitic infections is constrained by several issues such as the growing concern of possible residues in food products as well as environmental consequences (Hoste et al. 2012). In addition, the access of farmers to chemical anthelmintics is usually limited in developing countries where the livestock production is constantly increasing (Krecek and Waller 2006). Furthermore, the extensive use of synthetic anthelmintics against parasites has led to widespread resistance,

culminating in the formation of multi-resistant nematode strains (Kaplan 2004; Jackson 2009; Traversa et al. 2015).

As a consequence, there is an urgent need for alternative solutions to control ruminant parasites. One possible and promising approach is the utilization of bioactive plants as natural anthelmintics to at least partially replace the use of synthetic drugs (Niezen et al. 1995; Hammond et al. 1997; Hoste et al. 2015). It has been shown that by using tannin-rich forage it is possible to reduce the intestinal parasites of ruminants without any significant risk of resistance formation (Anthanasiadou et al. 2001; Hoste et al. 2006; Manke et al. 2015). However, it is still unknown what kinds of tannin structures are most efficient as natural anthelmintics and which chemical properties and structural features of the tannins cause the observed effects. While it is commonly accepted that these bioactivities are consequences of the interaction between tannins and proteins, the specific mode of action has remained unclear (Frutos et al. 2004; Mueller-Harvey 2006; Hoste et al. 2012). By understanding the mechanisms behind the anthelmintic effects of the plant tannins, it should be possible to predict which forage plants are the best choices as feed for ruminants.

The general interest in plant tannins has resulted in many efforts to provide sensitive and selective analytical tools for their detection and characterization (Prasain et al. 2004; Ignat et al. 2011; Flamini 2013). The more traditional tannin detection methods, which utilize the tendency of the functional groups to undergo characteristic reactions, are beginning to be replaced or accompanied by more sophisticated methods such as liquid chromatography combined with diode array detection (DAD) and/or mass spectrometric detection (LC-MS). The development of ultra-high performance liquid chromatography combined with tandem mass spectrometry (UHPLC-MS/MS) has further provided improvements in the performance of separation and detection, and reduced the time required for qualitative and quantitative analysis (Guillarme et al. 2010; Rodriguez-Aller et al. 2013). Nevertheless, due to the structural diversity and complexity of tannins, many methodological problems still exist (Cheynier and Fulcrand 2003; Salminen et al. 2011; Gu 2012). For example, in PA analysis, the time-consuming procedures of thiolysis and phloroglucinolysis are still the most commonly used methods for collecting qualitative and quantitative information on PA content, composition and mean degree of polymerization (mDP). Development of improved methods with high-throughput capabilities would be highly desirable.

2. LITERATURE REVIEW

2.1. Plant tannins

Tannins are the most abundant secondary metabolites made by plants and can be divided in three subgroups based on their structural features. Hydrolyzable tannins (HTs) and proanthocyanidins (PAs) are the two major groups of these bioactive compounds found in vascular plants. The third subgroup, phlorotannins, is a little studied group of secondary metabolites found only in marine brown algae (Stern et al. 1996; Kubanek et al. 2004; Amsler and Fairhead 2006) and is not included in this literature review.

2.1.1. Structural features of hydrolyzable tannins

HTs are a group of structurally diverse and complex molecules constructed around a central polyol, most often glucose, but occasionally glucitol, quinic acid, quercitol or shikimic acid (Mueller-Harvey 2001; Patra and Saxena 2010). The term hydrolyzable tannin (HT) stems from the significant feature of these tannins to be susceptible to hydrolysis by acids, bases or esterases. As a consequence, HTs yield hydrolysis products characteristic of their functional groups such as gallic acid and ellagic acid (Fig. 1A,B) (Haslam 1979; Bors et al. 2001; Patra and Saxena 2010). In the simplest HTs, only gallic acid units are esterified to the central polyol core whereas more complex HTs are formed by esterification and cross-linking of these gallic acid units (Gross 1992; Haslam 1998; Patra and Saxena 2010; Okuda and Ito 2011). Based on their structural features, HTs are divided into three main subclasses: galloylglucoses (GGs), gallotannins (GTs) and ellagitannins (ETs). Examples of the HT subgroups are presented in Figure 1. Simple gallic acid derivatives contain one to five gallic acid units i.e. galloyl groups esterified to the polyol core (Fig. 1C) while GTs contain six or more galloyl groups that are bound either to the central polyol or esterified to one of the galloyl groups forming a digalloyl group (Fig. 1D) (Gross 1992; Grundhöfer et al. 2001; Salminen and Karonen 2011). GGs and GTs are relatively simple molecules whereas the third group, ETs, is structurally highly diverse. ETs are further divided into six subgroups based on their structural features: primary ETs, dehydroETs, modified dehydroETs, *C*-glycosidic ETs, flavono-ETs and oligomeric ETs (Okuda et al. 2009).

Primary ETs contain one or two hexahydroxydiphenoyl (HHDP) groups and up to three galloyl groups (Fig. 1E). The HHDP group is biosynthetically formed through intramolecular, oxidative C–C bond formation between two galloyl groups. The position of the HHDP group can be O2–O4 and/or O3–O6 or O2–O3 and/or O4–O6. DehydroETs are formed when the HHDP group is oxidized to form a dehydrohexahydroxydiphenoyl (DHHDP) group (Fig. 1F) which can further be oxidized to modified dehydroETs (Fig. 1G) (Okuda et al. 2009; Yoshida et al. 2009, 2010). In DHHDP ETs, the central glucopyranose core adopts often $^{1}C_{4}$ conformation instead of the $^{4}C_{1}$ conformation characteristic for the primary ETs and in aqueous or alcoholic solutions, the DHHDP group equilibrates between six- and five-membered ring forms (Fig. 1F).

The characteristic feature of *C*-glycosidic ETs is the acyclic glucose core. *C*-glycosidic ETs can be categorized as: (1) castalagin-type (Fig. 1I), containing a nonahydroxytriphenoyl (NHTP) group that participates the *C*-glycosidic linkage and (2) casuarinin-type (Fig. 1K), in which an HHDP group participates the *C*-glycosidic linkage instead. *C*-glycosidic ETs with a β-hydroxyl group at the C1 of the acyclic glucose core show a unique reactivity that is reflected e.g. in the various condensates formed with other polyphenols. One such condensate is the subgroup of flavono-ETs that consist of a *C*-glycosidic ET and a flavan-3-ol (most often catechin or epicatechin) bound to each other via a carbon-carbon linkage between C1 of the acyclic glucose and C8 or C6 of the flavan-3-ol moiety [Fig. 1L (Yoshida et al. 2008, 2009, 2010)].

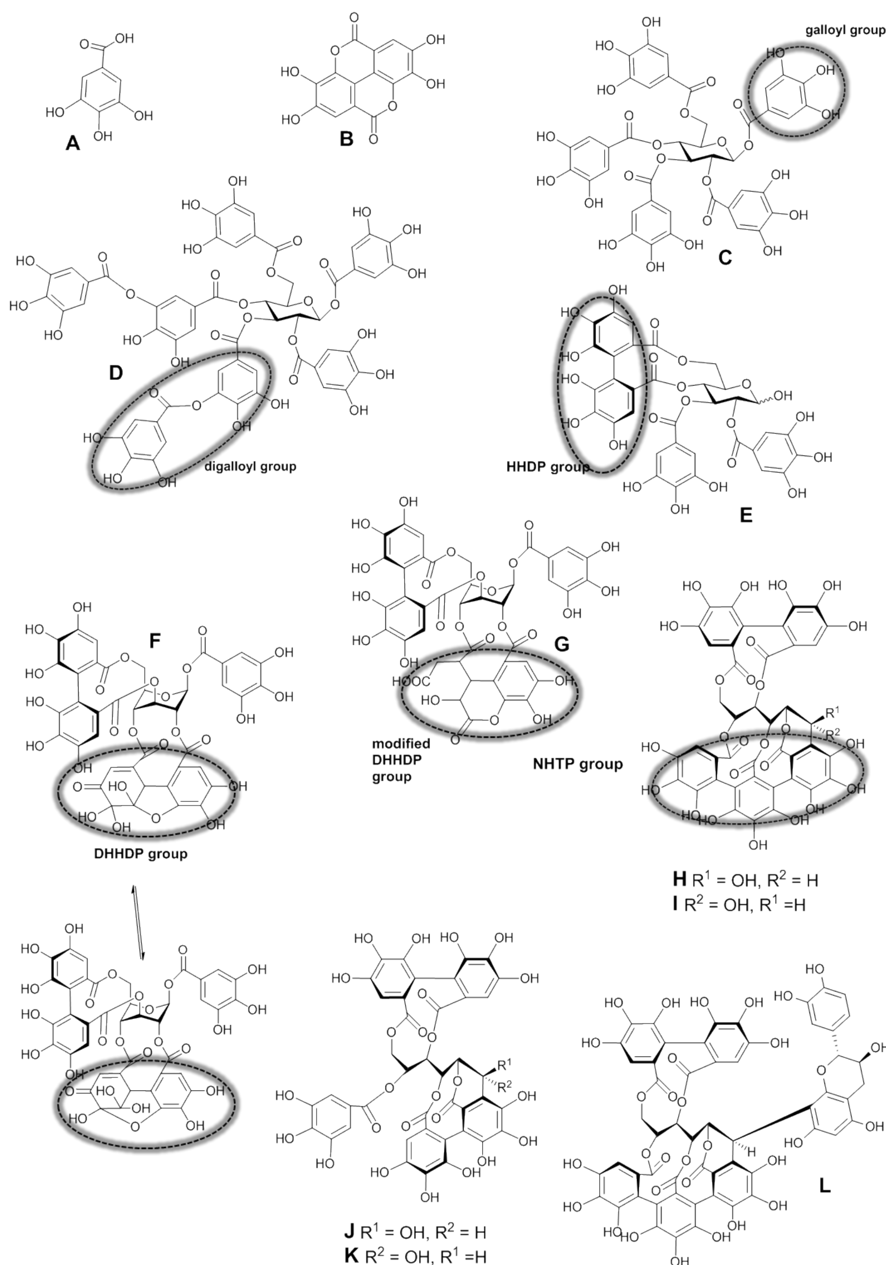

Figure 1. The structures of gallic acid (A), ellagic acid (B), pentagalloylglucose (C), heptagalloylglucose (D), tellimagrandin I (E), geraniin (F), chebulagic acid (G), vescalagin (H), castalagin (I), stachyurin (J), casuarinin (K) and acutissimin A (L). HHDP, hexahydroxydiphenoyl; DHHDP, dehydrohexahydroxydiphenoyl; NHTP, nonahydroxytriphenoyl.

Further diversity is brought by the last subgroup of ETs, oligomeric ETs, which are formed via intermolecular oxidative coupling of monomeric ETs. Oligomeric ETs can roughly be divided into three subgroups based on their structural features. The first group contains oligomers that are formed via single intermolecular C–O bonds between the monomeric units (Fig. 2A–C). The second group contains macrocyclic oligomers formed via two C–O bonds between the monomeric units (Fig. 2D). In the third group, C-glycosidic tannin oligomers are linked via intermolecular C–C bonds between the C1 of the acyclic glucose of one monomer and the HHDP or galloyl group of the other monomer (Fig. 2E) (Okuda and Ito 2011; Yoshida et al. 2009, 2010).

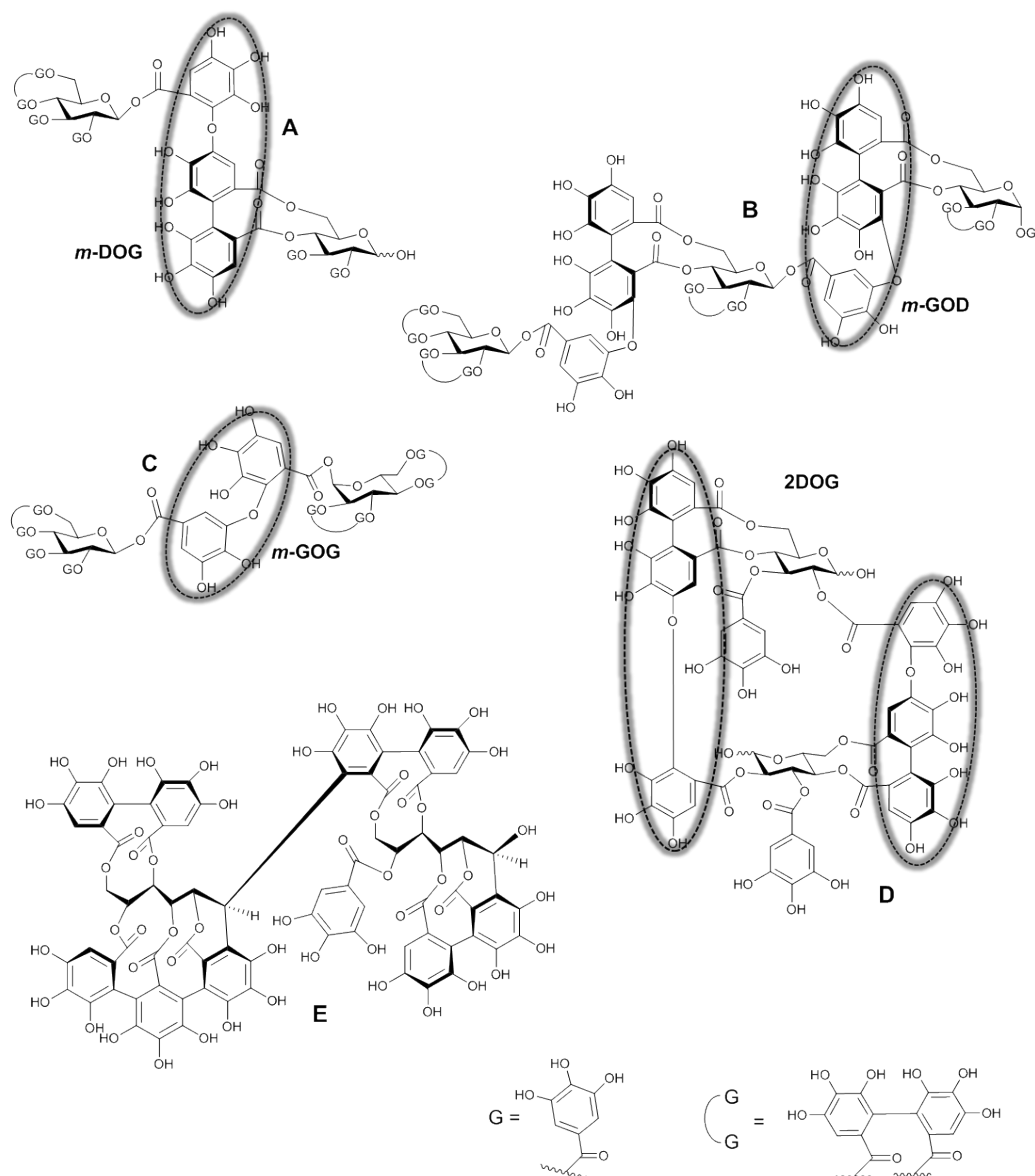

Figure 2. Structures of oligomeric ellagitannins rugosin E (A), lambertianin C (B), gemin A (C), oenothein B (D) and roburin A (E). *m*-DOG, valoneoyl group; *m*-GOD, sanguisorboyl group; *m*-GOG, dehydrodigalloylgroup; 2DOG, macrocyclic structure.

2.1.2. Structural features of proanthocyanidins

Proanthocyanidins (PAs, *syn.* condensed tannins) are oligomers and polymers consisting of flavan-3-ols units which are linked by carbon–carbon bonds (B-type PAs) or in some cases by additional ether-bonds (A-type PAs) (Porter 1989; Bors et al. 2001; Dixon et al. 2005; Ferreira et al. 2005). The origin of the term proanthocyanidin is that upon acid-catalyzed depolymerization and oxidation the extension units are converted to colored anthocyanidins (Porter 1989). The three rings of the flavan-3-ols units are labeled as A, B, and C (Fig. 3A). PAs differ structurally based on the number of hydroxyl groups on the A and B rings, and the stereochemistry of the asymmetric carbons of the C ring (Haslam 1977; Santos-Buelga and Scalbert 2000; Dixon et al. 2005). In most PAs, the absolute configuration of C2 is *R* whereas the absolute configuration of C3 can be either *R* or *S.* Accordingly, the stereochemistry of the molecule is either *cis* (2*R*, 3*R*) or *trans* (2*R*, 3*S*) (Santos-Buelga and Scalbert 2000). However, even more isomers can be found since the bond between the two monomers can be either in α or β configuration (Santos-Buelga and Scalbert 2000; Dixon et al. 2005; Buzzini et al. 2007; Barbehenn and Constabel 2011). The variable mean degree of polymerization is a further source of structural complexity of the PAs (Fig 3B).

In B-type PAs, the monomers are linked by C4–C8 or C4–C6 linkages. In addition to this, the A-type PAs contain C2‒O‒C7 or C2‒O‒C5 linkages [Fig. 3C (Hemingway et al. 1982; Porter 1989; Santos-Buelga and Scalbert 2000; Dixon et al. 2005; Ferreira et al. 2005; Buzzini et al. 2007; Barbehenn and Constabel 2011)]. The most common PAs are procyanidins (PCs) consisting of catechin and/or epicatechin units (Fig 3A), and prodelphinidins (PDs) consisting of gallocatechin and/or epigallocatechin units (Fig 3A). Also PAs consisting of both PC and PD units are common (Dixon et al. 2005; Ferreira et al. 2005; Buzzini et al. 2007). In addition to these main PA classes, some rarer PAs exist (e.g. propelargonidins, profisetinidins, prorobinetinidins and proguibourtinidins), but these are specific for certain plant species (Ferreira et al. 2005) and not discussed herein.

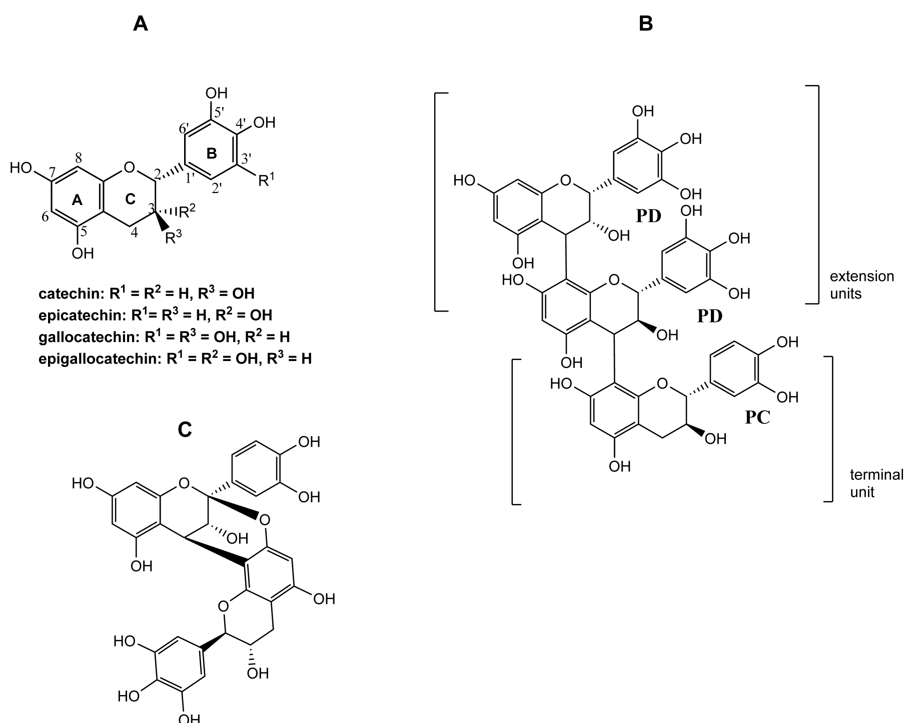

Figure 3. The most common proanthocyanidin monomeric units (A), an example of a trimeric proanthocyanidin with epigallocatechin and gallocatechin (PD) as extension units, and a catechin (PC) as terminal unit and an example of an A-type proanthocyanidin, procyanidin A1.

2.1.3. Tannin biosynthesis

A simplified scheme of the polyphenol biosynthesis pathway leading to HTs and PAs is presented in Figure 4. In the first steps of polyphenol biosynthesis, the carbohydrates such as sucrose and starch from the Calvin cycle are processed either by glycolysis to glyceraldehyde-3-phosphate or through the oxidative or reductive pentose phosphate pathway into erythrose-4-phosphate and glyceraldehyde-3-phosphate (Salminen and Karonen 2011). The first differences in HT and PA biosynthesis occur between the acetate ⁄malonate pathway and the shikimate pathway: while PA synthesis utilizes both of these pathways, HT biosynthesis relies solely on the shikimate pathway (Salminen and Karonen 2011). Regarding HT biosynthesis, 1-galloyl-*β*-*D*-glucose (*syn.* glucogallin) can be considered as the first intermediate on the HT biosynthetic pathway (Gross 1983a; Niemetz and Gross 1999). In the second step,

glucogallin functions as both an acyl acceptor and acyl donor, in the formation of digalloylglucose up to pentagalloylglucose (Gross 1983b; Gross 1999a,b; Niemetz and Gross 2005). The second biosynthetic pathway leads to GTs as the galloylation of pentagalloylglucose continues to hexa-, hepta-, octagalloylglucose etc. (Hoffman and Gross 1990; Niemitz and Gross 1998, 1999b, 2001; Gross 2008). The third pathway yields ETs, but in contrast to GT biosynthesis, the proposed ET pathway is mostly hypothetical, since only two steps of the ET pathway have been characterized by enzymatic studies (Gross 1999b; Niemetz et al. 2001; Niemetz and Gross 2003, 2005; Gross 2009; Okuda and Ito 2011). The other details have been deduced from tannin structures and their seasonal variation in the plant cell or their chemical synthesis (Salminen and Karonen 2011).

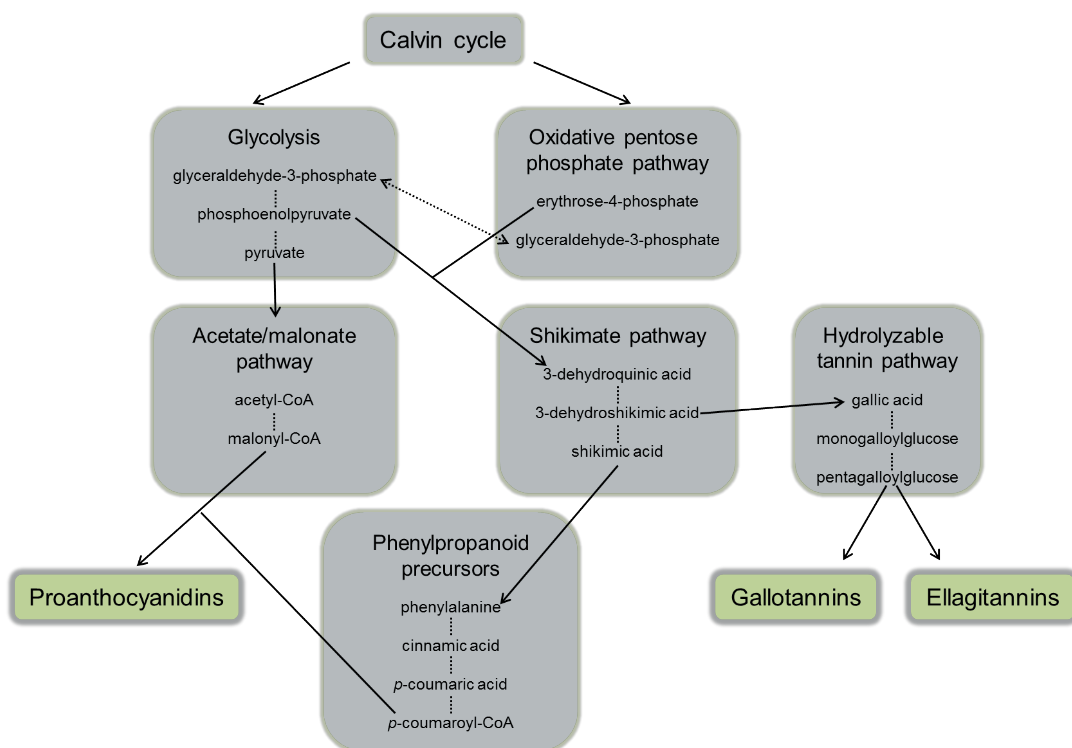

Figure 4. The biosynthetic pathway leading to proanthocyanidins and hydrolyzable tannins (adapted from Salminen and Karonen 2011).

PAs are the products of one of the several branches of the biosynthetic flavonoid pathway. The aromatic B-ring and the three carbon atoms of the heterocyclic C-ring are considered to originate from the amino acid phenylalanine, which is produced by the shikimate pathway and transformed into *p*-coumaroyl-CoA along the phenylpropanoid pathway (Haslam 1977; Strack 1997). The A-ring is formed by three units of malonyl-coenzyme A (malonyl-CoA), produced by the acetate/malonate pathway (Haslam 1977; Tsao 2010). The main steps of PA biosynthesis are presented in Figure 5. At the start of the flavonoid pathway, one coumaroyl-CoA molecule is condensed with three malonyl-CoA molecules to form tetrahydroxychalcone (Haslam 1977; Winkel-Shirley 2001; He et al. 2008; Tsao 2010). The closure of the C-ring converts it into flavanone naringenin which further transforms into dihydrokaempferol by the addition of a hydroxyl group to the C3 position of the C-ring (Holton and Cornish 1995). These dihydroflavonols are reduced to form (2*R*,3*S*,4*S*)-leucoanthocyanidins (flavan-3,4-diols) and further to (2*R*,3*S*)-flavan-3-ols catechin and gallocatechin (Stafford 1991; Winkel-Shirley 2001). Alternatively, the leucoanthocyanidin molecules can be oxidized to form anthocyanidins (Holton and Cornish 1995; He et al. 2008), which can further be converted into (2*R*,3*R*)-flavan-3-ols epicatechin and epigallocatechin (Xie and Dixon 2005; He et al. 2008). All three, (2*R*,3*R*)-flavan-3-ols, (2*R*,3*S*)-flavan-3-ols and (2*R*,3*S*,4*S*)-flavan-3,4-diols, could potentially function as precursors for the PAs (Dixon et al. 2005; Xie and Dixon 2005; Tian et al. 2010). However, so far, the polymerization step of PAs has remained unsolved (Dixon et al. 2005; He et al. 2008; Zhao et al. 2010; Jiang et al. 2015).

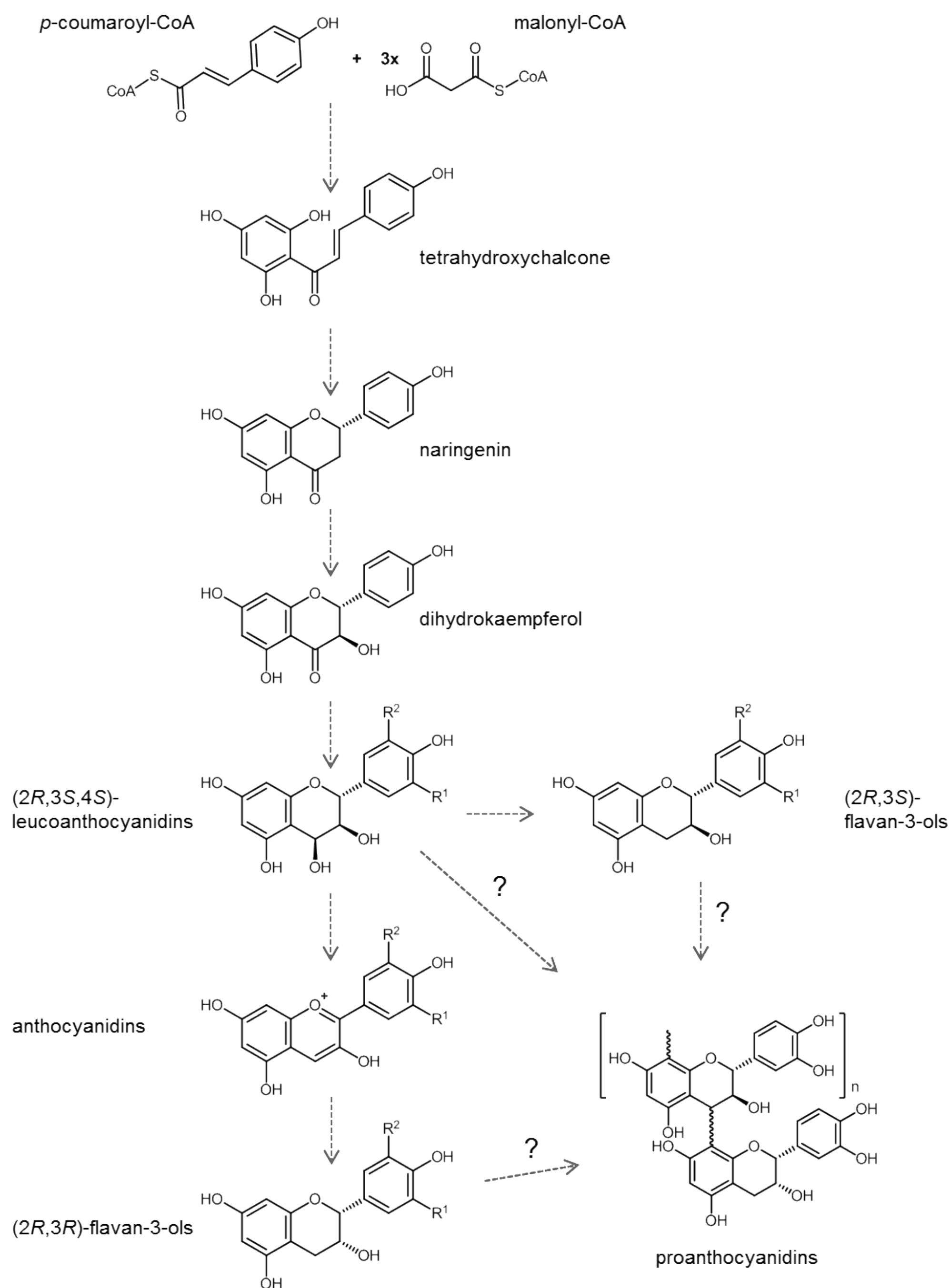

Figure 5. The main steps involved in the biosynthesis of proanthocyanidins.

The two types of terrestrial tannins seldom accumulate in high concentrations in the same plant tissues; while PAs accumulate in the vacuole (Stafford 1988; Dixon et al. 2005; Zhao et al. 2010), HTs appear to be concentrated in the cell wall of the plant tissues (Gross 1999a; Grundhöfer et al. 2001). An explanation for why PAs and HTs are rarely found simultaneously in high concentrations in same plant tissue can be found from the biosynthetic pathways of these tannins. As a consequence of the common building blocks in HT and PA biosynthesis, there is a potential “competition” for the formation of these compounds. If the glycolytic phosphoenolpyruvate is efficiently directed for the shikimate pathway, the production of pyruvate, which is utilized in the acetate/malonate pathway, is significantly reduced. This results in reduced PA biosynthesis, since it would need malonyl-CoA as one of their building blocks (Salminen and Karonen 2011). Similarly, PA biosynthesis is reduced by the efficient production of gallic acid from 3-dehydroshikimic acid as it negatively affects the synthesis of shikimic acid and thus the formation of the other PA precursor, phenylalanine (Salminen and Karonen 2011). The same holds for the two HT classes, GTs and ETs; they do not accumulate in the same plant tissue, presumably due to the common precursor, pentagalloylglucose (Salminen and Karonen 2011).

2.2. Liquid chromatography–mass spectrometry in tannin analysis

The growing interest towards plant tannins has resulted in many efforts to provide sensitive and selective analytical methods for their determination. Traditionally, tannin analysis has exploited the chemical reactivity of the tannin functional groups to undergo characteristic reactions that result in colorful chromophores to be detected by spectrophotometry. These types of methods include both the general polyphenol methods (Ciocalteau 1927) and the more selective functional group methods for the detection of PAs and HTs (Bate-Smith 1972; Porter et al. 1986; Hagerman and Butler 1989; Wilson and Hagerman 1990). While these spectrophotometric methods are rapid and simple, they lack the specificity for individual compounds. For this purpose, more sophisticated methods are needed. In addition to liquid chromatography coupled with a diode array detector, liquid chromatography coupled with mass spectrometry (LC-MS) is one of the most important tools in the characterization

of all organic compounds, including quantitation and identification of different tannins.

2.2.1. Liquid chromatography–mass spectrometry in general

LC is a separation technique in which a mobile phase is continuously passed over a stationary phase packed in a column. LC utilizes the interactions of the analyte with both the mobile and stationary phase; the stronger the interaction with the stationary phase compared to the mobile phase, the longer the retention time (Niessen 2007). Based on the polarity of the mobile and stationary phases, LC is divided into normal-phase LC and reversed-phase LC (Adrey 2003). In normal-phase LC, the stationary phase is more polar than the mobile phase (e.g. mobile phase hexane, stationary phase silica) whereas in reversed-phase LC, the mobile phase is more polar than stationary phase (e.g. mobile phase acetonitrile, stationary phase octadecylsilyl). LC utilizing very small particle sizes of the stationary phase (≤ 2 µm) combined with very high pressures (up to 1300 bar) is referred as ultra-high performance liquid chromatography (Guillarme et al. 2010; Rodriquez-Aller 2013). The advantages of UHPLC compared to its predecessor, high performance liquid chromatography (HPLC), include the improved chromatographic resolution, the increased sensitivity and the shorter analysis time (Guillarme et al. 2010).

When mass spectrometry is used in conjunction with a chromatographic technique, the compounds enter the ionization source based on their elution order from the chromatographic column used (Rodriguez-Aller et al. 2013). Mass spectrometry involves the following steps: ionization of the analytes at the ionization source, separation of the produced ions based on their mass to charge ratios (*m/z*) and detection of the ions (Adrey 2003). There are a number of ion sources available in mass spectrometry but currently most LC–MS systems utilize electrospray ionization (ESI) technique (Holčapek et al. 2012). In ESI, the sample is first injected into a capillary maintained at high voltage (Adrey 2003). Because of the high voltage, the liquid stream disperses into a mist of highly charged droplets that undergo desolvation due to the heated drying gas (often nitrogen) flow (Greavez and Roboz 2014). The droplet size decrease until the repulsive forces between the charges on the surface of the droplets overcome the surface tension and the so called Coulombic explosion occurs (Fenn et al. 1989; Adrey 2003; Kebarle and Verkerk 2009). A number of

smaller droplets are formed which further decrease in size until the droplet radius reaches ~10 nm, and the electric field starts to support direct ion evaporation (Niessen and Tinke 1995; Greavez and Roboz 2014). When this occurs, the droplets distort and the so called Taylor cone develops (Fig. 6). A Taylor cone is formed also at the tip of the ESI probe when the initial droplets are formed due to the nebulization of the solvent flow (Greaves and Roboz 2014). The released ions, i.e. analytes, are then transferred through a series of focusing lenses into the mass spectrometer (Adrey 2003; Holčapec et al. 2012). ESI is a soft ionization technique, in which analyte fragmentation remains moderate. However, due to the mechanism of ionization in ESI, larger molecules often become multiply charged, allowing their detection with common mass analyzers (Adrey 2003).

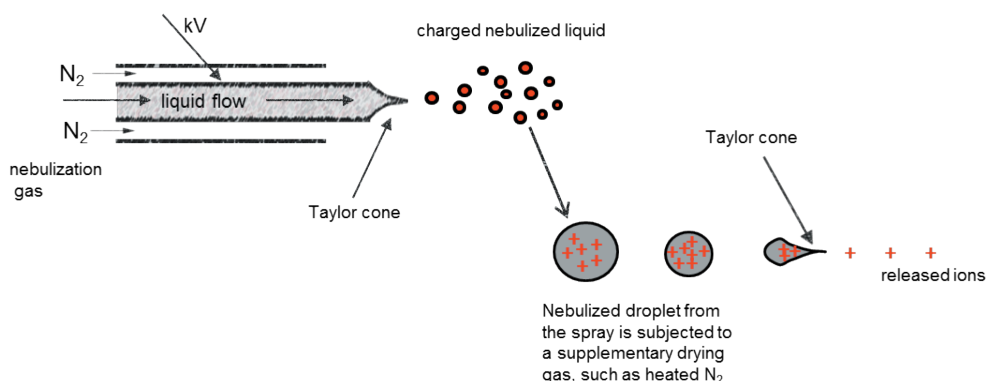

Figure 6. Taylor cone formation occurs both at the end of the ESI tube and when the droplet breaks up and releases ions (adapted from Greaves and Roboz 2014).

After the ions are formed, they are separated based on their *m/z* ratios. Several different mass analyzer types exist, and even if many of them differ significantly in the modes of operation, they all separate ions according to the *m/z* ratios (Greaves and Roboz 2014). Current mass analyzers include quadrupoles, quadrupole ion traps, time-of-flight analyzers, Fourier transform ion traps, ion mobility analyzers and magnetic sector analyzers (Gross 2011; Greaves and Roboz 2014). As such, these analyzers reveal the mass of the analyte, but limited information on the structure of the analytes is obtained. For this purpose, multi analyzers, i.e. tandem mass spectrometry (MS/MS) is

required. Several forms of MS/MS are available, but triple quadrupole (QqQ) is one the most widely used MS/MS instruments.

2.2.2. Triple quadrupole tandem mass spectrometry

As the name suggests, QqQ consists of three quadrupoles in series (Fig. 7). The first and third quadrupoles function as mass separation devices while the second quadrupole is a collision cell, in which fragmentation of the ions from the first quadrupole is carried out with a radio frequency voltage and collision gas, often argon. In some QqQ instruments, the second quadrupole is a hexapole, an octapole or an ion tunnel instead, but the principle remains the same (Niessen 2007; de Hoffman and Stroobrant 2008). The first and third quadrupoles are controlled by changing the radio frequency and direct current potentials to allow the transmission of ions of a single *m/z* ratio or a range of *m/z* values, depending on the application (Andrey 2003; Greavez and Robos 2014). Mass separation is based on the oscillation of the ions entering the electronic field of the quadrupole. Only ions with specific *m/z* values have stable oscillatory flight paths while other ions oscillate in an unstable pattern and are lost by collision to the rods of the quadrupole (Greaves and Roboz 2014).

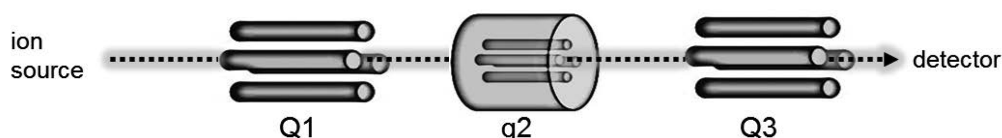

Figure 7. Principle of the triple quadrupole analyzer. Q1 and Q3 can operate in scanning mode or held static, depending on the application. In q2, the precursor ions are fragmented into product ions.

By keeping the two analyzers (Q1 and Q3) in either scanning mode or held static, different modes of data collection can be utilized, including full scan, product ion scan, precursor ion scan, neutral loss scan and selected/multiple reaction monitoring (Table 1). For full scan purposes, the direct-current and radio frequency voltages are changed progressively while keeping their ratio constant; this yields mass spectra comprised of all the different *m/z* values present in the sample. Due to the scanning technique, the QqQ analyzers have a limited resolution for full scan analysis and are categorized as low resolution

mass spectrometers, capable of measuring the *m/z* ratio of an ion to the nearest integer value (Adrey 2003). As a consequence of the low resolution, two ions with the same nominal mass but with a different exact mass cannot be separated.

Table 1. Different analysis modes in tandem mass spectrometry (modified from Niessen 2007).

Mode	Q1	Q3	Application
Full Scan	Scanning	Scanning	To obtain information on the sample composition
Product Ion Scan	Selecting	Scanning	To obtain structural information on the ions produced in the ion source
Precursor Ion Scan	Scanning	Selecting	To monitor compounds which give an identical fragment in CID*
Neutral Loss Scan	Scanning	Offset scanning	Q1 and Q3 scanning at fixed *m/z* difference: to monitor compounds that lose a common neutral species
Selected/ Multiple Reaction Monitoring	Selecting	Selecting	To monitor a specific CID* reaction

*CID: collision induced fragmentation, takes place in the collision cell q2

The true potential of QqQ analyzers is revealed when quantifying known compounds or when elucidating structural information concerning the structure of the molecule involved. As much as 100% of the instruments scanning capacity can be used for a selected ion, making the instrument highly sensitive when monitoring single *m/z* values (Hoffman and Stroobant 2008; Graves and Roboz 2014). In addition to the traditional MS/MS analysis, many instruments enable to utilize in-source fragmentation for selected and multiple reaction monitoring (SRM and MRM, respectively) methods. In this approach, ions are fragmented within the source of the mass spectrometer by application of a voltage in the nozzle-skimmer system through which the ions are sampled to

the analyzer (Adrey 2003; Hoffman and Stroobant 2008). The applied voltage increases the velocity of the ions exiting into the relatively high-pressure region between the nozzle and the skimmer. This increases the probability of the ions to collide with the residual gas molecules, leading to fragmentation of the analyte ions (Adrey 2003). These ions then travel to the Q1 where the MS/MS analysis continues as described above.

In general, QqQ instruments are well suited for both qualitative and quantitative analysis as they provide excellent sensitivity and selectivity, and high signal-to-noise ratios are obtained especially in SRM/MRM analyses. However, the number of MS/MS transitions in a single SRM/MRM segment is limited and thus, when applying many SRMs or MRMs a compromise between sensitivity and acquisition rate is unavoidable (Guillarme et al. 2010). When analyzing a large number of compounds, it is possible to divide the MS/MS method into several time-scheduled windows with different SRM/MRM channels and time segments (Guillarme et al. 2010). For example, an MRM method with a dwell time (time spent for particular transition) between 16 and 25 ms was used in UHPLC-MS/MS analysis of seven oligomeric ETs in willow herb leaves (Baert et al. 2015) but when analyzing 154 polyphenols in wine, it was reduced to 5 ms (Lambert et al. 2015). The partial limitation of this procedure arises from the possible variation in elution-time; with too many analytes to be detected, the time windows become very narrow and adjustments are required if the analyte retention times fluctuate outside the original time-window (Guillarme et al. 2010).

2.2.3. Tannin analysis by tandem mass spectrometry

Tandem mass spectrometry can be used for both qualitative and quantitative determination of tannins. The SRM/MRM methodology enables high sensitivity and selectivity for the quantitation of tannins while the other applications allow the analysis of their fragmentation patterns and thus the structural elucidation of tannins. In particular, ESI-MS/MS approach is a powerful tool for the identification and structural characterization of tannins. The different utilization modes (full scan, product ion scan, precursor ion scan, neutral loss as well as SRM/MRM) and combination of the data obtained with the different modes are very useful in structure elucidation (Bubba et al. 2012). Still, the most common method to study the fragmentation patterns is to use the product ion scan for a

selected precursor ion. This enables to tentatively identity the molecules and further, to select suitable parameters for the SRM/MRM methods (Zywicki et al. 2002). The fragmentation patterns of both HTs and PAs are well documented. Considering GGs and GTs, their fragmentation patterns have been suggested already by full scan MS (e.g. Salminen et al. 1999; Mämmelä et al. 2000) but utilization of MS^n has enabled a more detailed description of the fragmentation patterns. The fragmentation of GGs and GTs is rather straightforward as they contain only galloyl groups attached to the core polyol (GGs) and additional galloyls linked to the core galloyl groups via depside bonds (GTs). The depside bonds between two galloyls are less stable than the ester bonds between the core polyol and galloyl groups (Salminen et al. 1999), and thus the fragmentation of the former type of galloyl groups leads to the loss of 152 Da while the latter one gives a fragment ion at *m/z* 169. In principle, all the galloyl groups attached with depside bonds to the core galloyl groups are fragmented first and then the galloyl groups attached to core polyol start to fragment (Berardini et al. 2004; Regazzoni et al. 2013).

Figure 8 summarizes the current knowledge of the fragmentation patterns of galloylglucoses. The pentagalloylglucose shows consistent mass loss of galloyl moieties to tetra-, tri-, di- and monogalloylglucoses. This has been confirmed also by the neutral loss of gallic acid in case of pentagalloyl- to digalloylglucose (Tan et al. 2010). Interestingly, the fragmentation of digalloylglucose contributes not only to the loss of galloyl group but also two major product ions at *m/z* 439 and *m/z* 423 have been reported (Soong and Barlow 2005; Tan et al. 2010). While the former derives from decarboxylation, the latter has been suggested to derive from the removal of two formaldehyde moieties from the central glucose (Taylor et al. 2005; Tan et al. 2010). Similarly, the monogalloylglucose fragment ions at *m/z* 271 and *m/z* 211have been explained by the removal of one and two formaldehyde groups, respectively (Tan et al. 2010).

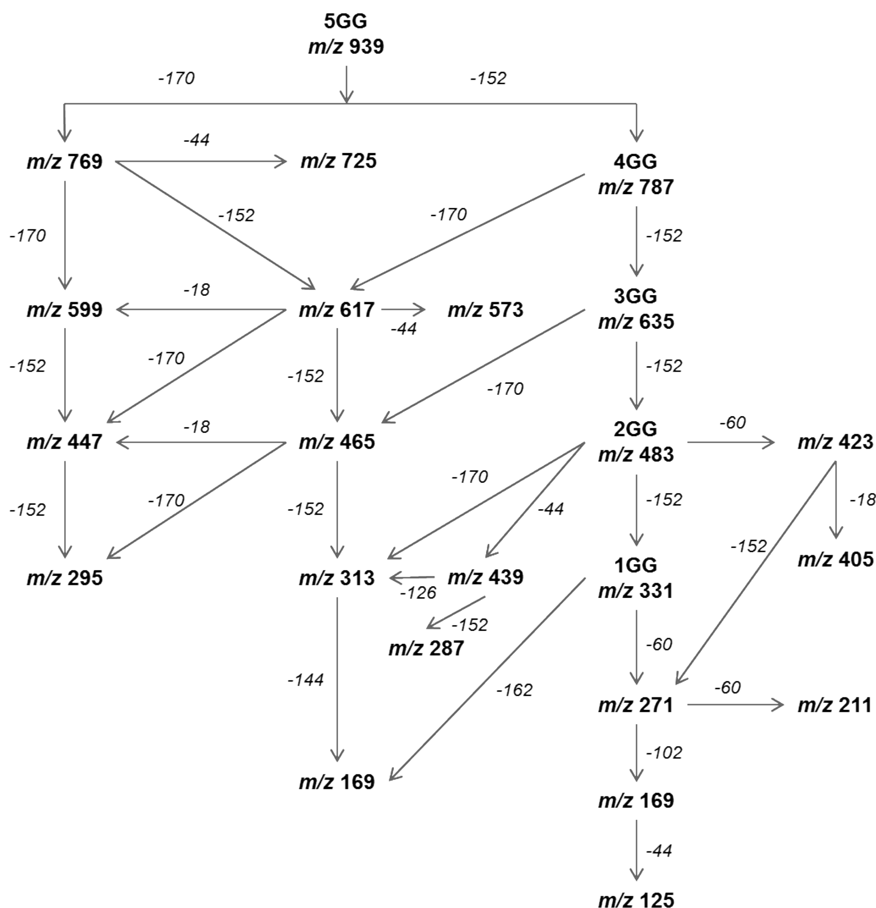

Figure 8. Fragmentation pathway of galloylglucoses in negative mode ESI-MS^n (Zywicki et al. 2000; Soong and Barlow 2005; Tan et al. 2010). 5GG, pentagalloylglucose; 4GG, tetragalloylglucose; 3GG, trigalloylglucose; 2GG, digalloylglucose; 1GG, monogalloylglucose.

ET structures are more complex than GG and GT structures, and correspondingly, their fragmentation is less straightforward. However, the fragmentation patterns do follow the same general rules and for monomeric ETs, the main fragments are often related to the functional groups such as the galloyl group and the HHDP group (Zywicki et al. 2002; Fracasetti et al. 2013; Regueiro et al. 2014). For example, tentative fragmentations of an acyclic ET, vescalagin, are presented in Figure 9. In case of oligomeric ETs, additional fragmentation pathways emerge due to their fragmentation into the monomeric units of which the ET consists of (Mullen et al. 2002; Hager et al. 2008; Bubba et al. 2012; Baert et al. 2015).

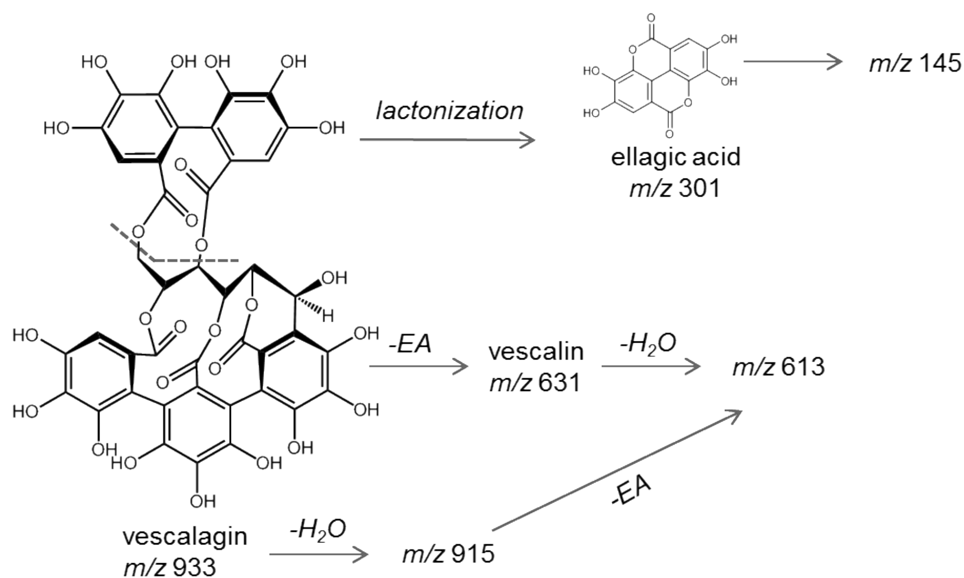

Figure 9. Fragmentation pathway of vescalagin in negative mode ESI-MS^n (Zywicki et al. 2002; Hager et al. 2008). EA, ellagic acid.

The last group of tannins, PAs, is structurally significantly different from HTs. For B-type PAs, the main fragmentation patterns include quinone-methide (QM) mechanism, heterocyclic ring fission (HRF) and retro-Diels–Alder (RDA) fragmentation (Gu et al. 2003; Callemien and Collin 2008; Pérez-Jiménez and Torres 2012) which are presented in Figure 10 for a PC dimer. With QM fragmentation it is possible to define the extension and terminal units of the oligomers and polymers. For example, in B-type PC dimers, the cleavage of the interflavanoid bond gives fragments (negative mode) with *m/z* 289 (terminal unit) and *m/z* 287 (extension unit). Trimeric PC gives fragments at *m/z* 577 and *m/z* 287 if the cleavage takes place between the upper units and *m/z* 289 and *m/z* 575 if it takes place between the lower units (Callemien and Collin 2008; Pérez-Jiménez and Torres 2012; Flamini 2013; Tala et al. 2013). For the PC dimer and trimer, HRF gives fragments at *m/z* 451 and *m/z* 739 while RDA fission results in a fragment at *m/z* 425 for the PC dimer and at *m/z* 713 for the PC trimer (Callemien and Collin 2008; Jaiswal et al. 2012; Pérez-Jiménez and Torres 2012; Teixeira et al. 2016). Further, losses of H_2O and CO are common and explain many of the other ions produced in the fragmentation of PAs (Callemien and Collin 2008; Li and Deinzer 2008; Delgado de la Torre et al.

2013). For PA gallates, the loss of the galloyl group (-152 Da) is characteristically observed (Pezet et al. 2011; Jaiswal et al. 2011; Pérez-Jiménez and Torres 2012). In general, same fragmentations occur also for A-type PAs and the *m/z* values of PAs with one A-type bond differ from the corresponding B-type PA by 2 Da. However, A-type interflavanoid linkages do not readily undergo RDA fissions because of the additional ether bond and thus, these differences can be used to distinguishing A-type and B-type PAs (Gu et al. 2003; Li and Deinzer 2008; Jaiswal et al. 2011; Flamini 2013).

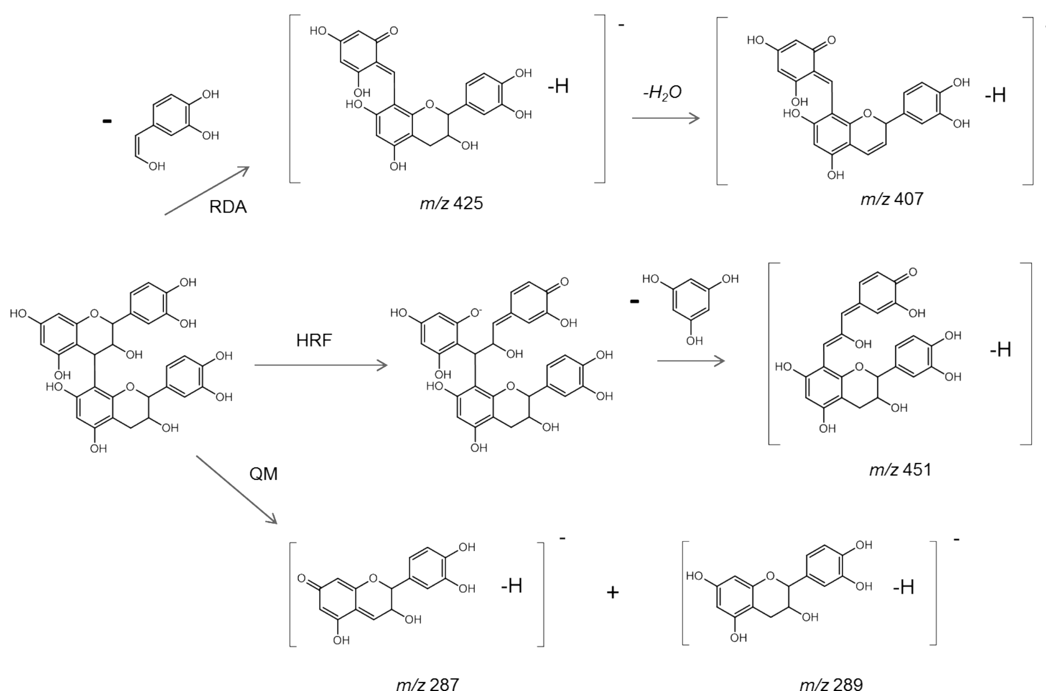

Figure 10. Fragmentation pathways of procyanidin dimer in negative ion mode (Gu et al. 2003; Callemien and Collin 2008; Pérez-Jiménez and Torres 2012). RDA, retro-Diels-Alder fragmentation; HRF, heterocyclic ring fission; QM, quinone methide cleavage.

Compared to the number of publications that have utilized MS/MS for tannin identification and to study the tannin fragmentation patterns, surprisingly few papers have used MS/MS for tannin quantitation. Examples of SRM and MRM methods used in the literature for the quantitation of tannins are listed in Table 2. In addition to adjusting the typical interface parameters, when utilizing

QqQ mass analyzers, the cone voltage, collision energy and the pressure of the collision gas are manually optimized (Sherwood et al. 2009). Usually, the most abundant fragment ion is selected for the SRM/MRM transition (Sherwood et al. 2009) and for smaller tannins, the selection of precursor ion is rather straightforward and often the singly charged molecular ions is chosen for that purpose. However, when the molecular weight of the tannin exceeds the mass range of the analyzer, multiple charged ions are utilized (Baert et al. 2015).

Table 2. Examples of selected and multiple reaction monitor transitions used in the literature for tannin quantitation. Multiple product ions indicate the use of quantifier and qualifier transitions.

Compound	MW	Precursor ion	Product ion	Cone voltage (V)	Collision energy (eV)	Reference
ellagic acid	302	301	145	55	35	Zywicki et al. 2002
gallic acid	170	169	125	25	15	Zywicki et al. 2002
monogalloylglucose	332	331	169	30	20	Zywicki et al. 2002
1-*O*-galloylglucose	332	331	169	34	22	Kårlund et al. 2014
digalloylglucose	484	483	169	35	30	Zywicki et al. 2002
trigalloylglucose	636	635	169	50	50	Zywicki et al. 2002
trigalloyl glucose	636	635	465	32	24	Kårlund et al. 2014
tellimagrandin I	786	785	301	44	55	Kårlund et al. 2014
pedunculagin	784	783	301	56	44	Kårlund et al. 2014
2,3-(*S*)-HHDP-glucose	482	481	301	26	26	Kårlund et al. 2014
galloyl-HHDP-glucose	634	633	301	46	32	Kårlund et al. 2014
casuarictin	936	935	633 / 301	66	26 / 46	Gasperotti et al. 2014
oentohein B	1569	783	765 / 301	30	20 / 40	Baert et al. 2015
oenothein A	2354	1176	301/ 275	46	52 / 62	Baert et al. 2015
tetrameric ET[1]	3138	1045	301/ 275	32	50 / 60	Baert et al. 2015
pentameric ET[1]	3923	1306	301/ 275	40	65 / 75	Baert et al. 2015
hexameric ET[1]	4707	1568	301/ 275	42	90 / 70	Baert et al. 2015
heptameric ET[1]	5492	1829	275/ 301	46	75 / 100	Baert et al. 2015
sanguiin H6	1870	934	633/ 301	34	22 / 38	Gasperotti et al. 2014
agrimoniin	1870	934	633 / 301	32	32 / 46	Gasperotti et al. 2014
agrimoniin	1870	934	301	38	46	Kårlund et al. 2014
catechin	290	289	109	40	25	Guillarme et al. 2010
catechin	290	289	245 / 205	45	10 / 15	Ortega et al. 2010

Compound	MW	Precursor ion	Product ion	Cone voltage (V)	Collision energy (eV)	Reference
epicatechin	290	289	109	40	25	Guillarme et al. 2010
epicatechin	290	289	245 / 179	45	10 / 15	Ortega et al. 2010
catechin gallate	442	441	169	40	25	Guillarme et al. 2010
epicatechin gallate	442	441	169	40	25	Guillarme et al. 2010
epigallocatechin	306	305	125	40	25	Guillarme et al. 2010
gallocatechin gallate	458	457	169	40	25	Guillarme et al. 2010
epigallocatechin gallate	458	457	169	40	25	Guillarme et al. 2010
proanthocyanidin B1	578	577	289 / 407 / 125	65	30	de la Torre et al. 2013
proanthocyanidin B2	578	577	289 / 407 / 125	65	30	de la Torre et al. 2013
proanthocyanidin A2	576	575	285 / 539 / 449	65	30	de la Torre et al. 2013
proanthocyanidin B2	578	577	289 / 425	45	20 / 45	Ortega et al. 2010
dimeric PA[2]	578	577	425	30	16	Kårlund et al. 2014
dimeric PA	578	577	425	30	16	Kårlund et al. 2014
trimeric PA	866	865	289	40	36	Kårlund et al. 2014
PA trimer	866	865	577 / 695	60	20 / 25	Ortega et al. 2010
PA tetramer	1154	1153	865 / 575	70	20 / 30	Ortega et al. 2010
PA pentamer	1442	1441	1028 / 1151	80	25 / 30	Ortega et al. 2010
PA hexamer	1730	1729	1153 / 863	80	30 / 30	Ortega et al. 2010
PA heptamer	2018	1008	865 / 575	60	20 / 20	Ortega et al. 2010
PA octamer	2306	1152	875 / 983	65	25 / 25	Ortega et al. 2010
PA nonamer	2594	1296	577 / 1152	50	30 / 30	Ortega et al. 2010

[1]Oligomeric ellagitannins based on tellimagrandin I units.
[2] PA: proanthocyanidin

2.3. Tannin–protein interactions

Tannin–protein interactions have great importance in numerous plant related domains, from the most basic functions in plant physiology and ecology to their utilization in agriculture, foods and medicine. The interest in tannin research has increased substantially during the past few decades and a particular interest has been on the role of tannins on the human and animal health and nutrition. Many of these nutritional and health effects have been associated with the ability of tannins to interact with proteins in biological systems. In order to understand the exact roles of tannins and proteins in these interactions, it has been essential to determine the nature of the interactions and the features affecting the complex formation.

2.3.1. Nature of tannin–protein interactions

The nature of the interactions between tannins and proteins can be divided into covalent and non-covalent bonding based on whether the molecules are irreversibly bound to each other or not, respectively. At low pH, tannins most often form non-covalent bonds with proteins, while at higher pH the formation of covalent complexes is favored (Prigent 2005; Hagerman 2012). Non-covalent bonds, more often referred as non-covalent interactions, enable tannins to bind specifically but transiently to other molecules. Tannins, as well as polyphenols in general, are amphiphilic molecules, containing both hydrophilic and hydrophobic characteristics. The hydrophilic character is caused by the phenolic hydroxyl groups whereas the aromatic ring itself is hydrophobic (Poncet-Legrand et al. 2006; Mueller-Harvey et al. 2007; Hagerman 2012). Although non-covalent bonds are weak, stable associations between tannins and proteins are formed when the interaction system forms multiple non-covalent bonds simultaneously (Haslam 1998). Almost all non-covalent interactions can be divided into subclasses of the electrostatically attractive interactions. From these interactions, π-π stacking, hydrogen bonding and hydrophobic interactions are the most important ones regarding tannin–protein interactions (Haslam 1998; Lodish et al. 2000; Dangles and Dufour 2006; McRae et al. 2011).

At neutral to alkaline pH, tannins may be oxidized to form quinones via unstable semiquinone radicals (Appel 1993). A scheme of oxidation of the HHDP group present in ETs is shown in Figure 11. Oxidation occurs most readily at a pH higher than the pKa of the tannin phenolic hydrogen, which

typically is in the range of 7–9 (Appel 1993; Hider et al. 2001). However, oxidation of the tannins by biological free radicals or reactive oxygen species is independent of the pH. The formed quinones are relatively unstable molecules and are easily quenched by a reaction with a protein to form covalently stabilized adducts (Appel 1993; Prigent et al. 2007; Quideau et al. 2011; Salminen and Karonen 2011). In this process, the electrophilic quinone is attacked by the lone-pair bearing amine or thiol of the protein and therefore may alter the structure and function of the protein (Ludlum et al. 1991; Prigent et al. 2007).

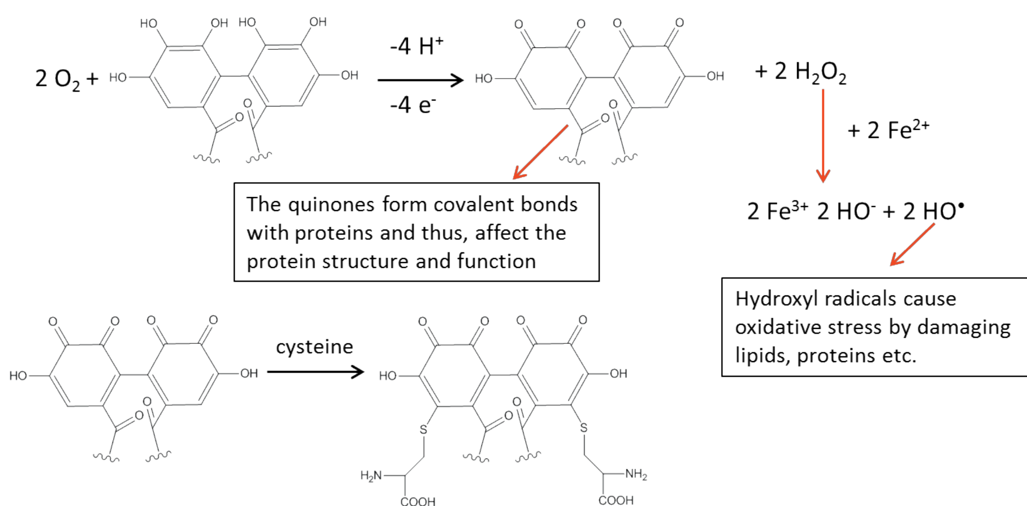

Figure 11. The oxidation of a hexahydroxydiphenoyl group to form a quinone, which further forms covalent bonds with proteins and as a consequence affect the structure and function of the protein. In addition, the byproducts cause oxidative stress by damaging lipids, proteins and other biomolecules (adapted from Salminen and Karonen 2011).

In addition to dividing the tannin–protein complexes to covalent or non-covalent, the nature of the interactions can be described by the solubility of the formed complexes. Even if the classical definition of tannin emphasizes their ability to precipitate proteins (Swain and Bate-Smith 1962), precipitation does not necessarily occur in conditions where the stoichiometry of the interacting species favors soluble complexes (Chen and Hagerman 2004; Hagerman 2012). Thus, even if a tannin does not precipitate proteins efficiently, it may have a

high affinity for the proteins. Formation of either soluble or insoluble complexes depends on many different features including the structural nature of the protein and the tannin as well as the conditions where the reaction takes place (Chen and Hagerman 2004; Kusuda et al. 2006; Hagerman 2012). The precipitation may occur via two different mechanisms, depending on the tannin (Fig. 12). Tannins with a strong hydrophobic character, such as pentagalloylglucose (PGG), precipitate proteins by forming a hydrophobic coat around the protein (Kawamoto et al. 1996; Chen and Hagerman 2004; Karonen et al. 2015). For more polar, hydrophilic tannins, such as PAs, the multidentate nature that enables the tannin to cross-link protein molecules plays an important role in the precipitation reaction (Charlton et al. 1996; Charlton et al. 2002; Simon et al. 2003; Jöbstl et al. 2004; Quideau et al. 2011). In general, soluble complexes are favored when the protein is in excess (Luck et al. 1994; Kusuda et al. 2006) and the number of tannin molecules required to precipitate protein is dependent on the size and structural flexibility of the tannin (Charlton et al. 2002). Together with these features the concentration of protein affects the initial interaction, while environmental factors including pH, ionic strength and temperature influence the precipitation of the complexes (McRae et al. 2011).

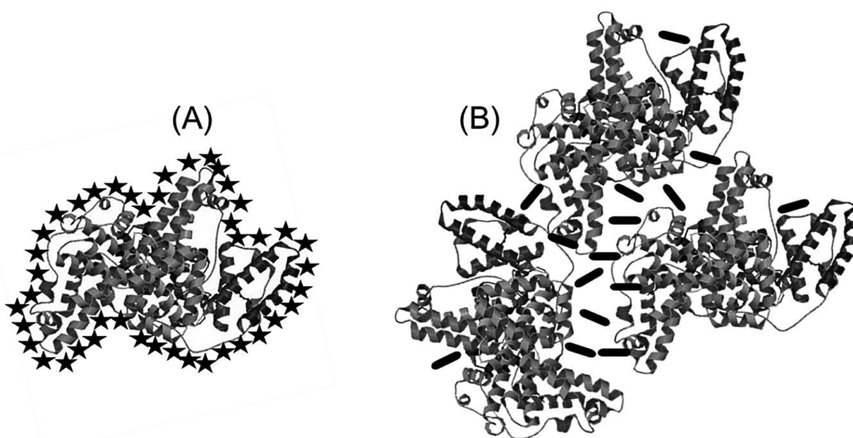

Figure 12. Protein precipitation may occur via two different mechanisms: hydrophobic tannins, such as PGG, precipitate proteins by forming a hydrophobic coat around the protein (A), while hydrophilic tannins, such as PAs, precipitate proteins by cross-linking the protein molecules (B). The model protein structure, BSA, is from RCSB protein data bank (www.rcsb.org, PDB ID: 4F5S, Bujacz 2012).

The third aspect regarding the nature of the tannin–protein interactions is the specificity of the interaction between the tannin and the protein. Specific interaction refers to drug/target-like interactions in which the polyphenol is bound to the protein as a substrate or as an inhibitor of that particular protein (Douat-Casassus et al. 2009; Fraga et al. 2010; Quideau et al. 2011). While non-specific mechanisms are related to the presence of the structural features commonly found among tannins, e.g. the phenolic groups, specific mechanisms are caused by a particular structural characteristic of the active tannin. For example, the structure of the protein may include a certain pocket, an active site, in which particular tannin fits perfectly as it structurally resembles the original molecule supposed to bind to the protein (Fraga et al. 2010; Jung et al. 2010). Non-specific mechanisms often require high tannin concentrations, but the specific mechanism may occur already in significantly lower tannin concentrations (Fraga et al. 2010; Gonçlaves et al. 2011; Quideau et al. 2011). The most studied polyphenols include different flavonoids but also PAs and ETs have been evaluated for their ability to specifically bind proteins (Zhu et al. 1997; Quideau et al. 2005, Mackenzie et al. 2008; Erlejman et al. 2008; Fraga et al. 2010; Gonçlaves et al. 2011; Quideau et al. 2011; Bellesia et al. 2015).

2.3.2. The effect of tannin and protein structures on their interactions

The structure of a tannin affects its covalent and non-covalent interactions with proteins considerably. Due to the differences of these two types of interactions, certain molecular features under defined conditions may favor one or the other mode of interaction. On one hand, the affinity of a tannin for a protein is determined by its ability to reach the protein and to form hydrogen bonds or hydrophobic interactions with it (Haslam 1996). On the other hand, covalent binding with a protein is dependent on the oxidation of the tannin, and thus, on the oxidative activity of the tannin (Appel 1993; Baxter et al. 1997; Salminen and Karonen 2011; Hagerman 2012). Regarding non-covalent complexes, tannin size and structural flexibility play an important role in its complexation with a protein (Haslam 1996; Deaville et al. 2007; Lorenz et al. 2013; Zeller et al. 2015; Kilmister et al. 2016). The effect of size on these complexes is partially explained by the increasing number of hydroxyl groups (Haslam et al. 1992; Baxter et al. 1997; Feldman et al. 1999; de Freitas and Mateus 2001; Aguie-Beghin et al. 2008) as the affinity for proteins increases with the number of phenolic groups and associated aromatic rings that enable the tannin to build

bridges between proteins and other tannin molecules (Charlton et al. 1996; Baxter et al. 1997; Dangles et al. 2001; Aguie-Beghin et al. 2008; Karonen et al. 2015). However, the correlation between tannin size and protein binding capacity may have an upper limit as the steric hindrance of large tannins may prevent access to binding sites (Baxter et al. 1997; Poncet-Legrand 2007).

The observed differences in binding characteristics between GTs, ETs (Deaville et al. 2007; Karonen et al. 2015) and PAs (Hagerman and Butler 1981; Hofmann et al. 2006; Ozdal et al. 2013) have further highlighted the importance of conformational flexibility of the polyphenol molecule. The HHDP groups characteristic of ETs constrain the structure, resulting in loss of conformational freedom and therefore higher ligand concentrations are needed to achieve protein aggregation. PAs in turn have flexible structures and are free to form cross-links between the molecules (Hofmann et al. 2006). Among HTs, the orientation of the substituent in the possible anomeric carbon of the sugar moiety is important in protein binding. For example, *α*-D-pentagalloylglucose appears to have a higher affinity for BSA than its natural stereoisomer *β*-D-pentagalloylglucose (Feldman et al. 1999), presumably because in the former the surfaces of the C1 and C2 galloyl rings are better exposed for hydrophobic interactions. Among PAs, the interflavanoid bond C4–C8 seems to be more favorable than the C4–C6 bond when comparing the affinities for salivary proteins. This has been suggested to be caused by the lesser steric rigidity of the C4–C8 bond (de Freitas and Mateus 2001; Cala et al. 2010; McRae et al. 2011). The protein affinity of PAs depends also on the stereochemistry of the heterocyclic ring hydroxyl group linked to the C3, on the stereochemistry of the interflavanoid bond (α or β) and on the degree of polymerization. The different affinities between isomers are mainly explained by the different conformations they adopt: a more extended conformation increases, and a compact and more rigid conformation decreases the affinity for the proteins (de Freitas and Mateus 2001; Cala et al. 2010).

Because tannins can be oxidized to form species which may covalently react with proteins, the oxidative activity of tannin could be indicative of the tendency of a tannin to covalently bind proteins (Appel 2003; Barbehenn and Constabel 2011; Salminen et al. 2011). Studies with the monomeric units of PAs, epigallocatechin gallate, epigallocatechin, epicatechin gallate, epicatechin and catechin have shown that the trihydroxylated B-ring is essential in the formation of covalent adducts with proteins (Poncet-Legrand 2007; Hagerman

2012). Considering this, PAs possessing a higher number of PD subunits should be more prone to form covalently stabilized complexes with proteins than PAs containing predominantly PC subunits. This is indirectly supported by the oxidative activity studies showing that PDs are more easily oxidized at high pH than PCs (Barbehenn et al. 2006). Regarding HTs, GGs and high-molecular-weight GTs have intermediate to low oxidative activities (Chen and Hagerman 2004; Barbehenn et al. 2006; Salminen et al. 2011; Hagerman 2012). On the contrary, ETs are more readily oxidized (Barbehenn et al. 2006; Moilanen and Salminen; Salminen and Karonen 2011). Detailed information about how the ET structure and their oxidative activities are related was reported by Moilanen and Salminen (2008). By comparing the structures of 27 ETs they showed that the oxidative activity can be predicted from the exact ET structures. The results showed that the order of how much the structural features affected the oxidative activities of ETs was: valoneoyl group > acyclic glucose having an α-OH like in castalagin > xylose / lyxose > NHTP group > valoneoyl group (with bound COOH) ≈ sanguisorboyl group > HHDP-group ≈ acyclic glucose having a β-OH like in vescalagin. The other structural details (cyclic glucose, galloyl, dehydrodigalloyl, catechin and methyl groups) either had no significant effect or had a slightly negative effect on the oxidative activity (Moilanen and Salminen 2008). Unfortunately, there have been relatively few studies on the covalent reaction between proteins and tannins, one difficulty being the characterization of the highly cross-linked adducts (Hagerman 2012). Similarly, the relation of the tannin oxidative activity and their tendency to form covalently stabilized complexes with proteins remains still unsolved and further studies are required to understand this biologically important aspect of tannin–protein interactions.

Similar to the effect of tannin structure, the interaction between tannins and proteins is affected by the size, structure, conformation and isoelectric point of the protein (Hagerman and Butler 1981; Charlton et al. 1996; Charlton et al. 2002). It appears that the conformational flexibility of the protein may be as important factor as the flexibility of the tannin for the complex formation and a flexible protein structure might decrease the importance of tannin flexibility. For example, the rigidity of the ET does not play a major role in determining binding strength with flexible proteins such as gelatin (Deaville et al. 2007) whereas it does impact the ability to bind to less flexible BSA (Hofmann et al. 2006; Deaville et al. 2007). Accordingly, the amino acid composition of the protein is a major factor in tannin–protein interactions. The presence of proline

is a common characteristic for proteins with high affinity for polyphenols. The tertiary amide carbonyl groups of the prolyl residue form hydrogen bonds with the phenolic groups of the tannin (Fig. 13A). Non-specific interactions are formed between the galloyl units of the tannin and the open, flat and rigid hydrophobic surface of the pyrrolidine ring of the proline (Fig. 13B, Hagerman and Butler 1981; Haslam 1998). Proline acts as a binding site but the high proline content also extends the protein structure and thus larger surface is available for the tannin (Baxter et al. 1997). It has been proposed that besides providing favorable binding sites, multiple repeated regions rich in proline make the structure of the protein more flexible. This in turn, enables the protein to fold and wrap around the polyphenol, and the multiple intermolecular interaction increases the association (Charlton et al. 1996; Santos-Buelga and de Freitas 2009).

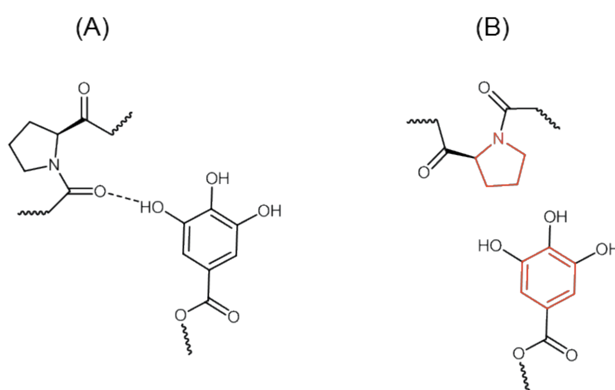

Figure 13. Hydrogen bonding of a galloyl group with the tertiary carbonyl group of a prolyl residue (A). Hydrophobic interactions may occur between the galloyl ring and the open, flat and rigid surface of the pyrrolidine ring (B) (modified from Haslam 1996).

In addition to the tannin and protein structures, the physicochemical conditions affect both the type of binding and the availability of both the tannin and the protein interacting with each other. These include the pH (Appel 1993; Hagerman et al. 1998; Bennick 2002; Li and Gu 2010; Adamczyk et al. 2012), the concentrations of both the protein and the tannin (Hagerman 1998; Frazier et al. 2006; Deaville 2007; Cala et al. 2010; Gonçalves et al. 2011), the solvent composition (Serafini et al. 1997; Hagerman et al. 1998; Pascal et al. 2007; Li

and Gu 2011), other ions and molecules present in the reaction mixture (Luck et al. 1994; Haslam 1998; de Freitas et al. 2003; Carvalho et al. 2006; Gonçalves et al. 2011; Chen et al. 2011), the ionic strength of the reaction solution (Luck et al. 1994, Kawamoto and Nakatsubo 1997; Carvalho et al. 2006) and the temperature (Haslam 1996; Kawamoto and Nakatsubo 1997; Hagerman et al. 1998; Charlton et al. 2002; Carvalho et al. 2006; Hofmann et al. 2006).

2.3.3. Analytical techniques for studying tannin–protein interactions

Numerous physicochemical methods have been developed over many years to study the tannin–protein interactions; aggregate formation, protein precipitation, binding affinities, stoichiometry of binding, conformational changes and kinetics, for example. These methods include electrophoretic methods, isothermal titration calorimetry, nephelometry, chromatography, mass spectrometry, NMR, and computational methods. All of these methods give complementary, but different types of information on tannin–protein complexes and the complex formation itself. Here, two commonly used methods for the verification of tannin–protein complexes and for the investigation of the stoichiometry of these complexes, gel electrophoresis and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), are briefly discussed.

All electrophoretic methods are based on the movement of ions in an electrophoretic field. Most often electrophoresis is carried out in a porous support matrix such as polyacrylamide or agarose. During the electrophoretic run, the gel serves as a size-selective sieve; due to the porous structure of the gel, smaller proteins travel more rapidly than larger proteins in the electric field (Barril and Nates 2012). Gel electrophoresis can utilize either so called continuous or discontinuous systems. In the former one, the separation matrix is uniform and yields protein bands that are rather diffuse and not well resolved. The latter one contains a large-pore stacking gel on the top of a small-pore resolving gel. Thus, when the proteins enter the resolving gel, the movement on the gel slows down, and tight protein bands are formed and improved resolution is obtained (Ahmed 2004). The two main gel electrophoresis techniques are native and sodium dodecyl sulfate polyacrylamide gel electrophoresis (native PAGE and SDS-PAGE, respectively). In native PAGE, the protein undergoes migration without denaturation (Shewry et al. 1995) while in SDS-PAGE, the proteins are fully denatured and dissociated from each other and adapt longitudinal, rod-like shapes instead of the usual complex tertiary conformation

(Laemmli 1970; Horie and Kohata 2000; Kusuda et al. 2006). Due to the SDS, SDS-PAGE is not useful method for detecting non-covalent tannin–protein complexes; also these interactions are dissociated by the SDS (Hagerman 2012). The proteins separated by the gel can be detected with various methods, staining being probably the most common technique. For detection of tannin–protein complexes, Coomassie blue and nitro tetrazolium blue staining techniques are often used (Paz et al. 1991; Gravel and Golaz 1996; Hagerman 2012).

MALDI-MS was first introduced by Karas and Hillenkamp (Karas and Hillenkamp 1988) and it has become a commonly used tool for the analysis of proteins, peptides and other biomolecules (Niesse and Falck 2015). MALDI is an ionization technique where the analyte is first co-crystallized with a matrix compound or mixture, such as sinapinic acid, having a strong absorption at the laser wavelength (de Hoffman and Stroobant 2008). The matrix has two important functions: (1) it heats rapidly when radiated with the laser and is vaporized together with the sample; (2) it protects the sample from being destroyed by the laser (Marvin et al. 2003). The three different mass analyzers used together with the MALDI source are a linear time-of-flight (TOF), a TOF reflectron, and a Fourier transform mass analyzer (de Hoffman and Stroobant 2008). In MALDI, ions are produced in a pulsed, non-continuous manner, and therefore it is well suited for the TOF analyzer (Marvin et al. 2003). Accordingly, TOF is the most commonly used mass analyzer for MALDI (Marvin et al. 2003; Hoffman and Stroobant 2008).

In TOF analysis, ions with constant kinetic energy are accelerated to a detector. The ions have the same kinetic energy, but ions with different mass have different velocities and thus they arrive to the detector at different times (Guilhaus 1996; Gross 2011). As the velocity is inversely proportional to the square root of m/z, the ions enter to the detector in order of increasing mass (Guilhaus 1996). Due to soft ionization, single charged ions and a broad mass range, the MALDI-TOF mass spectrometer is a powerful tool for analyzing tannin–protein complexes (Mané et al. 2007). Further advantage is achieved by the singly charged ions, which makes the interpretation of the tannin–protein complex spectra relatively simple (Chen and Hagerman 2004; Mané et al. 2007). However, the resulting data in MALDI-TOF depends highly on the choice of a suitable matrix and the solvents, as well as the analyte properties, sample purity, and sample preparation (Marvin et al. 2003; Hoffman and

Stroobant 2008). Also, MALDI suffers from some disadvantages such as low sample-to-sample and shot-to-shot reproducibility (Gusev et al. 1995). In addition, the signal level strongly depends on the laser beam homogeneity and irradiance, sample preparation (primarily the crystallization process) and substrate surface conditions (Gusev et al. 1995; Hoffman and Stroobant 2008).

2.4. Anthelmintic properties of plant tannins

2.4.1. Gastrointestinal nematodes of ruminants

Although there are many different species of nematode parasites that infect ruminant livestock, gastrointestinal nematode (GIN) infections represent the greatest threat for livestock production worldwide by causing numerous infectious diseases on their hosts (Sutherland and Scott 2010; Charlier et al. 2014; Preston et al. 2014). The most relevant GINs of ruminants are those belonging to the order of *Strongylida*, superfamily *Trichostrongyloidea* (Balic et al. 2000; Zajac 2006; Sutherland and Scott 2010). Among them, *Haemonchus contortus*, also known as the barber's pole worm, (Fig. 14A) is one of the species with greatest pathologic and economic importance (Whittier et al. 2009; Sutherland and Scott 2010; Gilleard 2013). *H. contortus* is a blood feeding abomasal parasite of sheep and goats but it has been found also in other ruminant species such as cattle and reindeer (Achi et al. 2003; Hrabok et al. 2006). An adult *H. contortus* is 2–3 cm in length and can easily be observed by eye on the abomasal content during post-mortem examination (Fig 14B). It is one of the most rapidly reproducing GINs and individual females can produce thousands of eggs per day (Loukas et al. 2005; Sutherland and Scott 2010; Roeber et al. 2013). In optimal conditions, this can lead to rapid contamination of the pasture with the larvae (Vlassoff et al. 2001). The fact that sheep are pack animals and naturally tend to graze close to each other and only on certain areas of the grass, makes them very susceptible to the larvae (Whittier et al. 2009; Roeber et al. 2013). The main pathogenic effects are caused by the L4 stage larvae and adult nematodes, which both feed on blood. As a consequence, the animal loses large quantities of blood and protein, which further causes general weakness and anemia (Fig 14C). A rapid infestation with the parasite may lead to sudden death of the animal due to excessive blood loss, even if they appear to be healthy and in good body condition. In case of a slower build-up of the

infection, the animals lose weight, suffer from anemia and their wool becomes brittle and may fall out (Zajac et al. 2006; Whittier et al. 2009; Roeber et al. 2013). The low protein levels can cause also a condition known as “bottle jaw”, in which fluid accumulates under the skin of the lower jaw (Fig. 14D, Whittier et al. 2009). Unlike with many other GINs, infection with *H. contortus* rarely results in diarrhea (Fig 14E) and therefore its effects are often not detected by routine observation (Zajac 2006). Thus, by the time symptoms appear, the larvae have already caused significant damage to the animal, and prompt action is necessary to prevent further consequences (Zajac 2006; Whittier et al. 2009).

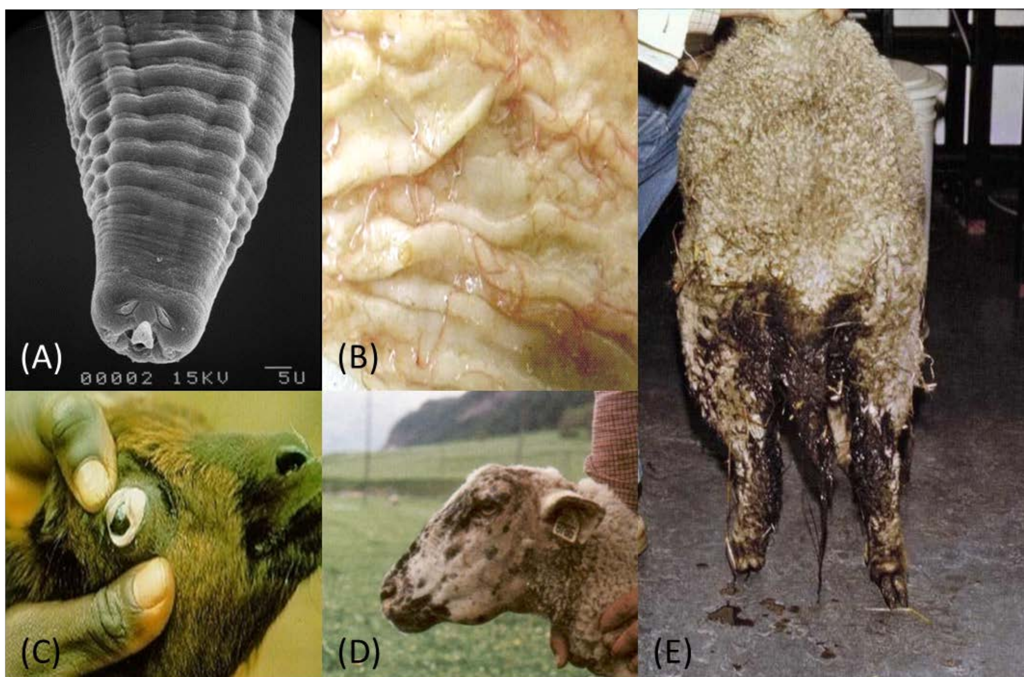

Figure 14. *Haemonchus contortus* nematode by scanning electron microscopy (A) and a direct view of a sheep abomasum full of *H. contortus* nematodes (B). Severe infestations with *H. contortus* cause anemia, which can be diagnosed e.g. from pale eyelids (C). The loss of protein may cause the condition called “bottle jaw“, where fluid accumulates under the skin of the lower jaw (D). Unlike many other gastrointestinal nematodes, infection with *H. contortus* rarely results in severe diarrhea (E). The images were kindly provided by Dr. H. Hoste.

Most GINs belonging to the order *Strongylida* share the same life-cycle (Zajac 2006). The life cycle of *H. contortus* was described in detail as early as 1915 (Veglia 1915). Each of the different larval stages includes two phases of development. The first phase is a period of activity during which the larva feeds and grows. The second phase is a period of inactivity, and this is when the structure of the larva changes substantially, resulting in the removal of the old cuticle through exsheathment (Nikolaou and Gasser 2006). The time required and the degree of growth in each developmental stage vary and are dependent on both the environment and the host (Rossanigo and Gruner 1996). The main stages of the life-cycle of *H. contortus* are illustrated in Figure 15. The cycle begins when the adult female nematode lays eggs in the abomasum of the ruminant. The development of the eggs requires oxygen and thus they are passed out in the feces. In the moist conditions of the feces, the eggs develop, hatch and continue to develop into L1 and L2 juvenile stages by feeding on bacteria in the dung. The L2 stage larva sheds its cuticle (exsheathment) and develops into a non-feeding L3 infective larva which then migrates to the top of a blade of grass to maximize its chances of meeting a host (Loukas et al. 2005). The ruminants become infected when they graze and ingest the L3 stage larvae which pass through the first three stomachs to the abomasum. There, the L3 larva exsheaths the protective cuticle and burrows into the internal layer of the abomasum, where it develops into an L4 preadult larva. Finally, the L4 larva develops into an L5 adult nematode via exsheathment (Loukas et al. 2005; Roeber et al. 2013). The adult nematodes, both male and female, live and mate in the abomasum where they feed on blood (Vlassoff et al. 2001; Nikolaou and Gasser 2006; Whittier et al. 2009).

The L3 larva may also stay in the abomasum and go into hypobiosis, a state of arrested development that occurs when the conditions do not favor completion of the life cycle (Gibbs 1982). This may occur e.g. during the winter months and the development into L4 and L5 stages continues in the early spring (Balic et al. 2000; Zajac 2006) as a response to changed conditions such as rising temperature and humidity (Gibbs 1982; Zajac 2006; Roeber et al. 2013). Also the immune system of the animal has a significant effect; e.g. relaxation of the immune system of periparturient animal (around the time of giving birth) makes them especially susceptible to nematode infections (Gibbs 1982; Zajac 2006). This phenomenon, called the periparturient egg rise, further exposes the vulnerable young lambs to highly infected pastures (Zajac 2006).

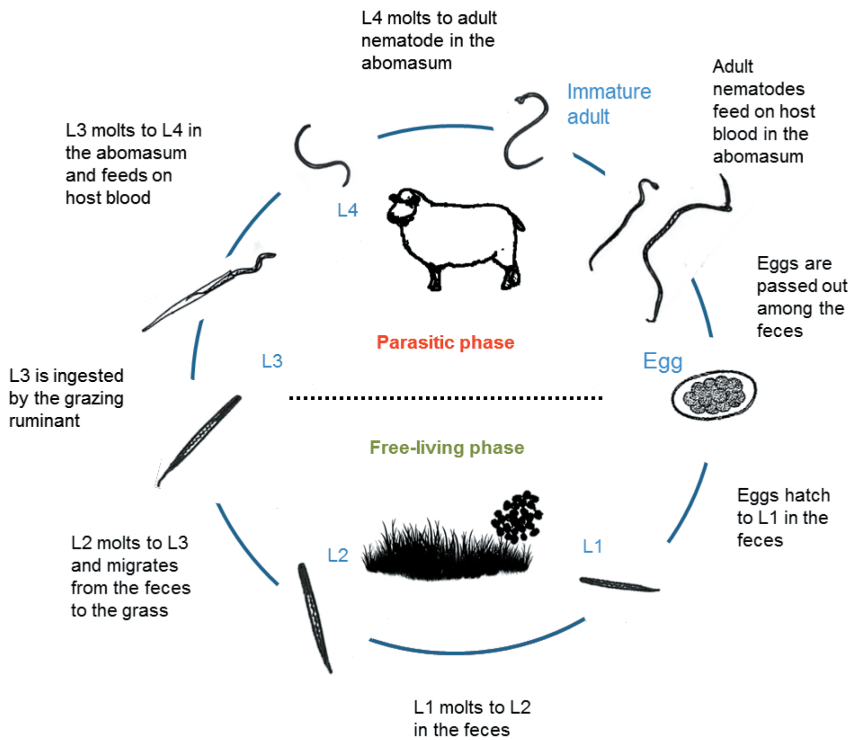

Figure 15. The life-cycle of *Haemonchus contortus*.

The control of GINs has relied for decades mainly on the use of synthetic anthelmintic drugs. One reason for this is the achievements on developing new anthelmintic drugs between the 1960s and 1990s (Kaplan 2004). At the same time, resistance of GINs against the synthetic anthelmintics started to emerge. The first reports of anthelmintic resistance are from the late 1950s and early 1960s with the first species of GIN with resistance formation being identified as *H. contortus* (Drudge et al. 1957; Conway 1964). Since then, the intensive use of anthelmintics for treating and controlling GINs has caused a global spread of parasite populations that are resistant to one or more classes of anthelmintics (Zajac et al. 2006; Ihler 2010; Kaplan and Vidyashankar 2014; Traversa and von Samson-Himmelstjerna 2015). Furthermore, multiple-resistant GINs are already highly prevalent in many parts of the world (Taylor et al. 2009; Gilleard 2013; McRae et al. 2015) and even situations in which the nematodes have developed resistance to all anthelmintic drugs available have been reported

(Cezar et al. 2010; Howell et al. 2008). Adding to this the growing concern of possible drug residues in food products as well as the environmental consequences (Beynon 2012) and the increasing national and international restrictions in the use of chemical anthelmintics (Kaplan 2013), it is clear that there is an urgent need for alternative solutions to control ruminant parasites. One promising approach is the utilization of bioactive plants as natural anthelmintics to at least partially replace the use of synthetic chemical drugs (Githiori et al. 2006; Hoste et al. 2015).

2.4.2. Methods for studying the antiparasitic effects of plant tannins

The methods used for testing the antiparasitic effects of plants and plant tannins include both *in vivo* and *in vitro* methods. The selection of the approach depends largely on the objective of the study. In more general studies, where the effect of certain plants is tested, the most realistic approach is to screen them *in vivo* by feeding infected ruminants with the plant. The reason for this is that the conditions change throughout the gastrointestinal track of the ruminant and it is impossible to accurately mimic these conditions *in vitro*. Thus, the true effect of plants on ruminants infected with nematodes can be obtained only *in vivo*. However, when studying the anthelmintic effects of large number of plants, or even more so, when utilizing purified tannin fractions or single compounds in order to learn about the tannin/nematode viability structure-activity relationship, it would not be feasible or even possible to test them all with animals. In such cases, several *in vitro* techniques are available for the primary screening (Table 3). With these methods, the effects of plant tannins can be tested on the different key stages of the GIN life cycle (eggs, infective larvae and adults). All the methods, except the larval exsheathment inhibition assay, have been adapted from the methods used to test synthetic anthelmintic drugs against ruminant GINs (Wood et al. 1995, Bahuaud et al. 2006; Jackson and Hoste 2010). Often, the more simple assays such as the egg hatch, larval migration inhibition and larval feeding inhibition assays are used for the first screening and the more time consuming and/or expensive assays such as the larval exsheathment, larval development and adult motility assays are used with the products for which some preliminary activity has been witnessed (Jackson and Hoste 2010). However, due to the difficulties in maintaining the complex life-cycles of parasitic nematodes, they are not ideal laboratory specimens (Holden-Dye and Walker 2007; Gilleard 2013). Furthermore, the tests with e.g.

adult *H. contortus* involve the killing and dissecting of an animal host to provide adults for the experiments. Therefore, more user-friendly model systems have been developed. One such a model system is *Caenorhabditis elegans*, a free living soil nematode that is often used as a model for veterinary parasites (Geary and Thompson 2001; Holden-Dye and Walker 2007), when screening the anthelmintic properties of different plant resources (Mori et al. 2000; Yamasaki et al. 2002; Katiki et al., 2011, 2013). The use of free-living nematodes as models for parasitic nematodes can be justified as the separation of nematodes into free-living and parasitic has been suggested to remain less relevant in comparison to how much the species have biologically in common (Rochfort et al. 2008). However, when using *C. elegans* as a model in parasitology research, significant emphasis should be made on detailed comparative analysis and careful experimental interpretation (Gilleard 2004).

Table 3. *In vitro* bioassays used in testing plant extracts for their anthelmintic activities (adapted from Jackson and Hoste 2010).

Bioassay	**Target stage**	**Process disrupted**
Egg Hatch Assay	Eggs	Hatching to L1 stage larva
Larval Migration Inhibition	L3	Locomotion of L3 stage larva
Larval Feeding Inhibition	L1	Feeding of L1 stage larva
Larval Exsheathment	L3	Exsheathment of L3 stage larva
Larval Development	Eggs→ L1	Development to L3 larva
Adult Motility	L5/adults	Motility of adult nematodes

One often utilized tool for studying the anthelmintic effect of plants is electron microscopy (Martínez-Ortíz-de-Montellano et al. 2013; Williams et al. 2014a; Peña-Espinoza et al. 2015). The two basic types of electron microscopy are scanning electron microscopy (SEM) and transmission electron microscopy (TEM). SEM is used for the high-resolution imaging of surfaces (Fig. 16A,B). In SEM, a beam of electrons with defined incident energy, usually between 1–40 keV, is generated in an electron column (Goldstein et a. 1992). The electrons produced are focused into a smaller beam by electromagnetic lenses, and scanning coils are used to direct and position the electron beam onto the sample surface (Egerton 2005). To produce a contrasting image, the electron beam is

scanned in a raster pattern over the surface of the sample. The actual image is generated when the emitted low energy electrons, i.e. secondary electrons, are detected with an electron detector and transferred into an image (Goldstein 1995; Egerton 2005). Depending on the instrument used, the object can be magnified roughly between 10 and 100,000 times (Goldstein 1992; Stadtländer 2007). In contrast to SEM, in TEM the electrons are projected through an ultrathin slice of the sample which produces a two-dimensional image (Egerton 2005, Fig. 16C,D). Thus, TEM gives information of cellular structures of the specimen (Stadtländer 2007). In TEM, magnifications between 500 to 500,000 times can be achieved. However, magnifications above 200,000 are rarely used in biology (Standländer 2007). Both SEM and TEM are operated under vacuum to enable the electron beam to travel in straight lines (Goldstein et al. 1992; Standländer 2007). The electron microscopes operate under the guidance of computers which makes the sample analysis rather straightforward and the main difficulty remaining is the sample preparation (Standländer 2007). SEM sample preparation includes cleaning of the surface, stabilization of the sample with a fixative, rinsing, dehydrating and drying the sample, mounting the specimen on a metal holder, and coating the sample with a layer of a material that is electrically conductive (Standländer 2007). Each preparation step is critical regarding the quality of the produced images (Goldstein et al. 1992; Egerton 2005; Standländer 2007).

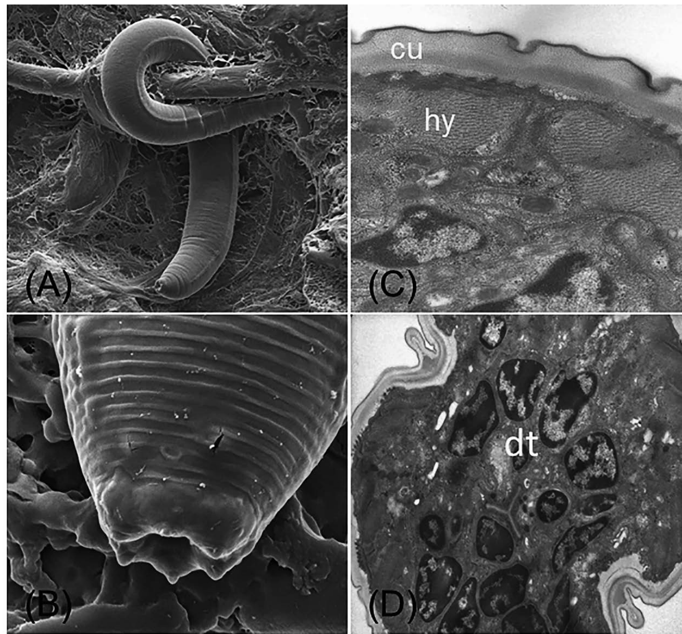

Figure 16. Scanning electron microscopy images of *Haemonchus contortus* L2 stage larva (A, B) and transmission electron microscopy images of cross-section of *H. contortus* L3 stage larva body (C, D). Panels C and D: "cu" indicates cuticle, "hy" indicates hypodermis and "dt" indicates digestive tract. The transmission electron microscopy images were kindly provided by Dr. H. Hoste.

2.4.3. Effects of tannins on the digestive parasites of ruminants

Plants and plant extracts have been used for centuries as de-wormers for both humans and livestock and a wide range of plants and their products around the world have been explored as alternative choices to synthetic anthelmintic drugs. A literature search with SciFinder Scholar® using research topic "plant" refined with "anthelmintic" resulted in 1942 references (dated 22.01.2016). The first reference available was a patent from 1868, an improved *vermifuge* against worms prepared of several plant materials (wormseed, aloes and catgut) (McKinsey 1868). Since then, numerous studies have been conducted to test the anti-nematode of different plants and plant materials, with the maximum number of papers published on the subject between 2011 and 2012 (Fig. 17).

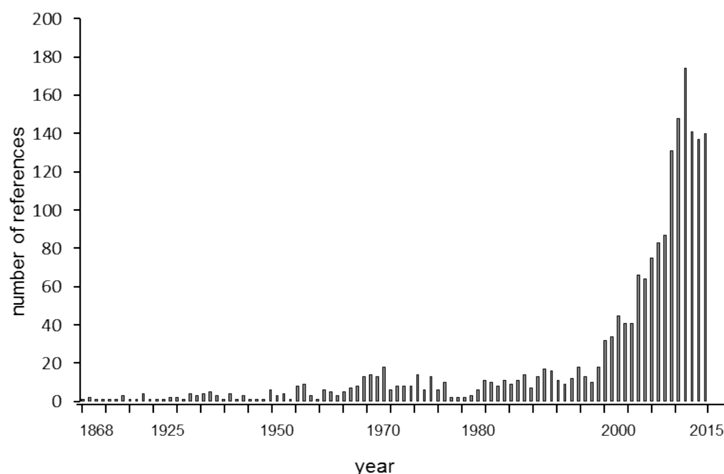

Figure 17. The number of references published per year obtained with SciFinder Scholar®, using "plant" as research topic and the results refined with "anthelmintic".

Several reviews exist describing the anthelmintic properties of different plant species on ruminant parasites (Rochfort et al. 2008; Mali and Mehta 2008; Manke et al. 2015). Unfortunately, most of these studies contain insufficient or no information on the chemical composition of the studied plants which makes the characterization of the active compounds impossible. Hoste et al. 2015 have suggested three main potential impacts on the GIN life cycle associated with the intake of tannin containing plants: 1) decrease in the establishment of the infective L3 stage larvae; 2) decrease in the excretion of nematode eggs by adult worms; and 3) reduction in the development of the nematode eggs into the third-stage larvae. In addition, partial paralysis and interference with the neurophysiology or neuromuscular coordination of the larvae have been witnessed (Molan et al. 2000; 2004).

To explain the mechanism by which plants act against GINs and to understand what types of compounds are the most active ones by this mechanism of action, pure compounds must be utilized in the activity tests. The better understanding on the structure−activity relationships between plant tannins and their anthelmintic properties would enable to select plant species with preferred tannin composition to be used as ruminant feeds or feed additives for the control of GINs. Hypothetically, this knowledge could be used to breed new plant varieties with enhanced tannin composition. Unfortunately, because of the difficulty of purifying individual PAs from plants, only few studies have

addressed the question about the effect of tannin structure on the anthelmintic effect. Molan et al. (2003) were the first ones to report that fodder species with higher PD/PC ratios resulted in more consistent anthelmintic activities than the species with lower PD/PC ratios. *In vitro* studies with the monomeric building blocks of PAs further supported this and it was shown that gallocatechins (PD building blocks) were usually more potent anthelmintic compounds than catechins (PC building blocks) (Molan et al. 2003; Brunet and Hoste 2006; Brunet et al. 2008; Williams et al. 2014b). Moreover, a comparison of the effect of galloylated versus non-galloylated PA building blocks has shown that the galloylation of flavan-3-ols results in higher activity against the different developmental stages of the nematodes (Molan et al. 2003; 2004; Brunet and Hoste 2006). Studies with the PA monomeric units have shown no or a small differences on the anthelmintic effect of the 2,3-*cis* versus 2,3-*trans* stereochemistry of the PA monomeric units (Molan et al. 2003; Brunet and Hoste 2006; Williams et al. 2014b). However, in a study where the effect of dimeric PCs B1 and B3 on *C. elegans* was compared, the dimer with 2,3-*trans*, 3,4-*trans* combination reduced motility more efficiently than the dimer with 2,3-*cis*, 3,4-*trans* combination (Mohamed et al. 2000). The effect of the PA size has been studied mostly with characterized PA fractions, and the results from these studies indicate that increase in mean degree of polymerization results in greater anthelmintic activity (Mohamed et al. 2000; Yamasaki et al. 2002; Williams et al 2014a, b; Klongsiriwet et al. 2015; Quijada et al. 2015; Desrues et al. 2016).

The other group of tannins found in terrestrial plants, HTs, has gained less attention, presumably due to their smaller presence at leguminous plants compared to PAs and the holding belief of HTs as toxic compounds (Patra and Saxena 2013). In reality, both PAs and HTs may reduce feed intake, feed digestibility or even cause toxicity if consumed in large quantities, but neither is toxic when consumed in moderation (Katiki et al. 2013). Several screening studies have been conducted with plants known to be rich in HTs (Chandrawathani et al. 2006; Mukai et al. 2008; Manolaraki et al 2010; Waterman et al. 2010) but no unambiguous evidence on the relationship between the HTs and the anti-parasitic properties of these plants have been established. However, the few studies with focus on HTs have indicated potential anthelmintic activity (König et al. 1994; Mori et al. 2000; Mohamed et al. 2000; Yamasaki et al. 2002; Katiki et al. 2011, 2013). Results in Katiki et al. (2013) suggested that both HT-rich and PA-rich plant extracts possess

anthelmintic activity but plant extracts rich in ETs were most lethal to soil nematode *C. elegans*. Further, König et al. (1994) showed that an ET rich fraction from *Quercus petraea* (sessile oak) bark inhibited the reproduction of the soil nematode *C. elegans* with a 50% lethal concentration (LC 50) of 500 µg mL^{-1}. Under the same conditions, the LC 50s for the synthetic anthelmintic mebendazole and a PA rich fraction from *Q. petraeu* were 10 µgmL^{-1} and 125 µg mL^{-1}, respectively (König et al. 1994). Unfortunately, these studies do not enable direct comparisons between the activities of different types of tannins as the extracts and fractions were not analyzed for a detailed tannin composition.

Thus far, the most comprehensive work on the effect of HT structures on their anthelmintic effect was done by Mori et al. (2000), Mohamed et al. (2000) and Yamasaki et al. (2002). Ironically enough, the objective of these studies were to provide evidence that tannins are essentially toxic for animals by exploring the effect of mono- to hexagalloylglucoses, a few ETs and a series of PAs on the motility and mortality of *C. elegans*. The results showed a correlation between the tannin size and the anthelmintic activity of galloylglucoses and PAs. A 1 mg mL^{-1} solution of mono- and digalloylglucoses had no or very little effect on either the motility or the mortality during a 72 hour incubation whereas trigalloylglucose solution with the same concentration significantly affected the motility after 42 hour incubation and the mortality after 72 hour incubation (Mohamed et al. 2000). The larger galloylglucoses, tetra- to hexagalloylglucoses were markedly more active; shorter incubation times resulted in decreased motility and increased mortality, and after 72 hours 100% mortality was observed (Mohamed et al. 2000). Regarding PAs, PC dimers and trimers decreased the motility in some extent but showed no lethal activity. Even for the PC tetramer, only a low mortality rate was observed while the motility steadily decreased to 60%. On the contrary, the polymeric PAs were significantly active and 72 hour incubation resulted in almost 100% mortality (Mohamed et al. 2000). However, the average molecular weight of the ETs did not correlate with the observed reduction in the motility or the increased mortality. Unfortunately, only two of the ten ET structures were confirmed, so no conclusion could be made on the effect of structural features other than molecular weight. In these studies, the LC 50 values for five day incubations obtained for pentagalloylglucose, tellimagrandin I, rugosin A methyl ester, the largest PA polymer (the average MW 4530) and ten unidentified ETs were 15 µgmL^{-1}, 88 µg mL^{-1}, 26 µg mL^{-1}, 5 µg mL^{-1}, and 26–83 µg mL^{-1}, respectively (Yamasaki et al. 2002). All in all, these studies indicated that both HTs and PAs possess *in vitro* anthelmintic activity.

Thus, in addition to PA-rich forages, the utilization of HT-containing forages to control ruminant GINs should be a fertile area for future research.

The mode of action behind the anthelmintic effect of tannins has been discussed, but is still largely unknown. Two main hypotheses, the direct and the indirect modes of action, could explain the effect of tannins against ruminant GINs. The direct mode of action presumes that tannins affect the biology of the nematode by a drug-like effect and thereby causes a dysfunction of the nematode (Hoste et al. 2012). A generally accepted hypothesis is that the direct effect of plant tannins is based on their interactions with the egg and larvae proteins vital for the development and biological functions of the larvae (Athanasiadou et al. 2001; Molan et al. 2010; Molan 2014). This direct hypothesis is supported by the results obtained from *in vitro* studies and also, some *in vivo* studies have suggested the direct effects of tannins against GINs (Athanasiadou et al. 2001, 2005; Hoste et al. 2006; Brunet et al. 2008). These functional modifications have been further supported by scanning and transmission electron microscopy experiments, in which major changes in the larval structure e.g. the cuticle, the digestive tract and the female reproductive tract have been observed (Hoste et al. 2006; Martinez-Ortiz-de-Montellano et al. 2013; Williams et al. 2014a; Yoshihara et al. 2015).

Alternatively, tannins may act indirectly by improving the response of the host immune system against the GINs. This can take place, for example, as a consequence of increased protein availability (Anthanasiadou et al. 2001). Because of the protein binding ability, tannins protect proteins from the microbial degradation in the rumen, thus making the non-degraded proteins available for digestion and absorption in the small intestine where the complexes are readily dissociated due to the increased pH (Frutos et al. 2004; Mueller-Harvey 2006). As a consequence, the resilience and resistance of the animal to parasites is enhanced indirectly via improved protein nutrition (Coop and Kyriazakis 2001). The tannins could also complex with the bacteria and nutrients that the non-parasitic larval stages feed on, thus inhibiting their availability and causing larval starvation and ultimately death (Anthanasiadou et al. 2001; Iqbal et al. 2007). Despite the numerous studies, the exact mode of action has remained obscure. However, it seems undisputed that it differs depending on the parasite, the stage of development and also the characteristics of the plant and the tannins causing the observed effects.

3. AIMS OF THE STUDY

The focus of this PhD-research was on development of an understanding of tannin bioactivity and their structure–activity relationships by investigating the chemical properties of those plant tannins that have greatest anthelmintic effects on ruminant parasites and to study the possible mechanisms of action between plant tannins and proteins on a molecular level. An additional aim of this research was to develop analytical methods for analysis of these bioactive tannins in plants extracts. The main topics under investigation were:

1. development of UHPLC-MS/MS methods for the qualitative and quantitative analysis of plant polyphenols, especially tannins (**I** and **II**);
2. establishment of a fast, sensitive and selective method for fingerprint analysis of the two most common proanthocyanidin types, procyanidins and prodelphinidins, including their ratios and mean degree of polymerization (**I**):
3. understanding the bioactivity of plant tannins (**III** and **IV**);
4. the effect of pH, tannin structure and tannin oxidative activity on the formation of tannin–protein complexes (**III**);
5. the effect of tannin structure on the in vitro anthelmintic activity (**IV**); and
6. the possible mode of action of plant tannins as anthelmintic substances (**IV**)

4. MATERIALS AND METHODS

4.1. Plant material, extraction and isolation

The plant materials (Fig. 18) used for the isolation of the studied HTs in articles **I-IV** were collected during the summer 2010 and 2011 from south-western Finland, including willowherb flowers (*Epilobium angustifolium*), silverweed leaves (*Potentilla anserina*), herb bennet leaves (*Geum urbanum*), English oak acorns (*Quercus robur*), purple loosestrife leaves and flowers (*Lythrum salicaria*), meadowsweet flowers (*Filipendula ulmaria*), raspberry leaves (*Rubus idaeus*), wood cranesbill leaves (*Geranium sylvaticum*), rose leaves (*Rosa rugosa*), and burnet rose leaves (*Rosa pimpinellifolia*). Sea buckthorn (*Hippophae rhamnoides*) and white birch (*Betula pubescens*) material was the same as used in Moilanen et al. (2015) and Salminen et al. (2002), respectively.

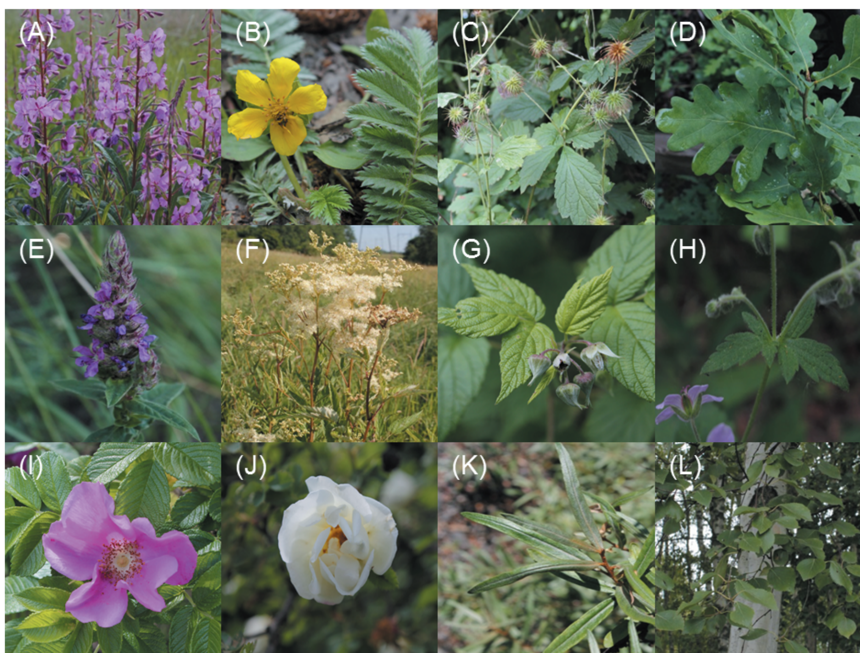

Figure 18. Plants sources utilized in compound purification: *Epilobium angustifolium* (A), *Potentilla anserina* (B), *Geum urbanum* (C), *Quercus robur* (D), *Lythrum salicaria* (E), *Filipendula ulmaria* (F), *Rubus idaeus* (G), *Geranium sylvaticum* (H), *Rosa rugosa* (I), *Rosa pimpinellifolia* (J), *Hippophae rhamnoides* (K), *Betula pubescens* (L). Images UTU Natural Chemistry Research Group.

The plant material was collected directly into 1 L glass bottles filled with pure acetone. Glass bottles were left to extract at 4˚C for 15 days. For the oak acorns, the shells were first removed and the nuts were crushed using a hand craft blender before addition of the acetone/water (4:1 v/v). In article **III**, after maceration, the extract was filtered, the acetone was evaporated and the remaining aqueous solution was frozen and lyophilized. In article **IV**, the extraction was further continued after 15 days of maceration by replacing the extract with 800 mL fresh 70% aqueous acetone and the plant material was crushed to fine grit with a high performance dispenser (IKA® VWR VDI 25 adaptable homogenizer, VWR, Illinois, US) and mixed with a reciprocating shaker for 48 hours. The extraction was repeated three times. Each time the extraction solution was changed, the samples were vacuum filtered and, after adding the new solvent, the samples were mixed with the high performance dispenser. The filtered samples were evaporated to remove the acetone, and the water-phases were extracted three times with ethyl acetate and re-evaporated to yield aqueous solutions. The water phases of all extracts were mixed with a slurry of Sephadex LH-20 material (in 100% water) and eluted with water, methanol/water (1:1 v/v), methanol, acetone/water (4:1 v/v) and acetone in a Büchner funnel (Ø = 240 mm) *in vacuo*. The organic solvents were evaporated from the fractions, and the remaining aqueous solution was frozen and lyophilized.

The fractionation was further continued with Sephadex LH-20 column chromatography. The samples were dissolved in 15 mL of ultrapure water, filtered (0.45 µm, PTFE) and applied on top of Sephadex LH-20 gel loaded into a glass column (40 × 4.8 cm i.d., Kimble-Chase Kontes™ Chromaflex™) and equilibrated with ultrapure water. The eluent profile depended on the HT to be isolated; the solvents used were ultrapure water, aqueous methanol, and aqueous acetone. Fractions were analyzed by UHPLC-DAD-MS (section 4.2.), concentrated to the water phase and lyophilized. Selected Sephadex fractions were further purified by preparative liquid chromatography.

The LC-DAD system used in the preparative and semipreparative LC consisted of a Waters Delta 600 liquid chromatograph, a Waters 600 Controller, a Waters 2998 Photodiode Array Detector and a Waters Fraction Collector III. In preparative LC, the column was manually filled with LiChroprep RP-18 (40-63 µm) material (Merck KGaA, Darmstadt, Germany) and a binary solvent system with methanol (A) and 1% aqueous formic acid (B) at a constant flow rate of 8 mL min^{-1} was used. The elution protocol depended on the composition

of the fractions; a typical gradient was as follows: 0–5 min, 100% B; 5–180 min, 0–40% A in B; 180–220 min, 40–60% A in B; 220–240 min, 60–80% A in B. The final purification of HTs was performed by semipreparative LC with a Gemini C18 column (150 × 21.2 mm, 10 µm, Phenomenex) and the eluents were acetonitrile (A) and 0.1% aqueous formic acid (B). Different gradients were used for different HTs; for example, a typical gradient for acyclic ETs was as follows: 0–5 min, 2% A in B; 5–51 min, 2–32% A in B; 51–55 min, 32–70% A in B. All steps in the preparative and semipreparative purifications were followed by UHPLC-DAD-MS (section 4.2).

4.2. UHPLC-MS/MS analysis

Sample analysis in articles **I-IV** was carried out with an Acquity UPLC system (Waters Corporation, Milford, MA, USA) coupled with a Xevo TQ triple quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA). The UPLC system consisted of a sample manager, a binary solvent manager, a column and a diode array detector. The column used was a 100 mm × 2.1 mm i.d., 1.7 µm, Acquity UPLC BEH Phenyl column (Waters Corporation, Wexford, Ireland). The flow rate of the eluent was 0.5 mL min^{-1}. The elution profile used two solvents, acetonitrile (A) and 0.1% aqueous formic acid (B): 0–0.5 min, 0.1% A in B; 0.5–5.0 min, 0.1–30% A in B (linear gradient); 5.0–5.1 min, 30–90% A in B (linear gradient); 5.1–8.5 min, column wash and stabilization. UV and MS data were collected from 0-6 min. Negative ionization mode was used for MS analyses. ESI conditions were: capillary voltage 2.4 kV, desolvation temperature 650 °C, source temperature 150 °C, desolvation and cone gas (N2) 1000 and 100 L h^{-1}, respectively and the collision gas was argon. Catechin (1 µg mL^{-1}) was used as a system standard to monitor the stability of the ionization efficiency of the mass spectrometer. All samples were filtered with a syringe filter (4 mm, 0.2 µm PTFE, Thermo Fisher Scientific Inc., Waltham, USA) prior to the UHPLC–MS analyses.

The optimization of cone voltages and collision energies in articles **I** and **II** was done by infusing aqueous solutions of different compounds and fractions directly into the ESI source with a syringe pump at a flow rate of 5−40 µL min^{-1}. For pure compounds, the concentrations of the aqueous solutions were 40 µg mL^{-1} and for the fractions 2 mg mL^{-1}. The range of cone voltage and collision energy varied between 10−170 V and 5−50 eV, respectively.

4.3. MALDI-TOF-MS analysis

In article **III**, the stability of tannin–protein complexes were studied by MALDI-TOF-MS. Tannin and protein were mixed in molar ratio 7:1 (polyphenol:protein) in pH 5 McIlvaine (citrate-phosphate) buffer and incubated at room temperature for 1 h. These control samples were analyzed before and after ultrafiltration. Alternatively, pH 7 buffer solution was added to adjust the final pH to 6.7 and the final tannin and protein concentrations to 0.32 and 0.045 mM, respectively. Samples were incubated at 37°C for 90 minutes and analyzed before and after ultrafiltration, which removes unbound or loosely bound polyphenol from the protein (Fig. 19). For ultrafiltration, samples were transferred to filter units (Amicon® Ultra -0.5 Centrifugal Filter Devices 30kDa MWCO) and repetitively washed with 100 mM KCl solution by adding 100 µL KCl solution and filtering the sample to 30 µl (16000x*g*, 5 min, 20 ºC). The final volume of all samples was adjusted to 300 µL with 100 mM KCl before mixing with sinapinic acid (10 mg ml^{-1} in 70% aqueous acetone) in 1:1 ratio (v:v) and spotting 2 µL on the steel target plate. The MALDI experiments were run with a Bruker Reflex III TOF mass spectrometer (Bruker, Billerica, MA) using nitrogen laser (337 nm, Laser Science, Franklin, MA). For ionization, 60−90% of the maximum laser power was used and the detection range was 10–140 kDa. Three replicates for each sample were run. BSA in sinapinic acid was used to calibrate the instrument. The data were handled by Compass DataAnalysis software (version 4.0; Bruker Daltonics).

Figure 19. The ultrafiltration technique is based on the equilibrium between the protein bound and unbound tannin during repetitive centrifugation with the filter unit. T: tannin, P: protein.

4.4. Gel electrophoresis

In article **III**, the effect of pH (pH 5, pH 6.7 and pH 7.6) on the formation of tannin–protein complexes was tested using Laemmli SDS-PAGE and native borate PAGE. Tannin and protein were mixed in molar ratio 7:1 (polyphenol:protein) in pH 5 McIlvaine (citrate-phosphate) buffer. After incubating at room temperature for 1 h, additional buffer (pH 5, 7 or 9) was added to adjust the final pH to 5, 6.7 or 7.6, and the final concentrations of polyphenol and protein to 0.32 mM and 0.045 mM, respectively. The samples were incubated at 37 °C for 90 min.

SDS-PAGE. After complex formation, samples were mixed with sample buffer, held at 100 ºC for 10 minutes and loaded on the gel in 2 µL aliquots. The resolving gel was 10% acrylamide and the stacking gel 3% acrylamide (Laemmli 1970). Gels were run at 160 V and stained with Coomassie blue or with nitroblue tetrazolium (NBT) after semidry electroblotting to transfer the analytes to nitrocellulose. While Coomassie blue reacts with protein, NBT specifically binds to the quinone forms of polyphenols. Markers (Fisher BioReagents, #BP3603500) comprised of 10 recombinant proteins covalently coupled to a blue chromophore plus 10 kDa and 72 kDa reference bands tagged with green and orange dyes, respectively.

Native PAGE. Borate was used to modify 10% native polyacrylamide gels. After complex formation, samples were mixed with sample buffer and loaded to the gel in 2 µL aliquots. Gels were run at 120 mV and stained with Coomassie blue. To analyze the gels, the relative mobilities were calculated based on the 34 kD marker band. The relative mobility for the band representing the tannin-complexed protein was expressed as a % of the relative mobility for the tannin-free control. Three independently prepared samples were run for each reaction condition.

4.5. Egg hatch assay and motility experiments

In article **IV**, the in vitro anthelmintic effect of HTs was tested by egg hatch assay (EHA) and motility experiments. Parasite eggs were freshly obtained from the feces of a donor sheep experimentally infected with *H. contortus*. To separate the eggs from the feces material, a water suspension of feces was filtered through a mesh (150 µm pore size) and transferred into 15 mL centrifuge tubes. The suspension was centrifuged (Heraeus Labofuge 400 R,

2500 rpm, 3 min, 20 °C) and the supernatant replaced with tap water. After repeating this three times, the supernatant was removed, replaced with saturated sugar solution and centrifuged. The eggs were collected from the top of the sugar solution into a 15 mL falcon tube, were washed with phosphate buffered saline solution (PBS) to remove the sugar solution residues, and the egg solution was diluted with PBS to a final concentration of 1000 eggs mL^{-1}. Then, 100 µL of the egg solution was pipetted into a 300 µL well of a 96 well plate together with 100 µL of tested HT solutions. After mixing, the samples were incubated for 48 h at 26 °C (Incucell - V 111, MMM Medcenter Einrichtungen GmbH, Gräfelfing, Germany). Thereafter, a drop of Lugol solution (1 g iodine + 2 g potassium iodide in 50 mL water) was added into each well to kill the larvae and to facilitate microscopic examination (Axio Scope.A1, Carl Zeiss Microscopy, LLC, US). The number of larvae, eggs with larvae inside and eggs with embryo inside was counted per well (Fig. 20). Tested HTs were dissolved in DMSO/PBS (4:96, v/v) and six different tannin concentrations, in four replicates, were tested: 2.0, 1.5, 1.0, 0.5, 0.25 and 0.125 mM. In addition, a positive control (thiabendazole at a concentration of 50 µg mL^{-1} in DMSO/PBS (10:90, v/v)) and a negative control (DMSO/PBS (4:96, v/v)), in five replicates, were included in the assay. The negative control was run for each batch of egg solution to take into account possible differences in the egg quality between different runs and days. As the egg hatching percentage had daily variation, the 0% egg hatching inhibition level was calculated for each batch separately. The percentage of inhibition was determined as follows:

$$\text{Inhibition (\%)} = \frac{A - (B \times \frac{1}{5}\sum_{i=1}^{5}\frac{Ci}{Di})}{B - (B \times \frac{1}{5}\sum_{i=1}^{5}\frac{Ci}{Di})} \times 100\% \qquad \text{(Eq.1)}$$

where A is the number of unhatched eggs; B is the total number of eggs and larvae; C is the number of unhatched eggs in control; D is the total number of eggs and larvae in control; and i refers to replicate (1–5). The final inhibition levels were presented as average egg hatch inhibition values and were calculated as an average from all the individual dose averages.

Figure 20. In egg hatch assay, the number of larvae, eggs with larvae inside and eggs with embryo inside was counted per well after the addition of Lugol solution. In addition, the visibility of empty eggs was evaluated.

The "motility after hatching" test was carried out similarly as in EHA except that approximately 150 eggs were pipetted per well and without adding Lugol solution prior to microscopic examination. After 48 h incubation, the number of motile and non-motile L1 and L2 stage larvae was counted per well. As the motility percentage of the controls varied between the different batches, the 0% motility inhibition level was taken into account in the calculations similarly as in EHA.

4.6. Scanning electron microscopy

Cryo-scanning electron microscopy (cryo-SEM) images were obtained with a FEG FEI Quanta 250 microscope (FEI Company, Eindhoven, Holland). The larvae and eggs obtained from the *in vitro* incubation in control or HT solutions (see section 4.5.) and were fixed with 2% glutaraldehyde in Sørensen buffer (0.1 M, pH = 7.4). The sample was deposited on a filter and the filter was placed to a stub using graphite mounting media. Then the sample was frozen in nitrogen slush at -200 °C and transferred under vacuum to the cryo-chamber apparatus (Quorum PP3000T Cryo Transfer System) at -140 °C. The temperature was then increased to -95 °C and maintained at this temperature during 1 hour for sublimation. The sample was then metalized with Pd (60 s, 10 mA) and introduced into the microscope chamber where it was maintained at -140 °C during the observation, operating at 5 kV accelerating voltage.

5. RESULTS AND DISCUSSION

5.1. Development of UHPLC-MS/MS methods

The developments in LC-MS/MS instruments have enabled utilization of compound-specific MRM methods for the identification and quantitation of single polyphenols. In these methods, MS/MS conditions are optimized separately for each precursor ion/product ion pair. Therefore, their use in screening large amounts of plant samples for their bioactive polyphenol content is laborious and time-consuming. However, since compounds belonging to the same polyphenol group typically share similar functional groups, it has been possible to measure the group-specific fingerprints for each polyphenol subgroup by utilizing parent ion and neutral loss scan modes. For example, galloylglucoses and gallotannins can be detected by the parent ion scan of *m/z* 169 (in negative mode) due to the fragmentation of gallic acid units (170 Da). Unfortunately, due to the scanning technique, each parent ion scan is rather time-consuming, depending on the selected *m/z* range. Thus, if including multiple parent ion scans for the simultaneous detection of several polyphenol sub-groups, the number of data points per single detection remains insufficient. To provide a solution for the current methodological problems in tannin analysis, non- scanning UHPLC-MS/MS methods measuring group-specific fingerprints for the two main tannin subgroups, HTs and PAs, and in addition for quinic acid derivatives, quercetin-, kaempferol- and myricetin-based flavonol glycosides were developed in **I** and **II**.

The MRM methods were optimized for different polyphenol subgroups by direct flow injection experiments with reference compounds and/or representative fractions for each polyphenol subgroup. Negative ion mode was preferred over positive for all polyphenol subgroups studied due to the increased sensitivity, less extensive fragmentation and clearer fragmentation patterns. As hypothesized, fragmentation of the functional groups of different polyphenol subclasses resulted in production of characteristic ions that were selected to serve as precursor ions for the group-specific MRM methods (Fig. 21). For gallic acid derivatives, ellagitannins, quinic acid derivatives, kaempferol-, quercetin- and myricetin-based flavonoids, the *m/z* values of these fragments corresponded to gallic acid, ellagic acid, quinic acid, and kaempferol, quercetin and myricetin aglycones, respectively. However, for PAs four types

of fragments were used due to the quinone methide cleavage; both PC and PD units yielded two different types of ions depending on whether they represented the extension or terminal units of the corresponding PA molecule.

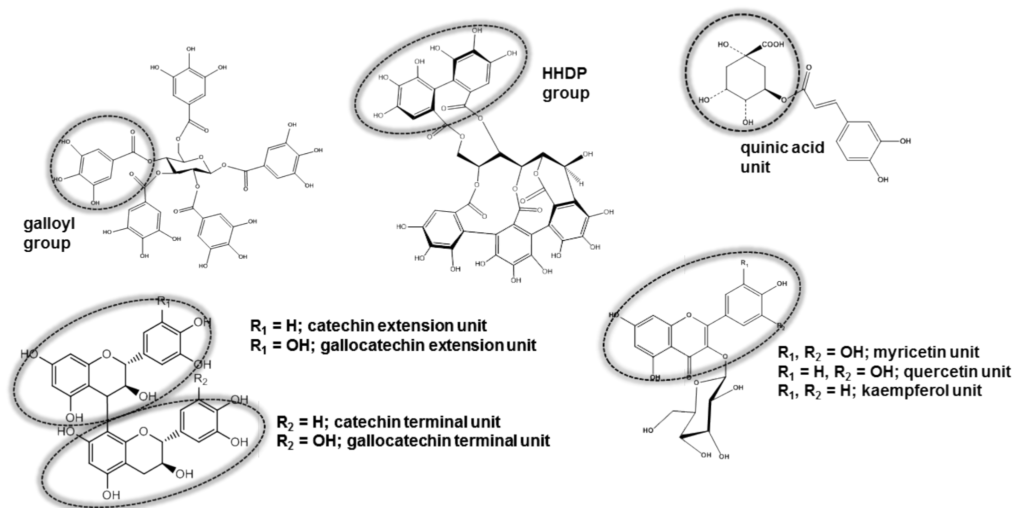

Figure 21. The functional group of each polyphenol subgroup which fragmentation was utilized in the polyphenol group-specific multiple reaction monitoring methods. HHDP, hexahydroxydiphenoyl.

The optimal cone voltage required for the maximal accumulation of the precursor ions of each polyphenol subgroup varied depending on the structural features of the studied molecules: e.g. the position of the functional unit, the number of selected functional units in the polyphenol structure, the size of the molecule and the general rigidness of the structure (see **I** and **II** for details). For example, for the monomer tellimagrandin I and its dimeric and trimeric macrocyclic oligomers, oenothein B and oenothein A, the optimal cone voltages for the accumulation of the selected precursor ion, *m/z* 301, were 90 V, 100 V and 130 V, respectively. Similarly, larger PAs required considerably higher cone voltages for the maximal accumulation of the precursor ions than smaller PAs, the optimal cone voltages ranging from 30 V to 150 V. Thus, it was not possible to utilize a single cone voltage for the maximal accumulation of the group-specific precursor ion for all different types of compounds in the same polyphenol subgroup. On one hand, increasing the cone voltages beyond the

optimum decreased the sensitivity due to extensive fragmentation of the ions before they entered the first quadrupole. On the other hand, use of cone voltages that were too low led to poor fragmentation of the original molecule and caused lower sensitivity. Therefore, a range of cone voltages was used to maximize the detection of variable compounds from the different polyphenol subgroups (Table 4). The variability of the optimal cone voltages was largest for PAs and finally, six different cone voltages were selected to be used in the method: the lowest cone voltage was used for the detection of the monomeric PA units and the five other cone voltages were selected to produce maximal precursor ion intensities for the various PA structures (Table 4).

After selection of suitable product ions, the collision energies were adjusted to produce maximal ion intensities. Herein, a slightly different approach was used in **I** and **II**; while **I** utilized only one precursor ion → product ion transition, in **II**, two transitions were selected for the detection of each group of compounds. The first transition (quantifier) was used for quantitation purposes and the second transition (qualifier) to distinguish possible false positives in the detection. The separation between true and false detection was done by calculating the ratio of the peak areas obtained by the qualitative and quantitative MRM transitions; their ratio should be constant for true detections while false positives result in a change in this ratio. The selection of optimal collision energy for the maximal accumulation of the selected product ion was straightforward due to the common precursor ions; only one optimal collision energy per transition was available. The precursor ions, product ions, cone voltages and collision energies utilized in **I** and **II** are presented in Table 4.

Table 4. Cone voltages (CV) and collision energies (CE) in the created multiple reaction monitoring methods for the detection of different polyphenol subgroups.

PA Unit	Precursor Ion (*m/z*)	Product Ion (*m/z*)	CV 1 (V)	CV 2 (V)	CV 3 (V)	CV 4 (V)	CV 5 (V)	CV 6 (V)	CE (eV)
PC extension	287	125	30	50	75	85	110	140	15
PC terminal	289	145	30	50	75	85	110	140	15
PD extension	303	125	30	55	80	110	130	150	20
PD terminal	305	125	30	55	80	110	130	150	20

Polyphenol Class	Precursor Ion (*m/z*)	Product Ion (quant/qual) (*m/z*)	CV (V)	CV Range (V)	CE (eV)	Qual : Quant (%)[a]
Gallic acid derivatives	169	125 / 107	80	50-130	15 / 20	32 ± 8
Ellagitannins	301	200 / 145	110	90-110	40 / 35	44 ± 3
Quinic acid derivatives	191	127 / 109	70	50-90	15 / 20	39 ± 3
Quercetin derivatives	301	151 / 179	85	60-110	20 / 20	46 ± 2
	300	271 / 255	85	60-110	25 / 20	48 ± 3
Kaempferol derivatives	285	229 / 257	80	60-110	20 / 25	27 ± 4
	284	255 / 227	80	60-110	25 / 25	99 ± 4
Myricetin derivatives	317	271 / 287	75	60-85	20 / 25	14 ± 1
	316	271 / 287	75	60-85	20 / 25	61 ± 2

With the methods developed, fingerprints of ETs, gallic acid and quinic acid derivatives, quercetin, kaempferol and myricetin glycosides as well as PAs can be recorded together with UV chromatograms and full scan mass spectra. Thus, a broad overview of fingerprints of eight common polyphenol classes can be achieved, and with correctly selected standards, this information can be transformed into quantitative data. In addition, as the PA MRM methods were based on the QM cleavage, it was possible to determine both the PC/PD ratios as well as the mean degree of polymerization for the PAs present in any studied sample. The latter feature was obtained by measuring the total peak areas obtained for PC and PD extension and terminal units separately, which enabled to develop an equation for the calculation of the PA mean degree of polymerization (see **I**). Furthermore, since PA fragmentation takes place after the chromatographical separation, the method can be used to create sample-specific fingerprints of the PA composition, PC/PD ratio and mean degree of polymerization throughout the chromatographic hump produced by the larger PA oligomers and polymers. This provides a straightforward visual tool, but also a quantitative comparison of different PA containing samples. For example, in Figure 22, PA concentration (Fig. 22A), PC and PD concentrations (Fig. 22B), PC to PD ratio (Fig. 22C) and mean degree of polymerization (Fig. 22D) of three plant samples are plotted against retention time (Salminen et al., unpublished material). This facilitates the use of the fingerprinting method to find differences or similarities in plant samples, which then can be grouped based on their chromatographic profiles.

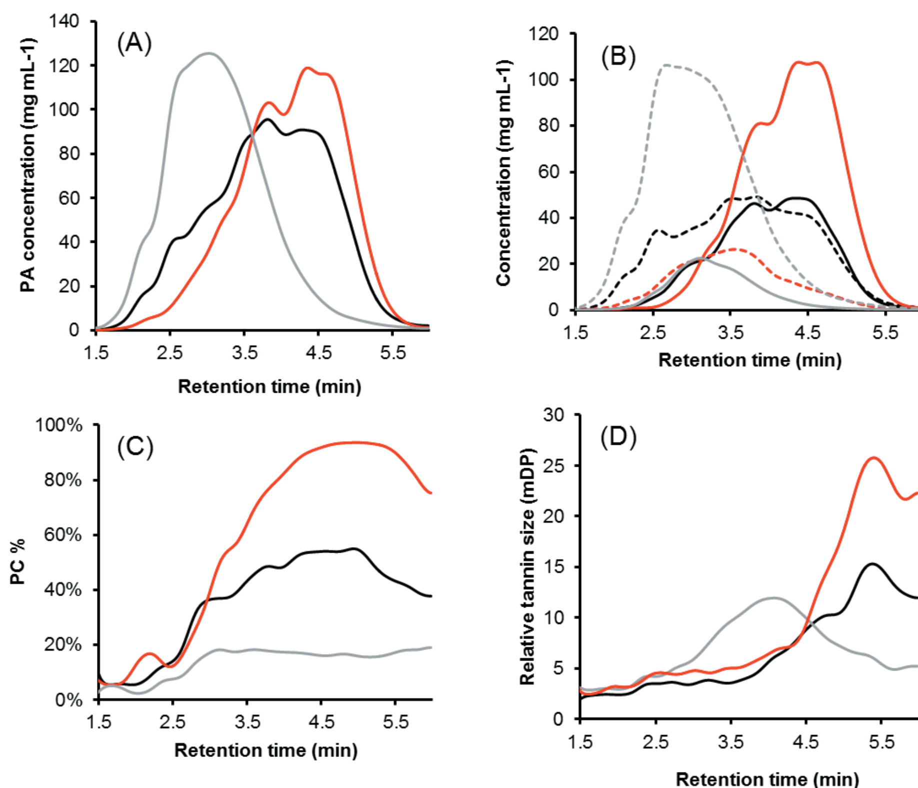

Figure 22. PA concentration (A), PC (B, solid line) and PD (B, dashed line) concentrations, PC to PD ratio (C) and mean degree of polymerization (D) as a function of retention time for *Rhododendron 'Cunningham's White'* (black lines), *Rhododendron canadense* (red lines) and *Thuja plicata* (grey lines). Salminen et al., unpublished material.

5.2. Analysis of tannin–protein interactions

It has been suggested that tannins may affect the protein structure and function by two different mechanisms: at low to neutral pH, tannins could reversibly bind to protein while at increased pH or in presence of oxidizing enzymes, the tannin-quinones formed could irreversibly bind to proteins (Hagerman 2012). Although the oxidative activity of tannins has been connected to their ability to form highly stabilized adducts with proteins (Appel, 1993; Barbehenn et al. 2006), this issue has not been verified by experimental studies. In **III**, the objective was to define the effect of pH on the formation of highly stabilized

tannin–protein complexes and to see how the tendency to form these adducts is related to tannin structure and oxidative activity.

The model compounds were selected to represent low oxidative activity (PGG), medium oxidative activity (tellimagrandin I, oenothein B and oenothein A) and high oxidative activity (vescalagin, castalagin, vescavaloninic acid and epigallocatechin gallate). To study the effect of rather moderate changes in pH on the formation of tannin–protein complexes, Laemmli SDS-PAGE and native PAGE experiments were performed for samples where tannin and BSA had been incubated at pH 5, pH 6.7 or pH 7.6. The results showed that already the change from pH 5 to pH 6.7 affected the formation of highly stabilized tannin–protein complexes. At pH 5, only BSA treated with epigallocatechin gallate or vescalagin resulted in a distinguishable response to NBT staining while at pH 6.7, the incubation of BSA with any of the studied compounds, except PGG, resulted in response to NBT. Further, more intensive responses to NBT were achieved after incubation at pH 7.8 than after the incubation at pH 6.7. However, as the NBT staining is based on cyclic redox reactions (Paz et al. 1991), this increase in intensity at higher pH did not explicitly indicate more tannin molecules were bound to BSA. In addition, one must also consider that the redox capacity of the tannins at different pH might have affected the response.

To obtain more comparable data, the incubated samples were analyzed with native PAGE which separates proteins according to their mass to charge ratio instead of only mass. Thus, the more tannin bound to the protein, the further the complex travels on the gel in comparison to native protein. Analysis of the gel shifts suggested that the pH induced differences in the tannin–protein complexes formed (Fig. 23A). At pH 5, a small shift was observed between native BSA and BSA incubated with tellimagrandin I, vescalagin or epigallocatechin gallate. At pH 6.7, no shift appeared for BSA treated with PGG, while for the other compounds the gel shifts varied between 3% and 8%. The incubation at pH 7.6 resulted in the most distinct differences in the gel shifts between the tannin treated and native BSA; for the less oxidatively active compounds, PGG, tellimagrandin I, oenothein B and oenothein A, the shifts were 4–5% while for the tannins with high oxidative activities, vescalagin, castalagin, vescavaloninic acid and epigallocatechin gallate, the gel shifts were 7–11%. This indicative relationship between the tannin oxidative activity and the gel shift of the tannin-treated BSA at pH 7.6 was confirmed by plotting these two measures against each other (Fig. 23B). By contrast, the mobility

shifts of tannin-treated BSA at pH 5 and pH 6.7 did not correlate with the measured oxidative activity (R^2 = 0.02 and 0.15, respectively). These results showed that the oxidatively more active compounds influence the mobility of the BSA to a greater extent, consistent with being more reactive with the protein. In other words, the compounds with low oxidative activities had a fixed stoichiometry that did not change with pH, while with compounds with high oxidative activity the stoichiometry was affected by the pH, so that more tannin was bound to BSA at increased pH.

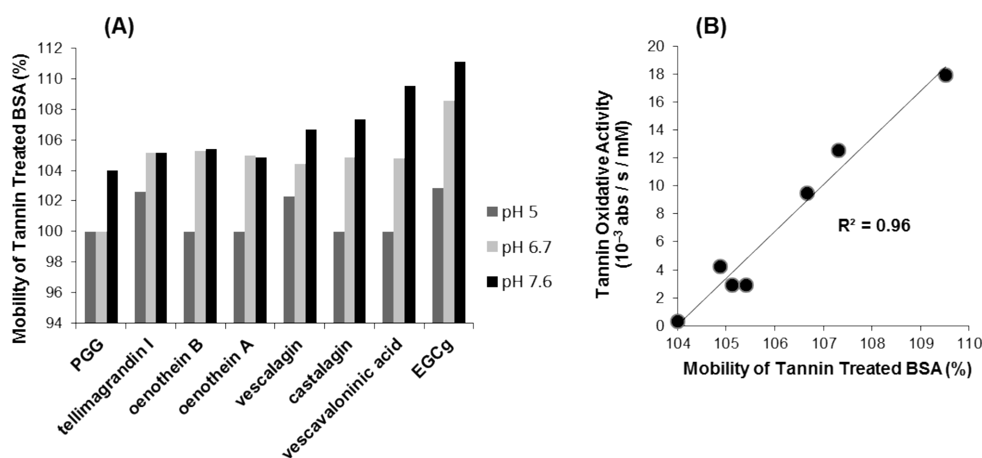

Figure 23. The relative mobility of the tannin treated BSA band in native PAGE at different pH, expressed as a % of the relative mobility for its matched untreated band (A) and the correlation between the BSA mobility at pH 7.6 and the tannin oxidative activity (B).

Other changes observed on the BSA bands after incubation with tannin were the width of the shifted bands, which indicated heterogeneity of the tannin–protein complexes. The variation was consistent with the gel shifts; the protein bands started to be broader at the pH values where the mobility of the complex increased. The broadness of the protein bands correlated somewhat with the oxidative activity of the polyphenol. The most oxidative polyphenols (vescavaloninic acid, epigallocatechin gallate, castalagin and vescalagin) had bands that were broader when pH was high compared to the polyphenols with medium (tellimagrandin I, oenothein B and oenothein A) or low oxidative activities (PGG).

Tannin–protein complexes formed at pH 5 and pH 6.7 and their stability was further studied by MALDI-TOF-MS. The extensive precipitation of BSA by PGG at both pH values and oenothein A at pH 5 interfered with the ionization process and thus no signals were detected for these samples. At pH 5, only oenothein B formed complexes with BSA that were not removed by ultrafiltration. The MALDI spectra obtained after the incubation of BSA with vescalagin, castalagin and vescavaloninic acid indicated unstable complexes as they were removed by ultrafiltration. The smallest compounds studied, tellimagrandin I and epigallocatechin gallate, did not form detectable complexes with BSA at pH 5. On the contrary, at pH 6.7, all the compounds studied formed detectable complexes with BSA. The MALDI spectra of BSA incubated with tellimagrandin I, oenothein B, vescalagin, castalagin and epigallocatechin were similar before and after the ultrafiltration, suggesting that the main tannin–protein complexes formed were highly stabilized. However, for BSA incubated with oenothein A and vescavaloninic acid, the ultrafiltration caused the peaks at the higher molecular weight to disappear, indicating that both stable and unstable tannin–protein complexes were present.

These results indicated that complex formation was affected by a combination of pH, tannin structure and tannin oxidative activity. The effect of tannin size effect could be seen when comparing the MALDI spectra of tellimagrandin I, oenothein B and oenothein A at pH 6.7 before and after the ultrafiltration (Fig. 24A,B). The largest complexes (clear signals) between BSA and oenothein B had an average molecular weight of ~70.9 kDa both before and after the ultrafiltration, which corresponded to complexes containing a maximum of three oenothein B molecules bound to one BSA. For oenothein A, the maximum distinguishable peak value at pH 7 was at *m/z* ~85.2 kDa before ultrafiltration, while after the ultrafiltration it was ~75.4 kDa. These values corresponded to complexes containing a maximum of eight and four oenothein A molecules bound to one BSA, respectively. Incubation of BSA with the monomeric precursor of both compounds, tellimagrandin I, did not result in additional peaks in the MALDI spectra but instead a slightly broadened hump was observed.

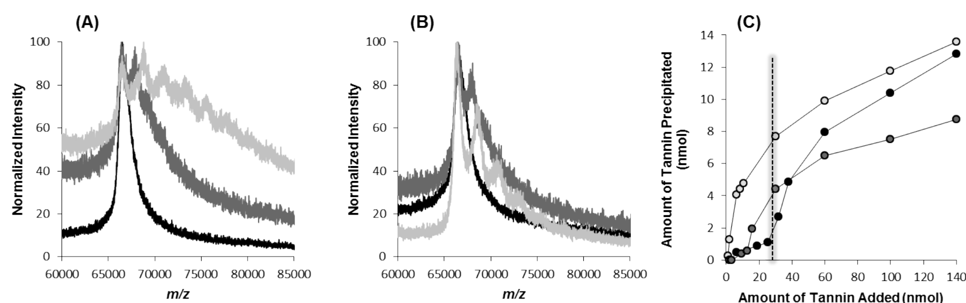

Figure 24. MALDI-TOF-MS spectra for tellimagrandin I (black line), oenothein B (dark grey line) and oenothein A (light grey line) before (A) and after (B) ultrafiltration. The protein precipitable phenolics method, in which the amount of protein bound tannin was measured by analytical HPLC after 30 min incubation at pH 7, showed differences in the ability of the three compounds to precipitate BSA (C). The dashed line shows the amount of tannin added in the MALDI experiments (added BSA 4.5 nmol).

The dimeric oenothein B consists of two tellimagrandin I monomers linked via two *m*-DOG (Yoshida et al. 2009) bonds, and thus is a macrocyclic structure in nature. In the trimeric oenothein A, the additional monomeric unit is attached by one *m*-DOG-type linkage, increasing both the size and the flexibility of the molecule. While the rigid structure of the former caused the formation of only highly stabilized complexes with BSA, the flexibility brought to the latter by the extra tellimagrandin I unit enabled the formation of both unstable and highly stabilized tannin–protein complexes. This is in agreement with a previous study with isothermal titration calorimetry, where a similar trend was observed for the complexation of the tellimagrandin I based oligomeric ET series with BSA (Karonen et al. 2015). In addition to the smaller molecular size, one explanation for the inefficient complex formation of tellimagrandin I with BSA could be the molar ratio used (32:4.5; tannin/protein). Experiments in which the amount of BSA bound tannin was measured as a function of tannin added to BSA solution (at pH 7) suggested that the tannin concentration selected for the MALDI-TOF-MS experiments was not ideal for tellimagrandin I to efficiently bind BSA (Fig. 24C).

The investigation of the MALDI-TOF-MS results in the light of oxidative activity indicated the same trend as the gel electrophoresis experiments; the oxidatively more active compounds were more sensitive to the change of pH. Altogether, it could be concluded that at low pH, the ability of tannins to non-

covalently bind with protein is an absolute pre-requisite for the formation of highly stabilized complexes; this seemed to occur with tannins having low or intermediate oxidative activities. At high pH, however, the tannin oxidative activity directly correlated with the formation of highly stabilized tannin–protein complexes. Although the results suggested the nature of the highly stabilized tannin–protein complexes to be covalent and the unstable complexes to be driven by non-covalent interactions, none of the methods utilized in **III** unambiguously describe the tannin–protein complexes as non-covalent or covalent. Thus, in future studies, emphasis will be put on determining whether covalent bonds stabilize the tannin-protein complexes formed at elevated pH.

5.3. Tannins as anthelmintics

In **IV**, 33 purified HTs and their most common hydrolysis product, gallic acid, were tested against the egg hatching of *H. contortus* to provide data on the structure–activity relationships between individual tannins and their *in vitro* anthelmintic activity. The HTs studied were selected to represent a wide variety of HT structures from ten different biosynthetic branches of the HT pathway (Table 5).

Table 5. The compounds studied in article **IV**.

Number	Compound	MW	Number	Compound	MW
1	gallic acid	170.1			
simple galloylglucoses			***macrocyclic m-DOG oligomers***		
2	monogalloylglucose	332.3	23	oenothein B	1569.1
3	tetragalloylglucose	788.6	24	oenothein A	2353.6
4	pentagalloylglucose	940.7	25	tetramer	3138.2
gallotannins			26	pentamer	3922.7
5	hexagalloylglucose	1092.8	27	hexamer	4707.2
6	heptagalloylglucose	1244.9	***m-DOG oligomers***		
7	octagalloylglucose	1397.0	28	rugosin E	1723.2
monomeric HHDP* esters			29	rugosin D	1875.3
8	tellimagrandin I	786.6	***m-GOG oligomers***		
9	tellimagrandin II	938.7	30	agrimoniin	1871.3
10	pedunculagin	784.5	31	gemin A	1873.3
11	casuarictin	936.7	***m-GOD oligomers***		
12	isostrictinin	634.5	32	sanguiin H6	1871.3
monomeric DHHDP* esters			33	lambertianin C	2805.9
13	geraniin	952.6	***C-glucosidic oligomers***		
14	carpinusin	952.6	34	salicarinin A	1869.2
C-glucosidic ET* monomers					
15	vescalagin	934.6			
16	castalagin	934.6			
17	vescavaloninic acid	1102.7			
18	castavaloninic acid	1102.7			
19	stachyurin	936.7			
20	casuarinin	936.7			
21	hippophaenin B	1104.8			
22	hippophaenin C	1104.8			

*HHDP: hexahydroxydiphenoyl, DHHDP: dehydrohexahydroxydiphenoyl, ET: ellagitannin.

The egg hatching inhibition percentages varied considerably between the compounds and the concentrations tested. Most commonly the anthelmintic effects are reported as EC50 (half maximal effective concentration) values but as only 16 of the tested HTs reached 50% inhibition levels, this could not be used to rank them. Instead, the activities were presented by taking the whole concentration range into account and an average inhibition value was calculated for each compound, and the compounds were ranked based on this (Fig. 25).

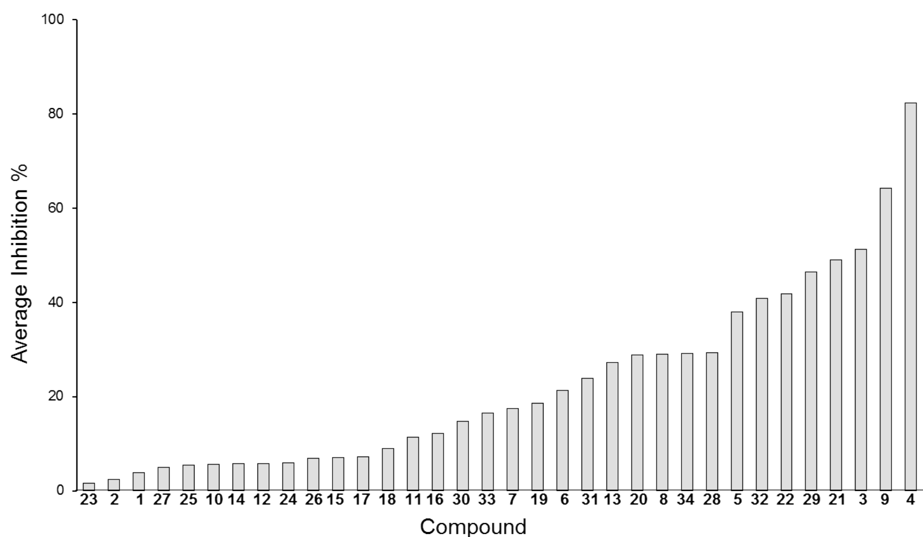

Figure 25. The studied 33 hydrolyzable tannins and gallic acid in the order of increasing average egg hatch inhibition activity. The average egg hatch inhibition was calculated as the average % unhatched eggs compared to control at the tested concentration range (2.0, 1.5, 1.0, 0.5, 0.25 and 0.125 mM). See compound identities in Table 5.

After calculating the average inhibition values for each compound, it was possible to compare those against the chemical characteristics of their structures. Comparison of the HT molecular weight against the *in vitro* anthelmintic activity showed that compounds with a molecular weight below 700 or above 2000 Da had no or very little effect on the egg hatching of *H. contortus*. On the contrary, the most active compounds had molecular weights relatively close to that of pentagalloylglucose, 940 Da, (Fig. 26A). However, this type of match did not guarantee a high activity, if the structures were biosynthetically distant from pentagalloylglucose. For instance, tellimagrandin II (MW 938 Da) is the immediate biosynthetic product from pentagalloylglucose and it was the second most active of the tested HTs. However, casuarictin, vescalagin and castalagin, which also have similar molecular weights to pentagalloylglucose (936 Da, 934 Da and 934 Da, respectively) but are biosynthetically distant from pentagalloylglucose, had significantly smaller activities (82% vs. ~10%). This dependence on the biosynthetic closeness to pentagalloylglucose could be seen also e.g. from the change in average activity of the monomeric HTs with cyclic glucose core along their proposed biosynthetic pathway (Fig. 26B).

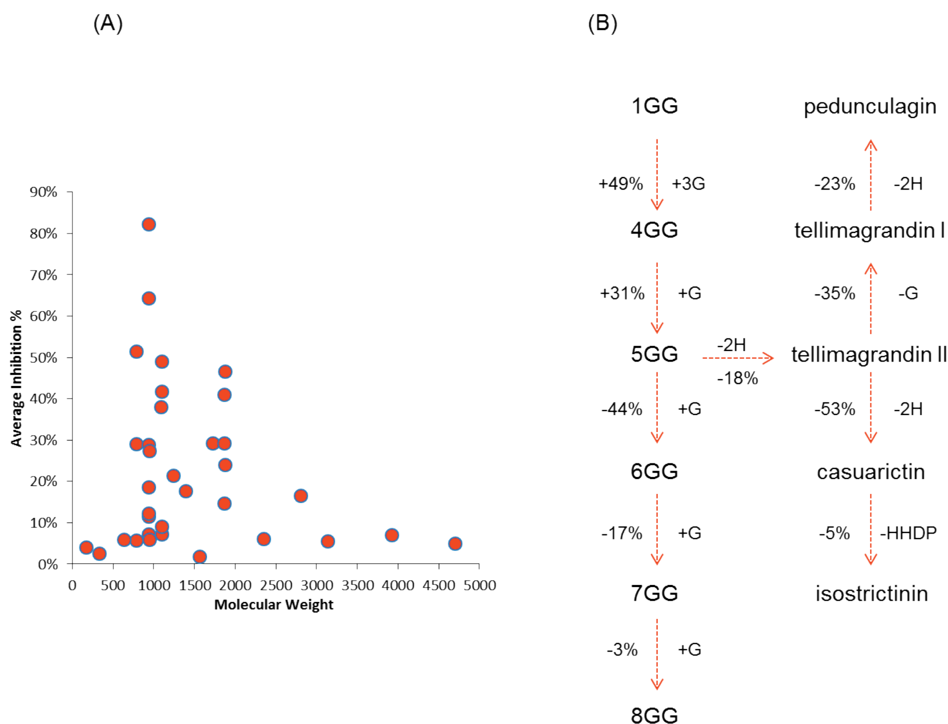

Figure 26. Average anthelmintic activity vs. molecular weight plot (A) and the change in the anthelmintic activity on the biosynthetic pathway of the studied monomeric HTs with cyclic glucose core (B). G, galloyl group; H, hydrogen; HHDP, hexahydroxydiphenoyl group. 1GG, 4GG, 5GG, 6GG, 7GG and 8GG indicate mono- to octagalloylglucose, respectively.

The structural differences between the 33 HTs were further compared compound-by-compound to reveal possible relationships between the tannin structure and their anthelmintic activity. This facilitated a tentative determination of the effect of a certain structural feature of an HT on the egg hatching inhibition activity (Fig. 27). Based on these comparisons, it was possible to create an equation for the estimation of the inhibitory activity on the egg hatching of *H. contortus*. To ensure enough degree of freedom in the equation, only those structural features that had a major effect on the activity were included and coefficients reflecting the relative impact of each structural feature were adjusted in Microsoft Excel to produce activity estimates that would best correlate with the measured activities. In comparison to the measured values, the final equation enabled an estimation of the average egg hatch inhibition activities of the studied HTs [R = 0.89 (**IV**)]. The equation

created provided a tool, if not to calculate the absolute activities, but especially to estimate whether plant HTs have low, moderate or high anthelmintic activities.

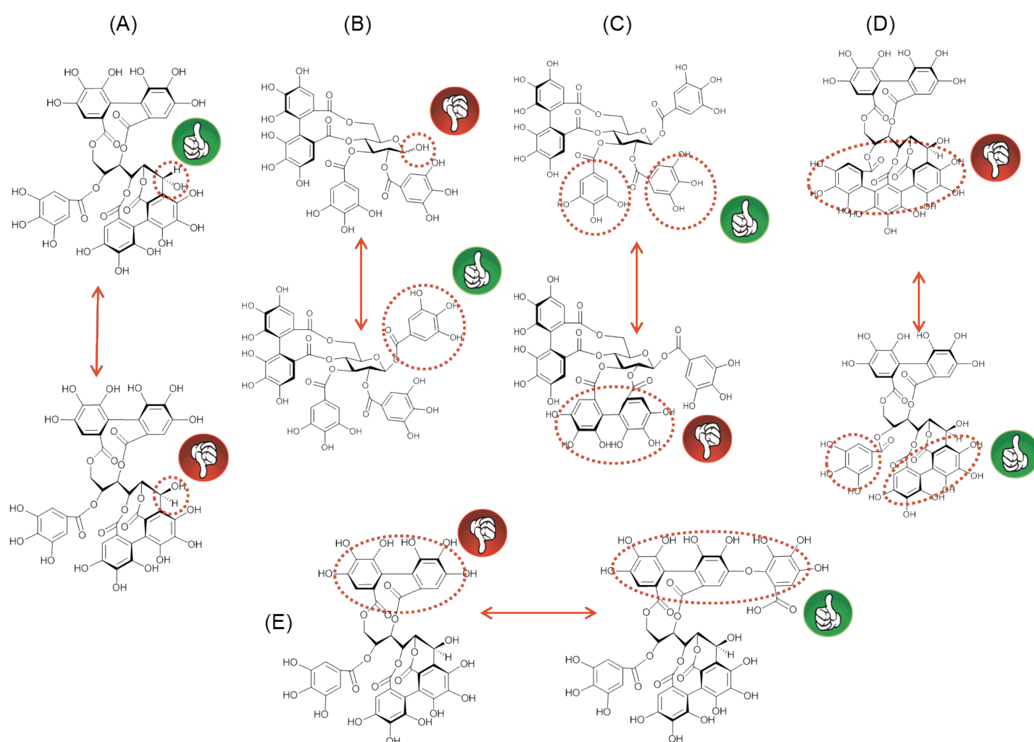

Figure 27. Compound-to-compound comparisons enabled to conclude whether a specific structural feature of a HT had a positive (green thumb) or negative (red thumb) effect on the egg hatching inhibition activity. For example, compound 20 vs. 19, α-OH vs. β-OH at C1 (A); 8 vs. 9, OH vs. a galloyl group at C1 (B); 9 vs. 11, 2 × galloyl group vs. hexahydroxydiphenoyl group (C); 15 vs. 19, nonahydroxytriphenoyl group vs. galloyl and hexahydroxydiphenoyl group (D); 19 vs. 21, hexahydroxydiphenoyl group vs. valoneoyl group. See compound identities in Table 5.

Based on the results from EHA, twelve structurally different compounds were selected for further testing against the motility of hatched L1 and L2 stage larvae of *H. contortus*. In general, the most effective egg hatching inhibitors also markedly decreased the motility and the least effective egg hatching inhibitors were also less active in the motility tests. However, all the compounds tested were more effective in inhibiting the larval motility than the egg hatching of *H. contortus* (Fig. 28). This was interesting especially when

considering *in vivo* conditions and the life cycle of *H. contortus*, in which the inhibition of motility may be more relevant than the inhibition of egg hatching. The motility inhibition values were calculated as the percentage of moving and non-moving larvae. In addition, all studied compounds decreased motility of the larvae already at the lower concentrations tested, progressively leading to the total immobilization at high concentrations.

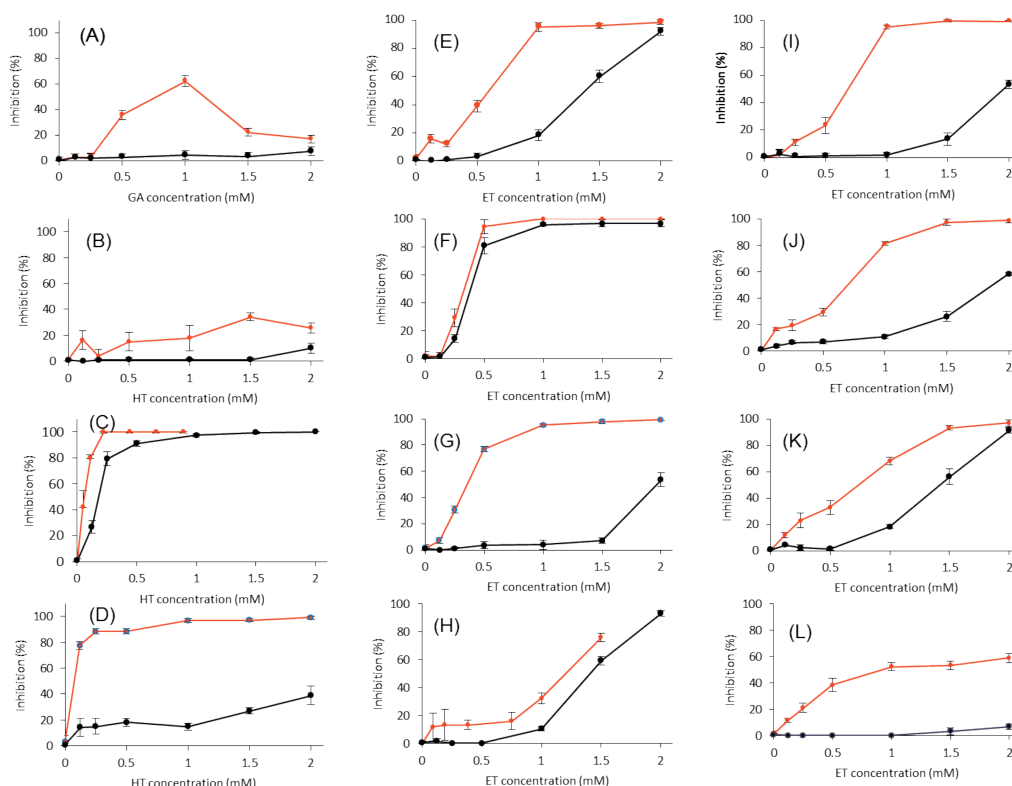

Figure 28. The inhibitory effect of selected HTs on the egg hatching (black lines) and motility (red lines) of *H. contortus*: gallic acid (A), monogalloylglucose (B), pentagalloylglucose (C), heptagalloyglucose (D), tellimagrandin I (E), tellimagrandin II (F), casuarictin (G), geraniin (H), castalagin (I), stachyurin (J), casuarinin (K) and oenothein B (L). GA, gallic acid; ET, ellagitannin; HT, hydrolyzable tannin.

Several observations suggested the possible modes of action by which the compounds inhibited the egg hatching and motility of *H. contortus*. Firstly, the amount of unhatched eggs with the embryo inside was constant in both control and HT solutions and only the amount of hatched vs. unhatched eggs varied depending on the tests. Secondly, before the addition of the Lugol solution,

most of the larvae were still moving inside the egg. Thirdly, the SEM results showed that when the eggs were incubated with pentagalloylglucose, the eggs were fully covered with chip-like layers of aggregates. On the contrary, no visible cover was observed when the eggs were incubated with the oxidatively more active casuarinin. For both compounds, aggregates located at the buccal capsule and the anterior amphidial channels of L1 and L2 stage larvae were observed after incubation, but for pentagalloylglucose these aggregates were more extensive and covered also parts of the surface of the other cephalic areas. Altogether, these observations indicated that the studied HTs were not prone to penetrate inside the eggs and disturb the development of the embryo directly. More likely, the HTs bound to the surface of the egg shell and either disturbed the proteins involved in the hatching process or the HTs changed the egg shell so that the penetration of the larvae through the shell was disabled. The partially different effect of the two compounds supported the assumption that different types of compounds may be effective via different modes of action. In regard to the hatched L1 and L2 stage larvae, the observed changes could suggest that the possible mode of action in inhibiting the motility occurred via binding to the surface of the larvae. Interestingly, it was observed in the cryo-SEM experiments that the eggs incubated with pentagalloylglucose had more often bacteria on their surfaces than the eggs incubated with casuarinin or in the control solution (unpublished results, Fig. 29). It was difficult to interpret this observation, but it could somehow be related to the anti-microbial properties of HTs (Buzzini et al. 2008); for example, pentagalloylglucose bound to the egg surface could bind bacteria and cause the observed effect.

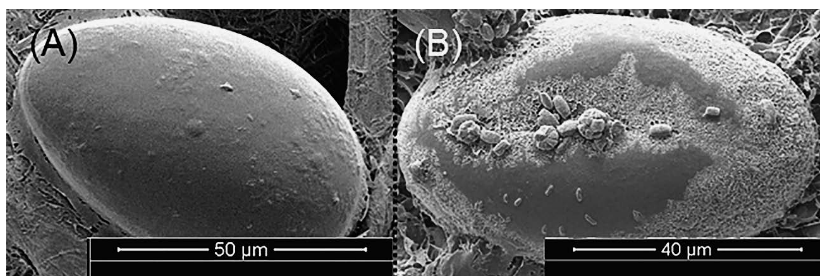

Figure 29. The egg of *H. contortus* incubated in control solution (A) and in the presence of pentagalloylglucose (B). In (B), clusters of microbes can be seen on the surface of the egg. Images are from cryo-scanning electron microscopy.

The prediction of the cause for the witnessed *in vitro* anthelmintic activity in **IV** was further complicated by the results that showed that the studied compounds underwent hydrolysis and oxidation during the 48h incubation time. The UHPLC-MS/MS analysis showed that for pentagalloylglucose, all degradation products were derivatives formed by the loss of galloyl group(s). With full scan MS it was possible to identify the main peaks as tetra-, tri- and digalloylglucoses which are common hydrolysis products of pentagalloylglucose. The results obtained with casuarinin indicated that both hydrolysis and oxidation occurred. These observations could partially explain the observed differences in the HT activities between EHA and motility inhibition assays. In EHA experiments, the HTs could bind to the egg shell immediately after adding the tannin to the incubation solution and before extensive degradation of the tannin occurred. On the contrary, in the motility inhibition experiments, the larvae were hatched first after ~24 hour incubation. Thus, at that point the possible degradation of the original tannin structures could have already occurred and the resulting hydrolysis and oxidation products be less or more active than the initial tannin. Altogether, three different modes of action by which HTs could disturb the egg hatching and the motility of the hatched larvae are suggested: (1) HTs with high protein precipitation capacity may bind to the eggs and larvae via non-covalent bonds, (2) HTs with high oxidative activity may be auto-oxidized and the oxidation products bind covalently to the egg and larvae and (3) HTs could undergo hydrolysis and the hydrolysis products may interact with the eggs and larvae via non-covalent or covalent interactions.

6. CONCLUSIONS

The methodological problems associated with tannin research were confronted by developing rapid UHPLC-MS/MS methods for the qualitative and quantitative fingerprint analysis of eight different polyphenol subgroups directly from crude plant extracts. These methods utilized rapid chromatography of different kinds of polyphenols achieved with UHPLC and their fragmentation into group-specific precursor and product ions for detection with multiple reaction monitoring methods. The developed methods provide a user-friendly, robust and fast addition to the chemical tools currently used for the qualitative and quantitative screening of large numbers of samples for their bioactive polyphenol types and contents.

The second main result in this thesis was the establishment of a relationship between the tannin structure and the *in vitro* anthelmintic activity. It was shown that tannin bioactivity against *H. contortus* can be predicted rather precisely from their structural features. In addition, the studies with scanning electron microscopy suggested the possible mode of action of tannins against nematodes to be their binding to the surface structures of both nematode eggs and larvae.

Regarding tannin–protein interactions, both tannin structure and pH have a strong influence on the formation of tannin–protein adducts and the stability of these adducts. While more basic pH had a stabilizing effect on the tannin–protein complexes, the tannin oxidative activity was directly linked with their tendency to form highly stabilized complexes with BSA at increased pH. Thus, it was concluded that tannin oxidative activity may in part determine the range of pH where the tannin is able to interact with proteins and express bioactivity.

To conclude, while this thesis provided many new findings regarding tannin bioactivity, at least equally as many new questions emerged. The mechanism of action by which tannins possess bioactivity provides an intriguing objective for the future studies. Also, more detailed work on the fate of plant tannins in different conditions and, correspondingly, the precise determination of the structural units that actually cause the observed bioactivity, will provide further evidence on their positive biological effects.

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