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Nicolas Baert

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ABSTRACT

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BAERT, NICOLAS JEAN-BAPTISTE: Oligomeric ellagitannins of *Epilobium angustifolium*: quantification and bioactivity assessment.

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Over the past few years there has been a growing body of evidence highlighting the fact that tannin-rich fodders could be beneficial to animal welfare as well as to the environment, by decreasing methane emissions from ruminants. However, the immense variety of tannin structures that exist within the plant kingdom has rendered difficult the study of their precise modes of action. Yet, understanding the structure-activity relationships that underlie the bioactivities of tannins would permit a targeted selection of optimal forages and thereby a more effective use of these natural resources. This work focuses on one particular aspect of the structure-activity relationships of ellagitannins: the degree of oligomerization. A series of oligomeric ellagitannins (ETs) isolated form fireweed (*Epilobium angustifolium*) was utilized to address the question of how the oligomeric size might affect the bioactivities of ellagitannins.

First, a method was developed to quantify these ETs using ultra-high performance liquid chromatography-diode array detector-tandem mass spectrometry (UHPLC-DAD-MS/MS). This method was then applied to investigate the distribution pattern of ETs and other phenolics within the plant and among several plant populations. Substantial differences were observed between leaves, flowers and stems but the interpopulational variability remained relatively low. The second part of this work was dedicated to the assessment of the relationships between oligomer size and bioactivity using *in vitro* assays. Oligomeric ETs were tested on an *in vitro* model of ruminal fermentation and on the adult stage of an intestinal nematode of goats and sheep. It was found that oligomeric ETs inhibited fiber degradation in a size-dependent manner. Their ability to specifically inhibit methanogenesis and protein degradation, however, reached an optimum with the trimer and the tetramer. The same optimum was also observed with the *in vitro* anthelmintic activity.

TIIVISTELMÄ

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BAERT, NICOLAS JEAN-BAPTISTE: Maitohorsman (*Epilobium angustifolium*) oligomeeriset ellagitanniinit: kvantitointi ja bioaktiivisuuden määritys.

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Maaliskuu 2017

Viimeisten vuosien aikana todisteet ovat osoittaneet tanniini-rikkaan rehun vähentävän märehtijöiden metaanipäästöjä ja tällä tavoin olevan hyödyksi sekä eläinten että ympäristön hyvinvoinnille. Johtuen kasvikunnassa esiintyvien tanniinirakenteiden suuresta moninaisuudesta, on tanniinien tarkan aktiivisuusmekanismin tutkiminen ollut kuitenkin vaikeaa. Tästä huolimatta olisi tärkeää oppia ymmärtämään, millainen vaikutus tanniinien rakenteella on niiden bioaktiivisuuteen; tämä mahdollistaisi tarkemmin valikoitujen rehujen käyttöönoton sekä kyseisten luonnovarojen tehokkaamman hyödyntämisen. Tässä työssä keskityttiin yhteen ellagitanniinien bioaktiivisuuteen vaikuttavaan rakenteelliseen tekijään: niiden oligomerisaatioasteeseen. Maitohorsmasta (Epilobium angustifolium) eristettyjen oligomeeristen ellagitanniinien avulla tutkittiin, kuinka ellagitanniinien koko vaikuttaa niiden bioaktiivisuuteen.

Työn ensimmäisessä osassa kehitettiin erittäin korkean erotuskyvyn nestekromatografia–tandemmassaspektrometriaan (UHPLC-MS/MS) perustuva menetelmä, jolla kyseisiä ellagitanniineja voitiin analysoida. Tätä menetelmää käytettämällä tutkittiin ellagitanniinien ja muiden fenolisten yhdisteiden esiintymistä sekä kasvin eri osissa että erillisissä kasvipopulaatioissa. Maitohorsman lehtien, kukkien ja varsien välillä havaitiin merkittäviä eroja, mutta populaatioiden väliset erot olivat pieniä. Työn toisessa osassa tutkittiin oligomerisaatioasteen vaikutusta ellagitanniinien bioaktiivisuuteen in vitro. Oligomeeristen ellagitanniinien bioaktiivisuutta tutkittiin märehtijöiden fermentaatiota matkivalla in vitro mallilla sekä lampaiden ja vuohien suoliston loisella. Tulosten perusteella käytetyt ellagitanniinit vähensivat kuitujen hajoamista, ja vaikutuksen suuruus riippui ellagitanniinien koosta. Toisaalta, ellagitanniinien kyky estää mikrobiperäistä metaanin muodostumista sekä proteiinien hajoamista oli tehokkainta trimeerisellä ja tetrameerisellä ellagitanniinilla. Myös tutkittujen ellagitanniinien vaikutus märehtijöiden loisia vastaan oli tehokkaintainta näillä oligomeereilla.

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ACKNOWLEDGEMENTS

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I would like to begin by thanking the man without whom none of this work would have been possible, the man who—more than anyone else—has shaped the scientist I can claim to be today, my supervisor, Professor Juha-Pekka Salminen. You have been such an outstanding mentor during those four years. You encouraged me to nurture and pursue my own ideas, yet you were always there whenever I needed your guidance and that is, to me, the best thing a supervisor can do for his student. Working with you did not feel like working, and you are, without a doubt, the main reason why the four years I have spent in Turku were such a bliss. Thank you, Juha-Pekka, for being an awesome boss and a great man.

I express my gratitude to my two other supervisors, Dr Maarit Karonen and Dr Jari Sinkkonen. Your help and feedback on my experiments and papers have been paramount to me. Maarit, I thank you warmly for the tremendous support that you have provided me inside and outside the University. You have done so much for my wife and children. I will be forever grateful to you.

During my PhD I had the fantastic opportunity to work in other laboratories, abroad. In the spring of 2013 I spent three months in Dr Wilbert Pellikaan's lab in Wageningen and from May to July 2014, I worked in Toulouse under the supervision of Dr Hervé Hoste. Moving away from Finland for such short periods of time with my family was challenging but was, at the same time, one the best thing I ever did during my PhD. It taught me the importance of interdisciplanary collaboration in science, it gave me the taste for travel and more importantly, it taught me that we (as a family) could go anywhere as long as we were together. And for that I would like to express my deepest gratitude to Wilbert, Hervé and all the people who made those experiences possible and fruitful.

Being part of the LegumePlus project was an incredible experience. It provided me with the best environment a PhD student could hope for. It also gave me the satisfying feeling of being part of something bigger. Something meaningful. Something that could have an impact on the world. And all that is primarily thanks to the hard work and intelligent

leadership of Professor Irene Mueller-Harvey. And of course this project would not have been possible without the contribution of all the participants—young researchers and supervisors alike—whom it would be too long to name individually in this book, but whose input was absolutely essential.

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I will never forget that all of this started thanks to you Cécile. You were the one who encouraged me to embark in a PhD. And from that day of July 2012 when left France, we have been living, happier than ever, travelling the world with our beautiful children, enjoying a life that I would not trade for all the gold in the world. Your love makes anything possible.

Eléonore, Gabriel and Thomas, this book is dedicated to you. May Life, Nature and Science be as wonderful to you as they are to me. Stay curious, strive to learn, experiment, try to remain a child at heart. That is my secret recipe for happiness.

Ithaca, May 2017

"Sometimes the best way to learn something is by doing it wrong and looking at what you did."
Neil Gaiman

"The most beautiful thing we can experience is the mysterious. It is the source of all true art and science. He to whom the emotion is a stranger, who can no longer pause to wonder and stand wrapped in awe, is as good as dead—his eyes are closed."
Albert Einstein

"Science and everyday life cannot and should not be separated."
Rosalind Franklin

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 Baert, N.; Karonen, M.; Salminen, J.-P. Isolation, characterisation and quantification of the main oligomeric macrocyclic ellagitannins in *Epilobium angustifolium* by ultra-high performance chromatography with diode array detection and electrospray tandem mass spectrometry. *J. Chromatogr. A.* 2015, 1419, 26–36.

II Baert, N.; Pellikaan, W.F.; Karonen, M.; Salminen, J.-P. A study of the structure-activity relationship of oligomeric ellagitannins on ruminal fermentation *in vitro*. *J. Dairy Sci.* 2016, 99, 8041–8052.

III Baert, N.; Kim, J.; Karonen, M.; Salminen, J.-P. Inter-population and inter-organ distribution of the main polyphenolic compounds of *Epilobium angustifolium*. *Phytochemistry* 2017, 134, 54–63.

IV Baert, N.; Hoste, H.; Karonen, M.; Salminen, J.-P. Comparison of the in vitro anthelmintic activities of dimeric to heptameric ellagitannins from *Epilobium angustifolium* against the adult stage of *Trichostrongylus colubriformis.* Manuscript submitted to *Veterinary Parasitology* as a short communication.

ABBREVIATIONS

ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
BCVFA	Branched-Chain Volatile Fatty Acid
DAD	Diode Array Detector
DM	Dry Matter
ESI	Electrospray Ionization
ET	Ellagitannins
GHG	Greenhouse Gas
HHDP	Hexahydroxydiphenoyl
HR	Hazard Ratio
HT	Hydrolysable Tannin
LC-MS	Liquid Chromatography-Mass Spectrometry
MRM	Multiple Reaction Monitoring
MS/MS	Tandem Mass Spectrometry
NAD^+/NADH	Oxidized/Reduced Nicotinamide Adenine Dinucleotide
NH_3-N	Ammonia Nitrogen
NPN	Non-Protein Nitrogen
OM	Organic Matter
PA	Proanthocyanidin
PBS	Phosphate Buffer Saline
P_i	Inorganic Phosphate
QqQ	Triple Quadrupole
R_T	Retention Time
SRM	Selected Reaction Monitoring
TCA	Trichloroacetic Acid
TVFA	Total Volatile Fatty Acid
UHPLC	Ultra-High Performance Liquid Chromatography
VFA	Volatile Fatty Acid

1. INTRODUCTION

Plant secondary metabolites are ubiquitous in the plant kingdom and, thus, have always been inevitably part of the diet of herbivorous mammals. These compounds can exert variable degrees of activity on mammalian physiology, beneficial or deleterious, acute or chronic (Acamovic and Brooker, 2005; Iason, 2005; Forbey et al., 2009). In certain cases, they can even be used to treat diseases such as intestinal parasites. In fact, the observation of animal behavior revealed that sick animals sometimes "seek treatment" by consuming plants that contain specific bioactive compounds that they otherwise avoid when not sick (Amit et al., 2013; Villalba et al., 2015).

A class of plant secondary metabolites that is of particular interest is tannins. Several *in vivo* and *in vitro* studies have shown that tannin-rich fodders could be beneficial to animal welfare as well as to the environment, by decreasing methane emissions from ruminants (Puchala et al., 2005; Bodas et al., 2012; Jayanegara et al., 2012; Piluzza et al., 2014). Moreover, there is evidence that feeding tannin-containing forages to lambs or dairy cows could improve meat and milk quality (Priolo and Vasta, 2007; Vasta et al., 2008; Morales and Ungerfeld, 2015). These observations fueled the idea that tannins (or tanniferous forages) could be used as feed additives for sheep and cattle. Since most tannins-rich fodders are relatively cheap to grow, their benefits could easily outweigh their costs, thus making them a very attractive solution to some of the problems that plague modern livestock productions, particularly in developing countries.

One of the major challenges that this area of research has been facing comes from the diversity of chemical structures the word "tannin" encompasses. Indeed, a single plant species can synthetize dozens—sometimes even hundreds—of different tannin molecules (Haslam, 2007). This vast structural diversity makes it difficult to pinpoint the precise mechanisms by which tannins exert their bioactivities, even more so in a system as complex as a mammalian organism. Although a largely accepted paradigm is that tannins exert most of their activities through their capacity to bind proteins, it has been demonstrated that this capacity is strongly dependent on the structure (Hagerman, 1989).

Therefore, in order to promote a more rational use of tannins in animal nutrition it is necessary to understand how the chemical features of tannins translate into bioactivity. For this purpose, *in vitro* assays using pure molecules (or chemically characterized fractionated extracts) are a relatively cheap and simple way to gather data as opposed to a costly systematic *in vivo* approach. Thus, i*n vitro* trials could be considered a preliminary step in the targeted selection of potentially bioactive forages. Once the patterns that link chemical structure and bioactivity are revealed, it could be theoretically

possible to predict the effects of any tannin-rich forage by simply assessing its tannin fingerprint.

Following that approach, this work endeavored to investigate the influence of one structural aspect of tannins on their bioactivity: the degree of oligomerization. In 1989, Field *et al.* showed that oligomeric proanthocyanidins (PAs) were more active than polymers and monomers against methanogenic bacteria. Based on these results, the authors theorized that "*They [oligomers] constitute the best compromise between being large enough for effective tanning quality and being small enough for penetration.*" (Field et al., 1989).

In terms of chemical diversity, the research on tannins in animal nutrition has remained largely limited to PAs for a very long time. The main reason behind that preference lies in the toxicity of some plant species that are known to contain high levels of HTs (e.g. *Quercus*). Reports of animal toxicosis (Zhu et al., 1992; Pérez et al., 2011) have given HTs a reputation of toxic or anti-nutritional compounds, although it is now known that the toxicity of oak, for instance, is mostly due to low molecular weight phenolics rather than tannins (Butler and Rogler, 1992). Nevertheless, some recent papers from the 2010's started to widen their focus and showed promising *in vitro* results with HTs on ruminal fermentation (Pellikaan et al., 2011b; Hassanat and Benchaar, 2013). Therefore, I wanted to test whether Field's theory held true for HTs as well.

In order to test the effect of size only, I needed a series of polymeric HTs constituted of the same monomeric unit, so as to eliminate the confounding influence of other structural differences. A recently identified series of oligomeric ellagitannins (ETs) from *Oenothera biennis* was the perfect test subject for this study (Karonen et al., 2010). Coincidentally, the same oligomeric ETs were just found in another species of *Onagraceae*: willowherb (*Epilobium angustifolium*).

This thesis aimed at achieving four goals: (1) developing a selective method for the quantification of willowherb's ETs, (2) using that method to assess the inter- and intra-individual distribution of these ETs in the plant, (3) testing the oligomeric ETs *in vitro* on a model of ruminal fermentation, and (4) evaluating the anthelminthic activity of oligomeric ETs on the adult stage of a parasitic nematode.

2. LITERATURE REVIEW

2.1. Oligomeric ellagitannins

The study of oligomeric ETs began in 1982 with the discovery of agrimoniin (a dimer of potentillin) in *Agrimonia pilosa* and P*otentilla kleiniana* (Okuda et al., 1982). From then on, numerous oligomeric ETs were discovered at an ever-accelerating pace, thus unveiling the incredible complexity of the chemistry of those polyphenolic molecules.

2.1.1. Oligomerization of ellagitannins by oxidative coupling

The oligomerization of ETs can occur either via C–O or C–C oxidative coupling. The latter being less common, it will not be reviewed in this work. The C–O coupling modes can be divided in three categories depending upon the nature of the *O*-donating and *O*-accepting units of each monomer.

- The GOG-type linkage:

It involves two galloyl groups and can result in an *m*-GOG or a *p*-GOG unit depending on whether the *O*-donating group is a *meta*- or *para*-hydroxyl group, respectively (Figure 1).

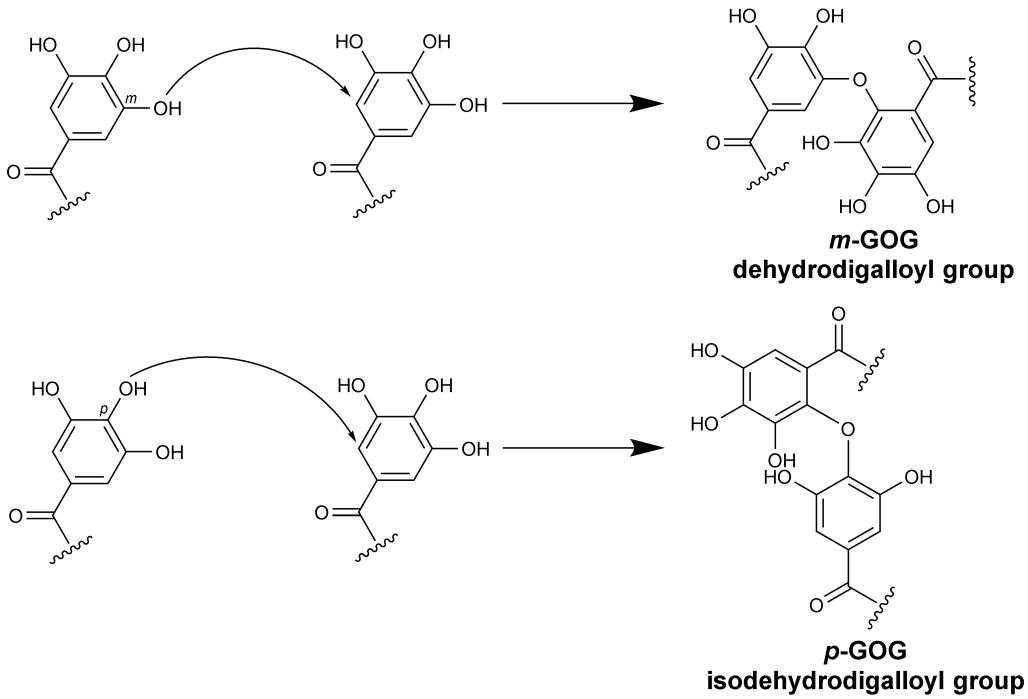

Figure 1. Formation of the *m*-GOG and *p*-GOG linking units.

- The DOG-type linkage

In this case, the *O*-donating group is an hexahydroxydiphenoyl (HHDP) moiety and the *O*-accepting group is a galloyl. As for the GOG-type, we distinguish the *m*-DOG group and the *p*-DOG depending on the position of the donating hydroxyl group on the benzoyl ring (Figure 2).

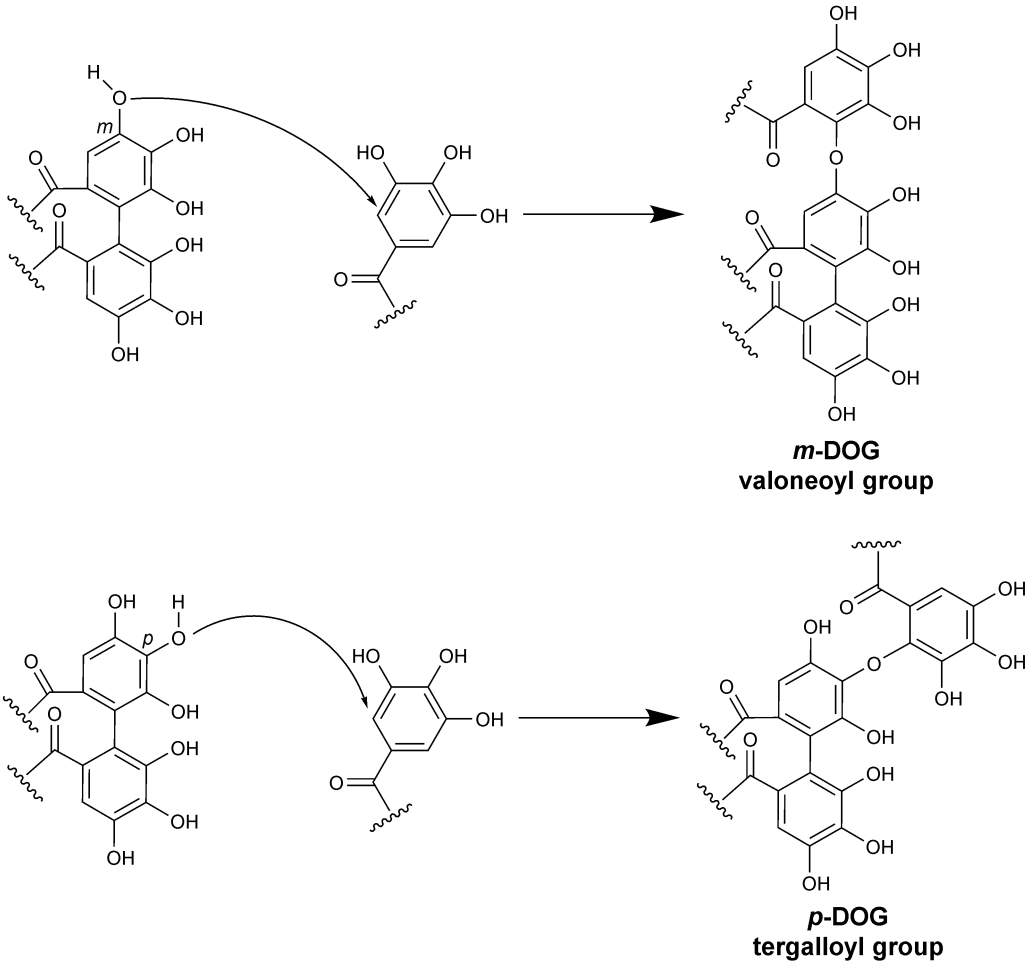

Figure 2. Formation of the valoneoyl group (*m*-DOG) and tergalloyl group (*p*-DOG).

- The GOD-type linkage

The *O*-donating unit is a galloyl group and the *O*-accepting unit is an HHDP moiety (Figure 3). So far, only *m*-GOD groups have been found in nature.

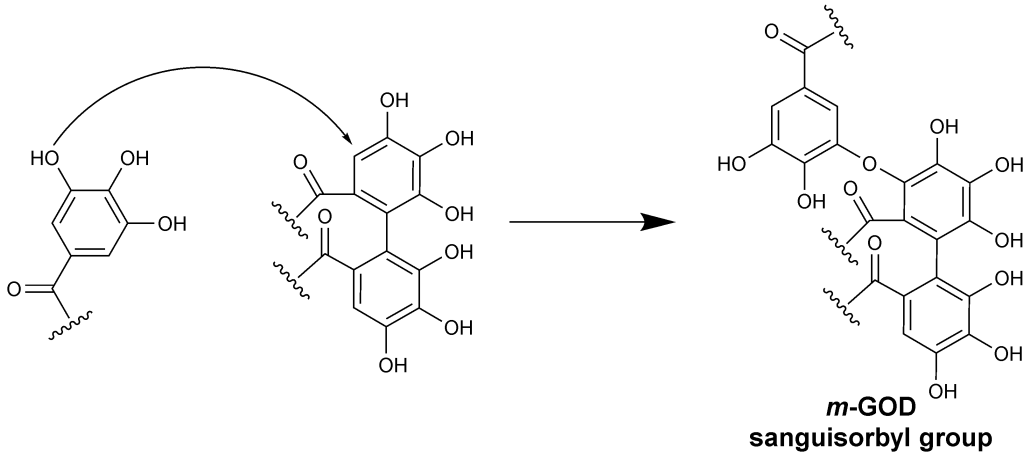

Figure 3. Formation of the sanguisorboyl group (*m*-GOD).

2.2. Ellagitannins of *Onagraceae*

In 2010, Karonen *et al*. extracted roots and leaves of *Oenothera biennis* and subsequently performed a careful fractionation of the extracts using column chromatography with Sephadex LH-20 gel. The fraction eluted with acetone/H_2O (7:3, v/v) was analyzed by HPLC-DAD-MS and revealed the presence of tellimagrandin I oligomers as large as heptamer.

Tellimagrandin I (monomer), oeonothein B (dimer) and oeonothein A (trimer) had been identified in *Oenothera* species in the early 1990s and their structures had been elucidated by NMR combined with chemical derivatization techniques (Hatano et al., 1989; Yoshida et al., 1991). Oeonothein B results from the dimerization of two tellimagrandin I monomers, linked together via two *m*-DOG bonds, thereby forming a macrocyclic structure (Figure 4). Oenothein A, on the other hand, arises from the formation of an *m*-DOG bond between the galloyl moiety in C2 position of a tellimagrandin I monomer and an HHDP moiety of oenothein B (Figure 4).

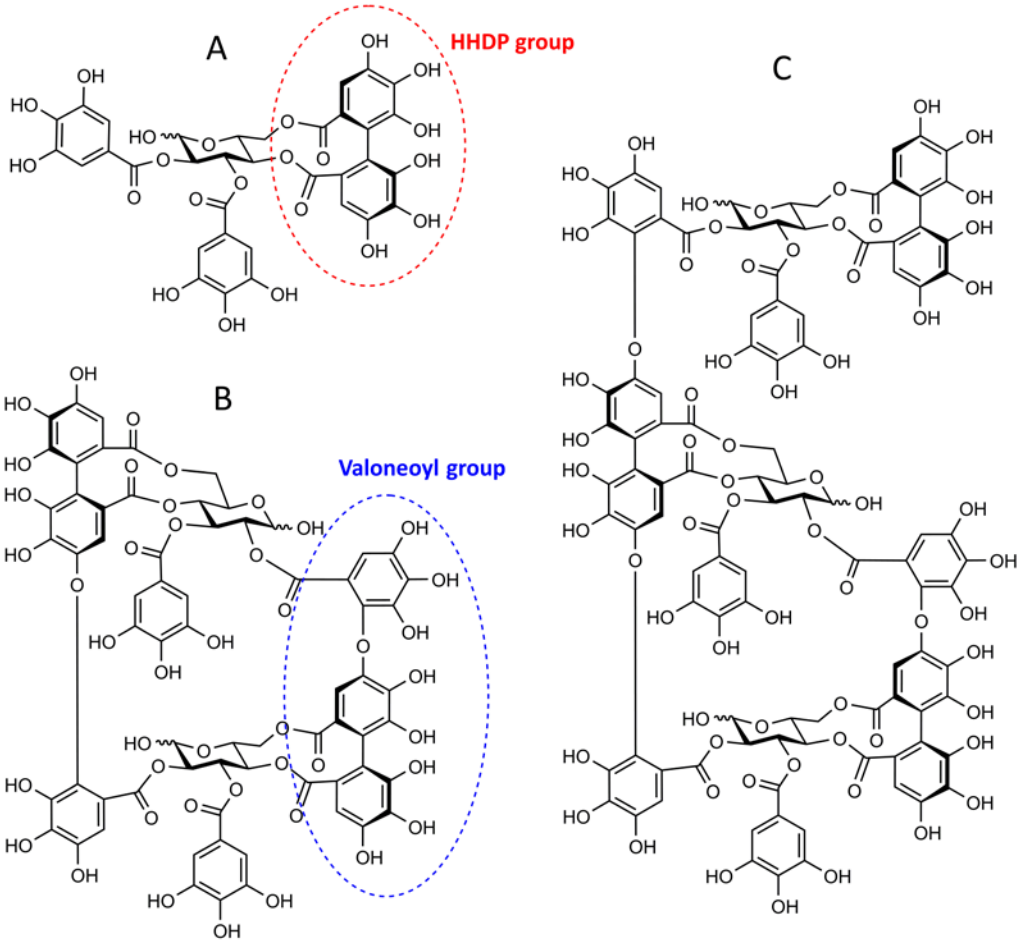

Figure 4. Structure of tellimagrandin I (**A**), oenothein B (**B**) and oenothein A (**C**).

The analyses of fragmentation patterns by high-resolution mass spectrometry revealed that larger oligomers were built by adding tellimagrandin I monomers via a single DOG-type bond. This process generates asymmetric oligomeric chains with a macrocyclic oenothein B core at one end (Figure 5).

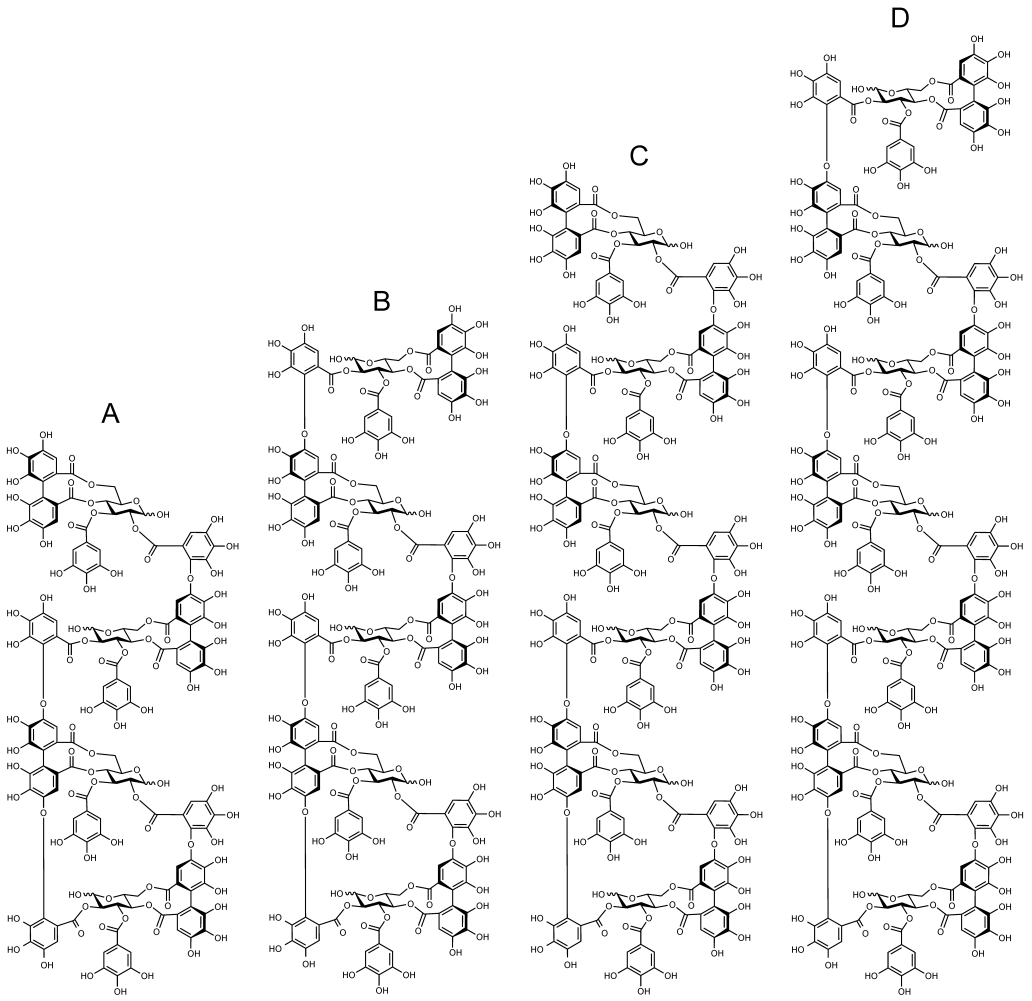

Figure 5. Structures of tetrameric (**A**), pentameric (**B**), hexameric (**C**) and heptameric (**D**) tellimagrandin I-based oligomers.

2.3. *Epilobium angustifolium*

Epilobium angustifolium L.—also known as *Chamerion angustifolium* L.—is a perennial herbaceous plant that belongs to the *Onagraceae* family (Figure 6). It is called “fireweed” in North America, “rosebay willow-herb” in Great Britain and “maitohorsma” in Finland. It is widespread throughout the Northern hemisphere and extends from Arctic regions to temperate areas of North America and Eurasia (Wagner et al., 2007).

Figure 6. A wild field of fireweed (Left). Details of the inflorescence (Right).

Fireweed extracts have been widely used in traditional medicine for the treatment of many ailments and the therapeutic properties of the plant have been closely investigated since the 2000s (Schepetkin et al., 2016). The medicinal properties of fireweed have been attributed to its high content in polyphenols and more particularly to the most abundant of its secondary metabolites: oenothein B (Ducrey et al., 1997; Kiss et al., 2004, 2006, 2011; Ramstead et al., 2012). Up until 2015, published studies about species within the *Epilobium* genus reported—as far as polyphenols are concerned—several flavonol glycosides, some simple galloyl- and HHDP-glucose derivatives and two macrocyclic ellagitannins: oenothein B and oenothein A: dimeric and trimeric tellimagrandin I, respectively (Granica et al., 2014).

2.4. Modern tools for the analysis of oligomeric ellagitannins

2.4.1. Liquid chromatography-tandem mass spectrometry

A significant part of the present work was dedicated to the detection and quantification of oligomeric ETs and various other polyphenols in fireweed tissue. In this regard, Ultra High Performance Liquid Chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) provided fast and effective separation of the analytes combined with sensitive and selective detection. The sensitivity of the method makes it possible to work with 10 mg of samples (and possibly less) and the short run time per sample (typically between 8 and 10 minutes) permits the analysis of up to one hundred samples a day. Excellent selectivity was achieved using the true potential of the triple quadrupole analyzer: Multiple Reaction Monitoring (MRM).

2.4.2. Multiple reaction monitoring-mass spectrometry

Multiple Reaction Monitoring—also called Selected Reaction Monitoring (SRM)—corresponds to a specific operating mode of the QqQ analyzer (Figure 7). In that mode, the first quadrupole (Q1) is held static and will only let ions with a selected *m/z* value go to the next quadrupole (i.e. the collision cell). Selected ions undergo collision-induced dissociation (CID) in the second quadrupole (q2). The third quadrupole (Q3) is also kept in static mode and acts as a filter that only allows fragments with selected *m/z* value(s) to hit the detector.

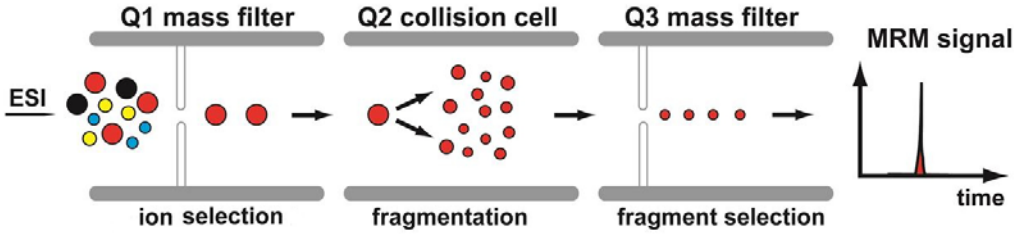

Figure 7. Diagram showing the principle of SRM/MRM with a triple quadrupole analyzer.

Thus, SRM/MRM provides excellent selectivity and sensitivity and is perfectly suited for quantification purposes. However, it requires preliminary characterization of the analytes in order to find, for each transition, the optimal cone voltage and collision energy that will maximize ionization and fragmentation, and will consequently yield a stronger signal. In mass spectrometry, a transition corresponds to the *m/z* values of a precursor ion and one of its fragments. It is noted precursor > fragment (e.g. 195 > 138 for caffeine in positive mode). The difference between MRM and SRM resides in the fact that MRM monitors several transitions for the same precursor ion whereas SRM monitors only one per ion. MRM/SRM allows the monitoring of several transitions at the same time, thus enabling the detection and quantification of co-eluting compounds. However, overlapping transitions divide the time that the instrument dedicates to acquiring data for each transition, which is called the dwell time. A higher dwell time ensures a higher number of data points per peak which increases signal/noise ratio and sensitivity, and ultimately results in a more accurate quantification. Therefore, the simultaneous analysis of several compounds by MRM-MS may come at the expense of some accuracy and sensitivity. Nevertheless, most modern instruments allow the monitoring of many simultaneous transitions while maintaining a number of data points per peak that is compatible with quantification purposes (Liebler and Zimmerman, 2013; Ehrhardt et al., 2014; Lambert et al., 2015).

2.5. Activities of tannins on ruminal fermentation

2.5.1. Greenhouse gas emissions from livestock

According to the Food and Agriculture Organization of the United Nations (FAO), agriculture generated 5.2 billion tons (as CO_2 eq.) of greenhouse gas (GHG), worldwide, in 2014 (FAO, 2014). Out of this total, 40% was CH_4 from enteric fermentation and 20% was in the form of N_2O that is produced by the decomposition of ammonia from manure (Gerber et al., 2013). Overall, the livestock sector is responsible for 14.5% of all anthropogenic GHG emissions (in CO_2 eq.) and is the largest contributor to anthropogenic CH_4 emissions (O'Mara, 2011; U.S. Environmental Protection Agency, 2015).

Livestock GHG mitigation strategies revolve around three main concepts (Knapp et al., 2014):

1) Feeding management and nutrition. The quality and composition of feeds have a large impact on methane production. The utilization of good-quality feeds can reduce methane production and increase feed efficiency.
2) Increasing animal production. By improving animal production (through genetics or management approaches) it is possible to decrease CH_4 per unit of product (e.g. milk and meat).
3) Rumen modifiers. This approaches consist in feeding active substances that alter ruminal fermentation.

The use of naturally occurring plant secondary metabolites as rumen modifiers has been extensively studied and has yielded some positive results (Patra and Saxena, 2011; Patra, 2012). Among the effective substances, tannins seem to offer promising prospects. However, there are still a few challenges to overcome before they can be safely used at the farm level, one of the main challenge being a better understanding of their modes of action.

2.5.2. Anatomy and physiology of the rumen of cows

The stomach of ruminants is divided into four compartments: the rumen, the reticulum, the omasum and the abomasum. The rumen (also called paunch) is the first and largest compartment and usually occupies about 65% of an adult cow's stomach volume. It contains about 100 L (depending on the size of the cow) of chewed-up feed mixed with water and saliva and is populated by a large collection of micro-organisms comprising bacteria ($\approx 10^{10}$/mL), ciliate protozoa ($\approx 10^{6}$/mL), fungi and yeast ($\approx 10^{3}$/mL). The main purposes of the rumen are i) to convert carbohydrates into assimilable sources of energy: volatile fatty acids (VFAs) and ii) to breakdown proteins into ammonia (NH_3). These

functions are performed by a combination of mechanical and biochemical processes. The mechanical actions aim at reducing the size of feed particles in order to increase their degradability by microbes. Cows spend little time chewing while they eat. The most effective chewing activity is performed through a process called cud-chewing (or rumination). During rest periods, cows regurgitate feed boli (cuds) into their mouths and rechew them. Another important role of rumination is to stimulate the production of saliva which is a key component of rumen liquor because it acts as a lubricant and a buffer that maintains the pH between 6.2 and 6.8 for an optimal digestion of feedstuff by micro-organisms. In a day, a typical dairy cow spends 7-10 hours ruminating, mostly while lying down, and produces about 200 L of saliva (Maekawa et al., 2002).

2.5.3. Anaerobic fermentation of carbohydrates and methanogenesis

The first step in the fermentation of carbohydrates into VFAs is the depolymerization of the various polysaccharides that compose the diet of the animal. Starch, cellulose, pectin and hemicellulose are depolymerized enzymatically by bacteria and protozoa called primary fermenters. Thereafter, simple sugars are utilized by both primary and secondary fermenters to produce pyruvate and energy (in the form of ATP) by glycolysis (Figure 8). Glycolysis is an oxidative process that requires the reduction of NAD^+ into NADH according to the following stoichiometry:

$$Glucose + 2\ ADP + 2\ P_i + 2\ NAD^+ \longrightarrow 2\ Pyruvate + 2\ ATP + 2\ NADH + 2\ H^+$$

Pyruvate is then converted into various end products, depending on the micro-organisms and the conditions inside the rumen. In cows, the three main products are (on molar proportion): acetate (≈ 65%), propionate (≈ 25%) and butyrate (≈ 10%) (Lin et al., 1985).

In order to maintain glycolysis, NADH has to be re-oxidized into NAD^+. In eukaryotic cells, and under aerobic conditions, the re-oxidation of NADH into NAD^+ takes place in the mitochondria and uses molecular oxygen (O_2) as the final electron acceptor. The rumen, however, is a fairly anoxic environment and the large majority of fermentative microorganism are prokaryotes. Therefore, the regeneration of NAD^+ in the rumen has to take other pathways.

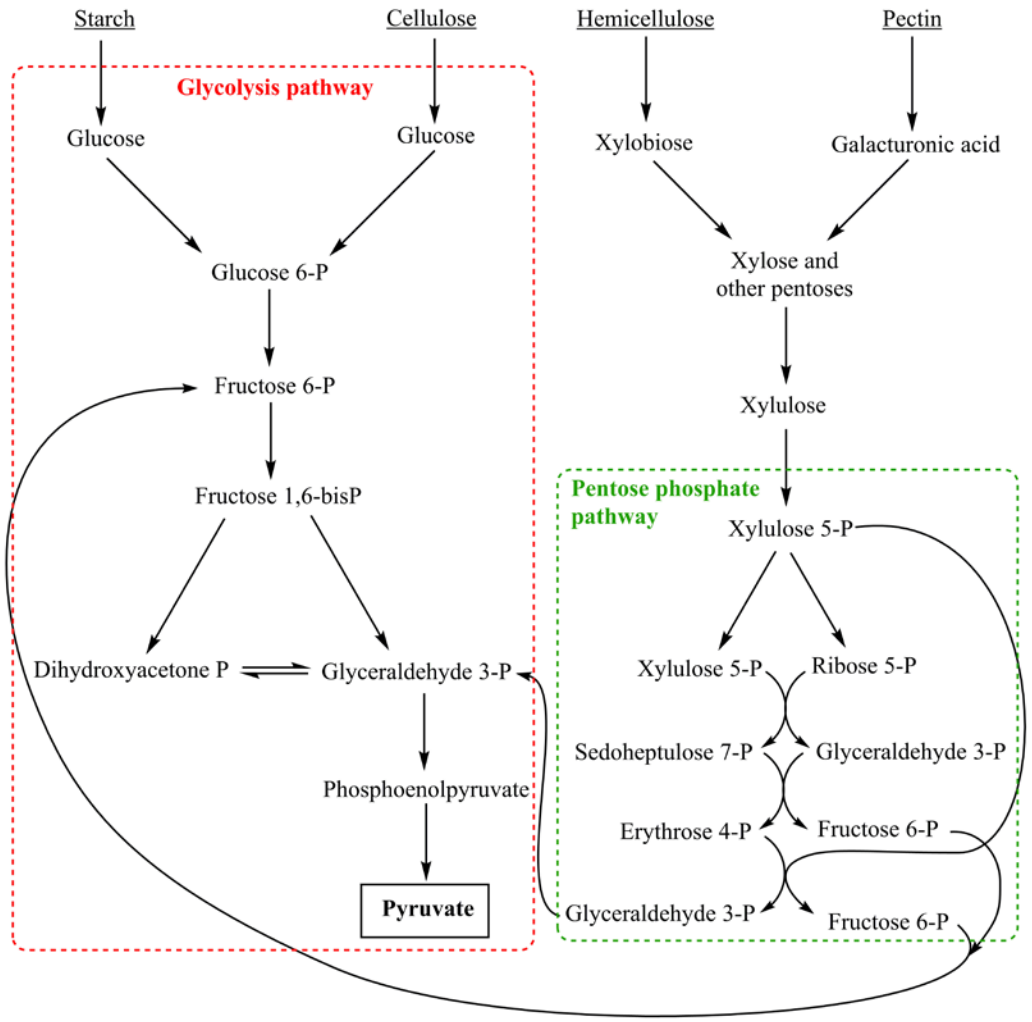

Figure 8. Fermentation pathways of polysaccharides in the rumen (adapted from Lin et al., 1985).

Fermentative microorganisms possess several biochemical pathways that can re-oxidize NADH into NAD^+. However, the most prevalent (and the most relevant with regards to methanogenesis) involves a ferredoxin oxidoreductase coupled with a hydrogenase (Figure 9). This chemical equilibrium is regulated by the partial pressure of H_2 (p_{H_2}). In a normal rumen (pH ≈ 6.8, T = 39°C), when p_{H_2} is low (< 10 Pa) the oxidation of NADH is thermodynamically favored. Conversely, high p_{H_2} (> 10 Pa) inhibits the hydrogenase activity and prevents the regeneration of NAD^+ (Liu and Whitman, 2008).

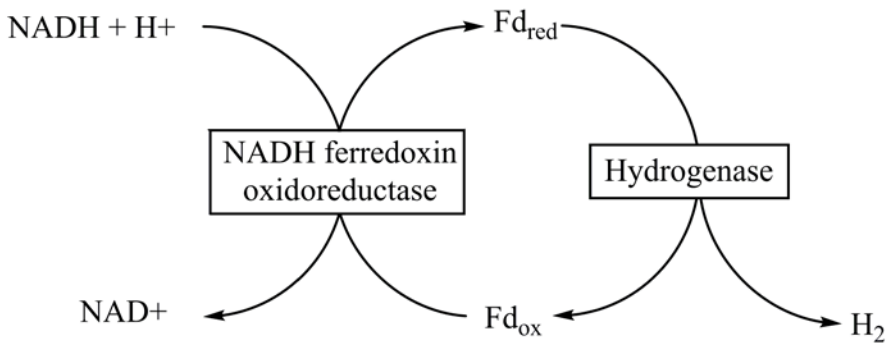

Figure 9. Regeneration of NAD^+. (Fd_{red}: reduced ferredoxin; Fd_{ox}: oxidized ferredoxin).

Consequently, the existence of metabolic hydrogen sinks in the rumen is critically important in order to maintain low p_{H_2} which enables the regeneration of NAD^+, and one the most predominant sink is methanogenesis.

With the exception of propionate, the fermentation of pyruvate into VFAs produces hydrogen and CO_2 as by-products (Figure 10):

$$Glucose + 2\,H_2O \longrightarrow 2\,Acetate + 8\,H + 2\,CO_2$$

$$Glucose \longrightarrow Butyrate + 4\,H + 2\,CO_2$$

$$Glucose + 4\,H \longrightarrow 2\,Propionate + 2\,H_2O$$

Hydrogen, however, does not accumulate in a normal rumen because it is rapidly used by methanogenic archaea for reducing CO_2 into CH_4 according to the following reaction:

$$CO_2 + 4\,H_2 \longrightarrow CH_4 + 2\,H_2O$$

The flow of H_2 from fermentative species to methanogens is called “interspecies hydrogen transfer”. By preventing the accumulation of H_2, methanogens indirectly enhance the degradation of cell wall polysaccharides by fibrolytic species (Bauchop and Mountfort, 1981; Ushida and Jouany, 1996). Interspecies hydrogen transfer can be facilitated by close, physical association between the two partners. Indeed, a significant fraction of ruminal methanogens live in symbiosis with ciliated protozoa, as both exo- and endosymbionts (Tokura et al., 1997). It has been estimated that these symbiotic methanogens are responsible for 9 to 25% of the methane produced in the rumen (Newbold et al., 1995).

Methanogenesis is very dependent on the rumen conditions and the type of feed. More precisely, fiber-rich diet tends to produce more methane than starch and soluble sugars (Beuvink and Spoelstra, 1992; Johnson and Johnson, 1995). The reason is that starch

and soluble sugars are preferably fermented into lactate which is a precursor of propionate (Figure 10). The propionate pathway being a hydrogen sink, it competes with methanogenesis for the use of H_2 and therefore decreases the amount of CH_4 per unit of glucose. More generally, the enhancement of the propionate pathway at the expense of acetate and butyrate will tend to reduce CH_4 production. For that reason, CH_4 production is closely associated with the ratio of acetate/propionate (C2/C3) (Moss et al., 2000).

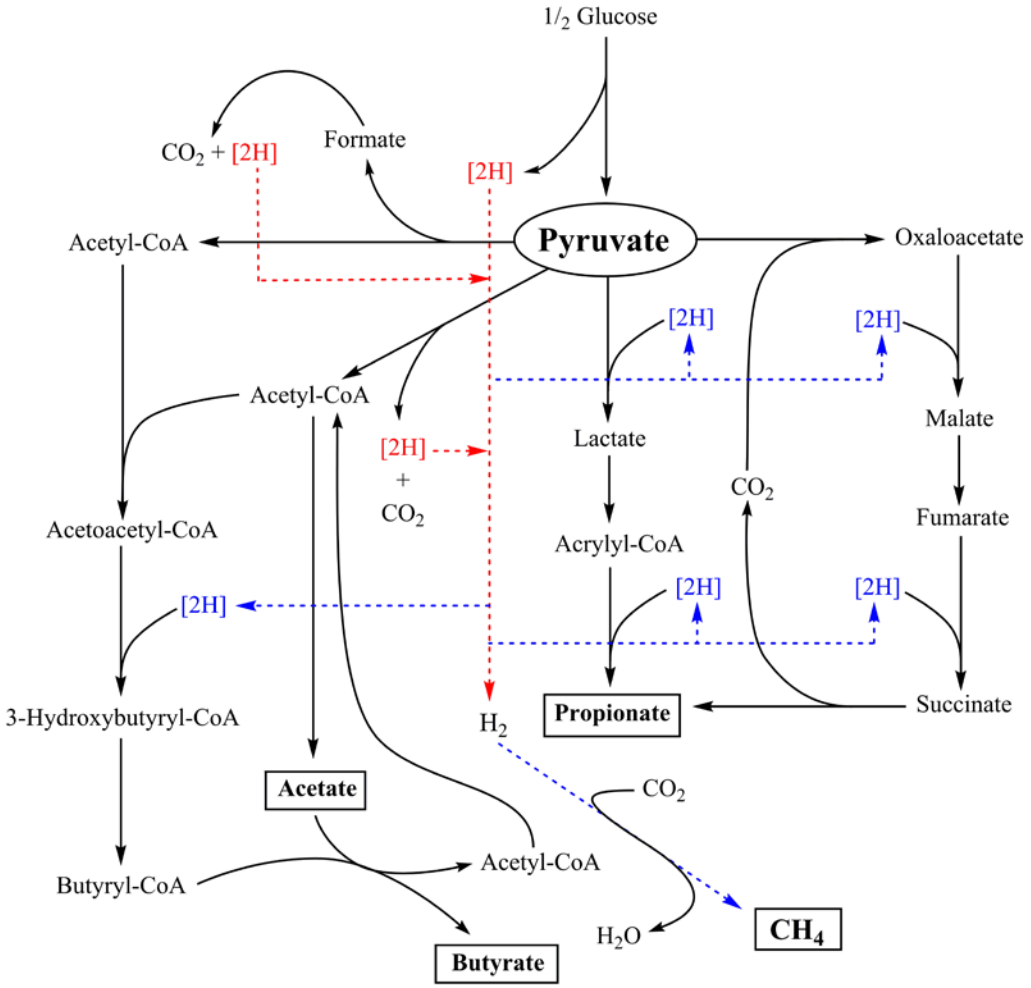

Figure 10. Metabolic pathways leading to the formation of acetate, propionate, butyrate and methane by the rumen microbiota. Dashed red lines represent metabolic sources of hydrogen, dashed blue lines represent hydrogen sinks (adapted from Mitsumori and Sun, 2008).

2.5.4. Nitrogen metabolism in the rumen

Feed proteins provide the main source of nitrogen and amino-acids needed to maintain vital functions and growth. In ruminants, dietary proteins undergo two different fates. Upon entering the rumen, the majority of feed proteins is degraded by bacteria which utilize that source of nitrogen for their own anabolism. A significant amount of these bacteria, however, flows to the duodenum and is degraded by digestive enzymes, thus rendering microbial proteins available to the animal. These ruminally synthetized microbial proteins represent the main source of amino acids absorbed by the animal (Bach et al., 2005). In addition to this primary source, a portion of the dietary proteins resists bacterial degradation and passes to the duodenum whereupon they are hydrolyzed by proteolytic pancreatic enzymes. This phenomenon has been dubbed “ruminal protein escape” (Figure 11). The amount of proteins that resist bacterial degradation varies greatly and mostly depends upon the chemical characteristics of feedstuff proteins (National Research Council, 2001). In addition to crude proteins, feedstuff sometimes also contains non-protein nitrogen (NPN). Inside the rumen, these compounds are hydrolyzed into ammonia by bacteria which can then incorporate it for their own amino acid synthesis. Amino acids and small peptides that reach the duodenum are absorbed and subsequently transported through the intestinal wall to the portal vein which carries them to the liver. In the liver, amino acids can be either catabolized into urea or transported to the general blood circulation and distributed to body cells that will utilize them for *de novo* protein synthesis.

In the rumen, free ammonia exists as an equilibrium between NH_3 and NH_4^+. Because pH inside the rumen is usually slightly below 7 and the pKa of NH_4^+/NH_3 is 9.2, ammonia is predominantly in the form ammonium. The basic form NH_3, however, has the ability to diffuse passively through the ruminal wall to the hepatic portal vein. Ammonia from the blood is then taken up and converted into urea by hepatocytes (Owens and Bergen, 1983). Part of the plasmatic urea is filtered out by kidneys and excreted into urine. The other part, however, can re-enter the rumen. This process, unique to ruminants, is called nitrogen-recycling (Lapierre and Lobley, 2001). Plasmatic urea can diffuse from the bloodstream to the ruminal lumen through the ruminal epithelium. Additionally, a fraction of plasmatic urea is taken up by salivary glands and subsequently excreted into saliva (Figure 11). The nitrogen-recycling process provides a steady source of NPN to ruminal microorganisms while decreasing urinary excretion of nitrogen. By recycling 23 to 92% of plasmatic urea, nitrogen-recycling is an effective way of saving nitrogen, especially in cases of low nitrogen diets (Kennedy and Milligan, 1980).

Degradation of dietary proteins by ruminal bacteria typically observes the following events: (1) extracellular proteolysis leading to small peptides and free amino acids, (2)

bacterial uptake of small peptides and free amino acids, (3) utilization of free amino acids for protein synthesis (4) deamination of free amino acids producing ammonia and carbon skeletons, (5) utilization of ammonia for resynthesis of amino acids, and (6) excretion of ammonia into the ruminal lumen (National Research Council, 2001).

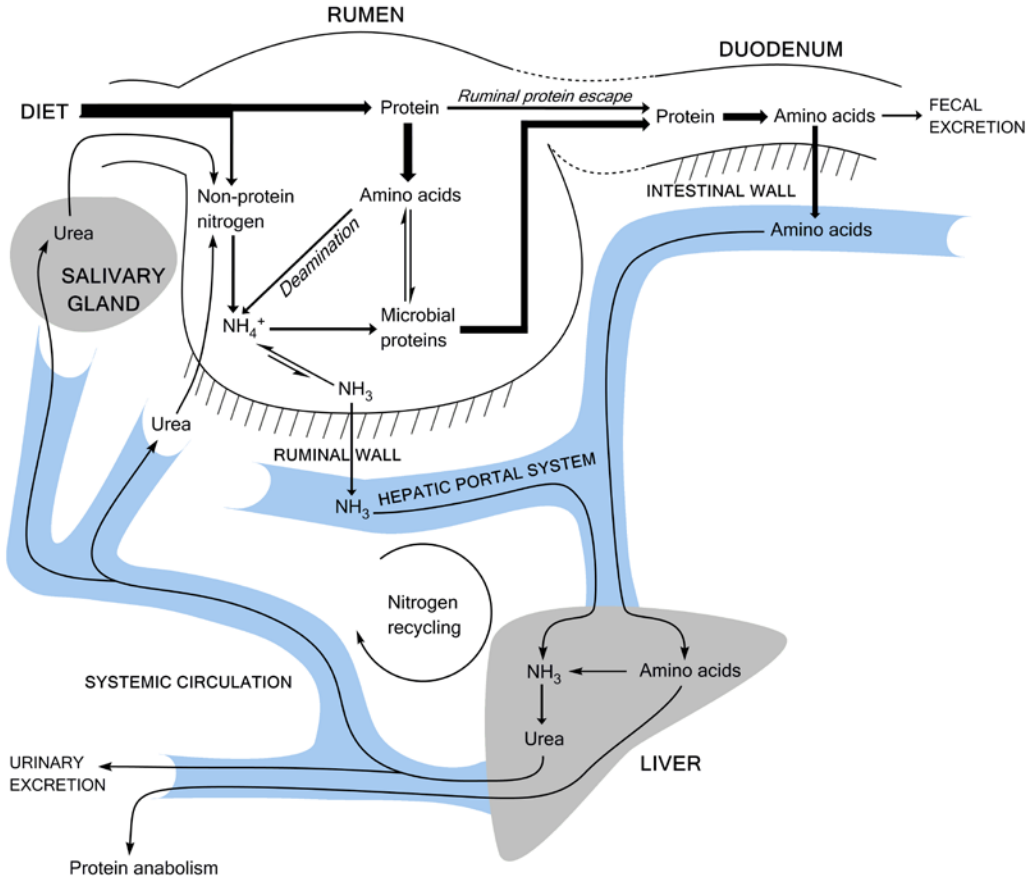

Figure 11. Metabolism of dietary proteins in ruminants.

Deamination of branched-chain amino acids valine, leucine and isoleucine by ruminal bacteria produces isobutyrate, isovalerate and 2-methylbutyrate respectively (Figure 12) (Menahan and Schultz, 1964; Robinson and Allison, 1969). These branched-chain volatile fatty acids (BCVFAs) are essential growth factors for several ruminal microorganisms. They can be utilized for re-synthesis of the branched-chain amino acids of which they derive or for synthesis of long-chain branched fatty acids and branched-chain aldehydes (Allison, 1969, 1978). They are considered good markers of bacterial proteolytic activity and are often monitored in *in vitro* studies to estimate the extent of dietary protein degradation by ruminal bacteria.

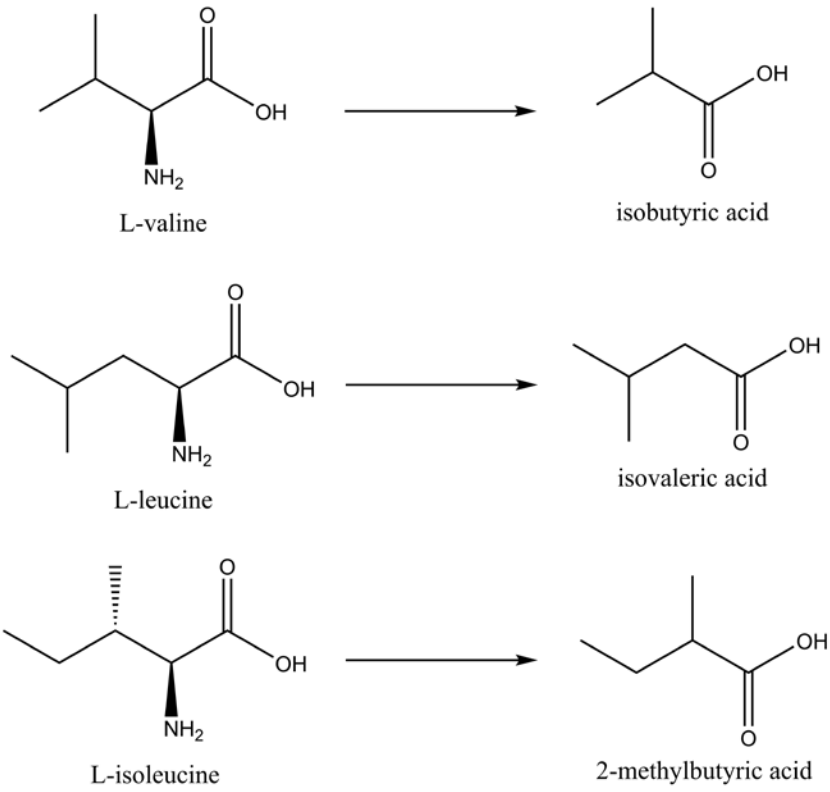

Figure 12. Structures of the branched-chain volatile fatty acids and their branched-chain amino acids precursors.

2.5.5. Direct and indirect actions of tannins toward methanogenesis

Despite having gained a somewhat exaggerated reputation of toxic, antinutritional compounds in the 1970s (Butler and Rogler, 1992), tannins recently showed that they could benefit ruminant nutrition, provided that they were used adequately. An optimal use of tannins, however, entails a comprehensive understanding of their modes of action. To date, this endeavor is still an ongoing process due to the large chemical diversity of tannins and the complexity of animal physiology. In this chapter I will focus on the ability of tannins to interact with various processes of ruminal fermentation.

In the early 2000s, various *in vivo* experiments started to show that proanthocyanidin-rich forages decreased methane emissions from goats and dairy cows (Woodward et al., 2001, 2002; Waghorn et al., 2002; Puchala et al., 2005). These promising results spurred the need to understand how tannins could inhibit methanogenesis and thus many *in vitro* and *in vivo* studies were conducted to answer that question.

The action of tannins on methanogenesis can be schematically divided into two categories: direct and indirect. Direct action implies that tannins suppress methanogenesis by acting directly on methanogens. Although the exact mechanisms of such inhibition remains unclear, this mode of action has been evidenced by *in vitro* experiments involving both PAs and HTs (Tavendale et al., 2005; Bhatta et al., 2009). By impeding the most prevalent hydrogen sink in the rumen, the direct inhibition of methanogens stimulates alternate hydrogen sinks such as the propionate pathway (Figure 10). Therefore, an increase in propionate and a decrease in the C2/C3 ratio are good indicators of direct inhibition of methanogens.

Indirect antimethanogenic action, on the other hand, corresponds to a decreased fermentation of organic matter—particularly fibers, resulting in a decreased production of H_2. With less H_2 available, methanogenesis is hampered. Tannins can inhibit fiber degradation by reducing the number of cellulolytic microorganisms, by inhibiting cellulolytic enzymes and by forming complexes with cellulose, thereby precluding contact between microorganisms and fibers (Śliwiński et al., 2002). Another indirect effect on methanogenesis is mediated via the action of tannins on ciliate protozoa. *In vitro* studies have shown a decrease in protozoal counts associated with a decrease in CH_4 production from rumen fluids treated with tannin-rich plant extracts (with both HTs and PAs) (Patra et al., 2006; Bhatta et al., 2009, 2015). The total or partial removal of protozoa from the rumen is known as defaunation. This process has been used as a method to study the roles of protozoa in ruminal fermentation. The action of defaunating agents on methanogenesis is twofold: it removes a source of H_2 and it eliminates the population of methanogens that are symbiotically associated with protozoa. Through these two mechanisms, defaunation can decrease CH_4 production. However, a meta-analysis has shown that CH_4 emission from experimentally defaunated animals varies greatly (from −49.6 to +11.1%) depending on the diet the animals were fed (Morgavi et al., 2010).

By acting directly and indirectly on methanogenesis, tannins have the ability to disrupt ruminal CH_4 production, at least partially. The main questions that need to be addressed now are: what are the precise molecular mechanisms by which tannins cause the above-mentioned activities? What are the chemical features responsible for these interactions? Can tannins decrease methanogenesis without impairing feed degradability?

2.5.6. Actions of tannins on nitrous oxide emissions

The emission of N_2O from manure originates from the oxidation of NH_4^+ by bacteria (Bothe et al., 2000; Kowalchuk and Stephen, 2001). Nitrogen in the feces is mostly in the form of bacterial and endogenous proteins (Bosshard et al., 2011) whereas urinary nitrogen largely consists of urea. In contact with soil bacteria, urea is rapidly hydrolyzed

into CO_2 and NH_4^+ which is further converted into NO_3^-, NO_2^-, NO and N_2O by microorganisms through various reactions of nitrification/denitrification (Firestone and Davidson, 1989; Cabrera et al., 1991). Fecal nitrogen, however, is more stable against bacterial degradation and therefore generates less N_2O than urine (Ussiri and Lai, 2012). By limiting ruminal protein degradation, tannins are able to decrease urinary nitrogen excretion and increase fecal excretion instead (Narjisse et al., 1995; McSweeney et al., 2001). This shift in the excreted form of nitrogen decreases N_2O emission from manure.

2.6. Anthelmintic activity of tannins

2.6.1. Gastrointestinal nematodiasis in ruminants

Gastrointestinal nematodiases affect livestock worldwide. They are arguably one of the biggest health challenge that ruminants have to face and can be responsible for massive financial losses in animal production, particularly in developing countries (Perry and Randolph, 1999). Those diseases are caused by parasitic round worms called nematodes. The most prevalent gastrointestinal nematodes of ruminants belong to the superfamily Trichostrongyloidea. The adult stage of the parasites thrives in the gastrointestinal tract of ruminants and causes damage to the mucosae (Barker and Beveridge, 1983; Hoste et al., 1988). Low worm burden is generally asymptomatic but as the number of worms increases subclinical symptoms start to appear. They may vary depending on the nematode species but usually comprise reduced weight gain and loss of appetite. Clinical nematodiasis occurs with heavier worm burdens and is characterized by diarrhea, weight loss, anemia and can lead to the death of the animal if not treated (Zajac, 2006).

2.6.2. Parasitic life cycle

Most gastrointestinal nematodes belonging to the Trichostrongyloidae superfamily share the same archetypal life cycle. Adult female parasites in the digestive tract produce eggs that are excreted along with the feces. The embryos develop in the fecal mass until they become first-stage larvae (L1) which hatch out of the eggs. Freshly hatched L1 feed on bacteria and undergo two molts thus reaching the third stage (L3) which corresponds to the infective larval stage. Third-stage larvae leave their habitat of fecal material and start migrating onto grass and forage where they can be ingested by grazing or browsing animals (typically sheep and goats). L3 larvae travel through the digestive tract of the host until they reach their site of predilection (e.g. abomasum or small intestine), whereupon they burrow into the crypts of the lining and complete their development. Adult male and female worms mate and, after a pre-patent period, female start to lay eggs, thereby perpetuating the cycle (Viney and Lok, 2007).

2.6.3. Veterinary anthelmintics

The first major progress in the treatment of gastrointestinal nematodiases was made in the 1960s with the discovery of thiabendazole (which led to the development of other benzimidazoles) and levamisole (an agonist of the nicotinic acetylcholine receptor). For the first time in history, veterinarians had access to molecules with broad-spectrum activity, good tolerance and *in vivo* efficacy at the level of milligram per kg of body weight (McKellar and Jackson, 2004). Another step forward was made in 1981 when ivermectin—a macrocyclic lactone—became commercially available. With its even broader spectrum of activity (which includes several ectoparasites) and microgram per kg dosage, ivermectin quickly became a "blockbuster" in veterinary medicine (Crump and Ōmura, 2011).

Unfortunately, the intensive use of anthelmintic drugs as the only strategy to control gastrointestinal nematodes quickly led to the emergence of resistances (Waller, 1994; Fleming et al., 2006). The first substantiated cases of resistance to thiabendazole were reported in *Haemonchus contortus* as early as 1964, only three years after the drug was marketed (Conway, 1964; Drudge et al., 1964). Other studies were conducted worldwide, and by the mid-1970s it became clear that benzimidazole resistance was present in several nematode species all over the world (Berger, 1975; Coles and Simpkin, 1977; Kelly and Hall, 1979). Few years later, scientists found that the same fate had befallen the other two classes of anthelmintic drugs (Sangster et al., 1979; van Wyk and Malan, 1988). The situation quickly escalated when, at around the same time, scientists started to report cases of multiple-drug resistance (Watson and Hosking, 1990; Várady et al., 1993; Praslička et al., 1994). To date, the problem threatens many animal productions worldwide and has become, in certain areas of the globe, one of the biggest obstacle to the success of these enterprises.

Though very successful for a while, the massive reliance on anthelmintic drugs for the control of gastrointestinal nematodes proved to be a shortsighted, unsustainable strategy. Fortunately, these drugs are by no means the only control method, and promising alternatives are being investigated (Stear et al., 2007). In brief, they consist in:

1) Grazing management: the principle of this method is to reduce the exposition of animals to contaminated pastures and to limit further contamination of clean pastures through various grazing schemes.
2) Vaccination.
3) Selective breeding: selection of animals with naturally enhanced resistance or resilience to nematodiases.
4) Nutritional supplementation: adapting the composition of the diet for infected animals can prevent or slow down the onset of clinical symptoms.

5) Biological control corresponds to the utilization of natural agents (e.g. fungi and plants) to disrupt certain key-stages of the parasitic life-cycle.

It has become increasingly evident that the complete elimination of the parasites from infected flocks and pastures was a pipe dream. Therefore, the focus of current strategies has shifted toward maintaining the disease at the asymptomatic or subclinical levels. By using an adequate combination of the abovementioned strategies, it may be possible to maintain low worm burdens and, ultimately, to keep symptoms at the subclinical level or below.

2.6.4. Why tannins?

The use of tannins—and of plant secondary metabolites in general—as a natural way to control gastrointestinal nematodiases has been investigated since the 1990s. *In vivo* trials showed that infested lambs fed forages containing proanthocyanidins experienced less severe symptoms than animals fed forages with no PAs (Niezen et al., 1993, 1995; Robertson et al., 1995). In those experiments, it was not possible to determine whether the observed effects of PA-rich forages came from a direct action of PAs on parasites or from an indirect action by enhancing the immune response of the host or, most likely, from a combination of both direct and indirect.

The indirect action hypothesis is supported by data from *in vivo* trials showing an increased local immune response in the abomasal mucosae in animals fed sainfoin hay or fresh red clover (Tzamaloukas et al., 2006; Ríos-de Álvarez et al., 2008). There is, however, no evidence regarding the mechanisms by which PAs trigger such response, though it has been hypothesized that that effect could come from an increased supply of amino acids mediated by the ability of PAs to increase ruminal protein escape (Niezen et al., 1995). The direct-action hypothesis, on the other hand, is based on a more conventional, pharmacological activity of tannins on the adult worms whereby tannins would disrupt vital functions of the worms, thus increasing their mortality and decreasing their fecundity. It is supported by several *in vitro* trials (Molan et al., 2000; Athanasiadou et al., 2001; Novobilský et al., 2013) but, to date, no *in vivo* study has been able to show this direct effect (partly because of the difficulty to rule out the indirect effect in an *in vivo* system).

Interestingly, the vast majority of the research on the anthelminthic activity of tannins and tannin-rich plants has focused on PAs, thus neglecting HTs (most probably on the account of their overrated anti-nutritional effect). At the time my research began, there were only two papers investigating the effect of HTs on nematodes, and both were conducted on the free-living soil nematode *Caenorhabditis elegans* (Katiki 2013, Mori 2000). Yet, both studies show promising results that are worth investigating on pathogenic species such as *Haemonchus contortus* and *Trichostrongylus colubriformis*.

Another lacking aspect of the research on the anthelminthic effect of tannins—and a fact that has been repeatedly pointed out by authors themselves—is the absence of endeavors to understand which chemical and structural features of tannins drive their activities. Indeed, the entirety of *in vivo* and *in vitro* studies conducted before 2016 have used crude extracts of varying purity, from various plant species. Although this approach has permitted the screening of several tannin-rich forages, it has likely been the source of large discrepancies in the results between studies and of the poor reproducibility of *in vivo* trials. The use of characterized extracts (*i.e.* of known tannin fingerprint) would increase the reproducibility of both *in vivo* and *in vitro* assays. Furthermore, it would allow to identify some structure-activity relationships by comparing the effects of extracts that differ slightly in their compositions. An even more controlled way to address that question is to compare the effects of pure molecules—similarly to what is done in drug development—and infer the chemical characteristics that determine the therapeutic effects (both direct and indirect) of tannins against nematodiases. However, due to the difficulty of separating PAs larger than trimer, the pure-molecule approach is virtually impossible to implement with this class of tannins. Hydrolysable tannins, on the other hand, are much easier to isolate and offer a vast chemical library of molecules which often feature minute structural differences (such as the absolute configuration of an asymmetric carbon atom).

3. AIMS OF THE STUDY

This work focused on several aspects of oligomeric ETs, from their detection and quantification in plants to their bioactivities *in vitro*. This piece of research was driven by two main ideas: i) testing whether willowherb could have some beneficial effects in animal nutrition and ii) understanding the chemical basis for those activities. In order to achieve those goals, I accomplished the following tasks:

1. To extract, isolate and purify dimeric to heptameric tellimagrandin I from *E. angustifolium* in order to create quantification standards and to have sufficient quantities of purified molecules for *in vitro* bioassays (Articles **I**–**IV**).

2. To characterize the purified oligomeric ETs by high-resolution MS (Article **I**).

3. To develop a UHPLC-MS/MS method based on MRM technology that can detect and quantify oligomeric ETs in extracts of *E. angustifolium* (Article **I**).

4. To adapt an *in vitro* technique of ruminal fermentation to the use of pure ETs and use it to understand how the degree of oligomerization of ETs affect their activities on ruminal fermentation (Article **II**).

5. To investigate the inter- and intra-individual variability of the concentration of oligomeric ETs in different populations of *E. angustifolium* along with other important polyphenols (Article **III**).

6. To test the anthelmintic activity of oligomeric ETs on the adult stage of *Trichostrongylus colubriformis*, an intestinal nematode of goats and sheep (Article **IV**).

4. MATERIALS AND METHODS

4.1. Isolation and purification of oligomeric ellagitannins

Fresh flowers of *E. angustifolium* (2 kg) were macerated in 10 L of acetone for 48 h at 4°C. The liquid phase was removed and replaced by an equal volume of acetone/water (4:1, v/v). Extraction went on for 8 weeks at 4°C. The extract was then filtered on a filter paper, the acetone was evaporated under reduced pressure and the remaining aqueous solution was frozen and lyophilized, yielding several hundred grams of extract.

Flower extract (30 g) was then mixed to a slurry of Sephadex LH-20 (in 100% water). The slurry was eluted successively with: 3 × 250 mL of water (fraction 1), 3 × 250 mL of methanol/water (1:1, v/v) (fraction 2), 3 × 250 mL of methanol (fraction 3), 6 × 250 mL of acetone/water (4:1, v/v) (fraction 4). Elution was carried out in a Büchner funnel (Ø = 240 mm) with a filter paper and using reduced pressure to accelerate the elution. An aliquot of each fraction was analyzed by UHPLC-DAD–ESI-MS/MS and the results showed that fraction 4 had the highest content in ETs. Fraction 4 was therefore used for the subsequent purification steps. Acetone was evaporated under reduced pressure and the remaining aqueous phase was frozen and lyophilized. This first fractionation step was repeated several times until it yielded a sufficient amount of material for the next steps.

Sephadex LH-20 gel was loaded into a glass column (40 cm × 4.8 cm internal diameter, Kimble-Chase Kontes™ Chromaflex™) and equilibrated with 1500 mL of ultrapure water at a flow rate of 5 mL min^{-1}. Three grams of fraction 4 were dissolved in 15 mL of ultrapure water, filtered (0.2 µm, PTFE) and applied on top of the gel. Elution started with 100% water and the proportion of acetone was gradually increased up to 60%. An aliquot of each fraction was analyzed by UHPLC-DAD–ESI-MS/MS and fractions with a similar composition were combined together. Acetone was evaporated under reduced pressure and the aqueous phase was frozen and lyophilized. This purification step was repeated several times in order to maximize the amount of each fraction and these Sephadex fractions were further purified by preparative HPLC.

The HPLC unit was constituted of a Waters 2535 Quaternary Gradient module coupled with a Waters 2998 Photodiode Array Detector. Collection was automatically operated by a Waters Fraction Collector III. I used a binary solvent system methanol/0.1% aqueous formic acid at a constant flow rate of 8 mL min^{-1}. The column (30 cm × 2.5 cm internal diameter) was packed with RP-18 (40–63 µm). Elution protocols were adjusted based on the composition of the fractions. The collected fractions were evaporated to dryness overnight using a Savant SC 210A SpeedVac Concentrator coupled with a

Savant RVT 5105 Refrigerated Vapor Trap from Thermo Scientific. Individual oligomeric ETs were lastly purified by semi-preparative HPLC.

The HPLC unit was the same as described above. The solvent system was acetonitrile/0.1% aqueous formic acid and was set at 16 mL min^{-1}. The column was a Gemini® 10 µm C18 110 Å AXIA™ packed (150 mm × 212 mm internal diameter) from Phenomenex. Different elution protocols were used depending on the composition of the fractions. The collected fractions were evaporated to dryness overnight using the evaporator described above.

4.2. UHPLC-DAD-ESI-MS/MS quantification methods

The UHPLC-DAD–ESI-MS/MS instrument used for all the sample analyses in the present study was an Acquity™ UPLC (Waters Corp., Milford, MA, USA) coupled with a XEVO™ TQ triple-quadrupole mass spectrometer (Waters Corp., Milford, MA, USA). The column was an Acquity UPLC BEH Phenyl 1.7 µm, 2.1 mm × 100 mm (Waters Corp., Wexford, Ireland). Elution was carried out with a binary solvent system consisting of acetonitrile (A) and 0.1% aqueous formic acid (B) at a constant flow rate of 0.5 mL min^{-1}. The elution pattern went as follows: 0–0.5 min: 0.1% A; 0.5–5.0 min: 0.1–30% A (linear gradient); 5.0–5.1 min: 30–90% A (linear gradient); 5.1–7.1 min: 90% A (washing); 7.1–7.2 min: 90–0.1% A (linear gradient); 7.2–8.5 min: 0.1% A (stabilization). UV–vis (190–500 nm) and MS data (*m/z* 100 to 2000) were recorded from 0 to 6 min. The electrospray was set on negative mode and the settings were: capillary voltage: 3.4 kV; desolvation temperature: 650°C; source temperature: 150°C; desolvation gas and cone gas (N2) flow rate: 1000 and 100 L h^{-1}, respectively; collision gas: argon.

4.3. Sample preparation for quantitative analysis

Sample extraction was carried out by adding 1400 µL of an acetone/water solution (4:1, v/v) onto 10 mg of freeze-dried plant material. Samples were then shaken for 3 h on a linear shaker in a cold room (4°C). After centrifugation at 13’500 × g for 10 min the supernatant was recovered and stored in a tube and the residue was extracted a second time following the same procedure. The two acetone/water extracts were combined and concentrated under reduced pressure (20°C) until complete evaporation of the acetone. The concentrated aqueous extract was frozen and lyophilized. The freeze-dried extract was dissolved in 500 µL of ultra-pure water and vortexed for 10 min. The resulting solution was then filtered using VWR® 0.2 µm PTFE syringe filters before analysis by UHPLC-DAD–ESI-MS/MS. In parallel, a fraction of the filtered solution was used to make a 1:31 (v/v) dilution which was also analyzed.

4.4. High-resolution mass spectrometry

High-resolution mass spectrometry measurements were performed with two instruments. For the characterization of pure oligomeric ETs, I used an ESI-micrOTOF-Q mass spectrometer (Bruker Daltonics, Bremen, Germany). Approximately 3 mg of each ET (dimer to heptamer) was dissolved in 500 µL of an acetonitrile/H_2O mixture (1:4, v/v), filtered (0.2 µm, PTFE) and analyzed via direct infusion. The mass spectrometer was controlled by Compass micrOTOF control software (Bruker Daltonics) and operated in negative mode. The capillary voltage was maintained at +4000 V with the end plate offset at −500 V. The nebulizer gas (N_2) pressure was set at 0.4 bar, the flow rate of the drying gas (N_2) was 4.0 L min^{-1} and its temperature was set at 200°C. Masses were scanned from m/z 100 to 2000. The calibration of the instrument was performed with a 5 mM sodium formate solution injected by direct infusion before the sample analyses in order to provide high-accuracy mass measurements. Data were processed by Compass DataAnalysis software (version 4.0, Bruker Daltonics). For each ET, I selected the major molecular ion and performed MS/MS fragmentation of it by gradually increasing the collision energy in the collision chamber (argon was used as collision gas). A software-assisted (Bruker Daltonics DataAnalysis 4.0) calculation of the chemical formula was also performed using the monoisotopic peak of the molecular ion.

For the identification of the oxidized derivative of oenothein A from extracts of *E. angustifolium*, I used a UHPLC-DAD-ESI-Orbitrap-MS. The UHPLC unit, DAD, column and elution gradient were identical to those described in paragraph 4.2. The mass spectrometer was a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer with an ESI source set on negative mode. Spray voltage was 3.0 kV; capillary temperature 380°C and S-lens RF level 60. Full MS scan and MS^2 of the main ions were recorded using the Full MS / dd-MS^2 (Top5) procedure of the Thermo Q Exactive software.

4.5. Sample collection for population study

Willowherb samples were collected from 10 distinct populations located around the Aura River in Turku (Southwest Finland) in July 2012. From each population, 10 individual plants were harvested. Shortly after collection, samples were brought back to the laboratory. For the study of interpopulational variations, 5 leaves and 5 flowers were cut off each individual plant from populations #1 to #10. For the study of polyphenol distribution across organs I utilized plants from population #6 only and those individuals were subjected to further sampling. Additionally, to leaves and flowers, I also collected 5 flower buds and 5 pieces of stem. Stem pieces were 5 cm long and were collected at 5 different heights along the stem. They were labelled with letters “A” to “E”. Stem A was

taken from the apex of the plant. Stem B corresponded to the bottom of the inflorescence. Stem C, D and E corresponded to the vegetative part of the stem; they were equally spaced from each other and spanned from the middle of the stem to its very bottom. Every piece of stem was thoroughly cleared of petioles and pedicels. Before extraction, all plant samples were frozen, lyophilized and ground to a fine homogeneous powder with a ball-mill. Leaf, flower and stem samples were subsequently extracted according to the protocol described in section 4.3.

4.6. Model of *in vitro* ruminal fermentation

4.6.1. Source of rumen fluid

Rumen fluid was collected from four rumen-cannulated non-lactating Holstein-Friesian dairy cows. Donor cows were fed on a mixture of grass silage and maize silage (on a 1:1 ratio, based on dry matter). The animals were fed once a day in the morning. The diet was provided *ad libitum* and animals had free access to water at all times. The handling of the animals was approved by the Institutional Animal Care and Use Committee of Wageningen University (Wageningen, the Netherlands) and was done in accordance with the European Union directive 2010/63/EU and the Dutch legislation on use of experimental animals. Rumen fluid was collected before the morning feeding into pre-warmed and CO_2-flushed thermos flasks and transported quickly to the laboratory. Two independent mixtures (dubbed A and B) were then created by mixing equal volumes of fluid from two cows. Rumen fluid mixtures were filtered through two layers of cheesecloth into two separate glass bottles and kept under a constant flow of CO_2 and in a 39°C water bath. Rumen fluid mixtures A and B were then diluted in a 1:15 ratio (v/v) with a freshly prepared buffer medium following the procedure of Williams et al. (2005) and Pellikaan et al. (2011b). The buffer medium that I used in this study was a slightly modified version of that described by Williams et al. (2005) from which I only removed the cysteine.

4.6.2. In vitro gas production technique

Gas production was measured using an automated gas production system as described by Cone et al. (1996) and further modified by Pellikaan et al. (2011a) to allow headspace sampling for measurement of CH_4 concentration (Figure 13). About 250 mg of ryegrass silage was weighed in each bottle (except for 2 blank samples). The bottles were pre-warmed at 39°C and flushed with CO_2 to remove oxygen and were then filled with 30 mL of buffered rumen fluid. Before sealing the bottles, 1 mL of ET solution was added to achieve a final concentration in the buffered rumen fluid of 0.1 mM. Finally, bottles were sealed, connected to the electronic pressure transducer units and placed in a water bath maintained at 39°C with a gentle and constant shaking. Gas production was

recorded over 72 h. The choice of the operating concentration of 0.1 mM was made on the basis of a preliminary experiment in which dimer and trimer were tested (with the same experimental setup) at 0.5, 0.2, 0.1, 0.05 and 0.02 mM. The concentration 0.1 mM was chosen because it induced a moderate inhibition of total gas and CH_4 production.

Figure 13. Full experimental setup for the *in vitro* gas production technique (Left). Details of the glass bottles with side port and butyl rubber tubing (Right).

4.6.3. Experimental design

The experiment followed a randomized block design that consisted of two blocks (one for each rumen fluid mixture). Each block consisted of 1 blank (i.e. buffered rumen fluid alone), 5 controls (i.e. buffered rumen fluid + substrate without ET) and 4 replicates for dimer, trimer, tetramer, pentamer, hexamer and polymers (i.e. buffered rumen fluid + substrate + ET). In the case of heptamer, only 3 replicates from one source of rumen fluid were performed because the purification process of heptamer was difficult and only yielded a limited amount of pure compound. Total cumulative gas production was expressed in mL/g OM.

4.6.4. Measurement of methane concentration

Methane concentration in the headspace of the fermentation bottles was measured at 0, 2, 4, 6, 9, 11, 23, 27, 32, 36, 48, 52, 60 and 72 h. The headspace was sampled via a side port sealed with a screw cap and an air-tight septum and CH_4 was quantified by gas chromatography as described in Hatew et al. (2015).

4.6.5. Volatile fatty acids determination

After 72 h of incubation, samples from the fermentation fluid were collected and analyzed for VFA. From each bottle, 600 µL of fluid was pipetted and mixed with 600 µL of an internal standard solution containing 19.68 mM of 4-methylpentanoic acid in

0.42% aqueous ortho-phosphoric acid. Samples were vigorously shaken, centrifuged at 14'000 × g for 5 min and the clear supernatants were pipetted into crimp neck vials. Analyses were carried out by gas chromatography coupled with a flame ionization detector. The column was a HP-FFAP (30 m, 0.53 mm i.d., 1.00 µm film thickness) from Agilent. The oven temperature program started at 80°C for 1 min, then increased to 120°C at a rate of 20°C min^{-1} and further increased at a rate of 6.1°C min^{-1} to 205°C and remained at that temperature for 2 min. Split injection mode was used with a split ratio of 1:9 and an injection volume of 0.1 µL. Injection temperature was set at 260°C. Dihydrogen was used as carrier gas at a constant pressure of 25 kPa. The base temperature of the detector was set at 260°C. Each sample was analyzed in duplicate for determination of acetic acid, propionic acid, butyric acid, isobutyric acid (2-methylpropanoic acid), valeric acid and isovaleric acid (3-methylbutanoic acid). Concentrations of those analytes in the samples were determined by using an external calibration solution which was analyzed before and after every set of 10 samples. Internal standard (4-methylpentanoic acid) was used for correction of peak area and for peak identification of the individual VFA acetate (C2), propionate (C3), butyrate (C4) and valerate (C5) and the branched-chain VFA isobutyrate (iC4) and isovalerate (iC5).

4.6.6. Ammonia nitrogen analyses

After 72 h of incubation, 750 µL of fluid was pipetted from each bottle and deproteinized by addition of 750 µL of a trichloroacetic acid (TCA) solution (100 g L^{-1} TCA, neutralized with NaOH). Samples were then centrifuged for 10 min at 14'000 × g. Ammonia concentration was determined from the supernatant by colorimetry (Weatherburn, 1967) using a UV spectrophotometer (Evolution 201 - Thermo Scientific). Each sample was analyzed in duplicates and the retained value for the concentration of NH_3-N was the average of the two determinations.

4.7. Assessment of the anthelmintic effect of oligomeric ellagitannins

4.7.1. Source of adult Trichostrongylus colubriformis

Trichostrongylus colubriformis adult worms were obtained from a donor goat experimentally infected *per os* with a pure strain of 6000 *T. colubriformis* L3-stage larvae. Four weeks after infection, the goat was euthanized and, immediately after death, the small intestine was collected, opened, briefly washed and placed in a Baermann apparatus with saline at 37°C. After 2 h, worms that had migrated into the saline were collected and promptly transferred into the multiwell-plates. The *T. colubriformis* strain was susceptible to the main anthelmintic drugs.

4.7.2. Adult motility inhibition assay

Adult *T. colubriformis* were placed in 48-multiwellplates at a concentration of two to ten worms per well. The worms were first washed for in PBS at 37°C. After 1 hour, the washing medium was removed and replaced by 1 mL of the different ET solutions or PBS as a negative control. The experimental design consisted of seven 48-wellplates (one per ET plus one for levamisole). On each plate, I tested one ET at five different concentrations and included a negative control (PBS only). Each treatment was replicated in 4 wells. Levamisole was used as positive control and was tested at the following concentrations: 0.1, 0.5, 0.25 and 0.125%. The supernatant was renewed after 24 h of incubation. The motility of adult worms was noted by careful observation under a stereomicroscope at ×40 magnification after 0, 16, 24, 40 and 47h. Death or paralysis of worms was ascertained by the absence of motility for an observation period of 10 s. The plates were maintained at 37°C throughout the whole experiment, except during the periods of microscopic examination, in which case they stayed at room temperature (~22°C) for a few minutes.

The effect of oligomer size was assessed at each concentration by fitting the nematode survival curves with Cox proportional-hazards regression model (using the “survival” package for R). Hazard ratios (HR) were thus calculated for each treatment (*i.e.* oligomer size) and represent the relative odds of paralysis/death for the treated nematodes compared to the control group.

5. RESULTS AND DISCUSSION

5.1. Isolation and purification of oligomeric ellagitannins

The crude acetone/water extract of fireweed flowers appears to be quantitatively dominated by oenothein B (R_T = 2.93 min) and oenothein A (R_T = 3.27 min) (Figure 14). A small hump containing larger oligomeric ETs is visible between 3.5 and 4.5 min.

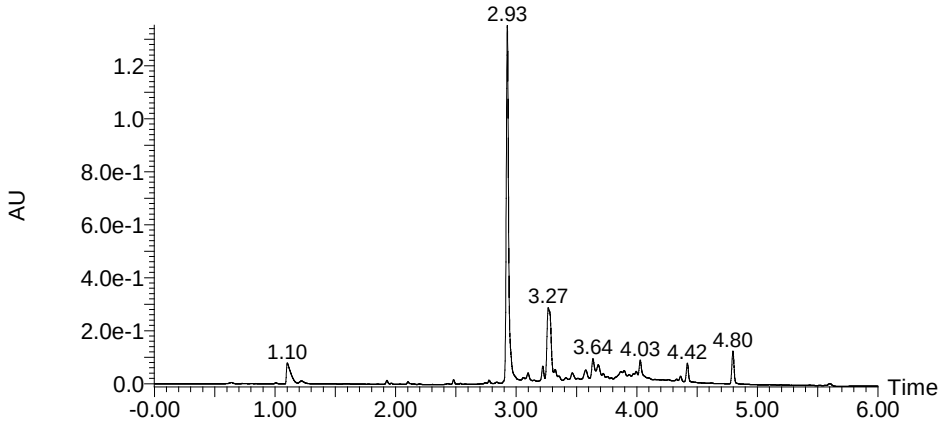

Figure 14. UV chromatogram (280 nm) of the crude acetone/water (4:1, v/v) extract of *Epilobium angustifolium* flowers.

A first fractionation by Sephadex LH-20 allows to accumulate the compounds that were only visible as an unresolved hump in the crude extracts. The acetone/water (4:1, v/v) eluate is now almost exclusively constituted of ellagitannins (Figure 15). The several isomers of the tetramer can be seen around R_T = 3.62 min

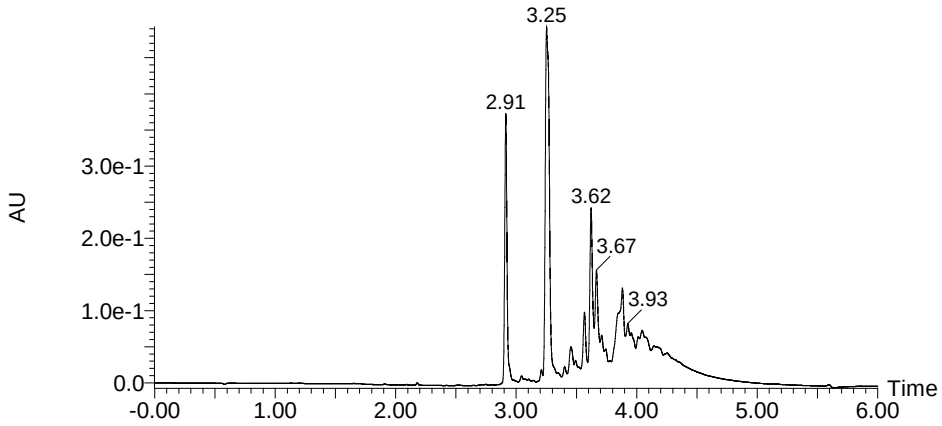

Figure 15. UV chromatogram (280 nm) of the acetone/water (4:1, v/v) fraction of *Epilobium angustifolium* flower.

The careful fractionation of that acetone/water eluate by column chromatography allowed to recover oenothein B and oenothein A as almost pure compounds. Larger ellagitannins, however, typically eluted as mixtures of two or three successive oligomers. Figure 16 shows, for instance, a fraction containing tetramer (between R_T = 3.50 and 3.67 min), pentamer (around R_T = 3.89 min) and hexamer (around R_T = 4.1 min).

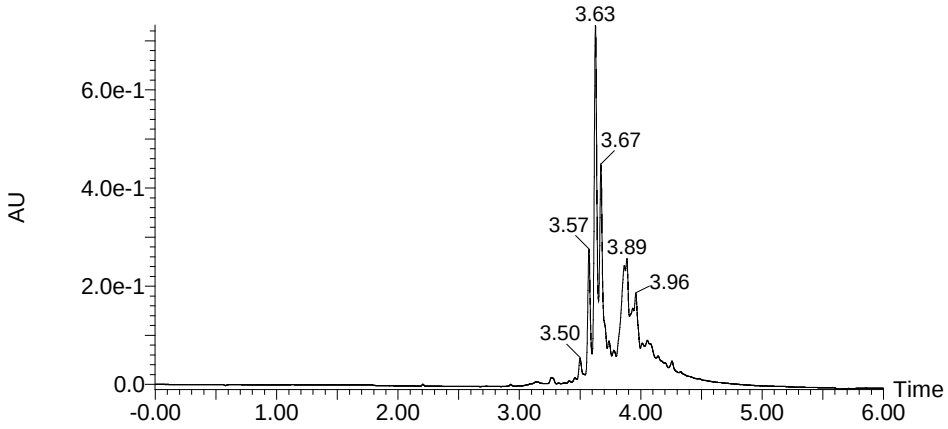

Figure 16. UV chromatogram (280 nm) of a Sephadex fraction containing a mixture of tetrameric, pentameric and hexameric tellimagrandin I.

This type of fraction thus required further purification in order to separate the compounds. This was done by preparative and/or semipreparative HPLC and resulted in the isolation and accumulation of pure oligomeric tellimagrandin-I from dimer to heptamer. Figure 17 shows the UV chromatogram of the pure tetramer as an example.

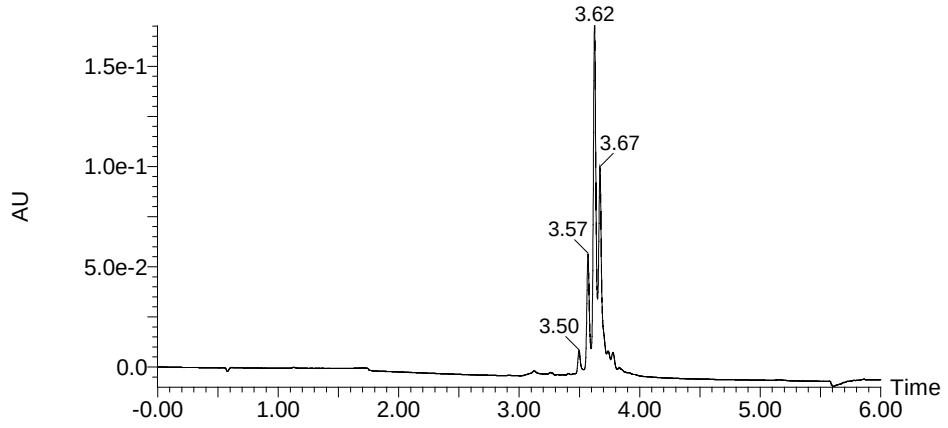

Figure 17. UV chromatogram (280 nm) of pure tetrameric tellimagrandin I.

5.2. Development of a quantification method

Once the oligomeric ETs had been isolated and purified, it became possible to start developing a quantification method. This was done in two major steps. The first one consisted in optimizing ionization and fragmentation for each ET so as to maximize the sensitivity of detection. This was done by adjusting cone voltage and collision energy until they produced maximum parent and daughter ions, respectively. The final result of this optimization process is shown in Table 1. Two transitions were monitored for each oligomer in order to limit the risk of false positive detection. The transition that gave the stronger signal was used for quantification (Figure 18) whereas the other one was used as a means of confirming the detection. The second step in the development of the quantification method was the construction of calibration curves for each compound (see **I**).

Table 1. Parameters of the multiple reaction monitoring transitions for the main oligomeric ellagitannins of *Epilobium angustifolium*.

Compound	Molar mass ($g\ mol^{-1}$)	MRM scan time window (min)	MRM1 quantification (*m/z*)	CV/CE	MRM2 confirmation (*m/z*)	CV/CE
Oenothein B	1569.1	2.80–3.37	783.1 > 765.3	30/20	783.1 > 301.0	30/40
Oenothein A	2353.6	3.13–3.58	1175.5 > 301.0	46/52	1175.5 > 275.0	46/62
Tetramer	3138.2	3.42–3.95	1044.8 > 301.0	32/50	1044.8 > 275.0	32/60
Pentamer	3922.7	3.71–4.20	1306.4 > 301.0	40/65	1306.4 > 275.0	40/75
Hexamer	4707.3	3.70–4.70	1567.9 > 301.0	42/90	1567.9 > 275.0	42/70
Heptamer	5491.8	3.80–4.80	1829.3 > 275.0	46/75	1829.3 > 301.0	46/100

MRM, Multiple Reaction Monitoring
CV, cone voltage (V) ; CE, collision energy (eV).

The combination of UHPLC and MRM-MS permitted the creation of a fast and sensitive method with an analysis time of about 10 min per sample and limits of quantification below the micromolar level. Yet this method possesses some limitations. First and foremost, it requires the purification of the ETs because there are no commercially available standards to build the calibration from. Secondly, like all MS-based quantification method using electrospray ionization, it is susceptible to the matrix effect. This effect corresponds to a decrease in the efficiency of ionization of the analytes due to co-eluting compounds (Kebarle and Tang, 1993; King et al., 2000). There are several techniques that can be used to overcome the matrix effect or at least to limit its detrimental impact on the quantification. The matrix-matched calibration, for instance, consists in building external calibration curves in a matrix similar to that of the samples instead of pure solvent. However, this technique requires a blank matrix (i.e. a matrix that is devoid of the analytes). In our case, this was not possible as it would have required a fireweed extract deprived of oligomeric ETs (the matrix-matched calibration technique

is typically applied to the quantification of exogenous molecules in biological matrices such as plasma and urine, in which case a blank matrix is easy to obtain). Another way to compensate for the matrix effect is called the standard addition technique. This technique consists in spiking the sample with known concentrations of the analytes. This allows to build internal calibration curves within the sample matrix. The standard addition technique is a very effective way to overcome the matrix effect but comes at the expense of time and pure analytes as each sample must be analyzed at least two or three times, each time with a different spiking concentration (typically: no-spike, low spike and high spike). Unfortunately, these requirements were hardly compatible with the difficulty of obtaining pure oligomeric ETs.

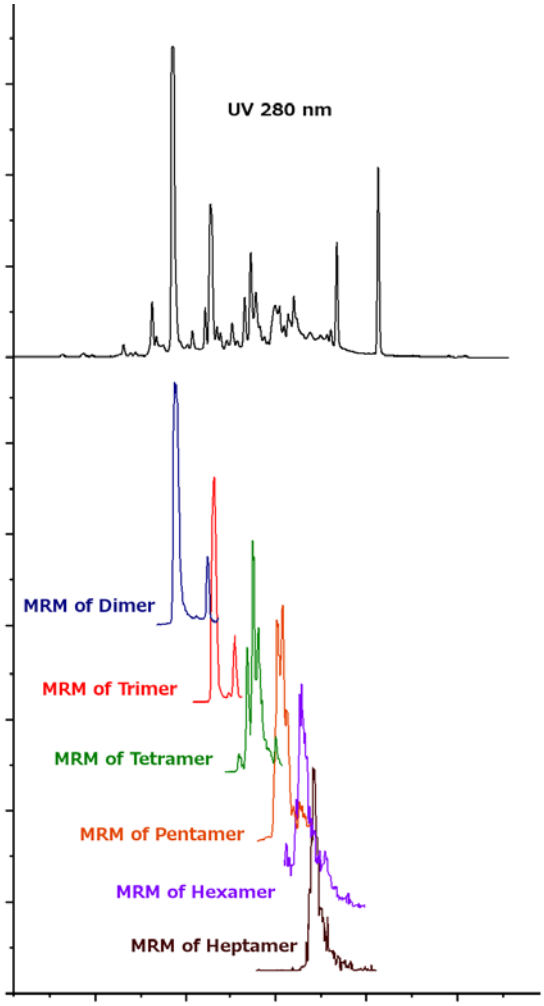

Figure 18. Overlay of the UV chromatogram and Multiple Reaction Monitoring transitions obtained from the analysis of a fireweed flower extract by UHPLC-DAD-ESI-MS/MS.

Finally, instead of trying to compensate for the matrix effect it is possible to mitigate it by removing undesired molecules during the extraction process. This can be achieved by cleaning up the extract using solid phase extraction techniques. With the appropriate material, it may be possible to selectively wash out some of the undesired matrix components thereby reducing the interference they might cause in the ion source. Such clean-up phase could be envisaged in order to mitigate the matrix effect in fireweed samples and to overall improve the sensitivity of the method. This would be particularly beneficial for the quantification of the large oligomers for which I witnessed a strong abatement of the signal (up to 73%) due to matrix effect. Despite this drawback, the quantification of oligomeric ETs by UHPLC-DAD-ESI-MS/MS proved to be highly selective, fast and repeatable.

5.3. Structure-activity relationships of oligomeric ellagitannins

Structure-activity relationships were investigated using two *in vitro* assays: ruminal fermentation and anthelmintic activity against adult nematodes. While the latter was relatively easy to interpret (owing to the simplicity of its design), the former showed unexpected levels of complexity. The main difficulty came from the fact that rumen fluid is a complex mixture in which many interdependent biochemical reactions take place, in a delicate equilibrium. Adding ETs to the medium disrupted several (if not all) of those processes, thus making it difficult to pinpoint the exact targets of ETs. However, by monitoring several indicators of ruminal fermentation is was possible to make reasonable assumptions regarding the potential modes of action of oligomeric ETs on ruminal fermentation.

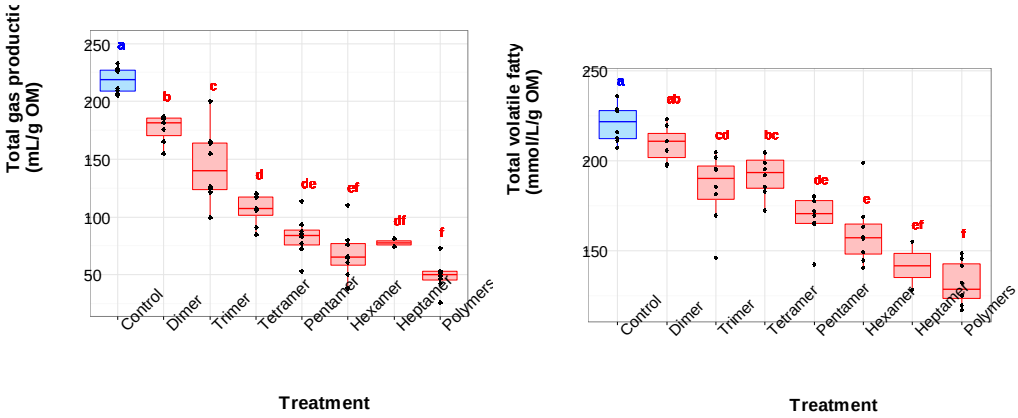

Figure 19. *In vitro* total gas production for each treatment at 48 h (left) and concentration of total volatile fatty acids in the fermentation fluid (right).

Total gas and TVFA production decreased proportionally to the size of ETs (Figure 19). Given that gas and VFAs are end products of the fermentation of carbohydrates, this

result evoked an action of ETs on the degradation of OM (which, in this experiment, largely consisted of fibers). However, it was not possible to determine whether ETs directly disrupted fibrolytic microorganisms or if they simply decreased the degradability of fibers by forming complexes with them, or a combination of both mechanisms. Regardless of the mechanism, the inhibition of OM degradation is to be considered an adverse effect and should be as low as possible *in vivo*. In addition to acting on OM fermentability, ETs also decreased the production of CH_4 (Figure 20), but the size-effect relationship was not linear. One of the difficulties of interpreting CH_4 data is that part of the observed decrease is a direct consequence of the inhibition of OM degradation. As we saw in section 2.5.3, methanogenesis requires hydrogen which happens to be a product of the fermentation of carbohydrates. Therefore, the values of CH_4 production, alone, do not bring much insight as to what ETs might be doing the methanogens, given that these values might in fact be the result of a decreased hydrogen production rather than a direct inhibition of methanogenic bacteria.

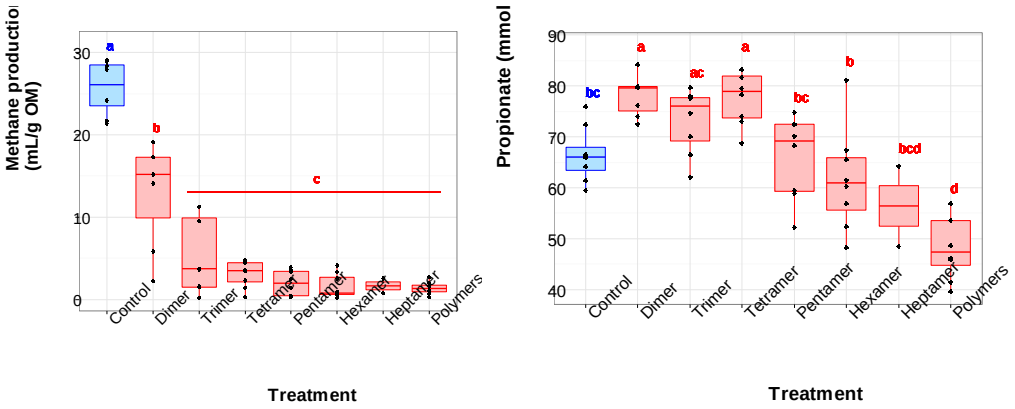

Figure 20. *In vitro m*ethane production for each treatment at 48 h (left) and propionate concentration in the fermentation fluid (right).

Looking at the production of VFAs, however, allows a more insightful interpretation of the possible action of ETs on methanogenesis. More specifically, propionate production showed a significant increase with the dimer and the tetramer (and a marginally significant increase for the trimer) compared to control (Figure 20). Like methanogenesis, propionate synthesis requires hydrogen and therefore, in physiological condition, those two reactions compete with one another for hydrogen. Thus, a likely explanation for the observed increase in propionate production is the inhibition of methanogenesis. Indeed, if the uptake of hydrogen by methanogenic bacteria is impeded, hydrogen will be more readily available for propionate synthesis. The fact that we observed an increase in the concentration of propionic acid despite the general inhibition of OM fermentation is a strong indication that ETs inhibited methanogenesis. Contrary to the inhibition of OM fermentation, the inhibition of methanogenesis by oligomeric

ETs was not strongly dependent on the oligomeric size as highlighted by the assessment of the acetate/propionate ratio (C2:C3) (Figure 21).

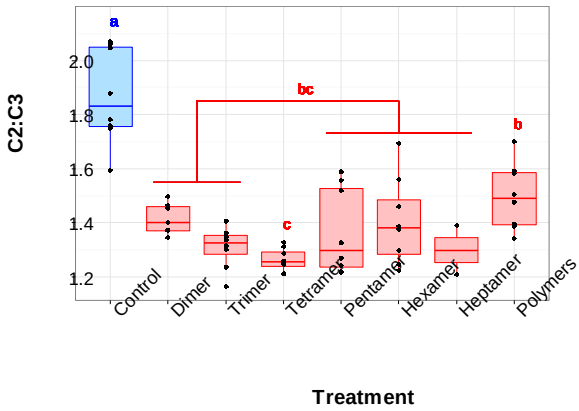

Figure 21. Ratio of the concentrations of acetate/propionate (C2:C3) in the fermentation fluid at the end of the experiment for each treatment.

The inhibition of ruminal protein degradation was estimated by measuring NH_3-N and BCVFA concentrations (Figure 22). Results indicate that all the tested ETs were able to decrease protein degradation by ruminal bacteria. Furthermore, dimer and polymers showed a weaker effect than all the other intermediate-size oligomers.

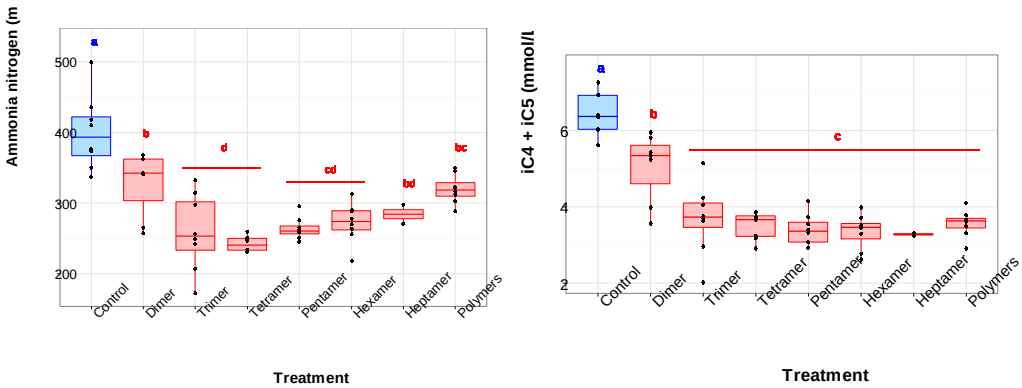

Figure 22. Ammonia nitrogen concentration in the fermentation fluid (left) and sum of isovalerate and isobutyrate concentrations (right).

Finally, the bioactivity of fireweed's oligomeric ETs was tested on the adult stage of *T. colubriformis*. I observed a lack of consistency in the results between the different concentrations that were tested. It was not possible to discern a clear structure-activity pattern as was the case with the ruminal fermentation experiment. However, despite the

shortcomings of that assay, it is possible to notice that trimer and tetramer scored the highest anthelmintic activity at almost all the tested concentrations (Figure 23). Furthermore, they remained active at a very low concentation (0.39 µM).

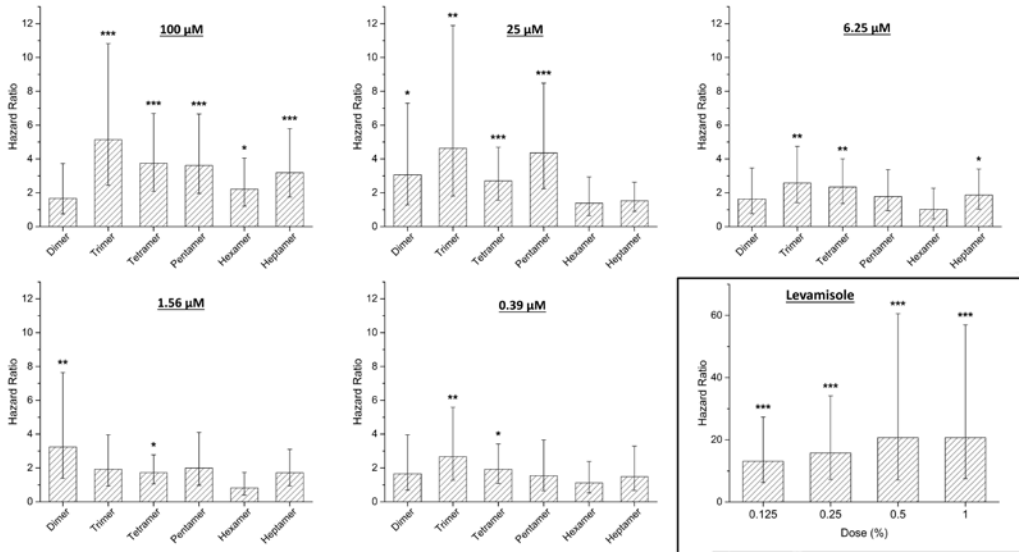

Figure 23. *In vitro* activity of oligomeric ellagitannins against *Trichostrongylus colubriformis* compared to control (phosphate buffer). Hazard ratios have been calculated using Cox proportional-hazard regression model. (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001).

The results from these *in vitro* experiments clearly demonstrate that the oligomeric size is a determining factor for the bioactivity of fireweed's ETs. Additionally, I have shown that the relationship between size and activity was far from a simple linear one. Nevertheless, these results do raise some questions, particularly concerning the modes of action of those oligomeric ETs. Are these molecules active in their native form or does the activity come from their degradation products? The stability of these compounds was only briefly investigated. Preliminary results show that, at least in PBS, they tend to decay rapidly (see **IV**). Therefore, it seems reasonable to assume that activities which were observed after 24, 48 or even 72 hours of incubation were most likely caused by the action of metabolites rather than the original ETs. However, I cannot rule out the possibility that, once bound (e.g. to fibers or proteins), oligomeric ETs could be less prone to hydrolysis and oxidation and could therefore remain intact for a longer period of time.

5.4. Distribution of polyphenols in *Epilobium angustifolium*

The study of the intra-individual distribution of the main polyphenols of *E. angustifolium* (Table 2) taught us that there are large qualitative and quantitative differences between plant tissues (differences between leaves and flowers are illustrated in Figure 24). Conversely, variations between populations proved to be relatively small and even smaller within each population.

Table 2. Main phenolic compounds of *Epilobium angustifolium.*

Peak #	Compound name	R_T (min)	UV absorption maxima (nm)	$[M–H]^-$ (*m/z*)	Main product ions (*m/z*)
1	Neochlorogenic acid	2.48	245, 324	353	191
2	Unknown (oxidized oenothein A derivative)	2.74	224, 259sh	1182.1[b]	765, 301
3	Oenothein B	2.94 ; 3,23[a]	222, 262	783.1[b]	765, 301
4	Oenothein A	3.33 ; 3.50[a]	227, 261	1175.1[b]	765, 301
5	Tetrameric tellimagrandin I	3.58–3.79	225, 266	1044.4[c]	301, 275
6	Pentameric tellimagrandin I	3.81–4.02	223, 267	1305.8[c]	301, 275
7	Hexameric tellimagrandin I	4.05–4.24	223, 269	1567.1[c]	301, 275
8	Heptameric tellimagrandin I	4.12–4.30	223, 269	1828.5[c]	301, 275
9	Myricetin-3-*O*-glucoside	3.63	259, 355	479	316, 317
10	Myricetin-3-*O*-glucuronide	3.66	259, 353	493	316, 317
11	Quercetin-3-*O*-(6´´-galloyl)-galactoside	3.83	264, 352	615	300, 301
12	Quercetin-3-*O*-galloylhexoside	3.89	266, 352	615	300, 301
13	Myricetin-3-*O*-rhamnoside	3.98	258, 350	463	316, 317
14	Quercetin-3-*O*-galactoside	4.01	265, 353	463	300, 301
15	Quercetin-3-*O*-glucuronide	4.06	255, 353	477	300, 301
16	Quercetin-3-*O*-arabinoside	4.31	253, 354	433	300, 301
17	Kaempferol-3-*O*-glucuronide	4.38	265, 349	461	284, 285
18	Quercetin-3-*O*-rhamnoside	4.43	255, 349	447	300, 301
19	Kaempferol-3-*O*-rhamnoside	4.82	263, 344	431	284, 285

[a]isomer peak
[b]$[M-2H]^{2-}$
[c]$[M-3H]^{3-}$

The largest interpopulational variability observed, concerned the ratio of oenothein A/oenothein B (OA/OB). I found that the analyzed populations could be divided into three distinct categories as far as this ratio was concerned: low, high and intermediate OA/OB (see **IV**). This result likely has some ecological implications. Indeed, it has been previously shown that selection pressure by insect herbivores on *Oenothera biennis* favored the emergence of phenotypes with a higher OA/OB ratio in the fruits (Agrawal et al., 2012). This suggests that oenothein A—and most presumably the larger oligomers too—act as defensive compounds against herbivores and are more effective than the

dimer. The fact that the highest concentration of those molecules was found at the apex of the inflorescence shoot is in accordance with the optimal defense hypothesis whereby plants invest more defensive resources in a way that maximizes their fitness (Stamp, 2003). Investing more resources to protect floral meristems against insect herbivores might be an adaptive strategy aimed at maximizing the number of flowers that reach maturity, and ultimately, the number of seeds.

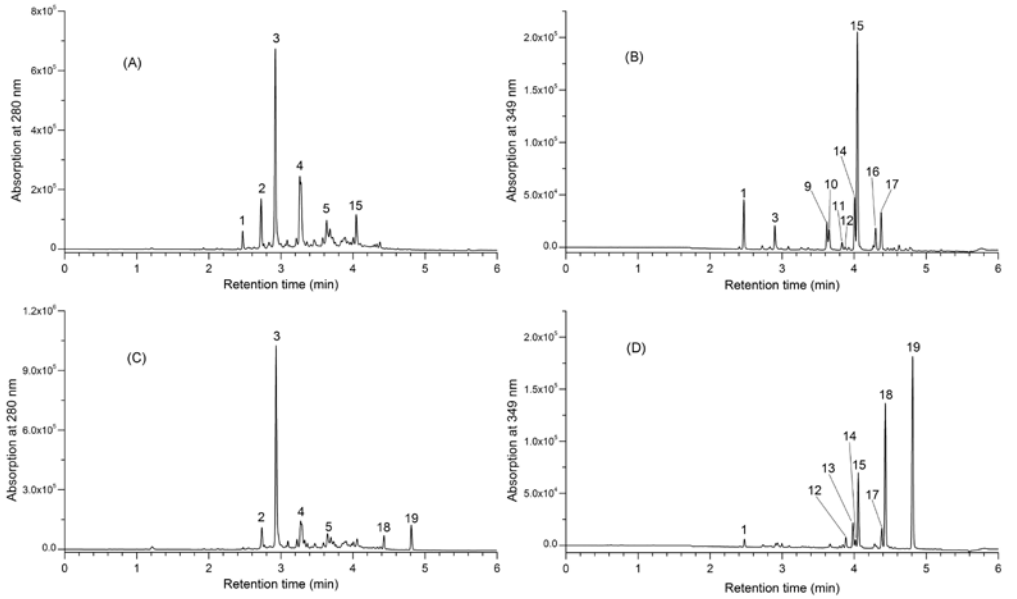

Figure 24. UV chromatograms of *Epilobium angustifolium* leaf extracts (A and B) and flower extracts (C and D) at 280 and 349 nm, respectively.

6. CONCLUSIONS

The main aim of this thesis was to investigate how the degree of oligomerization affected the bio-activity of oligomeric ETs. Two *in vitro* bioassays were conducted to answer that question: a simulation of ruminal fermentation and an adult motility inhibition assay on the parasitic nematode *Trichostrongylus colubriformis*. Both assays confirmed the hypothesis that the degree of oligomerization influences the activity. However, they revealed different patterns depending upon the target. Oligomeric ETs inhibited the degradation of dietary fibers by the ruminal microbiota in a size-dependent manner. Methanogenesis and protein degradation, however, showed a maximum inhibition with the trimer and the tetramer. The evaluation of the *in vitro* anthelminthic activity revealed a similar pattern, although the effect was not as clear. Even though these are only *in vitro* tests, they highlight the fact that—at least as far as oligomers of tellimagrandin I are concerned—trimer and tetramer are the best candidate for potential *in vivo* activities whereas large oligomers should be avoided on account of their adverse effect toward OM fermentation.

Another part of this work consisted in the development of an LC-MS/MS based method for the quantification of these oligomeric ETs. This method was then utilized to investigate how these ETs (and other phenolic compounds) are distributed in the plant and how the level of these molecules varies between populations. A large contrast was found between leaves and flowers, both in terms of ET concentration and flavonoid profile. Differences between populations, however, were small.

To conclude, this work shows that the size of oligomeric ellagitannins plays a crucial role in their bioactivities. This observation highlights the importance of a thorough assessment of the tannin fingerprint of potentially bioactive forages. Furthermore, it shows that *E. angustifolium* is a good candidate for methane mitigation and anthelmintic activity *in vivo*.

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