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Preface

The report “Bark in feed – for improved feed utilization and animal health” is based on the work from a pilot study that was funded by “Regionalt forskningsfond Midt-Norge” (project number ES504464-227179) and the participating institutions own funding. The project was a collaboration between the research institutions Bioforsk- Norwegian Institute for Agricultural and Environmental Research (the divisions Organic Food and Farming and Plant Health and Plant Protection), Norwegian University of Science and Technology (Department of Chemistry), Norwegian Institute of Wood Technology, MTT Agrifood Research Finland and Scotland’s Rural College. The project was co-ordinated by Bioforsk Organic Food and Farming. Marit Almvik from Bioforsk Plant Health and Plant Protection received a short-term scientific mission grant from the Cost Action FA1006 “PlantEngine”. Financial contributions from “Regionalt Forskningsfond Midt-Norge” and Cost Action FA1006 are acknowledged.

Content

Summary

Condensed tannins (CT) in the diet of ruminants may reduce feed intake and digestibility, and thereby production. However, dietary CT may increase protein utilization, reduce enteric methane loss and reduce gastrointestinal nematodes. The cultivation of tannin containing forages are limited in Norway, but bark of tree species that is harvested may have high quantities of CT. The objectives of the present study were to 1) assess the value of CT as feed additive; 2) assess the availability and the condition of Norwegian bark resources; 3) characterize the concentration, variation and type of CT in bark sampled from commercial sawmills and in fresh bark from logging sites; 4) evaluate the possibility for isolation and concentrating CT from bark, and 5) quantify the anthelmintic activity of selected bark extracts *in vitro*.

- Few studies have tested bark or CT from bark *in vivo*. The literature review revealed that the studies conducted mainly focused on hydrolysable tannins, extracted from the wood of a chestnut species, and CT from the bark and wood of the tropical tree species (mimosa and quebracho). Anthelmintic activity of CT extracted from Scotts pine (*Pinus sylvestris*) has been demonstrated in laboratory test, and the addition of bark in the diet of goats of an American pine species (*Pinus taeda*) resulted in increased growth rate and reduced fecal egg count.

- Total annual bark volume in Norway is estimated to be 642,665 m^3. About 60% of the bark is burned, which provides about 40% available for alternative use. A challenge is contamination of sand and soil.

- The content of CT in bark samples taken from tree species in Middle Norway varied with the age of the tree at sampling, but on average, dry bark of pine contained 4.9, spruce 3.9 and birch 3% CT, respectively. Procyanidins were the dominant CT in all species (100% pine bark and 93% in spruce bark), but birch contained in addition on average 14% prodelfinidins. The average degree of polymerization, i.e. the size/chain length of the CT polymer, was 6.7, 9.0 and 5.8 in pine, spruce and birch, respectively.

- We tested methods for isolation and purification of bark CT. Extraction with ethanol and acetone were compared, and 70% acetone gave the highest yield.

- In selected bark samples, we tested their effect on egg hatching and motility inhibition in larvae of a parasitic nematode common in small ruminants (*Teladorsagia circumcincta*). Crude extract (water) and acetone extract was used. The results showed that both the crude and acetone extracts had an effect and reduced the egg hatching and impaired larvae motility. The birch extract seemed strongest, while extracts from spruce weakest. In pine, the crude extract had stronger effect than the than the extracts form acetone, which suggests that there may be other substances than CT that are anthelemintic.

Samandrag

- Vi har skrive ei litteratursamanstilling om kondenserte tannin (CT) i dietten til drøvtyggarar og effekten det er har på ernæring og dyrehelse. Kondenserte tannin i dietten kan auke nitrogenunyttinga, redusere metantapet og redusere problemet med mage- og tarmnematodar. Men CT kan også redusere fôropptaket og redusere fordøyelegheita av fôret og dermed produksjon. Det er få studiar der ein har testa bark eller CT i frå bark.

- Vi har gjennomført ei vurdering av mengde og kvalitet av bark tilgjengelig i Norge. Totalvolum er årleg estimert til å være 642,665 m3. Om lag 60% av barken blir brent, noko som gir om lag 40 % tilgjengeleg for alternativ bruk. Ei utfordring er forureining av sand og jord.

- Vi har tatt ut prøver av bark frå bjørk, gran og furu, både i frå sagbruk (gran og furu) og frå tømmer rett etter hogst, og analysert for innhaldet av CT. CT-innhaldet varierte med alderen til trea, men i gjennomsnitt inneheld furu 4,9, gran 3,9 og bjørk 3% CT i tørr bark. Procyanidiner var dominerande typen CT i alle artar (100 % i furubark og 93% i granbark), men bjørk inneheld og prodelfinidiner (i gjennomsnitt 14%). Gjennomsnittleg polymeringsgrad, dvs. mål på storleiken av CT-polymerane, var i gjennomsnitt 6,7 hos furu, 9,0 hos gran og 5,8 hos bjørk.

- Vi gjorde også testing av isolering av og reingjering av CT. Ekstrahering med etanol og aceton blei samanlikna, og 70% aceton var det som gav størst utbytte.

- Vi testa i nokre av barkprøvane våre for deira effekt på egg-klekkinga og rørslehemming hos larvane av ein parasittisk nematode vanleg hos småfe (*Teladorsagia circumcincta*). Både råekstrakt (vatn) og acetonekstrakt blei brukt. Resultata viste at både råekstrakt og acetonekstrakt virka og reduserte både egg-klekkinga og hemma rørslene til larvane. Bjørkestrakta virka sterkast, medan ekstrakta frå gran svakast. Hos furu verka råekstrakta sterkare enn acetonekstrakta, noko som kan tyde på at det kan vere andre substansar enn CT som verkar anthelmintisk (nematodehemmande).

Introduction

Dairy milk and sheep meat production are the major agricultural activities in Middle Norway. Dairy milk production has intensified and milk yield by dairy cows has increased considerably the last decade. Much of this improvement is due to increased level of concentrates in the diet, particularly increased use of soy and rapeseed protein imported from other regions and countries. Although forage produced in Middle Norway contains high amounts of protein, forage protein is poorly utilized by ruminants due to its high rumen degradability. As a consequence, and to maintain high milk yield, there is a requirement for increased reliance on high quality protein (bypass) from soy and rape seed, with detrimental financial and environmental consequences. In their report on research and development needs in Middle Norway, "Grønn Forskning i Midt Norge" (2012) has concluded that there is a requirement to promote research on how to improve the dairy farm self-sufficiency of protein and protein utilization. One way of achieving these would be to include condensed tannins (CT) in the diet of ruminants. Condensed tannins are compounds found in plants that have been known to exert effects contrary to optimum nutrition, specifically by reducing feed intake and digestion in ruminants, and have therefore been regarded as anti-nutritional factors. However, when ingested in small quantities (2-4 % of DM), CT may actually be beneficial for ruminants, as they bind to dietary protein and thereby reduce rumen protein degradation without reducing the amount of protein synthesized by the rumen microbes; they have also been shown to inhibit the growth of proteolytic bacteria, which may further reduce proteolysis (see reviews by (Broderick, 1995; Min et al., 2004; Patra and Saxena, 2011). The reduced ruminal protein degradation increases the dietary protein bypass or non-ammonia nitrogen and dietary amino acids flow to the small intestine and, thus, improves protein utilization by the animal. Condensed tannins in the diet may also decrease methanogenesis and enteric methane emission from ruminants by decreasing the activity of methanogenic bacteria in the rumen (Hess et al., 2006; Jayanegara et al., 2012). Improved protein utilization and reduced methane emissions in ruminants are both seen as important measure to lower livestock contribution to pollution of particular greenhouse gasses. In addition, dietary CT may also have a positive effect on animal health as they have been shown to reduce the problems associated with gastrointestinal parasites (Athanasiadou et al., 2001). This may be mediated through an increase in protein supply as described above, which improves their immune response, but also, through a direct antiparasitic effect, which reduces the worm burden and nematode hatchability in sheep and goats (see reviews by Hoste et al., 2006; Min and Hart, 2003). Recent research has shown that there is high treatment frequency with anthelmintics in lambs in Norway, particularly in coastal areas, and the authors conclude that there is a real danger for development of anthelmintic resistance (Domke et al., 2011). It is therefore necessary to develop sustainable measures for gastrointestinal parasitism control and dietary inclusion of CT may be one of them.

The inclusion of CT rich forages, such as birdsfoot trefoil (*Lotus corniculatus*), sulla (*Hedysarum coronarium*), sericea lespedeza (*Lespedeza cuneata*) and sainfoin (*Onobrychis viciifolia*), as CT source for animal production in Middle Norway is likely to have limitations; with the exception of birdsfoot trefoil, these species are not native to Norway, and the existing varieties are not adapted to local climatic conditions which will result in low yields, as it has been shown for other countries, e.g. Scotland (Athanasiadou et al., 2005). However, Middle Norway has a strong forest and sawmill industry, and bark is a by- product from sawmills and is currently mainly used as fuel. Bark has high concentration of CT (Hellström and Mattila, 2008; Matthews et al., 1997), and recent research from North America has shown that inclusion of pine bark meal into the diet of male goats increased the animal's protein retention and reduced the faecal egg count (Min et al., 2012). The pine bark used in North America was from a species not found in Norway, and, consequently, the results cannot directly be related to the species and conditions available in Middle Norway. Using a combined approach (literature review and experimental work)

we will assess if condensed tannins in bark of Scots pine (*Pinus sylvestris*), Norway spruce (*Picea abis*) and birch (*Betula pubescens*) can be utilized as feed supplement.

The objectives of the current work wer to: 1) assess the value of CT as feed additive; 2) assess the availability and the condition of the bark resources; 3) characterize the concentration, variation and type of CT in bark sampled from commercial sawmills and in fresh bark from logging sites; 4) Evaluate the possibility for isolation and concentrating CT from bark, and 5) quantify the anthelmintic activity of selected bark extracts *in vitro*.

For references see chapter 1.6

1. Effects of condensed tannins on protein utilization, enteric methane production and anthelmintic effects – literature review

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1.1 Introduction

Tannins are polyphenolic secondary metabolites (PSM) of higher plants (Khanbabaee and van Ree, 2001a) that are broadly classified into either hydrolysable or condensed tannins (CT= proanthocyanidins) based on their molecular structure (Min and Hart, 2003). Some classify them into even more groups (e.g. gallotannins, ellagitannins, complex tannins and CT) on the basis of their diverse structural characteristics (Khanbabaee and van Ree, 2001). Condensed tannins are the most widespread (Mangan, 1988) with all oligomeric and polymeric proanthocyanidins formed by linkage of C-4 of one catechin with C-8 or C-6 of the next monomeric catechin/flavan-3-ols (Khanbabaee and van Ree, 2001; Chung et al., 1998a; Zucker, 1983; Mangan, 1988).

The CT, like all others, are produced in adaptation to plant defence mechanisms towards mammalian and insect herbivory achieved through deterrence and/or toxicity (Barbehenn and Peter Constabel, 2011; Lindroth and St.Clair, 2013) and also serve various ecological functions (Iason, 2005) - like the inhibition of microbial activities and altering nutrient dynamics in soil (e.g. rate of N mineralization). They show diversity in structure (Zhou et al., 2011; Mueller-Harvey, 2006), relative astringency (Bate-Smith, 1973), concentrations in different plant species (Mueller-Harvey, 2006), and different accessions and parts of a given plant species (Azuhnwi et al., 2012) along with seasonal changes in response to plant phenology (Theodoridou et al., 2011) and environmental conditions (Iason et al., 2012; Tiemann et al., 2010; Barry and Duncan, 1984) and, therefore, in function (Lorenz et al., 2013; Mueller-Harvey, 2006). As such, their effects on livestock feeding/grazing with varied concentrations from different sources have been reported either as positive [e.g. improved protein utilization, reduced parasitism and reduced bloating; see reviews by Min et al.(2003) and Mueller-Harvey (2006)], negative [e.g. toxic to an animal (Ben Salem et al., 2003), reduced dry matter intake (Mueller-Harvey 2006), reduced growth and wool production- especially at higher concentrations (Dschaak et al., 2011; Min et al., 2003)], or even neutral in some cases (Dschaak et al., 2011). Such complexity in response, among other things, has led to the sustained interest among different disciplines for looking into the effects of CT of kinds and from various sources, both in vitro and in vivo, for example: on animal feed utilization (Min et al., 2005a; Broderick, 1995;Dschaak et al., 2011), against gastro-intestinal nematode (GIN) parasites (Min et al., 2004; Min et al., 2005b; Lisonbee et al., 2009; Tibe et al., 2013; Min and Hart, 2003), greenhouse gas emission, and also in human foods and drinks (Chung et al., 1998a; Versari et al., 2013; Chung et al., 1998b). Hereunder, we provide a short literature review on the current knowledge on CT from different plant sources and their effects on animal nutrient utilization and growth, effects on parasitism and effects on greenhouse gas emission. Furthermore, we look into the potential of bark of Scots pine (*Pinus sylvestris*), Norway spruce (*Picea abis*) and birch (*Betula pubescens*) from Norway on CT production and utilization in relation to the above mentioned effects towards improved livestock production.

1.2 CT and animal nutrition

Protein nutrition in ruminants is a complex process where the overall aim of diet formulation is to meet proper rumen function and to make adequate supply of proteins carrying balanced amino acids as desired in the duodenum. However, microbial activity in the rumen and its protein degradation of dietary feed sources are the most important factors influencing intestinal amino acid supply to ruminants. As such, the profile of amino acids absorbed from the ruminant digestive tract depends on complex interactions governing the extent to which dietary protein is degraded in the rumen, the rate of rumen microbial growth and the rate of outflow from the rumen (Mangan, 1988). High quality by-pass protein, for example from soybean, is often fed to high producing dairy animals in a view to meet the protein requirement of these animals. However, these are either expensive or have to travel a considerable distance before being available.

The natural occurrence of CT in feeds grazed/browsed or processed for these animals could provide a direct nutritional benefit by protecting protein from rumen degradation. However, the outcome of feeding CT to an animal has also been associated with negative consequences. The CTs from different plant species have different physical and chemical properties (Porter, 1992; Mangan, 1988) and as such show very diverse biological properties (Zucker, 1983). They form complexes with macromolecules (example: proteins, cell walls, starch, pectin, digestive enzymes etc.) through multiple type of bonding, with hydrogen-bond suggested to be the main one in CT-protein complex (Zucker, 1983). This binding of CT with proteins renders the protein un-degradable in the rumen with beneficial effects once reaching duodenum in its form (Mangan, 1988). In view of the complexity of the outcome of consumption CT containing food by ruminants, there two are broad expectations: (a) beneficial - when the overall effect promotes nutrient intake, utilization and production and (b) negative/anti-nutritional - when the consumption adversely affects dry matter (DM) intake, DM digestibility, health and production efficiency.
For herbivores, the outcome of ingesting a feed with CT and level of intake by preference depend, among other things, on their structure (Lorenz et al., 2013; Clausen et al., 1990). Clausen et al.,(1990) suggest that differences in tannin structure can lead to different depolymerized products and rates of depolymerisation, both of which may affect herbivore preferences and intake. In general, at modest levels of intake (2-4% of DM) (Acamovic and Brooker, 2005, Min and Hart, 2003), CT may actually benefit ruminants by reducing rumen protein degradation without adversely affecting the amount of protein synthesized by the rumen microbes and inhibiting the growth of proteolytic bacteria which may further reduce proteolysis (Patra and Saxena, 2013; Broderick, 1995; Ben Salem et al., 2003). In addition to the CT structure, feed protein type (Lorenz et al., 2013; Cortés et al., 2009) and the relative concentration of protein and CT (Zucker, 1983) are also important in the precipitation process and reducing the degree of proteolysis. For CT, the factors promoting the formation of complexes include their relatively high molecular weight and their great structural flexibility (Hagerman et al., 1992) and also delphinidin:cyanidin ratio (relative astringency) (Mangan, 1988; Molan et al., 2003). For proteins, the affinity for tannins correlates with relatively large, hydrophobic and flexible structure and richness in proline (Hagerman et al., 1992; Kumar and Singh, 1984).

At higher levels of intake, if achieved from browsing/grazing –especially from CT rich tropical browse species (for concentrations see (Berard et al., 2011) - the CT can impact animal DM and energy intake, digestibility and production parameters (Barry and Duncan, 1984; Silanikove et al., 1994). They can influence the digestion kinetics of not only proteins but also cell wall and cell contents forming complex under favourable gut conditions (Silanikove et al., 1994; Barry and Manley, 1984) whereby they exert their anti-nutritional effects. However, in temperate grazing conditions to attain such a level that can negatively affect DMI and DM utilization may be not be a concern as the CT content of the crops far below their tropical counterparts.
Recently, attention has been given to forages containing CT and their practical use under housed or grazing conditions. These include, but not limited to, trees/shrubs (*Acacia spp., Calliandra calothyrsus, Picea abies,*

Schinopsis lorentzii), herbaceous materials (*Lespedeza striata, Onobrychis viciifolia* Scop,*),* cereals *(*e.g. *Sorghum Spp.)* and legumes *(Medicago sativa* L, *Dalea purpurea, Trifolium spp., Lotus corniculatus* and *Coronilla varia);* see Table 1.1 for summary and refer to others (Berard et al., 2011) for specific CT concentrations of some tropical and temperate crops.

Table 1.1. Summary of plant species with plant secondary metabolites (PSM) and observed effects (feed intake, nitrogen retention, growth, anti-parasitism, methane emission) under different conditions

Plant type	Plant part	Type of PSM	Type of trial	Reported effects	References
Herbs/legume crops					
Lespedeza striata (Kobe Lespedeza)	Whole plant fed with forages low in CT	CT	With goats	↓methane production ↔total rumen bacterial count	(Animut et al., 2008)
Onobrychis viciifolia (sainfoin)	Sainfoin leaves and stems	CT	With sheep	↓rumen fluid soluble N ↓rumen NH_3-N	(Theodoridou et al., 2011)
▫	Sainfoin plant (?) incubated at different mixing ratios with cocksfoot	CT	*In vitro* - rumen fluid from sheep	↓NH_3 production ↓ protein degradation ↑DM digestibility from the mixtures	(Niderkorn et al., 2012)
▫	Sainfoin hay	CT	With Parasitized goats	↓Nematode egg excretion ↑DMI from hay ↓ 50% intestinal worm population ↑resilience ↓ mortality (goats)	(Paolini et al., 2003; Paolini et al., 2005)
▫	Leaves and stems (var. Cotswold Common)	CT extract	*In vitro* test on cattle nematode	↓exsheathment of cattle GI larvae	(Novobilsky et al., 2011)
*Hedysarum coronarium (*sulla)	Whole plant (?)	CT extract	*In vitro* test	↓migration of larvae of GI nematodes	(Molan et al., 2000a)
Lotus spp.	Leaves and stems	CT extract	*In vitro* test on cattle nematode	↓exsheathment of cattle GI larvae	(Novobilsky et al., 2011)
Shrubs/trees					
Calliandra calothyrsus (calliandra)	Leaves	CT	With Parasitized goats	↓N digestion/retention ↑ faecal N	(Hove et al., 2001)
Acacia karoo (Acacia)	Leaves	CT extract	With Parasitized goats	↓faecal egg count ↓abomasal parasite burden	(Kahiya et al., 2003)
Castanea sativa (Chestnut tree)	External tegument of fruits	Crude extract	*In vitro* test	↓exsheathment (led to total inhibition)	(Bahuaud et al., 2006)
Pinus sylvestris (Pine tree)	Leaves	Crude extract	*In vitro* test	↑time of exsheathment (delayed process)	(Bahuaud et al., 2006)
Peltophorum africanum Sond.	Barks, leaves and roots	Crude extract	*In vitro* test	↓egg hatching and larval development	(Bizimenyera et al., 2006)
Leucaena leucocephala (Leucaena)		CT extract	*In vitro* test	↓*In vitro* DM and N disappearance increasing CT ↓ methane and total gas production increasing CT ↓ total VFA with increasing CT	(Tan et al., 2011)
*Acacia mearnsii (*Black Wattle tree)	Barks	CT extract	Lactating dairy cows at low and high dose level vs control	↓methane production ↓milk production at high CT intake ↓fat and protein yield at high CT intake ↓nitrogen loss in urine in dose dependent manner ↓energy digestibility in dose dependent manner	(Grainger et al., 2009)

▫= reduction; ▫ =increment; ▫ = no change of a parameter relative to controls

* cows were grazing ryegrass pasture supplemented with 5 kg per day of triticale and given twice daily with 0, 163 or 326 g CT per day

In summary, the beneficial effects of CT containing crops can be utilized either in situ, harvested or processed when included in the diet of target animals. However, there are limitations; for example, lack of unified protocol as to how and how much of a CT containing crop could be used under typical production system, lack of consistency in the outcome of reported effects (Athanasiadou and Kyriazakis, 2004), form of CT included (for example, crude extract, purified commercial form or part of plant deemed to contain CT) and also the geographical limitation of the crops to some areas. For example, in the Nordic agriculture the share of such crops is either limited or non-existent.

1.3 CT and animal health (GIN parasitism)

In addition to the protein nutrition, dietary CT may also have positive effects on animal health as they have been shown to reduce the problems associated with GIN parasites (Min et al., 2005b; Athanasiadou and Kyriazakis, 2004). The mechanisms for these effects are still elusive, but there are at least a couple of mechanism postulated: direct anti-parasitic effects and altered protein nutrition along with modified gut environment of the parasitized host or the combination of both.

The direct anti-parasitic effects of CT from forages containing varying concentrations of CT have been reported in many field, housed and laboratory conditions (Iqbal et al., 2007; Athanasiadou et al., 2001; Paolini et al., 2003; Paolini et al., 2005). The in vivo effects are mainly assessed by feeding parasitized animals with feeds differing in CT concentration, or addition of purified CT along with control group that either gets a CT free diet or a diet with polyethylene glycol (PEG- this neutralizes the effects of CT) and looking into the reduction in parasite egg count in faeces, reduction in GIT worm count after necropsy, reduction in the development of parasites into an adult worm, assessing the trajectory of protective immune responses, monitoring clinical signs and also assessing growth/production of the host. Such assessments involved mainly small ruminants (e.g., goats (Min et al., 2012; Paolini et al., 2005; Paolini et al., 2003; Kahiya et al., 2003), sheep (Rojas et al., 2006; Athanasiadou et al., 2005; Molan et al., 2000b) but also cattle (Novobilsky et al., 2011) under varied conditions. In vitro laboratory effects assess inhibition of parasite egg hatching, larval viability and migration/motility when incubated at varying concentrations of purified or crude extracts of CT containing plant materials (Molan et al., 2000c; Molan et al., 2003; Athanasiadou et al., 2001; Iqbal et al., 2007; Moreno-Gonzalo et al., 2013b; Moreno-Gonzalo et al., 2013a; Kozan et al., 2013; Macedo et al., 2012; Hounzangbe-Adote et al., 2005; Bizimenyera et al., 2006).

The indirect effects of CT are said to be mediated through an increased/improved protein nutrition of a parasitized host. This is due to the fact in a parasitized animal protein would be diverted from a productive processes into repair of the gastrointestinal tract, synthesis of plasma proteins and mucoprotein production (Coop and Kyriazakis, 1999). Most of the immune effector arms are protein in nature and improved protein supply to the host tissue will increase the rate of acquisition of immunity, increase resistance to reinfection and this has been associated with an enhanced cellular immune response in the gastrointestinal mucosa (Coop and Kyriazakis, 1999).

Recent research reports from Norway show that there is high anthelmintic treatment frequency in small ruminants and also about the existence of drug resistance by parasites (Domke et al., 2011). This may, therefore, necessitate the need to develop alternative sustainable gastrointestinal parasite control strategies where the use of CT from available sources within Norway could be looked into.

1.4 CT and greenhouse gas emission

Condensed tannins in the diet may also decrease methanogenesis and enteric methane emission from ruminants as observed *in vivo* and *in vitro* (Hess et al., 2006; Jayanegara et al., 2012; Huang et al., 2011; Tan et al., 2011; Pellikaan et al., 2011; Grainger et al., 2009). Improved protein utilization (reduced feed nitrogen loss) and reduced methane emissions in ruminants are both seen as important measures to lower livestock contribution to pollution of greenhouse gasses. However, in some instances works indicated that CT could also reduce DM digestibility and VFA production in a dose dependent manner (Tan et al., 2011) suggestive of reduced DMI, DM and energy digestibility if ingested by animals beyond a certain threshold level as observed with dairy cows fed ryegrass and supplemented with varying concentration CT extract from bark of Black wattle tree (Grainger et al., 2009). In an *in vitro* gas production experiment with CT from *Leucaena leucocephala* hybrid-Rendang , Tan et al (2011) reported that with different graded levels of CT inclusion, total gas production, CH4 production and total VFA concentration decreased at a decreasing rate with increasing levels of CT and so were the in vitro DM degradation and N disappearance. Estimates of rumen methanogenic archaea and protozoa populations later showed linear reductions in total methanogens and total protozoa with increasing levels of CT (Tan et al., 2011) and this may justify the observed outcome.

1.5 Barks as a potential source of CT in animal production

The inclusion of CT rich forages, for example - the likes of *L. corniculatus* and *O. viciifolia*, as CT source for animal production in Norway is likely to have limitations as these species are not native to Norway, and the existing varieties are not adapted to local climatic conditions which will result in low yields, as it has been shown for other countries (e.g. Scotland (Athanasiadou et al., 2005). However, Norway has a strong forest and saw-mill industry, and bark is a by- product from the saw-mills that currently is mainly used as fuel. Bark has high concentration of CT (Matthews et al., 1997).

The dominating CT types in coniferous bark are procyanidins (PC) and prodelphinidins (PD). In PC the flavan-3-ol units are either the trans-flavanol catechin (C) or its cis isomer epicatechin (EC), whereas trans-flavanol gallocatechin (GC) and its cis isomer epigallocatchin (EGC) make up PD. The dominating CT in Norway spruce (93%) and Scotch pine (100%) bark is PC, with B-type linkage (>98%) between the flavan monomers and high degree of polymerization (on average 6.7 in pine and 9.0 in spruce, own data). The main commercial CT products are extracted from the tree species quebracho (*Schinposis balansae*), wattle (*Acacia mearnsii*) and chestnut (*Castanea Sativa*), and they are mainly used in leather processing (Feng et al., 2013). However, only a few studies have been carried out to test bark and bark CT for their nutritional and veterinary effects *in vivo*. Dietary inclusion of bark from *Pinus taeda* L. improved animal performance and reduced faecal egg count and ruminal ammonia concentration in growing male goats (Min et al., 2012). To our knowledge, there are no records of nutritional effects and only a few records of veterinarian effects of bark and CT extracted from tree species found in Scandinavia. Williams et al. (2014) found direct anthelmintic effects of CT from pine bark against a parasitic nematode (*Ascasis suum)*. We have *in vitro* data from a preliminary study (not yet published), where anthelmintic activity of water and acetone extracts of bark samples from downy birch (*Betula pubescens*), Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*) was observed against the ovine nematode *Teladorsagia circumcincta*. This indicates that Norwegian bark sources may have a potential as a feed additive for improved livestock health. This is particularly interesting as high anthelmintic treatment frequency is required to control parasites in lambs in Norway, which has been associated with the development of anthelmintic resistance (Domke et al., 2011).

1.6 References

Acamovic,T., and J.D. Brooker. 2005. Biochemistry of plant secondary metabolites and their effects in animals. Proceedings of the Nutrition Society 64:403-412.

Animut, G., R. Puchala, A.L. Goetsch, A.K. Patra, T. Sahlu, V.H. Varel, and J. Wells. 2008. Methane emission by goats consuming diets with different levels of condensed tannins from lespedeza. Animal Feed Science and Technology 144:212-227.

Athanasiadou, S., I. Kyriazakis, F. Jackson, and R.L. Coop. 2001. Direct anthelmintic effects of condensed tannins towards different gastrointestinal nematodes of sheep: in vitro and in vivo studies. Veterinary Parasitology 99:205-219.

Athanasiadou,S., O. Tzamaloukas, I. Kyriazakis, F. Jackson, and R.L. Coop. 2005. Testing for direct anthelmintic effects of bioactive forages against Trichostrongylus colubriformis in grazing sheep. Veterinary Parasitology 127:233-243.

Athanasiadou, S., and I. Kyriazakis. 2004. Plant secondary metabolites: antiparasitic effects and their role in ruminant production systems. Proceedings of the Nutrition Society 63:631-639.

Azuhnwi, B.N., B. Thomann, Y. Arrigo, B. Boller, H.D. Hess, M. Kreuzer, and F. Dohme-Meier. 2012. Ruminal dry matter and crude protein degradation kinetics of five sainfoin (Onobrychis viciifolia Scop) accessions differing in condensed tannin content and obtained from different harvests. Animal Feed Science and Technology 177:135-143.

Bahuaud, D., C.M.-O. de Montellano, S. Chauveaus, F. Prevot, F. Torres-Acosta, I. Fouraste, and H. Hoste. 2006. Effects of four tanniferous plant extracts on the in vitro exsheathment of third-stage larvae of parasitic nematodes. Parasitology 132:545-554.

Barbehenn,R.V., and C. Peter Constabel. 2011. Tannins in plant-herbivore interactions. Phytochemistry 72:1551-1565.

Barry, T.N., and S.J. Duncan. 1984. The role of condensed tannins in the nutritional value of Lotus pedunculatus for sheep 1. Voluntary intake. British Journal of Nutrition 51:485-491.

Barry, T.N., and T.R. Manley. 1984. The role of condensed tannins in the nutritional value of Lotus pedunculatus for sheep. 2. Quantitative digestion of carbohydrates and proteins. British Journal of Nutrition 51:493-504.

Bate-Smith, E.C. 1973. Haemanalysis of tannins: The concept of relative astringency. Phytochemistry 12:907-912.

Ben Salem, H., I.B. Salem, A. Nefzaoui, and M.S.B. Said. 2003. Effect of PEG and olive cake feed blocks supply on feed intake, digestion, and health of goats given kermes oak (Quercus coccifera L.) foliage. Animal Feed Science and Technology 110:45-59.

Berard, N.C., Y. Wang, K.M. Wittenberg, D.O. Krause, B.E. Coulman, T.A. McAllister, and K.H. Ominski. 2011. Condensed tannin concentrations found in vegetative and mature forage legumes grown in western Canada. Can. J. Plant Sci. 91:669-675.

Bizimenyera, E.S., J.B. Githiori, J.N. Eloff, and G.E. Swan. 2006. In vitro activity of Peltophorum africanum Sond. (Fabaceae) extracts on the egg hatching and larval development of the parasitic nematode Trichostrongylus colubriformis. Veterinary Parasitology 142:336-343.

Broderick, G.A. 1995. Desirable characteristics of forage legumes for improving protein utilization in ruminants. Journal of Animal Science 73:2760-2773.

Chung, K.T., C.I. Wei, and M.G. Johnson. 1998a. Are tannins a double-edged sword in biology and health? Trends in Food Science & Technology 9:168-175.

Chung, K.T., T.Y. Wong, C.I. Wei, Y.W. Huang, and Y. Lin. 1998b. Tannins and Human Health: A Review. Critical Reviews in Food Science and Nutrition 38:421-464.

Clausen, T.P., I.F.D. Provenza, E.A. Burritt, P.B. Reichardt, and J.P. Bryant. 1990. Ecological implications of condensed tannin structure: a case study. Journal of Chemical Ecology 16:2381-2392.

Coop, R.L., and I. Kyriazakis. 1999. Nutrition-parasite interaction. Veterinary Parasitology 84:187-204.

Cortés, .E., B. Moreno, M.L. Pabón, P. Avila, M. Kreuzer, H.D. Hess, and J.E. Carulla. 2009. Effects of purified condensed tannins extracted from Calliandra, Flemingia and Leucaena on ruminal and postruminal degradation of soybean meal as estimated in vitro. Animal Feed Science and Technology 151:194-204.

Domke, A.V.M., C.Chartier, B. Gjerde, N. Leine, S. Vatn, O. Østeras, and Tuen S. 2011. Worm control practice against gastro-intestinal parasites in Norwegian sheep and goat flocks. Acta Veterinaria Scandinavica 53:29.

Dschaak, C.M., C.M. Williams, M.S. Holt, J.S. Eun, A.J. Young, and B.R. Min. 2011. Effects of supplementing condensed tannin extract on intake, digestion, ruminal fermentation, and milk production of lactating dairy cows. Journal of Dairy Science 94:2508-2519.

Grainger, C., T. Clarke, M.J. Auldist, K.A. Beauchemin, S.M. McGinn, G.C. Waghorn, and R.J. Eckard. 2009. Potential use of Acacia mearnsii condensed tannins to reduce methane emissions and nitrogen excretion from grazing dairy cows. Can. J. Anim. Sci. 89:241-251.

Hagerman, A.E., C.T. Robbins, Y. Weerasuriya, T.C. Wilson, and C. McArthur. 1992. Tannin chemistry in relation to digestion. Journal of Range Management 45:57-62.

Hess, H.D., T.T. Tiemann, F. Noto, J.E. Carulla, and M. Kreuzer. 2006. Strategic use of tannins as means to limit methane emission from ruminant livestock. International Congress Series 1293:164-167.

Hounzangbe-Adote, M.S., V. Paolini, I. Fouraste, K. Moutairou, and H. Hoste. 2005. In vitro effects of four tropical plants on three life-cycle stages of the parasitic nematode, Haemonchus contortus. Research in Veterinary Science 78:155-160.

Hove, L., J.H. Topps, S. Sibanda, and L.R. Ndlovu. 2001. Nutrient intake and utilisation by goats fed dried leaves of the shrub legumes Acacia angustissima, Calliandra calothyrsus and Leucaena leucocephala as supplements to native pasture hay. Animal Feed Science and Technology 91:95-106.

Huang, X.D., J.B. Liang, H.Y. Tan, R. Yahya, and Y.W. Ho. 2011. Effects of Leucaena condensed tannins of differing molecular weights on in vitro CH4 production. Animal Feed Science and Technology 166-167:373-376.

Iason,G. 2005. The role of plant secondary metabolites in mammalian herbivory: ecological perspectives. Proceedings of the Nutrition Society 64:123-131.

Iason, G.R., M. Dicke, and S.E. Hartley. 2012. The Ecology of Plant Secondary Metabolites: From Genes to Global Processes. pp 335. Cambridge University Press.

Iqbal, Z., M. Sarwar, A. Jabbar, S. Ahmed, M. Nisa, M.S. Sajid, M.N. Khan, K.A. Mufti, and M. Yaseen. 2007. Direct and indirect anthelmintic effects of condensed tannins in sheep. Veterinary Parasitology 144:125-131.

Jayanegara, A., F. Leiber, and M. Kreuzer. 2012. Meta-analysis of the relationship between dietary tannin level and methane formation in ruminants from in vivo and in vitro experiments. Journal of Animal Pysiology and Animal Nutrition 96:365-375.

Kahiya, C., S. Mukaratirwa, and S.M. Thamsborg. 2003. Effects of Acacia nilotica and Acacia karoo diets on Haemonchus contortus infection in goats. Veterinary Parasitology 115:265-274.

Khanbabaee ,K., and T. van Ree. 2001. Tannins: classification and definition. Natural Product Reports 18:641-649.

Kozan, E., S.A. Anul, and I.I. Tatli. 2013. In vitro anthelmintic effect of Vicia pannonica var. purpurascens on trichostrongylosis in sheep. Experimental Parasitology 134:299-303.

Kumar, R., and M. Singh. 1984. Tannins: their adverse role in ruminant nutrition. J. Agric. Food Chem. 32:447-453.

Lindroth, R.L., and S.B. St.Clair. 2013. Adaptations of quaking aspen (Populus tremuloides Michx.) for defense against herbivores. Forest Ecology and Management 299:14-21.

Lisonbee, L.D., J.J. Villalba, F.D. Provenza, and J.O.Hall. 2009. Tannins and self-medication: Implications for sustainable parasite control in herbivores. Behavioural Processes 82:184-189.

Lorenz, M.M., L. Alkhafadji, E. Stringano, S. Nilsson, I. Mueller-Harvey, and P. Udén. 2013a. Relationship between condensed tannin structures and their ability to precipitate feed proteins in the rumen. Journal of the Science of Food and Agriculture (wileyonlinelibrary.com) DOI 10.1002/jsfa.6344.

Macedo, I.T.F., C.M.L. Bevilaqua, L.M.B. de Oliveira, A.L.F. Camurç-Vasconcelos, S.M. Morais, L.K.A. Machado, and W.L.C. Ribeiro. 2012. In vitro activity of Lantana camara, Alpinia zerumbet, Mentha villosa and Tagetes minuta decoctions on Haemonchus contortus eggs and larvae. Veterinary Parasitology 190:504-509.

Mangan, J.L. 1988. Nutritional Effects of Tannins in Animal Feeds. Nutrition Research Reviews 1:209-231.

Matthews, S., I. Mila, A. Scalbert, and D.M.X. Donnelly. 1997. Extractable and non-extractable proanthocyanidins in barks. Phytochemistry 45:405-410.

Min, B.R., G.T. Attwood, W.C. McNabb, A.L. Molan, and T.N. Barry. 2005a. The effect of condensed tannins from Lotus corniculatus on the proteolytic activities and growth of rumen bacteria. Animal Feed Science and Technology 121:45-58.

Min, B.R., T.N. Barry, G.T. Attwood, and W.C. McNabb. 2003. The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review. Animal Feed Science and Technology 106:3-19.

Min, B.R., and S.P.Hart. 2003. Tannins for suppression of internal parasites. Journal of Animal Science 81:E102-E109.

Min, B.R., S.P. Hart, D. Miller, G.M. Tomita, E. Loetz, and T. Sahlu. 2005b. The effect of grazing forage containing condensed tannins on gastro-intestinal parasite infection and milk composition in Angora does. Veterinary Parasitology 130:105-113.

Min, B.R., W.E. Pomroy, S.P. Hart, and T. Sahlu. 2004. The effect of short-term consumption of a forage containing condensed tannins on gastro-intestinal nematode parasite infections in grazing wether goats. Small Ruminant Research 51:279-283.

Min, B.R., S. Solaiman, N. Gurung, J. Behrends, J.S. Eun, E. Taha, and J. Rose. 2012. Effects of pine bark supplementation on performance, rumen fermentation, and carcass characteristics of Kiko crossbred male goats. Journal of Animal Science 90:3556-3567.

Molan, A.L., R. Alexander, I.M. Brooks, and W.C. McNabb. 2000a. Effects of an extract from sulla (Hedysarum coronarium) containing condensed tannins on the migration of three sheep gastrointestinal nematodes in vitro. New Zealand Society of Animal Production 60:21-25.

Molan, A.L., W.C. McNabb, S.O. Hoskin, and T.N. Barry. 2000b. Effect of condensed tannins extracted from four forages on the viability of the larvae of deer lungworms and gastrointestinal nematodes. Veterinary Record 147:44-48.

Molan, A.L., L.P. Meagher, P.A. Spencer, and S. Sivakumaran. 2003. Effect of flavan-3-ols on in vitro egg hatching, larval development and viability of infective larvae of Trichostrongylus colubriformis. International Journal for Parasitology 33:1691-1698.

Molan, A.L., G.C. Waghorn, B.R. Min, and W.C. McNabb. 2000c. The effect of condensed tannins from seven herbages on Trichostrongylus colubriformis larval migration in vitro. Folia Parasitologica 47:39-44.

Moreno-Gonzalo, J., F. Manolaraki, P. Frutos, G. Hervás, R. Celaya, K. Osoro, L.M. Ortega-Mora, H. Hoste, and I. Ferre. 2013. In vitro effect of heather (Ericaceae) extracts on different development stages of Teladorsagia circumcincta and Haemonchus contortus. Veterinary Parasitology 197:235-243.

Mueller-Harvey, I. 2006. Unravelling the conundrum of tannins in animal nutrition and health. Journal of the Science of Food and Agriculture 86:2010-2037.

Niderkorn, V., I. Mueller-Harvey, A. Le Morvan, and J. Aufrere. 2012. Synergistic effects of mixing cocksfoot and sainfoin on in vitro rumen fermentation. Role of condensed tannins. Anim. Feed Sci. Technol. 178:48-56.

Novobilsky, A., I. Mueller-Harvey, and S.M. Thamsborg. 2011. Condensed tannins act against cattle nematodes. Veterinary Parasitology 182:213-220.

Paolini, V., F. De La Farge, F. Prevot, P. Dorchies, and H. Hoste. 2005. Effects of the repeated distribution of sainfoin hay on the resistance and the resilience of goats naturally infected with gastrointestinal nematodes. Veterinary Parasitology 127:277-283.

Paolini, V., P. Dorchies, and H. Hoste. 2003. Effects of sainfoin hay on gastrointestinal nematode infections in goats. Veterinary Record 152:600-601.

Patra, A.K., and J.Saxena. 2013. Exploitation of dietary tannins to improve rumen metabolism and ruminant nutrition. Journal of the Science of Food and Agriculture 91:24-37.

Pellikaan, W.F., E. Stringano, J. Leenaars, D.J.G.M. Bongers, S.v.L. Schuppen, J. Plant, and I. Mueller-Harvey. 2011. Evaluating effects of tannins on extent and rate of in vitro gas and CH4 production using an automated pressure evaluation system (APES). Animal Feed Science and Technology 166-167:377-390.

Porter, L. 1992. Structure and Chemical Properties of the Condensed Tannins. p. 245-258. In R.Hemingway, and P. Laks (ed.) Plant Polyphenols. Basic Life Sciences. Springer US.

Rojas, D.K., J. López, I. Tejada, V. Vàzquez, A. Shimada, D. Sànchez, and F. Ibarra. 2006. Impact of condensed tannins from tropical forages on Haemonchus contortus burdens in Mongolian gerbils (Meriones unguiculatus) and Pelibuey lambs. Animal Feed Science and Technology 128:218-228.

Silanikove, N., Z. Nitsan, and A. Perevolotsky. 1994. Effect of a Daily Supplementation of Polyethylene Glycol on Intake and Digestion of Tannin-Containing Leaves (Cerutoniu siliquu) by Sheep? J. Agric. Food Chem. 42:2844-2847.

Tan, H.Y., C.C. Sieo, N. Abdullah, J.B. Liang, X.D. Huang, and Y.W. Ho. 2011. Effects of condensed tannins from Leucaena on methane production, rumen fermentation and populations of methanogens and protozoa in vitro. Animal Feed Science and Technology 169:185-193.

Theodoridou, K., J. Aufrère, D. Andueza, A. Le Morvan, F. Picard, E. Stringano, J. Pourrat, I. Mueller-Harvey, and R. Baumont. 2011. Effect of plant development during first and second growth cycle on chemical composition, condensed tannins and nutritive value of three sainfoin (Onobrychis viciifolia) varieties and lucerne. Grass and Forage Science 66:402-414.

Tibe, O., I.A. Sutherland, L. Lesperance, and D.R.K. Harding. 2013. The effect of purified condensed tannins of forage plants from Botswana on the free-living stages of gastrointestinal nematode parasites of livestock. Veterinary Parasitology 197:160-167.

Tiemann, T.T., L.H. Franco, G. Ramìrez, M. Kreuzer, C.E. Lascano, and H.D. Hess. 2010. Influence of cultivation site and fertilisation on the properties of condensed tannins and in vitro ruminal nutrient degradation of Calliandra calothyrsus, Flemingia macrophylla and Leucaena leucocephala. Animal Feed Science and Technology 157:30-40.

Versari, A., W. du Toit, and G.P. Parpinello. 2013. Oenological tannins: a review. Australian Journal of Grape and Wine Research 19:1-10.

Zhou, H.C., Y.M. Lin, S.D. Wei, and N.F. Tam. 2011. Structural diversity and antioxidant activity of condensed tannins fractionated from mangosteen pericarp. Food Chemistry 129:1710-1720.

Zucker, W.V. 1983. Tannins: Does Structure Determine Function? An Ecological Perspective. The American Naturalist 121:335-365.

2. Availability and the condition of Norwegian bark resources

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2.1 Bark volume produced in Norway

To estimate the volume of bark available from timber production in Norway, the annual volume of logging needs to be determined. In 2013, a total of 9 million m^3 of wood has been harvested and processed in Norway. This volume is mainly composed of the softwoods spruce 6.7 mega (M) m^3 (Norway spruce (*Picea abies*), small quantities of Sitka spruce (*Picea sitchensis*) in the coastal areas of western Norway, and 1.9 M m^3 Scots pine (*Pinus sylvestris*). A total of 1.7 M m^3 hardwood was harvested in Norway during 2013 (Statistics Norway 2014).This volume has been quite stable throughout the last five years (Figure 2.1). Additionally, a considerable amount of hardwood is logged for use as fuel wood which is burned with bark. This volume cannot be estimated reliably and will therefore be neglected in this report. The utilization of the logged volume is summarized in Table 2.1.

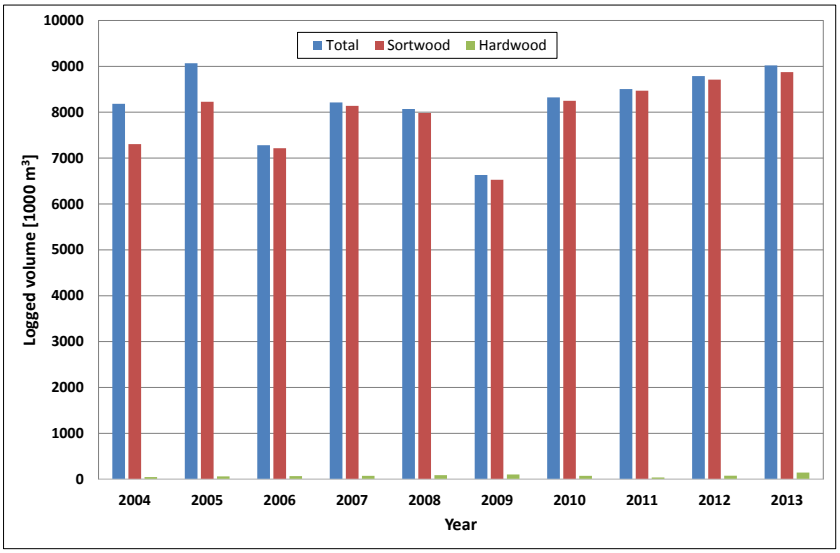

Figure 2.1. Volume of logging [1000 m3] for 2004-20013. Final numbers for 2013 are available 09/2014 (Source: Statistics Norway 2014).

Table 2.1. Utilization of the logged volume [1000 m^3] in 2013 (Source: Statistics Norway 2014).

Assortment	Spruce	Pine	Hardwood
Timber	3,478	1,154	3
Wood for pulping	3,249	808	142
Mixed	166	19	-
Subtotal	6,893	1,951	145
TOTAL		9,019	

The percentage of bark per volume unit of wood is estimated to be 5.5% and 9%, by Rødland (2009) and Tellnes et al. (2011) respectively. These figures are based on reports and official statistics. In the Norwegian forest sector, the average volume percent of bark for the softwoods spruce and pine is estimated to be 8%.
Based on this average figure and the volume of softwoods logged in Norway in 2013, the volume of softwood bark from trees logged in Norway in 2013 is calculated to be 707,520 m^3.

It is assumed that birch represents- the major part of hardwood logged in Norway. Therefore, the total bark volume of hardwood logged in Norway is calculated to be 23 m^3, based on the average bark volume of birch at 20% of stem height, 15.9% (Vadla 1999).

Not all the timber produced in Norway is processed here, since timber is a commodity, too. The estimated import and export is given in Table 2.2. Based on these values, and assuming the same share of softwood and hardwood as in Norway, the total volume of bark available in Norway in 2013 amounts to 707,327 m^3 (707,305 m^3 from softwood and 22 m^3 from birch).

Table 2.2. Estimated import and export of logs [m3] during 2010-2013. The numbers are calculated as value of logs imported and exported, divided by the average price for timber logged in Norway in 2013, 305 NOK/m3 (Source: Statistics Norway 2014).

Year	2010	2011	2012	2013
Import	2285	2251	1454	1191
Export	1390	1595	2595	3888
Balance	895	656	-1141	-2697

In 2013, one of the main customers for pine pulp wood in Norway, Södra Cell Tofte AS, closed its production site in Norway. The production plant was moved to the Värö in southern Sweden. The logging activities in 2013, however, remained unaffected of this. One reason for that could be that the wood chips were needed for pulp production were still produced in Norway and exported to Södra Cell Värö pulp mill in Sweden. Still it is uncertain to which extent this situation will affect logging activities in 2014. Following a conservative calculation approach, the author of this report assumes that the logging volume in 2014 will be reduced by the volume of pine pulp wood logged in 2013.

The Borregaard bio refinery in Sarpsborg does not utilize the bark of the softwood purchased for refinement. According to Borregaard (2012), Borregaard produced a total of 35,760 ton dry bark from 1 million m^3 logs (71% from Norway) in 2012. This bark was mainly sold for use as horticultural products and as biofuel. Thus it is considered available on the general bark marked in Norway.

Assuming an otherwise stable timber marked in Norway, the total volume of bark produced in Norway in 2014 is estimated to be 642,665 m^3 from softwood and 22 m^3 from birch. The bark from birch, however, is considered negligible because of its small volume and the scattered occurrence.

2.2 Contamination of bark

Contamination and impurities of bark potentially cause problems during processing of the bark and the extraction of the tannins. Thus, mechanical sorting and purification have to be included in processing plants. The degree of contamination determines the required purification efforts.

Potential causes for contamination of the bark in the process chain of logging are felling and skidding of the trees and storage of logs and bark in the sawmill. During logging, the trees fall to the ground and the rough surface of the bark takes up some of the underground. Therefore, the season influences the extent of contamination at this process step.

In Scandinavia, most of the logging is fully mechanised, this means that the logs are skidded from the forest with the help of a forwarder. Thus, they are not exposed to contamination by the forest floor, but stacked on a trailer and transported to an intermediate storage place. From there, they are transported to the sawmill. This logging technique contributes little to the contamination of the bark. At most sawmills, logs and bark are stored on concreted areas, minimizing the contamination.

Each processing step and factor influence the degree of contamination of bark at a sawmill. Therefore, a high variation of contamination is expected. Some information characterizing the bark resource is compiled in Table 2.3 and Table 2.4. The moisture content of bark is especially important for its transport and storage stability. Increasing moisture content increases the mass of a given bark volume and reduces the storage stability due to biological degradation. The ash content describes the mineral content of bark. Compared to the value of plain sawdust of 0.3, the high ash content of bark potentially shows mineral contamination of bark due to manipulation and/ or storage. The contamination of bark with sand, soil and stones can be estimated by the silicon-content of the bark samples (Table 4); this value is prominent in sample A. As expected, the range of the elemental composition of the samples is very wide. Due to the structure of the bark, removal of the contaminants from the bark in industrial purification processes is expected to be complex and labour intensive.

Table 2.3. Key data on three samples of spruce bark collected at Norwegian sawmills (Source: Norsk Treteknisk Institutt).

	Sample		
	A	B	C
Moisture content [%]	74.1	56.4	49.5
Basic density [kg/m³]	88.4	150.2	103.0
Ash content [%]	2.4	2.2	2.6
Calorific value [kWh/kg]	5.6	5.6	5.5

Table 2.4. Main elementary composition [g/kg oven-dry mass] of three samples of spruce bark collected at Norwegian sawmills (Source: Norsk Treteknisk Institutt).

	Sample		
	A	B	C
Calcium	322.5	9.4	11.3
Potassium	70.2	2.7	3.0
Magnesia	37.7	0.8	1.0
Phosphorous	22.0	0.5	0.6
Sulphur	6.3	0.3	0.3
Silicon	24.0	0.1	0.7
Zinc	6.9	0.2	0.2

2.3 Availability and uses of bark

It is assumed that only bark from the softwoods spruce and pine is available in volumes economically relevant for refinement. The estimated total volume of bark produced in Norway in 2014 amounts to 642,665 m^3. This amount of bark accumulates in vicinity to wood processing plants, for example sawmills. Since the monetary value of bark nowadays is relatively low, transportation costs are limiting the percentage of bark available for alternative utilization. The profitability of selling bark depends on the distance between production site and refinement site or end marked, respectively.

Traditionally, bark is mainly combusted for energy production directly at the sawmill. Sawdust and wood shavings are mixed into the bark to reduce the moisture content of the burning fuel and to minimize unburned residues. According to Rødland (2009) and Tellnes et al. (2011), the main share of bark is used for combustion (70% and 57%, respectively), leaving 30% or 43%, respectively, for alternative uses.

After shredding and sifting, bark is used horticultural products. For this purpose, it is either used pure to cover the soil and to prevent weeds from growing, or mixed with compost to fertilize garden soil.
Shredded and sifted bark is also used as litter/ bedding in stables for horses and small pets. Ground bark is also sold as sanitary bark for use in outhouses.

Bark can be utilized as absorbing medium for liquids, for example in case of liquid spills in industrial operations. For this purpose, the bark is sifted and ground. Product examples are Miljøbark from Bergene Holm AS, and the systems developed by *MOSE Innovation* AS.

2.4 Price

The price for bark clearly depends on the purchased volume. The biggest supplier of bark for refinement and trade in Norway indicated to sell bark for 100-120 NOK/ lm^3 (loose cubic metre). Additionally it is indicated that the price for Pine bark is higher than that for Spruce bark. Accurate figures about the difference in price could not be found.

Based on an assumed moisture content of bark of 50%, one loose cubic metre equals approximately 550 kg of dry bark. This price is applicable with an annual purchase of several thousand lm^3 of bark which is sifted into fractions of 0-15 mm or 15-40 mm.

Therefore, the price threshold for the availability of bark for the production of additives to ruminant fodder is 120 NOK/ lm^3 or 240 NOK/ ton of dry bark.

2.5 References

Borregaard 2012. Bærekraft og samfunnsansvar i Borregaard. Report, downloaded from www.borregaard.no 25. januar 2014.

Rødland, K. A. 2009. Tømmer og trebaserte produkt i Noreg - virkesstraumar og verdiskaping i den trebaserte verdikjeda. [Logs, wood based products and pulp & paper products in Norway – product flows and value added in the wood based value chain] Master thesis. Department for Ecology and Natural Resource Management, Norwegian University of Life Sciences. 75 p.

Statistics Norway .2014. Commercial roundwood removals, 2013, preliminary figures, Published: 22 January 2014. http://www.ssb.no/en/jord-skog-jakt-og-fiskeri/statistikker/skogav.

Tellnes, L., Flæte, P. O. and Nyrud, A. Q. 2011. Material flows in the Norwegian sawmilling industry. Proceedings of the 7th meeting of the Nordic-Baltic network in wood material science and engineering (WSE), pp. 113-118.

Vadla, K. 1999. Virkesegenskaper hos bjørk, osp og gråor i Troms. Rapport fra skogforskningen 5/99: 48 s. Norwegian Forest and Landscape Institute.

3. Concentration and variation in concentration and type of condensed tannins in bark from Middle Norway

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3.1 Introduction

Bark - a sawmill by-product - could be an important raw material for the production of bio pesticides or feed additives. Bark contain condensed tannins (CT; also known as proanthocyanidins) that are flavanoid polymers believed to play an important role in a tree's defence towards external invasions by wood-rotting fungi, microbes or insects. Condensed tannins have also been shown to prevent infections of parasitic worms and to have nutritional benefits when ingested by animal-s. Before any utilization of bark, we need to a definition of condensed tannins, how they behave and how they can be analysed and quantified.

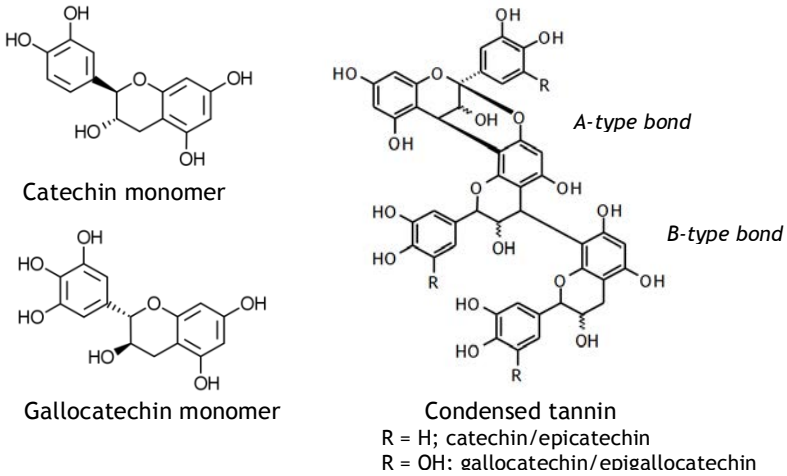

Figure 3.1. Condensed tannins in bark are predominantly polymers of catechin and gallocatechin and their epimers, epicatechin and epigallocatechin, interlinked with double bonds (A-type) or single bonds (B-type).

Condensed tannins are complex flavanoid polymers widely distributed in higher plants and especially rich in gymnosperm bark. They consist of flavan-3-ol monomer units linked primarily through carbon-carbon bonds (B-type bonds), but carbon-oxygen bonds may also occur (A-type bonds). A key feature of CT is that they yield anthocyanidins upon heating in acidic media, hence their name. CT in bark are largely polymers of procyanidins (catechin and its epimer) and to a lesser extent, prodelphinidins (gallocatechin and its epimer) (Figure 3.1). The polymeric nature of condensed tannins makes their analysis and estimation in bark difficult. Free CT in the bark are solvent extractable and referred to as free or "extractable" CT.

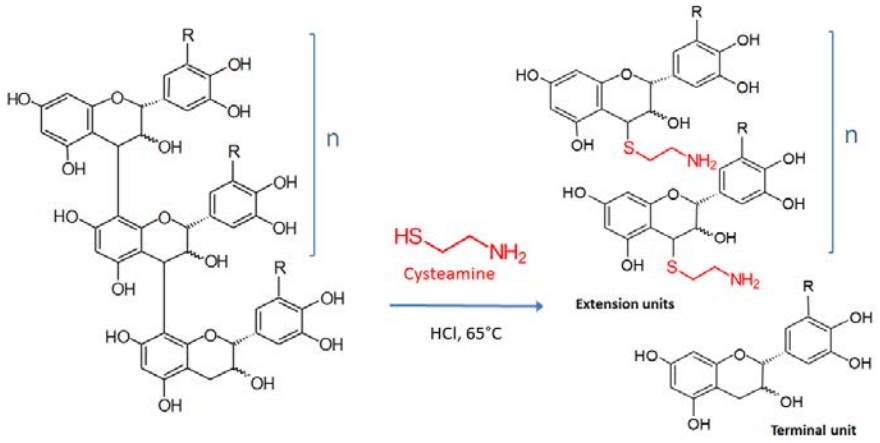

Figure 3.2. After thiolysis of bark with the nucleophile cysteamine, the CT interflavan links are broken and extension units are released as flavanol-cysteamine adducts and terminal units are released as free flavanols.

CT that are protein-bound or fibre-bound in the bark need to be released by thiolysis, i.e. an acid-catalysed depolymerisation reaction in the presence of a strong nucleophile (e.g. cysteamine) before analysis (Figure 3.2). Such CT are referred to as bound or "non-extractable" CT.

The depolymerisation of CT with cysteamine yields free monomeric flavanols (terminal unit/chain ending unit) and flavanol-cysteamine (extension units), which can be determined individually with HPLC-PDA and summed into total CT content. Only the B-type bonds are broken during thiolysis; the A-type bonds are intact and produce dimers and trimers which display chromatographic elution profiles different from the monomeric flavanols. As the CT chains in the bark are cleaved during thiolysis, the nominal CT chain lengths - or degree of polymerization (DP) – cannot be determined, but the average degree of polymerization can be calculated by dividing the total CT amount with the amount of terminal units.

3.2 Material and methods

Bark of Scots pine (*Pinus sylvestris*), Norway spruce (*Picea abies*) and Downey birch (*Betula pubescens*) was sampled by Bioforsk from trees cut from January to April in 2013 at different locations in the municipality of Tingvoll (Table 3.1). Samples were taken from lower and upper part of the trunk, and the age of the tree was estimated by tree-ring dating. In addition, bark samples of Scots Pine and Norway spruce were provided by the sawmills MøreTre AS (Surnadal) and Kjeldstad Sagbruk & Høvleri AS (Selbu), respectively. All samples were collected in April 2013. The bark was composed of both inner and outer bark (not separated). The bark samples were cut into smaller pieces, dried at 65°C for 48 hours and homogenized to pass a 0.5 mm screen using a rotor mill (Retsch Ultra Centrifugal Mill ZM 200) (Figure 3.3).

Table 3.1 Bark sample id, sample location, tree species, and estimated age of tree, diameter of trunk and location of bark sample on trunk

Sample id	Species	Location/ Source	Cutting date	Sampling date	Tree age (years)	Trunk diameter (cm)	Location on trunk
1	Scots Pine	Holmeide	Mar.13	03.apr.13	60	35	lower
2	Scots Pine	Holmeide	Mar.13	03.apr.13	40	20	upper
9	Scots Pine	Storset	Mar.13	04.apr.13	70	34	lower
12	Scots Pine	Storset	Mar.13	04.apr.13	41	33	upper
33	Scots Pine	Sellanrå	Jan.13	04.apr.13	63	20	upper
36	Scots Pine	Sellanrå	Jan.13	04.apr.13	95	44	lower
41a	Scots Pine	MøreTre AS	Mars/Apr.13	05.apr.13	-	-	-
41b	Scots Pine	MøreTre AS	Mars/Apr.13	05.apr.13	-	-	-
42a	Scots Pine	MøreTre AS	Mars/Apr.13	05.apr.13	-	-	-
43	Scots Pine	Gjørsvik	Apr.13	08.Apr.13	72	39	lower
46	Scots Pine	Gjørsvik	Apr.13	08.Apr.13	75	19	upper
7	Norway Spruce	Holmeide	Mar.13	03.Apr.13	42	30	lower
8	Norway Spruce	Holmeide	Mar.13	03.Apr.13	24	15	upper
15	Norway Spruce	Storset	Mar.13	04.Apr.13	60	24	upper
16	Norway Spruce	Storset	Mar.13	04.Apr.13	65	26	lower
27	Norway Spruce	Sellanrå	Jan.13	04.Apr.13	25	15	upper
29	Norway Spruce	Sellanrå	Jan.13	04.Apr.13	70	37	lower
56	Norway Spruce	Gjørsvik	Apr.13	10.Apr.13	14	15	lower
57	Norway Spruce	Gjørsvik	Apr.13	10.Apr.13	12	15	upper
61a	Norway Spruce	Kjeldstad AS	Mars/Apr.13	11.Apr.13	-	-	-
61b	Norway Spruce	Kjeldstad AS	Mars/Apr.13	11.Apr.13	-	-	-
62a	Norway Spruce	Kjeldstad AS	Mars/Apr.13	11.Apr.13	-	-	-
62b	Norway Spruce	Kjeldstad AS	Mars/Apr.13	11.Apr.13	-	-	-
3	Downey Birch	Holmeide	Mar.13	03.Apr.13	40	25	lower
4	Downey Birch	Holmeide	Mar.13	03.Apr.13	19	10	upper
21	Downey Birch	Sellanrå	Jan.13	04.Apr.13	18	30	lower
24	Downey Birch	Sellanrå	Jan.13	04.Apr.13	13	10	upper
49	Downey Birch	Gjørsvik	Apr.13	08.Apr.13	38	22	lower
52	Downey Birch	Gjørsvik	Apr.13	08.Apr.13	16	8	upper

Figure 3.3. Dried and homogenized bark samples of Scots pine, Norway spruce and Downey birch.

Total CT in bark (sum of bound and free CT) were determined by HPLC-PDA after depolymerization of the CT in the presence of a nucleophile (Hellström et al. 2008), at the Biotechnology and Food Research laboratory of MTT Agrifood Research Finland. In short, 10 mg homogenized bark sample was weighed into an Eppendorff tube and 1 ml thiolysis reagent (3 g cysteamine + 56 ml methanol + 4 ml aqueous (37%) HCl) was added. The sample was put in a water bath at 65°C for 60 min, and then cooled on ice for 5 minutes. The sample was homogenized using a Whirli mixer and filtrated (0.45 µm regenerated cellulose syringe filter) into an HPLC vial and analysed immediately. Each sample was analysed in triplicate by HPLC-PDA on an Inertsil ODS-3 (GL Sciences Inc., Torrance, CA) reversed phase column (150 × 4.0 mm i.d., 3 µm). The mobile phase consisted of (A) 50 mM phosphoric acid (aqueous), pH 2.5, adjusted by NaOH and (B) acetonitrile. Elution was started isocratically with a constant flow of 5% B in A, 5 min; followed by 5-20% B in A, 5-35 min; 20-50% B in A, 35-45 min; and 50% B in A, 45-50 min. Separation was monitored by PDA (λ1 = 270 nm, λ2 = 280 nm). External standards derived from authentic compounds were used for the quantification of flavan-3-ols (λ1 = 280 nm for (epi)catechin; λ2 = 270 nm for (epi)gallocatechin) and their cysteaminyl derivatives. A representative chromatogram is given in Figure 3.4.

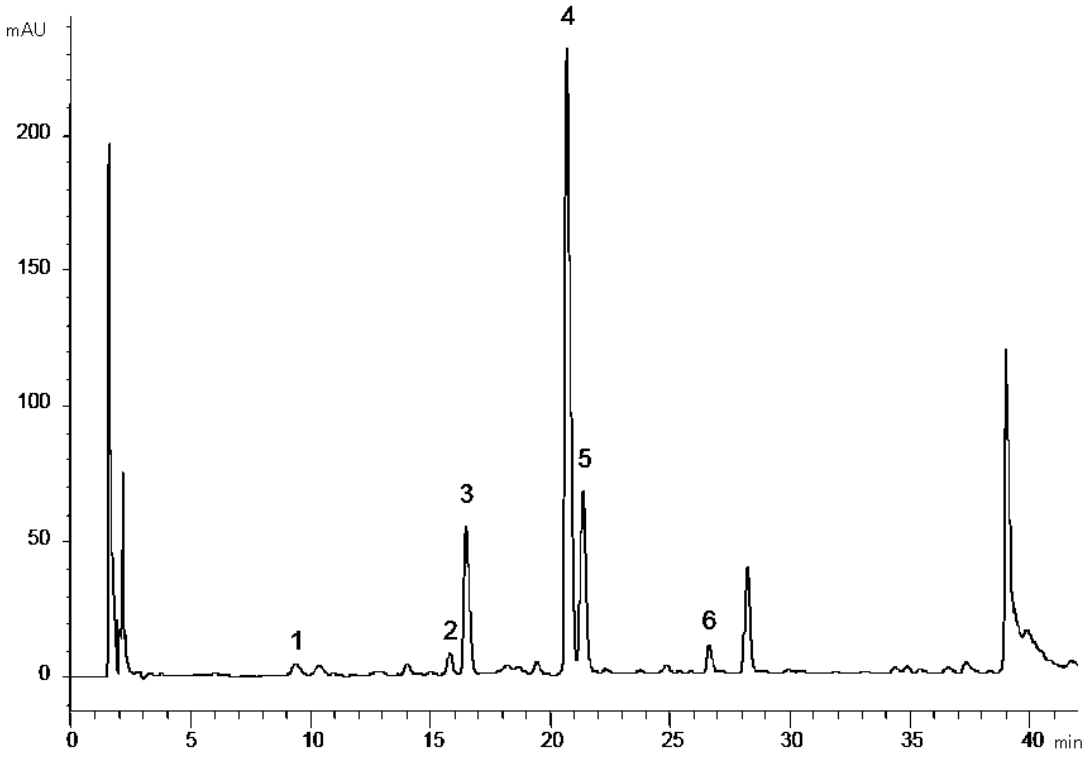

Figure 3.4. HPLC-PDA (λ = 280 nm) chromatogram of birch bark (sample id 3) after thiolysis. Peaks 1, 2, 3 and 4 indicate cysteaminyl thioethers of gallocatechin, epigallocatechin, catechin and epicatechin, respectively. Peak 5 corresponds to catechin and peak 6 to epicatechin.

3.3 Results and Discussion

Thiolysis allows the determination of both free and bound CT (extractable and non-extractable CT) in bark. In pine and spruce, the CT content was highest in bark from the younger trees (≤ 40 years) and was seen to decline in older trees (Figure 3.5, Table 3.2). This result could be due to the fact that the younger trees contained less outer bark than the older trees, as observable from Figure 3.6, and the dead outer bark naturally has less CT - or less available and thiol-reactive CT - than the inner bark (Matthews et al 1997). There will be a decline in free

and soluble CT with increasing tree age as the CT in the dead outer bark is being bound to lignocellulose. Inner and outer bark were not analysed separately in this pilot study, but were mixed into one homogenous sample. The highest total CT content (8.5%) was found in pine bark, whereas up to 6-7 % CT was found in birch and spruce bark. Our data are in agreement with data published previously; levels of 5.5-8.2% CT are reported in Scots pine inner bark and 4.7% in outer bark (Matthews et al. 1997; Hellström et al. 2008). Birch bark had much more variability in the CT content, which seemed to be related to growth site-specific effects.

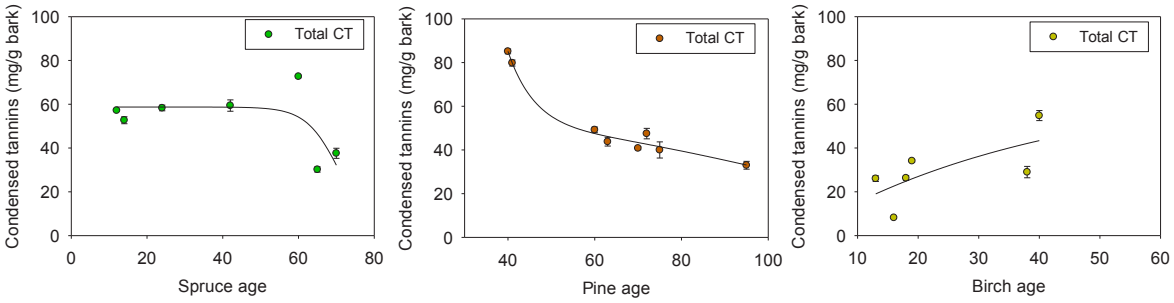

Figure 3.5. The content of condensed tannins (mg CT/g, average ± standard deviation, n = 3) in bark of Norwegian pine, spruce and birch varied with the age of the tree.

Figure 3.6. A higher content of inner bark gave the bark from 40 year old pine (no. 2 and 12) a more yellow appearance, as compared to the brown, predominantly outer bark from the older trees.

Prodelphinidins ((epi)gallocatechin) were detected in spruce (max. 19.9 %) and birch (max. 34 %). Pine contained only procyanidins ((epi)catechin), as also reported by others (Porter 1989; Matthews et al. 1997).

The average size of the CT molecules, given as the average degree of polymerization, was approximately 7-8 in spruce bark, 6-7 in pine bark and 5-6 in birch. The DP level did not vary much with tree age (Figure 3.), in contrast to an expected increase in DP with age due to oxidative polymerization of tannins into larger polyphenols (Viriot et al 1994). DP of spruce bark from Kjeldstad Sagbruk & Høvleri AS in Selbu were not plotted in the figures, as the tree age was not known, but these bark samples displayed the highest DP of all samples (Table 3.2) They also had a higher level of prodelphinidins than the other spruce bark samples.

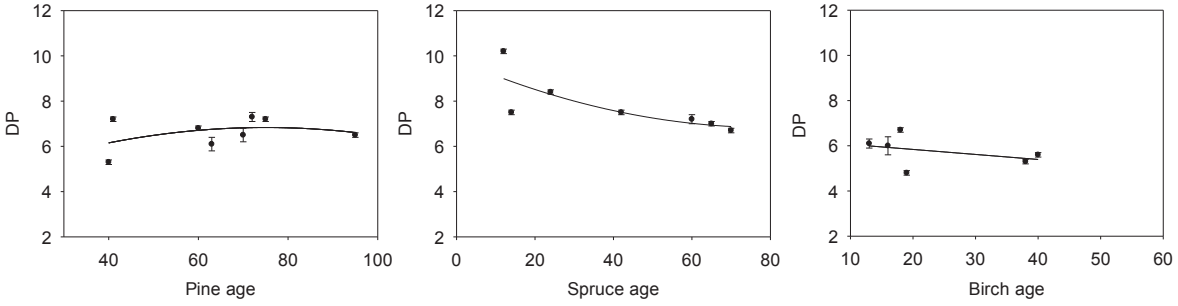

Figure 3.7. Average degree of polymerization (DP) in pine, spruce and birch.

Table 3.2. Condensed tannin amount in bark samples (average ± standard deviation, n = 3), average degree of polymerization (DP), content of procyanidins ((epi)catechin) and prodelphinidins ((epi)gallocatechin) and interflavan bond type of bark CT.

Sample id	Species	Age (years)	Total CT (g/100g)	Average DP	Procyanidins (%)	Prodelphinidins (%)	B-type bonds (%)	A-type bonds (%)
1	Pine	60	4.92 ± 0.13	6.8 ± 0.1	100	0	> 99	< 1
2	Pine	40	8.51 ± 0.11	5.3 ± 0.1	100	0	98.1	1.1
9	Pine	70	4.08 ± 0.07	6.5 ± 0.3	100	0	> 99	< 1
12	Pine	41	7.98 ± 0.14	7.2 ± 0.1	100	0	100	0
33	Pine	63	4.38 ± 0.20	6.1 ± 0.3	100	0	97.8	2.2
36	Pine	95	3.30 ± 0.18	6.5 ± 0.1	100	0	> 99	< 1
41a	Pine	-	3.56 ± 0.12	6.3 ± 0.1	100	0	98.7	1.3
41b	Pine	-	3.21 ± 0.12	7.4 ± 0.2	100	0	86.8	3.2
42a	Pine	-	4.68 ± 0.26	7.1 ± 0.2	100	0	> 99	< 1
43	Pine	72	4.75 ± 0.24	7.3 ± 0.2	100	0	98.1	1.1
46	Pine	75	4.00 ± 0.37	7.2 ± 0.1	100	0	> 99	< 1
7	Spruce	42	5.94 ± 0.26	7.5 ± 0.1	94.4	5.6	100	0
8	Spruce	24	5.83 ± 0.13	8.4 ± 0.1	94.2	5.8	100	0
15	Spruce	60	7.27 ± 0.06	7.2 ± 0.2	100	0	100	0
16	Spruce	65	3.02 ± 0.12	7.0 ± 0.1	100	0	100	0
27	Spruce	25	0.84 ± 0.04	10.8 ± 0.2	100	0	100	0
29	Spruce	70	3.76 ± 0.23	6.7 ± 0.1	100	0	100	0
56	Spruce	14	5.27 ± 0.16	7.5 ± 0.1	94.9	5.1	100	0
57	Spruce	12	5.72 ± 0.03	10.2 ± 0.1	96.6	4.4	100	0
61a	Spruce	-	2.61 ± 0.03	10.1 ± 0.3	83.3	16.7	100	0
61b	Spruce	-	2.01 ± 0.06	9.7 ± 0.5	85.5	14.5	100	0
62a	Spruce	-	1.92 ± 0.06	11.3 ± 0.3	80.1	19.9	100	0
62b	Spruce	-	2.10 ± 0.15	11.3 ± 0.5	81.4	18.6	100	0
3	Birch	40	5.48 ± 0.23	5.6 ± 0.1	84.9	15.6	100	0
4	Birch	19	3.41 ± 0.05	4.8 ± 0.1	90.3	9.7	98.8	1.2
21	Birch	18	2.63 ± 0.11	6.7 ± 0.1	91.9	8.1	98.6	1.4
24	Birch	13	2.60 ± 0.13	6.1 ± 0.2	90.2	9.8	99.2	< 1
49	Birch	38	2.90 ± 0.26	5.3 ± 0.1	94.7	5.3	98.6	1.4
52	Birch	16	0.82 ± 0.04	6.0 ± 0.4	65.8	34.2	100	0

3.4. Conclusion

This study confirmed that CTs in Scots Pine bark are essentially procyanidins, while in Norway spruce and Downey Birch bark, also prodelphinidins can be found. The highest CT levels was found in bark from young (<40 years old) pine trees. The levels were in agreement with other published data and the extraction method used (acidic methanol and depolymerisation of the CTs in the presence of the nucleophile cysteamine) seemed to extract both bound and free CTs in the bark well.

The study has given insight into proanthocyanidin levels in Norwegian resources and highlighted challenges upon their measurement. The obtained results present an important step toward further large scale production of PAs from barks and a basis for further studies of their potential as bio pesticides and feed additives.

3.5 References

Hellström, J. K. and P. H. Mattila. 2008. HPLC determination of extractable and unextractable proanthocyanidins in plant materials. Journal of Agricultural and Food Chemistry 56: 7617-7624.Matthews, S., I. Mila, A. Scalbert, and D. M. X. Donnelly. 1997. Extractable and non-extractable proanthocyanidins in barks. Phytochemistry 45: 405-410.

Viriot C, Scalbert A, Hervé du Penhoat CLM, Moutounet M (1994). Ellagitannins in woods of sessile oak and sweet chestnut-dimerization and hydrolysis during wood ageing. Phytochemistry 36, 1253-1260

4. Isolation and purification of condensed tannins from bark

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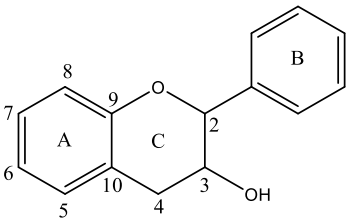
Abstract

The report gives a literature review of the published methods for extraction and analysis of CT. In addition, two most commonly used solvents, 70% acetone and 80% ethanol, were compared in terms of efficiency and yield on the sample marked as **"1".** The crude extract obtained by each solvent is further treated with Sephadex LH-20 and Yb(III)-acetate in order to separate CT from the mixture. The yield is determined by the gravimetric method.

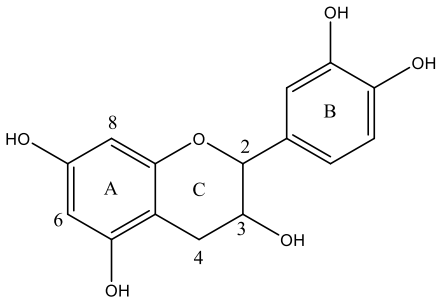
Based on the obtained results and complexity of the extraction procedure, the method consisting of extraction with 70% acetone and precipitation of CT with Yb (III) ions was chosen for determination of CT in the selected samples. The obtained results are given in the Table 4.4. The results and methods were discussed in terms of limitations and applicability on a large scale.

4.1 Structure of CT

CT are a complex group of organic compounds. They are polyhydroxy-flavan-3-ol oligomers and polymers, with C-C bond between monomer units (Fig. 4.1). Variations in number and/or positions of OH groups in flavan-3-ol as a parent compound lead to a variety of CT, as it is shown on Fig. 4.1.



Flavan-3-ol



Proanthocyanidin (PA)

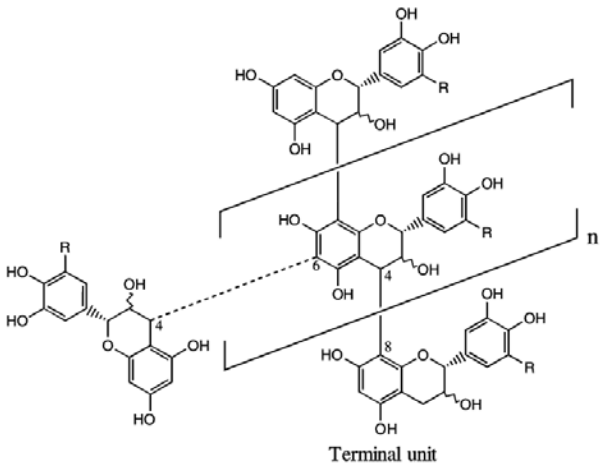


Figure 4.1. Flavan-3-ol and model structure of a condensed tannin. For R=H the structure is procyanidin, for R=OH it is prodelphinidin. The dotted line shows an alternative interflavan bond. The terminal unit is at the bottom of the structure (Schofield et al., 2001).

4.2 Methods for Extraction and Determination of CT

Sample Collection and Preparation

Sample collection and preparation have significant effects on CT analysis. Some of the factors which have to be taken into account and controlled as far as possible during the procedure of collection and preparing the sample for extraction are:

1. Stress; It affects metabolic state of the plant, which can further influence the tannin (and also other metabolites) composition. With the cell's death, cellular integrity is lost and enzymes can come in contact with substrates they are normally not exposed to in living cells.
2. Tannins are, after the cell's death, more exposed to oxidizing agents and they oxidize faster. Oxidation gives quinones, which usually undergo polymerization reactions (Makkar, 2003).

In order to minimize unwanted effects of the above mentioned factors to the tannin composition, it is suggested to:

a. Freeze the plant material shortly after collection, best with liquid nitrogen. This will shut down the metabolism and prevent unwanted enzymatic transformations. Transport and storage should be under dark conditions (prevents photo-induced free radical oxidation).
b. Freeze-dry the samples without thawing (by using a lyophilizer). If the lyophilizer is not available, drying can be done with a forced air oven at 50-52°C. Temperatures higher than 55°C should be avoided, since it can lead to oxidation of phenolics and/or decrease of their extractability (Makkar, 2003).
c. Grind the samples. If frozen with liquid nitrogen, the samples can be ground in homogenizers, taking care that the temperature does not rise during homogenization. Since tannins are usually extracted by aqueous organic solvents, the moisture in the fresh material has to be taken into account while preparing solvents for extraction (Makkar, 2003).

CTs are mixtures of many different compounds and their composition depends on the plant species, age, phase of development, part of the plant, as well as on ecological factors, such as location, local climate and soil characteristics (Schofield et al., 2001). Therefore, those parameters have to be specified during collection of the samples in order to facilitate results interpretation, comparison with other species and drawing according conclusions.

Extraction and Determination Methods

Generally used solvents for extraction of CT are aqueous acetone (70%), aqueous methanol (50%) (Makkar, 2003), or aqueous ethanol (80%) (Hagerman, 2002). The choice of the solvent usually depends on the type of tannins present in the plant, but also on the method used for determination.

There are several analytical methods used for quantifying tannins in plants. They are based on:
- Oxidative depolymerisation of PA,
- Reactions of the ring A with an aromatic aldehyde,
- Oxidation-reduction reactions,
- Acid cleavage reactions,
- Precipitation reactions,
- Enzyme and microbial inhibition and
- Gravimetric methods (Schofield et al., 2001).

It is important to mention that for most of the methods there is still a lot of work to be done in order to improve the efficiency, develop new methods and understand tannin biology and biochemistry.

Acid cleavage reactions

There are several assays based on acid-catalysed cleavage of interflavan bonds of CT. The reaction mechanism and the degree of bond cleavage depend on the reagent used, heterogeneity of tannins and reaction conditions. The most commonly used methods are acid-butanol assay, vanillin assay, thiolysis and phloroglucinol degradation.

Acid-Butanol Assay

The assay is based on acid-catalysed oxidative depolymerisation of CT, yielding red coloured anthocyanidins, which are determined calorimetrically (Fig. 4.2). The reaction is diagnostic for polyflavan structure.

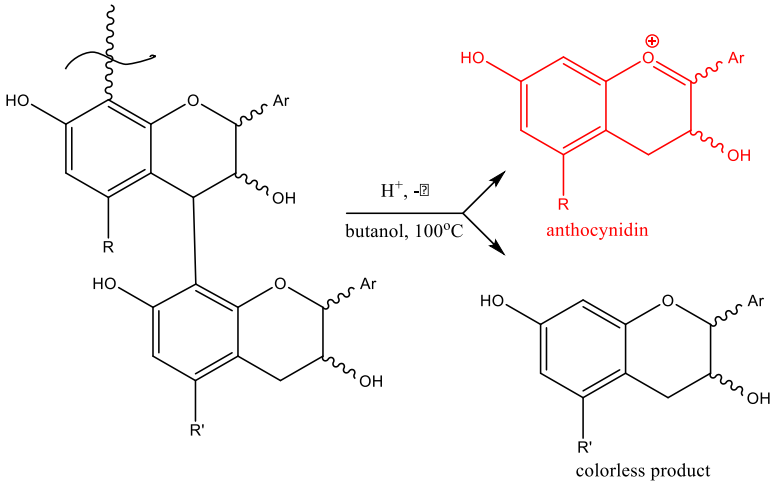


Figure 4.2. Acid - butanol reaction gives red-coloured anthocyanidin and colourless product from the terminal unit (Haslam, 1989).

There are certain issues associated with this method. For example, it is found that the amount of water in the reaction medium affects strongly colour formation and thus accuracy of quantitative determination of PAs. If the water content increases from 2-6% in methanol as a solvent, the anthocyanidin yield of CT is suppressed. The amount of water may lead also to variable colour development for different plant species (Dalzell and Kerven, 1998).

It was also found that interflavan 4®6 bonds are more resistant to cleavage than usual 4®8 bonds (Fig. 4.1). In addition, the nature of substituents of A ring affects interflavan bonds stability in acidic cleavage reaction (Giner-Chavez et al., 1997).

The wavelength of the absorption maximum and extinction coefficients of anthocyanidin products depend on the number of OH groups attached to rings A and B. For example, cyanidin and delphinidin, with 2 and 3 OH groups in the ring B (Fig. 4.3) have l_{max} at 545 and 557 nm, respectively (Hemingway, 1989).

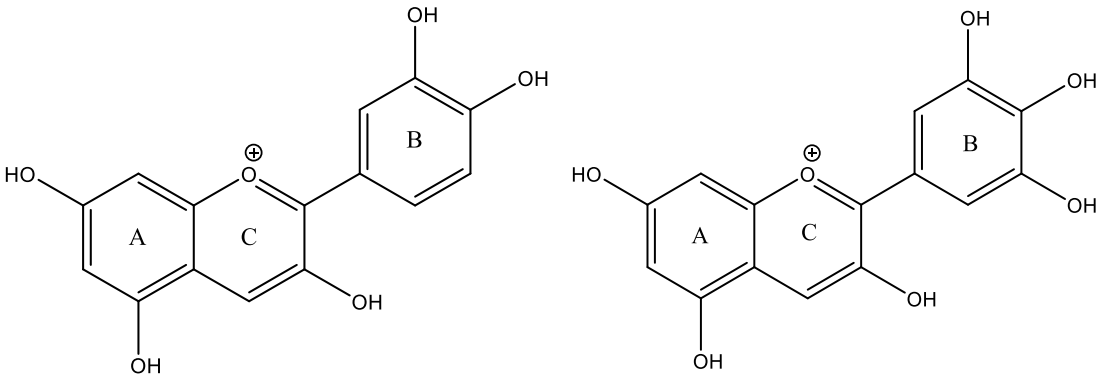


Figure 4.3. Structures of cyanidin and delphinidin

An important fact related to this method is that colour yield is not always linear with the amount of tannin present. Larger amounts could give a lower unit colour yield (Waterman et al., 1994).

Although it was confirmed that the presence of transition metal ions in the medium can influence the colour development, inconsistent results from different research groups call for additional work to clarify the role of metal ions in anthocyanidin formation (Schofield et al., 2001).

The ratio of acid-butanol to sample medium in the reaction mixture is another important factor. When the ratio of reagent to sample increases from 4:1 to 6:1, the colour yield decreases (Dalzell and Kerven, 1998). The temperature and reaction time are also very important factors which can affect the accuracy of the measurement and therefore have to be strictly controlled (Scalbert, 1991).

The choice of standards has been still an issue, due to the heterogeneity of CT. Cyanidin, for example, is an appropriate standard for PAs, but not for prodelphinidins (Scalbert, 1992, Giner-Chavez et al., 1997). Because of considerable variations of CT structures, using a wrong standard can severely under- or overestimate the CT content. It was, therefore, suggested to use internal standards, derived from the investigated plant material (Giner-Chavez et al., 1997).

The acid-butanol method is used to estimate to content of soluble PAs. However, not all PAs are soluble in the common solvents used for extraction. For example, it was reported that after extraction with 70% of aqueous acetone, up to 20 % of PAs remained in the insoluble fraction. This can lead to a significant under-estimation of the tannin content (Makkar et al., 1999).

The method's advantage is the ability to confirm the presence of a polymeric interflavan structure. It is not suitable for hydrolysable tannins and therefore it should be applied with caution as a quantitative assay. However, despite of limitations mentioned above, it remains the most commonly used method for determination of PAs in plants (Schofield et al., 2001).

Vanillin Assay

The method is based on the reaction of vanillin with CT, which yields coloured complexes (Fig. 4.4). Critical parameters for successful use of this method are the type of solvent, used acid and its concentration, the reaction time, temperature, vanillin concentration and type of reference standards (Schofield et al., 2001).

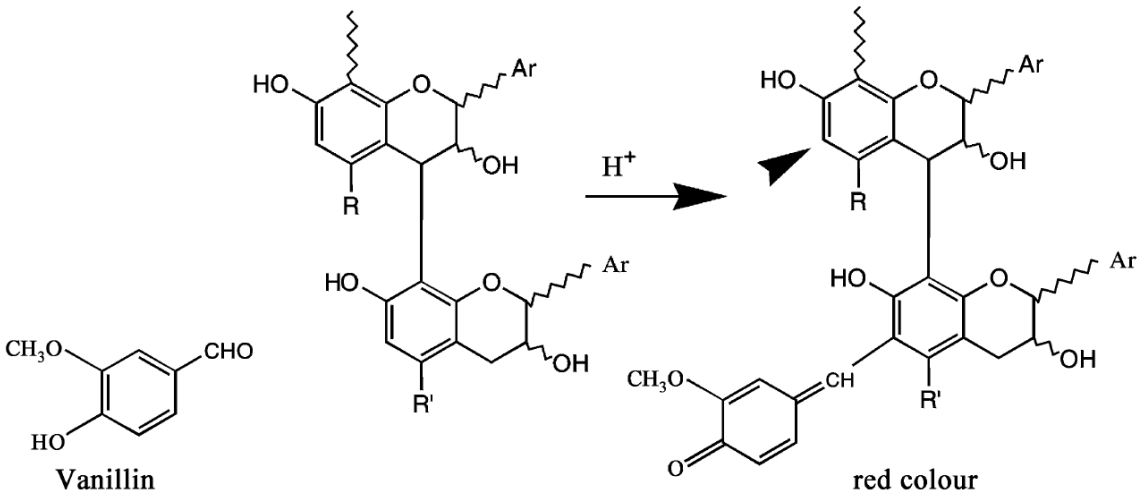


Figure 4.4. The reaction of vanillin with a CT. The arrow shows second reactive site.

Most of already mentioned issues with acid-butanol assay apply to this method as well. The main cause of it lays in complexity and variability of the CT structures (Schofield et al., 2001).

Thiolysis

If CTs are heated in the presence of acid and benzyl mercaptan (toluene-a-thiol), the acid catalysed condensation reaction occurs (Fig. 4.5).

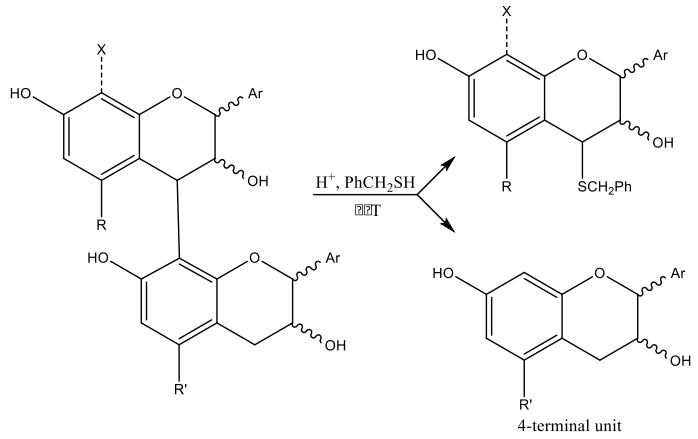


Figure 4.5. Thiolysis of CT. The terminal unit is released with no substituents attached.

If the product mixture is analysed by HPLC, both chain length and composition of a tannin can be determined. Heterogeneity of the analysed CTs could lead to incomplete thiolysis (Matthews et al., 1997). There have been reported also higher yields of thiolysis (88%, (Labarbe et al., 1999), but it is still unclear how the reaction conditions and structure of CT affect the yield.

Phloroglucinol degradation

Phloroglucinol reacts with CT very similarly to benzyl mercaptan (Fig. 4.6), giving, however, much lower yield (3-10%, (Matthews et al., 1997)) than thiolysis. It is therefore that thiolysis is a more preferred reaction.

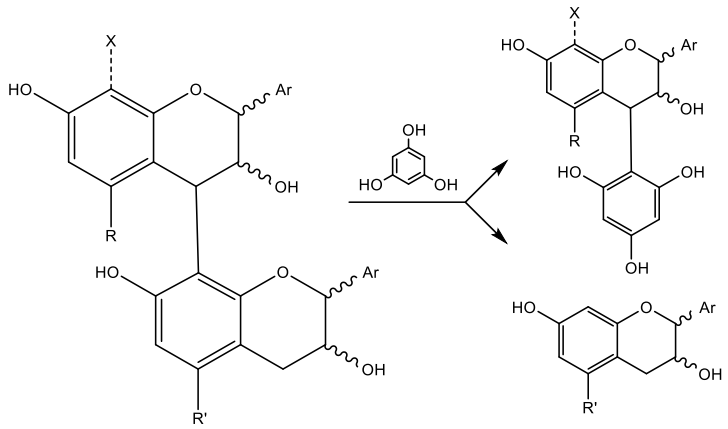


Figure 4.6. Degradation reaction of CT with phloroglucinol

Certain CTs, like prorobinetinidins and profisetinidins, behave abnormally in all acid cleavage reactions. The reason is more cleavage-resistant interflavan bond in the absence of a 5-OH group (Fig. 4.7).

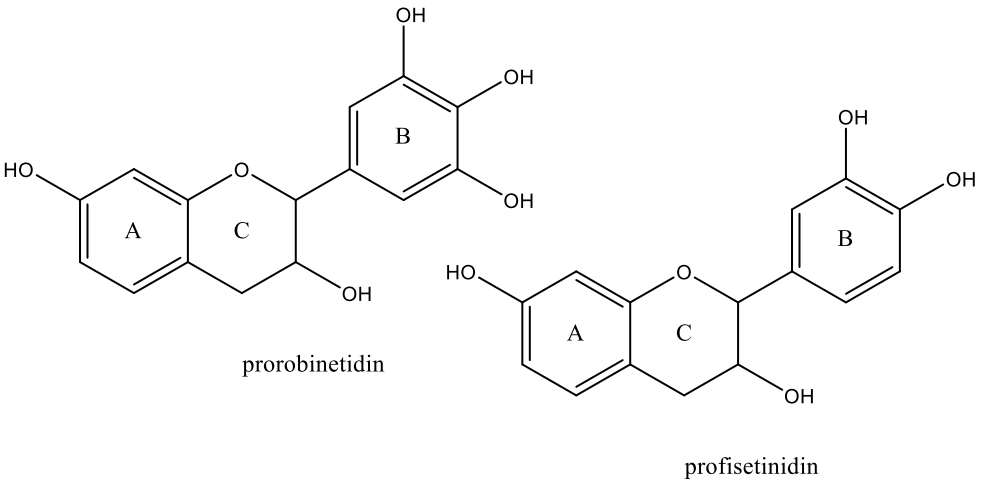


Figure 4.7. Structures of prorobinetidin and profisetinidin. Lack of OH group at position 5 in the A ring makes interflavan bond more resistant to acid-cleavage reactions

Colorimetric assay for total phenolics

There are two most commonly used methods for determination of total phenolics: Prussian Blue assay (Price and Butler, 1977) and the Folin-Denis method (Folin and Denis, 1912a, Folin and Denis, 1912b). The latter has been later modified by Folin and Ciocalteau (Folin and Ciocalteu, 1927). The methods are not specific for any particular group of phenolic compounds. They serve just to quantify the total concentration of phenolic groups in the investigated sample.

Gravimetric methods

There are two gravimetric approaches for quantification of phenolic compounds in plant materials. The first is based on use of trivalent ytterbium to precipitate soluble phenolics (Reed et al., 1985). The second method uses polyvinylpyrrolidone (PVP) to bind tannins, which are then determined by measuring the increase of PVP weight (Makkar et al., 1993). The main advantage of gravimetric methods is that they do not require standards. On the other side, they are less sensitive than colorimetric methods (Makkar et al., 1993). Since Yb (III)-precipitation was the assay used in this WP, more details about it are given below.

Ytterbium (III)-precipitation assay

The method was developed to precipitate CTs in order to use them later as standards in the acid-butanol assay (Giner-Chavez et al., 1997). It was validated by comparison of CTs precipitated by Yb(III) with those purified with commonly used Sephadex LH-20 (Hagerman, 1998). The two extracts were used as internal standards and they gave similar results. Yb(III)-method can be used itself for quantification, by ashing the dry precipitate and by deducting the amount of the remaining ytterbium-oxide.

The method is convenient, faster and less expensive than the Sephadex LH-20 (Giner-Chavez et al., 1997). It was found, however, that certain non-polymeric phenolics precipitate with Yb(III) ions at lower degree, or do not precipitate at all. Therefore, it was concluded that the method is not suitable for determination of total phenolics (Lowry and Sumpter, 1990). However, if the molar ratio of phenolics to Yb(III) ions is controlled, most model phenolics do precipitate (Giner-Chavez, 1996).

Assays Based on Enzyme Activity Changes

Tannins inhibit the activity of many enzymes (Goldstein and Swain, 1965). Methods based on this property have the advantage of giving information about what may be an important bioactivity of the investigated tannins. An assay is influenced by many parameters, the most important being type of enzyme, pH, ionic strength, temperature and reaction time.

Enzyme inhibition by tannins has been studied a lot and there are many publications about it. Among many enzymes which can be used for this study, worthy of mentioning are ß-glucosidase, trypsin, amylase, cellulose and alkaline phosphatase.

Enzyme assays were not given priority in WP4 activity and therefore no detailed review of those methods will be given in this survey.

Protein Precipitation Assays

By definition, tannins are protein-binding and precipitating agents. Therefore tannin assays based on protein precipitation have been very widely used. More information about details of such assays can be found in publications of Waterman and Mole (Waterman et al., 1994), Asquith and Butler (Asquith and Butler, 1985) and Hagerman (Hagerman, 1987).

Polyethylene glycol (PEG) Binding Assay

PEG has a high affinity to bind with a wide range of CTs and hydrolysable tannins, forming a stable complexes in the pH range 2-8.5 (Jones, 1965). The tannin-PEG complex is insoluble in boiling water, neutral and acid detergents and many organic solvents (Jones, 1965, Jones and Mangan, 1977). In addition, PEG can disrupt tannin-protein complexes because of its much higher affinity to tannins. Thus PEG can be used for *in-situ* determination of tannins, even in samples where they are strongly bound to proteins, where extraction with common solvents gives low tannin yields. ^{14}C-labeled PEG 4000, which is used for this assay, is radioactive, which rises safety issues related to work and disposal of such materials (Silanikove et al., 1996).

Analysis of PA with High Performance Liquid Chromatography (HPLC)

HPLC has been extensively used for quantification of CT. Both normal- and reversed-phase (RP) columns have been used for the separation of CT (Cheynier et al., 1999, Lazarus et al., 1999, Waterhouse et al., 1999). Individual CT from monomers to tetramers can be separated on reversed-phase C18 columns, but polymeric CT are better separated by normal-phase HPLC of which CTs display a clear dependence of retention times on the degree of polymerization (Karonen et al., 2011).

Detection method used for HPLC of CT is usually PDA (Waterman et al., 1994). Since it is not specific for CT, other phenolics present in the sample can make the detection difficult. Therefore, use of alternative detectors may be necessary, such as electrochemical and fluorescence detectors (Waterman et al., 1994, Lazarus et al., 1999). For identification of CT oligomers, mass spectrometry, nuclear magnetic resonance and chemical hydrolysis were used (Hammerstone et al., 1999). However, if CT in samples are depolymerized by thiolysis before HPLC-PDA analysis, interferences with other plant constituents are avoided due to the no ambiguous identification of CT-derived products (Matthews et al. 1997; Hellström et al. 2008).

LC-MS methods are also used for analysis of tannins, using various ionization methods. For example, catechin-based CT polymers with more than 10 units have been analysed by MALDI-TOF MS and FAB-MS (Ohnishi-Kameyama et al., 1997).

Inhibition of Microbial Growth

Tannins can inhibit microbial growth, which leads to reduced fibre digestion in ruminants and to decreased infection of plants by plant pathogens. The antimicrobial activity of tannins is usually assessed either by comparisons of microbial growth with and without tannins (Nelson et al., 1997), or by measuring differences in substrate disappearance. Another method is based on determination of fibre digestibility between treated and untreated tannin-containing samples (Giner-Chavez, 1996). Treatment consists of washing the sample with neutral detergent in order to remove non-fibre-bound tannins. The difference in fibre digestibility of treated and untreated samples gives a relative index of inhibition of microbial fibre digestion by tannins. Assessment of antimicrobial activity can be also done by measuring binding of tannins to bacteria, similarly to the protein-binding assays (Jones et al., 1994, Nelson et al., 1997). In this assay tannins are added to bacterial cultures grown without tannins in the medium. The cells are then centrifuged to form a pellet, which is rinsed with buffer and the cells analysed for tannins (Nelson et al., 1997).

Choice of Assay

When choosing an assay for CT determination, it is suggested to get answers to following questions:

1. Are tannins present – yes or no?

A quick answer can be obtained by spot tests, like acid-butanol, or total phenolic assay. Note that the answer might not be certain.

2. What is the amount of material with tannin-like properties?

Consider limitations of certain methods, especially when acid-butanol method is used for quantification. Have in mind importance of using appropriate standard. Internal standard, extracted and purified from the investigated plant material would be maybe the best approach. Any of the colorimetric or precipitation methods can be used.

3. What is the biological activity of this material?

It might be difficult to answer this question. Some of the protein precipitation and enzyme inhibition methods could give some answers. Also, colorimetric method for total phenolics can give indirect information about the bioactivity. It can be designed to react only to ortho-di or tri-phenolic compounds, and there are already evidences that the ortho-phenolic OH groups are associated with the bioactivity.

4. Which group the tannins belong to, hydrolysable, or condensed, or both?
The acid-butanol and vanillin assays are good diagnostic methods for CT. Hydrolysable tannins can be detected by the rhodanine assay for gallate esters (Inoue and Hagerman, 1988).

5. What are the molecular sizes/structures of tannins present?
Physicochemical methods based on thiolysis and GC-MS could provide detailed answers to this question.
The methods for CT analysis presented in this review are also briefly summarized in the Table 4.1.

Table 4.1. A brief summary of the methods for analysis of CT (Schofield et al., 2001)

Assay name	Assay type	Pro	Con	Comments
Acid-butanol (AB)	Chemical, colorimetric	Specific for condensed tannins	Requires internal standard. Colour yield varies with tannin structure	Classical method. Not well suited to quantification
Vanillin	Chemical, colorimetric	Specific for *meta*-diphenols	Same as AB. Simple diphenols also react	
Prussian Blue	Chemical, colorimetric	General test for phenols	Depending on conditions, all phenols can react. Reducing agents react also	Gives best correlation with biological activity
Folin-Ciocalteu	Chemical, colorimetric		Complex chemistry. All phenols react	Prussian Blue method gives better reading of total phenolic content
Thiolysis	Chemical, needs HPLC	Good for structure determination	Requires pure tannin	Benzyl mercaptan is unpleasant to handle
Phloroglucinol	Chemical, needs HPLC		Product yields tend to be low	Thiolysis gives better yields of cleavage products
Ytterbium precipitation	Gravimetric	No standard needed	Yield may vary with Yb:tannin ratio. Sample must be ashed	Can be used to prepare standards for colorimetric analysis
Enzyme assay	Enzymatic inhibition	Gives a more biological evaluation	Some enzymes much more susceptible than others	Does not rely on protein precipitation
Protein precipitation	Precipitation	Reflects a biologically important process	Results depend on many variables such as choice of protein	Can be done in agar plates (Hagerman)
PEG precipitation	Precipitation	Can assay protein-bound tannin	Requires ^{14}C-PEG	
HPLC	HPLC	For polymers up to 7-8 units long	Some condensed tannins bind irreversibly	Better reserved for structural studies
Microbial growth inhibition	Toxicity	A good biological assay	Choice of bacteria and medium composition will affect results	Requires relatively high tannin levels because of competing tannin-binding agents

4.3 Comparison and Selection of the Most Suitable Extraction Method

From a number of published methods for extraction of CT in lab conditions we selected two: 1) Yb(III)-precipitation and 2) isolation of CT by selective adsorption on Sephadex LH-20. As mentioned in Section 4.2, both methods are primarily developed for extracting tannins from a sample for later use as internal standards in a colorimetric assay (e.g. acid-butanol). Colorimetric assays are, of course, more sensitive, but for quick estimation of CT content in a sample, gravimetric approach can be used as well. Gravimetric methods do not require standards, allowing for faster and simpler CT determination, which is in our case a big advantage.

Yb(III)-precipitation is relatively fast and inexpensive method. In our assays we used typically 0.7 g Yb(III)-acetate for 1 g of the sample. With a price of 511 NOK per 10g of Yb(III)-acetate×4H_2O, it seems to be a rather affordable method, at least for small scale assays. Time typically needed for completion an assay is two days.

The drawback of the method is that it is necessary to optimize the amount of added Yb(III) according to the content of CT in a particular sample. If the concentration ratio between CT and Yb(III) ions is too high, the precipitation will be incomplete, leading to under-estimated values for CT content (Giner-Chavez et al., 1997). In addition, if the precipitated CTs should be further used, it is necessary to develop a suitable procedure for their releasing from the Yb-complex and purification.

The Sephadex method is similar to solid phase extraction, because the principle is basically the same: the extract solution containing solvent of low eluting strength (80% ethanol) is brought in contact with Sephadex. CTs are adsorbed on Sephadex and other phenolics washed out with 95% ethanol. By applying the solvent of higher elution strength (50% aqueous acetone) the CTs are eluted. The obtained CTs look very clean, and after lyophilisation have a white, fluffy form. An important advantage of Sephadex is that it can be re-used, which is very important having in mind its relatively high cost. Sephadex was used in our assays in amount of 25g per 1 g of sample and its price was about 6000 NOK per 100g package. Another drawback of Sephadex is relatively long time needed for an assay completion. It takes usually 3 days and the most of the time is consumed by long lasting lyophilisation or freeze-drying processes for removing water. In those 3 days it was not encountered time for regeneration (washing) of Sephadex after use.

A brief summary of the main properties of the methods for extraction and determination of CT used in this WP of the project is given in the Table 4.2.

Table 4.2. A summary of the main properties of the methods used in WP4 of Bark in Feed project

Method	Pro	Contra	Comments
Yb(III)-precipitation	- faster (2 days per assay) - cheaper (511 NOK per 10g)	- necessity to optimize the CT/Yb(III) ratio for every sample - the complexing reaction is slow (12 h at 5°C) - need for developing procedure for releasing CTs from the Yb-complex and purifying, if they are needed as pure for other purposes	Method suitable mostly for analytical purposes.
Sephadex LH-20 selective adsorption	- processes of binding and releasing of CTs to Sephadex are fast - gives clean CTs – a product ready to use - re-usable	- time consuming – 3 days per assay in lab conditions - costly (~6000 NOK for 100 g package) - consumption: typically 25 g per 1 g of sample - regeneration (washing) needed	The most time consuming steps are: 1) water-removing (lyophilisation / freeze-drying), 2) equilibration of Sephadex and 3) regeneration of Sephadex after usage

For initial extraction we compared 70% acetone, as the most common solvent for extraction of tannins, and 80% ethanol. After extraction with either solvent, both Yb(III)-precipitation and Sephadex LH-20 were used for CT determination (Fig. 4.8). The comparison was done on bark Sample 1.

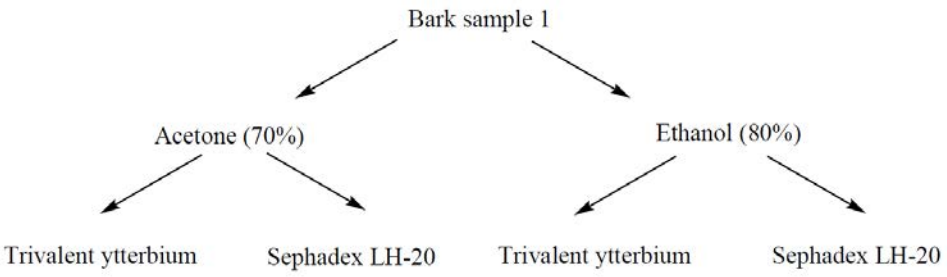


Figure 4.8. Scheme for comparison of solvents for initial extraction

As shown in the Table 4.3, differences between Yb(III) and Sephadex methods were minor, which is in agreement with earlier findings of Giner-Chavez and co-workers (Giner-Chavez et al., 1997). Differences in the CT yield between the solvents are, however, significant and can be explained only by better property of acetone as a solvent for CTs in Sample 1.

When selecting acetone as a solvent for initial extraction, choice of the isolation method was, in a way, limited. In order to adsorb and retain CTs from the crude extract, Sephadex is used in the form of slurry in 80% ethanol. Presence of acetone as a solvent of higher elution strength in this stage would prevent binding of CTs to Sephadex and therefore it is necessary to dry the crude extract. Since it contains 30% of water, freeze-drying is necessary. That process typically requires 12-24 h, which will additionally prolong already long lasting Sephadex assay. Having in mind limited time and resources allocated for this WP, the only rational choice was combination of 70% acetone as a solvent and Yb(III)-precipitation for CT determination.

Table 4.3. Total amount of CTs in Sample 1

Solvent	Method	Yield of CT in Sample 1	
		mg/g	%
70% acetone	Yb(III)-precipitation	9.3	0.93
"	Sephadex LH-20	8.3	0.83
80% ethanol	Yb(III)-precipitation	1.8	0.18
"	Sephadex LH-20	2.6	0.26

4.4 Results of Extraction and Determination of Total CT in the Selected Samples

The chosen extraction method was applied to 12 selected samples and the results are given in the Table 4.4.

Table 4.4. Yield of CTs in the selected samples determined by the gravimetric Yb(III)-precipitation method

Sample ID	Appearance/colour of the extract	CT yield	
		mg/g	%
1	Orange	9.3	0.93
2	Yellow	17.5	1.75
3	Orange	24.6	2.46
4	Yellow	10.3	1.03
7	Orange	6.3	0.63
8	Yellow	18.4	1.84
9	Orange	11.7	1.17
12	Yellow	13.8	1.38
15	Yellow	17.7	1.77
16	Pale yellow	10.5	1.05
42a	Pale orange	13.2	1.32
61a	Orange/brown	35.0	3.50

As mentioned before, gravimetric methods are less sensitive then colorimetric ones. That can explain differences between our results and results I received from Håvard, probably obtained by a colorimetric method (the acid-butanol assay?).

In the description of the Yb(III)-precipitation assay above, it was mentioned that the ratio of CT and Yb(III) had to be controlled, as excess of tannins could lead to incomplete precipitation. Such an optimization has not been carried out in this WP, which could be associated with a measurement error.

4.5 Experimental

All the bark samples were freeze-dried for 24 h before extraction.

Ethanol extraction

The bark (1 g) was suspended in 10 mL of ethanol and kept in the fridge overnight. The following day the extract was filtered. For the sample prepared for the ytterbium experiment, the ethanol was removed (Giner-Chavez et al., 1997).

Acetone extraction

The bark (1 g) was suspended in 20 mL of acetone, sonicated in ice-water for 20 minutes and the extract filtered through a Gooch crucible. The filtrate was transferred to a flask on ice. This was repeated two more times. After the last extraction the bark was washed with 40 mL of acetone. Acetone was then removed under vacuum (Hagerman, 1998).

Isolation of condensed tannins using Sephadex LH-20

The plant extract was mixed with slurry of Sephadex LH-20 (25 g) in ethanol (80%), stirred for 3 minutes and filtered through a Gooch crucible without vacuum. Non-tannin phenolics were separated from the gel by eluting with ethanol (95%) until absorbance at 280 nm was close to zero. Approximately 200–300 mL solvent was needed. Tannin polymers were released from the gel by elution with acetone (50%). Acetone was removed and the aqueous solution was washed 3 times with equal volume ethyl acetate. Traces of ethyl acetate were removed, and the aqueous solution was freeze dried (Hagerman, 1998).

Isolation of condensed tannins using Yb(III)-acetate

The aqueous solution after removing ethanol/acetone was washed 3 times with petroleum ether. Traces of solvent in the water phase were removed under vacuum before washing it 3 times with equal amounts of ethyl acetate. Traces of ethyl acetate in the aqueous phase were removed under vacuum. The remaining solution was separated into portions of 50 mL. Ytterbium(III)-acetate (1 M, 2mL) was added to the aqueous solution and stored over night at 5°C. The next day the stored solution was centrifuged at rpm= 3590 for 10 min at 5°C. After the pellet was washed and re-centrifuged twice with acetone (70%) and once with pure acetone, it was dried under vacuum. The dry CT-ytterbium precipitation was weighed and ashed at 800°C with a burner, above the oxidizing flame, until the black powder turned into grey ash. Before measuring the weight of the ash, the dish was cooled down to room temperature in a desiccator (Giner-Chavez et al., 1997). The total amount of CT is corrected for Yb(III) ash.

4.6 Conclusions

The obtained results indicate that 70% aqueous acetone is a good choice for CT extraction from bark samples. It is easy to handle, relatively cheap and convenient. Concerning the isolation method, Yb(III)-precipitation is convenient for laboratory use. However, when isolation of CT as a final product is the goal, especially on a large scale, the Yb(III) method has certain drawbacks. The overall process would have two main steps: a) complexing-precipitation and b) releasing CTs from the complex. That would very likely impose certain limitations when

planning a large scale production. Namely, a method of releasing CT from the Yb(III)-complex and isolation of pure CT fraction has not been described in literature yet. Although it should not be a serious chemical problem[1], it will be still a considerably long process in industrial scale. In addition, the complexing reaction is very slow (takes approximately 12 h at 5°C), which might be unacceptable for a large scale production.

On the other hand, Sephadex method has drawbacks in length of time needed for separation and high costs. However, it should be mentioned that most of that time is consumed by removing water by freeze-drying/ lyophilization processes during changing solvents, and not by the separation process itself. Sephadex, though, has two big advantages, worth of considering when a large scale production is in the matter. It is re-usable and the release of bound CTs is fast and easy. The obtained CTs are clean and ready for further use.

Disadvantages of Sephadex might be alleviated by using, for example, flow reactors with columns of Sephadex. Continual flow can shorten the time needed for separation, as well as the time for Sephadex equilibration and regeneration after each use. Water removal during switching solvents can be done by conventional freeze dryers, used in food processing and pharmaceutical industry. The nature of the water removal process is such that it will probably have to be a discontinued process, like those in batch reactors.

Those are just few of many aspects that have to be taken into consideration when planning to scale up the process of CT extraction from bark. Nevertheless, the information about chemistry behind those processes, described in this report, can contribute to one of the project goals – assessment of techniques, equipment, efficacy and costs of industrial concentration – processes for condensed tannins as stock food additives.

4.7 References

Asquith, T. N. and Butler, L. G. 1985. Use of dye-labeled protein as spectrophotometric assay for protein precipitants such as tannin. Journal of Chemical Ecology 11: 1535-44.

Cheynier, V., Souquet, J.-M., Le Roux, E., Guyot, S. and Rigaud, J. 1999. Size separation of condensed tannins by normal-phase high-performance liquid chromatography. Methods in Enzymology 299: 178-184.

Dalzell, S. A. and Kerven, G. L. 1998. A rapid method for the measurement of Leucaena spp proanthocyanidins by the proanthocyanidin (butanol/HCl) assay. Journal of the Science of Food and Agriculture 78: 405-416.

Folin, O. and Ciocalteu, V. 1927. Tyrosine and tryptophan determinations proteins. Journal of Biological Chemistry 73: 627-50.

Folin, O. and Denis, W. 1912a. Tyrosine in Proteins as Determined by a New Colorimetric Method. Journal of Biological Chemistry 12: 245-51.

Folin, O. & Denis, W. 1912b. Phosphotungstic-phosphomolybdic Compounds as Color Reagents. Journal of Biological Chemistry 12: 239-43.

Giner-Chavez, B. I. 1996. *Condensed tannins in tropical forages (arachis, desmodium, gliricidia, manihot). Thesis. Cornell University, Ithaca, NY.*

Giner-Chavez, B. I., Van Soest, P. J., Robertson, J. B., Lascano, C. and Reed, J. 1997. A method for isolating condensed tannins from crude plant extracts with trivalent ytterbium. Journal of the Science of Food and Agriculture 74**:** 359-368.

Goldstein, J. L. and Swain, T. 1965. Inhibition of enzymes by tannins. *Phytochemistry* 4: 185-92.

Hagerman, A. E. 1987. Radial diffusion method for determining tannin in plant extracts. Journal of Chemical Ecology 13: 437-49.

1 Acid hydrolysis would probably work for releasing the CT from the complex, while Yb(III) and acetate ions could be removed by ion exchange resin; follows solvent/water removal until pure CTs are obtained

Hagerman, A. E. 1998. Tannin Analysis.

Hagerman, A. E. 2002. The Tannin Handbook. Miami University, Oxford OH 45

Hammerstone, J. F., Lazarus, S. A., Mitchell, A. E., Rucker, R. and Schmitz, H. H. 1999. Identification of procyanidins in cocoa (Theobroma cacao) and chocolate using high-performance liquid chromatography/mass spectrometry. Journal of Agricultural andfood Chemistry 47: 490-496.

Haslam, E. 1989. *Plant Polyphenols*, Cambridge University Press.

Hemingway, R. W. 1989. Reactions at the interflavanoid bond of proanthocyanidins. In: Hemingway, R.W and J.J. Karchesy (eds.). Chemistry and Significance of Condensed Tannins, Plenum Press, New York, pp 265-83.

Hellström, J. K. and P. H. Mattila. 2008. HPLC determination of extractable and unextractable proanthocyanidins in plant materials. Journal of Agricultural and Food Chemistry 56: 7617-7624

Inoue, K. H. and HAGERMAN, A. E. 1988. Determination of gallotannin with rhodanine. Analytical Biochemistry, 169, 363-369.

Jones, D. E. 1965. Banana tannin and its reaction with polyethylene glycols. *Nature* 206: 299-300.

Jones, G. A., McAllister, T. A., Muir, A. D. and Cheng, K. J. 1994. Effects of sainfoin (Onobrychis viciifolia scop.) condensed tannins on growth and proteolysis by four strains of ruminal bacteria. Applied and Environmental Microbiology 60: 1374-8.

Jones, W. T. and Mangan, J. L. 1977. Complexes of the condensed tannins of sainfoin (Onobrychis viciifolia Scop.) with fraction 1 leaf protein and with submaxillary mucoprotein and their reversal by poly(ethylene glycol) and pH. Journal of Agricultural and Food Chemistry 28: 126-36.

Karonen, M., Liimatainen, J. and Sinkkonen, J. 2011. Birch inner bark procyanidins can be resolved with enhanced sensitivity by hydrophilic interaction HPLC-MS. Journal of Separation Science 34: 3158-3165

Labarbe, B., Cheynier, V., Brossaud, F., Souquet, J.-M. and Moutounet, M. 1999. Quantitative Fractionation of Grape Proanthocyanidins According to Their Degree of Polymerization. Journal of Agricultural and Food Chemistry 47: 2719-2723.

Lazarus, S. A., Adamson, G. E., Hammerstone, J. F. and Schmitz, H. H. 1999. High-performance liquid Chromatography/Mass spectrometry analysis of proanthocyanidins in foods and beverages. Journal of Agricultural and Food Chemistry 47: 3693-701.

Lowry, J. B. and Sumpter, E. A. 1990. Problems with ytterbium precipitation as a model for determination of plant phenolics. Journal of Agricultural and Food Chemistry 52: 287-8.

Makkar, H. P. S. 2003. Quantification of tannins in tree and shrub foliage: A laboratory manual. Kluwer Academic Publishers. 102 p.

Makkar, H. P. S., Bluemmel, M., Borowy, N. K. and Becker, K. 1993. Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. Journal of Agricultural and Food Chemistry 61: 161-165.

Makkar, H. P. S., Gamble, G. and Becker, K. 1999. Limitation of the butanol-hydrochloric acid-iron assay for bound condensed tannins. Food Chemistry 66: 129-133.

Matthews, S., Mila, I., Scalbert, A., Pollet, B., LaPierre, C., Herve du Penhoat, C. L. M., Rolando, C. and Donnelly, D. M. X. 1997. Method for Estimation of Proanthocyanidins Based on Their Acid Depolymerization in the Presence of Nucleophiles. Journal of Agricultural and Food Chemistry 45: 1195-1201.

Nelson, K. E., Pell, A. N., Doane, P. H., Giner-Chavez, B. I. and Schofield, P. 1997. Chemical and biological assays to evaluate bacterial inhibition by tannins. Journal of Chemical Ecology 23: 1175-1194.

Ohnishi-Kameyama, M., Yanagida, A., Kanda, T. & Nagata, T. 1997. Identification of catechin oligomers from apple (Malus pumila cv. Fuji) in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and fast-atom bombardment mass spectrometry. Rapid Communications in Mass Spectrometry 11: 31-36.

Price, M. L. and Butler, L. G. 1977. Rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. Journal of Agricultural and Food Chemistry 25: 1268-73.

Reed, J. D., Horvath, P. J., Allen, M. S. & Van Soest, P. J. 1985. Gravimetric determination of soluble phenolics including tannins from leaves by precipitation with trivalent ytterbium. Journal of the Science of Food and Agriculture 36: 255-61.

Scalbert, A. 1991. Antimicrobial properties of tannins. Phytochemistry 30: 3875-83.

Scalbert, A. 1992. Quantitative methods for the estimation of tannins in plant tissues. Basic Life Science 59: 259-80.

Schofield, P., Mbuguda, D. M. and Pell, A. N. 2001. Analysis of condensed tannins: a review. Animal Feed Science and Technololgy 91: 21-40.

Silanikove, N., Shinder, D., Gilboa, N., Eyal, M. and Nitsan, Z. 1996. Binding of Poly(ethylene glycol) to Samples of Forage Plants as an Assay of Tannins and Their Negative Effects on Ruminal Degradation. Journal of Agricultural and Food Chemistry 44: 3230-3234.

Waterhouse, A. L., Price, S. F. and McCord, J. D. 1999. Reversed-phase high-performance liquid chromatography methods for analysis of wine polyphenols. Methods in Enzymology 299: 113-121.

Waterman, P. G. and Mole, S. 1994. Analysis of Phenolic Plant Metabolites. Blackwell Scientific Publications. 248 p.

Quantify anthelmintic activity of bark extracts in vitro

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5.1 Introduction

Dietary condensed tannins (CT) may have a positive effect on animal health as they have been shown to reduce the problems associated with gastrointestinal parasites (Athanasiadou et al., 2001). This may be mediated through an increase in protein supply, which improves their immune response, but also, through a direct antiparasitic effect, which reduces the worm burden and nematode hatchability in sheep and goats (see reviews by Hoste et al. (2006) and Min and Hart (2003)). The inclusion of CT rich forages, such as birdsfoot trefoil (*Lotus corniculatus*), sulla (*Hedysarum coronarium*), sericea lespedeza (*Lespedeza cuneata*) and sainfoin (*Onobrychis viciifolia*), as CT source for animal production in Middle Norway is likely to have limitations; with the exception of birdsfoot trefoil, these species are not native to Norway, and the existing varieties are not adapted to local climatic conditions which will result in low yields, as it has been shown for other countries e.g. Scotland (Athanasiadou et al., 2005). However, Middle Norway has a strong forest and saw-mill industry, and bark is a by- product from the saw-mills that currently is mainly used as fuel. Bark has high concentration of CT (Hellström and Mattila, 2008; Matthews et al., 1997), and recent research from North America has shown that inclusion of pine bark meal into the diet of male goats increased the animal's protein retention and reduced the faecal egg count (Min et al., 2012). The pine bark used in North America was from a species not found in Norway and as a consequence, the results cannot be directly related to the species and conditions available in Middle Norway. Our objective here was to evaluate *in vitro* the anthelmintic activity of selected bark extracts and associate if possible, this activity with certain types of plant secondary metabolites.

5.2 Material and Methods

Plant extracts

Ten bark samples were shipped to Scotland in dried and pulverised form, for anthelmintic activity screening. The selection of bark samples was based on their origin, content and structure of plant secondary compounds. The 10 samples and brief chemical descriptions are seen in Table 5.1. Two types of extracts were prepared from each sample. For the water extracts, 1g of the bark sample was added to 20ml of water, heated at 80°C and stirred on a magnetic stirrer for 30 minutes. The homogenate was then filtered through Miracloth (Millipore, UK); the extract was re-suspended in 20mls of 1% DMSO. For the acetone extracts, 1g of the bark sample was added to 20mls of 70% acetone and sonicated (probe sonicator) in ice for 20sec. This was followed by stirring for 4 min. This procedure was followed 5 times and the homogenate was filtered through Miracloth (Millipore, UK). The filtrate was then placed in a rotary evaporator until all acetone had evaporated; the extract was re-suspended in 20mls of 1% DMSO. Water and acetone extracts from each sample were tested with the assays described below.

Table 5.1. Composition of bark samples shipped to Scotland for anthelmintic screening

Species	Latin	Id	Location	position	Age	Total CTs*	Degree of polymerisation	PCs*	PDs*
Pine	P. sylvestris	2	Holmeide	Upper	40	8.51	5.3	100	0
	P. sylvestris	9	Storset	Lower	70	4.08	6.5	100	0
	P. sylvestris	12	Storset	Upper	41	7.98	7.2	100	0
	P. sylvestris	41a	Møre Tre AS	.		3.56	6.3	100	0
Spruce	P. abies	7	Holm	Lower	42	5.94	7.5	94.4	5.6
	P. abies	15	Storset	Upper	60	7.27	7.2	100	0
	P. abies	27	Sellanrå	Upper	25	0.84	10.8	100	0
	P. abies	61a	Selbu	.		2.61	10.1	83.3	16.7
Birch	B. pubescens	3	Holmeide	Lower	40	5.48	5.6	84.9	15.6
	B. pubescens	52	Gjørsvik	upper	16	0.82	6	65.8	34.2

CTs: condensed tannins
PCs: proanthocyanidins
PDs: prodelphinidins

Egg hatch assays

An egg hatch assay was used to test the anthelmintic efficacy of selected bark extracts. *T.circumcincta* eggs were isolated with a flotation technique (Jackson and Christie, 1982) from freshly collected faeces deriving from donor sheep monospecifically infected with the abomasal nematode. Following their isolation, eggs were washed with distilled water to remove any debris and quantified per ml suspension. From 100-150 eggs were added in each well of 24-well plates, at volumes that did not exceed 500 µl; depending on the egg concentration in the suspension, distilled water was added to make up the appropriate volumes. Equal volume of the bark extract of a given concentration was added to each well. In the negative controls, distilled water was added instead of a bark extract. The plates were cultured at 20°C for 48hr. At the end of this period hatching was stopped by adding Lugol in the samples and the number of eggs and first stage larvae present was counted in each well. Each plant extract was tested in triplicate at 2% concentration (1g of extract in 20mls of 1% DMSO). The % hatching was calculated as: number of first stage larvae / number of first stage larvae + number of eggs per well.

Larval motility assays

A recently modified (Athanasiadou et al, 2013), high throughput larval motility assay was used to test the anthelmintic efficacy of selected bark extracts. The DP xCELLigence Real Time Cell Analyser (Figure 5.1), which measures electrical impedance-based signals across interdigitated microelectrodes integrated on the bottom of tissue culture e-plates (Figure 5.1) has been previously developed to diagnose anthelmintic resistance (Smout et al, 2010). It has been used over the last 2 years in our lab to screen more than 100 plant extracts originating from medicinal plants from Ethiopia (Athanasiadou et al 2013). Third stage (infective larvae) *T.circumcincta* larvae were recovered from faecal cultures of monospecifically infected donor sheep after a 10-day incubation period at 20 °C. Larvae suspension (3,000 L3 per 100µl of PBS) was added in the wells of E-plates and the impedance was monitored every 15 sec, for 24h at 37°C. At the end of this period, bark extracts were added in all wells except the negative control wells, where 1%DMSO was added; these wells served as quality controls and the expectation was that these larvae will be maintained alive until the end of the experiment. In addition to the negative quality controls, positive controls (dead larvae) were also included in 3 wells. The wells with dead larvae received bark extracts at 24h, to enable the testing of our hypothesis: the motility of larvae incubated in bark extracts was statistically the same as the motility of the dead larvae. Impedance signals were monitored in total for 48h (24 prior to and 24h post extract addition) and then the experiments were stopped. Each treatment was run in triplicate. Impedance

data were analysed and a motility index was calculated as described by Smout et al, 2010. Statistical analysis and imaging representation of the larval motility results was performed by the R Project for statistical computing (www.r-project.org/). *T-test* was performed to analyse the egg hatching data.

Figure 5.1. Real Time Cell Analyser and E-plates (Acea, USA)

5.3 Results

Egg hatch assays

The percentage of egg hatching under control conditions was between 95-99% 48h post incubation; it was significantly reduced when water bark extracts were added in the culture. It varied from 0%, which implies complete inhibition of egg hatching to 15% inhibition of hatching 48h post incubation. *P.sylvestris* species (*Pinus*) appeared to be the most effective whereas P. *P. abies* (*Picea*) species were the least effective, but still achieved considerable reduction in egg hatching (Figure 5.2). Acetone extracts of selected plants also showed significant activity, by inhibiting hatching up to 100% in some cases (Figure 5.3).

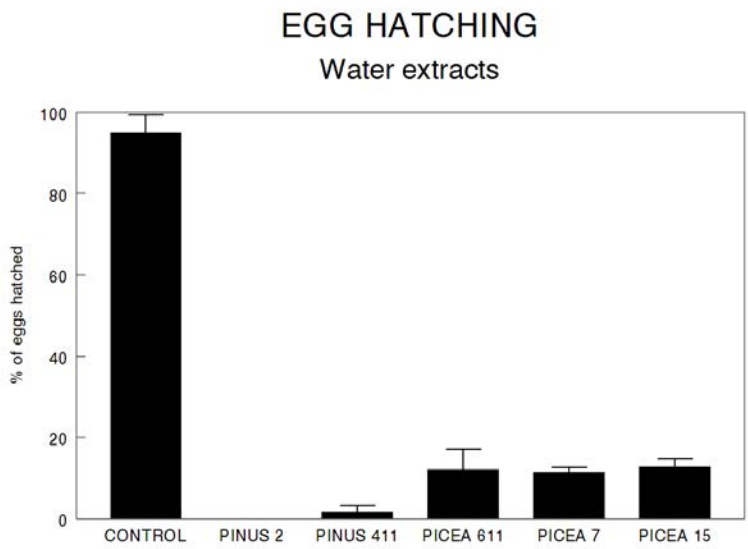


Figure 5.2. Percentage of T.circumcincta egg hatching following incubation for 48h in solutions that contain selected water bark extracts.

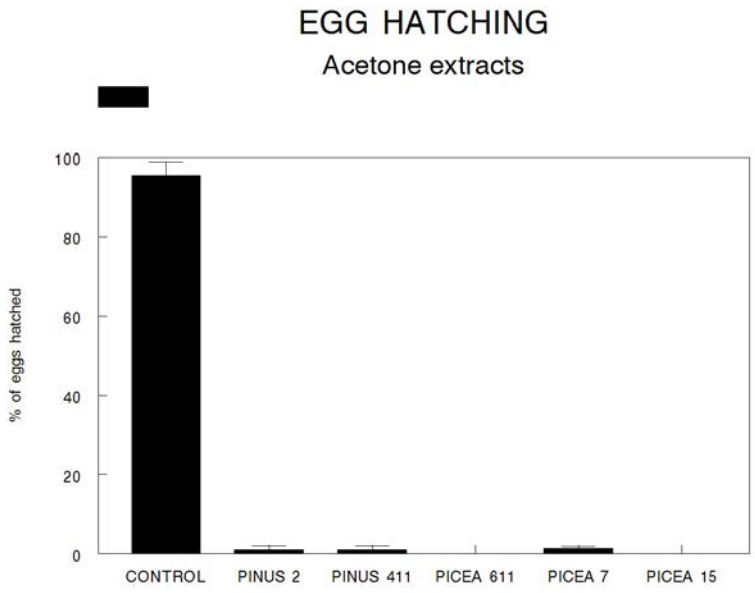


Figure 5.3. Percentage of T.circumcincta egg hatching following incubation for 48h in solutions that contain selected acetone bark extracts.

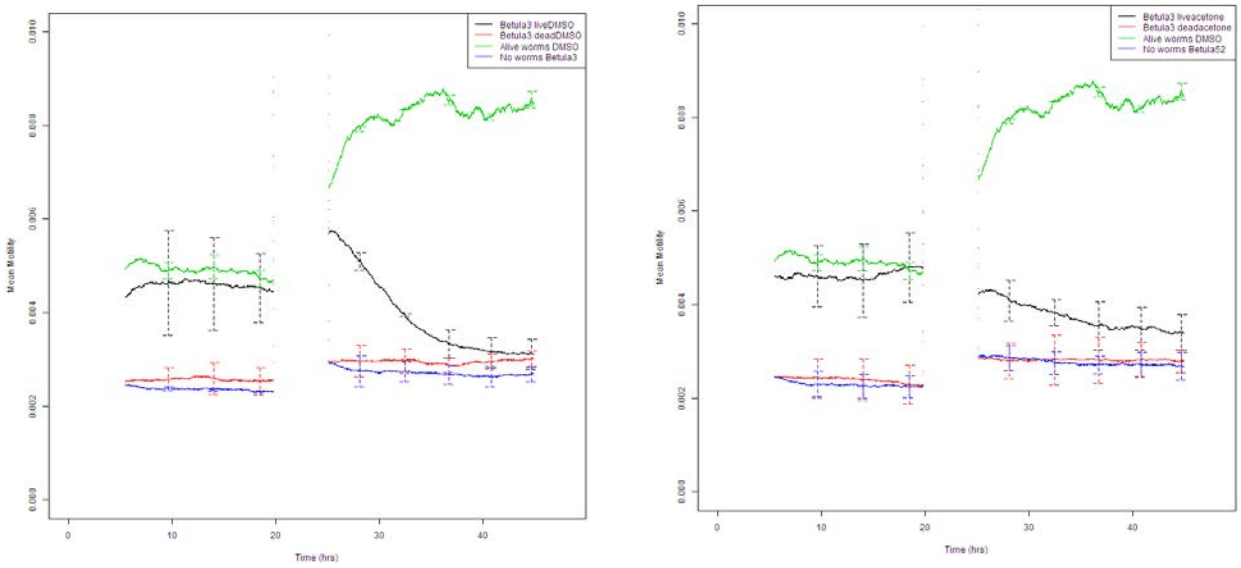
Larval motility assays

Alive control larvae in most cases showed higher motility in the second 24h compared to the first 24h of the experiment; this was likely attributed to the addition of 1% DMSO in the wells, as this appeared to have changed the ionic environment in these wells and as a consequence impedance measurements. The larval motility results indicated that the activity of the extracts is variable between species and between extracts (Table 5.2). Water extracts from both *Betula* species demonstrated consistently high efficacy, in most cases approaching 100% of motility inhibition (Figure 5.4a). Acetone extracts also showed anthelmintic activity, although it appeared weaker compared to water extracts (Figure 5.4b). Similarly, water extracts from the *P.sylvestris* species showed higher activity than the acetone extracts from the same species. Neither water nor acetone extracts from *P. sylvestris* 9 and 12 showed any inhibitory activity on the motility of L3 (Figure 5.5). Acetone extract from the *P. abies* 61a sample showed strong activity; water extracts from two *P. abies* samples (sample 7 and 27) also showed high anthelmintic activity (Figure 5.6).

Table 5.2. Overall results of larval motility assay for water and acetone extracts

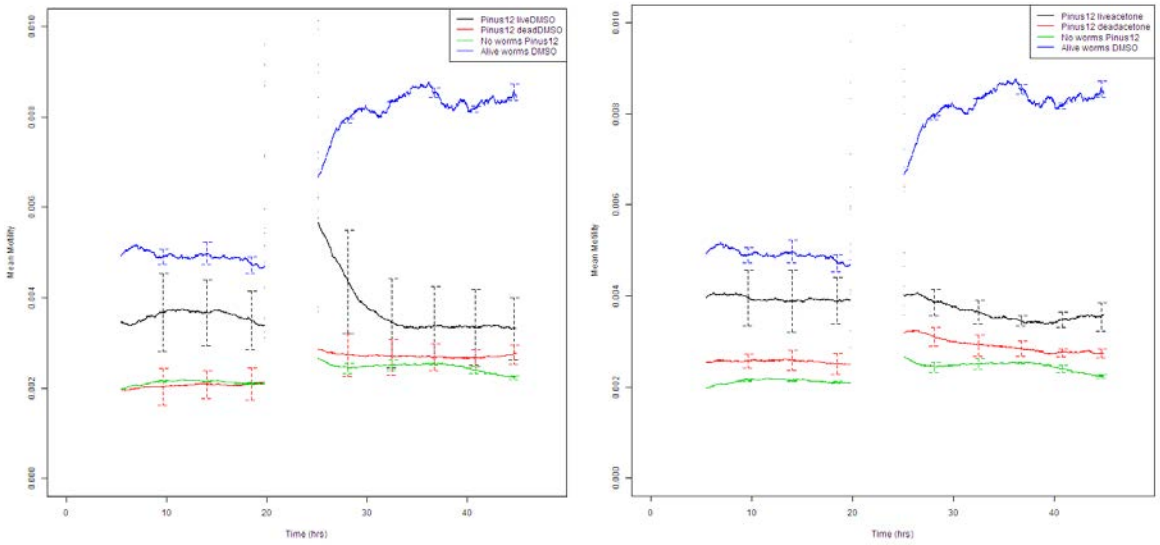
Latin names	Id	Location	Age	Total CTs* g/100g	Degree of poly-merisation	PCs*	PDs*	Water	Acetone
P. sylvestris	2	Holmeide	40	8.51	5.3	100	0	**	NS
P. sylvestris	9	Storset	70	4.08	6.5	100	0	NS	NS
P. sylvestris	12	Storset	41	7.98	7.2	100	0	NS	NS
P. sylvestris	41a	Møre Tre AS	-	3.56	6.3	100	0	*	NS
P. abies	7	Holm	42	5.94	7.5	94.4	5.6	*	NS
P. abies	15	Storset	60	7.27	7.2	100	0	*	NS
P. abies	27	Sell	25	0.84	10.8	100	0	*	NS
P. abies	61a	Selbu	-	2.61	10.1	83.3	16.7	NS	*
B. pubescens	3	Holmeide	40	5.48	5.6	84.9	15.6	**	*
B. pubescens	52	Gjørsvik	16	0.82	6	65.8	34.2	**	*

CTs: condensed tannins
PCs: proanthocyanidines
PDs: prodelphinidins



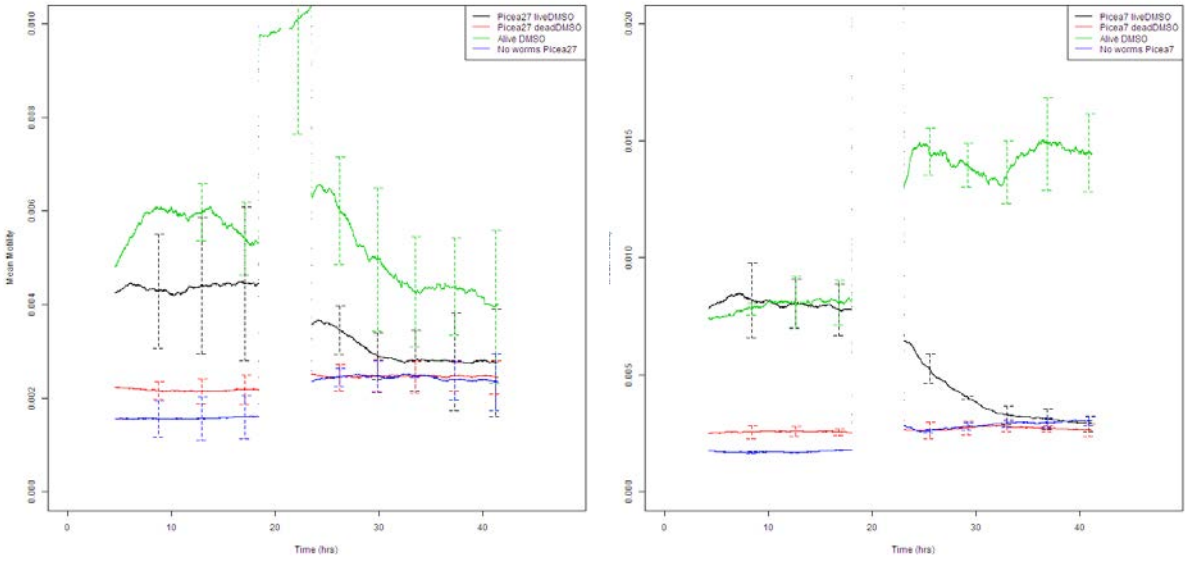
(a) (b)

Figure 5.4. Motility of T.circumcincta L3, during a 48h incubation period prior and following the addition of water (a) and acetone (b) extracts of B. pubescens 3. During the first 24h, all larvae were treated the same; bark extracts were added during the second 24h period



(a) (b)

Figure 5.5 Motility of T.circumcincta L3, during a 48h incubation period prior and following the addition of water (a) and acetone (b) extracts of P.sylvestris 12. During the first 24h, all larvae were treated the same; bark extracts were added during the second 24h period



(a) (b)

Figure 5.6. Motility of T.circumcincta L3, during a 48h incubation period prior and following the addition of water extracts of P.abies 27 (a) and P.abies 7 (b). During the first 24h, all larvae were treated the same; bark extracts were added during the second 24h period

5.3 Discussion and Conclusions

Overview of egg hatching assay results

Percentage of hatching was severely affected by egg incubation in bark extracts; acetone extracts appeared to have higher efficacy compared to water extracts, but incubation in both types of extracts significantly reduced hatching compared to controls. The main metabolites present in the acetone extracts are expected to be condensed tannins, so it is possible that the anthelmintic activity observed is attributed to those. However, other compounds may also be partly responsible, as not all bark extracts tested here had high CT content (e.g. Pinus 41a).

Overview of motility results

Larval motility assay results have demonstrated that there is variation in the anthelmintic activity observed in bark samples, with some samples reducing the motility of larvae to the level of dead worms (100% inhibition). We have also shown that water as well as acetone extracts show anthelmintic activity. Acetone extracts from *P. abies* 61a, *B. pubescens* 3 and 52, all three of which have a high content in PDs (Table 5.1), showed high anthelmintic activity. This is consistent with the literature, where PDs appear to be the type of CTs with anthelmintic activity (Hoste et al, 2006). This is further supported by the fact that acetone extracts from *P. Sylvestris* 2 and 9 and *P. abies* 15, which had the highest overall CT content, but no PDs at all, did not show significant anthelmintic activity. Interestingly, despite the fact that *B. pubescens* 3 and 52 samples differed in their total CT content, acetone extracts of both showed significant anthelmintic activity, likely attributed to the high PD content in both. *P. Sylvestris* 9 sample originated from the oldest tree, whereas *B. pubescens* 52 sample originated from the youngest tree; the expectation was that older trees may show stronger activity compared to younger trees, which was not confirmed in our case. The degree of polymerisation was the greatest in *P. abies* 27 and 61a, whereas it was low in the *B. pubescens* samples; anthelmintic activity did not appear to be related with degree of polymerisation, as acetone extracts from all the above samples showed high biological activity with the exception of *P. abies* 27, where water extract was active. Our data did not support previous evidence where polymer size

appeared to have a positive correlation with anthelmintic activity (Novobilsky et al, 2013). Furthermore, site alone also did not appear to have an effect in anthelmintic activity; *P. Sylvestris* 2 and 12 with high overall CTs content but from different sites both didn't show significant activity. It is important to note that in our experiments, sample size was small and it was not possible to statistically correlate each factor with the strength of the anthelmintic activity present.

In some cases where anthelmintic activity was present in water but not in acetone extracts, such as *P.albies* 7 and 15, and *P. sylvestris* 2, it is possible that the anthelmintic compound is other than condensed tannins. This observation requires further investigation. Furthermore, the anthelmintic efficacy of certain extracts was different against egg hatching and larval motility. For example, acetone extracts from Pinus 2 sample were active to inhibit the hatching of the eggs but did not affect the motility of the larvae. However, as infective larvae are the most resistant form of the parasite during their life-cycle, this was not unexpected. In conclusion, these result clearly showed that extracts from bark samples originating from Norway have anthelmintic activity in vitro. Whether this activity is also observed in vivo to a level that can benefit the animal remains to be seen

5.4 References

Athanasiadou, S., I. Kyriazakis, F. Jackson, and R. L. Coop. 2001. Direct anthelmintic effects of condensed tannins towards different gastrointestinal nematodes of sheep: in vitro and in vivo studies. Veterinary Parasitology 99: 205-219.

Athanasiadou, S., O. Tzamaloukas, I. Kyriazakis, F. Jackson, and R. L. Coop. 2005. Testing for direct anthelmintic effects of bioactive forages against Trichostrongylus colubriformis in grazing sheep. Veterinary Parasitology 127: 233-243.

Athanasiadou, S., Terry S, Tolera A, Debela E, Tolossa K, Connelley T, Burgess S, Houdijk JGM. 2013. *In vitro* validation of the antiparasitic properties of medicinal Ethiopian plant extracts (Proceedings of the British Society of Animal Science).

Christie M, Jackson F. 1982 Specific identification of Strongyle eggs in small samples of sheep faeces. Research in Veterinary Science, 32:113-117.

Hellström, J. K., and P. H. Mattila. 2008. HPLC Determination of Extractable and Unextractable Proanthocyanidins in Plant Materials. Journal of Agricultural and Food Chemistry 56: 7617-7624.

Hoste, H., F. Jackson, S. Athanasiadou, S. M. Thamsborg, and S. O. Hoskin. 2006. The effects of tannin-rich plants on parasitic nematodes in ruminants. Trends in Parasitology 22: 253-261.

Matthews, S., I. Mila, A. Scalbert, and D. M. X. Donnelly. 1997. Extractable and non-extractable proanthocyanidins in barks. Phytochemistry 45: 405-410.

Min, B. R., and S. P. Hart. 2003. Tannins for suppression of internal parasites. Journal of Animal Science 81: E102-E109.

Min, B. R. et al. 2012. Effects of pine bark supplementation on performance, rumen fermentation, and carcass characteristics of Kiko crossbred male goats. Journal of Animal Science 90: 3556-3567.

Novobilský A, Stringano E, Hayot Carbonero C, Smith LM, Enemark HL, Mueller-Harvey I, Thamsborg SM. 2013. *In vitro* effects of extracts and purified tannins of sainfoin (Onobrychis viciifolia) against two cattle nematodes. Veterinary Parasitology 196(3-4): 532-537.

Smout M.J., Kotze A.C., McCarthy J.S., Loukas, A. 2010. A novel high throughput assay for anthelmintic drug screening and resistance diagnosis by real-time monitoring of parasite motility. PLoS Neglected Tropical Diseases 4(11), e885.

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