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Tannins and Tannin Agents

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12.1 Introduction

Tannins are plant phenolics, which is one of the most widespread and complex group of chemical compounds in the plant kingdom. Tannins are constitutive polymeric polyphenols, and fairly recently were also found to be induced by damage (e.g. Constabel, 1999). They have relatively high molecular mass with a typical aromatic ring stucture with hydroxyl substituents. These chemically reactive components easily form conjugations with other biomolecules via their hydroxyl moieties. They are able to precipitate proteins, enzymes, carbohydrates and alkaloids, and are used industrially for millenia to convert raw animal hides into leather. The most effective molecular weight for a tanning process is 500–2000 (3000) and the binding activity is dependent on the tannin structure (Seigler, 1998).

In plants two different groups of tannins are found: hydrolysable tannins (HT) and proanthocyanidins (syn. condensed tannins, CT). Brown algae produce phlorotannins, which consist of units of phloroglucinol (e.g. Porter, 1989; Waterman and Mole, 1994). Evolutionarily older CTs (about 370 million years) preceded hydrolysable tannins and are found in ferns, gymnosperms and angiosperms, especially in dicotyledonous grasses, shrubs and trees (Harborne, 1984; Seigler, 1998). CTs tend to be correlated with a woody growth form of higher plants (e.g. Waterman and Mole, 1994). HTs are found widely in angiosperms and also in some green algae (Harborne, 1984; Waterman and Mole, 1994). Several species also contain complex mixtures of both tannins, such as *Acasia*, *Acer*, *Quercus* and *Betula* (e.g. Reed and Mueller-Harvey, 1987; Julkunen-Tiitto *et al*., 1996; Tikkanen and Julkunen-Tiitto, 2003).

Tannins are found most often in plant cell vacuoles, and are concentrated in epidermal tissues (e.g. Siegler, 1998). Depending on the species, a high proportion of tannins are also found in weakly soluble or insoluble forms, obviously mainly bound into cell wall material

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(e.g. Strack *et al*., 1989; Keski-Saari *et al*., 2005, 2007). Tannins are found in wood, bark, leaves, buds, floral parts, seeds and roots, and usually at high constitutive levels by dry weight of plants. HTs are especially abundant in roots of several woody species (e.g. Keski-Saari and Julkunen-Tiitto, 2003; Keski-Saari *et al*., 2007). A high content of tannins is found in galls induced in plants by herbivores (e.g. Nyman and Julkunen-Tiitto, 2001). The content of tannins will vary over plant phenology and ontogeny. Often young juvenile plants/organs will contain higher amounts of soluble HTs while CTs predominate to the

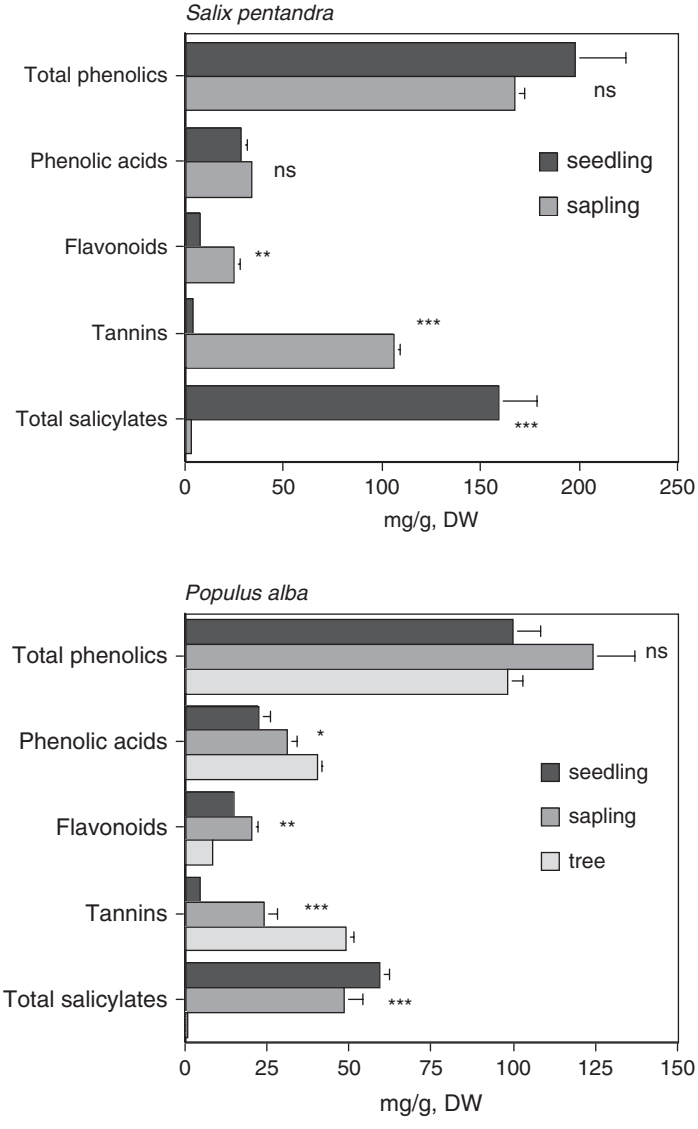


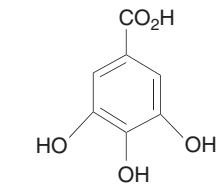
Figure 12.1 *Ontogenetic change of phenolics in the leaves of Salix pentanda and Populus alba*

end of the growing season and in later ontogenetic phases (e.g. Feeny, 1970; Scalbert and Haslam, 1987; Salminen *et al*., 2001; Tikkanen and Julkunen-Tiitto, 2003). Even a linear increase was found in CTs in willows (*Salix sericea* and *S. eriocephala*) with the age of the seedlings (Fritz *et al*,, 2001). Interestingly, the content of soluble CTs is increased in the leaves of two to three year old deciduous *S. pentandra* and *Populus alba* (Salicaceae) saplings, followed by a strong decrease of smaller molecular weight salicylates and flavonoids (Figure 12.1). Moreover, the genotype-dependent tannin metabolism in plants is modified by different environmental factors, such as soil nutrients (e.g. Mansfield *et al*., 1999; Keski-Saari *et al*., 2005). For instance, nitrogen fertilization is found to decrease CTs in several woody species (Mattson *et al*., 2004), while CO_2 elevation alone enhances the accumulation of CTs in *Betula pendula* and *S. myrsinifolia* (e.g. Julkunen-Tiitto *et al*., 1993; Mattson *et al*., 2004). The enhancement of temperature alone reduces CTs in the leave*s of B. pendula* and *B. pubescens* and are unaffected in the leaves of *S. myrsinifolia* (Veteli *et al*., 2002; Veteli, 2003). The interaction effect between different environmental factors has been reported for CTs. In *B. pendula* and *S. myrsinifolia* leaves, CO_2 elevation enhanced CT levels mostly at the highest level of nitrogen fertilizer (Julkunen-Tiitto *et al*., 1993; Mattson *et al*., 2004), while temperature elevation had an opposite effect on CTs at elevated CO_2 and fertilizer level. Though condensed tannins have some absorption in the region of ultraviolet-B (UV-B) radiation they seem not to have a response in the leaves of *Salix* sp. and *Betula* sp. leaves on enhanced UV-B radiation (Tegelberg *et al*., 2003; Keski-Saari *et al*., 2005).

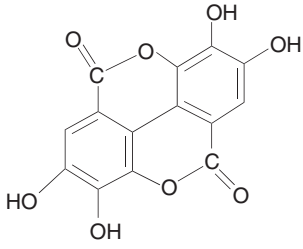
Generally, tannins have a great importance at cellular level as well as in plant physiology and ecology. They may be fytotoxic, antimicrobic, antimycotic, antiviral, allelopathic, phytoalexins, signal molecules and also free radical scanvengers.

12.2 Chemical Structure, Biosynthesis and Degradation

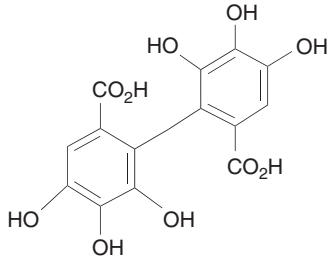
Plants produce a high variety of tannins, leading to an unlimited number of different chemical profiles in different plant organs. The variation in structure of tannins within plant genera may be as great as the variation among genera (Ayres *et al*., 1989). Of three major types of tannins HTs are grouped as gallotannins or ellagitannins, the former are mainly gallic (**1**) and the latter ellagic acids (**2**), yielding on hydrolysis tannins having Mr 500–2000 (Figure 12.2). Both gallic and ellagic acids or other degradation products have also been detected upon hydrolysis of some HTs. Gallic acids in gallotannins are esterified with the hydroxyl groups of central polyol moiety, which is very often glucose but also in some species such as shikimic acid, quinic acid, glucitol, quercitol or cyclitol (Gross, 1992; Salminen, 2002). The simplest and most widespread gallotannin is pentagalloylglucose (ß-1,2,3,4,6-pentagalloyl-*O*-D-glucopyranoside (PGG, **3**), which is proposed to be the precursors of both higher gallo- and ellagitannins. Ellagitannins are esters of hexahydroxydiphenic acids (HHDP, **4**), and are formed by oxidative C–C coupling (most common between C-4/C-6 and C-2/C-3) between the adjacent galloyl residues of related gallotannins (**5**). Hexahydroxydiphenic acid occurs together with gallic acids in certain hydrolysable tannins (in oak and chestnut). In gallotannins additional galloyl residues are attached by depside bonds, usually at the C-3 position of gallic acid (Gross, 1992; Siegler 1998).



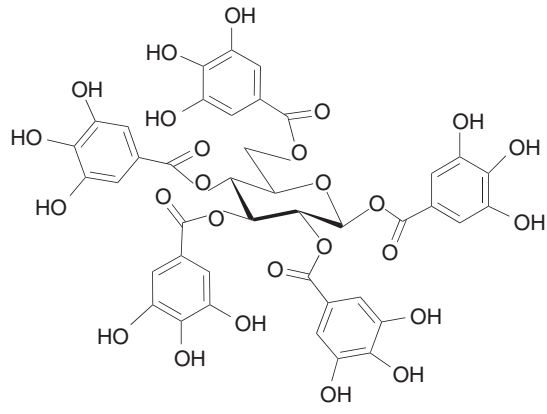
gallic acid (1)



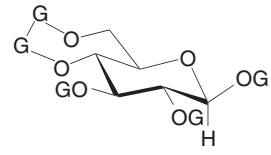
ellagic acid (2)



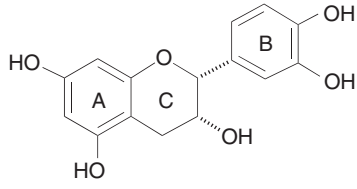
hexahydroxydiphenic acid (4)



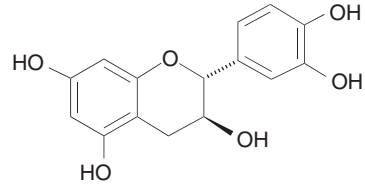
pentagalloylglucose (3)



ellagitannin eugeniin (5)
G = gallic acid
G-G = 4



(–)-epicatechin (6)



(+)-catechin (7)

Figure 12.2 *Some monomers of hydrolysable and condensed tannins and β-pentagalloylglucose*

CTs are oligomers or polymers of 15-carbon polyhydroxyflavan-3-ol monomer units (mostly (-)-epicatechin or (+)-catechin) (**6, 7**) linked by an acid labile C–C bond between C-4 and C-8, which gives linear structures, or between C-4 and C-6, which gives a branched globular structure (Figure 12.2) (Porter, 1992). CTs with C-4/C-8 linkages are more widespread that those of C-4/C-6 (Hagerman and Butler, 1991; Haslam, 1998).

Recently, Cheynier (2005) has reported ether bonds between O-7 and C-2 and between O-5 and C-2. C-3 hydroxylated monomers are the most common (Porter, 1992). CTs are able to be distinguished based on their hydroxylation pattern in the A- and B-rings. The procyanidin unit (3′,4′-hydroxyl substitution in the flavan B-ring) is the most widespread, while propelargonidin polymers (4′-hydroxyl substitution in the flavan B-ring) are rare (Haslam, 1998). Mixed procyanidin and prodelfinidin have been found to co-occur when procyanidin units predominate. Generally, proanthocyanidin polymers are polydisperse and may contain molecules with degrees of polymerization of 2 to more than 50. They are able to bind with ellagitannins as well as gallotannins (Porter, 1992; Haslam, 1998).

Due to their complex chemical nature (oligomers, polymers) to reveal the biosynthesis and polymerization pathways of tannins has been and still is a challenging task. However, most of the main biosynthetic pathways leading to tannins are now well established and many of the enzymes have been cloned (e.g. Peter and Constabel, 2002). Current knowledge indicates that precursors for the biosynthesis of hydrolysable tannins are supplied most probably via the shikimic acid pathway as 3-dehydroshikimic acids (e.g. Gross, 1992; Ossipov *et al.*, 2002). The direct aromatization of 3-dehydroshikimic acid gives rise to gallic acid (3,4,5-trihydroxyl benzoic acid), which is esterified to a core polyol. Monogalloylglucose is the starting compound, which is activated by UDP glucose. UDP glucose activated gallic acid, ß-*O*-glucogallin, functions as both donor and acceptor in the sequential addition of galloyl units to the hydrolysable tannin (Gross, 1992) (Figure 12.3).

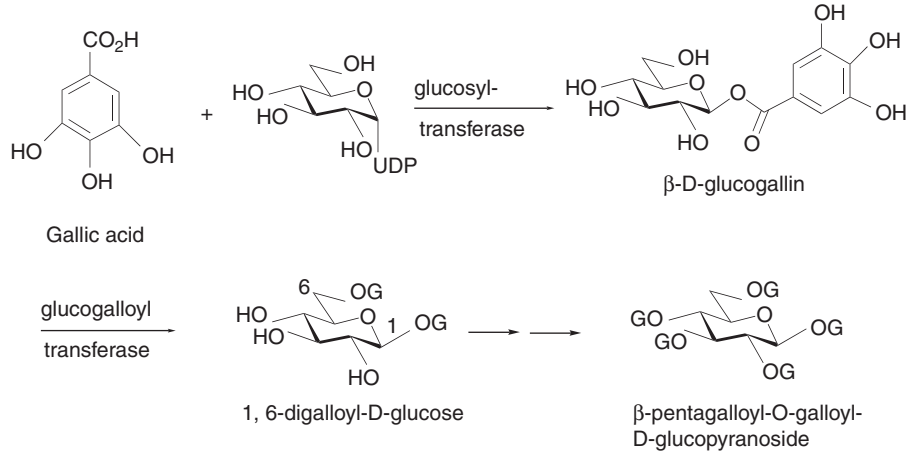


Figure 12.3 *Biosynthesis of hydrolysable tannins*

The biosynthesis of CTs occurs through two different pathways: the phenylpropanoid pathway and the polyketide pathway. The polyketide pathway supplies malonyl moieties for A-ring formation in flavonoid biosynthesis. Via the phenylpropanoid pathway aromatic amino acid, L-phenylalanine is nonoxidatively deaminated to E-cinnamate by phenylalanine ammonia-lyase (PAL). Chalcone synthase catalyses the formation of the chalcone by condensing 4-coumaroyl-CoA with three molecules of malonyl-CoA. This C_{15} skeleton product possesses a monohydroxyl B-ring, typical for nearly all flavonoids, giving rise to

CTs. The chalcone isomerase catalyses the ring closure of chalcone to give rise to flavanone, which is transformed by flavanone 3-hydroxylase to dihydroflavonol and further to leucoanthocyanidin (flavan-3,4-*cis*-diol) catalysed by dihydroflavonol 4-reductase. Flavan-3,4-diol is finally reduced by flavan-3,4-diol 4-reductase to catechin (flavan-3-ol) or via anthocyanidin by anthocyanidin reductase to epicatechin (flavan-3-ol) (e.g. Strack, 1997; Petersen *et al*., 1999; Xie *et al*., 2003). Flavan-3-ols serves as a starting unit for still weakly clarified polymerization for CTs (Waterman and Mole, 1994; Haslam, 1998) (Figure 12.4).

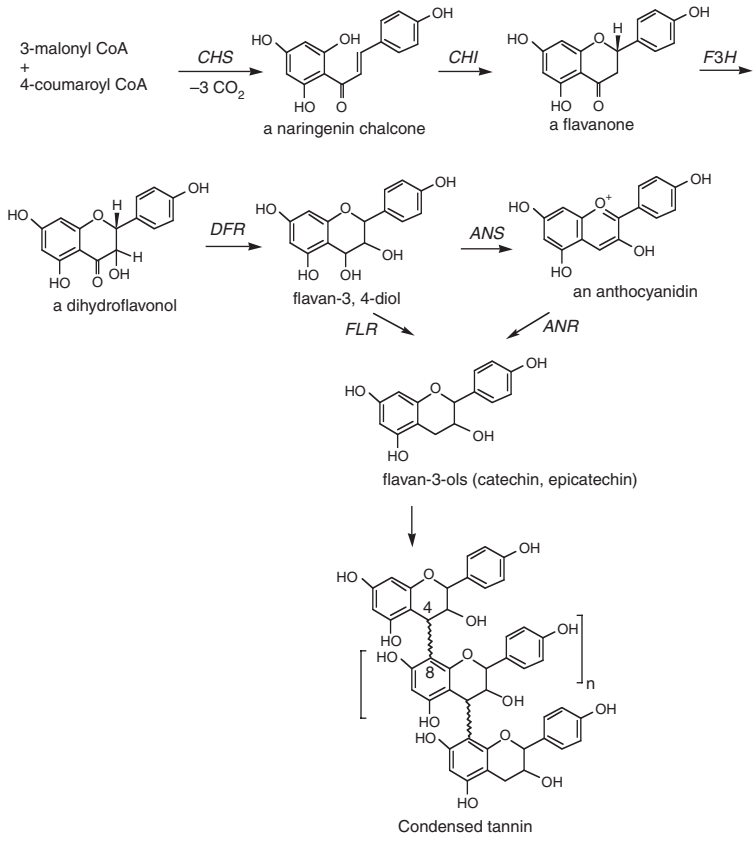


Figure 12.4 *Biosynthesis of condensed tannins*

According to Xie *et al*. (2003) the critical step in the synthesis of flavan-3-ol (epicatechin, leading to CTs production) is that it is catalysed by anthocyanidin reductase (ANR) enzyme encoded by the *Banyuls* gene. This discovery made the epicatechin-based CT production also possible in plant tissues, which do not usually synthesize these compounds, assuming that the tissue contains the necessary precursors, i.e. a functional anthocyanin biosynthetic pathway. Anthocyanin and/or CT pathways have been found to be controlled by over 25 transcription factors belonging to MYB, bHLH, WD40, WKRY, WIP,

homeodomain and bMADS protein families (Dixon *et al*., 2005). Recently, Xie *et al*. (2006) succeeded in metabolic engineering of CT in tobacco through co-expression of anthocyanidin reductase gene and the PAP1 MYB transcription factor.

CT and its precursor, flavan-3-ols, readily ionize in aqueous solution, and solubility of CTs is dependent on phenolate ion formation (Porter, 1992). In mild acid solutions, CTs decompose easily to give flavan-3-ols and quinone methide, while in stronger acid solutions quinone methide is in equilibrium with a carbocation. Carbocation will change to anthocyanidins in hot alcohol solutions (Porter, 1992). CTs are relatively unstable polymers, which may even decompose at natural pH of their aqueous solutions (pH 3–4) (Porter, 1992). Similarly, HTs are susceptible to oxidation at high pH. The ester bonds in HTs are also base and enzyme labile (Halsam, 1989). HTs are readily degraded to gallic acid or hexahydroxydiphenic acids (HHDP) and the core polyol. HHDP will spontaneously form the internal ester ellagic acid (Hagerman and Butler, 1989).

12.3 Properties of Tannins

The presence and intensity of activities underlined for tannins are determined by the structure of each tannin and structural type of tannin (Okuda *et al*., 1989). The use of the chemical properties of tannins obviously dates back to the unwritten history of mankind. Tanning processes to make leather from animal skin was well developed in ancient civilizations from Babylon to China, and leather making belongs to the oldest human activities. Historically tannin use to cure leather dates back to 5000–6000 BC. One example is a leather funeral tent of an Egyptian queen dated at 1100 BC in a museum in Cairo (Hegert, 1989). Tannins (such as hemlock, chestnut, oak, quebracho, mangrove, wattle, etc.) are still important today in the leather industry.

Both CTs and HTs are able to bind and precipitate proteins. The astringent sensation (puckeriness and dryness, loss of lubrication in the mouth and throat) produced in the mouth is due to binding of tannins with mucoproteins (Bate-Smith, 1973; Haslam, 1989, 1998). The formation of soluble and insoluble tannin–protein complexes is caused by crosslinking the separate protein molecules by the phenol (association with hydrogen bonding between hydroxyls of tannins and the polar group of proteins).

Tannins seem to be most astringent to mammals, leading to antinutritional or even toxic effects; tannin–protein complexes are less digestible than free proteins. Tannins may also act by interacting with the gut wall and so affect the passage of nutrients (Hagerman *et al*., 1992). Herbivores show adaptation to tannins; they are able to modify the gut environment to inhibit tannin reactions (high pH and surfactants, or even degrade the compounds). If gut reactions are not enough or have failed, tannins are able to be degraded after being absorbed in the liver (McArthur *et al*., 1991; Buttler, 1993). One of the best-characterized defence mechanisms in animals is tannin-binding protein (TBP) of herbivore saliva (Mehansho *et al*., 1987). Binding of tannins by TBP in the digestive tract could protect the inhibition of digestive enzymes and other essential physiological activities (Mole *et al*., 1990). TBP is shown to contain an extraordinarily high content of proline, as well as glutamine and glycine, and is found in root vole (*Microtus oeconomus*), European moose and North American moose (*Alces alces*) (Juntheikki *et al*., 1996).

The protein precipitating ability of different tannins varies widely and determines most of the physiological activities of herbal medicines, the taste of foodstuffs and beverages and the nutritional value of feeds and herbivore food selection and physiology (e.g. Harborne, 1988). Condensed tannins apparently function against the action of microbes and hydrolysable tannins act as a deterrent against chewing phytophagous insects or animals (e.g. Seigler, 1998). Tannins may also have positive effects as phagostimulants and feeding cues (Schults, 1989). Moreover, tannins are able to inhibit the activity of pathogens by complexing extracellular enzymes produced by pathogens or may interfer with the metabolism of the pathogen itself (Laks and McKaig, 1988; Seigler, 1998). In soil, tannins are reported to have a central role in humus formation by producing complexes that are resistant to microbial decay (Ya *et al*., 1989).

CTs also have an important agronomic trait which protects ruminant animals from potential pasture bloat caused by high-leaf-protein-containing forages. By binding proteins in the rumen condensed tannins reduce the rate of fermentation and subsequent reduction in methane production and better nitrogen nutrition. The recent genome and transcriptome level information of CT biosynthesis and regulation may potentially produce biotechnological approaches to breeding CT-containing forages (such as alfalfa) (Xie *et al*., 2006). However, the recent study failed to reduce enteric methane emissions from growing cattle when the feed contained up to 2 % of the dietary DM as quebarcho tannin extract, although the protein-binding effect of quebracho tannin extract was evident (Beauchemin *et al*., 2007).

In addition to protein, CTs and HTs are able to bind alkaloids, carbohydrates and metals, such as iron, aluminium, copper, vanadium and calsium (Gaffney *et al*., 1986; Porter, 1992; Haslam, 1998). Metal complexes will form readily in an aqueous solution of tannins. Metal complexation of tannins is a distinctive feature and in the past it has been a very important, but poorly investigated, feature. Over many centuries, the blue-black iron tannate complex was the main source of writing inks and also ancient Egyptians used the complex as a hair dye (Slabbert, 1992). Ferric ion complexes with *o*-diphenols are stable, highly coloured and may have negative features in leather manufacture where tannins are used. Contact with iron surfaces, iron-contaminated water or other tannin chemicals results in unsightly blue/grey stains (Slabbert, 1992). Aluminium (III) and boron (III) complexes with tannins (wattle) are colourless. Boric acid complex is reported to be water soluble while iron and aluminium complexes are insoluble. The complex of titanium (IV) with *o*-diphenols is highly yellow-coloured and is used to caste a golden colour on leather.

12.4 Chemical Activities of Tannins

Chemical activities of CTs are determined by the properties of A- and B-rings of the molecules. The A-ring is primarily responsible of the lability of the interflavonoid bond and the use of adhesives and the B-ring determines the properties of formation of metal complexes and antioxidant characteristics of tannins (Laks, 1989). Especially, a 5,7-dihydroxy A-ring is susceptible to interflavonoid bond cleavage under acidic or basic conditions (Hemingway, 1989). Also the interflavonoid bond between C-4 and C-8 is more easily cleaved (**5**) than that between C-4 and C-6, and CTs with the upper units having the

2,3-*cis* configuration (**6**) are more readily decomposed compared with their 2,3-*trans* analogues (Scalbert, 1992). Hydroxylation in the B-ring reflects functionally phenol, catechol and pyrogallol having one, two or three hydroxyls, respectively. Often the reactivity and complexity of reactions increase with addition of hydroxyls to B-rings. Acidity and reactivity to electrophiles and reducing power will increase if comparing phenol to catechol or pyrogallol (e.g. Laks, 1989).

12.5 Analysis of Tannins

12.5.1 Sample Preservation

The research of chemically and biochemically unstable tannins has suffered from methodological difficulties. Care should be taken in sample collection, prehandling, preservation and also quantification of tannin content found in plants. There are several studies available where preservation methods of plant material for tannin analyses have been screened. Generally, whenever possible, intact fresh plant material should be preferred for tannin extraction and analyses (e.g. Harborne, 1984; Julkunen-Tiitto, 1985; Julkunen-Tiitto and Sorsa, 2001; Mueller-Harvey, 2001). However, if analytical procedures necessitate sample preservation, Hagerman (1988) has recommended freeze-dryed or frozen material for CTs, while Orians (1995) preferred vacuum drying. Both freeze drying and vacuum drying were found more appropriate methods than air or oven drying for HTs of *Betula* leaves (Salminen, 2003). The degree of chemical changes during different drying methods is also strongly dependent on plant organs. Oven drying (less than 50 °C) did not change CT content of *Salix* bark but was deleterious for whole twig drying (Julkunen-Tiitto and Tahvanainen, 1989). Instead, heat drying (at higher than 60 °C) of *Salix* leaves induced a strong decrease in soluble CTs and (+) catechins (Julkunen-Tiitto, 1985; Julkunen-Tiitto and Sorsa, 2001), as did oven drying (at 40 °C) for CTs of *Crassula argentea* (succulent species) (Bate-Smith, 1977). If air drying is the only possibility direct sunlight should be avoided (Okuda *et al*., 1989). Moreover, the storage time of dried material will have a strong effect on the extractable yield of tannins. For instance, Scalbert (1992) has reported that the storage of dried sorghum grain for 18 days decreased 15 % of the CT content and 4 years of storage of oak wood decreased 40 % of the ellagitannins yield. Opposite results were obtained for the outbuilding storage of *S. myrsinifolia* dried shoots: there were no change in CT content after one year of storage (Julkunen-Tiitto *et al*., 2005).

12.5.2 Extraction and purification

Generally, tannins are regarded as water soluble. However, the solubility of the higher molecular weight tannins are difficult for any solvent (Hagerman and Butler, 1991). The complex formation is suggested to be one reason for the difficulties of isolation or insolubility of tannins (Porter, 1992). The soluble and insoluble fractions vary among species, sometimes the insoluble fraction may be even higher than the soluble one and is seen as giving a high anthocyanidin (butano/HCl method) or ellagic acid (acid degradation)

yield on hydrolysis of the extracted residue (Porter, 1992; Scalbert, 1992; Keski-Saari *et al*., 2007). However, the extraction conditions, especially temperature, will have a marked effect on tannin extractability. Though most tannins are water soluble, water is not the best extraction solvent for tannins (Scalbert, 1992). Aqueous organic solvent mixtures are recommended for extraction of tannins (e.g. Hagerman and Butler, 1994). The most often used are acetone (e.g. 50 or 70 %) and methanol (e.g. 50 %) as extractants. Aqueous acetone is often shown to be a better extractant than aqueous methanol (Julkunen-Tiitto, 1985; Scalbert, 1992; Hagerman and Butler, 1994). Especially, methanol is not recommended for gallotannins because it may cleave the pepside bonds, even at room temperature, but not in acidified methanol (Haslam *et al*., 1961). Instead, methanol is reported to be a better solvent for tannins of low molecular mass and if plant tissues contain a high amount of enzymes (Mueller-Harvey, 2001). Generally, tannin yields obtained by the solvent mixtures are variable and are dependent on plant species, prehandling of plant material and also on individual tannin structures (Salminen, 2003).

Tannin extracts often contain complex mixtures of other phenolics and nonphenolics (polysaccharides and proteins) (Scalbert, 1992). Solvent fractionation with diethyl ether has been used to separate small molecular mass phenolics (such as gallic acid or catechin) from HTs or CTs (Singleton, 1974). The more feasible method is to use sorption of tannins by Sephadex LH20, Toyopearl HW-40 or Diaion HP-20 (Okuda *et al*., 1989; Hagerman and Butler, 1994). However, it has been reported that on Sephadex, labile oligomeric HTs may be degraded and large molecular mass CTs and HTs may be bound so strongly that they cannot be eluted with acetone (Okuda *et al*., 1989; Mueller-Harvey, 2001). After isolation HTs are fairly stable at room temperature (in the air), while CTs (as well as catechin and epicatechin units) will slowly oxidize to give reddish colours (Okuda *et al*., 1989).

12.5.3 Quantification of Tannins

There are general and specific functional group assays and binding assays to measure the content of tannins in plant material (Hagerman and Butler, 1989, 1991; Porter, 1989). When using colorimetric tests their nonspecificity for tannins should be considered, as recommended by Hagerman and Butler (1989). When the mixture of CTs is measured by colorimetric tests this will at best give results only for semi-quantitative comparisons (Mueller-Harvey, 2001).

General assays for CTs and HTs include the Folin–Dennis (1912), Folin–Ciocalteau (1927) and Prussian blue (Price and Butler, 1977) assays, which are nonspecific and detect all other phenolics and other oxidizable compounds in samples. The Folin–Ciocalteau method gives about 30 % more colour than the Folin–Dennis assay (e.g. Julkunen-Tiitto, 1985), while proteins interfere only weakly in the Prussian blue method (Hagerman and Butler, 1991). Both of the latter methods are recommended for total phenolic determination (Waterman and Mole, 1994).

The most often used and the more specific functional group assay for CTs is anthocyanidins assay (Porter *et al*., 1986) and vanillin addition assay (Broadhurst and Jones, 1978). In anthocyanidin assay (or acid butanol assay) proantocyanidins are depolymerized oxidatively via the interflavan bond by hydrochloric acid in butanol, and subsequent

autooxidation of formed carbocations yields red antocyanidins (Porter *et al*., 1986). Colour formation is sensitive to water and acetone in the reaction mixture (Waterman and Mole, 1994). Transition metals (such as Fe(III)) as autooxidation elicitors enhance colour intensity (Scalbert, 1992). The colour yield is roughly proportional to the nonterminal flavonoid units in CTs, and it also is dependent on the stability of the interflavan bond (Hagerman and Butler, 1991, 1994).

In the vanillin (aromatic aldehyde) test, vanillin forms a red-coloured complex with the meta-substituted ring of CTs and flavanols (such as catechin and epicatechin). Vanillin forms a complex via the flavonol A-ring at C-6 and essential for the reaction is the double bond between C-2 and C-3 and the absence of carbonyl at C-4 (Sarkar and Howarth, 1976). Colour formation is very sensitive to temperature, light and the presence of water. A small amount of water and less than 1.5 ^{o}C difference in temperature may drastically alter the colour yield of the reaction (Porter, 1989; Hagerman and Butler, 1994; Waterman and Mole, 1994). Waterman and Mole (1994) recommended the use of some other methods in addition to the vanillin test for CTs.

Rhodanine and potassium iodate tests have been developed for quantification of gallotannins. The rhodanine test is specific for gallic acid and gives a red colour (Inoue and Hagerman, 1988). The amount of gallic acid is determined before and after hydrolysis of the gallotannins. The weakness of the method is that different gallotannins in plants contain different numbers of gallic acid units, and ellagitannins may also contain gallic acids (Mueller-Harvey, 2001). The potassiun iodate test yielding a pink colour (Bate-Smith, 1977) is shown to be unreliable for complex mixtures of tannins or other polyphenols (Hagerman *et al*., 1997). Hartzfeld *et al*. (2002) have improved the method and introduced a methanolysis step followed by oxidation of formed methyl gallate with KIO_3. The colour test for ellagitannin is based on the hydrolysis and reaction of ellagic acid with sodium nitrite to give a red nitrosylated chromophore (Wilson and Hagerman, 1999). The method is oxygen, glassware purity and time sensitive.

The ability of tannins to precipitate proteins has also been exploited in quantitative determinations and in biological activity assays of the tannin (containing samples) (Hagerman and Buttler, 1989). The quantification is based on the amount of tannin precipitated by standard protein protocols and in biological activity assays; either the amount of precipitated protein is determined or the quality of the complexes formed is analysed (e.g. Makkar *et al*., 1987). Protein-binding responses are dependent on tannin and protein size and structure and reaction conditions (pH, temperature, reaction time, among others) (Hagerman and Butler, 1978; Martin and Martin, 1982; Scalbert, 1992). Proline-rich proteins have a very high affinity for tannins (e.g. Seigler, 1998). The precipitation recovery is also dependent on protein/tannin ratios in the reaction mixture (Hagerman and Robbins, 1987). According to Hagerman and Butler (1989), precipitation assays must be interpreted cautiously. Instead, the simple radial diffusion method developed by Hagerman (1987) is recommended for determining insoluble tannin–protein (bovine serum albumin) complexes where the tannin sample diffuses through protein-containing agar gel. The area of the ring is proportional to the amount of tannin used.

Modern high-performance liquid chromatography (HPLC) accompanied with diode array (DAD) and electrospray ionization (ESI) mass spectrometry (MS) are the powerful methods used for quantification of hydrolysed units of tannins (such as gallic acid, ellagic acid and procyanidins) and also HTs (e.g. Bianco *et al*., 1988; Salminen *et al*., 1999).

Normal (NP) and reversed phase (RP) columns can be used for the separation of mixtures of tannins. RP-HPLC is an appropriate method only for shorter-chain CTs, up to trimers. RP-HPLC used for CTs of *Acacia* sp., *Euclea schimperi* and *Pterolobium stellatum* showed poorly resolved hills eluting between 10 and 20 min (Mueller-Harvey, 2001). Recently, Karonen *et al*. (2006) have developed a quantitative NP-HPLC method of CTs for *Betula* leaf extracts as a late eluting peak in the chromatogram. Salminen (2003) successfully used RP HPLC-ESI-MS (positive and negative modes) for the quanification of HTs in *B. pubescens* leaves. He also compared the rhodanine colour test with the HPLC-MS method and found that the colour test will give an underestimated total concentration of galloylglucoses in an extract. The high selectivity of HPLC-ESI-MS also effectively excludes possible false detection in a complex mixture where HTs may form as a minor fraction together with other more abundant phenolics (Salminen, 2002).

12.6 Use, Toxicology and Safety Aspects of Tannins

The role of tannins in plants is under active research, which has widened their role from the originally presented metabolic by-products to more specific roles. For instance, different tannins may result in different responses within the same herbivore species and the same tannin structure may cause different responses in different herbivores (Rautio *et al*., 2007). These results underline the importance of considering the potential toxicological effects of tannins or tannin residues on a case by case basis.

Tannins are also a part of our everyday life. They are present in many common plant foods such as sorghum, barley, cranberry, wine and tea (Chung *et al*., 1998; Foo *et al*., 2000; Dixon *et al*., 2005). They are important quality factors with a gastronomic value, e.g. in wines, but they are also considered because of their therapeutic or functional food properties. Tannins are strong antioxidants protecting against oxidative tissue damage (Shi *et al*., 2003, and references therein), they protect animal cells against UV radiation induced damage (Carini *et al*., 2000) and they have also been found effective in urinary tract infections (Foo *et al*., 2000). Chung *et al*. (1998) have pointed out that the overall effects of tannins on human health is vast, but sometimes also conflicting. In the review of Chung *et al*. (1998), the authors indicate that the incidences of certain cancers have been related to consumption of tannin-rich foods, while other reports indicate that carcinogenic activity might rather be related to components associated with tannins. Vice versa, CTs have also been shown to prevent the growth of human cancer cells *in vitro* (Tamagawa and Fukushima, 1998; Ye and Krohn, 1999). In addition, the health-promoting role of tannins has been increasingly studied and it has been shown that tannins are able to reduce the levels of serum lipids and blood pressure as well as to improve brain health (Jerez *et al*., 2007). These findings indicate a wide commercial potential of plant-derived tannins.

Tannins are used for very many applications in the pharmaceutical (as gallic acid, pyrogallic acid, sulfanilamide drugs), food (as clarifying agent and flavour stability for beer, wine), feed, dyestuff (as fixing agents), cosmetic and chemical (slurry-treating agents in the petrochemical industry, in treating agents for various industrial manufactures) industries. There are a number of commercial suppliers for different tannin preparations (e.g. http/www.biomatnet.org/secure/Fair/S340.htm, http://www.ferco-dev.com/home.htm,

http//www.indofinechemical.com/). Furthermore mimosa bark (*Acacia mearnsii*), quebracho wood (*Schinopsis balansae*), pine bark (*Pinus radiata*), oak bark (*Quercus* sp.), grape (*Vitis vinifera*) and wattle (*Acacia mearnsii*), are used for industrially produced tannin extracts and preparations (Cadahía *et al*., 1998, Garnier *et al*., 2000; Vivas *et al*., 2003). At present the most intensive use of tannins is in the manufacture of wood adhesives and leather (Taiwo and Ogunbodede, 1995; Garnier *et al*., 2000; López-Suevos and Riedel, 2003; Li *et al*., 2004).

Natural tannin-based adhesives for wood composites have been commercially available for several decades and, especially, the antimicrobial role of tannins has been exploited in the wood composite industry and adhesives (Chow, 1977; Pizzi, 1982; Li and Maplesden, 1998). Building materials such as plywood, other laminated veneer products, fibreboard and fibreglass insulation are major uses of adhesives (Lu and Shi, 1995). Tannin binders are hardened by formaldehyde or other chemicals to form resins (e.g Chow, 1977; Taiwo and Ogunbodede, 1995; Pizzi, 2006). Petrochemical-derived formaldehyde-based wood adhesives are still today the dominating ones in European markets. However, formaldehyde emissions, their suspected carcinogeny (Sowumni *et al*., 2000; Li *et al*., 2004) and increasing costs due to high petroleum prices and natural gas are directing a search to find alternative adhesive materials. A formaldehyde-free adhesive has been constructed by mixing condensed tannin and polyethyleneimine together, providing a suitable adhesive for the ply-wood composite having properties of high water resistance (Li *et al*., 2004). Moreover, new technologies such as nanotechnology are developing in order to solve critical problems related to adhesion of tannins in wood material.

The use of natural tannins in leather production has an ancient history and the old methods are still valid in the present-day industry. In addition to natural leather tanning agents, toxic chrome salts, aldehydes, sulfonated polymers and other resins are also in use (http//www.bionatnet.org/secure/FP5/S1180.htm; Haslam, 1998). Based on the conclusions of an EU project, chrome salts and natural tannins cover more than 90 % of the total leather manufactured, of which the former covers about 70 %. Chrome salts are generally used to manufacture soft leather. Natural tannins have a high protein precipitation ability and strong astringent effect, which give possibilities to manufacture the most fine leather for different kinds of accessories, among other things (Haslam, 1998).

Employment in the leather and leather manufacturing products industry has been associated with various diseases caused by biological, toxicological and carcinogenic agents. During the tanning process, infections may occur as the hide serves as a medium for micro-organisms and yeasts (http://www.ilo.org/encyclopedia/). Skin disorders and allergic dermatitis have also been diagnosed. Tannery workers have the potential for exposure to numerous known or suspected occupational carcinogens (reviewed in http://www.ilo.org/encyclopedia/), and, for instance, excess risks for pancreatic, testicular, sinonasal and bladder cancers have been reported. In the leather industry especially, tannins have been regarded as possible etiological agents for nasal cancer (Battista *et al*., 1995).

Considering the safety aspects, chemical usage as well as waste disposal practices have been improved since the early history of the leather industry (Durant *et al*., 1990), the wastewater treatment plants have improved practices and chemical regulations are more stringent than they used to be. Also technological improvements are in progress. One of the attempts has been to prepare tanning agents by copolymerization of vegetable tannins

and/or waste lignocellulosic materials with acrylics and to apply these pre-polymerized tanning agents for leather fabrication. During the research a novel, high-performance melamine–urea–formaldehyde (MUF) resin was successfully used in copolymerization, producing leather products of a quality corresponding to that of the chrome-treated leather (http://www.biomatnet.org/secure/FPS/F1180.htm). Also the biological wastewater treatment has found to minimize environmental damage and to cause a decrease in overall effluent toxicity (Vijayaraghavan and Murthy, 1997).

In the textile industry heavy metal ions or natural or synthetic tannins have been used as assisting chemicals or auxiliary substances to be attached to the fibres permanently (so-called mordant dyes). The mordants allow many natural dyes, which would otherwise wash out, to be utilized by the industry. In recent work Koyunluoglu *et al*. (2006) studied the effect of pre-ozonation of commercial textile tannins on their biodegradability and toxicity. The results indicated no significant changes in acute toxicity for natural tannin whereas the inhibitory effect of synthetic tannin could be completely eliminated after 40 min ozonation at a rate of 1000 mg/h and a fair biodegradability improvement was found.

Similar to the leather industry, occupational exposure to tannins and tannin agents potentially leading to health problems also may occur in the wood industry from wood dust, in such work as mill work and furniture, cabinet and pattern making. The health problems may include inhalation and oral intake of wood dust or dermal exposure (e.g. Mark and Vincent, 1986). Hardwood dust exposure is associated with the risk of sinonasal cancer (Nylander and Dement, 1993) and in the European Union all hardwood dust is considered carcinogenic (Council of European Union, Directive 1999/38/EC, 1999).

In conclusion, plant tannins, utilized for a variety of applications, are derived from diverse plant sources characterized by different chemical structures. In many applications synthetic tannins are also utilized. In the leather tannin industry most of the toxic effects of the tanning procedures are caused especially by chrome and other hazardous ions needed by the industry. Therefore, when considering the toxicity and safety aspects of the applications where either tannins or tannin agents are used, they should be evaluated case by case.

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