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EXTRACTABLE AND NON-EXTRACTABLE PROANTHOCYANIDINS IN BARKS

SARA MATTHEWS, ISABELLE MILA, AUGUSTIN SCALBERT* and DERVILA M. X. DONNELLY†

Laboratoire de Chimie Biologique (INRA), INA-PG, 78850 Thiverval-Grignon, France; † Department of Chemistry, University College Dublin, Dublin 4, Ireland

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Abstract—Proanthocyanidins were analysed in the bark of several European conifer and broad-leaved tree species by thiolysis and gel permeation chromatography. They are largely procyanidins with varying proportions of catechin/epicatechin units and average degrees of polymerization ranging from 3 to 8. Some were not extracted by methanol–water and were directly analysed in the residue of extraction by thiolysis. These non-extractable proanthocyanidins represent up to 97% of the total proanthocyanidins in the outer bark exposed to rainwater. Their contribution to tree defence is discussed. ©1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Bark tannins have been used extensively in the manufacture of leather. In Europe, leather was traditionally made by alternating layers of oak bark and hide in pits [1]; tannins would slowly diffuse from the bark in to the hide during periods as long as a year. American colonists would use extracts of either hemlock, oak or chestnut bark [2]. Today, the main bark used for tannin extraction is that of black wattle (*Acacia* spp.) grown in South Africa and Brazil (other major sources of commercial tannins are heartwoods from either quebracho or chestnut).

Tannin content in bark may exceed 50% in some species [3]. They are principally proanthocyanidins (PAs) [4, 5], although some tree species belonging to *Quercus*, *Castanea* or *Eucalyptus* geni may also accumulate ellagitannins. In the present paper, the nature and content of PAs in barks of some European tree species previously found to be rich in tannins [6] are determined. Both extractable and non-extractable PAs are considered. Non-extractable PAs were described more than 20 years ago in leguminous plants [7], but have received little attention since due to the lack of suitable methods of analysis. Thiolysis, recently developed as a method for PA estimation [8], is used here to study PAs both in extracts and in solid extraction residues.

* Author to whom correspondence should be addressed.

RESULTS AND DISCUSSION

Content of proanthocyanidin extractives

Methanol–water extracts account for 5–27% of the dry weight of the various barks analysed (Table 1). The major part of these extractives is soluble in water and insoluble in diethyl ether. PAs, estimated in these various extracts by thiolysis, represent 15–39% of the water-soluble extractives in gymnosperm barks, 13% in birch and less than 1.5% in chestnut and oak. These values are underestimated partly because water has been added in the reaction mixture to fully solubilize the extract but, more probably, because some PAs resist degradation by thiolysis, particularly in aged tissues, such as bark [8]. The presence of other solutes, such as sugars or non-proanthocyanidin polyphenols, would also reduce these values. In chestnut and oak, an extra peak corresponding with ellagic acid was found in thiolysis chromatograms. In these two species, the bark is relatively rich in ellagitannins and other esters of phenolic acids but poor in PAs [9].

Nature of proanthocyanidin extractives

PAs in bark are largely procyanidins and, to a more limited extent, prodelphinidins (Table 1). This is in agreement with data published previously [4, 5]: Douglas fir and larch, as well as Scots pine, contain no prodelphinidin; most other species analysed contain no or less than 10% delphinidins. However, some

Table 1. Total extractives, extractable and non-extractable proanthocyanidins in bark of various gymnosperm and angiosperm tree species

	Total extractives		Proanthocyanidins					
	Soluble	Soluble	Extractable					
Bark	in Et_2O (% bark)	in H_2O (% bark)	Procyanidins (% bark)	Prodelphinidins (% bark)	2,3-*cis*/2,3-*trans* procyanidins	X_n*	X_w	Non-extractable† (% bark)
Picea abies	4.1	21.3	3.6	0.08	80:20	N.D. (4.6)	N.D.	0.8
Picea sitchensis	1.4	26.3	5.4	n.d.	78:22	N.D. (6.0)	N.D.	0.6
Pinus contorta	3.3	10.9	1.34	0.25	60:40	5.1 (4.7)	15.8	1.6
Pinus pinaster	3.6	11.1	3.1	n.d.	62:38	6.6 (4.1)	24.2	1.3
Pinus radiata	1.9	19.6	7.0	0.61	27:73	7.9 (7.8)	26.2	2.0
Pinus sylvestris	0.5	4.7	1.0	n.d.	79:23	5.3 (5.6)	16.4	3.1
Pseudotsuga menziesii	1.3	9.4	2.2	n.d.	81:19	6.2 (3.7)	18.4	1.1
Larix decidua	1.2	24.4	5.6	n.d	84:16	4.5 (3.1)	8.8	0.8
Betula sp.	0.8	4.6	0.6	n.d.	44:66	6.2 (6.4)	20.7	1.1
Castanea sativa	0.5	15.1	0.08	0.15	—			n.d.
Quercus sp.	0.3	9.7	0.09	< 0.04	—	—	—	n.d.

* X, degree of polymerization; values in brackets determined by thiolysis.

† Procyanidins + prodelphinidins.

N.D., non-determined due to the presence of predominant stilbene peak.

n.d., non-detected.

discrepancies exist for *Pinus contorta* previously reported to contain 69% prodelphinidins [5]. This could be explained by a different ratio of inner and outer bark in the analysed samples as the procyanidin:prodelphinidin ratio is known to differ in both tissues [5].

Thiolysis also gives a direct insight on the stereochemistry of carbons 2 and 3 in PA units. Large variations in the 2,3-*cis*/2,3-*trans* procyanidin (epicatechin/catechin) ratio were found between species (Table 1). This ratio was also determined after reduction of the flavanol thioethers by Raney Ni: the values obtained (72:28, 29:71 and 66:54 for *Picea sitchensis*, *Pinus radiata* and *Pinus sylvestris*) compare well with those in Table 1 (analysis with Raney Ni was not carried out more extensively due to some instability of the products after removal of the catalyst and to some difficulty to observe the expected gallocatechin with samples containing prodelphinidins). Here again, a good agreement was found with data found in the literature [4, 5]: spruce, Douglas fir, larch and Scots pine barks contain 78–85% epicatechin units. Other pines and birch contain higher proportions of catechin units. The highest proportion was found in *P. radiata* bark (73% catechin units), a figure identical to that reported for a *P. radiata* outer bark sample [5] (outer bark formed the bulk of the bark sample analysed).

Molecular weights of PAs in bark extracts were determined by gel permeation and by thiolysis. The extractives soluble in water were peracetylated and analysed on Ultrastyragel columns. A broad peak corresponding with PA polymers was observed [Fig. 1(a)]; the surface below the peak was used to calculate the number and weight average M_r. An additional narrow peak was often observed [Figs 1(b) and (d)] corresponding with some polar phenolic compounds, stilbene glycoside in spruce [10, 11] or flavonoid glucosides in Douglas fir [12], larch [13] and possibly lodgepole pine and Scots pine [4, 14]. In spruce species, the narrow peak was too important to allow calculation of the PA M_r. In the four other species, calculation was made on the broad peak by smoothing the curve below the narrow peak. The chromatogram of *Castanea sativa* bark showed numerous peaks corresponding with the various ellagitannins, galloyl esters and phenol glycosides recently identified in this species [Fig. 1(c)] [9].

Close values in number average M_r were found for several bark species using gel permeation and thiolysis methods (Table 1). Some discrepancies exist for other species, possibly explained by the presence of polymeric polyphenols non-exclusively based on PA units [8, 15] or by an incomplete extraction with diethylether of free (+)-catechin and (−)-epicatechin identified in the bark of many conifers [4] before thiolysis of the aqueous-soluble PAs. The range of polymerization varies from 4.5 to 7.9 (3.1 to 7.8 by thiolysis) according to bark species. Values are significantly lower than those previously reported (7–10) [4, 5], probably because the authors made their measurement on PAs purified on Sephadex LH-20 and not on the whole water-soluble extracts. This same difference in experimental procedure should also explain why no significant dependence of M_r on bark species could be established by comparison of the present data with those in the literature [4, 5]. Dispersivity (X_w/X_n) varies between 2.0 and 3.7 according to species.

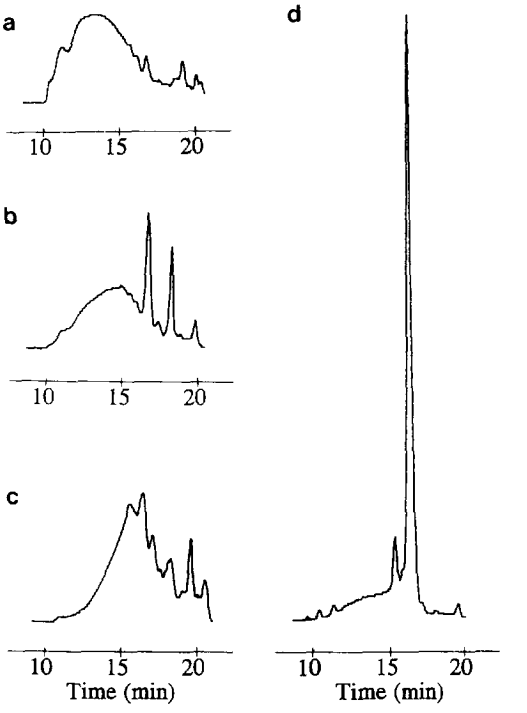

Fig. 1. Gel permeation chromatograms of peracetylated extractives of barks. (a) *Pinus radiata*; (b) *Pseudotsuga menziesii*; (c) *Castanea sativa*; (d) *Picea sitchensis*.

Non-extractable proanthocyanidins

Thiolysis was applied to the residue of extraction to detect and estimate non-extractable PAs. Chromatographic profiles were identical to those obtained with extractable PAs. They were found in the bark of all studied conifers and birch (Table 1). Their content varies between 0.6 and 3% of the bark dry weight. They account for more than 50% of the total PAs in several bark samples, e.g. Monterey pine, 50%, Scots pine, 75% and birch, 65%.

Inner bark and outer bark of two pine species were analysed separately. The amount of extractives and, in particular, of PAs soluble in aqueous methanol is much reduced in the dead outer bark compared with the inner bark; the amount of non-extractable PAs in outer bark consequently increases (Table 2). Secondary reactions during outer bark formation and ageing affect PAs and reduce their solubility. Similar effects have been discussed in detail for heartwoods [16–19]. The total amount of PAs (extractable and non-extractable) measured in outer bark is also reduced. The same secondary reactions diminish the

Table 2. Total extractives, extractable and non-extractable proanthocyanidins in inner and outer bark of two gymnosperm tree species (% dry wt. bark). Values in brackets determined on fresh bark

Bark	Total extractives	Proanthocyanidins	
		Extractable	Non-extractable
Pinus sylvestris			
Inner bark	22.9 (22.5)	5.6 (5.3)	2.6 (3.6)
Outer bark	1.4 (1.3)	0.12 (0.11)	4.6 (4.4)
Pinus nigra			
Inner bark	29.1 (29.7)	4.2 (1.0)	1.2 (3.7)
Outer bark	3.3 (2.1)	0.7 (0.3)	2.5 (2.1)

reactivity of PAs with thiols. A similar reduction of reactivity was observed during storage of purified PAs [8].

Extraction yields of PAs will thus depend on the extent of these secondary reactions. Both an increase of the proportion of outer bark in the bark sample and storage of bark should reduce these yields. Commercial extraction of bark tannins is often carried out in hot sodium sulphite solutions [20]. This reagent, known to cleave intermonomeric bonds in PA polymers [21], should contribute to solubilize PAs non-extractable with aqueous solvents.

The effect of drying bark samples on the extractability of PAs was further studied. Freshly collected barks were compared with freeze-dried barks. No effect was observed with either *Pinus sylvestris* bark or *Pinus nigra* outer bark. Adversely, with *Pinus nigra* inner bark, drying resulted in an increase of the extractability of PAs (Table 2). Such an increase of PA extractability upon drying at room temperature has previously been described with sorghum grain [22] and Douglas fir bark [23] (see ref. [24] for a general discussion on the effect of sample preparation on tannin extractibility).

Thiolysis and determination of proanthocyanidins

Thiolysis allows the determination of both extractable and non-extractable PAs in plant materials. One of the limitations of the method is that part of the polymer resists degradation with thiols and is not taken into account. No chemical methods have so far been proposed to estimate this fraction of the polymer. Measurements on purified PAs have suggested that over one third of the polymer may resist degradation, particularly when PAs originate from aged tissues, such as outer bark [8]. This resistant fraction can be compared with the 'condensed' fraction of lignins (the other major phenolic polymer in biomass), which also resists depolymerization in acids [25]. It should be noted here, that the meaning of the word 'condensed' differs in 'condensed tannins' (PAs) and in 'condensed lignins'. In PAs, monomers are condensed, because they are linked through carbon–carbon bonds (easily cleaved in acids), whereas in lignins, condensed linkages are those which are not cleaved by acids (principally biaryl ether or biphenyl linkages), as opposed to the labile alkyl aryl ether linkages.

This limitation of thiolysis also applies to the depolymerization of PA into anthocyanidins and to the reaction of PAs with vanillin, two reactions largely used for their determination. The yield of anthocyanidin formed from purified PAs [26], as well as the reactivity with vanillin [8], decreases upon storage, probably due to some secondary reactions which similarly affect thiolysis yields. However, thiolysis offers the advantage of providing at the same time some information on the nature of the building units of the polymer (hydroxyl substitution, stereochemistry at C-2 and C-3). It is also more specific and sensitive and particularly well-suited for the analysis of samples containing either low amounts of PAs or non-extractable PAs.

Resistance of bark proanthocyanidins to rainwater leaching

PAs in bark contribute to protect the underlying living tissues (phloem, cambium and xylem) against invasion by pathogens or rots [27, 28]. For such a defence to be effective, PAs must resist leaching by rainwater. Non-extractable PAs, firmly bound to the lignocellulosic matrix (Fig. 2) and particularly abundant in the more exposed outer bark, resist leaching. Extractable PAs are only partially leached by water despite their water solubility; only one-third of PA extractives in *Pinus sylvestris* bark could be solubilized by stirring the bark powder in cold water overnight. Leaching resistance of PA extractives could be explained by the existence of weak chemical linkages, either hydrogen bonds or π-π interactions, with non-extractable PAs (Fig. 2). It was shown recently that tannin–tannin interactions are in fact stronger than the better known tannin–protein interactions [29].

PA-containing bark can be compared with leather tanned with vegetable tannins. They share two fea-

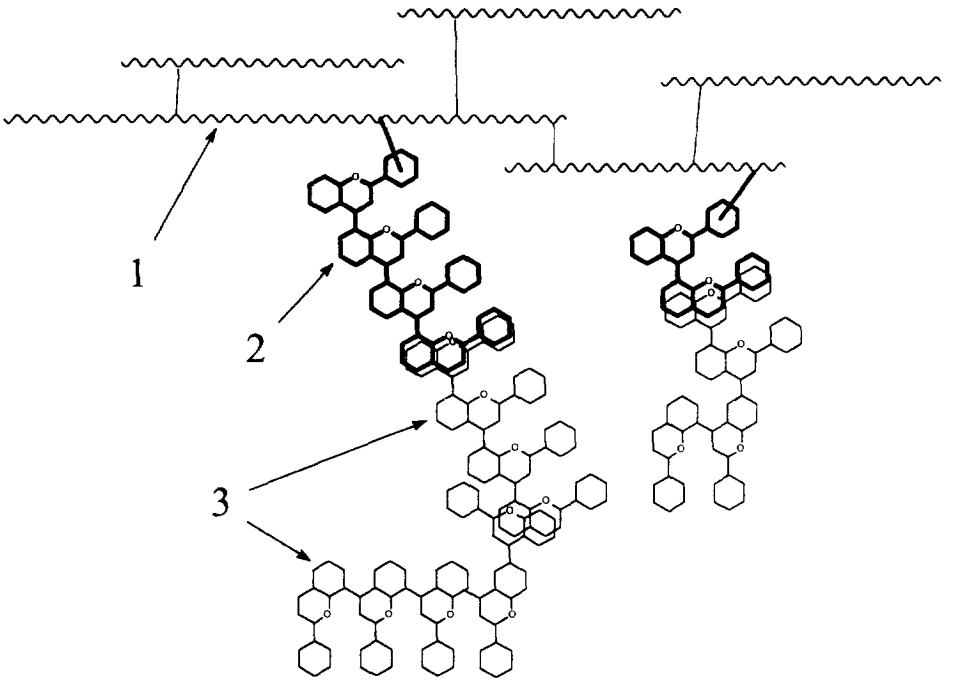

Fig. 2. Direct linkage of proanthocyanidin with the cell wall matrix and proanthocyanidin–proanthocyanidin weak interactions in bark limit proanthocyanidin leaching by rain water. (1) cell wall matrix; (2) non-extractable proanthocyanidin; (3) proanthocyanidin extractable with aqueous methanol, but not with water.

tures in common: (i) leather contains high amounts of tannins (30–50%) [1]; (ii) just as PAs in bark, tannins in leather can be stripped by aqueous organic solvents but are not solubilized by plain water, at least in the mild acidic conditions prevailing in bark [30, 31]. Resistance of leather tannin to water-leaching could not be explained by direct linkage of all tannin molecules to the collagen matrix due to their too high content [32, 33]. Following a scheme similar to that of Fig. 2, it is suggested that extractable tannins would be retained in the matrix through weak interactions with the tannin molecules bound to collagen fibres.

EXPERIMENTAL

Plant material. Barks were collected from mature trees. Unless otherwise mentioned, no attempt was made to separate inner and outer bark. The *Betula* sample was an undefined mixture of *B. pendula* and *B. pubescens*, the *Quercus* sample, an undefined mixture of *Q. robur* and *Q. petraea*. Most samples were air-dried and ground in a mill (particle-size less than 60 mesh). Others were analysed fr. or freeze-dried. Fresh inner and outer barks were frozen in liquid N_2 within 30 min of collection and ground to fine powders under liquid N_2 in a vibratory ball mill (*ca* 2 min.). They were either extracted directly or freeze-dried. Moisture content in inner and outer bark was, respectively, 60 and 30% for *P. sylvestris* and 62 and 21% for *P. nigra*.

Extraction. Bark samples (5 g) were extracted with MeOH–H_2O (1:1) (3 × 200 ml for 2 hr). MeOH was removed under red. pres. and the aq. soln extracted with Et_2O (3 × 140 ml) to provide an aq. extract containing 'extractable tannins' and freed from most low M_r phenols. Both extracts and extraction residues were freeze-dried.

Thiolysis. Bark aq. extracts (6 mg) or bark residues of extraction (12 mg) were added to EtOH–H_2O (4:1, 2 ml) containing toluene-α-thiol (60 μl) and HOAc (30 μl). Thiolysis was carried out under N_2 in sealed tubes at 105° during 24 hr. Thioether products were analysed by reverse-phase HPLC. 4β-Benzylthioepicatechin, used as a standard, was prepd by prep. thiolysis of bark (see ref. [8] for more details). Degree of polymerization was measured by calculating the molar ratio of thioether adducts and (+)-catechin and (−)-epicatechin terminal units.

Gel permeation was carried out on peracetylated samples in THF with Ultrastyragel column [34].

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