Method for Estimation of Proanthocyanidins Based on Their Acid Depolymerization in the Presence of Nucleophiles

Sara Matthews,[†] Isabelle Mila,[†] Augustin Scalbert,*[,†] Brigitte Pollet,[†] Catherine Lapierre,[†] Catherine L. M. Hervé du Penhoat,[‡] Christian Rolando,[‡] and Dervilla M. X. Donnelly[§]

Laboratoire de Chimie Biologique (INRA), Institut National Agronomique Paris−Grignon, 78850 Thiverval-Grignon, France, Département de Chimie, URA 1679, Ecole Normale Supérieure, 24 rue Lhomond, 75231 Paris Cedex 05, France, and Department of Chemistry, University College Dublin, Dublin 4, Ireland

Depolymerization of proanthocyanidins in the presence of phloroglucinol or benzyl mercaptan nucleophiles was studied to develop a method for their quantitative determination. Degradation products were separated and quantified by either GC or HPLC and the reaction conditions optimized. The high depolymerization yields obtained with benzyl mercaptan make the method suitable for proanthocyanidin determination. The method is more specific and sensitive than the colorimetric methods classically used. It is particularly well suited for the analysis of samples containing either low amounts of proanthocyanidins or insoluble proanthocyanidins.

Keywords: *Proanthocyanidins; tannins; estimation; thiolysis; phloroglucinol*

INTRODUCTION

Proanthocyandins [PA; synonym, condensed tannins; e.g. **1** (see Chart 1)] are polymers made of flavan-3-ol units linked together by carbon−carbon bonds. They are the most widespread polyphenols in plants after lignins and can be found in leaves, fruits, woods, barks, or roots, often in high concentration (over 50% in some barks). Many food products such as fruits, cereals, legume seeds, wine, and cider contain PAs, and their astringency is due to the ability of the PAs they contain to form complexes with proteins in the mouth (Haslam, 1989).

Several methods have been proposed for their estimation [see Scalbert (1992) for a review]. The most largely used are colorimetric assays relying either on the reaction of the A-ring with an aromatic aldehyde (vanillin and dimethylaminocinnamaldehyde) or on their oxidative depolymerization into anthocyanidins. PAs with 5,7-dihydroxy A-rings (procyanidins, prodelphinidins) undergo facile cleavage under acidic or basic conditions (Hemingway, 1989). Acid-catalyzed degradations have given rise to the butanol/HCl assay in which the chain extender flavan-3-ol units are oxidized to give anthocyanidins absorbing in the visible region. The reaction mechanisms are complex, and the yields are not quantitative (Swain and Hillis, 1959; Porter et al., 1986).

Other depolymerization reactions in the presence of nucleophiles are frequently employed for the structural analysis of PAs (Hemingway, 1989). The nucleophile forms adducts (e.g. **3**−**7**) with the chain extender units in the polymer, which are purified or analyzed by chromatography. Their structure, once established, can be used to determine the nature of the monomer units within the polymer. Numerous nucleophiles have been used including thiolacetic acid (Betts et al., 1967), benzene-*p*-sulphinic acid, benzenethiol (Brown and Shaw, 1974), benzyl mercaptan (Thompson et al., 1972), 2-mercaptoethanol (Tanaka et al., 1994), or phloroglucinol (Foo and Porter, 1978). Various factors have dictated their choice, most particularly the ease of separation of the products and their recovery yields. Phloroglucinol is sometimes preferred to the sulfur nucleophile because it is odorless.

Surprisingly, depolymerization in the presence of a nucleophile has never been used for the determination of PA content in plant samples. Such a reaction presents various potential advantages that could make it particularly useful for such an application. Associated with the quantitative analysis of the products by chromatography, it may simultaneously offer information on the nature of the PAs and on their content in plant tissues. In contrast to colorimetric methods, interferences with other plant constituents are avoided due to the nonambiguous identification of the PA-derived products. In comparison to the butanol/HCl depolymerization, the reaction preserves the stereochemistry at the C2−C3 positions of the polymer units. Finally, the use of a nucleophile limits the occurrence of side reactions that could affect recovery yields of the products.

We develop here the first chromatographic method for PA estimation. The two most widely used nucleophiles, benzyl mercaptan and phloroglucinol, are compared. Separation of the products by gas chromatography (GC) and high-performance liquid chromatography (HPLC) is described and reaction conditions are optimized. Factors that may explain the low yields obtained with some PAs such as those isolated from bark are discussed. Finally, the method developed is compared to the widely used vanillin assay.

MATERIALS AND METHODS

General. Total phenols and proanthocyanidins were estimated according to the Folin−Ciocalteu and the vanillin/H_2SO_4 assays, respectively, as previously described (Scalbert et al., 1989). Yields of depolymerization with either phloroglucinol or benzyl mercaptan were calculated on a flavan-3-ol

* Author to whom correspondence should be addressed [telephone (33) 1 30 81 54 62; fax (33) 1 30 81 53 73; e-mail scalbert@cardere.grignon.inra.fr].

[†] INRA, Grignon.

[‡] ENS, Paris.

[§] UCD, Dublin.

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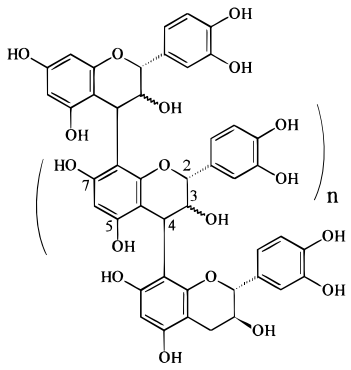
Chart 1



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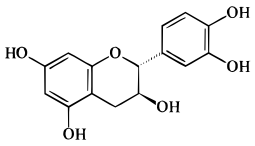

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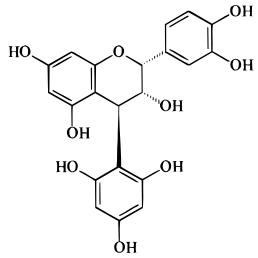

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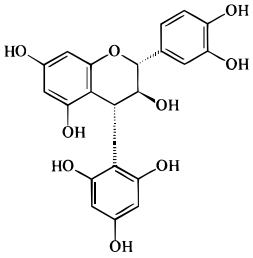

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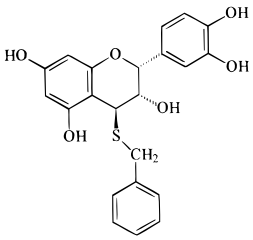

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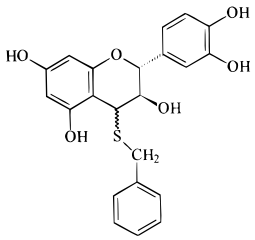

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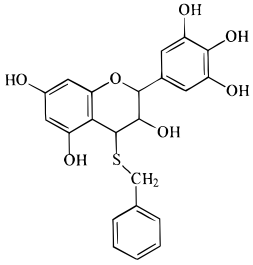

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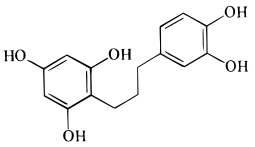

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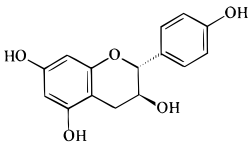

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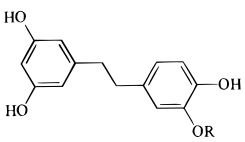

11 R = CH_3

12 R = H

basis (M = 288). NMR assignments were based on COSY and HETCOR and INAPT (Bax et al., 1985) spectra.

Tannin Isolation. Typical procedure for PA isolation from bark is exemplified as follows: *Picea sitchensis* bark powder (250 g) was extracted with acetone/water 7:3 (3 × 1.5 L) with magnetic stirring during 2 h at room temperature. Acetone was eliminated from the filtrate under reduced pressure and the residual aqueous phase extracted with ethyl acetate (3 × 0.7 L). The aqueous phase was freeze-dried (60 g). A fraction (27 g) was dissolved in methanol/water 1:1 (50 mL) and loaded onto a 50 cm × 5 cm i.d. Sephadex LH20 (100 μm, Pharmacia) column. The column was washed with 4 L of water and 5 L of methanol/water 1:1 to eliminate sugars and sugar-contaminated PAs. More PAs were eluted with 3 L acetone/water 1:1 (30% of the loaded sample). Fractions (100 mL) were collected and their purities assessed by the vanillin/HCl assay (Czochanska et al., 1980): $E^{1\%}_{1cm}$ values varied between 166 and 284.

Phloroglucinol Degradation. PAs (25 mg), phloroglucinol (8 mg), and dioxane/aqueous HCl 0.2 M, 1:1 (0.5 mL) were added in a tube which was sealed and heated at 80 °C during 20 min. An aliquot was then diluted exactly with methanol/water 1:1 to reduce the concentration of the main phloroglucinol adduct below 0.5 mg/L before analysis by chromatography.

Thiolysis. Benzyl mercaptan (90 μL) and acetic acid (45 μL) were added in a tube to 3 mL of a 0.7 mg/mL solution of PAs in ethanol. The mixture was purged with nitrogen and the tube sealed and heated in an oil bath at 105 °C during 4.5 h. All reactions were duplicated. Thiolysis was also carried out on insoluble plant materials and the suspension stirred throughout the reaction (Matthews et al., 1997). Before HPLC analysis, MeOH/H_2O 1:1 (2 volumes) was added to avoid the formation of asymmetrical peaks and improve their chromatographic resolution. All of the quantitative data presented in this paper were obtained by HPLC. For GC analysis, water (2 mL) was added to 1 mL of the reaction mixture, and the thioethers were extracted by ethyl acetate (2 mL). Water was removed from the organic phase with sodium sulfate. An aliquot of this solution (10 μL) was then mixed with *N,O*-bis-(trimethyl)trifluoroacetamide (100 μL) and GC grade pyridine (10 μL) in a 200 μL screw cap vial. The vial was sealed and left overnight at room temperature.

HPLC Analysis. Degradation products were analyzed on a LiChrospher 100 RP-18 column (5 μm, 250 mm × 4 mm i.d.; Merck). Addition of sufficient water to the sample solutions prevents autoassociation and improves the separation (Scalbert et al., 1990). The elution conditions were as follows: solvent A, water/phosphoric acid 999:1; solvent B, methanol; linear gradient 0−90% B (phloroglucinol adducts) or 30−90% (thiolysis adducts) in 30 min; flow rate, 1 mL/min; detection, 280 nm.

GC and GC/MS Analyses. Trimethylsilylated samples (2 μL) were introduced with a moving-needle type injector onto a wall-coated open tubular (WCOT) polydimethylsiloxane fused silica capillary column (SP B1 30 m × 0.2 mm i.d.; film thickness, 1 μm). The carrier gas was helium at a pressure of 1 bar. The column temperature was programmed from 200 to 290 °C at 10 °C/min. The injector temperature was 250 °C and the flame ionization temperature 270 °C. Mass spectra were recorded with a Nermag R10-10H quadrupole mass spectrometer at 70 eV in the positive ion mode, on-line with a gas chromatograph operating in the same conditions as above. The temperature of the transfer line was 270 °C and that of the ion source 180 °C. MS spectra of **3**−**5** are given below. Similar spectra (TMS derivatives) were obtained for 3,4-*trans*-benzylthioepicatechin (**5**) and its two isomers **6** and **7**. TMS derivatives of benzylthioepigallocatechin isomers: EI-MS, *m/z* 860 (tr) $[M]^+$, 737 (14), 709 (2), 649 (2), 443 (4), 456 (29), 368 (4), 355 (4), 281 (2), 267 (5), 207 (5), 196 (4), 181 (2), 147 (6), 121 (12), 91 (100), 73 (56).

Purification of Phloroglucinol Adducts. Purified *Pinus pinaster* bark tannin (5 g) was treated with 1.5 g of phloroglucinol (Prolabo) in 100 mL of dioxane/aqueous HCl 0.2 N, 1:1 during 20 min at 80 °C. The solution was partially concentrated under reduced pressure, 100 mL of water was added, and the whole mixture was extracted with ethyl acetate (4 × 100 mL). Ethyl acetate was removed, and the solid materials (2.5 g) were resolubilized in ethanol/water 10:1 (25 mL) and fractionated over a Sephadex LH20 column (28 cm × 2.5 cm i.d.) with ethanol as eluent. Phloroglucinol (290 mg), (+)-catechin (35 mg), and phloroglucinol adducts (two fractions, 180 and 250 mg) were successively eluted. Phloroglucinol adducts solubilized in methanol/water 1:2 were further purified by reversed-phase preparative HPLC with methanol/water

8:92 as eluent (Scalbert et al., 1990). Various fractions differing in the proportions of phloroglucinol adduct isomers were recovered as white powders. Epicatechin 4-phloroglucinol and catechin 4-phloroglucinol were the main isomers in the Sephadex fractions eluted first and second, respectively. Epicatechin (4β→2)-phloroglucinol (**3**): ^{1}H NMR (400 MHz, acetone-d_6) δ 7.14 (d, J = 2 Hz, H-2′), 6.92 (d, J = 8 Hz, H-5′), 6.87 (dd, J = 8, 2 Hz, H-6′), 6.18 (2H, AB, J = 2 Hz, H-6, H-8), 6.07 (2H, br, H-3″, H-5″), 5.2 (br s, H-2), 4.75 (br d, J = 2 Hz, H-4), 4.17 (br d, J = 5.5 Hz, H-3), 3.90 (d, J = 5.5 Hz, OH-3); ^{13}C NMR (100 MHz, acetone-d_6) δ 156.24 (2C, C-2″, C-6″), 156.87−156.03 (4C, C-5, C-7, C-8a, C-4″), 143.63, 143.47 (C-3′, C-4′), 130.70 (C-1′), 117.45 (C-6′), 113.76 (C-5′), 113.44 (C-2′), 105.14, 98.87 (C-4a, C-1″), 94.57 (3C, C-6, C-3″, C-5″), 94.08 (C-8), 75.24 (C-2), 71.62 (C-3), 35.13 (C-4); EI-MS (TMS derivative), m/z 990 (2) [M]$^+$, 900 (7), 695 (5), 607 (3), 533 (51), 368 (13), 147 (18), 73 (100). Catechin (4α→2)-phloroglucinol (**4**): ^{1}H NMR (400 MHz, acetone-d_6) δ 7.14 (d, J = 2 Hz, H-2′), 6.99 (dd, J = 8, 2 Hz, H-6′), 6.94 (d, J = 8 Hz, H-5′), 6.06 (br, H-3″, H-5″), 6.04 (2H, AB, J = 2 Hz, H-6, H-8), 4.63 (m, H-2, H-4), 4.57 (∼dt-second-order system, J = 10, 4 Hz, H-3); ^{13}C NMR (100 MHz, acetone-d_6) δ 156.14 (C-6″), 156.02 (C-2″), 156.75−155.59 (4C, C-5; C-7, C-8a, C-4″), 143.62, 143.46 (C-3′, C-4′), 130.69 (C-1′), 119.01 (C-6′), 114.13 (C-5′), 113.79 (C-2′), 105.5, 104.04 (C-4a, C-1′), 95.54 (C-8), 95.47 (C-6), 94.00 (2C, C-3″, C-5″), 82.2 (C-2), 70.76 (C-3), 36.43 (C-4); EI-MS (TMS derivative), m/z 990 (5) [M]$^+$, 900 (5), 695 (5), 648 (20), 607 (4), 533 (44), 450 (4), 368 (10), 267 (3), 147 (9), 73 (100).

Purification of Benzylthioepicatechin. *P. sitchensis* tannin (2 g) was refluxed with benzyl mercaptan (20 mL) and acetic acid (10 mL) in ethanol (40 mL) under an argon atmosphere during 20 h. After concentration under reduced pressure, water (300 mL) was added and the mixture was extracted with diethyl ether (4 × 150 mL). The organic phase was dried under reduced pressure and fractionated on a silica column (22 cm × 2.5 cm i.d.). Elution with toluene removed all benzyl mercaptan. The thioadducts were then eluted with increasing acetone concentrations (10−50% in toluene). Elution was followed on silica TLC with toluene/acetone/formic acid 5:5:1 eluent and $FeCl_3/K_3Fe_3(CN)_6$ spray reagent. Thioadducts in combined fractions were further purified on a Sephadex LH20 column (32 cm × 2 cm i.d.) with ethanol as eluent. An orange oil fraction (460 mg) redissolved in methanol was finally purified by reversed-phase preparative HPLC with methanol/water 9:11 as eluent (Scalbert et al., 1990). This led to the isolation of 157 mg of 3,4-*trans*-benzylthioepicatechin (**5**): ^{1}H NMR (270 MHz, acetone-d_6) δ 7.48−7.20 (5H, m, phenyl), 7.04 (s, H-2′), 6.83−6.76 (2H, m, H-5′, H-6′), 6.03 (d, J = 2.2 Hz, H-8), 5.91 (d, J = 2.2 Hz, H-6), 5.28 (s, H-2), 4.09−4.00 (4H, m, H-4, H-3, SCH_2); ^{1}H NMR (270 MHz, pyridine-d_5) δ 7.61−7.54 (3H, m, H-2′, H-5′, H-6′), 7.35−7.18 (5H, m, phenyl), 6.74 (d, J = 2.6 Hz, H-8), 6.63 (d, J = 2.2 Hz, H-6), 6.04 (s, H-2), 4.97 (d, J = 2.2 Hz, H-4), 4.64 (m, H-3), 4.2 (2H, s, SCH_2); ^{13}C NMR (67.8 MHz, acetone-d_6) 158.9 (C-7), 154.4 (C-5), 157.1 (C-8a), 145.5 (C-4′), 145.3 (C-3′), 139.9 (C-1″), 131.9 (C-1′), 129.8, 129.2 (C-2″, C-3″, C-5″, C-6″), 127.6 (C-4″), 119.2 (C-6′), 115.5, 115.3 (C-2′, C-5′), 99.8 (C-4a), 96.7 (C-6), 95.6 (C-8), 75.4 (C-2), 71.1 (C-3), 43.4 (C-4), 37.2 (SCH_2); ^{13}C NMR (67.8 MHz, pyridine-d_5) δ 160.4 (C-7), 160.2 (C-5), 158.2 (C-8a), 147.6 (C-4′), 147.4 (C-3′), 140.4 (C-1″), 132.7 (C-1′), 130.1, 129.5 (C-2″, C-3″, C-5″, C-6″), 127.6 (C-4″), 119.8 (C-6′), 117.0, 115.7 (C-2′, C-5′), 101.1 (C-4a), 97.7 (C-6), 96.3 (C-8), 76.6 (C-2), 71.8 (C-3), 44.9 (C-4), 38.1 (SCH_2); FAB-MS (glycerol), m/z 413 (4) [M + 1]$^+$, 289 (23), 271 (15), 163 (18), 151 (9), 139 (23), 123 (26), 93 (35), 91 (100); EI-MS (TMS derivative), m/z 649 (9) [flavylium]$^+$, 621 (4), 559 (6), 395 (3), 379 (1), 368 (14), 355 (9), 324 (1), 281 (1), 267 (4), 207 (6), 179 (5), 147 (6), 121 (5), 103 (7), 91 (23), 73 (100); CI-MS (NH_3), m/z 773 (100) [M + H]$^+$, 651 (35), 369 (67).

Preparation and Analysis of the Thiolysis-Resistant Fraction (TRF). *P. sitchensis* tannin (2 g) was mixed with benzyl mercaptan (8.7 mL) and acetic acid (4.35 mL) in ethanol (290 mL). The mixture was purged with nitrogen, sealed, and heated at 105 °C for 6 h. After reaction, the volume was reduced to 20 mL under reduced pressure and water (100 mL) was added. The mixture was successively extracted with hexane and diethyl ether and the residual aqueous phase freeze-dried to provide the TRF (320 mg). TRF (10 mg) was further degraded by hydrogenolysis (75 bar hydrogen) over Pd/C (5 mg) in ethanol (1.5 mL) at 105 °C for 3 h under stirring. The suspension was filtered over Celite, reduced in volume, and extracted with ethyl acetate. Solutes in the organic phase were trimethylsilylated and analyzed by GC/EI-MS as above. Compound **9**: m/z 636 (13) [M]$^+$, 621 (1), 604 (3), 562 (5), 413 (3), 369 (3), 355 (34), 318 (2), 294 (11), 281 (17), 268 (42), 205 (6), 193 (7), 179 (22), 147 (5), 73 (100). Compound **10**: m/z 562 (16) [M]$^+$, 355 (34), 280 (100), 267 (12), 205 (12), 192 (25), 133 (12), 73 (28). Compound **11**: m/z 476 (13) [M]$^+$, 461 (1), 267 (2), 252 (2), 223 (3), 209 (100), 193 (4), 179 (12), 149 (1), 147 (1), 89 (1), 73 (41). Compound **12**: m/z 534 (14) [M]$^+$, 519 (2), 267 (100), 251 (2), 237 (1), 223 (1), 193 (4), 179 (29), 149 (2), 147 (3), 133 (2), 73 (93). The degree of polymerization was established by gel permeation chromatography of the peracetylated TRF using an Ultrastyragel column and THF as eluent (Viriot et al., 1994): ^{13}C NMR (100 MHz, acetone-d_6/D_2O) δ 159.6−156.0 (C-5, C-7, C-8a), 146.0−144.3 (C-3′, C-4′), 131.5 (C-1′), 120.0−119.1 (C-6′), 116.2−115.0 (C-2′, C-5′), 108.7−107.1 (C-8), 101.4−100.8 (C-4a, C-1″), 96.4−95.6 (C-6), 82.0, 77.2−76.8 (C-2, C-3″, C-5″), 74.0 (C-2″), 72.7 (C-3), 70.5 (C-4″), 67.8, 61.8 (C-6″), 37.5 (C-4).

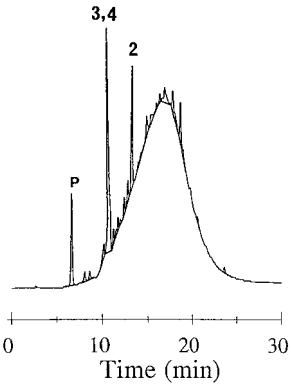

Figure 1. HPLC chromatogram of a *P. pinaster* bark tannin degraded in the presence of an acid and phloroglucinol: P, phloroglucinol; peak numbers refer to the structures given in Chart 1.

RESULTS AND DISCUSSION

Phloroglucinol Degradation. When a *P. pinaster* bark tannin **1** was submitted to acid degradation in the presence of phloroglucinol, two main narrow peaks were observed by HPLC superposed to an intense broad peak. The two narrow peaks were identified as being (+)-catechin **2** and phloroglucinol adducts (**3** and **4**) (Figure 1). The broad peak, by far the most important quantitatively, corresponds to the fraction resistant to the depolymerization. The peak corresponding to the phloroglucinol adducts could be resolved into two peaks by using an isocratic mode of elution (Figure 2a). Both phloroglucinol adducts were more easily separated as their TMS derivatives by GC/MS (Figure 2b). Their MS spectra were consistent with their being epicatechin (4β→2)-phloroglucinol (**3**) and catechin (4α→2)-phloroglucinol (**4**).

The reaction was carried out on a larger amount of the same *P. pinaster* bark tannin, and the phloroglucinol adducts were purified by Sephadex LH20 chromatography and reversed-phase HPLC. Various fractions differing in the ratio of the phloroglucinol adduct isomers were obtained. Two of them were shown by ^{1}H- and ^{13}C-NMR spectroscopy to contain, respectively, epicatechin (4β→2)-phloroglucinol (**3**) and catechin (4α→2)-phloroglucinol (**4**) as the main products. Spectra were very similar to those published recently (Foo et al., 1996), and all signals were assigned using homo- and heteronuclear 2D NMR methods (COSY, HETCOR,

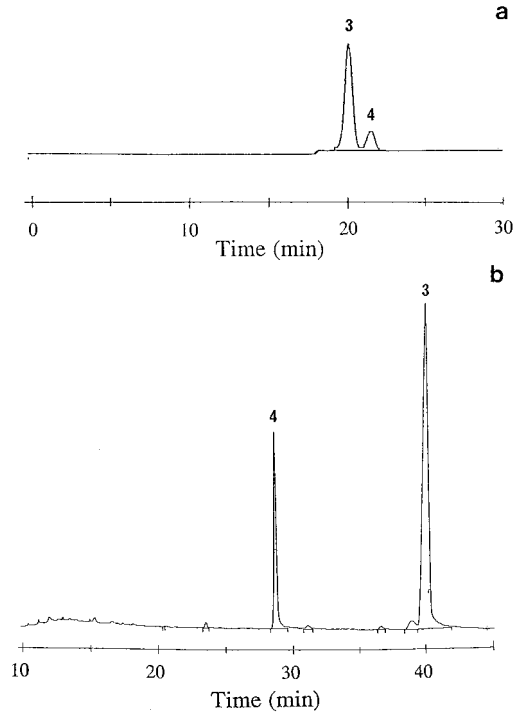

Figure 2. Chromatograms of a mixture of epicatechin (4β→2)-phloroglucinol (**3**) and catechin (4α→2)-phloroglucinol (**4**): (a) HPLC (isocratic elution with methanol/water/phosphoric acid 80:919:1); (b) GC.

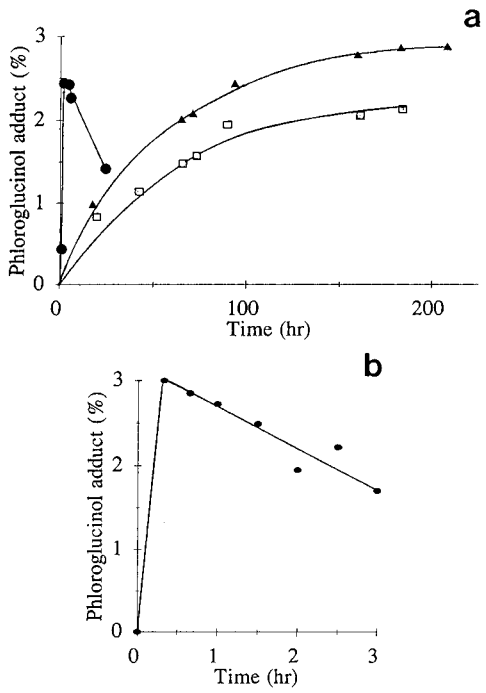

Figure 3. Kinetics of the acid degradation of a *P. pinaster* bark tannin in the presence of phloroglucinol: (a) HCl concentration = 0.05 M; (b) HCl concentration = 0.1 M. Temperature: (□) room temperature; (▲) 37 °C; (●) 80 °C.

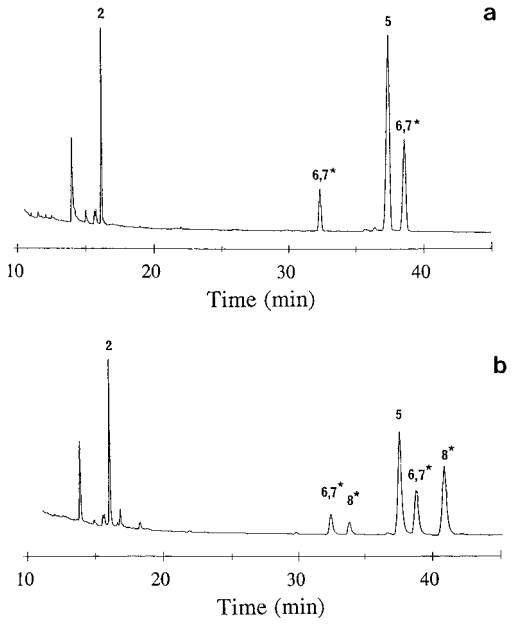

Figure 4. GC chromatogram of the thiolysis products of bark tannins: (a) *P. pinaster* bark tannin; (b) *P. contorta* bark tannin. Peak numbers refer to the structures given in the text; *, the exact stereochemistry for peaks corresponding to compounds **6**−**8** was not determined.

INAPT) (see Materials and Methods). These partially purified fractions allowed the identification of the two isomers on HPLC and GC chromatograms (Figure 2). The order of elution by HPLC is opposite to the one reported previously using similar chromatographic conditions (Koupai-Abyazani et al., 1992).

A fraction containing 80% **3** and 20% **4** was used as a standard to measure and optimize yields of depolymerization. Tannin concentration was shown to affect the reaction. At a low concentration similar to that used for thiolysis (0.7 mg/mL; see below) or even higher (5 mg/mL), no peak corresponding to the phloroglucinol adducts could be observed. A 50 mg/mL concentration was needed to observe their formation. Yields were also influenced by temperature and acid concentration. At room temperature, more than 6 days was required to reach a plateau, whereas the phloroglucinol adduct concentration reached a maximum within 2 h at 80 °C (Figure 3a). By doubling the HCl concentration, the yield was further increased and the reaction duration reduced to 20 min (Figure 3b). The same results were obtained under air or nitrogen. The yields of phloroglucinol adducts obtained with procyanidins isolated from various barks varied between 3 and 10% and are much lower than those reported in the literature for thiolysis degradation. Thiolysis was thus further explored.

Thiolysis: Chromatographic Separation of the Products. Typical GC and HPLC chromatograms of thiolysis products are shown in Figures 4 and 5. *P. pinaster* bark tannin gave four main peaks (Figures 4a and 5a). The compound corresponding to the major peak was purified by Sephadex LH20 chromatography and reversed-phase HPLC. NMR spectra showed that it is 3,4-*trans*-benzylthioepicatechin (**5**) (Thompson et al., 1972). Peak 2 corresponds to (+)-catechin. The mass spectra of the last two peaks were very similar to that of 3,4-*trans*-benzylthioepicatechin. Haslam and collaborators (Thompson et al., 1972) have shown that epicatechin internal units in procyanidins, when treated by thiolysis, give 3,4-*trans*-benzylthioepicatechin (**5**) and no 3,4-*cis* isomer, whereas catechin internal units give both 3,4-*trans*- and 3,4-*cis*-benzylthiocatechins (**6** and **7**, respectively). It was thus suggested that the two last peaks correspond to these benzylthiocatechin isomers. This was confirmed by comparing the chromatograms obtained with two PAs differing in their 2,3-stereochemistry: *P. sitchensis* bark PA with predominant 2,3-*cis* units and *Pinus radiata* bark PA largely made of 2,3-*trans* units (Matthews et al., 1997).

Pinus contorta bark tannin gave two extra peaks upon thiolysis (Figure 4b). Their MS spectra were characteristic of benzylthioethers of (epi)gallocatechin **8** de-

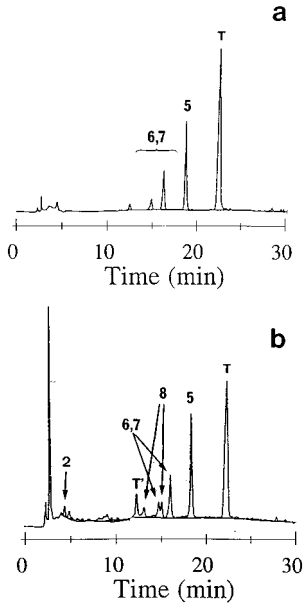

Figure 5. HPLC chromatogram of the thiolysis products of bark tannins: (a) *P. pinaster* bark tannin; (b) *P. contorta* bark tannin. Peak numbers refer to the structures given in the text; *, the exact stereochemistry for peaks corresponding to compounds **6**−**8** was not determined. T, benzyl mercaptan; T′, product derived from benzyl mercaptan.

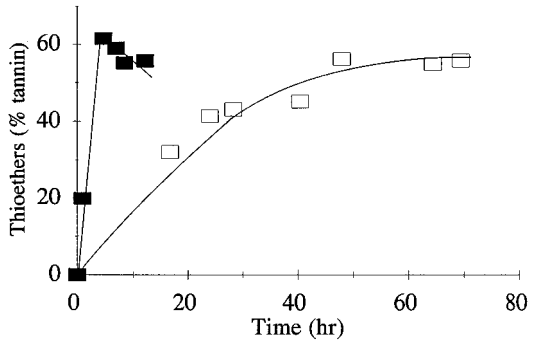

Figure 6. Kinetics of thiolysis of a *P. sitchensis* bark tannin. Temperature: (□) 70 °C; (■) 105 °C.

rived from prodelphinidin internal units. Their stereochemistry has not been determined.

Thiolysis products could also be separated by HPLC (Figure 5). Peak identity on HPLC chromatograms was established by cochromatography with available standards and by comparison of their relative area in both GC and HPLC chromatograms using various bark species. The elution order is the same as that reported by Samejima and Yoshimoto with a similar HPLC system (Samejima and Yoshimoto, 1981).

Parameters Affecting the Thiolysis Reaction. Some parameters of the reaction were optimized to maximize thioether yields. When tested on a *P. sitchensis* bark tannin, increasing the temperature from 70 to 105 °C increased the thioether yields from 56 to 63% and the reaction time needed to reach this maximum was reduced from 70 to 4.5 h (Figure 6).

Thiolysis is influenced by the solvent. It is usually carried out in ethanol (Thompson et al., 1972; Kolodziej, 1990), but crude phenolic extracts are often poorly soluble and water must be added to fully solubilize the sample. Using an ethanol/water 4:1 mixture instead of pure ethanol in thiolysis of *P. sitchensis* and *P. pinaster* bark tannin resulted in 24 and 36% yield reductions, respectively. Thiolysis can also be applied to insoluble plant residues containing nonextractable PAs; chromatograms similar to those of Figures 4 and 5 were

Table 1. Analytical Data of a *P. sitchensis* Tannin and Its TRF

	tannin	TRF
thiolysis yield (%)	62.9	3.2
phenol content (mg/g)	786	734
reactivity with vanillin/HCl ($E^{1\%}_{1cm}$)	231	79
polymerization degree		
X_n	3.9	2.9
X_w	11.9	7.2

obtained and used to estimate the proportion of tannins nonextractable by aqueous MeOH in various bark samples (Matthews et al., 1997).

Thiolysis Yields. Total yields of thioether derivatives, measured on purified PAs, were found to be 34% for a *P. sitchensis* bark tannin and 48 and 63% for two *P. pinaster* bark tannins. These yields were about 4 times higher than those obtained by degradation with phloroglucinol. Few authors have compared yields obtained with phloroglucinol and benzyl mercaptan nucleophiles. Gupta and Haslam (1978) have treated sorghum procyanidins by the two methods in nonoptimized conditions and purified the main products. Yields (recalculated as flavan-3-ol equivalents) were similarly lower with phloroglucinol (25%) than with benzyl mercaptan (38%).

Even in the present optimized reaction conditions, degradation yields are not quantitative. This could be due (i) to some inaccuracy in yield measurement, (ii) to the presence of phenolic or nonphenolic impurities in the PA samples, or (iii) to the existence of some thiolysis resistant bonds involving the polymer units.

Some instability of the thiolysis products under the conditions of the reaction could contribute to underestimate the depolymerization yields. Benzylthioepicatechin was stable (<1% degraded). (+)-Catechin and (−)-epicatechin formed from the less abundant terminal units of the polymer are partially degraded and epimerized during thiolysis into (+)-epicatechin and (−)-catechin, respectively, which are not separated from their enantiomers by HPLC or GC (Haslam, 1975). Under our experimental conditions, only 11% (+)-catechin and 5% (−)-epicatechin were degraded and small amounts of their epimers (3% for both) were formed. These values are of the same order as those reported by Prieur et al. (1994), although the reaction conditions they used were much milder. An underestimation of thiolysis yields thus appears unlikely.

A TRF was then isolated after thiolysis of a *P. sitchensis* bark tannin. Its reactivity and molecular weight were compared to those of the tannin before thiolysis (Table 1). When treated again by thiolysis, the yield of thioethers recovered from TRF was 20 times less than for the untreated tannin. The phenolic group content of TRF measured according to the Folin−Ciocalteu method was close to the one of untreated tannin. The main signals in the ^{13}C NMR spectrum of TRF were typical of procyanidins, which suggests that polyphenols in TRF are largely derived from procyanidins. TRF still reacted with vanillin, although its reactivity was significantly reduced as compared to the untreated tannin. Gel permeation of TRF showed that it is largely polymeric with a polymerization degree lower than that of the untreated tannin.

The ^{13}C NMR spectrum of TRF also showed prominent signals in the 60−80 ppm region with high relative intensities as compared to the untreated tannin. The majors signals in this region (δ = 101.4, 77.0, 76.8, 74.0, 70.5, 61.8) identical to those of methyl β-glucoside (Azuma and Koshijima, 1981) are attributed to glucose

linked as a β-glucoside. TRF thus appears richer in sugars than the untreated tannin (which may explain the lower phenolic group content of TRF; see Table 1). These sugars are attached to PAs either directly (Achmadi et al., 1994) or indirectly via the condensation of PAs with some phenolic glycosides (e.g. stilbenes in *P. sitchensis*; see below).

Attempts were made to further degrade TRF polyphenols. Catalytic hydrogenation over palladium (Jacques et al., 1974; Foo, 1982) led to the formation of some procyanidin characteristic products. Several reaction conditions have been tested: at room temperature and atmospheric pressure (Jacques et al., 1974), no products were formed. At a higher temperature (110 °C) and pressure (120 bar), a complex mixture of relatively volatile compounds was formed but none of them could be used for diagnosis. Finally, intermediate conditions (105 °C, 75 bar) led to the formation of (−)-epicatechin, (+)-catechin, and several other compounds tentatively identified by their MS spectra: 1-(3,4-dihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propane (**9**), which is a known hydrogenation product of (+)-catechin, (−)-epicatechin and procyanidin dimers (Jacques et al., 1974), and a minor compound that could correspond to afzelechin (**10**). Afzelechin has a hydroxylation pattern uncommon in barks but identified in *P. sitchensis* bark tannins in the form of propelargonidins (Hergert, 1989).

Two other peaks in the hydrogenolysis chromatogram correspond to the TMS derivatives of dihydroisorhapontigenin (**11**) and dihydroastringenin (**12**), the expected hydrogenolysis products of the stilbene glucosides isorhapontin and astringin, well-known constituents of *P. sitchensis* bark (Aritomi and Donnelly, 1976). Association of stilbenes with PAs has been previously documented (Steynberg et al., 1983; Hergert, 1989, 1992). Likely, it is the consequence of some secondary reactions occurring during the formation and aging of secondary tissues in bark (Hergert, 1992). Thus, both the presence of non-PA compounds and some secondary reactions leading to the formation of thiolysis-resistant linkages in the polymer can be inferred to explain the limits in thiolysis yields.

Double linkages between polymer units characteristic of A-type PAs occur to variable extents in PAs of various botanical origins (Porter, 1992). These linkages resist thiolysis (Thompson et al., 1972; Baldé et al., 1991) but are cleaved by hydrogenolysis (Jacques et al., 1974). These or other thiolysis-resistant intermonomeric linkages may contribute to limit thiolysis yields.

Secondary reactions that may affect thiolysis yields should be particularly important in aged tissues such as bark and heartwood and may also occur during the preparation of the plant sample or the extraction and isolation of PAs. Decreases of reactivity with vanillin (Butler, 1982) and of yield of depolymerization in butanol/HCl (Porter et al., 1986) upon drying and storage have been reported. In our own experience, storage of purified *P. sitchensis* bark tannin in dry conditions during 12 months in a sealed tube resulted in a 7% decrease in the thiolysis yield and a 16% decrease in the vanillin/HCl reactivity.

Comparison of Thiolysis and the Vanillin/H_2SO_4 Assay for Proanthocyanidin Estimation. Thiolysis was compared to the vanillin/H_2SO_4 assay, a well-established method for PA estimation (Scalbert et al., 1989). A good correlation was observed between the values obtained for both methods with raw bark extracts prepared from different tree species (Figure 7). Some discrepancies may exist and could be explained by differences in molecular weight. In our experimental

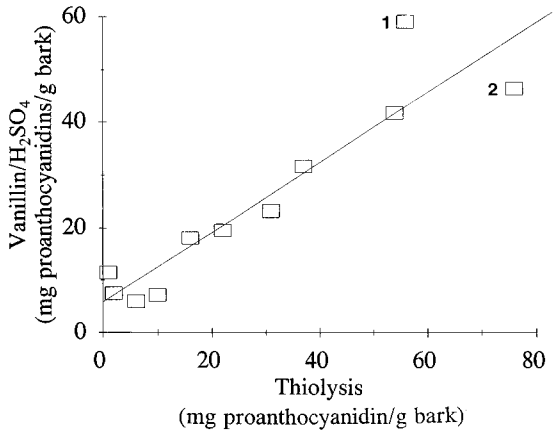

Figure 7. Comparison of proanthocyanidin contents determined by the vanillin/H_2SO_4 and thiolysis methods. Data correspond to raw bark extracts prepared from various tree species. (+)-Catechin and benzylthioepicatechin were used as standards for vanillin/H_2SO_4 and thiolysis methods, respectively. Points 1 and 2 correspond to *L. decidua* and *P. radiata*.

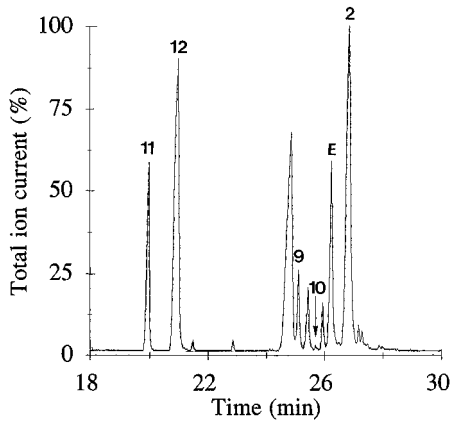

Figure 8. GC chromatogram of hydrogenolysis products of a *P. sitchensis* bark tannin. Products are analyzed as their TMS derivatives. Detection was by total ion current. Peak numbers refer to the structures given in the text; E, (−)-epicatechin.

conditions, vanillin reacts presumably with all units of the polymer (Butler et al., 1982), whereas only chain extender units give thioethers by thiolysis. The higher is the proportion of terminal units (i.e. the lower is the molecular weight), the higher will be the value determined by the vanillin assay as compared to the thiolysis value. This is consistent with the fact that *Larix decidua* tannin (point 1 in Figure 7) with the lowest degree of polymerization among all barks tested (X_w = 8.8) (Matthews et al., 1997) appears above the curve (relatively high vanillin value), whereas *P. pinaster* (point 2 in Figure 7) with the highest polymerization degree (X_w = 26.2) appears below the curve.

For bark extracts with low PA content, no correlation exists between values obtained by both methods. This is explained by the poor sensitivity and selectivity of the vanillin colorimetric assay. This method gives with these extracts ill-defined absorption maxima and relatively high background absorbance. For such samples, a selective chemical reaction such as thiolysis coupled with a chromatographic method is recommended.

Conclusions. Depolymerization in the presence of a nucleophile can be advantageously applied to the estimation of PAs. The use of benzyl mercaptan nucleophile rather than phloroglucinol is recommended due to the higher yields of depolymerization. Depolymerization products can be easily separated and quantified by GC or HPLC. As compared to the vanillin or

butanol/HCl colorimetric assays commonly used today for PA estimation, the present thiolysis assay is more specific and sensitive. It is particularly well suited for the analysis of samples containing either low amounts of PAs or insoluble PAs.

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