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Thermally Assisted Hydrolysis and Methylation of Purified Tannins from Plants

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A collection of tannins extracted from various plant species was subjected to thermally assisted hydrolysis and methylation (THM) using tetramethylammonium hydroxide. The products obtained included 1,3,5-trimethoxybenzenes, derived from A rings of condensed tannins (CTs), and 1,2-dimethoxybenzene, 1,2,3-trimethoxybenzene, and derivatives as major products from the B ring. 1,2,4-Tri- and two tetramethoxybenzenes were also detected in most analyses. Correlation analyses revealed that they were derived from the B ring, with 1,2,4-trimethoxybenzene being derived from a procyanidin (PC) B ring and 1,2,3,5-tetramethoxybenzene from a prodelphinidin (PD) ring. Tannins from species that contained both CT and hydrolyzable tannin (HT) produced mainly permethylated gallic acid moieties upon THM, and the products derived from CTs were less abundant. Most likely, the ester-bound HTs are more easily transmethylated than the cleavage and methylation of the C−C and C−O linked CTs. Statistical analyses of the THM products and ^{13}C NMR data of the tannins showed good correlations between the B ring hydroxylation and the di- and trimethoxybenzenes observed. Using the ratio of the methyl esters of 3,4-dimethoxybenzoic acid and 3,4,5-trimethoxybenzoic acid only provided good correlations of the percent PC as well. Furthermore, the 1,3,5-trimethoxybenzenes may serve as good markers of tannins in plant, soil, sediment or other samples as analyzed by THM.

Tannins encompass the fourth most abundant biopolymer in terrestrial plants and can be more abundant than lignin in foliage and bark.[1,2] Two main types of tannins can be distinguished: condensed tannins (CTs) and hydrolyzable tannins (HTs). The first consist of flavanol moieties, which are connected by C4−C8 linkages and sometimes C4−C6 links, while the latter ones have a sugar core to which gallic acids are bound through ester bonds (Figure 1).

Despite tannins' apparent key function in many ecological and biogeochemical processes, such as herbivore defense, pedogenesis, metal complexation, and nutrient dynamics,[3,4] their fate in soils is far from clear. While pure tannin fractions can be extracted from plants, tannins are difficult to measure in litter and humus, and especially in mineral soil samples when using assays developed for total phenols (e.g., Folin−Ciocalteu and Prussian Blue) and condensed tannins (e.g., HCl−butanol and vanillin assay)[5,6] in plants. Tannin-enriched fractions extracted from humus also contained lignin-derived structures,[2,7] which are also phenolic compounds that can interfere with tannin quantification. From mineral soil samples, no tannins could be obtained even after addition of tannins followed by various extraction procedures.[8]

At the more advanced molecular level, characteristic peaks for tannins in litter and soil organic matter samples can be detected by solid-state ^{13}C nuclear magnetic resonance spectroscopy, mainly the two peaks in the phenolic region at 145 and 155 ppm.[2,7,9,10] However, the phenolic carbons of lignin also occur in this region, and other important tannin resonances are interfered by lignin and polysaccharides (C2 and C3 of tannins at 75 ppm with those of C2, C3, and C5 of cellulose at 73 and 75 ppm; C10 and C8 in C4−C8 linkages of tannins at 105 ppm with the anomeric C1 of cellulose and aromatic C of lignin at 105 ppm). Therefore, it is difficult to obtain detailed information on tannin amounts, structures, and possible transformations.[7,9,11] Recently, an acid depolymerization technique was developed to analyze CTs in environmental samples.[12] In addition, MALDITOF-MS has been applied to determine the chain length of CTs and their monomer sequence.[13]

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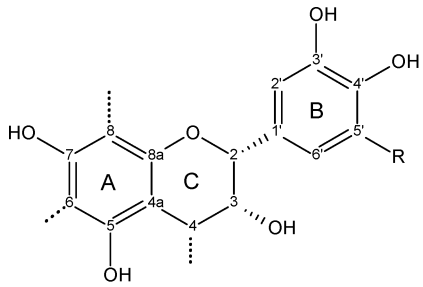
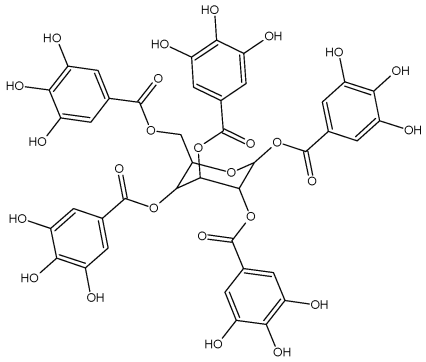


R=H: Epicatechin

R=OH: Epigallocatechin



Tannic acid

Figure 1. Structures of epi(gallo)catechin and tannic acid.

Thermally assisted hydrolysis and methylation (THM) using tetramethylammonium hydroxide (TMAH) is a relatively new technique that has received increasing attention through its application without time- and reagent-consuming sample preparations and subsequent derivatization that are needed in most degradative procedures (hydrolysis, oxidation, etc.).[14,15] It has been applied in both on-line and off-line systems analyzing a variety of biopolymers, such as lignin,[16] cutin and suberin,[17,18] and soil organic matter.[14,19,20] With THM, hydrolyzable bonds are cleaved and the resulting carboxylic acid and hydroxyl groups are in situ transformed into their corresponding methyl esters and methyl ethers. In the past, analytical pyrolysis and THM, sometimes also referred to as thermochemolysis, have also been used to analyze CTs in wine and archaeological vine samples.[21−23] Using THM, a few papers already mentioned the presence of tannins in soil organic matter.[24,25] To date, however, no purified tannins from plants have been analyzed with this technique. In this paper, we analyze a number of purified tannins from plants, both pure CTs and mixtures of CTs and HTs, to determine their THM products and to assess their potential as markers in complex mixtures, such as soil organic matter.

EXPERIMENTAL SECTION

Tannin Collections. Some of the purified tannins were prepared in earlier studies: Corsican pine,[26] balsam fir and pecan,[10] bishop pine, bolander pine, cypress, evergreen huckleberry, labrador tea, manzanita, rhododendron, and wax myrtle.[27] The tannins from kalmia (sheep laurel), black spruce, jack pine, Douglas fir, oval-leaved blueberry, salal, western hemlock, and western red cedar were prepared using a similar procedure as described in Preston,[2] and chemical characteristics for these tannins have not been reported previously. These tannins originated from boreal forest sites in Quebec (kalmia), Manitoba, and Saskatchewan (black spruce, jack pine), Vancouver Island coastal forests (Douglas fir, oval-leaved blueberry, salal, western hemlock, western red cedar), and the PFC site in Victoria (Scots pine, the only species growing in an exotic location). Tannins were prepared from foliage, except that both foliar and root tannins were prepared for kalmia, and the balsam fir tannin was prepared from branch tips (twig with attached needles). Methods for tannin extraction and purification differed only in minor details. Briefly, dried and ground plant material was pre-extracted by hexane to remove lipids. The residue obtained was extracted by acetone/water (7:3 v/v). After evaporation of the acetone, the aqueous solution was successively washed with dichloromethane and ethyl acetate. The solution obtained was freeze-dried, redissolved in methanol/water (1:1 v/v), and loaded onto a Sephadex LH-20 column. Tannin monomers and other phenols were separated from the crude mixture using methanol/water as the eluate. The CTs were obtained after switching the eluate to aqueous acetone. From this solution, acetone was removed by rotary evaporation and the final product was freeze-dried, yielding the purified CT powder. See Table 1 for structural data obtained by ^{13}C NMR. Epicatechin, epigallocatechin (Sigma), and tannic acid (Merck) were purchased commercially.

Solution ^{13}C NMR Spectroscopy. Purified tannins (100−200 mg) were dissolved in a mixture of 1.5 mL of acetone and 1.5 mL of D_2O and transferred through sintered glass filters into 10-mm-diameter NMR tubes. Spectra were obtained at 75.47 MHz on a Bruker MSL 300 spectrometer, using inverse-gated decoupling, 45° pulse, 0.4-s acquisition time, and up to 40 000 scans. Spectra for the tannins reported in Kraus et al.[27] were run with 2.6-s relaxation delay. Tannins from salal, Corsican pine, and kalmia

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Table 1. Characteristics of Tannins as Determined by ^{13}C NMR

species	Latin name	% CT	% PC	cis	chain length	fraction of trans terminal units
balsam fir	*Abies balsamea*	100	21.1	71.7	4.5	0.55
bishop pine	*Pinus muricata*	100	27.0	72.9	4.2	0.51
black spruce	*Picea mariana*	100	85.7	75.9	5.4	0.69
bolander pine	*Pinus contorta ssp. Bolanderi*	100	36.7	72.2	4.7	0.47
Corsican pine	*Pinus maritima var. nigra*	100	37.3	85.3	6.6	0.55
cypress	*Cupressus goveniana ssp. Pygmaea*	100	88.5	76.2	4.6	0.67
Douglas-fir	*Pseudotsuga menziesii*	100	63.3	76.8	4.1	0.58
evergreen huckleberry	*Vaccinium ovatum*	100	83.2	91.8	3.4	1
jack pine	*Pinus banksiana*	100	23.0	71.3	5.0	0.49
kalmia foliar	*Kalmia angustifolia*	100	80.2	82.0	2.3	0.73
kalmia root	*Kalmia angustifolia*	100	77.3	81.6	2.2	0.65
Labrador tea	*Ledum glandulosum*	57	90	81	4.8	0.5
manzanita	*Arctostaphylos nummularia*	37	35	84		>0.5
oval-leaved blueberry	*Vaccinium ovalifolium*	100	93.9	93.7	3.7	0.66
pecan	*Carya illinoinensis*	100	23.7	50.0	5.5	0.15
w. red cedar	*Thuja plicata*	100	27.4	37.7	3.8	0.32
rhododendron	*Rhododendron macrophyllum*	68	96	85	4.5	<0.5
salal	*Gaultheria shallon*	100	27.8	74.8	4.8	0.59
Scots pine	*Pinus sylvestris*	100	49.5	77.5	6.3	0.63
tannic acid		0				
wax myrtle	*Myrica californica*	40	0	>85		>0.5
western hemlock	*Tsuga heterophylla*	100	21.6	76.4	5.9	0.59

root and leaf were run with 4.5-s delay, and Scots pine and pecan tannins were run with 3.6-s delay. Balsam fir, western red cedar, western hemlock, Douglas fir, jack pine, and black spruce tannins were run with 3.6- and 10.0-s delay and the results averaged as there were no consistent differences between the two delay times. New spectra were run for the balsam fir and pecan tannins reported in Preston et al.[10]

Subspectra expanded to 1 or 2 ppm/cm with minimal line broadening (8 Hz) were analyzed using standard methods[13,28,29] to determine the proportion of C2 in cis versus trans units, average chain length from the ratio of C3 extender and terminal units, and proportion of PC versus PD units (% PC) from the ratio of the peak areas at 145 (C3′ and C4′ of PC) and 146 ppm (C3′ and C5′ of PD).

In Kraus et al.,[27] the percent PC was determined from the ratio of two peak heights at (116 ppm/106 ppm). This approach was developed to determine percent PC for tannins that were mixtures of CT and HT, so that the 144−145 ppm region could not be used for this purpose. For this study, the original spectra from Kraus et al.[3] were reanalyzed for percent PC using the 144−145 ppm region, so that values reported here differ slightly. A calibration curve (not shown) for ratio versus percent PC was produced from NMR data for condensed tannins, except for western red cedar, which was high in trans units, and the two kalmia tannins, which had an apparent very short chain length, and were also found to include other structures. Based on this, we also report here slightly revised values of percent PC for the mixed tannins. While the method of analyzing percent PC from the ratio of peak heights at 116 to 106 ppm was found to be satisfactory for this group of tannins, it is only recommended for obtaining an estimate where there is interference by HT.

Thermally Assisted Hydrolysis and Methylation-Gas Chromatography/Mass Spectrometry. Prior to THM, purified tannin samples were pressed onto Curie-point wires, after which a droplet of a 25% solution of TMAH in water was added to the samples, which were subsequently dried by a 100-W halogen lamp. THM was performed by heating the sample for 5 s at 600 °C. The Horizon Instruments Curie-Point pyrolyzer was connected to a ThermoQuest Trace GC 2000 gas chromatograph, and the products were separated by a fused-silica column (J & W, 30 m, 0.32-mm i.d.) coated with DB-1 (film thickness 0.50 μm). Helium was used as carrier gas. The oven was initially kept at 40 °C for 1 min; next it was heated at a rate of 7 °C/min to 320 °C and maintained at that temperature for 10 min. The column was coupled to a Finnigan Trace MS quadrupole mass spectrometer (electron ionization (EI), ionization energy 70 eV, mass range m/z 45−600, cycle time 1 s). Identification of the compounds was carried out by interpretation of their EI spectra using a NIST library, by their GC retention times, and by comparison with literature data.

Statistical Treatment. Relative quantification of the compounds was performed by integrating the corresponding peaks in the chromatograms. The resulting relative contribution of each compound is presented in Table 2. Basic (bivariate/zero-order) correlation analysis (Pearson) was performed with SPSS 11.0 for Windows. Proximity matrices (Euclidean distance) were used to compare tannin compositions.

RESULTS AND DISCUSSION

Monomers: Epicatechin and Epigallocatechin. THM of epicatechin and epigallocatechin (see Figure 1 for structures) yielded 1,3,5-trimethoxybenzene and 2-methyl-1,3,5-trimethoxybenzene as the main products (Figure 2). Both compounds are derived from the A ring of these monomers.[23,30] A relatively small peak of 2-ethyl-1,3,5-trimethoxybenzene was also present in the THM chromatogram of both monomers. We only found trace

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Table 2. THM Product Distribution (in %) of Purified Tannins[a]

compound[b]	mass fragments (*m/z*)	balsam fir	bishop pine	black spruce	bolander pine	Corsican pine	cypress	Douglas fir	evergreen huckleberry	jack pine	kalmia (foliar)	kalmia (root)
benzoic acid, methyl ester	51, 77, 105, 136		1.3		0.2							
1,2-dimethoxybenzene[B2]	77, 95, 123, 138	3.6	6.3	11.8	0.8	1.9	1.5	0.3	1.8	0.3	2.9	0.5
1,4-dimethoxybenzene	123, 138											
4-methyl-1,2-dimethoxybenzene[B2]	137, 152	2.9	5.7	8.3	1.0	1.7	1.8	1.4	2.3	0.6	3.5	2.0
1,2,3-trimethoxybenzene[B3]	95, 110, 125, 153, 168	9.8	15.1		2.2	3.9	1.7	0.6			0.9	0.8
1,2,4-trimethoxybenzene[B2]	125, 153, 168	1.3	2.0	7.2	2.0	3.1	10.0	3.8	9.2	3.3	5.2	7.8
4-methoxybenzoic acid, methyl ester	135, 166									1.1		
5-methyl-1,2,3-trimethoxybenzene[B3]	167, 182	14.8	23.6		2.8	3.8	1.5	2.4		0.0		0.4
1,3,5-trimethoxybenzene[A]	139, 168	20.9	25.1	20.3	31.8	39.5	32.0	23.7	39.6	31.3	29.5	26.4
3,4-dimethoxybenzaldehyde[B2]	165, 166	1.4		6.2	5.0	2.3	4.1	4.2	0.8		1.8	5.1
2-methyl-1,3,5-trimethoxybenzene[A]	153, 182	13.9	11.7	19.7	30.2	18.0	15.5	27.7	29.9	35.9	18.7	24.9
1,2,3,4-tetramethoxybenzene[B3]	140, 183, 198	3.0							0.3		0.2	
2-ethyl-1,3,5-trimethoxybenzene[A]	165, 181, 196	2.1	1.3	5.2	2.8	4.6	5.0	3.4	3.2	5.5	3.6	2.3
1,2,3,5-tetramethoxybenzene[B3]	155, 183, 198	4.2	1.2	4.7	3.6	3.3	2.3	8.0	0.9	8.8	3.9	6.8
3,4-dimethoxyacetophenone[B2]	165, 180	0.4		1.2	3.1			1.8	0.4		1.5	0.8
3,4-dimethoxybenzoic acid, methyl ester [B2]	165, 196	4.3	1.3	14.8	4.3	4.6	22.8	8.8	9.6	3.5	8.7	13.3
3,4,5-trimethoxybenzaldehyde[B3]	181, 196	1.0		0.2	1.0	0.6	0.2	1.4	0.5			0.6
3,4,5-trimethoxybenzoic acid, methyl ester[B3]	155, 195, 211, 226	18.1	5.4	0.4	9.1	12.6	1.5	12.3	0.5	9.7	2.8	2.9
4-methoxycinnamic acid, methyl ester	165, 192										16.9	1.5
3,4-dimethoxycinnamic acid, methyl ester	191, 207, 222											4.0
% PC NMR		21.1	27.0	85.7	36.7	37.3	88.5	63.3	83.2	23.0	80.2	77.3
% PC-THM[c]		21.5	25.3	90.3	46.4	36.2	84.6	45.1	91.8	29.5	75.1	72.0
% PC-acid[d]		19.3	19.7	97.2	32.3	26.9	93.7	41.7	95.5	26.7	75.7	82.1

compound	mass fragments (*m/z*)	Labrador tea	manzanita	oval-leaved blueberry	pecan	w. red cedar	rhodo-dendron	salal	Scots pine	western hemlock	wax myrtle
benzoic acid, methyl ester	51, 77, 105, 136										
1,2-dimethoxybenzene[B2]	77, 95, 123, 138	2.1		3.6	0.3	0.3	4.7	0.7	4.4		
1,4-dimethoxybenzene	123, 138			3.0							
4-methyl-1,2-dimethoxybenzene[B2]	137, 152	3.4		3.6	1.4	0.8	2.4	1.8	2.6		
1,2,3-trimethoxybenzene[B3]	95, 110, 125, 153, 168	8.2	0.9		2.1	1.3	5.9	2.0	3.8		15.4
1,2,4-trimethoxybenzene[B2]	125, 153, 168	1.3	0.6	5.2	0.9	0.9	4.0	1.8	2.4	3.3	
4-methoxybenzoic acid, methyl ester	135, 166										
5-methyl-1,2,3-trimethoxybenzene[B3]	167, 182	9.8	0.2			12.8	3.1	19.1	2.2		
1,3,5-trimethoxybenzene[A]	139, 168	15.6	7.5	34.4	35.6	23.6	25.4	20.8	34.9	47.1	16.2
3,4-dimethoxybenzaldehyde[B2]	165, 166	0.5		2.8	2.3	1.3	1.4	1.7	2.5		
2-methyl-1,3,5-trimethoxybenzene[A]	153, 182	6.2	5.4	28.1	25.7	27.2	11.5	19.3	28.7	21.0	2.7
1,2,3,4-tetramethoxybenzene[B3]	140, 183, 198					0.2		1.5			
2-ethyl-1,3,5-trimethoxybenzene[A]	165, 181, 196	0.3	1.5	4.8	3.5	3.4	0.9	2.8	2.8	1.6	0.2
1,2,3,5-tetramethoxybenzene[B3]	155, 183, 198	0.7	1.6	0.6	12.0	7.6	3.1	4.8	5.0	2.7	
3,4-dimethoxyacetophenone[B2]	165, 180			0.4	1.2	1.7		1.3	0.6		
3,4-dimethoxybenzoic acid, methyl ester[B2]	165, 196	2.6	1.1	13.1	1.6	1.2	7.5	4.6	3.8	4.4	0.2
3,4,5-trimethoxybenzaldehyde[B3]	181, 196			0.1	0.8	5.1	0.2	1.4	0.3	2.9	
3,4,5-trimethoxybenzoic acid, methyl ester[B3]	155, 195, 211, 226	49.3	81.1	0.3	12.4	12.5	29.9	16.3	5.8	17.1	65.4
4-methoxycinnamic acid, methyl ester	161, 192										
3,4-dimethoxycinnamic acid, methyl ester	191, 207, 222										
% PC NMR		90	35	93.9	23.7	27.4	96	27.8	49.5	21.6	0
% PC-THM[c]		13	2	96.5	22.4	13.6	32	21.0	48.9	25.1	0
% PC-acid[d]		5	1	97.5	11.8	8.7	20	22.2	40.0	20.3	0

[a] For compounds at <0.1%, no values are given. [b] Codes: (A) compound originating of the A ring; (B2) compound originating of a PC B ring; (B3) compound originating of a PD B ring. [c] % PC-THM is based on all compounds related to di- and trihydroxy B ring. [d] % PC-acid is based on di- and trimethoxybenzoic acid, methyl esters only.

amounts of 3,4-dimethoxybenzoic acid methyl ester and no other compounds that could be related to the B ring after THM of epicatechin. By contrast, small peaks of 1,2,3-trimethoxybenzene, 5-methyl-1,2,3-trimethoxybenzene, and 3,4,5-trimethoxybenzaldehyde and a relatively high peak of 3,4,5-trimethoxybenzoic acid methyl ester were present in the chromatogram of epigallocatechin, reflecting the trihydroxy pattern of the B ring. Also, 1,2,3,5-tetramethoxybenzene was identified.

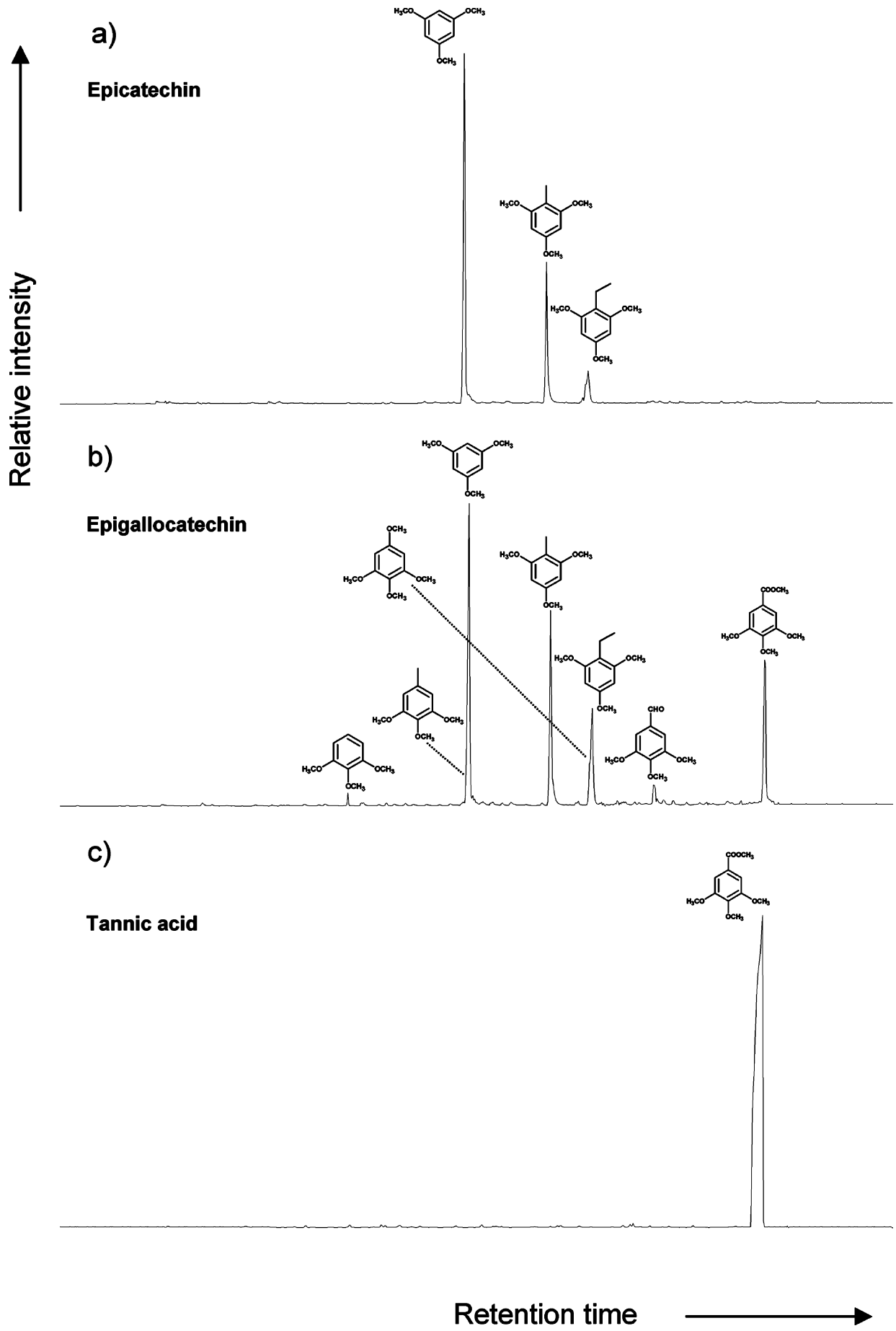

Figure 2. Gas chromatogram of products released upon THM of (a) epicatechin, (b) epigallocatechin, and (c) tannic acid.

The A ring-derived compounds were by far the two dominant products upon THM of both the monomers, and as such, the results agree well with the data of Garnier et al.[23] However, the B-ring derived compounds did not resemble that of Garnier et al.,[23] i.e., similar to Galletti and Bocchini[30] for catechin; we found very small traces of 3,4-dimethoxybenzoic acid methyl ester in the THM-GC trace of epicatechin whereas Garnier et al.[23] found significant amounts of this compound. Conversely, 3,4,5-trimethoxybenzoic acid methyl ester in the THM-GC trace of epigallocatechin was not observed by them, while we found relatively large amounts. Moreover, the presence of 2-ethyl-1,3,5-trimethoxybenzene was observed by Galletti and Bocchini[30] and us, but not identified by Garnier et al.[23] Compared with Galletti and Bocchini,[30] we did not find the isomers of (2-methoxyethenyl)-3,4-dimethoxybenzene and products with higher molecular weights. All these differences may, to some extent, be related to a difference in pyrolysis tools and operating conditions (temperature and pyrolysis time) producing a different distribution of compounds. Apart from that, it is striking that epicatechin hardly showed a B ring product in our analysis, while considerable amounts of 3,4,5-trihydroxybenzene derivatives were produced upon THM of epigallocatechin. It would suggest that three OH groups at the B ring stimulated the formation of acids while two OH groups did not, but there seems no reasonable explanation for that.

Tannic Acid. Tannic acid, the structure of which is considered as composed of a glucose core with five gallic acids units connected through ester linkages[27] (Figure 1), produced only the methylated version of gallic acid, 3,4,5-trimethoxybenzoic acid methyl ester after THM (see Figure 2). The absence of other compounds suggests a virtually complete hydrolysis and methylation of the esterified gallic acid units without side reactions.

Condensed Tannins. All purified CTs produced 1,3,5-trimethoxybenzene and 2-methyl-1,3,5-trimethoxybenzene as main and, generally, as predominant compounds upon THM (as shown in Figure 3 for balsam fir, Scots pine, and black spruce). Such relatively high amounts and omnipresence makes them the best possible markers of tannins in soil organic matter when analyzed by THM under the restriction that no other biopolymers produce them. As far as we know, only one paper about the characterization of cutan, a nonhydrolysable aliphatic biopolymer isolated from the cuticles of some plants, by TMAH thermochemolysis has revealed these compounds.[31] It is very striking that in this paper the distribution and predominance of both compounds resembles that of the analyzed tannins. In fact, some other aromatic compounds that were suggested to be derived from cutan were also found with tannins. The purification method of cutan did not include a special tannin removal step, which could imply that they were present in the cutan isolate. Apart from that, as cutan is suggested to be present in drought-adapted plants only,[32] the 1,3,5-trimethoxyaromatics can at least serve as tannin markers in environmental samples that lack input of such plants.

Other compounds that were identified included the methyl esters, ethers, or both of benzoic acid, *p*-methoxybenzoic acid, 3,4-dimethoxybenzoic acid, and 3,4,5-trimethoxybenzoic acid. Next to these, compounds identified were the dimethoxybenzene derivatives 1,2-dimethoxybenzene, 1,4-dimethoxybenzene, 3,4-dimethoxytoluene, 3,4-dimethoxybenzaldehyde, and 4-acetyl-1,2-dimethoxybenzene; the trimethoxybenzene derivatives 1,2,3-trimethoxybenzene, 1,2,4-trimethoxybenzene, 5-methyl-1,2,3-trimethoxytbenzene, and 3,4,5-trimethoxybenzaldehyde; and two tetramethoxybenzenes, i.e., the 1,2,3,4 and 1,2,3,5 isomers (identified using the mass spectra of Paszczynski et al.).[33] Only in the case of kalmia, both the foliar and the root tannins contained *p*-coumaric acid and ferulic acid, caffeic acid, or both and also produced 4-vinyl-1,2-dimethoxybenzene upon THM.

Apart from 1,2,4-trimethoxybenzene and the two tetramethoxybenzenes, all these compounds were identified when lignin-containing samples were subjected to THM, such as wood.[16] Obviously, the lignin products are derived from 2-methoxyphenols and 2,6-dimethoxyphenols, whereas tannins contain phenolic moieties lacking methoxy groups.

1,2,3-Trimethoxybenzene was previously identified as a THM product of ellagic acid.[30] This compound, a dimer of gallic acid, is not expected in CTs, but may reflect a similar structure in CTs. 1,2,4-Trimethoxybenzene was previously identified as a THM marker of polysaccharides.[34] Since the tannin samples used were free of polysaccharides as observed by ^{13}C NMR, and no other polysaccharide-derived THM compounds were identified, this compound is also a CT marker. As far as we know, the two tetramethoxybenzenes have not been observed earlier in THM studies of other biopolymers, which suggest that they also can be used as markers of CTs.

Contrary to all other tannins, *p*-coumaric acid and trace(s) of ferulic acid, caffeic acid, or both were identified in the foliar tannins from kalmia (Figure 4). In the root tannin, ferulic acid/caffeic acid was relatively more abundant than in the foliar tannin, and only a very small contribution of *p*-coumaric acid was observed. In addition, a rather small contribution of 4-vinylmethoxybenzene was found in the foliar tannins, and most likely this compound is a decarboxylation product of *p*-coumaric acid. The root tannins showed a small, but a clear peak of 4-methoxybenzoic acid methyl ester, which was virtually absent from the foliar tannins.

In a repurified kalmia foliar tannin (after an extra removal of low molecular weight compounds by ethyl acetate), the *p*-coumaric acid peak was reduced ~50% in intensity compared with the other compounds, suggesting that part of the *p*-coumaric acid was not bound to high molecular weight tannins and possible only as free acids (Figure 4) as found for water extracts of plants and litter.[35,36] In addition, other compounds, such as (4-methyl-) 1,2-dimethoxybenzene, 1,2,3-trimethoxybenzene, 3,4-dimethoxybenzaldehyde, and methylated ferulic acid/caffeic acid decreased relatively compared with other compounds produced upon THM. Similar to *p*-coumaric acid, these products are likely to be partly involved in low molecular weight compounds that do not make up the high molecular weight tannins. The presence of these components may partially explain the apparent short-chain length (~2.2) found for all kalmia tannins (greater intensity at 63−67 ppm).

Mixtures of Condensed and Hydrolyzable Tannins. All the chromatograms after THM of tannins of the four species that

(31) McKinney, D. E.; Bortiatynski, J. M.; Carson, D. M.; Clifford, D. J.; de Leeuw, J. W.; Hatcher, P. G. *Org. Geochem.* **1996**, *24*, 641−650.
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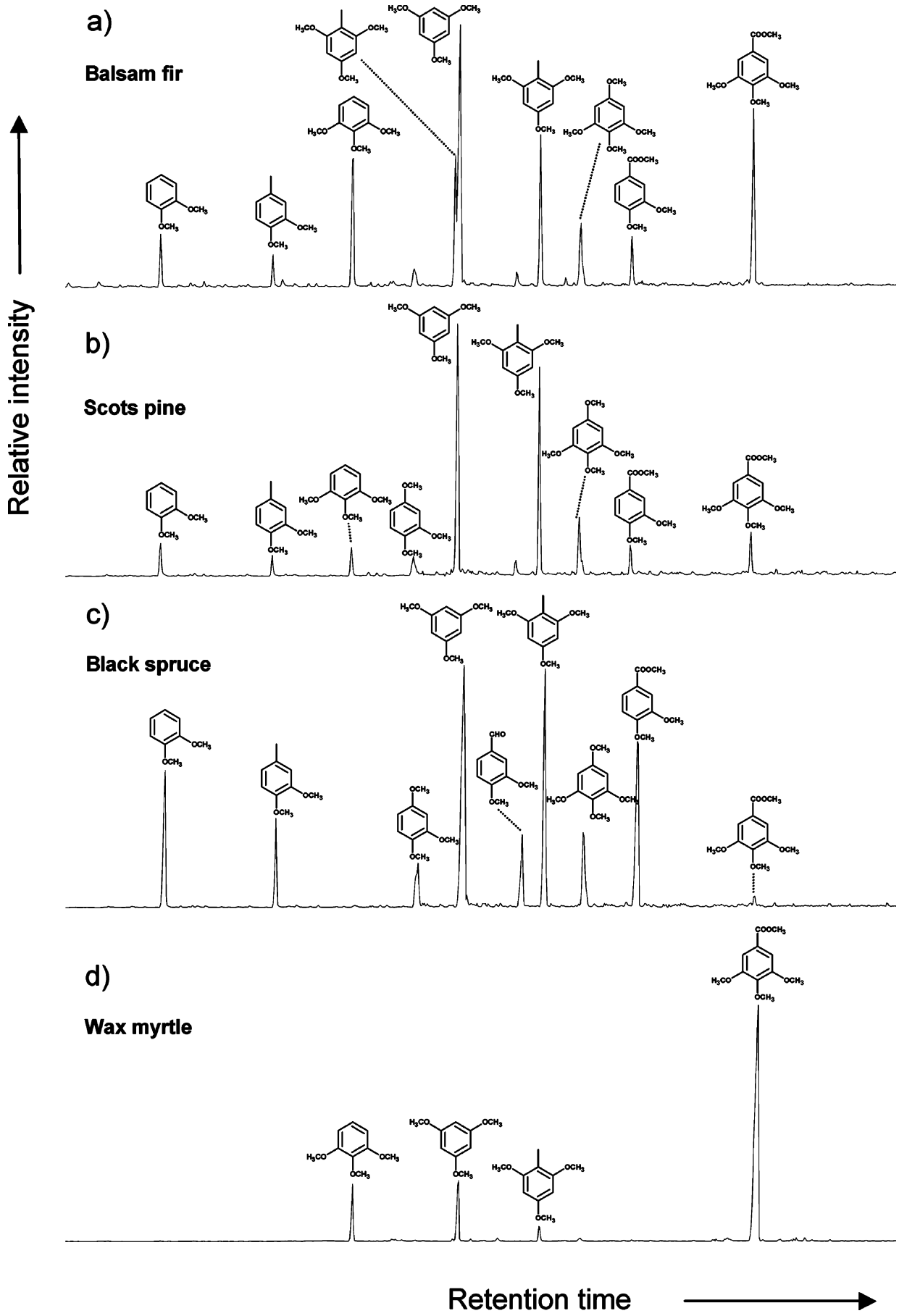

Figure 3. Gas chromatogram of products released upon THM of tannins of (a) balsam fir, (b) Scots pine, (c) black spruce, and (d) wax myrtle. Note that only the main peaks are assigned.

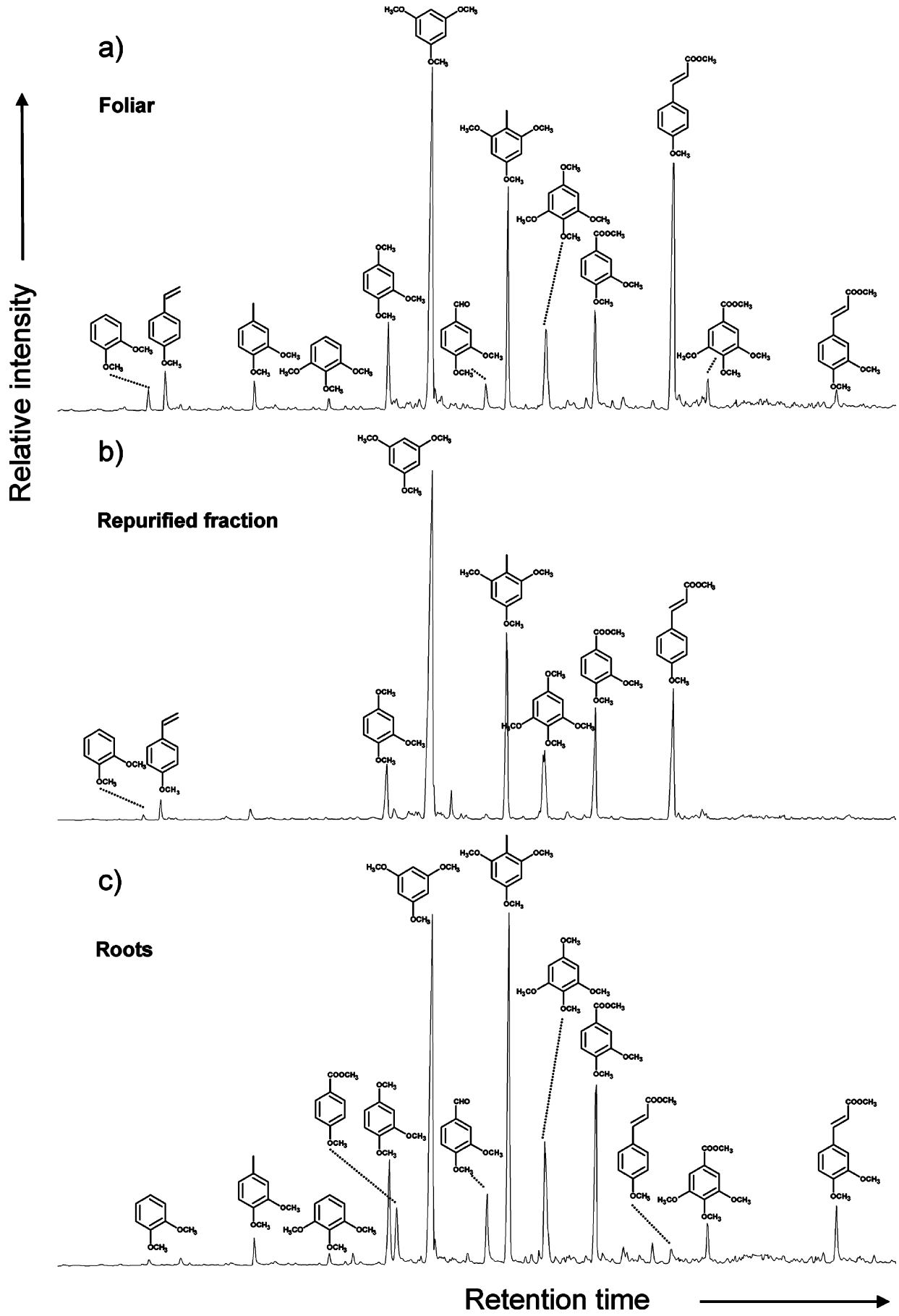

Figure 4. Gas chromatogram of products released upon THM of tannins of (a) kalmia foliar, (b) repurified tannin fraction of kalmia foliar, and (c) kalmia roots. Note that only the main peaks are assigned.

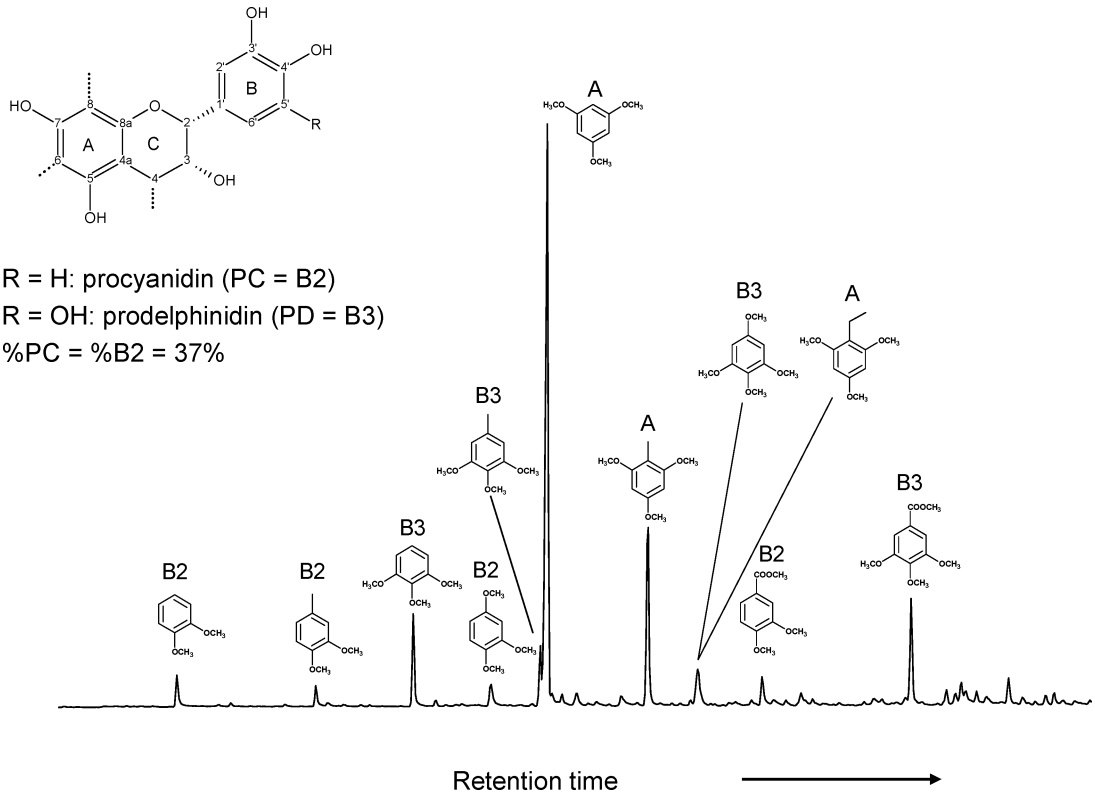

Figure 5. Relation between THM products and precursor fragments of condensed tannins as shown for Corsican pine tannin.

contain both condensed and hydrolyzable tannins showed a very dominant peak attributed to the methyl ester of 3,4,5-trimethoxybenzoic acid as shown for wax myrtle in Figure 3. As indicated earlier, tannic acid, as an example of a pure HT, produced only this compound after THM (see Figure 2). With this in mind, the predominance of the methyl ester of 3,4,5-trimethoxybenzoic acid of mixed tannins is an obvious result, as condensed tannins produce many different compounds upon THM. As hydrolyzable tannins are expected to produce only the methyl ester of 3,4,5-trimethoxybenzoic acid, it is impossible to calculate the amount of HT in a given tannin mixture as this compound is also produced by prodelphinidin (PD) containing CTs. Apart from the intense gallic acid peak, the same compounds observed for pure CTs were present in the thermochemolysates. The higher the proportion of HTs, the less abundant the THM products of CTs were in the thermochemolysates.

In the case of mixtures of a pure CT with tannic acid, the permethylated gallic acid peak was always by far the dominant compound. Even in a 3:1 mixture of black spruce tannin (a pure CT with 86% procyanidin (PC)) with tannic acid, the gallic acid-derived peak represented more than 50% of the peak areas and tannic acid was up to 3 times more sensitive to THM than CTs (data not shown). This suggests that the ester bonds in tannic acid are more easily cleaved and methylated than the C−O or C−C bonds in CTs. Probably, ester hydrolysis and (trans)-methylation of the HTs is mainly a temperature-assisted chemolytic process, while the cleavage of the C−C and C−O links of CTs is a more true pyrolysis process with subsequent methylation.

Comparison of Monomers versus Polymers. All compounds produced upon THM of monomers were also found in the thermochemolysates of the purified CTs. Additional compounds, such as the methyl ester of 3,4-dimethoxybenzoic acid, 1,2,4-trimethoxybenzene, and 1,2,3,4-tetramethoxybenzene, which were only produced by the polymers, likely reflect products that were derived from connected monomers. However, monomers are only linked via A and C rings, while all additional products are related to B rings. For example, 3,4-dimethoxybenzoic acid methyl ester is associated with the B ring, but not produced upon THM of epicatechin. Apparently, the 4−8 linkage promotes the cleavage of the A ring as an (alkyl)-1,3,5-trimethoxybenzene and to oxidation at C2 of the C ring. In addition, THM is known to promote the formation of such acids as was demonstrated for lignin.[37]

Correlation between THM Fragments. Table 2 summarizes all identified compounds and their relative abundance in the THM data of all analyzed tannins. Using only the NMR (percent PC, chain length, cis, terminal trans) and THM data of pure CTs ($n = 17$), a first correlation analysis was used to determine whether 1,2,4-trimethoxybenzene and 1,2,3,4- and 1,2,3,5-tetramethoxybenzene were A or B ring products, or both. To perform this, all 1,2-dimethoxybenzenes were classified as PC B ring products. Similarly, all 1,2,3-trimethoxybenzenes were assigned as PD B ring products, while the three 1,3,5-trimethoxybenzenes were attributed to the A ring. All products that were only found once or twice upon THM of tannins were omitted in the correlation analysis. It appeared that 1,2,4-trimethoxybenzene correlated positively (0.85, level of significance (P) < 0.001) with percent PC (NMR), while 1,2,3,4-tetramethoxybenzene correlated negatively (−0.54, P < 0.03) with the total of A ring products. As a consequence, the first compound was assigned as a PC B ring product, and the

(37) Hatcher, P. G.; Minard, R. D. *Org. Geochem.* **1995**, *23*, 991−994.

latter one from a PD B ring. 1,2,3,5-Tetramethoxybenzene had a weak negative correlation (−0.37, with a significance of 0.14) with percent PC (NMR), was found as THM product of epigallocatechin in a very small amount, and was therefore also assigned as a PD B ring product (see Figure 5).

The relative contribution of 1,2-dimethoxybenzene was always in the same range as that of the 4-methyl analogue in all samples. Their mutual correlation is 0.96 ($P < 0.001$), strongly suggesting that they have the same origin. No other correlations of these two compounds with other products with $P < 0.05$ were found, but with $P < 0.10$, a negative correlation with the methyl ester of 3,4,5-trimethoxybenzoic acid and the percent A ring products was noticed. Similarly, 1,2,3-trimethoxybenzene correlated well with 5-methyl-1,2,3-trimethoxybenzene (0.76, $P < 0.001$), and both, as expected, negatively with percent PC, and they also exhibited a negative correlation with the A ring products (for both, $P < 0.01$). This was also found for 1,2-dimethoxybenzene and 4-methyl-1,2-dimethoxybenzene, suggesting that both compound "duos" have a similar mechanism of production.

The products that were oxidized at the 4- and 5-position of 1,2-dimethoxybenzene and 1,2,3-trimethoxybenzene, respectively, indicate that during THM C2 of the C ring that connects to the B ring was susceptible to oxidation although it does not bear an oxygen that is involved directly. The O atom of the C ring that is linked with the A ring was always found back in the A ring products (1,3,5-trimethoxybenzenes); otherwise we would have found 1,3-dimethoxybenzenes, which we did not. Also, C2 as such was disconnected, and oxidation leading to an aldehyde or an acid took place. The cleavage and subsequent oxidation of C2−C3 and the cleavage of C8a−O−C2 may be explained by an intramolecular epoxide formation followed by various reactions leading to acids, methyl ketones, and aldehydes as proposed for the β-O-4 cleavage of lignin,[38] which is similar to the C8a−O−C2 linkage of CTs. Other fragmentations and oxidations may have lead to 1,2,4-trimethoxybenzene or to 1,2,3,5-tetramethoxybenzene.

Based on correlation analysis, the type of THM product of the B ring can be associated with two "mechanisms". First, the B ring can be disconnected from the C ring by cleavage of the C1′−C2 linkage or by cleavage within the C ring between C2 and C3. Generally, the latter pathway correlates negatively with the A ring products 2-methyl- and 2-ethyl-1,3,5-trimethoxytoluene. By contrast, the B1′−C2 degradation pathway leads to higher contributions of the A ring derivates with (m)ethyl extension. Hence, cleavage of the C1′−C2 linkage rather than cleavage at C2−C3 yielded A ring products with a (m)ethyl group. The second mechanism is the oxidation of the B ring products. The A ring products contained one or two C atoms of the C ring especially if the B ring product was oxidized. Combined, this caused a positive correlation between A ring extension degree and 1,2,3,5-tetramethoxybenzene and 1,2,4-trimethoxybenzene, whereas all other B ring derivatives (apart from the acids) have negative correlations with the A ring products. As PD tannins were less easily cleaved at the C1′−C2 linkage than PC tannins, PD tannins yielded relatively low amounts of A ring products (all B2 products in Table 2, $P = 0.37$; all B3 products, $P = 0.02$).

(38) Filley, T. R.; Minard, R. D.; Hatcher, P. G.; *Org. Geochem.* **1999**, *30*, 607−621.

As virtually all CTs consist of phloroglucinol A rings, the relative amount of B ring products depended on the hydroxylation pattern of the B ring, and perhaps the chain length, the location (end member or not) of the monomer, and the stereochemistry at C2−C3. Although our results suggest that the variety in B ring contributions is associated with the relative abundance of PD B ring products, more knowledge about the effects of the other variables is necessary to elucidate the effect of tannin composition on the percent A ring products, especially in view of the positive correlations of the percent PC (NMR and THM) with the percent trans terminal units and percent cis.

Percent PC. All assigned B ring products (see Table 2) were used to calculate the percent PC as determined by THM. The correlation with percent PC (NMR) was 0.97 ($P < 0.001$), suggesting that THM is well able to determine the hydroxylation pattern of pure CTs. Those calculated for mixed tannins did not agree well as the HT-derived gallic acids interfered the calculation.

If we use only the methyl esters of 3,4-dimethoxybenzoic acid and 3,4,5-trimethoxybenzoic acid as indicators of the di- and trihydroxy-substituted B rings of pure CTs and calculate their relative proportions as percent PC, the obtained results match as well as those that used all B ring compounds (Table 2). Apart from kalmia root, where PC as calculated by THM is 5% higher than that by NMR, the tannins that did not match with the NMR data have a much lower percent PC with THM. As the monomers produced much more PD B ring products (epigallocatechin) than PC B ring products (epicatechin) upon THM, it may be a preferential (epi)catechin monomer as an end member. Another explanation may be a difference in branching, i.e., partial 4−6 connections next to the more common 4−8 linkages.

Pine Condensed Tannins. The five analyzed pine tannins produced a rather similar product distribution upon THM, apart from a relatively high amount of 1,2,3-trimethoxybenzene and 5-methyl-1,2,3-trimethoxybenzene for bishop pine.

The rather uniform distribution of THM products (confirmed by a similarity matrix) suggests that the pines studied have a similar tannin composition. It appeared that balsam fir produces tannins that are chemically more similar to those of the pines (especially jack and bishop pine) than to Douglas fir CT.

The effects of B ring hydroxylation on the type of A ring product described above is particularly recognizable for pine CTs, as they have similar tannin compositions (fraction trans terminal and cis units). For the pines, the A ring product 2-ethyl-1,3,5-trimethoxybenzene is associated with the contribution of 1,2,4-trimethoxybenzene (0.94, $P < 0.02$) (Table 3) but not with the 2-methyl analogue (0.33, $P = 0.59$). However, 1,2,3,5-tetramethoxybenzene correlated well with the 2-methyl- (0.87, $P = 0.05$) and with 2-ethyl-1,3,5-trimethoxybenzene (0.79, $P = 0.11$). These observations confirm that PC units indeed promote the formation of 2-ethyl-1,3,5-trimethoxybenzene upon THM. Moreover, the negative correlation between the nonoxidized (4-methyl-) 1,2-dimethoxybenzenes with the A ring products, and especially those with a (m)ethyl group, supports this mechanism (Table 3).

The difference in origin of the nonoxidized B ring products (methyl) 1,2-dihydroxybenzene and (methyl) 1,2,3-trihydroxybenzene with the seven oxidized B ring products identified for the pine CTs is illustrated by the positive correlations between all nonoxidized compounds (4* $P < 0.10$) and the negative correla-

Table 3. THM Product Correlation Analysis (Pearson's *R*) of Pine Tannins[a,b]

compound	1,3,5-trimethoxybenzene[A]	2-methyl-1,3,5-trimethoxybenzene[A]	2-ethyl-1,3,5-trimethoxybenzene[A]
1,2-dimethoxybenzene[B2]	−0.44 (0.46)	−0.72 (0.17)	−0.79
4-methyl-1,2-dimethoxybenzene[B2]	−0.59 (0.29)	−0.81 (0.10)	−0.79
1,2,4-trimethoxybenzene[B2]	0.57 (0.32)	0.33 (0.59)	0.94 (0.02)
1,2,3-trimethoxybenzene[B3]	−0.62 (0.26)	−0.87 (0.06)	−0.79
5-methyl-1,2,3-trimethoxybenzene[B3]	−0.71 (0.18)	−0.83 (0.08)	−0.74 (0.15)
1,2,3,5-tetramethoxybenzene[B3]	0.21 (0.73)	0.87 (0.05)	0.79

[a] Values in parentheses indicate levels of significance. [b]Codes: (A) compound originating of the A ring; (B2) compound originating of a PC B ring; (B3) compound originating of a PD B ring.

tions between the nonoxidized and the oxidized compounds. Finally, we found a remarkable yet inexplicable correlation between the chain length of the pine CTs with the contribution of 1,3,5-trimethoxybenzene (0.93, $P = 0.02$), which was not the case for the other A ring products. Perhaps the long-chained CTs, which consist of a relatively large amount of C4−C8 linked monomers, and are therefore more subject to cleavage at C4, yielded more A ring products with a (m)ethyl group upon THM. This idea is supported by the relatively large fraction of (m)ethyl A ring products obtained from the epi(gallo)catechin monomers, which obviously did not undergo interflavanoid breaking at C4.

Implications for Organic Matter Studies. In organic matter studies using conventional pyrolysis, the presence of (modified) tannins was usually related to alkylphenols and catechol moieties, but at the same time, these pyrolysis products could have multiple sources such as (modified) lignin, proteins, and polypeptides.[24,39,40] As aforementioned, the use of THM in organic matter studies attributed methoxyaromatics generally to lignin. Our results for purified tannins shows that most lignin-derived aromatics can also have a tannin origin. Using ^{13}C-TMAH, which labels the OH groups with a ^{13}C methyl group, could help to distinguish lignin-derived THM products from those of tannin-derived products in plant material containing both lignin and tannin.[41] However, during decay, lignin demethylates and THM products of such demethylated lignin moieties will then produce the same ^{13}C-labeled products as those of tannin. Only the 1,3,5-(2-alkyl)trimethoxybenzenes and both tetramethoxybenzenes have not been found with lignin, so they can be used as markers for tannins. Although 1,3,5-(2-alkyl)trimethoxybenzenes have also been found with THM of cutan,[31] cutan is suggested to be present in drought-adapted plants only,[32] which are not present in most ecosystems, making these compounds excellent markers of tannins when using THM. Even in the case when only small samples are available, such as in archaeology, THM is able to detect tannins, as already shown by Garnier et al.,[23] as THM needs only (sub)milligrams of sample in contrast to many other analytical techniques.

CONCLUSIONS

THM of purified CTs yielded a suite of mono-, di-, tri-, and tetramethoxyalkylbenzenes and methyl esters of benzoic acid, mono-, di-, and trimethoxybenzoic acids and mono- and dimethoxycinnamic acids. 1,3,5-Trimethoxybenzene and its 2-methyl and 2-ethyl derivatives were always found and serve therefore as good markers for CTs. Both tetramethoxybenzenes and, particularly, the 1,2,3,5 isomer appear to be typical of tannins as well. The hydroxylation pattern of the B ring of most CTs agrees excellently with those as determined by NMR. The large variety of products obtained from the B ring, either a PC or a PD, ranging from a "bare" B ring to oxidized forms, suggests that their formation upon THM is very complex. The differences in product distribution between THM of monomers and that of the purified polymers indicate that the linkages between monomers induces additional fragmentation mechanisms.

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