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Methylation, acetylation and gel permeation of hydrolysable tannins

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ABSTRACT

Different derivatization methods for hydrolysable tannins were compared and assessed for calibration of gel permeation systems. Methylation of ellagitannins such as vescalagin or castalagin with either diazomethane or dimethyl sulphate yields small amounts of the expected permethylated products together with several by-products resulting from the cleavage of some ester bonds. Acetylation with acetic anhydride–pyridine mixture of the same ellagitannins gives a unique product from each compound, which is partially degraded if the excess of reagent is destroyed by addition of water or methanol. In contrast, application of the same methods to β-penta-O-galloyl-D-glucose and gallic acid gives a unique permethylated product or a relatively more stable peracetate. The best calibration graph for gel chromatography on Styragel columns with tetrahydrofuran as eluent was obtained with peracetylated derivatives.

INTRODUCTION

Tannins are usually considered to be water-soluble polyphenols of molecular mass varying between 500 and 3000. They can be classified in two groups: proanthocyanidins (condensed tannins) and hydrolysable tannins. Hydrolysable tannins include galloyl esters (gallotannins) and hexahydroxydiphenoyl esters (ellagitannins) [1]. The molecular mass upper limit value of 3000 is probably largely underestimated as proanthocyanidin polymers with a degree of polymerization as high as 50 ($M_r = 15\,000$) have been described [2]. The largest molecules of hydrolysable tannins which have been characterized are tetramers ($M_r = 4000$) [3,4]. It is unknown if larger polymeric hydrolysable tannins exist naturally in plants.

The structural characterization of such complex molecules often requires derivatization by either methylation or acetylation. Phenolic and aliphatic hydroxyl groups react with methyl donors to form methoxyl groups. The methylated derivatives, much less reactive than the crude tannins, have been used in chemical degradation studies in which ester bonds of

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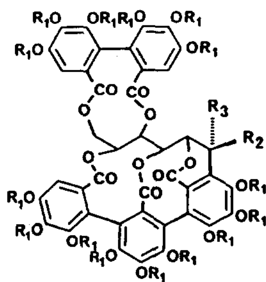
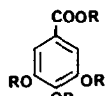


1 (R_1 = H, R_2 = H, R_3 = OH)
2 (R_1 = H, R_2 = OH, R_3 = H)
3 (R_1 = Me, R_2 = H, R_3 = OH)
4 (R_1 = Me, R_2 = H, R_3 = OMe)
5 (R_1 = Ac, R_2 = H, R_3 = OAc)
6 (R_1 = Ac, R_2 = OAc, R_3 = H)



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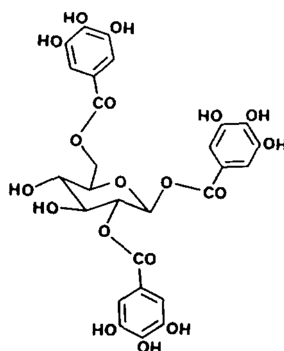

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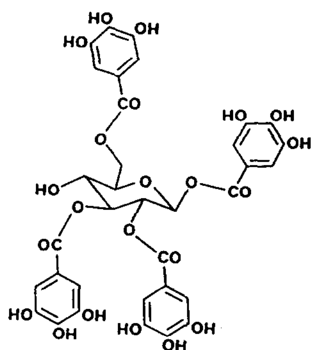

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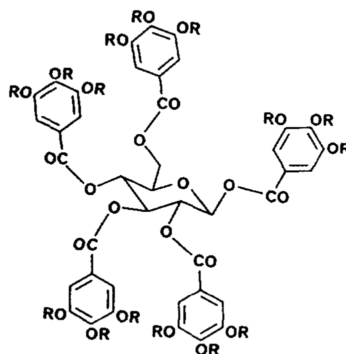

11 (R = H)
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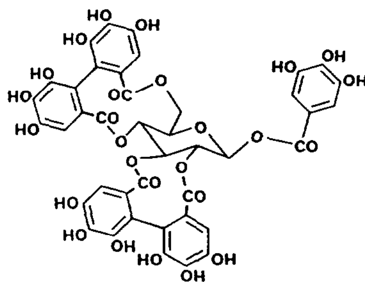

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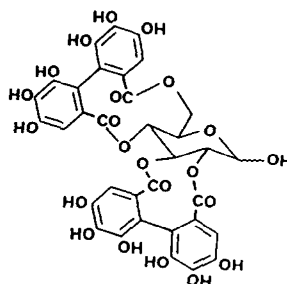

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hydrolysable tannins are cleaved by methanolysis [5].

Methylation (by diazomethane) followed by acetylation can be used to determine the number of phenolic and aliphatic hydroxyl groups in a purified molecule [5,6].

Methylation and acetylation are often used to modify the solubility properties of tannins. Such reactions limit tailing effects in chromatography on silica (purification) [6] or styrene–divinylbenzene copolymers (gel permeation) [7].

Various methods have been proposed for determining the molecular masses of tannins. Fast atom bombardment mass spectrometry is undoubtly the most accurate method for pure compound or simple mixtures of a few compounds [8,9]. Other methods such as ebulliometry [10], ultracentrifugation [11,12], ^{13}C NMR spectroscopy [13], vapour pressure osmometry [14], low-angle laser scattering [14] and chemical methods [15,16] have been used to determine average molecular masses or molecular mass distributions of more complex mixtures of condensed tannins.

Normal-phase high-performance liquid chromatography with a mixture of hexane, methanol (or isopropyl alcohol), tetrahydrofuran (THF) and formic acid as eluent usually gives a positive and linear relationship between molecular mass and retention time for condensed [17] and hydrolysable [3,18,19] tannins. It requires proper tannin standards and can probably be used exclusively for the comparison of closely related molecules. This method has so far been applied to the comparison of purified tannins and to the analysis of simple mixtures of structurally well defined tannins with molecular masses not exceeding 4000. It is unlikely that such a method could be applied to higher molecular mass tannins, particularly those of indefinite polymeric structure. For these reasons, gel permeation is often preferred for studies of molecular mass distributions of condensed [7] and hydrolysable tannins [19,30].

In this paper, particular problems arising with the methylation, acetylation and gel permeation of hydrolysable tannins are reported and partially solved.

EXPERIMENTAL

Materials

The following C-glucosidic ellagitannins were purified from oak heartwood: four monomers [castalagin (**1**), vescalagin (**2**), grandinin and roburin E] and four dimers (roburin A, B, C and D) [21,22]. The structures of C-glucosidic ellagitannins given here have been revised according to refs. 23 and 24. β-1,2,6-Tri-O-galloyl-D-glucose (**9**), β-1,2,3,6-tetra-O-galloyl-D-glucose (**10**), β-penta-O-galloyl-D-glucose (**11**) and galloyl methyl ester were purified from methanolysed tannic acid (Fluka) [25,26]. Tellimagrandin II (**13**) and pedunculagin (**14**) were purified from oak leaves [27–29]. The identities of all the tannins were established by comparison of their ^{1}H and ^{13}C NMR spectra with those in the literature.

Methylation with diazomethane

Diazomethane was generated by reaction of N-methyl-N-nitroso-*p*-toluenesulphonamide (Aldrich) (2 g in 20 ml of diethyl ether) with potassium hydroxide [5 g in 18 ml of 55% (v/v) aqueous methanol] and recovered by distillation. The etheral solution of diazomethane was added to a methanolic solution (1 ml) of pure tannin (2 mmol of diazomethane per 1–5 mg of tannin). After standing overnight, the mixture was directly analysed by HPLC.

Methylation with dimethyl sulphate

Castalagin (56 mg), K_2CO_3 (550 mg) and dimethyl sulphate (0.5 ml) were added to acetone (3 ml) and refluxed for 3.5 h under nitrogen. The mixture was filtered. The reaction products in the filtrate were dried under reduced pressure.

Acetylation

Tannins (1 mg) were acetylated with acetic anhydride–pyridine (1:1) (500 μl) at room temperature overnight. Reagents were removed under reduced pressure after addition of toluene.

Analytical HPLC

Non-derivatized, permethylated and peracetylated tannins were analysed on a 10-μm μPorasil column (300 mm × 3.9 mm I.D.) (Waters). The elution conditions were as follows: linear gradient from 0 to 95% B from 0 to 30 min, solvent A = hexane–MeOH–THF (72:21:7), solvent B = MeOH–THF (75:25) with citric acid (0.25%) added to both solvent mixtures; flow-rate, 1 ml/min; detection, UV at 280 nm.

Purification of methylated tannins by preparative HPLC

Castalagin (50 mg) was methylated with diazomethane as above and fractionated on a 7-μm LiChrosorb Si 60 column (25 cm × 25 mm I.D.) (Merck). Elution was carried out in the isocratic mode with hexane–MeOH–THF (72:21:7) at a flow-rate of 20 ml/min and the eluate was monitored at 280 nm. Compounds eluted with the three main peaks were collected (5.0, 8.4 and 7.5 mg).

Castalagin (56 mg) permethylated with dimethyl sulphate as above was fractionated by HPLC under the same conditions. Two fractions (15.4 and 32.5 mg) were collected.

Gel permeation chromatography

Polystyrenes and acetylated tannins (1–2 mg/ml in THF, injection volume 20 μl) were analysed on two columns of Ultrastyragel, 500 and 1000 Å (300 mm × 7.8 mm I.D.) (Waters), connected in series. THF (Chromasol SDS) was delivered at a flow-rate of 1 ml/min. Detection was carried out at 280 nm.

Mass spectroscopy

Mass spectroscopy was carried out on a Nermag R 10.10C instrument operating in the chemical ionization mode. The reagent gas was ammonia, the source temperature was 80°C and the pressure of the ionization chamber was 10^{-4} Torr (1 Torr = 133.322 Pa).

^{1}H NMR spectroscopy

Spectra were measured on a Bruker AM 400-MHz spectrometer in a quantitative manner (acquisition time, 1.14 s; recycle time, 61 s) with DMSO-d_6 as the solvent. Galloyl methyl ester, δ 7.16 (s, 2H), 3.96 (s, 3H); tetra-O-methyl gallate (**8**), δ 7.46 (s, 3H), 4.06 (s, 3H), 4.05 (s, 6H), 3.95 (s, 3H).

RESULTS

Methylation

When gallic acid (**7**) and a galloyl ester, β-penta-O-galloyl-D-glucose (**11**), are methylated with diazomethane in a diethyl ether–methanol mixture, they give a unique product as seen by HPLC on a silica column (Fig. 1a). These products were identified by mass spectrometry as the tetra-O-methyl gallate (**8**) and pentadeca-O-methylpentagalloylglucose (**12**).

When castalagin (**1**), an ellagitannin, is methylated under the same conditions, it gives several products (Fig. 1b). The chromatographic profile was not changed by repeated methylations. Similar results were obtained with vescalagin (**2**).

In order to check that the multiple products were not due to incomplete methylation (of either phenolic or aliphatic hydroxyl groups), other reagents known to methylate both phenolic and aliphatic hydroxyl groups were tested. Neither the addition of $BF_3 \cdot Et_2O$ nor $HBF_4 \cdot Et_2O$ catalysts to diazomethane or the use of dimethyl

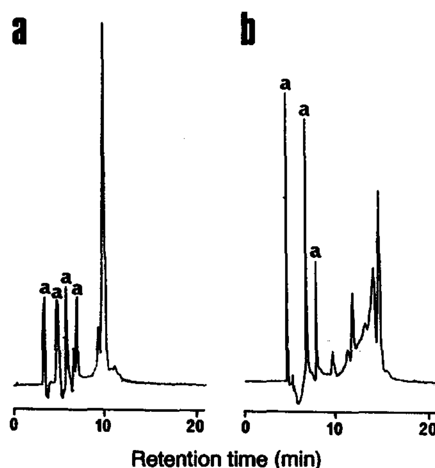

Fig. 1. Normal-phase HPLC of reaction mixtures after diazomethane methylation of hydrolysable tannins. (a) β-Penta-O-galloyl-D-glucose; (b) castalagin. a = Artefacts due to the chromatographic system.

sulphate resulted in the formation of a unique product.

The crude mixture obtained by diazomethane methylation of castalagin was analysed by mass spectroscopy. A complex spectrum was obtained (Fig. 2) which shows the formation of several compounds, most of them having a molecular mass higher than that of the expected pentadeca-O-methyl derivative of castalagin (**3**) (M_r = 1144).

The various products obtained by methylation of castalagin with diazomethane were partially purified by preparative HPLC on silica. Three fractions were obtained. Three products could be detected in the first fraction, with molecular masses of 1144 [m/z 1144 (M^+), 1162 ($(M+NH_3+H)^+$], 1190 [m/z 1190 (M^+), 1208 ($(M+NH_3+H)^+$] and 1208 [m/z 1208 (M^+), 1226 ($(M+NH_3+H)^+$]. The second fraction contained a producet with a molecular mass of 1190 together with one of molecular mass 1176 [m/z 1176 (M^+), 1194 ($(M+NH_3+H)^+$]. The mass spectrum of the third fraction showed only the presence of the compound with a molecular mass of 1176. Analysis by ^1H NMR spectroscopy showed that this compound has 16–17 methoxyl groups with signals varying from 3.52 to 4.14 ppm. Such methoxyl groups might be attached to aromatic or carbonyl carbons [see proton chemical shifts of galloyl methyl ester and tetra-O-methyl gallate (**8**) under Experimental].

Dimethyl sulphate methylation of castalagin also resulted in a complex mixture which was again fractionated by preparative HPLC on silica. Two main fractions were obtained. The first showed major ions corresponding to a compound of molecular mass 1202 [m/z 1202 (M^+), 1220 ($(M+NH_3+H)^+$] and minor ions corresponding to the expected hexadeca-O-methyl derivative (**4**) of castalagin with a molecular mass of 1158 [m/z 1158 (M^+), 1176 ($(M+NH_3+H)^+$]. The second fraction contained two species, a major one of molecular mass 1144 and a minor one of molecular mass 1190 (molecular ions as above).

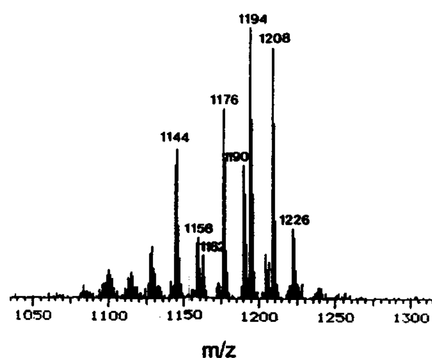

Fig. 2. Mass spectrum of diazomethane-permethylated castalagin.

Acetylation

Acetylation with acetic anhydride in pyridine gave only one product for all the compounds tested, *viz.*, gallic acid (**7**), β-penta-O-galloyl-D-glucose (**11**), castalagin (**1**) or vescalagin (**2**) (Fig. 3). The stability of the resulting acetates varies markedly, however. When the excess of acetic anhydride was destroyed by addition of water or methanol, gallic acid and β-penta-O-galloyl-D-glucose peracetates were not affected; castalagin peracetate (**5**) was partially degraded by the treatment as shown by the several peaks observed by HPLC (Fig. 4a). A decrease in temperature during the degradation of acetic anhydride did not lead to better results.

The best way to remove the reagents was finally found to be direct evaporation under vacuum. Under these conditions only one peak was observed by HPLC of the peracetylated castalagin (Fig. 4b).

Gel permeation

The different tannins and their permethylated and peracetylated derivatives were analysed by gel permeation chromatography on Styragel columns with THF as the eluent. The variations in the retention times of the underivatized tannins, monomers (15.4–15.6 min) and dimers (15.7–15.9 min), were too small to be used for molecular mass determination. Chromatography of dimethyl sulphate permethylated tannins gave a better separation of dimers from monomers (not shown). However, the best results were obtained with peracetylated tannins (Fig. 5): a linear relationship between retention times and the logarithms of molecular masses was obtained for peracetates of castalagin (**5**), vescalagin (**6**) and

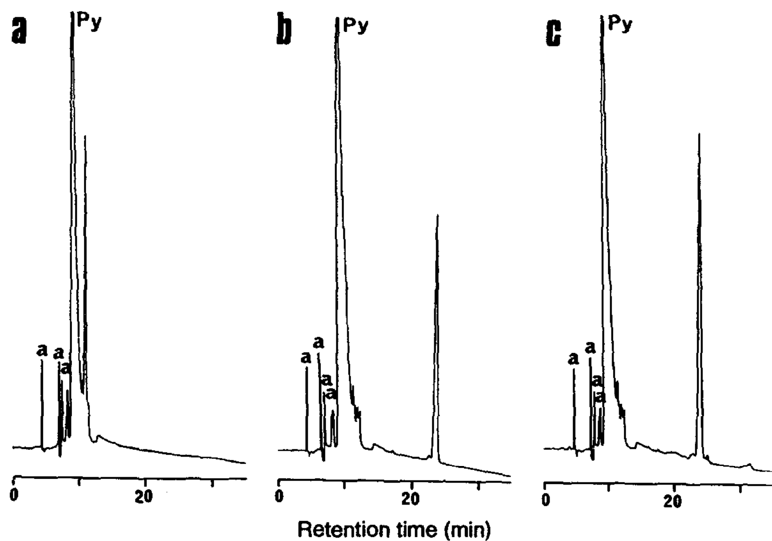

Fig. 3. Normal-phase HPLC of reaction mixtures after acetylation of hydrolysable tannins and derivatives. (a) Gallic acid; (b) β-penta-O-galloyl-D-glucose; (c) castalagin. Py = Pyridine; a = artefacts due to the chromatographic system.

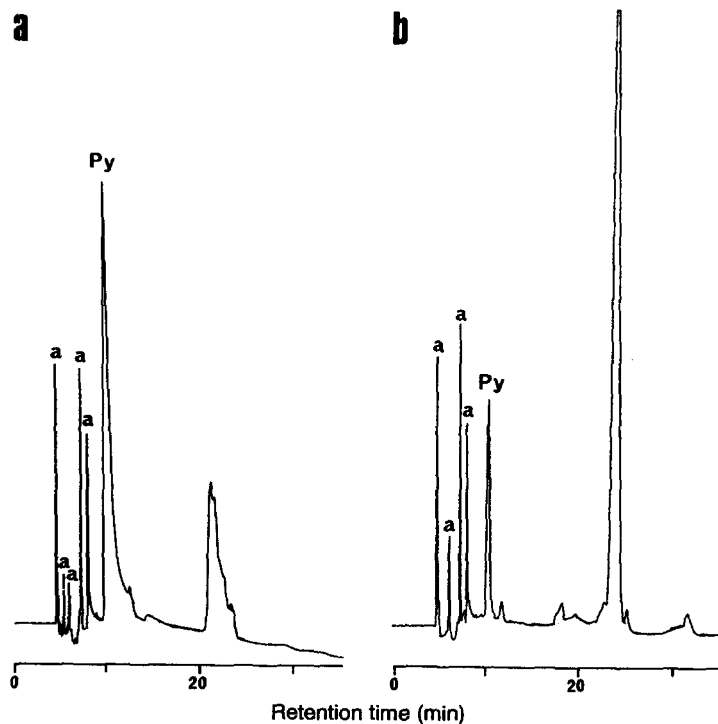

Fig. 4. Normal-phase HPLC of peracetylated castalagin (a) after addition of water to the acetylation reaction mixture and (b) after removal of reagents by evaporation and dissolution in chloroform. Py = Pyridine; a = artefacts due to the chromatographic system.

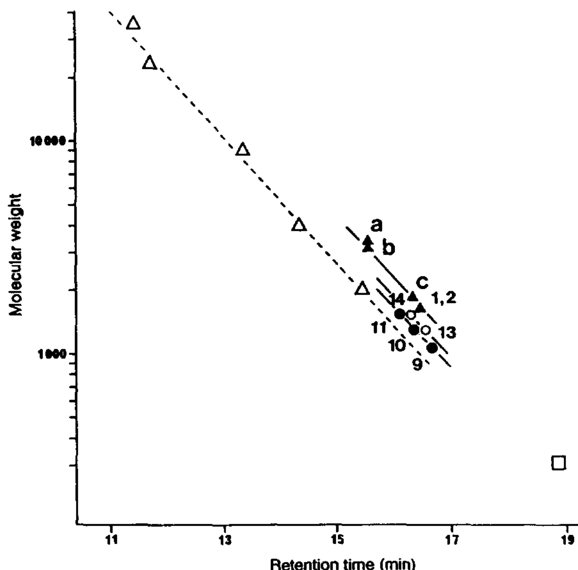

Fig. 5. Calibration graphs for gel permeation of peracetylated hydrolysable tannins on a Styragel column with THF as the eluent. △ = Polystyrenes; □ = galloyl methyl ester peracetate; ● = gallotannin peracetates; ▲ = C-glucosidic tannin peracetates; ○ = other ellagitannin peracetates. Numbers refer to the structures shown in the text; a = roburin B and C; b = roburin A and D; c = roburin E and grandinin.

six of their derivatives, monomers or dimers (all C-glucosidic ellagitannins with a carbon–carbon linkage between the first carbon of the aliphatic chain and one aromatic carbon). The plot is parallel to but not identical with that obtained with polystyrenes.

Gallotannins **9**, **10**, **11** and other ellagitannins [tellimagrandin II (**13**) and pedunculagin (**14**)] gave, for a given molecular mass, retention times intermediate between those of polystyrenes and C-glucosidic ellagitannins.

DISCUSSION

Methylation

Several methods of methylation have been applied previously to hydrolysable tannins. Some of these methods will methylate both phenolic and aliphatic hydroxyl groups (dimethyl sulphate [30], methyl iodide with silver oxide [31], diazomethane with boron trifluoride [30,32]) and others will only methylate phenolic hydroxyl groups (diazomethane) [5] or hemiacetal hydroxyl groups (methyl carbonate) [30]. In general, these methods when applied to ellagitannins give a crude oil composed of several permethylated products.

Permethylation of castalagin (**1**) with diazomethane gave low yields of the pentadeca-O-methyl derivative **3** (M_r = 1144), together with several other products. Most of them differ from the pentadeca-O-methyl derivative by a mass increment of 32 (M_r = 1176, one extra methoxyl group observed in the ^{1}H NMR spectra) or 2 × 32 (M_r = 1208). These products result from methanolysis of one or two ester linkages. Similar side-reactions have been reported previously with diazomethane methylation of ellagitannins [32]. Partial methylation of the aliphatic hydroxyl group is also observed; the resulting hexadeca-O-methyl derivative **4** (M_r = 1158) is eventually degraded by methanolysis of one ester linkage (M_r = 1190).

Methanolysis of ellagitannins has been explained by the presence of water in the diazomethane reaction mixture and avoided by carefully drying the solvents (methanol and diethyl ether) [32]. Here, methylation of castalagin, either as a powder or in methanol solution, with diazomethane in diethyl ether gave the same mixture of products as observed by HPLC.

Permethylation of castalagin with dimethyl sulphate gave small amounts of the hexadeca-O-methyl derivative **4** (M_r = 1158) together with significant amounts of the pentadeca-O-methyl derivative **3** (M_r = 1144), resulting from difficulties met in methylating the aliphatic hydroxyl group. By-products were also formed. The mass 1190 (1158 + 32) can be explained by methanolysis of one ester bond in the hexadeca-O-methyl derivative. Similar by-products have previously been encountered in the permethylation of ellagitannins with dimethyl sulphate [33,34] and the yields were not improved by using dry acetone and anhydrous potassium carbonate [35].

Acetylation

Acetylation of tannins is generally performed with an acetic anhydride–pyridine mixture and the excess of reagent is destroyed by addition of

water. Incomplete acetylation or partial deacetylation has not been previously reported and most workers have not reported any particular problems with the acetylation of flavonoids [35] and proanthocyanidins [7]. With gallic acid (**7**), β-penta-O-galloyl-D-glucose (**11**) or ellagitannins such as castalagin (**1**) or vescalagin (**2**), acetylation is apparently complete as only one product is observed by HPLC. However, HPLC shows the formation of several peaks on addition of water to the ellagitannin reaction mixtures. This can be explained by the relative instability of castalagin and vescalagin peracetates (**5** and **6**) compared with peracetates of gallic acid or β-penta-O-galloyl-D-glucose. A suitable method for the removal of reagents was found to be their direct evaporation under reduced pressure.

Gel permeation

Several gel permeation systems have been used for the determination of molecular mass distributions of tannins. Most workers use divinylbenzene polymer (Shodex K [36]) or styrene–divinylbenzene copolymer columns (TSK-H type [3], HSG-15 [37], Styragel [7]) with THF as the eluent for the analysis of either underivatized [36], permethylated [3] or peracetylated [7] tannins.

When applied to pure underivatized monomeric and dimeric ellagitannins, no significant separation according to molecular mass was observed. The best results were obtained with peracetylated ellagitannins.

The application of such a method to the determination of molecular masses requires proper standards for calibration. The calibration graph established with acetylated hydrolysable tannins differs significantly from that obtained with polystyrenes. The use of polystyrenes as standards will result in a 40% underestimation of the molecular mass of some ellagitannins, particularly those of the C-glucosidic type.

The retention time depends on the structure of hydrolysable tannins. It increases with the degree of oxidation of the tannin molecule (number of biphenyl bonds between aromatic rings), as illustrated with the three following hydrolysable tannins of nearly identical molecular mass; 16.10 min for β-penta-O-galloyl-D-glucose (no biphenyl bond), 16.37 min for tellimagrandin II (one biphenyl bond) and 16.48 min for castalagin (three biphenyl bonds).

Biphenyl linkages decrease the conformational mobility of hydrolysable tannins and reduce their apparent hydrodynamic radii. As a result, for a given molecular mass, gallotannins (with no biphenyl linkage) have relatively short retention times when compared with ellagitannins and flexible linear polystyrenes are particularly ill-suited for calibration of oxidized ellagitannins.

The range of calibration with ellagitannins has been limited to dimers (this work) or to tetramers. No larger molecule has so far been purified and characterized [38]. Determinations of molecular masses higher than those of tetramers by gel chromatography relies on extrapolation of the calibration graph and should thus be interpreted with care [39].

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