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Analysis of hydrolysable tannins

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Abstract

This review deals with the main issues relating to the analysis of hydrolysable tannins (HTs). It covers briefly their distribution in the plant kingdom and describes their main structural features. HTs have often been ignored because they are apparently more difficult to analyse than condensed tannins. Meaningful analytical data are critically dependent on appropriate sample preparation, storage and extraction techniques. This requires some understanding of the reactivities of hydrolysable tannins. Mixtures of HTs have been measured by general tannin assays, such as precipitation with metals or proteins, and by colorimetric assays for total phenols. Some HTs can also be measured by more specific colorimetric tests. Although colorimetric assays are widely used for tannin analyses, they generally do not provide accurate quantitative data. At best, they provide data for comparative purposes. Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) are useful tools to screen samples for the different types of tannins, hydrolysable or condensed tannins. In addition, there are physicochemical techniques, such as nuclear magnetic resonance (NMR) and mass spectrometry (MS), which are used to identify pure tannin compounds. More recently, other MS techniques have been developed that are capable of yielding molecular weights of crude tannin mixtures.

Keywords: Extraction; Structures; Colorimetry; TLC; HPLC; NMR; MS

1. Occurrence and structures of hydrolysable tannins (HTs)

Table 1 illustrates that hydrolysable tannins are synthesized by a wide variety of plants and trees (Haslam, 1981; Okuda et al., 1990; Kumar and Vaithiyanathan, 1990) and several of these have been used as animal feeds (Le Houerou, 1980). They can occur in wood, bark, leaves, fruits and galls. It is worth noting that some species produce either gallotannins or ellagitannins, whilst others produce complex mixtures containing

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Table 1
Occurrence of hydrolysable tannins in plants and trees

Family	Species	Types of tannins
Anacardiaceae	*Rhus* sp.	Gallotannins, ellagitannins
Leguminosae	*Caesalpinia* sp.	Gallotannins, ellagitannins
	Acacia sp.	Gallo-, catechin gallates
Fagaceae	*Quercus* sp.	Gallotannins, ellagitannins
	Castanea sp.	Ellagitannins
Combretaceae	*Terminalia* sp.	Ellagitannins
Myrtaceae	*Eucalyptus* sp.	Ellagitannins

gallo-, ellagi- and condensed tannins. For example *Acacia, Acer* and *Fagaceae* are well known for having both condensed and hydrolysable tannins (Bate-Smith, 1977; Mueller-Harvey et al., 1987; Ishimaru et al., 1987).

Fig. 1 shows the biosynthetic relationships between different HTs compounds. The central compound, pentagalloylglucose, is the starting point for many complex tannin

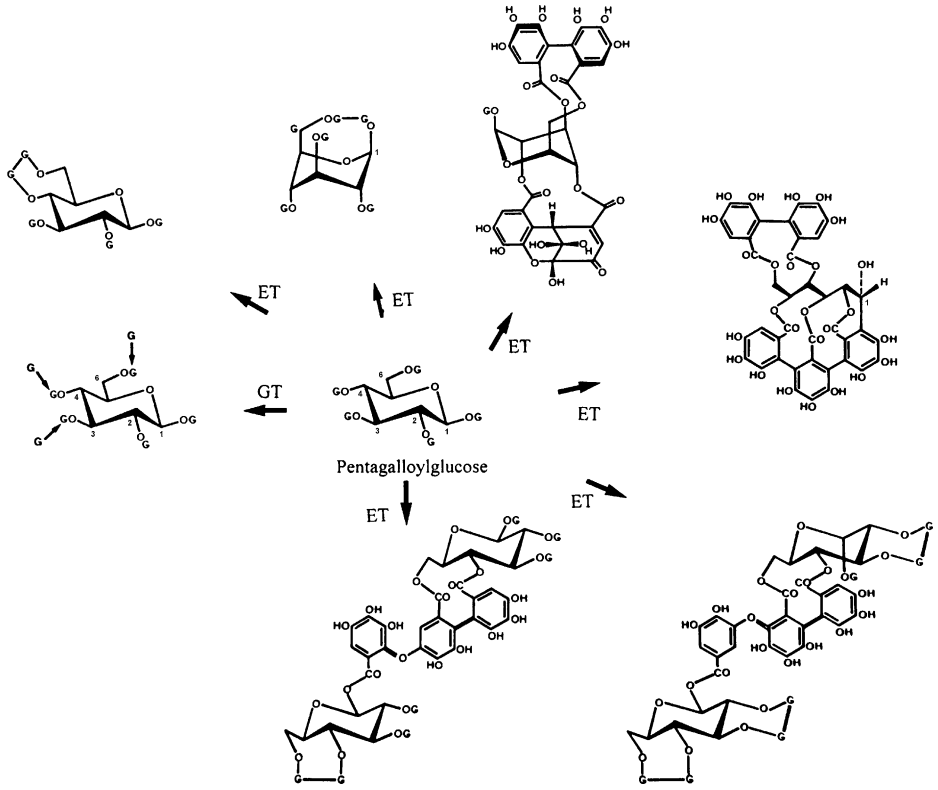

Fig. 1. Metabolism of gallic acid (G) in hydrolysable tannins (after Self et al., 1986): pentagalloylglucose is the precursor of gallotannins (GT) and ellagitannins (ET).

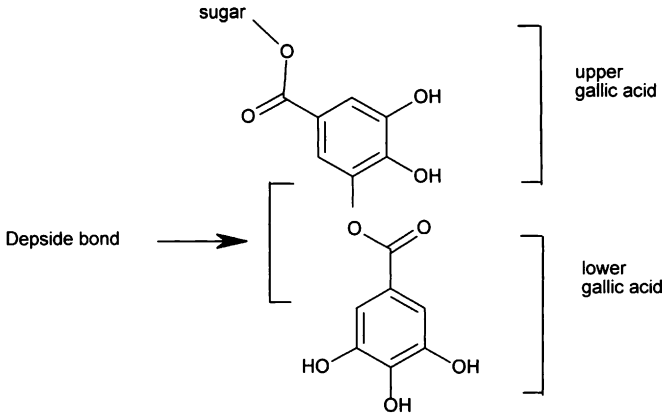

Fig. 2. Example of a depside bond which is formed between the phenolic group of the upper and the acid group of the lower gallic acid units.

structures. This compound belongs to the so-called gallotannins. These consist of a central polyol, such as glucose, which is surrounded by several gallic acid units. Further gallic acid units can be attached through a depside bond (Fig. 2). Simple gallotannins are relatively rare in nature but tend to be the main constituents of commercially available tannic acids, e.g. Chinese tannins from *Rhus semialata* (Hagerman et al., 1997). Gallotannins occur in *Acer*, *Quercus*, *Rhus* and *Pelargonium* species (Haslam, 1986; Self et al., 1986). More complex tannins, such as the ellagitannins, are also derived biosynthetically from pentagalloylglucose by oxidative reactions between the gallic acid units.

A very large number of HTs molecules exist in nature. The structural variation amongst these compounds is caused by oxidative coupling of neighbouring gallic acid units or by oxidation of aromatic rings (Nonaka, 1989; Okuda et al., 1990). Figs. 3 and 4 give examples of these two types of reactions. Such oxidative coupling reactions can produce large and complex HTs. Fig. 5 shows a dimeric HTs formed by the oxidative coupling between two gallic acids, each of which is attached to a different glucose core. It is worth stressing at this point that most colorimetric HTs assays only detect the galloyl or hexahydroxydiphenoyl (HHDP) groups, but not the more complex reaction products depicted in Figs. 3 and 4. As a result, many HTs compounds can escape quantification in colorimetric tests designed for HTs!

2. Extraction of HTs

There has been much debate about whether fresh, freeze-dried or air-dried samples should be analysed. The general recommendations are that fresh samples are the preferred option for extracting tannins. However, this is often not practical or feasible. The next best option is to transport the samples cold (e.g. on ice or carbon dioxide) before freeze-drying. However, if samples are to be air-dried, it is recommended that they be dried in the shade. Okuda et al. (1989) demonstrated that some phenolic compounds decompose quite rapidly in direct sunlight or if dried at elevated temperatures. Oven drying at low

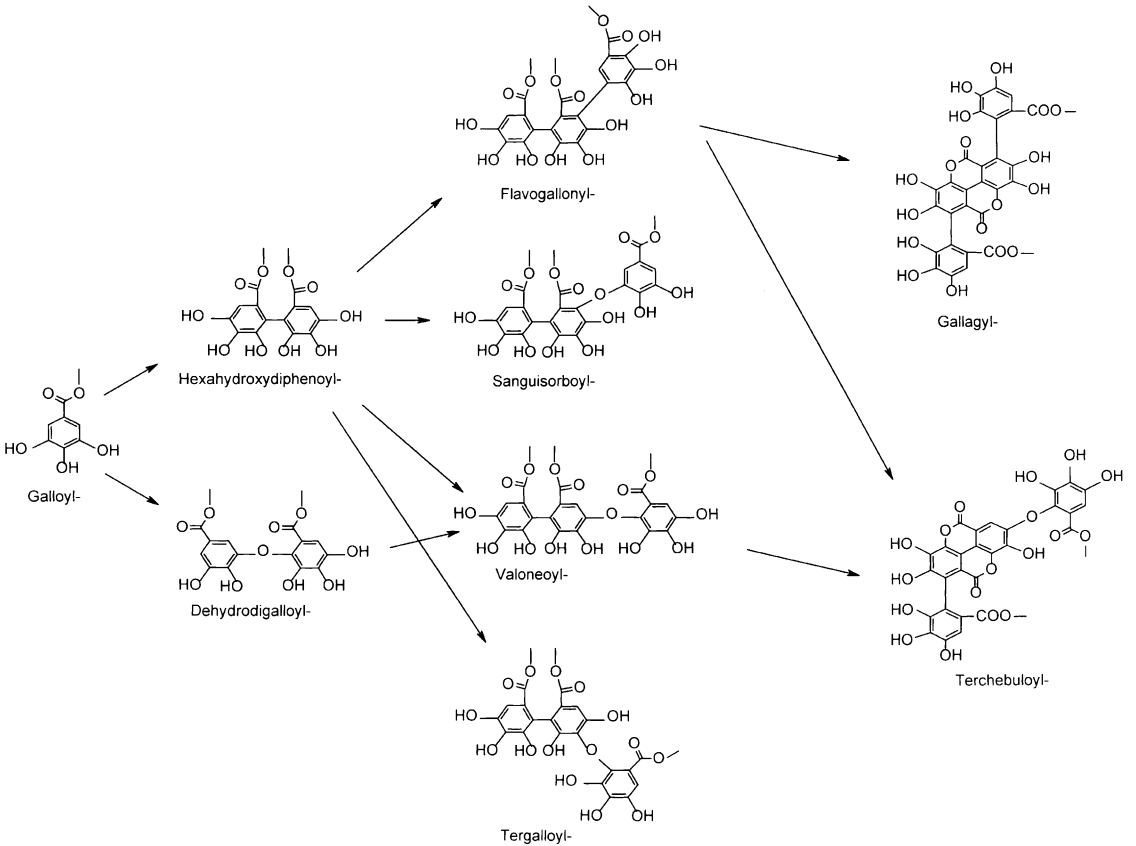

Fig. 3. Oxidative coupling reactions between gallic acid units can lead to larger phenolic residues in hydrolysable tannins (after Nonaka, 1989).

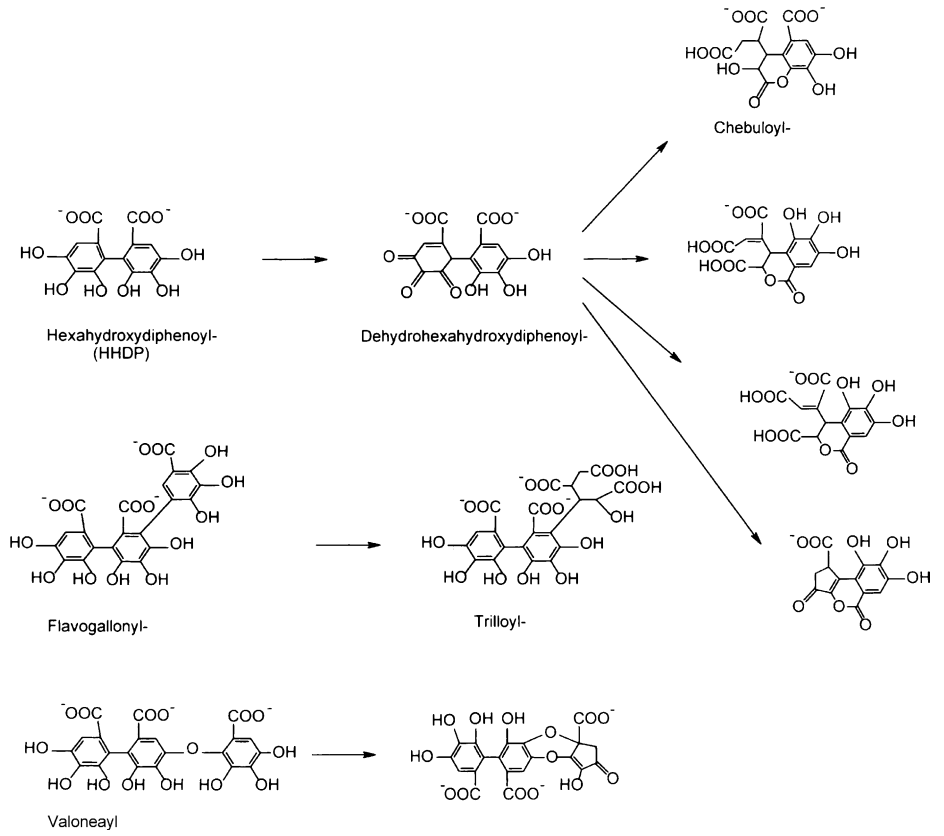

Fig. 4. Oxidation reactions of aromatic rings in ellagitannins and other hydrolysable tannins (after Nonaka, 1989).

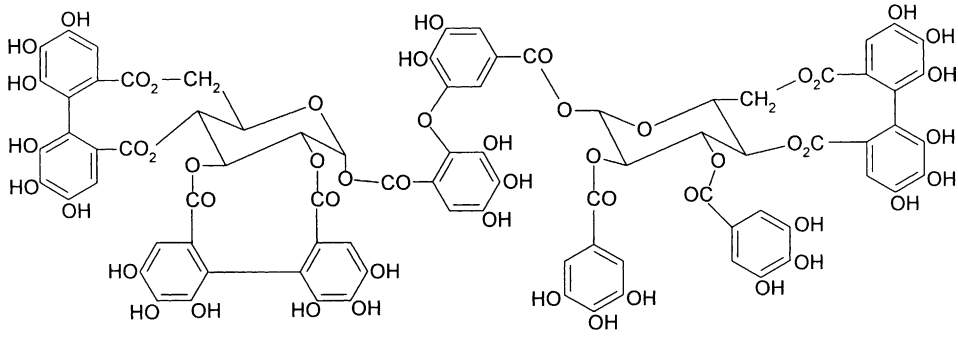

Fig. 5. Example of a so-called dimeric hydrolysable tannin as it contains two sugar units, each of which is surrounded by gallic acid or its metabolites (after Yoshida et al., 1985).

temperatures (<40°C) in a fan-assisted oven may be the only feasible option in humid environments. Clearly, it is not possible to recommend a single optimal procedure for all types of samples or tannins (Hagerman, 1988; Hagerman et al., 1997). Extractability of tannins can also depend on the seasonal maturity of tissues and on the type of plants or tannins. Torti et al. (1995) found that a Polytron homogeniser achieved higher extraction efficiencies and lower coefficients of variation than an ultrasonic bath. Okuda et al. (1989) commented that isolated HTs tend to be fairly stable in air at room temperature (RT), whereas condensed tannins tend to oxidise under the same conditions.

Several solvents have been used to extract HTs. Hexane or dichloromethane can be used initially to remove lipids and chlorophyll. All extractions of HTs should be done at or below RT despite the fact that higher temperatures have often been used (Okuda et al., 1990). HTs have been extracted with water (RT or 90°C), 50% methanol (RT or boiling) and 50–70% aqueous acetone (4°C or RT) (Hagerman, 1988; Waterman and Mole, 1994). Methanol tends to be the better solvent for tannins of low molecular weight or if tissues contain large amounts of enzymes (i.e. bark or fruits). However, acetone is often the preferred solvent as it is less liable to react with HTs than water or methanol (see below). The solubility of HTs is surprisingly variable; for example, castalagin and vescalagin are highly water-soluble, whereas pentagalloylglucose is only sparingly water-soluble but highly soluble in ethylacetate (Haslam, 1996). On a cautionary note: ethylacetate is often used to remove non-tannin components from crude tannin extracts and may thus cause significant losses of some HTs.

3. Stability and reactions of HTs

HTs are more likely to react with the extracting solvent than condensed tannins. For example, methanol cleaves the depside bonds in gallotannins (Fig. 6) at neutral pH and RT (Tedder et al., 1972; Porter, 1989), but acidified methanol (pH < 3) will not cleave these bonds (Haslam et al., 1961). Large and complex tannins are easily degraded into smaller tannins by water or dilute acids especially at elevated temperatures in just 30 min (Beasley et al., 1977; Okuda et al., 1989, 1990). Water at 60°C is likely to liberate gallic

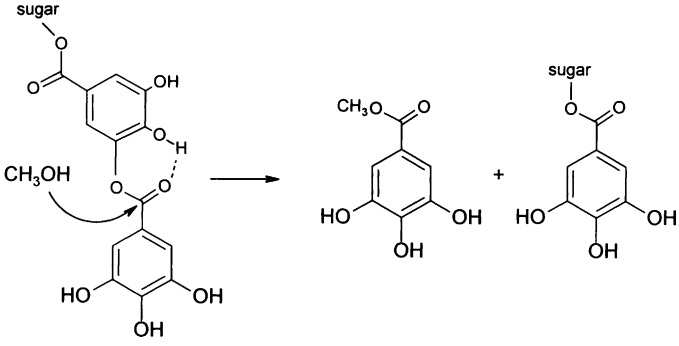

Fig. 6. The dissolution by methanol, or methanolysis, of a depside bond.

acid from the anomeric C-1 position of glucose (Tedder et al., 1972). Water at 100°C may also release ellagic acid from ellagitannins (Nishimura et al., 1986) and cleave the ether bond (see Fig. 3) in the valoneoyl group (Okuda et al., 1990). Extraction of *A. nilotica* with hot ethanol produced ethylgallate as an artifact from catechin gallates (Lowry et al., 1996). Although some of the reactions happen under fairly mild conditions, not all bonds in HTs are readily degraded. It may take up to 26 h to hydrolyse gallic or ellagic acids from some HTs with dilute acids (Porter, 1989; Wilson and Hagerman, 1990). Some researchers employed the tannase enzyme to remove gallic or ellagic acid from HTs (Yoshida et al., 1989a,b; Okuda et al., 1989). This enzyme is commercially available from ICN Biomedicals, (Basingstoke, UK), Juelich Enzyme Products GmbH, (Heidelberg, Germany) or Wako BioProducts, (Neuss, Germany).

Following extraction, tannins are frequently subjected to further purification in order to serve as a 'crude' tannin standard in subsequent colorimetric assays. Sephadex LH20, Toyopearl HW-40 or Diaion HP-20 (Okuda et al., 1989) tend to work well with most HTs. However, some labile oligomeric HTs can be degraded during purification on Sephadex LH20 (Yoshida et al., 1989a,b; Okuda et al., 1990). It is well known, yet rarely mentioned, that Sephadex LH20 tends to absorb large molecular weight CTs, and possibly some large HTs, so strongly that they cannot be eluted subsequently (Okuda et al., 1989).

4. Assays for the determination of total phenols

There are several methods to measure the contents of total phenols in plant materials (Hagerman and Butler, 1989). Most of these assays suffer a major defect despite the fact that they are very widely used: every phenolic compound produces a different colour yield per unit mass in the colorimetric assays. As the quantitative composition of an unknown mixture of phenolics is by definition not known, it is clearly impossible to use a single 'standard phenol' on which to base such assays. Therefore, colorimetric tests should only be used for semi-quantitative comparisons.

The Folin–Ciocalteau (1927) assay is a modification of the Folin–Denis reagent as it produces less interfering precipitates which can be problematic if samples are high in K^+-ions (Waterman and Mole, 1994). The Folin–Ciocalteau reagent also has the advantage of being commercially available (Sigma Chemicals, Poole, UK). The Folin–Ciocalteau assay and the Price and Butler (1977) assay, as modified by Graham (1992), are the recommended colorimetric tests for total phenolics (Waterman and Mole, 1994).

An alternative procedure was developed by Reed et al. (1985) in which phenolic compounds are precipitated by ytterbium acetate. The fact that the phenolics are weighed is a distinct advantage of this method and it does not require a 'standard' phenolic compound. In our laboratory, this procedure completely precipitated all phenolics if they accounted for more than 15% of the DM. At lower phenolic concentrations, precipitation was incomplete. This assay, therefore, shows promise for the determination of total phenolics but would benefit from further improvement. Indeed, the precipitation conditions have been slightly modified in a more recent publication by this group (Krueger et al., 2000).

5. Colorimetric assays for HTs

5.1. KIO_3-reagent for gallo- and ellagitannins

Bate-Smith (1977) described a procedure for measuring gallo- and ellagitannins in *Acer* species, which yield a pink reaction product with the KIO_3-reagent. Hagerman et al. (1997) noted, however, that this assay was not suitable for complex mixtures of tannins as they tended to give brown rather than pink products. Furthermore, this is not a robust assay as development of the colour is critically dependent on the temperature and duration of the reaction. Hagerman et al. (1997) suggests that pentagalloylglucose be used as the standard tannin (see Fig. 1) Hagerman (2001) describes a method for isolating it from commercial tannic acid. This site is highly recommended for this purpose. More recently, Willis and Allen (1998) re-examined this assay in detail and suggested an improved protocol for the determination of gallo- and ellagitannins.

5.2. Rhodanine reagent for gallotannins

Gallotannins can be detected quite specifically by the rhodanine test that was developed by Inoue and Hagerman (1988). The success of this assay depends critically on the absence of oxygen and is performed either under nitrogen or in a vacuum in a sealed test tube at RT. Rhodanine reacts only with gallic acid and not with galloyl esters, ellagic acid, ellagitannins or other phenolics (Porter, 1989). Gallotannins are measured by determining the quantity of gallic acid before and after hydrolysis of the tannins. It is important to recognise that this assay cannot provide an absolute quantification of gallotannins as the number of gallic acid units differs between the various gallotannin molecules that occur in nature (Inoue and Hagerman, 1988). A further complication arises from the fact that many so-called ellagitannins also contain gallic acid, i.e. there is no clear distinction between gallo- and ellagitannins.

5.3. $NaNO_2$-reagent for ellagitannins

Wilson and Hagerman (1990) reported an improved $NaNO_2$ assay that was first described by Bate-Smith (1972) for measuring ellagitannins. The new assay is selective for ellagic acid only (Fig. 7); gallic acid, gallotannins, ellagitannins, condensed tannins, flavonoids do not interfere. It is advisable to analyse extracts and whole tissues, as ellagitannins are often very insoluble. Unfortunately, this assay is also sensitive to oxygen and therefore needs to be carried out under nitrogen. Furthermore, the authors recommend that new glass tubes be used as residues from glass washing can inhibit the reaction. Clearly, this test would benefit from further development.

It is suggested, therefore, that HPLC analysis of gallic or ellagic acid might be more convenient as these can be released by tannase or dilute acids from gallo- and ellagitannins. None of the phenolic constituents of HTs depicted in Figs. 3 and 4 having molecular weights larger than ellagic acid will react with the above reagents. This means that not all HTs can be detected by these colorimetric assays.

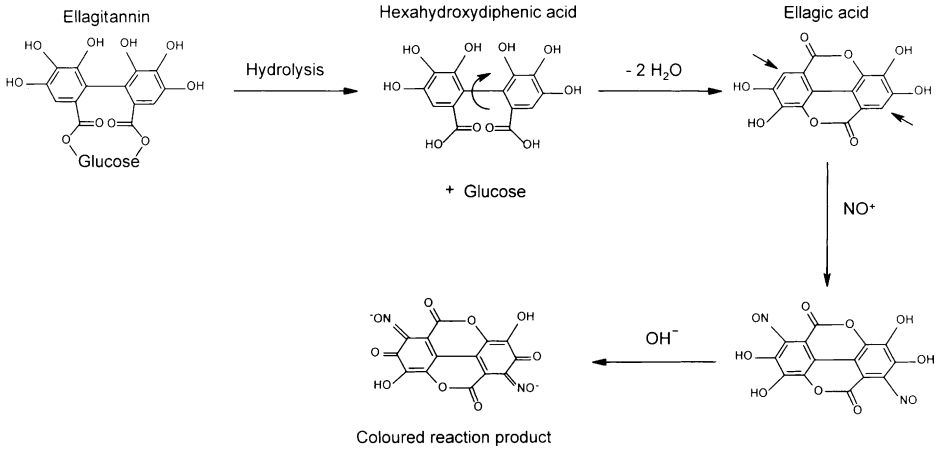

Fig. 7. The formation of a coloured reaction product derived from ellagitannins by the $NaNO_2$-reagent (after Wilson and Hagerman, 1990).

6. Surveys for HTs and condensed tannins (CTs) by thin layer chromatography (TLC)

TLC is a relatively cheap but powerful technique to screen plant extracts for the presence of different types of tannins. Extracts are spotted onto layers of cellulose or silica gel that are attached to glass plates or plastic sheets. The bottom of the plate is then placed into a solvent. As the solvent migrates up the plate, the various phenolic compounds are separated. When the solvent has reached the top, the plate is removed and the solvent is dried off. The plate can then be turned by 90° and placed into a second solvent in order to achieve separation of HTs and CTs. This is a brief description of two-dimensional TLC. Typical solvents are butan-2-ol/acetic acid/water (14:1:5, upper phase, v/v), *t*-butanol/acetic acid/water (3:1:1, v/v) or butan-1-ol/acetic acid/water (60:15:25, v/v) for the first direction and acetic acid/water (2:98 or 6:94, v/v) for the second direction (Porter, 1989; Mueller-Harvey et al., 1987). The relative positions, or R_f-values, of the phenolic spots can be used as a first indication of the presence of HTs or CTs (Fig. 8). Under UV-light, galloyl esters and gallotannins appear as violet fluorescent spots that are enhanced on fumigation with ammonia vapour. Ellagic acid produces a violet spot that darkens on exposure to ammonia vapour.

Plates can then be sprayed with various reagents to detect the tannin compounds (Haslam, 1965; Gupta et al., 1982; Haddock et al., 1982; Yazaki and Okuda, 1990). The $FeCl_3/K_3Fe(CN)_6$-spray reveals all phenolic compounds, including tannins, as blue spots (note: all tannins are phenolics, but not all phenolic compounds are tannins!). The blue background of the cellulose plates can be minimised by soaking the plates immediately after applying the Fe-sprays first in dilute HCl (0.2–2 M) and then in water. The vanillin/HCl spray gives red/pink spots with flavan-3-ols and CTs. The KIO_3-spray gives orange pink or brown spots with gallic acid and gallotannins and, lastly, the $NaNO_2$ also produces orange–brown spots with ellagic acid and ellagitannins. It would appear that

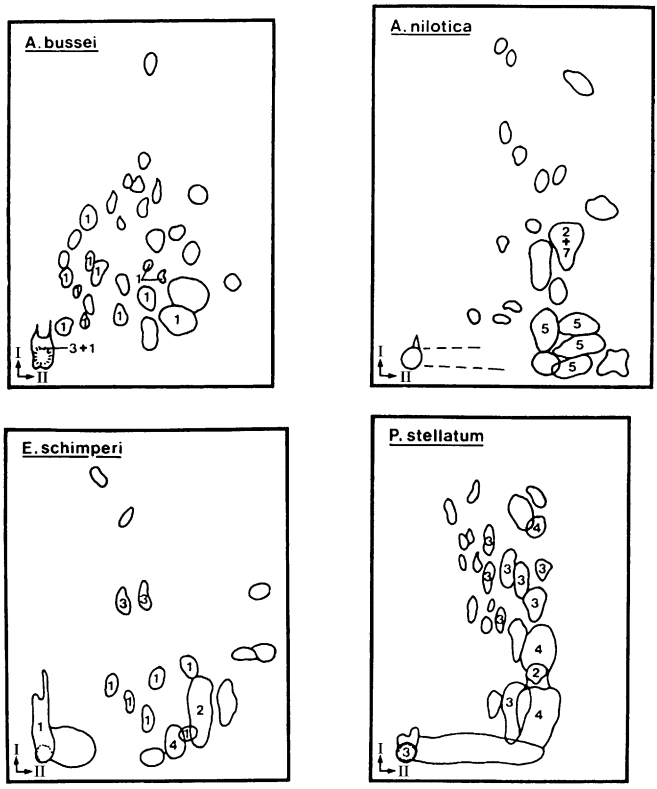

Fig. 8. TLC of condensed and hydrolysable tannins extracted from *Acacia bussei*, *A. nilotica*, *Euclea schimperi* and *Pterolobium stellatum* (Mueller-Harvey et al., 1987). 1: condensed tannins, 2: gallic acid, 3: gallic acid esters, 4: ellagic acid esters, 5: catechin gallates, 7: catechin.

high molecular weight tannins tend to absorb strongly to the origin of cellulose plates or appear as a broad band near the origin (unpublished).

Finally, it is also possible to separate and isolate tannins from TLC plates by using slightly thicker layers of cellulose (0.5–1 mm), so-called preparative TLC. If such layers have been impregnated with a fluorescent indicator (excitation at 254 nm), tannins will appear as dark spots under a short wavelength UV lamp. Such plates are particularly useful for preparative work because they do not require destructive visualisation sprays. The dark areas are simply scraped off and tannins are eluted with appropriate solvents.

7. Surveys for HTs and CTs by high performance liquid chromatography (HPLC)

Mixtures of tannins can be separated on two types of HPLC columns. In normal phase HPLC, the stainless steel columns are tightly packed with polar silica particles (<5 μm).

In reverse phase HPLC, the silica particles are coated with non-polar alkane chains, e.g. C_8 or C_{18} chains. As a first approximation in reverse phase HPLC, CTs often appear as poorly resolved 'humps' eluting between 10 and 20 min, whereas mixtures of HTs appeared in later eluting 'humps' under the conditions employed by Mueller-Harvey et al. (1987) (see Fig. 9). Several workers (Beasley et al., 1977; Tanaka et al., 1984; Okuda et al., 1989; Hagerman et al., 1992) found that normal phase HPLC was a useful technique for separating HTs by their molecular weights (Fig. 10a and b). Interestingly, Tanaka et al. (1984) showed that if HTs are chromatographed on reverse phase columns, the chromatograms were much more complicated (Fig. 10c). This was attributed to anomeric isomers and acetal formation in the alcoholic HPLC eluants (Okuda et al., 1989). It is also worth noting that a commercially available gallotannin had much lower molecular weights than the naturally occurring HTs in fireweed flowers (Fig. 10b; Hagerman et al., 1992).

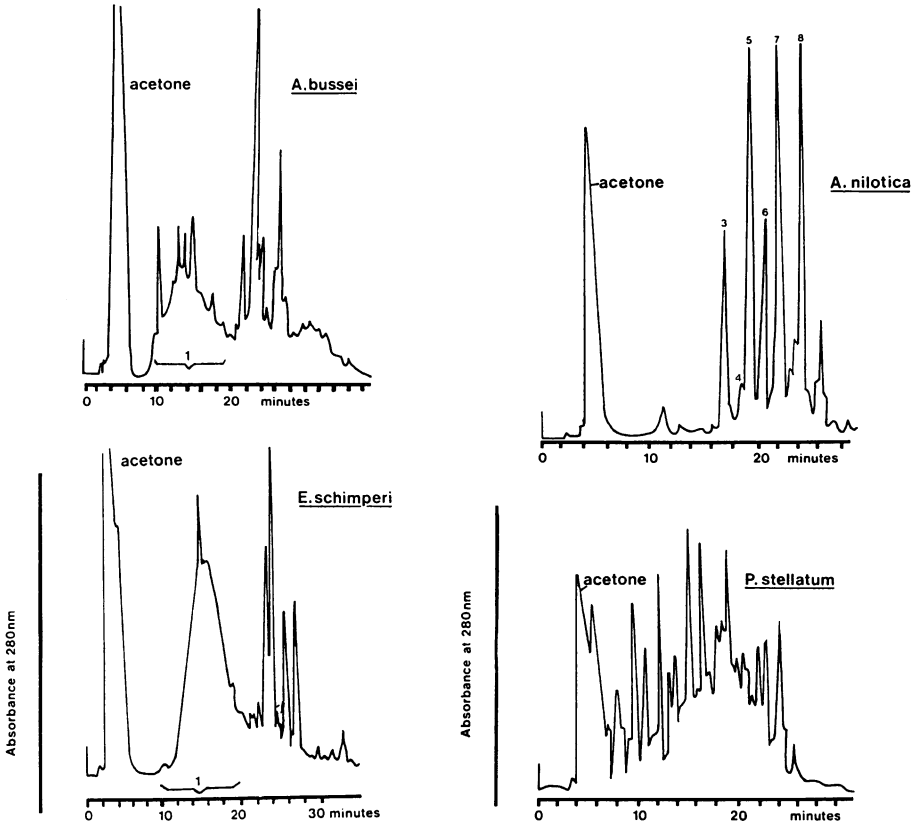

Fig. 9. Reverse phase HPLC of condensed and hydrolysable tannins extracted from *Acacia bussei*, *A. nilotica*, *Euclea schimperi* and *Pterolobium stellatum* (Mueller-Harvey et al., 1987). 1: gallic acid, 2: catechin, 3–7: catechin gallates, 8: a catechin gallate and quercitrin.

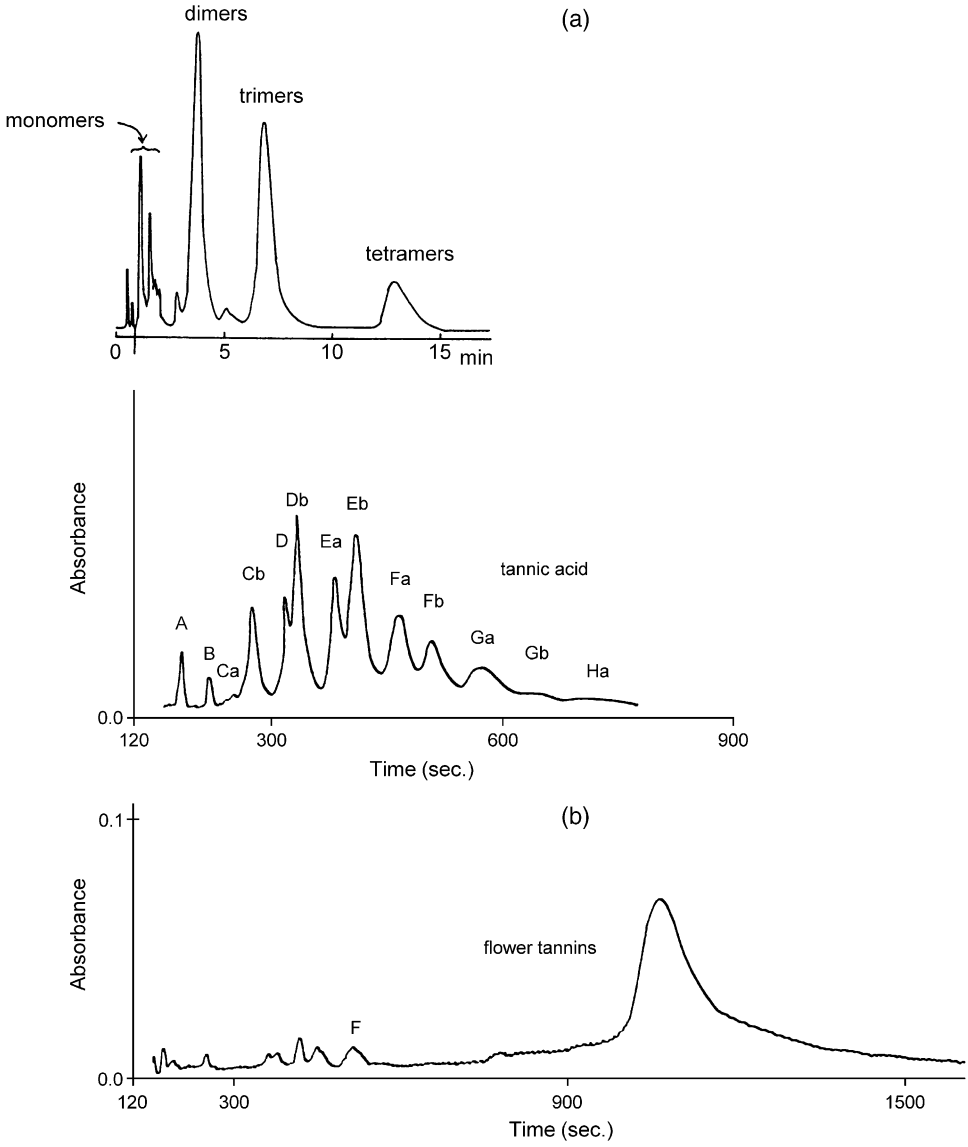

Fig. 10. Normal phase HPLC of hydrolysable tannins from (a) *Heterocentron roseum* (after Okuda et al., 1989), (b) top: commercial tannic acid (Mallinkrodt), bottom: fireweed flower tannins (after Hagerman et al., 1992) and (c) penta- to undecagalloylglucoses from leaves of *Mangifera indica* (after Tanaka et al., 1984).

8. Nuclear magnetic resonance (NMR) for identification of tannins

NMR and mass spectrometry is the main technique for identifying individual compounds and requires ca. 1–5 mg of pure tannin compounds. The compound is then dissolved in 1–2 ml of a deuterated solvent such as water, methanol, acetone or dimethyl

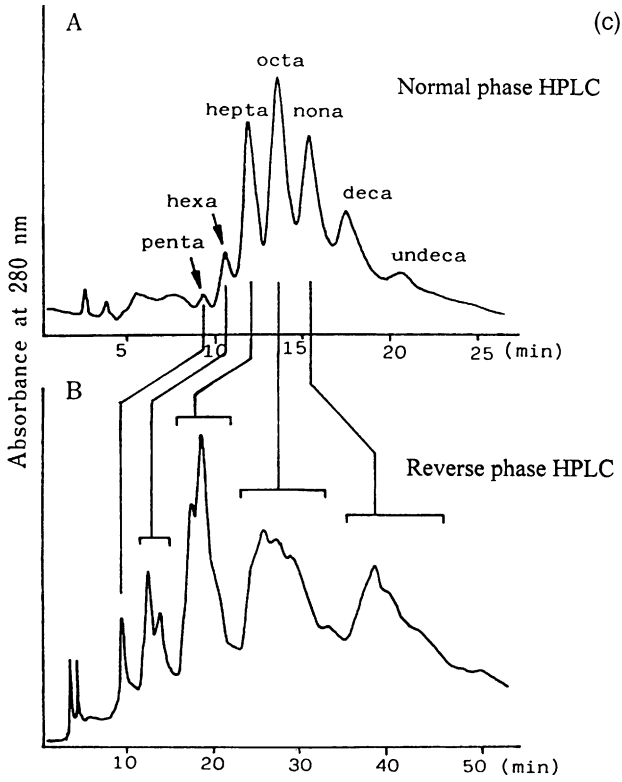

Fig. 10. (*Continued*).

sulfoxide before the NMR spectrum is recorded. The absorption of protons (^{1}H NMR) or carbons (^{13}C NMR) yields chemical shifts which provide detailed information about the chemical environment of each individual atom. This allows the recognition of chemical groups such as esters, alcohols, double bonds, etc. Fig. 11 shows an example of a complex tannin (1), which is composed of two subunits, a hydrolysable (2) and a condensed tannin (3). The ^{13}C NMR spectrum of (3) is almost identical with the combined NMR spectra of (2) and (3) (Ishimaru et al., 1988). Such detailed chemical studies have provided insights into the complexity of tannin chemistry (Ferreira and Bekker, 1996; Okuda et al., 1989) and it is, therefore, not surprising that simple colorimetric tests are unable to differentiate between the tannins from different plants or to quantify 'tannins'.

9. Mass spectrometry (MS) of HTs and CTs

Several different types of MS techniques exist and significant progress has been achieved in the past 10–15 years in this field (Okuda et al., 1989). Modern MS techniques can be applied to microgram quantities of labile, polar and large molecular weight compounds without prior derivatisation. Briefly, the compounds of interest are ionised

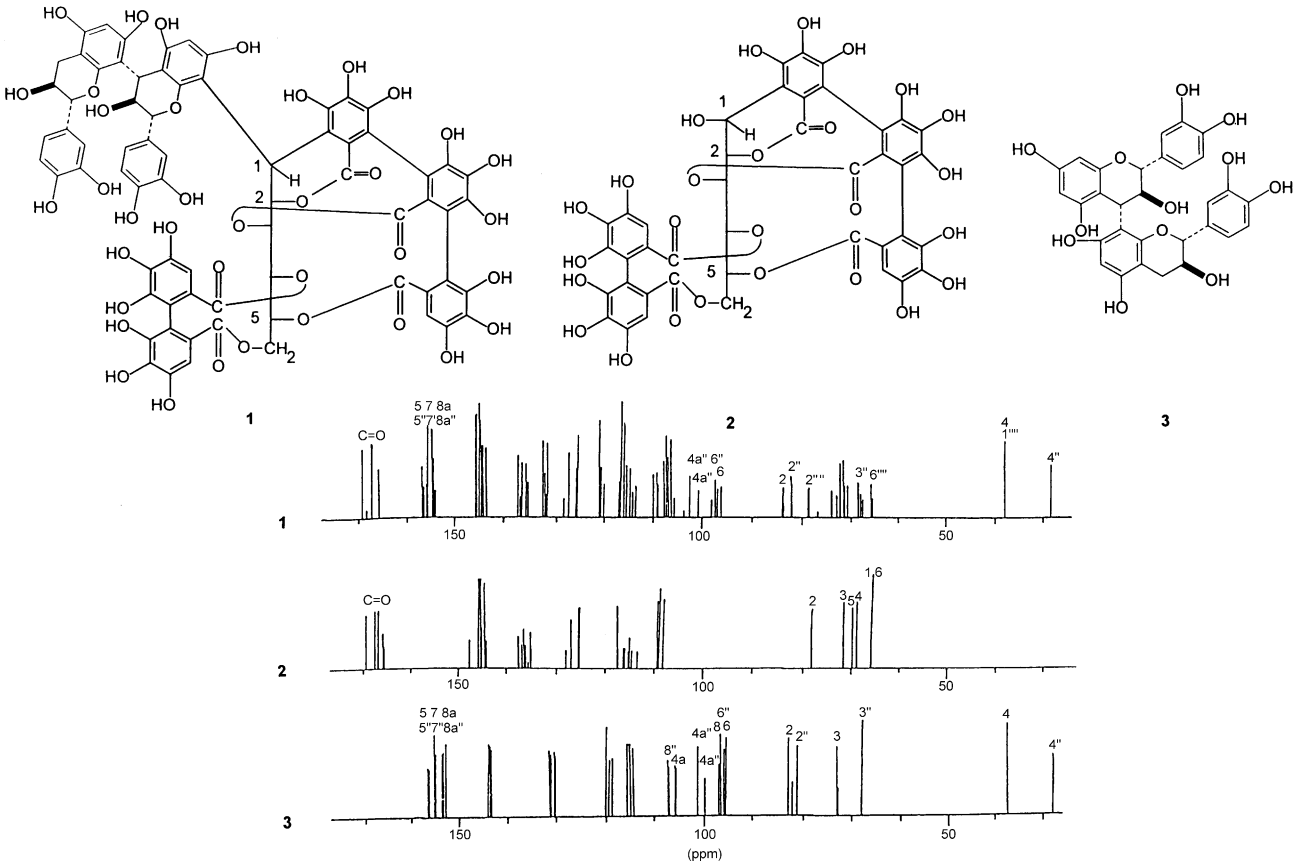

Fig. 11. ^{13}Carbon NMR spectroscopy of (1) monolicanin, (2) vescalagin and (3) procyanidin B-3 tannins which were isolated from *Quercus mongolica* (after Ishimaru et al., 1988).

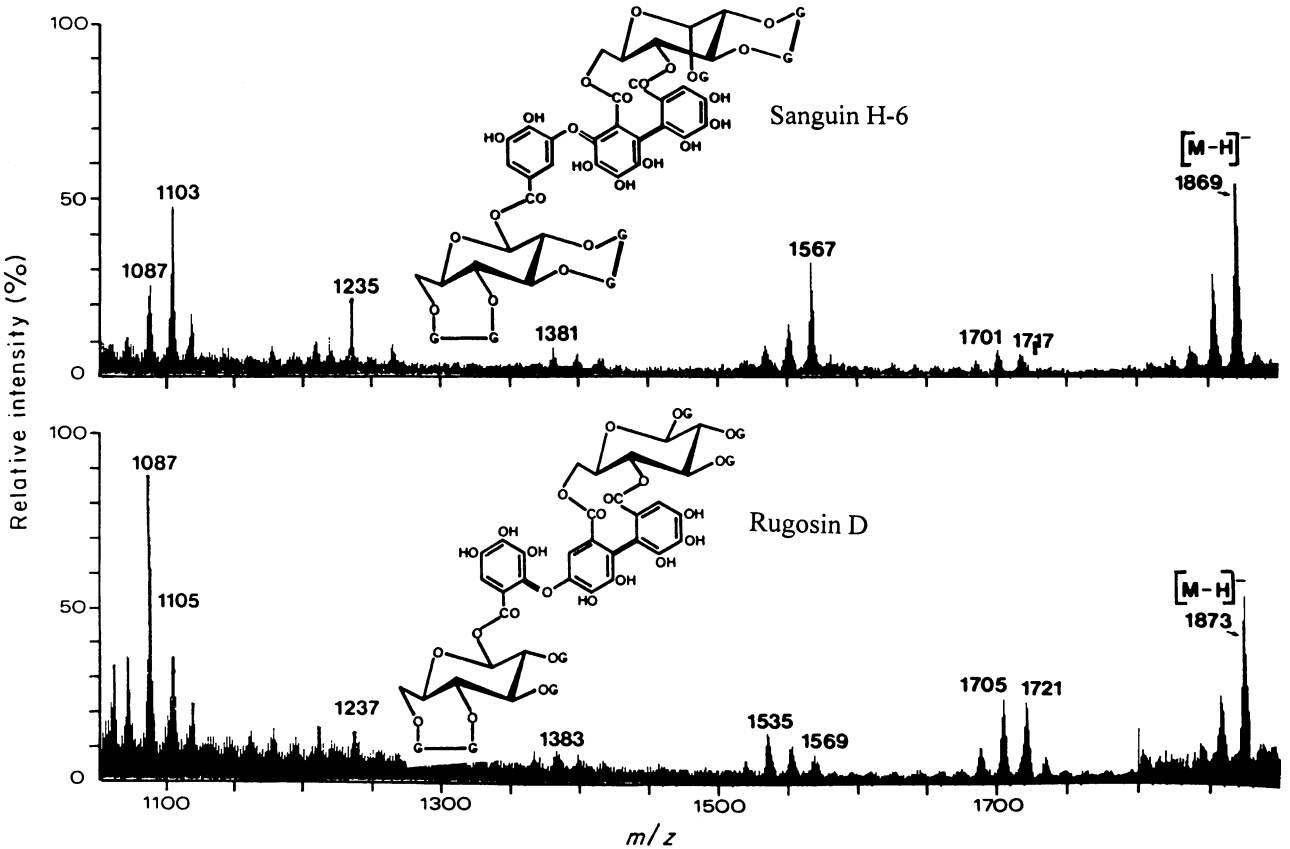

Fig. 12. Fast atom bombardment MS of two hydrolysable tannins, sanguin H-6 and rugosin D (Self et al., 1986). The spectrum reveals the molecular ions $[M–H]^-$.

and then yield significant structural information such as molecular ions or characteristic fragment ions. Fast atom bombardment MS in the negative ion mode was particularly successful at identifying a range of pure HTs and CTs compounds up to 1900 Da (Fig. 12; Self et al., 1986; Porter, 1989). Characteristic losses from the molecular ion $[M–H]^-$ of 152 and 170 were attributed to gallic acid units. More recently, new MS techniques have been used to study crude tannin mixtures. Electronspray ionisation MS succeeded in identifying tannins in mixtures up to 2300 Da (Guyot et al., 1997) and recent work by Krueger et al. (2000) and Hedqvist et al. (2000) provided highly detailed information on a range of crude tannins using MALDI-TOF MS. These two techniques hold great promise for studying the chemistry of tannins, but success at this stage seems to be an art. MS techniques can yield information on the components and molecular weights of tannins. However, the linkages between the components are best studied by NMR techniques.

10. Which method to choose for tannin analysis?

Unfortunately this question does not have an easy answer — yet answers are needed in order to assess the nutritional value of different plants or tree fodders or for screening new varieties in breeding programmes. Our lack of understanding as far as tannin structure/activity relationships are concerned is a serious obstacle to recommending any particular analytical method at the present. Therefore, it is suggested that wherever possible an array of methods be used to characterise tanniniferous feeds (Table 2). In addition to these chemical assays, biochemical and biological assays (see other contributions to this symposium) should also be considered wherever possible in conjunction with animal feeding trials in order to unravel the effects of tannins on animal nutrition. Only when the active components or properties of tannins are known will it be possible to focus the analytical techniques more accurately.

Table 2
Methods for characterising tannins in animal feeds

Types of tannins/tannin properties	Reagent/technique	Reference
Total phenolics	Phosphomolybdic acid	Folin and Ciocalteau (1927)
	$FeCl_3/K_3Fe(CN)_6$	Price and Butler (1977), Graham (1992)
	$Yb(acetate)_3$	Reed et al. (1985)
Condensed tannins	HCl/butanol	Porter et al. (1986)
Gallotannins	Rhodanine	Inoue and Hagerman (1988)
Gallo- and Ellagitannins	KIO_3	Willis and Allen (1998)
	HPLC determination of gallic and ellagic acids after hydrolysis	Mueller-Harvey et al. (1987)
All types of tannins	TLC	Mueller-Harvey et al. (1987)
Molecular weights	ESI or MALDI-TOF MS for HTs and CTs; normal phase HPLC for HTs	Guyot et al. (1997), Krueger et al. (2000), Hedqvist et al. (2000); Tanaka et al. (1984), Okuda et al. (1989), Hagerman et al. (1992)

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