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MALDI-TOF-CID for the Microstructure Elucidation of Polymeric Hydrolysable Tannins

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ABSTRACT: High molar mass wood tannin extracts are complex mixtures that are distributed in both molar mass and chemical composition. Hydrolysable tannins from tara, Turkey gall, and chestnut woods were analyzed and compared using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Although MALDI-TOF MS reveals the oligomer structure of the tannins, this method cannot distinguish between isomers with isobaric masses and, therefore, ambiguous structural assignments were made in a number of cases. To determine the actual microstructures present, MALDI-TOF-CID (collision induced dissociation) experiments were conducted. MALDI-TOF-CID enables monomer sequence determination and positive assignments of isobaric structures can be made. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

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INTRODUCTION

Tannins are divided into two major groups, namely, condensed and hydrolysable tannins. Condensed tannins (also known as polyflavonoids or proanthocyanidins) are based on flavonoid units that undergo condensation and polymerization reactions to form oligomers with varying degrees of polymerization.[1] Hydrolysable tannins differ from condensed tannins as they are derivatives of gallic acid and are usually esterified to a carbohydrate core, mainly glucose.[1–4] However, these tannins often occur as complex mixtures of simple phenols (e.g., pyrogallol), gallic and digallic acids as well as esters of sugars and other structures (e.g., three-dimensional networks) formed as a result of oxidative coupling and further esterification of the galloyl groups. The simplest of the hydrolysable tannins are gallotannins that are made up of polygalloyl esters of glucose such as pentagalloyl glucose (PGG).[4,5]

These tannins are found in various plants and trees such as chestnut, tara, Chinese gall, turkey gall, sumac, and oak tannin. Monomers that form gallotannins are pentagalloylglucopyranose and the precursor for ellagitannins is trigalloyl-HHDP-glucopyranose.[5–9] The different types of hydrolysable tannins structures are shown in Figure 1. In nature, however, there exist different oxidation products and polymerized forms of the molecules described. In the analysis of tannin extracts, other higher oxidation structures were isolated, such as castalin/vescalin and vescalagin/castalagin, and the structures are shown in Figure 2.[6]

In the analysis of both condensed and hydrolysable tannins, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) was used very successfully to determine the chemical composition of complex plant extracts.[10–15] The analysis of tannins using MALDI-TOF is a relatively new technique and provides additional information on the structure and molar mass distributions in a single experiment. Pasch and coworkers[13] applied MALDI-TOF to analyze polymeric tannins. In the analysis of a chestnut extract which is a hydrolysable tannin, the presence of vescalin/castalin and vescalagin/castalagin was conclusively determined.[12,15] MALDI-TOF has been successfully applied offline to analyze fractions obtained from a chromatographic separation, information obtained from this technique is very valuable as it can reach very high molar masses.[15–18] electrospray ionization (ESI)-MS as an alternative method tends to form multiply charged molecules while MALDI-TOF provides mostly singly charged molecules thus making the spectra easier to interpret. One major problem of MALDI-TOF in particular for higher oligomers is that a given mass peak only provides the elemental composition. The sequence of these units in the macromolecule, however, cannot be obtained by MALDI-TOF.

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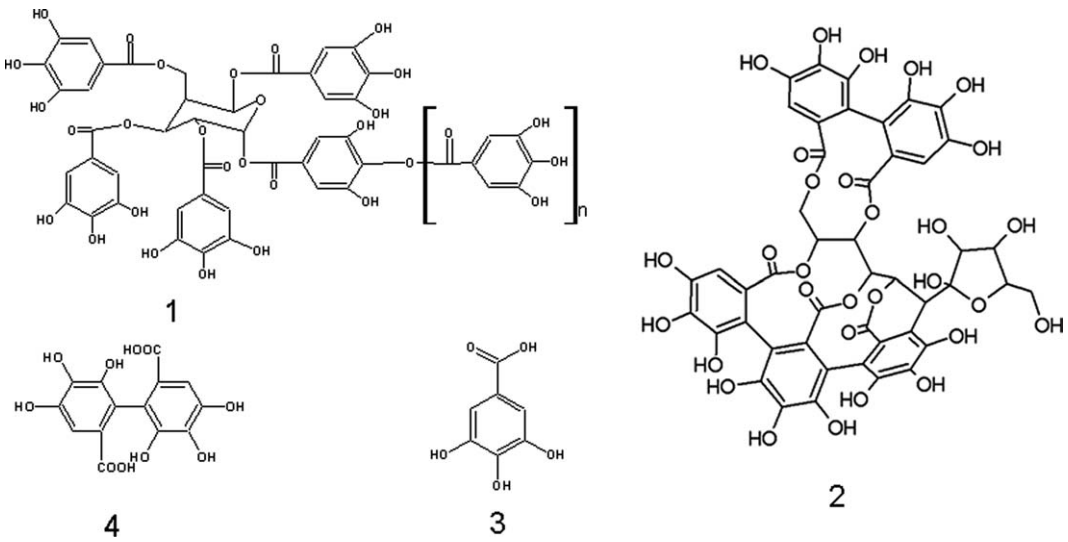

Figure 1. Typical structures of hydrolysable tannins: gallotannin (1), ellagitannin (2), gallic acid (3), hexahydroxydiphenic acid (HHDP) (4).

A new promising technique for the analysis of tannin monomer sequences is post source decay (PSD) fragmentation whereby a specific ion is selected from a MALDI-TOF spectrum and is subjected to higher laser intensities that result in fragmentation of the "mother" ion and the detection of the resulting fragments.[16,19] When the ions fragment in the field free region, the process is termed PSD.[16,20] Behrens et al.[16] was the first to show the applicability of this method to tannins and condensed tannins from lime and spruce. Another method that performs a similar type of analysis is collision induced dissociation (CID) whereby the precursor ion is selected from the first TOF analyzer and introduced into a collision cell whereby it collides with inert gas molecules. The fragments formed are then reflected and analyzed in the second TOF analyzer.[20,21] Although this technique has not been used for the analysis of tannins it has been shown to be applicable to biopolymers as well as synthetic polymers and thus shows great promise for analysis of tannins.[20,21] Recently, ion trap mass spectrometry has been used for the analysis of tannins. Corke and coworkers[22,23] identified phenolic antioxidants in Chinese roses and galls while Zhang and Lin analyzed condensed tannins in Pacific oak leaves.[10]

In a first publication, we reported on the analysis of condensed tannins by MALDI-CID.[24] The aim of this study is to elucidate the microstructure of hydrolysable tannins that were obtained from various wood extracts. It is known that hydrolysable tannins from different origins have different chemical compositions. Chestnut tannins are known to contain digalloylglucose while tara tannins contain ellagic acid. To elucidate their complex composition in detail, three samples were selected for this study, namely a chestnut tannin, a tara tannin, and a Turkey gall tannin. It shall be demonstrated that MALDI-TOF-CID is a unique method to determine the topology of the oligomers and the arrangement of gallic acid moieties around the sugar core.

EXPERIMENTAL

Materials

The description of the samples and the types of tannins involved are provided in Table I.

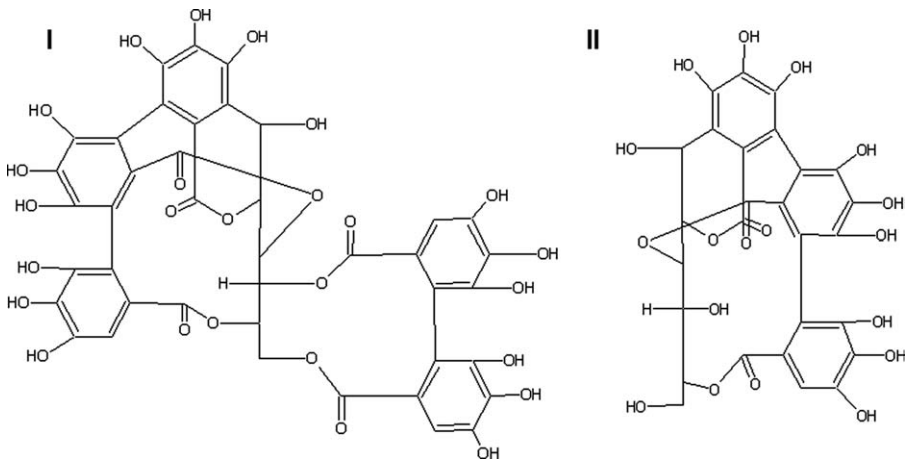

Figure 2. Structures of (I) castalagin/vescalagin and (II) castalin/vescalin positional isomers extracted from chestnut tannin [3].

Table I. Sample List with Description and Tannin Type

Sample	Description	Type of tannin
1	Tara, solvent-extracted	Hydrolysable tannin
2	Turkey Gall, solvent-extracted	Hydrolysable tannin
3	Chestnut, water-extracted	Hydrolysable tannin

Sample Preparation

Chestnut Tannin. The chestnut tannin is an extract of the wood of chestnut tree (*Castanea sativa*) that grows naturally in central Europe. The wood, after being separated from the bark, is chipped into small pieces of 1–4 cm and is charged in an extractor battery where it is extracted with water at 105–115°C. This first raw extract, that contains around 5% of dry material, is concentrated to 10% of dry material with a multiple effect concentrator. At this concentration, the hemicelluloses and gums are flocked and eliminated. After this purification step, the extract is concentrated to 50% of dry material and spray-dried to powder.

Turkey Gall Tannin. The Turkey gall tannin is an extract of Turkey galls that are produced by larvae of Gallic bee insects (*Cynips gallae-tinctoriae*) who are parasitized in young branches of the Fagaceae Gallic tree (*Quercus infectoria*) growing in the oriental Mediterranean coasts. The galls are manually collected, air dried, and stored. Then the galls are milled and extracted several times with organic solvents like acetone, ethanol, ethyl acetate and their mixtures with water to regulate the yield and the purity. In a concentrator, the solvents are stripped with vacuum and with several additions of water. The obtained water-dissolved extract is filtered and purified. In this step sugars, hemicelluloses, gums, natural resins, and chlorophylls are eliminated. The purified tannin solution is concentrated to 50% of dry material and spray-dried to powder.

Tara Tannin. The Tara tannin is an extract of the pods of Peruvian Tara (*Caesalpinia spinosa*). The pods are manually collected from the trees of Tara twice a year, air dried, separated from the seeds, milled, and stored followed by multiple extraction with organic solvents like acetone/water or ethanol/water mixtures. In a concentrator, the solvents are stripped with vacuum and with several additions of water. The obtained water-dissolved extract is filtered and purified. In this step sugars, salts and polysaccharides are eliminated. The purified tannins solution is concentrated to 50% of dry material and spray-dried to powder.

MALDI-TOF and MALDI-TOF-CID Analysis

MALDI-TOF-CID experiments were performed on an AximaTOF^2 spectrometer (Shimadzu Biotech, Manchester, UK), equipped with a nitrogen laser (337 nm), an ion gate for the selection of precursor ions and a collision cell. The windows for separation of precursor ions were approximately 4 Da. Argon has been used as the collision gas. The pressure within the collision cell was 8×10^{-6} mbar. The pulsed extraction ion source accelerated the ions to a kinetic energy of 20 keV. All data have been obtained in positive ion linear mode applying the accumulation of 441 scans per spectrum. The calibration of the linear mode as well as the reflectron mode for CID analysis was done using PEG in mass range up to 2000 Da. The accuracy of the product ion calibration is ±0.5 Da. The mass calibration was conducted based on the average masses. Compared to the monoisotopic masses and depending on the accuracy of the calibration, mass differences of 1–2 Da may be observed. This inaccuracy, however, does not disturb the peak assignment. Higher resolution can be obtained by using the reflectron mode.

The samples were dissolved in acetone/water 50/50 (% v/v) at a concentration of 4 mg/mL. The sample solutions were mixed with a 10 mg/mL solution of the matrix in the same solvent. 2,5-dihydroxy benzoic acid was used as the matrix. NaCl was added as the salt to enhance ion formation. The sample and matrix were then combined at a ratio of 1 : 1. 5 microL of the resulting solution was spotted on a 384 well MALDI-TOF plate, followed by evaporation of the solvent at ambient temperature without any assistance. The MALDI-TOF target was then analyzed to give the resulting spectra. In all cases, for the MALDI as well as for the MALDI-CID experiments, a very good reproducibility of the spectra was obtained both regarding the ion peaks and their positions.

RESULTS AND DISCUSSION

The hydrolysable tannins were analyzed by MALDI-TOF MS and as illustrated before this technique is successful in determining both the molar mass and the chemical composition. MALDI-CID was subsequently used to elucidate the molecular topology and the arrangement of the different gallic acid units in the oligomers. The tara and turkey gall tannins are both gallotannins in the polygallic form, see Figure 3.[6]

MALDI-TOF of Tara and Turkey Gall Tannins

Figure 4(a, b) show the MALDI-TOF spectra of the hydrolysable tannins from tara and turkey gall, respectively. As expected, similar repeat units of 152 Da are observed for both tannins confirming the general structure as shown in Figure 3. The structural assignments were done based on the work of Pizzi et al.[6]

The peaks at m/z of 521, 673, 825, 978, 1130, 1282, and 1435 in the tara tannin represent the major series with the mass increments of 152 Da belonging to gallic acid units. As has been shown previously, tara tannin is based on an ellagic unit that is decorated with different numbers of gallic acid units. In addition, the ellagic acid contains an ester group (—COO—) which contributes an additional mass of 44 Da to the oligomer mass. The ester group stems from hydrolysis reactions that take place

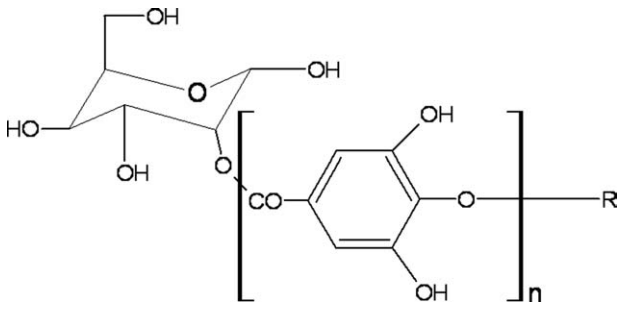

Figure 3. Proposed structure of gallotannins extracted from tara and turkey gall.

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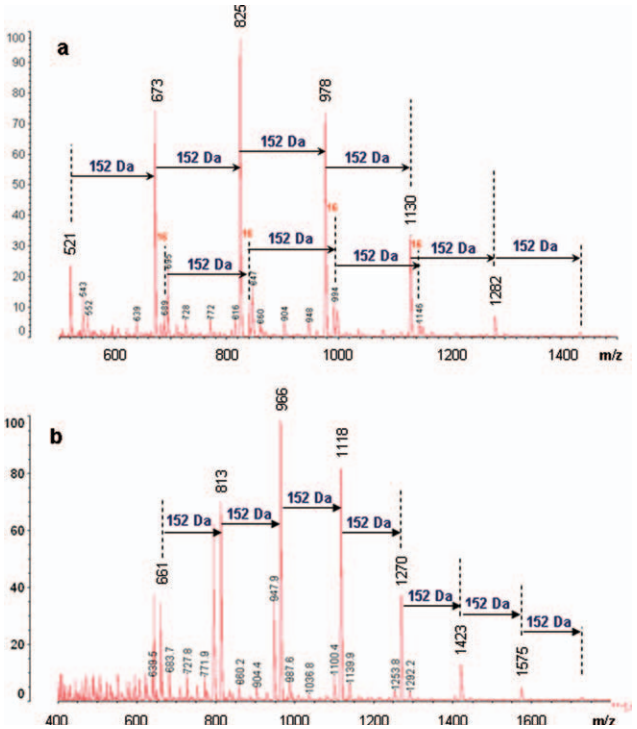


Figure 4. MALDI-TOF spectra of solvent-extracted tara (a) and turkey gall (b) tannins with repeat units of 152 Da as indicated. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

during the extraction process. Thus, the following equation was used to construct Table II: $M + Na^+ = 23\ (Na^+) + 304 - 1H$ (ellagic unit) + 152 (gallic unit) + 44 (COO). According to Figure 4, oligomers are detected up to heptamers. The first oligomer peak is detected at 521 Da. This peak corresponds to the sodium adduct of an ellagic acid with one gallic acid unit attached. All subsequent peaks are formed due to the addition of further gallic acid units.

A series of peaks with lower intensity appears adjacent to the major series also having repeat units of 152 Da. However, this minor series is shifted by 16 Da from the major series and thus is made up of different but related structures. These peaks correspond to the major oligomer series plus an OH-group.

Table II. Dominant Oligomer MALDI-TOF Peaks and Description of Structures Present in the Solvent-Extracted Tara Tannin

Calculated $M + Na^+$	Experimental $M + Na^+$	Description $M + Na^+$
522	521	Ellagic acid + COO + 1 gallic acid
674	673	Ellagic acid + COO + 2 gallic acid
826	825	Ellagic acid + COO + 3 gallic acid
978	978	Ellagic acid + COO + 4 gallic acid
1130	1130	Ellagic acid + COO + 5 gallic acid
1282	1282	Ellagic acid + COO + 6 gallic acid
1434	1435	Ellagic acid + COO + 7 gallic acid

Table III. Dominant Oligomer MALDI-TOF Peaks and Description of Structures Present in the Solvent-Extracted Turkey Gall Tannin

Calculated $M + Na^+$	Experimental $M + Na^+$	Description $M + Na^+$
659	661	Glucose + 3 gallic acid
811	813	Glucose + 4 gallic acid
963	966	Glucose + 5 gallic acid
1115	1118	Glucose + 6 gallic acid
1267	1270	Glucose + 7 gallic acid
1419	1422	Glucose + 8 gallic acid
1571	1575	Glucose + 9 gallic acid

A look at the turkey gall tannin also shows a simple polygallic structure. The basic structure of the turkey gall is quite similar to that of the tara tannin but does not include any ellagic acid groups in its basic structure. The first member of the main series of peaks is observed at m/z 661 representing the sodium adduct of three gallic acid units attached to glucose, see Figure 4(b). It is calculated using the following equation: $M + Na^+ = 23\ (Na^+) + 180$ (glucose) + 3 × 152 (gallic unit). The dominant oligomer peak at 965.9 Da is believed to represent PGG followed by oligomers that contain even more gallic acid units. A tentative peak assignment is presented in Table III.

MALDI-TOF-CID of Tara and Turkey Gall Tannins

As has been demonstrated, MALDI-TOF reveals the oligomer composition and the presence of different chemical moieties and building blocks. However, the arrangement of the different molecular fragments in a given molecule cannot be provided. As has been shown previously, only CID experiments can provide detailed information on the topology of the different oligomers. In the present case, the interesting question is how the gallic acid units are arranged around the core fragment which is a ellagic acid for tara and a glucose for Turkey gall.

For further investigation of the oligomer structures presented in the tara tannin, the precursor ions with m/z 824.5 and 839.8 were selected for fragmentation experiments. The first to be considered will be the trimer with m/z 824.5. The MALDI-CID

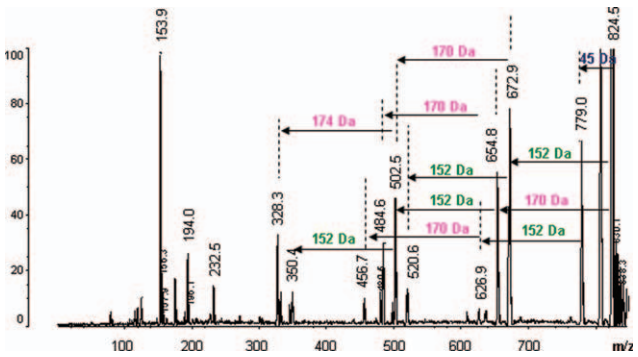


Figure 5. MALDI-TOF-CID spectrum of the trimer ion at m/z 824.5 from tara tannin, the fragmentation pattern is illustrated by indicating the mass loss incurred from the precursor ion. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

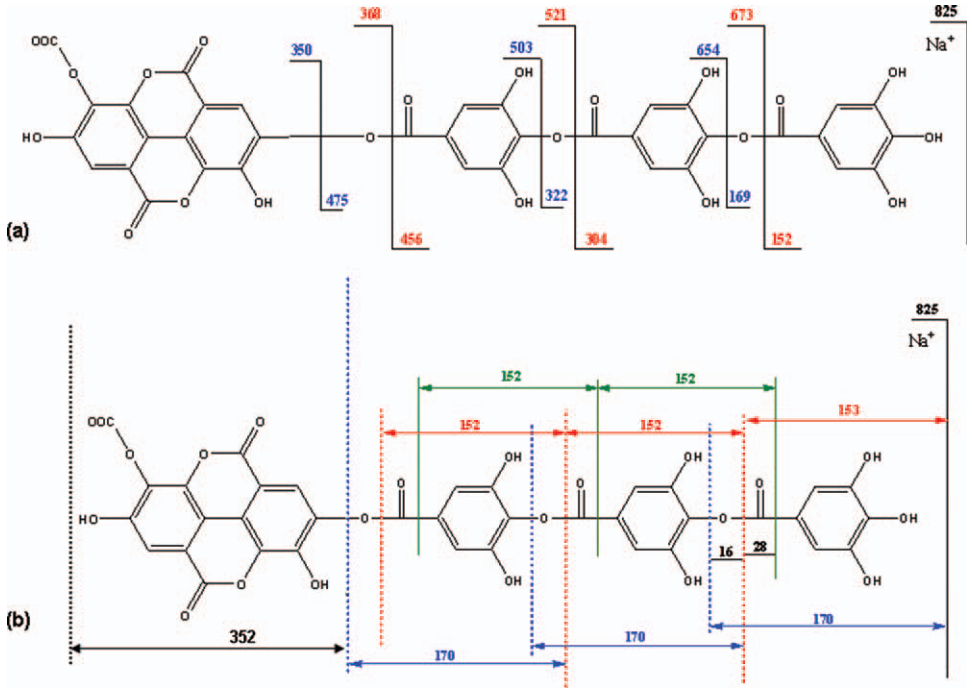


Figure 6. Proposed structures and fragmentation patterns for the parent ion at m/z 824.5 observed in the MALDI-TOF spectrum of the tara tannin, (a) observed fragment masses, (b) structures that form the observed mass increments (repeat units). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

spectrum for this oligomer is presented in Figure 5; the mass loss incurred by the precursor ion and the product ions are also indicated. The fragmentation pattern of the oligomer is relatively simple and the dominant ions appear at m/z 673, 502, 328, and 154. These peaks belong to different oligomer series, for example, m/z 824-673-520, 655-502-350, and so forth showing losses of 152 Da (gallic acid) or 170 Da (gallic acid + —O—). The peak at 328 Da is obtained from the oligomer at m/z 502 by the loss of one gallic acid unit and an oxygen. The loss of units of 152 Da occurs three times, representing the loss of 3 gallic acid units that form the oligomer structure. Once formed, the fragment ions may lose —O— or C=O groups, shown by mass losses of 16 and 28 Da, respectively. These types of fragments show that the galloyl ester bond is the most labile, and thus the fragmentation may occur before or after the carbonyl group of the ester bond. However, as observed in the spectrum, there are no fragment ions resulting from the simultaneous loss of the —O— and C=O groups.

The loss of the 3 gallic acid units results in the formation of the fragment ion at m/z 350 which can be assigned to a proton adduct of ellagic acid that still contains the ester group. The fragment at m/z 301 can then be assigned to the proton adduct of a single ellagic acid unit. The proposed structure with an ellagic acid unit at the terminal end of the molecule is supported by the present fragmentation pattern.

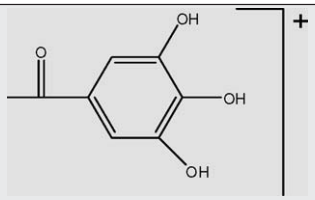
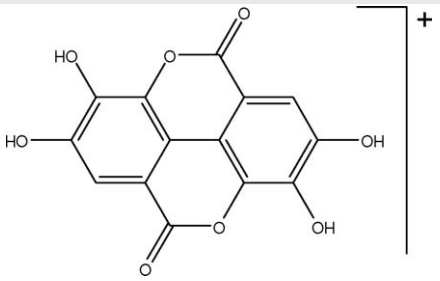
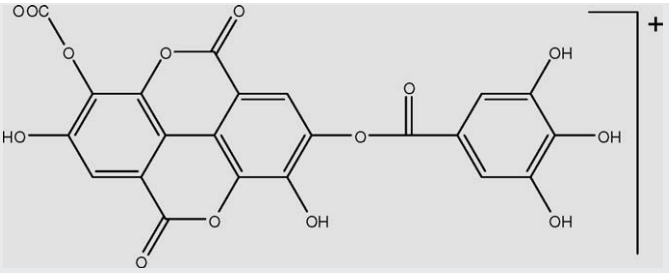
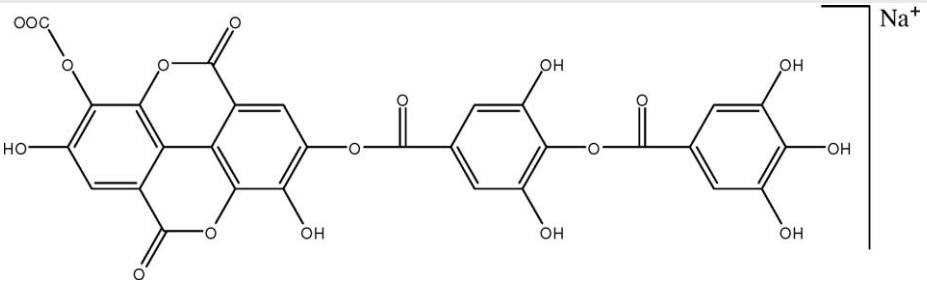
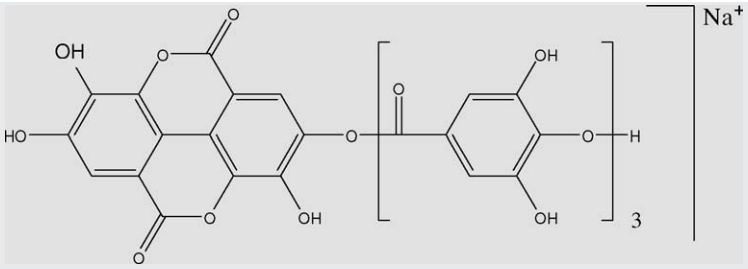
The precursor ion (m/z 824) additionally loses a mass of 44 Da arising from the ester group to form the ion m/z 779 which then undergoes further fragmentation by loosing gallic acid units. This confirms the presence of the postulated 44 Da mass, and the presence of the m/z 350 ion indicates that this group is attached to ellagic acid unit as there are no fragments observed that indicate its attachment to the gallic acid units. As the exact position at the ellagic acid cannot be determined, an arbitrary position was selected for the location of the ester group (Figure 6).

A summary of some typical fragmentation products is given in Table IV.

Further investigations were conducted on the parent ion at m/z 839.8 which appears in the MALDI-TOF spectrum only as minor peak. This oligomer is related to the preciously determined m/z 824 structure by an additional mass of 16 Da. This ion peak was tentatively assigned to the sodium adduct of a trigalloyl diglucose trimer, see Figure 7. This form of structure may be present due to the polygallic form of the oligomers present in the tara tannin extract. As can be seen, the MALDI-CID spectra of the two ions show some similarities in the higher mass region but distinct differences in the lower mass region indicating that the two ions have different molecular structures of the core, compare Figures 5 and 8.

The MALDI-CID spectrum of the ion at m/z 839 in Figure 8 shows mass losses of 152 and 170 Da which indicate losses of gallic acid units in a rather simple fragmentation pattern. The loss of two gallic acid units can be observed although it was postulated that the ion contains three gallic acid units as is shown in Figure 7. A possible interpretation is that gallic acid units do not form a trimer that is attached to one glucose side but rather a dimer and a monomer that are attached to different glucose sites. Assuming that all ester bonds of the gallic acid units have the same stability, this interpretation would fit the observed fragmentation pattern.

Table IV. Structures of Selected Fragment Ions Observed in the MALDI-CID Spectrum of the Parent Ion at m/z 824 from Tara Tannin

m/z (observed)	m/z (calc.)	Fragment ion
154	153	
301	302	
502	499	
673	672	
779	778	

A more complex fragmentation pattern is obtained in the mass range <300 Da. The observed fragment peaks are assumed to be a result of the fragmentation of the core molecule in the present case the diglucose. Fragmentation may occur via internal fragmentations of the glycosidic linkage, the presence of these internal fragments may be indicated by a mass loss of 134 Da. These types of fragmentations are commonly observed during MALDI-CID of carbohydrates.[25] The internal fragments

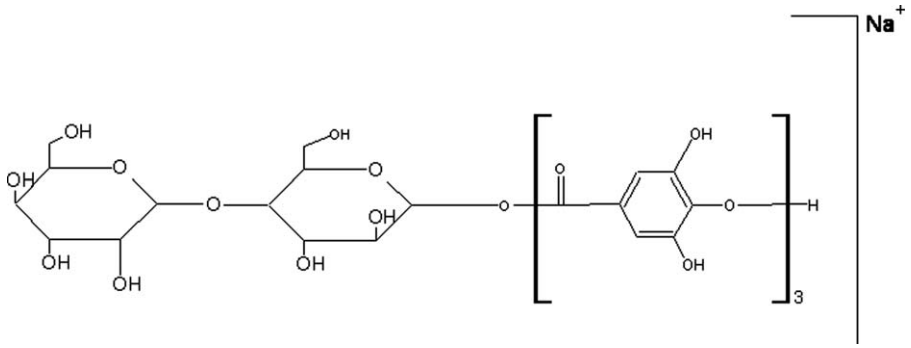


Figure 7. Proposed structure for the trimer at m/z 839 as postulated from the bulk sample MALDI-TOF analysis.

result from cleavage of the glucose ring at bonds adjacent to the cyclic ether group. The gallic acid units may be located at any of the OH groups present in the glucose structure; however, due to the presence of the internal fragments from the glucose molecules, exact locations of these groups may be determined. In the present case, one of the gallic acid units must be located at the terminus of the glucose unit; this is shown by the presence of a mass loss of 288 Da observed in the spectrum. This mass can only be formed by the loss of a gallic acid unit attached to a 134 Da fragment from the glucose monomer. This assumption is further confirmed by the loss of a fragment of 272 Da, see Figures 8 and 9.

In the case of the Turkey gall tannin, it was assumed that the gallic acid units are attached to a glucose core. To investigate the structure of the Turkey gall tannin in detail, the parent ion at m/z 811.6 which is assumed to be a tetramer of four gallic acid units attached to glucose was analyzed by CID. The fragmentation spectrum is shown in Figure 10. As can be seen there, the fragmentation pattern is relatively simple and resembles closely the type of fragmentation observed for the m/z 824.5 ion from the tara tannin. In the fragment spectrum of the ion at m/z 811.6 there are no fragments resulting from the glucose monomer. This is due to the fact that only a single glucose monomer is present and thus higher energies would be required to form the 134 Da mass fragment previously shown for glucose oligomers. In addition, the glucose molecule is more stable against fragmentation than the galloyl esters and, therefore, fragmentation preferentially occurs at these positions.

The fragmentation of the oligomer at m/z 811.6 takes place as a sequential loss of two gallic acid units to form the fragment at m/z 469.1 which is obviously the glucose monomer with two gallic acid units attached. The structure forming the m/z 811.6 ion can thus be assigned as a branched structure with two gallic acid dimers linked to different positions at the glucose monomer, see Figure 10.

As a second parent molecule, the ion at m/z 964.2 was investigated by MALDI-TOF-CID, see Figure 11. This molecular ion was tentatively assigned to PGG. The CID spectrum shows sequential losses of fragments of 152/170 Da corresponding to gallic acid units. Comparing Figure 5 with Figure 11 it is obvious that the arrangement of the gallic acid units must be different. While in Figure 5 the consecutive loss of at least three units can clearly be seen, in Figure 11 the loss of only two gallic acid units can be identified. This is similar to the behavior of the parent ion at m/z 811.6. Therefore, it is assumed that the gallic acid units are not arranged as a trimers or tetramers attached to one position of the core molecule but rather as monomers or dimers attached to different positions of the core molecule. The fragment peak at m/z 642 would most likely

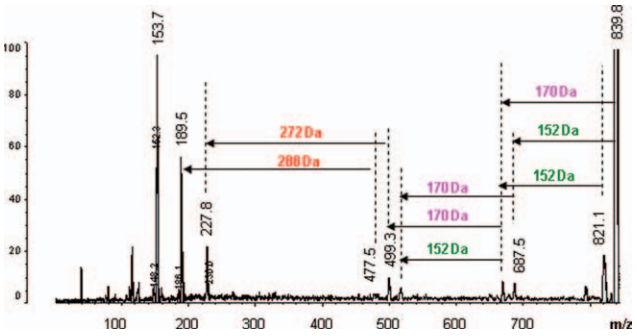


Figure 8. MALDI-TOF-CID spectrum of the parent ion at m/z 839.8 of the tara tannin. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

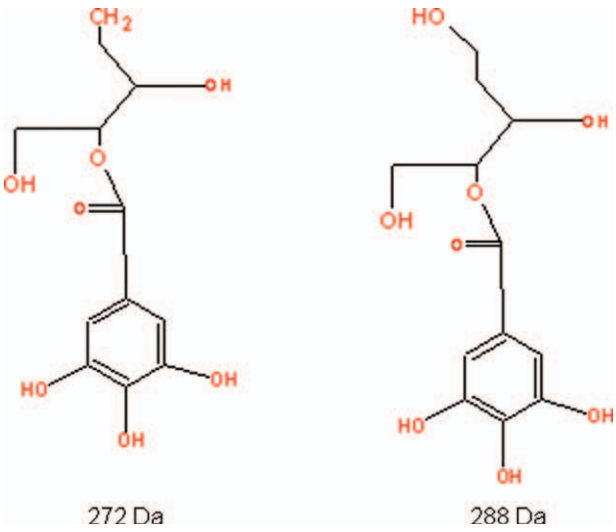


Figure 9. Fragments observed in the MALDI-TOF-CID spectrum of the tara tannin trimer m/z 839.8. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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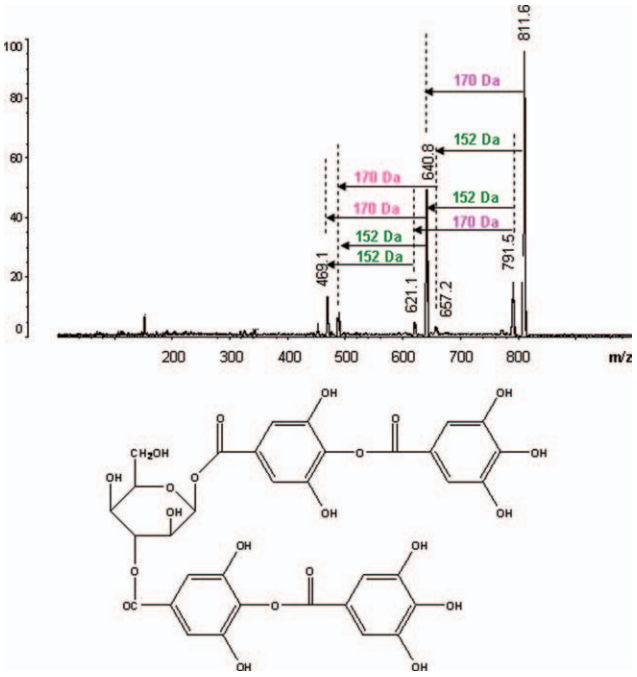

Figure 10. MALDI-TOF-CID spectrum of the parent ion at m/z 811.6 from solvent-extracted Turkey gall tannin and proposed structure of the parent ion. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

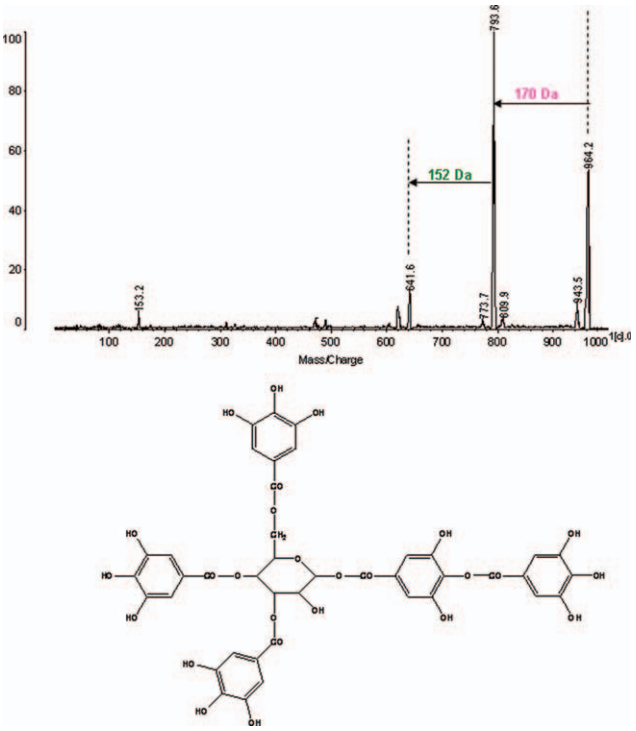

Figure 11. MALDI-TOF-CID spectrum of parent ion at m/z 964.2 from solvent-extracted Turkey gall tannin and proposed structure of the parent ion. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

correspond to a trigalloylglucose, see Figure 11 for the topology of the parent ion.

Further investigations were performed on a pentamer related to the previous oligomer. As shown for the tara tannin, a small difference in masses between related oligomers can display very different structures. This point is further illustrated by analysis of the parent ion at m/z 947.2, the MALDI-CID spectrum of which is shown in Figure 12. In this case, consecutive losses of four gallic acid units is observed. This is a clear indication that these units must have been arranged in the parent molecule as a tetramer. The topology given in Figure 12 is proposed for this oligomer.

MALDI-TOF of Chestnut Tannin

The structures of natural water-extracted chestnut tannin and the chestnut tannin treated with 3.5% ammonium sulfite for use in leather tanning obtained by MALDI-TOF have been reported before.[6,11] The analysis of both extracts proved the oligomeric nature of the extract *in situ* in the wood, the tannin was shown to be composed of PGG oligomers with up to 16 units.[6,11] In the water-extracted chestnut tannin, the PGG oligomers were detected up to trimers whereas in the sulfited sample, glucose oligomers stripped of its galloyl units were detected.[6] The linear mode spectrum of a water-extracted chestnut tannin is shown in Figure 13 being markedly different from the previously reported spectra for this tannin. The peak assignments were made based on Pizzi et al.[6,11] The mode of extraction clearly affects the composition of this tannin. The repeat unit in this case is 162 Da which cannot be explained by the theories previously presented for the structures present in this extract, thus this warrants an in-depth discussion. The repeat unit of 162 Da is similar to the one observed for Chinese gall tannin. The explanation for this distribution was given as oligomers formed by esters of gallic and digallic units attached to glucose.[6] However, it cannot be determined whether two gallic acid units are attached to each other or independently on

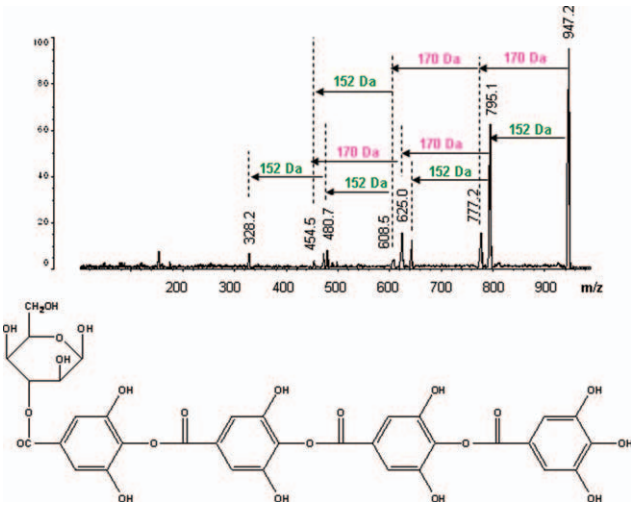

Figure 12. MALDI-TOF CID spectrum of the parent ion at m/z 947.2 representing a pentamerfrom solvent-extracted turkey gall tannin and proposed parent ion structure. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

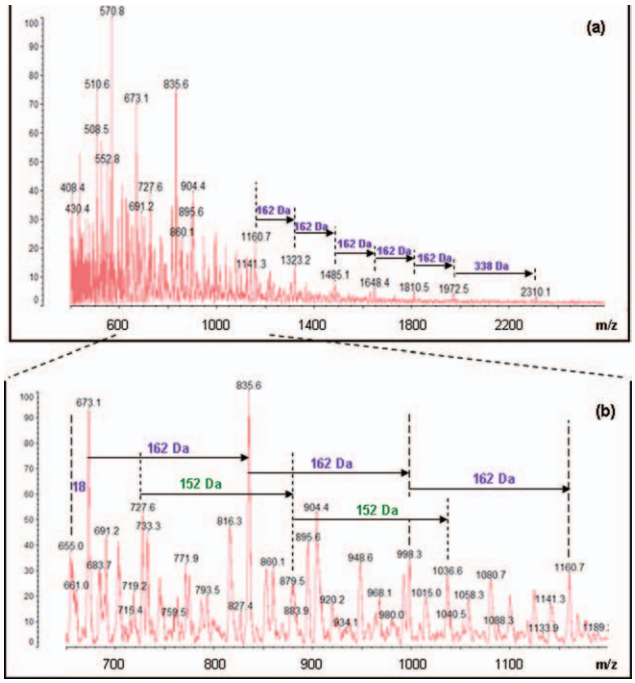

Figure 13. MALDI-TOF spectrum of water-extracted chestnut tannin (linear mode). Mass range: (a) 400–2600 Da (b) 650–1200 Da. The peak assignments are indicated and the details shown in Table V. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the pyranose ring of the glucose. In addition, ellagic acid units may be present. The proposed structure of the oligomer is shown in Figure 14.

The most abundant oligomer distribution is observed with the series of peaks beginning at 508 Da. This peak is known to be a digalloylglucose monomer (digalloylglucose = 23 (Na^+) + 152 × 2 + 180 = 507 Da), the series with 162 Da is completed at 1972 Da. The 162 Da increment represents an additional glucose unit being added to the oligomer structure. A list of peaks and assignments is shown in Table V. As can seen there are a number of units that are indicated as "stripped glucose." These units are glucose units that lost some of the original OH groups together with a loss of gallic acid units. Such reactions may take place during the extraction process as has been discussed previously. The mass of these units is 132 Da.

In the present sample, a dominant series is composed of glucose units that are attached to two galloyl units. These may also be present as an ellagic acid unit. The MALDI experiment alone cannot give a conclusive answer here. The distributions that are seen in the spectrum indicate that the main composition here is long chains of glucose units (up to 9 units) with the terminal group being a digalloyl glucose unit. The peak observed at 438 Da can be assigned to a single galloyl unit attached to two stripped glucose units. This forms another distribution of peaks at m/z 438, 727, 879, and 1036, which have a mass increment of 152 Da indicating galloyl units. The difference of 388 Da observed between the peaks at m/z 1972 and 2310 indicates the presence of an ellagic acid unit attached to a OCO group added to the oligomer chain, (2310 Da = 23 (Na^+) + 2 × 152 + 180 + 162 × 9 + 302 + 44). This assignment may also be made according to Pizzi et al.[6] as 2 additional galloyl units being added to the stripped glucose skeleton structure. The former is less likely, however, it is possible that these structures may be present as the chestnut tannin is a known ellagitannin.[6]

MALDI-TOF-CID of Chestnut Tannin

The chestnut tannin is composed of glucose units, gallic acids, and possible ellagic acids. The main constituents in this tannin are galloyl unit oligomers stripped from their glucose core. The applicability of the MALDI-CID has shown to be able to distinguish between the various oligomer structures and thus it will be applied here to note the sequence distribution of the oligomers in this extract. The fragmentation spectrum for the oligomer peak at 672.3 Da is shown in Figure 15.

There are only two fragments formed from the oligomer at m/z 672. The fragmentation patterns have been discussed and analyzed for the tara and Turkey gall tannins. However, the present fragment spectrum for the chestnut tannin does not resemble any of the previously discussed spectra. The fragment mass that is lost by the precursor ion is not consistent with gallic acid

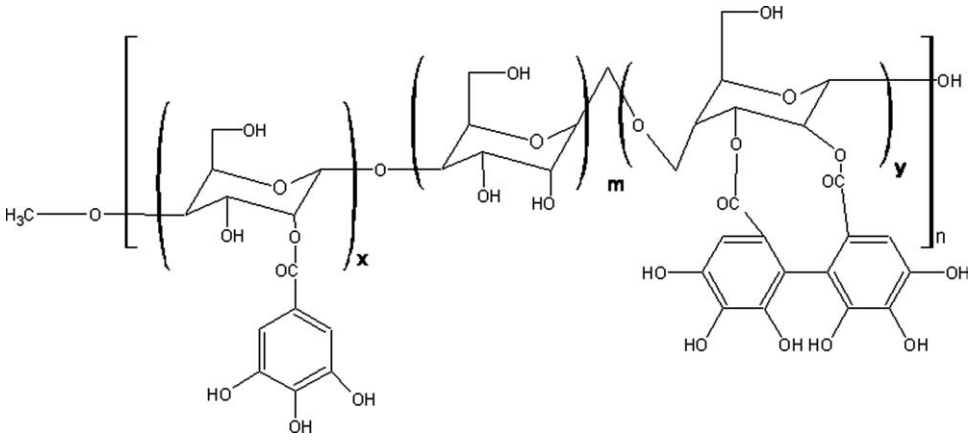

Figure 14. Representation of the possible oligomer structure present in chestnut tannin. With the values of x, m, and y differing according to chemical structure, n represents the total chain length.

Table V. Observed and Calculated Oligomer Peaks from the Linear Mode MALDI-TOF Spectrum of Water-Extracted Chestnut Tannin

Experimental ($M + Na^+$)	Calculated ($M + Na^+$)	Description	Oligomer
508[a]	507	Digalloyl glucose	Monomer
571[a]	571	1 Galloyl unit + 3 stripped glucose	Trimer
655[a]	659	Trigalloyl glucose	Monomer
673[a]	669	Digalloyl glucose + glucose	Dimer
836[a]	835	1 Galloyl unit + 5 stripped glucose	Pentamer
968	967	1 Galloyl unit + 6 stripped glucose	Hexamer
998	993	Digalloyl glucose + 3 glucose	Tetramer
1161[a]	1155	Digalloyl glucose + 4 glucose	Pentamer
1233	1232	1 Galloyl unit + 8 stripped glucose	Octamer
1323[a]	1317	Digalloyl glucose + 5 glucose	Hexamer
1364	1364	1 Galloyl unit + 9 stripped glucose	Nonamer
1485[a]	1479	Digalloyl glucose + 6 glucose	Heptamer
1630	1627	1 Galloyl unit + 11 stripped glucose	Undecamer
1648[a]	1641	Digalloyl glucose + 7 glucose	Octamer
1764	1759	1 Galloyl unit + 12 stripped glucose	Dodecamer
1810[a]	1803	Digalloyl glucose + 8 glucose	Nonamer
1972[a]	1965	Digalloyl glucose + 9 glucose	Decamer
2310[a]	2307	2 Galloyl units + 15 stripped glucose	Pentadecamer

[a]Dominant oligomer peaks.

units linked via a galloyl ester bond. The most likely structure represented by the fragment at m/z 312 is a diglucose that is completely stripped from gallic or ellagic acid. A proposed fragmentation is shown in Figure 15 being consistent with the expected structure in Table V and the fragment mass of 312 Da which would be the protonated stripped diglucose. The mass loss of 360 Da corresponds to the mass of the ellagic acid residue plus the sodium cation.

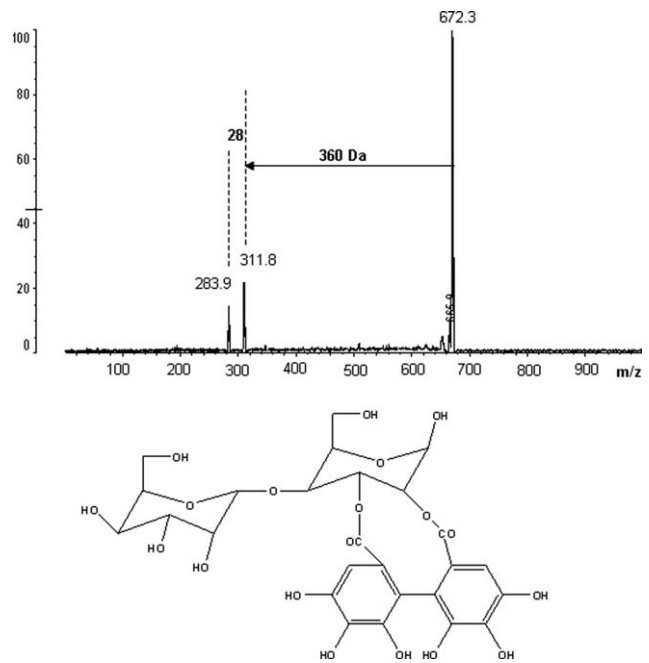

Figure 15. MALDI-TOF-CID spectrum of the parent ion at m/z 672.3 representing a dimer from chestnut tannin and proposed parent ion structure.

CONCLUSION

MALDI-TOF-CID experiments have been demonstrated to be uniquely powerful for the monomer sequence determination and positive assignments of oligomer structures in wood-based hydrolysable tannin mixtures. Although standard MALDI-TOF MS measurements reveal the oligomer structure of these tannins, this method cannot distinguish between isomers and, therefore, ambiguous structural assignments are obtained in a number of cases. In most cases, the actual microstructures present can unambiguously be identified by MALDI-TOF-CID. In other cases, most likely structures can be proposed.

Three representative hydrolysable tannins were investigated, namely tannins from tara, chestnut, and Turkey gall wood extracts. It has been shown that the tara and Turkey gall tannins show a number of similarities in their MALDI-TOF spectra. MALDI-TOF-CID, however, proofed that the tara tannins contain ellagic acid while the Turkey gall tannins exhibit arrangements of gallic acid units around a glucose core. The chestnut tannin has been found to be composed mainly of digalloyl glucose, glucose, and gallic acid units.

REFERENCES

1. Pizzi, A. Wood Adhesives Chemistry and Technology; Marcel Dekker: New York, **1983.**

2. Pizzi, A. Advanced Wood Adhesives Technology; CRC Press: New York, **1994.**
3. White, T. *J. Sci. Food Agric.* **1957,** *8*, 377.
4. Hartzfeld, P. W.; Forkner, R.; Hunter, M. D.; Hagerman, A. E. *J. Agric. Food Chem.* **2002,** *50*, 1785.
5. Haslam, E. Practical Polyphenolics from Structure to Molecular Recognition and Physiological Action; Cambridge University Press: New York, **1998.**
6. Pizzi, A.; Pasch, H.; Rode, K.; Giovando, S. *J. Appl. Polym. Sci.* **2009,** *113*, 3847.
7. Pizzi, A. In Monomers, Polymers and Composites from Renewable Resources; Belgacem, M., Gandini, A., Eds.; Elsevier, **2008.**
8. Mueller-Harvey, I. *Anim. Nutr. Feed Technol.* **2001,** *91*, 3.
9. Salminen, J.-P.; Ossipov, V.; Loponen, J.; Haukioja, E.; Pihlaja, K. *J. Chromatogr. A* **1999,** *864*, 283.
10. Zhang, L. L.; Lin, Y. M. *Molecules* **2008,** *13*, 2986.
11. Pasch, H.; Pizzi, A. *J. Appl. Polym. Sci.* **2002,** *85*, 429.
12. Pasch, H.; Pizzi, A.; Rode, K.; *Polymer* **2001,** *42*, 7531.
13. Navarette, P.; Pizzi, A.; Pasch, H.; Rode, K.; Delmotte, L. *Ind. Crops Prod.* **2010,** *32*, 105.
14. Oo, C. W.; Pizzi, A.; Pasch, H.; Kassim, M. J. *J. Appl. Polym. Sci.* **2008,** *109*, 963.
15. Perret, C.; Pezet, R.; Tabacchi, R. *Phytochem. Anal.* **2003,** *14*, 202.
16. Behrens, A.; Maie, N.; Knicker, H.; Koegel-Knabner, I. *Phytochemistry* **2003,** *62*, 1159.
17. Raust, J. A.; Bruell, A.; Moire, C.; Farcet, C.; Pasch, H. *J. Chromatogr. A* **2008,** *1203*, 207.
18. Montaudo, G.; Garozzo, D.; Montaudo, M. S.; Puglisi, C.; Samperi, F. *Macromolecules* **1995,** *28*, 7983.
19. Hanton, S. D. *Chem. Rev.* **2001,** *101*, 527.
20. Belgacem, O.; Bowdler, A.; Brookhouse, I.; Brancia, F. L.; Raptakis, E. *Rapid Commun. Mass Spectrom.* **2006,** *20*, 1653.
21. Jackson, A. I. T.; Jennings, K. R.; Scrivens, J. H. *J. Am. Soc. Mass Spectrom.* **1997,** *8*, 76.
22. Cai, Y. Z.; Xing, J.; Sun, M.; Zhan, Z. Q.; Corke, H. *J. Agric. Food Chem.* **2005,** *53*, 9940.
23. Zhu, F.; Cai, Y. Z.; Xing, J.; Ke, J.; Zhan, Z.; Corke, H. *Rapid Commun. Mass Spectrom.* **2009,** *23*, 1678.
24. Radebe, N.; Rode, K.; Pizzi, A.; Pasch, H. J. Appl. Polym. Sci. **2012;** to appear.
25. Spina, E.; Sturiale, L.; Romeo, D.; Impallomeni, G.; Garozzo, D.; Waidelich, D.; Glueckmann, M. *Rapid Commun. Mass Spectrom.* **2004,** *18*, 392.