Considerations on the Macromolecular Structure of Chestnut Ellagitannins by Matrix-Assisted Laser Desorption/Ionization–Time-of-Flight Mass Spectrometry

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ABSTRACT: The rather novel picture of chestnut wood tannin, *in situ* in the wood, which emerges from matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF-MS) is that of a pervasive and extended random tridimensional macromolecular network, formed by pentagalloylglucose clusters linked to each other, to form chains and encrust the wood constituents matrix. Up to pentagalloylglucose trimers were identified in the MALDI-TOF analysis of the commercial chestnut tannin extract, which were clearly degradation products of more extensive chains likely to be present *in situ* in the wood before extraction, in the network through polygallic/polyellagic chains or flavogallonic acid bridges. The hydrolyzable chestnut tannin network is capable of being extracted, to yield the commercial chestnut tannin extract exclusively by its degradation, a degradation that is possible only because of the susceptibility to hydrolysis of the ester bridges holding the network together. Internal rearrangements of the fragments formed by the extraction appear to occur readily and with ease, to yield a variety of structures characterized by the presence of ellagic acid residues, flavogallonic acid residues, and also, but less readily, nonahydroxytriphnoic acid residues. The other main constituents of the commercial tannin extract, castalagin and vescalagin, are shown to be simply the more stable degradation plus internal rearrangement products derived from the hydrolysis of polypentagalloylglucose chains.
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Key words: mass spectrometry; MALDI; supramolecular structure; networks; tannins; hydrolyzable tannins; ellagitannins; ellagic acid; chestnut; structure

INTRODUCTION

Vegetable tannins have been used to tan leather, either alone or accompanying other tanning agents, for thousands of years. They are natural products obtained from plants and are very diffuse in the whole plant kingdom. The term *natural vegetable tannins* is used loosely to define two broad classes of chemical compounds of a mainly phenolic nature: condensed or polyflavonoid tannins and hydrolyzable tannins. To the recognized oligomeric nature of condensed tannins[1–4] corresponds the allegedly nonpolymeric nature of hydrolyzable tannins.[2–4] These latter tannins, including chestnut, myrabolans, divi-divi, tara, algarobilla, valonea, oak, and several other

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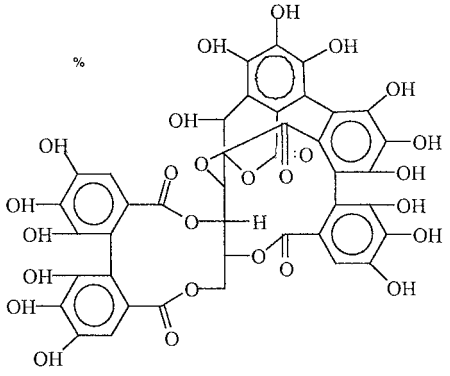
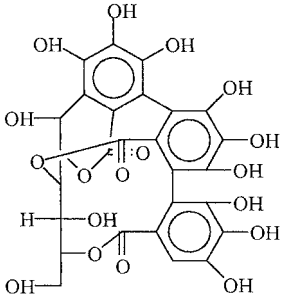
commercial tannin extracts, are reputed to be mixtures of simple phenols such as gallic and ellagic acids and of esters of a sugar, mainly glucose, with gallic and digallic acids, and with more complex structures containing ellagic acid. Despite their alleged lack of a polymeric structure, complex structures can be formed. Thus, several studies[1,2] identified that the main constituents of the main commercial hydrolyzable tannin—chestnut tannin extract, an ellagitannin—are castalagin and vescalagin, positional isomers of identical 935 mass, composing, respectively, 14.2 and 16.2% by mass of chestnut tannin, the structure of which (**I**) is as follows:



(**I**)

The rest of the tannin is composed of 6.6% castalin and vescalin (**II**) (positional isomers, the structure of which follows),[1,2] 6% gallic acid, and 3% pentagalloyl glucose.



(**II**)

It must be pointed out, however, that the authors of the study advancing relative composition percentages[2] clearly state that the disadvantage of the chromatographic technique they used is its strong adsorption of several types of tannins, particularly tannins composed of large molecules. This limitation might well slant the percentages of lower molecular weight to higher molecular weight components in the analysis of chestnut extract that have been presented.[2] Despite such a limitation, however, two classes of compounds have mass predominance in chestnut tannins: 28.8% of small molecules (the formula of which is shown in Fig. 1) and 25.4% (or higher; see above-stated reasons) of an unknown and difficult to isolate fraction of apparently significantly higher molecular mass[2] and very low retardation factor R_f, determined by thin-layer chromatography (TLC). This fraction appears to be composed of a number of closely related components, giving a continuous TLC smudge of R_f value between 0 and 0.33. It was in the hope of identifying the nature of this higher molecular mass fraction and the manner in which the different components are bound in it, that commercial chestnut tannin extract was examined by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).

Since its introduction by Karas and Hillenkamp in 1987,[5] MALDI-MS has greatly expanded the use of mass spectrometry toward large molecules and has revealed itself to be a powerful method for the characterization of both synthetic and natural polymers.[6–12] Fragmentation of analyte molecules upon laser irradiation can be substantially reduced by embedding them in a light-absorbing matrix. As a result intact analyte molecules are desorbed and ionized along with the matrix and can be analyzed in a mass spectrometer. This soft ionization technique is mostly combined with time-of-flight (TOF) mass analyzers, which is the case, given that TOF-MS presents the advantages of being able to provide a complete mass spectrum per event, its virtually unlimited mass range, the small amount of analyte necessary, and the relatively low cost of the equipment.

EXPERIMENTAL

Tannin Types

Two types of commercial hydrolyzable tannin extracts were used for MALDI-TOF analysis: (1) sumach (*Rhus coraria*) leaves commercial natural tannin extract, a gallotannin, and (2) chestnut (*Castanea sativa*) wood commercial natural tannin extract, an ellagitannin.

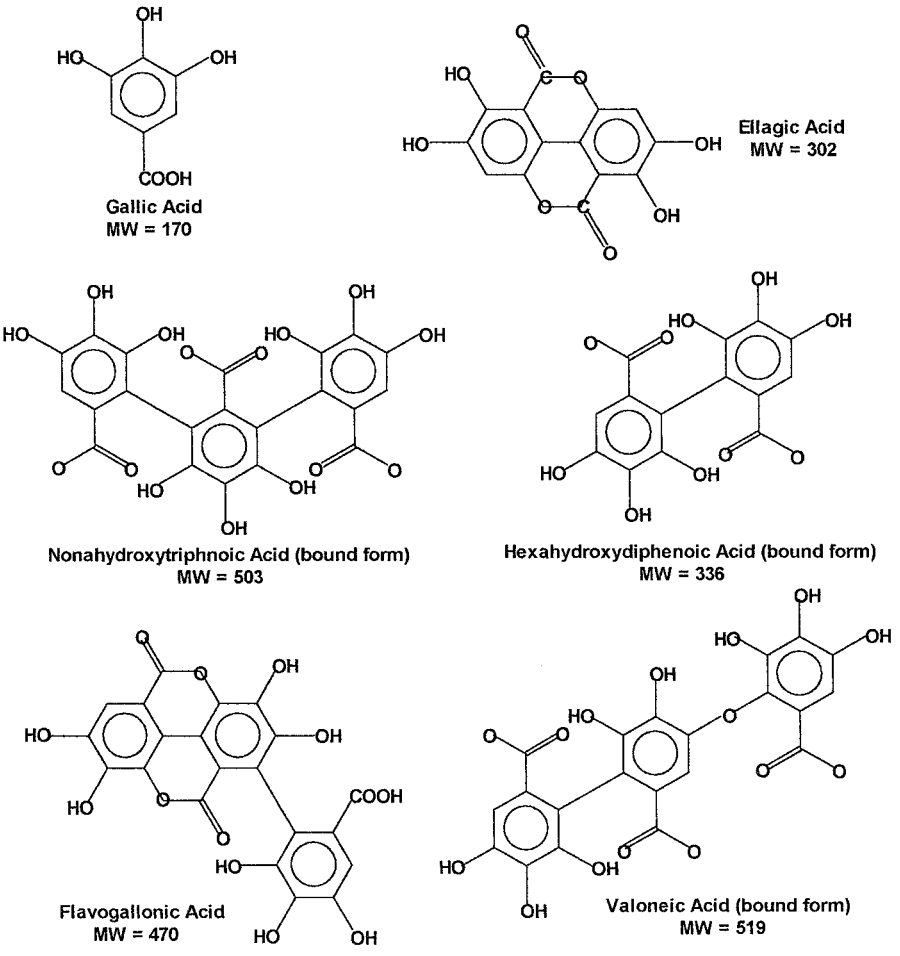


Figure 1 Chemical species characteristic of the low molecular weight fraction of hydrolyzable tannins (Tang et al., 1991[2]).

MALDI-TOF-MS

The spectra were recorded on a Kratos Kompact MALDI 4 instrument (Kratos Analytical Instruments, Ramsey, NJ). The irradiation source was a pulsed nitrogen laser with a wavelength of 337 nm. The length of one laser pulse was 3 ns. The measurements were carried out using the following conditions: positive polarity; linear flight path; high mass (20 kV acceleration voltage); 100–150 pulses per spectrum. The delayed extraction technique was used, applying delay times of 200–800 ns.

MALDI-TOF Sample Preparation

The samples were dissolved in acetone (4 mg/mL). The sample solutions were mixed with an acetone solution (10 mg/mL acetone) of the matrix, for which 2,5-dihydroxy benzoic acid was used. For the enhancement of ion formation NaCl was added to the matrix. The solutions of the sample and the matrix were mixed in equal amounts and 0.5 to 1 μL of the resulting solution were placed on the MALDI target. After evaporation of the solvent the MALDI target was introduced into the spectrometer.

RESULTS AND DISCUSSION

To evaluate the differences that can be seen by MALDI-TOF between different types of hydrolyzable tannins, that is, ellagitannins and gallotannins (the latter of which, in their polygallic form, are also often simpler oligomeric structures derived by the polymerization of gallic, digallic, and

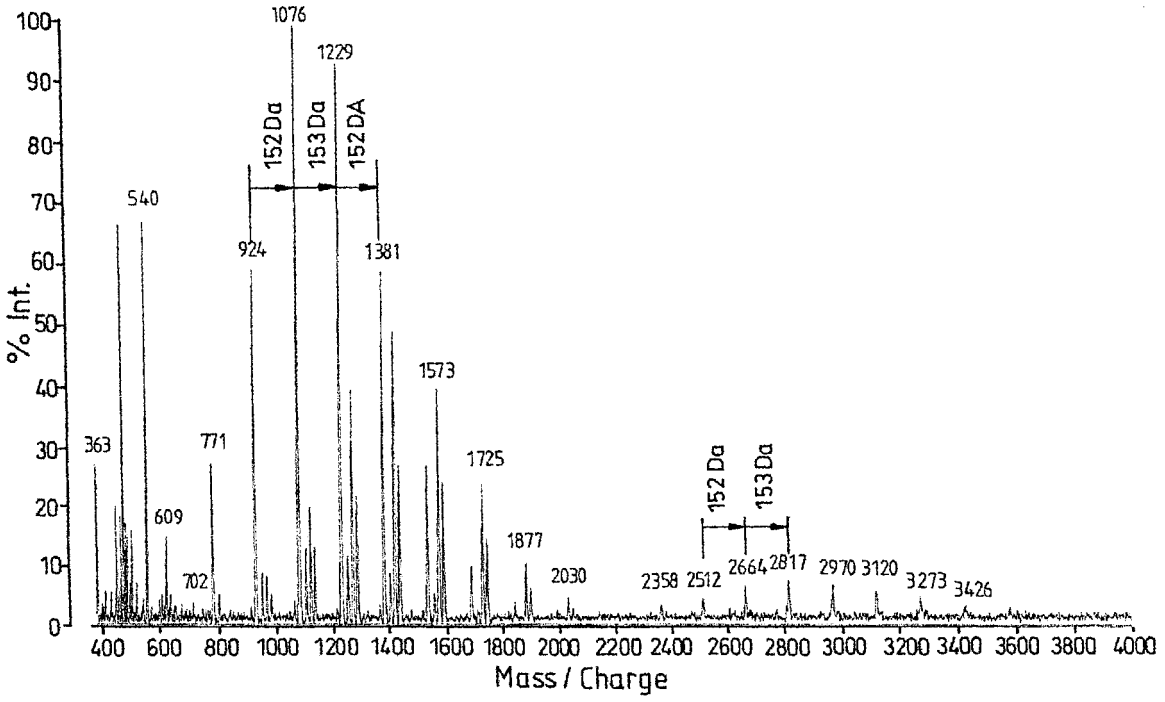
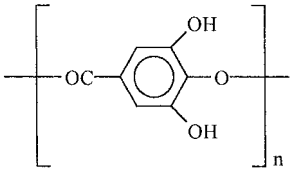


Figure 2 MALDI mass spectrum of natural sumach tannin extract.

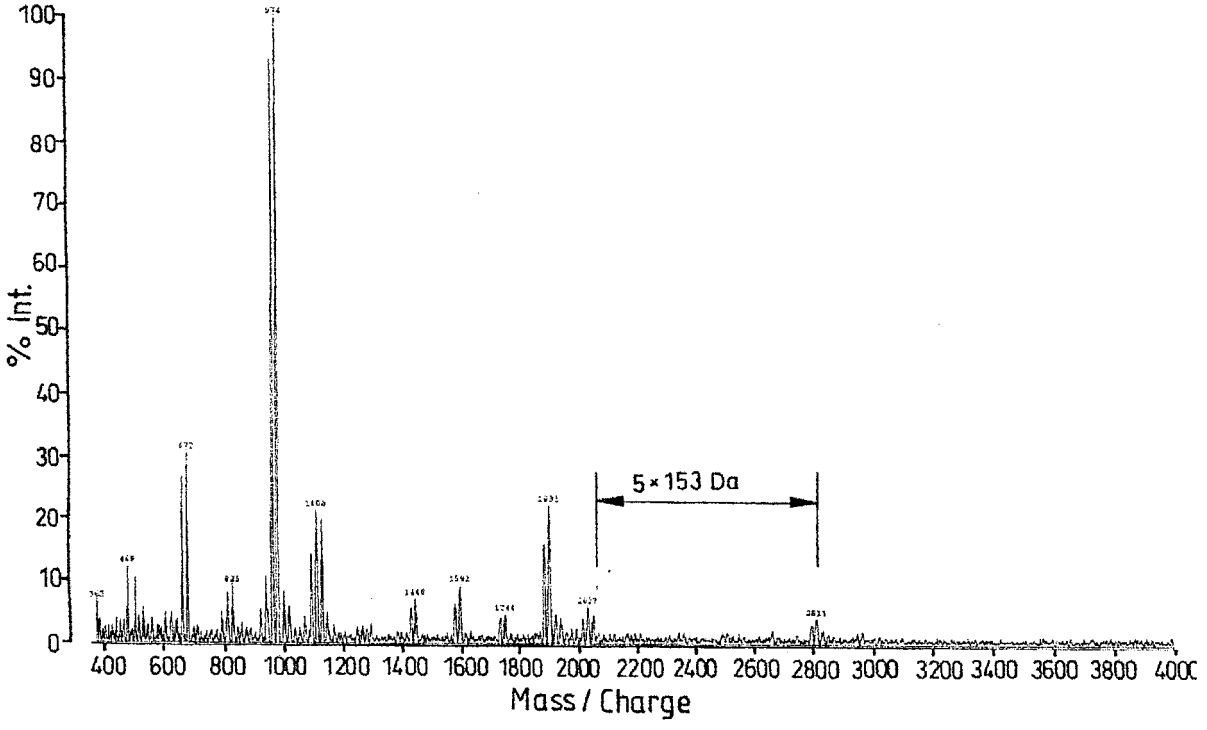
ellagic acids), two hydrolyzable tannins were also investigated under the same conditions, that is, (1) sumach tannin extract, a polygallotannin, and (2) the main commercial type of hydrolyzable tannin, chestnut wood tannin extract, an ellagitannin.

Sumach tannin extract is a polygallic tannin and its MALDI-TOF spectrum shows a major peak series exhibiting a mass increment of 152 Da (Fig. 2), corresponding to what is known with regard to the structure of polygallic tannins with the repeat unit being (**III**).[13–15]

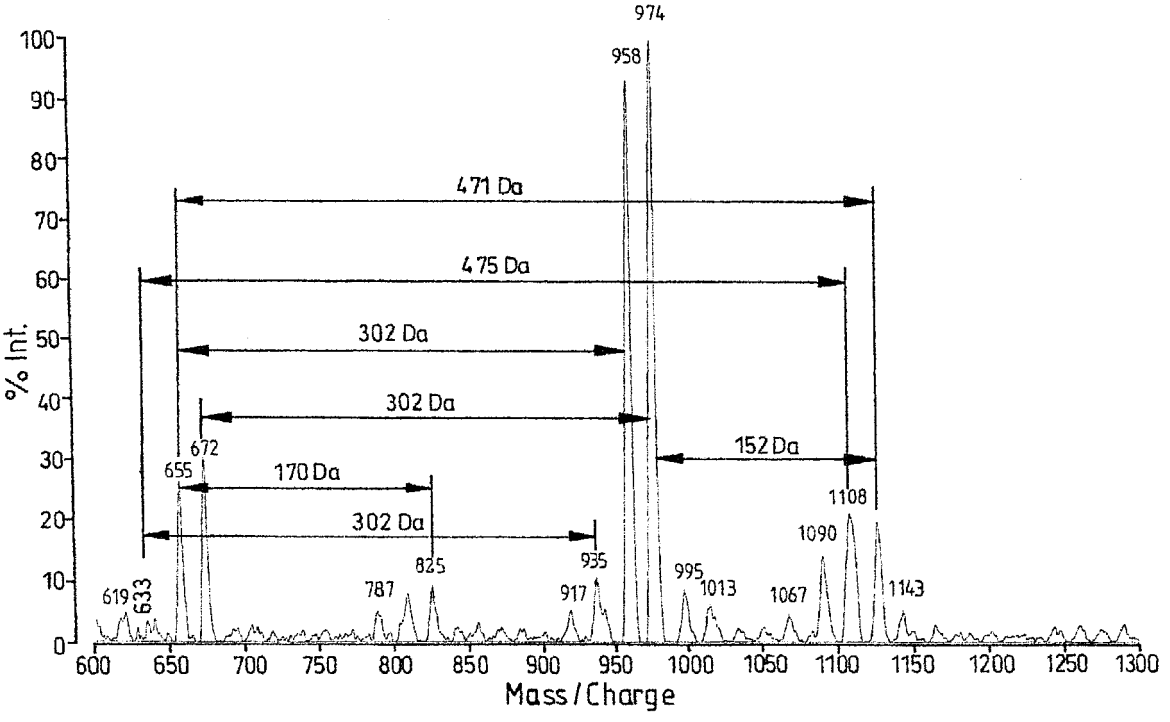


(**III**)

The most interesting result of the two hydrolyzable tannins is the spectrum obtained for the chestnut tannin extract [see Fig. 3(a)–(c)]. Two main patterns can be distinguished. The first one, attributed to the relatively lower-abundance series of peaks 935–1877–2811, is a series of pentagalloylglucose oligomers, the peaks of which are those corresponding to the masses of pentagalloylglucose monomer, dimer, and trimer, respectively. The structure of pentagalloylglucose trimers and dimers, which are oligomeric compounds formed by the tree in an enzymatically catalyzed manner, although already found and elucidated by ^{13}C-NMR for euphorbia tannins,[16,17] is known, although it is the first time that these oligomers have been shown to exist also in chestnut tannin. The structure of the chestnut pentagalloylglucose trimer (**IV**) is:

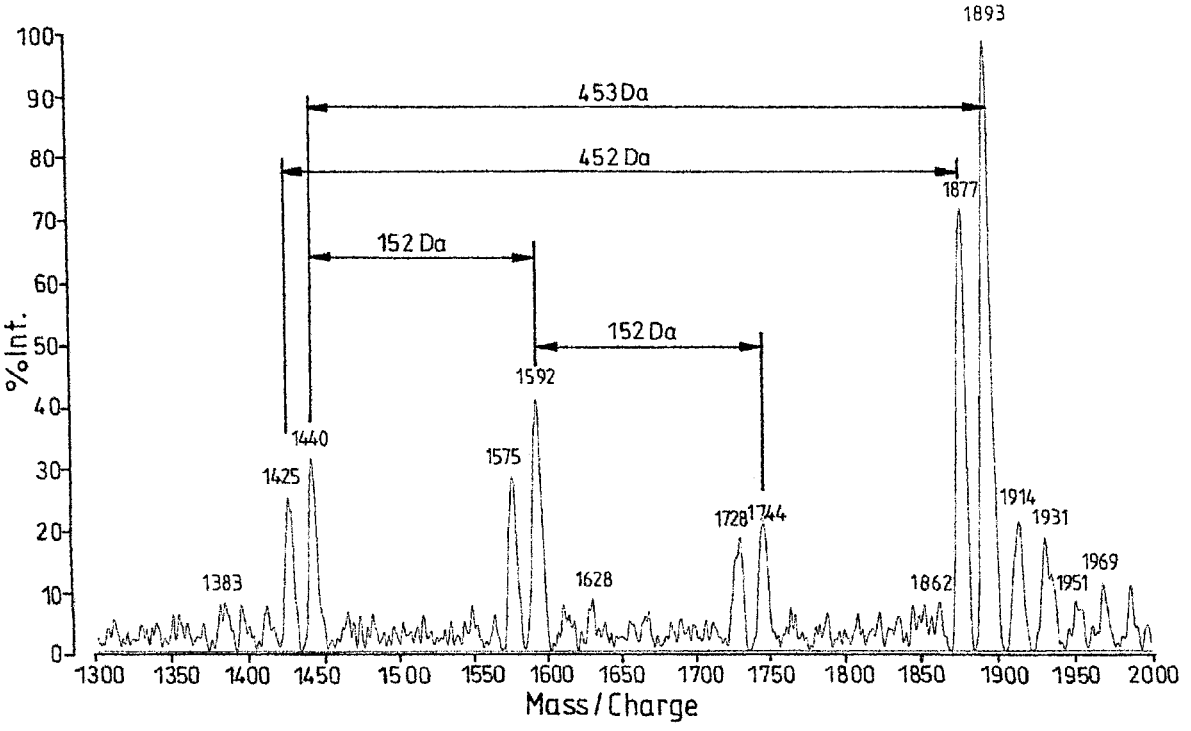


(a)



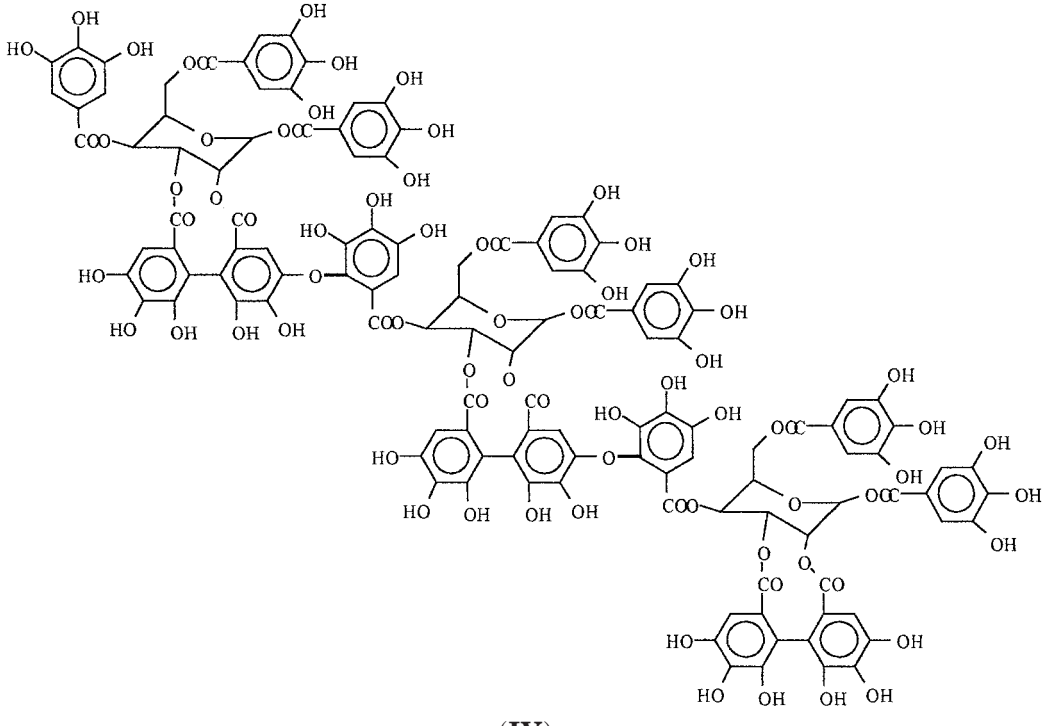
(b)

Figure 3 MALDI mass spectrum of (a) natural commercial chestnut tannin extract. (b) Details of the 600- to 1300-Da range with indication of the relevant mass fragmentation patterns. (c) Details of the 1300- to 2000-Da range with indication of the relevant mass fragmentation patterns and 152- and 302-Da repeat units.



(c)

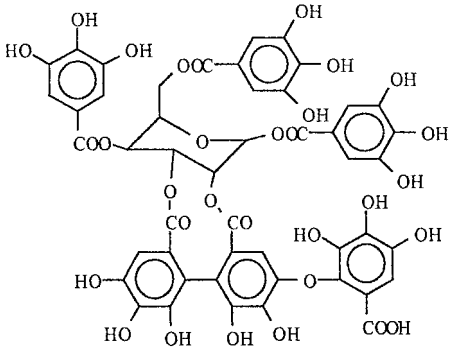
Figure 3 (*Continued*)



(**IV**)

The pattern is completed by the 1893-Da peak, slightly more prominent than the 1877-Da peak, consisting of a pentagalloylglucose dimer presenting an additional hydroxy group.

The second and dominant pattern is more complex. In Figure 3(b) one can note a pure vescalin/castalin peak at 633 Da and the rather noticeable peak at 935 Da, corresponding to both pentagalloylglucose but also pure castalagin/vescalagin. The 655 and 672 peaks are obtained from the 958- and 974-Da peaks by loss of an ellagic acid structure. The major peaks at 958 and 974 are, respectively, the 935-Da castalagin or pentagalloylglucose to which has been added a C—O—C grouping from a group that has split off (974) (**V**) and a 935 Da to which, again, a C—O—C remains attached and an –OH has been subtracted (958).



(**V**)

This means that pentagalloylglucose, castalagin, and vescalagin, although reputed to be the main known constituents of chestnut extract (there are more important unknown constituents[2]) are, rather, the more stable products derived from the degradation of a larger structure. The 1090 (1087) peak corresponds to either (1) a castalagin (935 Da) structure added by a gallic acid residue that esterifies the only free alcoholic –OH group of the structure [at the oxygen of the higher sugar carbon (C1) in the figure] or equally, but most likely, (2) a monomeric pentagalloylglucose added by a gallic acid residue that esterifies one of the gallic residues of pentagalloylglucose: it is thus a degradation product of a pentagalloyl glucose dimer, yielding a pentagalloyl + 1 gallic fragment (1090) and a smaller fragment of a tetragalloylglucose (the smaller peak of which is found at 787 Da) and smaller fragments. The 1108-Da peak is the same but more likely presents an –OH group. The 1126-Da peak is the 974-Da structure more likely added by gallic acid. The 1108-Da peak is a castalagin or pentagalloylglucose bound to a further gallic acid residue that presents an added –OH group on the structure.

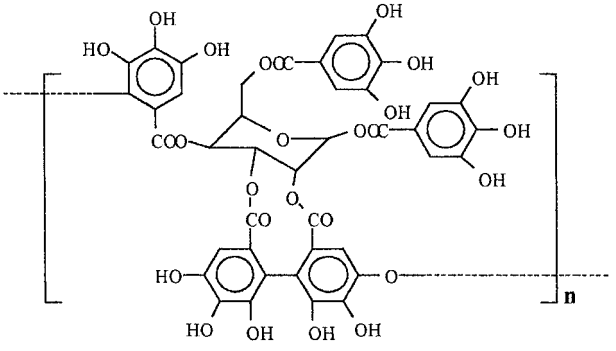
One can proceed in this series with the peak at 1440 Da corresponding to a castalagin/pentagalloylglucose bound to a nonahydroxytriphnoic acid residue through a single ester bond [Fig. 3(a) and (c): 935 + 503 + 2 = 1440]. Addition of a further ester-bound gallic acid residue (+152 Da) yields the peak at 1592. Addition to the same structure of a single bound ellagic acid residue yields the peak at 1893 Da, with the intermediate 1744 Da peak attributed to the addition of just a single, ester-bound gallic acid residue. There are further clear structures as the peak at 2046 (a further bound gallic acid residue, obviously on a pentagalloylglucose dimer structure) and more important and more significantly at 2811 Da, which is the consequence of the addition of 5 other 153 gallic acid residues, to yield the pentagalloylglucose trimer already shown.

It is rather likely that in this polygallic acid residues network even more complex and longer chains, based on a pentagalloylglucose repeat unit, exist in the wood itself. In the commercial extract, obtained by radical hydrolysis of the original material in wood, only up to trimers are observed by MALDI analysis.

Examination of the spectrum at higher masses starts to give the first indication of what is likely to be the type of structure that exists: the commercial chestnut ellagitannin is composed of a high proportion of pentagalloylglucose trimer, dimer, and all sorts of degradation and oxidation products thereof. The evident presence of flavogallonic residues linked within some of the higher molecular weight fragments indicates that extraction-induced degradation leads readily to internal molecular rearrangements of the fragments creating flavogallonic acid, ellagic acid, and nonatriphnoic acid structures. It is also evident that castalagin and vescalagin are apparently readily obtained by internal rearrangements of fragments obtained by higher molecular weight structures. That this is the case is shown by the fact that the dominant MALDI peaks of the whole extract are indeed the castalagin/vescalagin ones added by a few residual atoms, thus of slightly greater mass than that of castalagin/vescalagin, derived by internal rearrangements of a larger molecule. Thus, as suspected, the castalagin/vescalagin structure is simply the most stable one obtained by degradation of the original mo-

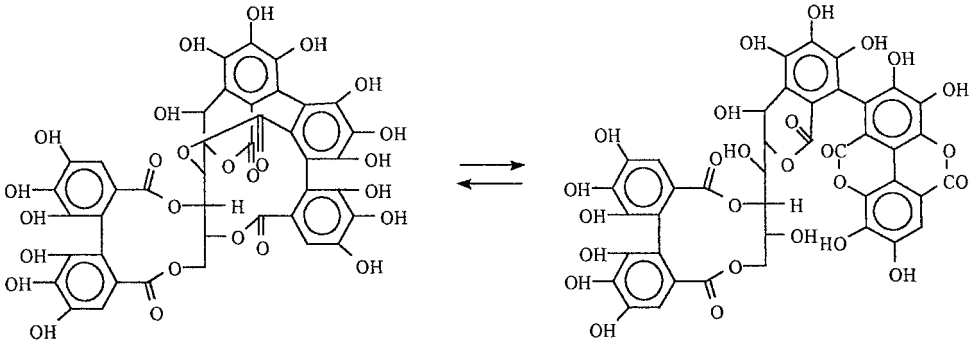
lecular species present in the timber. Of course, the mechanism appears in reverse in the fractionation generated by MALDI.

From the visualization of the mechanism from the above formula it starts to become apparent why it has proved impossible to isolate as a single compound the major constituent of chestnut tannin (up to 60% of chestnut tannin). This is the case because the component is nothing other than a very high molecular weight random series of pentagalloylglucose oligomers, situated within the wood, and of undefined number-average molecular mass, presenting the generalized structure (**VI**), a type of structure already elucidated by ^{13}C-NMR for other hydrolyzable tannin oligomers[16,17]:



(**VI**)

The component in the tannin extract itself is formed by a series of degradation products, the greater number-average degree of polymerization of which is of 3 in the sample examined. It is also understandable how flavogallonic acid and ellagic acid structures within molecules of higher molecular weight are formed by internal rearrangement of some of the degradation products that form, induced by the extraction of the tannin itself. Thus the difference of 452 Da between the 1893 and 1440 peaks and the same difference between the 1877 and 1425 peaks indicate clearly the presence of heavily linked flavogallonic acid residues formed by internal rearrangement within a larger fragment structure. For flavogallonic acid residues the same is true for the peak differences 1126–655 Da and 1108–633 Da, and for ellagic acid residues for the differences 974–672 Da and 958–655 Da, for linked residues formed by internal rearrangement within larger fragment structures. Flavogallonic acid residues, alone, are also clearly observed [469 Da, Fig. 3(a)], indicating the relative ease of such a rearrangement. Equally, the 1592-Da peak can belong only to a castalagin or pentagalloylglucose to which are linked not only an additional gallic acid residue but also a nonahydroxytriphnoic acid residue (935 + 153 + 503 Da) (Fig. 1), indicating that even nonahydroxytriphnoic acid is produced by an internal rearrangement, although that is perhaps slightly less common. The rearrangement mechanism can be easily visualized between pentagalloylglucose structures shown above and castalagin/vescalagin as well as within a pentagalloylglucose structure and within the castalagin/vescalagin structures, the latter, for example, as in (**VII**) and (**VIII**):



(**VII**) (**VIII**)

CONCLUSIONS

MALDI-TOF analysis appears to confirm previous knowledge about the generalized structures involved in chestnut ellagitannin and to give some further insights in its macromolecular nature both *in situ* in the wood as well as after extraction in the commercial tannin extract. The

rather novel picture of chestnut wood tannin, *in situ* in the wood, which emerges from the MALDI-TOF analysis is that of a pervasive and extended random tridimensional macromolecular chains network, perhaps not dissimilar to the morphological wood-encrusting appearance of a lignin, present in the wood and capable of being extracted exclusively by its degradation, a degradation that is possible only because of the susceptibility to hydrolysis of the ester bridges holding the network together. This situation is very different from what occurs instead in condensed polyflavonoid tannins. Internal rearrangements of the fragments formed by the extraction appear to occur readily and with ease, to yield a variety of structures characterized by the presence of ellagic acid residues, flavogallonic acid residues, and also, but less readily, nonahydroxytriphnoic acid residues. The other main constituents of the commercial tannin extract, castalagin and vescalagin, are shown to be simply the more stable degradation plus internal rearrangement products derived from the hydrolysis of polypentagalloylglucose chains.

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