ELSEVIER

Polymer 42 (2001) 7531–7539

polymer

www.elsevier.nl/locate/polymer

MALDI–TOF mass spectrometry of polyflavonoid tannins

H. Pasch[a,*], A. Pizzi[b], K. Rode[a]

[a]Deutsches Kunststoff-Institut, Schlossgartenstr. 6, D-64289 Darmstadt, Germany
[b]ENSTIB, University of Nancy 1, 27 Rue du Merle Blanc, F-88000 Epinal, France

Received 24 January 2001; received in revised form 6 March 2001; accepted 11 March 2001

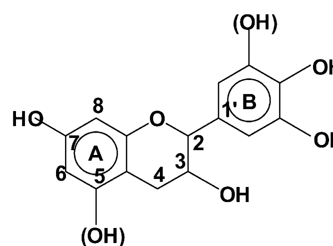
Abstract

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI–TOF) appears to be a suitable method for examining polyflavonoid tannin oligomers. It appears capable to determine aspects of the structure and characteristics of polyflavonoid tannins, which are otherwise too difficult to determine by other techniques. It has been possible to determine by MALDI–TOF for the two major industrial polyflavonoid tannins which exist, namely mimosa and quebracho tannins, and some of their modified derivatives that: (i) mimosa tannin is predominantly composed of prorobinetinidins while quebracho is predominantly composed of profisetinidins, that (ii) mimosa tannin is heavily branched due to the presence of considerable proportions of 'angular' units in its structure while quebracho tannin is almost completely linear. These structural differences also contribute to the considerable differences in viscosity of water solutions of the two tannins. (iii) the interflavonoid link is more easily hydrolysable, and does appear to sometime hydrolyse in quebracho tannin and profisetinidins, partly due to the linear structure of this tannin, and confirming NMR findings that this tannin is subject to polymerisation/depolymerisation equilibria. This showed that the decrease of viscosity due to acid/base treatments to yield tannin adhesive intermediates does also depend in quebracho from a certain level of hydrolysis of the tannin itself and not only of the carbohydrates present in the extract. This tannin hydrolysis does not appear to occur in mimosa tannin in which the interflavonoid link is completely stable to hydrolysis. (iv) sulphitation has been shown to influence the detachment of catechol B-rings much more than pyrogallol-type B-rings. (vi) the distribution of tannin oligomers, and the tannins number average degree of polymerisation obtained by MALDI–TOF appear to compare well with the results obtained by other techniques. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: MALDI; Mass spectrometry; Polyflavonoids

1. Introduction

Polyflavonoid tannins are natural polyphenolic materials which can be hardened both by reaction with formaldehyde [1] or by induced autocondensation [2]. They have now been used for over 20 years as industrial thermosetting tannin-formaldehyde adhesives for wood products [1]. Industrial polyflavonoid tannin extracts are mostly composed of flavan-3-ols repeating units, and smaller fractions of polysaccharides and simple sugars. Two types of phenolic rings having different reactivities with formaldehyde are present on each flavan-3-ol repeating unit, namely A-rings and B-rings, with each repeating unit being linked 4,6 or 4,8 with the units, which precede and follow it.



Recently, the radical and ionic mechanisms of the reaction of autocondensation and networking to hardened resins of polyflavonoid tannins induced by bases and by weak Lewis acids has been described [2–8]. The application of such a reaction for the application to wood adhesives of tannins hardened without the use of an aldehyde has also been examined [9]. The autocondensation reaction of tannins to yield resins was found to contribute under any set of circumstances to both network formation as well as increased solution viscosity and holds some interest as

* Corresponding author. Tel. +49-6151-163407; fax: +49-6151-29-2855.

E-mail address: hpasch@dki.tu-darmstadt.de (H. Pasch).

0032-3861/01/$ - see front matter © 2001 Elsevier Science Ltd. All rights reserved.
PII: S0032-3861(01)00216-6

bonded wood products which do not emit formaldehyde (as none has been added) can be produced [9,10]. Different polyflavonoid tannins however present different structures and different average molecular masses, and as a consequence often present peculiarly different behaviour in their application [10]. The most common method of examination of the relative structures of polyflavonoid tannins, and of their differences, is by ^{13}C NMR [10]. Since its introduction by Tanaka [23] and Karas and Hillenkamp [11], Matrix-Assisted Laser Desorption/Ionization (MALDI) mass spectrometry (MS) has greatly expanded the use of mass spectrometry towards large molecules and has revealed itself to be a powerful method for the characterization of both synthetic and natural polymers [12–17]. 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 analysed in a mass spectrometer. This soft ionization technique is mostly combined with time-of-flight (TOF) mass analysers. This is so as TOF–MS present the advantage of being capable to provide a complete mass spectrum per event, for its virtually unlimited mass range, for the small amount of analyte necessary and the relatively low cost of the equipment.This paper investigates how useful as a tool in defining polyflavonoid tannin structures is Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI–TOF) MS and compares the results obtained for the most common industrial polyflavonoid condensed tannins when using this technique.

2. Experimental

2.1. Samples

Four types of commercial flavonoid tannin extracts were used, namely (i) Pecan (*Carya illinoensis*) nut membrane tannin extract, from the USA (ii) Mimosa (*Acacia mearnsii*, formerly *mollissima*, de Wildt) bark tannin extract from Brazil (iii) natural Quebracho (*Schinopsis balansae*) wood tannin extract, from Argentina, and (iv) Quebracho tannin extract modified for use in wood adhesives according to procedures already reported [18], (v) Quebracho tannin extract modified by solvent extraction to complete eliminate the carbohydrate fraction of the extract.

2.2. MALDI–TOF–MS

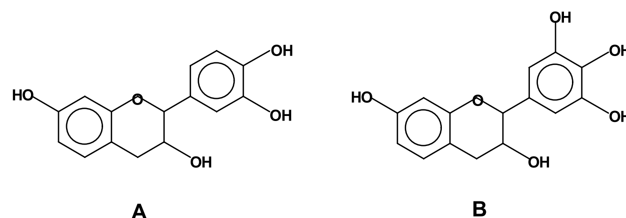
The spectra were recorded on a KRATOS Kompact MALDI 4 instrument. The irradiation source was a pulsed nitrogen laser with a wavelength of 337 nm. The length of one laser pulse was 3 ns. The measurements were carried out using the following conditions: polarity-positive, flight path-linear, mass-high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

2.3. 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. As the matrix 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 0.5–1 μl of the resulting solution were placed on the MALDI target. After evaporation of the solvent the MALDI target was introduced into the spectrometer.

3. Results and discussion

The profisetinidin/prorobinetinidin type of polyflavonoid tannins are the most common extracted industrially [1]. Quebracho tannin and Mimosa tannin are the two main exponents of this class. Quebracho gave clear spectra showing the degree of polymerization of the building units and oligomer series with masses of the repeat units of 272.3 and 288.3 Da (Fig. 1a; Table 1). For each oligomer, substructures with mass increments of 16 Da appear, indicating different combinations of various substructures. As quebracho is mainly based on combinations of resorcinol, catechol and pyrogallol building blocks the following monoflavonoids and their oligomers can be expected to be present:



The masses of units A and B are 274.3 and 290.3 Da, respectively. Combinations of these masses can be used to calculate the masses of the oligomer peaks in the spectra according to the expression $M + Na^+ = 23.0(Na) + 2.0$ (endgroups, $2 \times H$) + 272.3A + 288.3B (Table 1). As can be seen in the spectra, there are more peak series, which are due to different endgroups. They have the same repeat units, for example 683–956 and 1555–1827 Da in Fig. 1. The peak at 683 is very near to a result of 688 Da which would be obtained by the loss of both a B-ring plus the three-carbons chain from the heterocycle of the lower terminal repeat unit, be this of type B or of type A, to yield two flavonoid units linked to a resorcinol phenoxy anion.

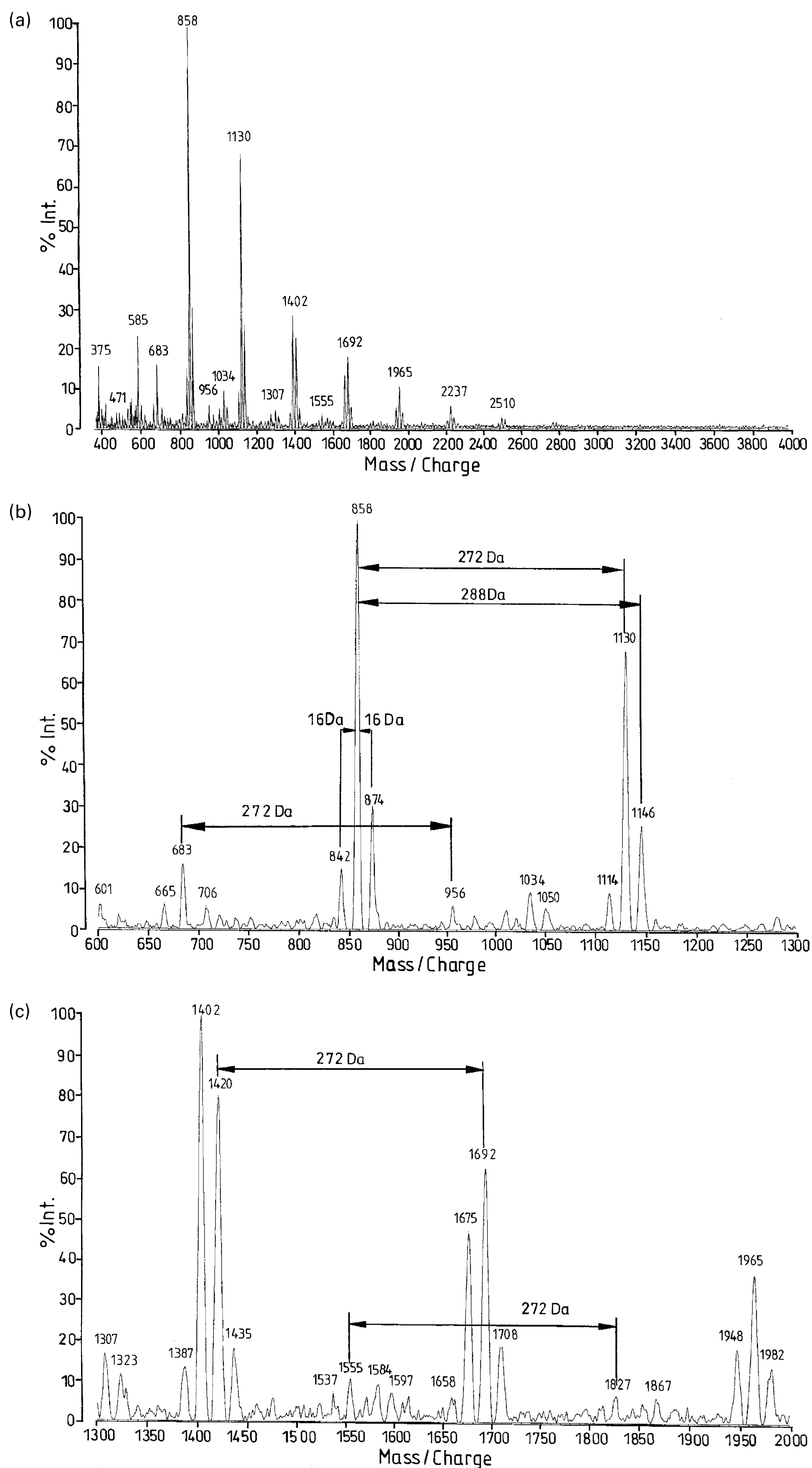
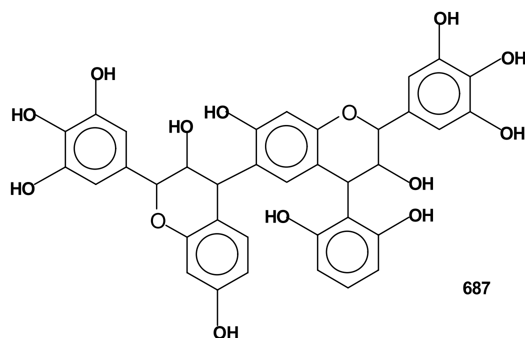


Fig. 1. MALDI mass spectrum of: (a) natural sulphited quebracho tannin extract; (b) details of the 600–1300 Da range with indication of the relevant 272 Da repeat unit; (c) details of the 1300–2000 Da range with indication of the relevant 272 Da repeat unit.

Table 1
MALDI peaks for industrial quebracho tannin extract. Note that the predominant repeat units in this tannin is 272 Da, indicating that this tannin is predominantly a profisetinidin.

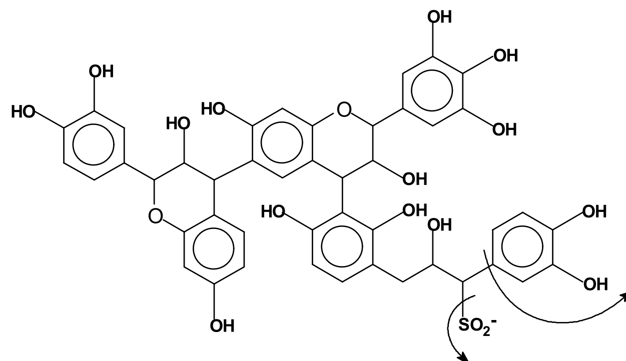
$M + Na^+$	$M + Na^+$	Unit type	
Experimental	Calculated	A	B
Dimers			
585	585.6	1	1
601	601.6	–	2
Trimers			
842	841.9	3	–
857[a]	857.9	2	1
874	873.9	1	2
Tetramers			
1114	1114.2	4	–
1130[a]	1130.2	3	1
1146	1146.2	2	2
Pentamers			
1387	1386.5	5	–
1402[a]	1402.5	4	1
1420	1418.5	3	2
1435	1434.5	2	3
Hexamers			
1658	1658.8	6	–
1675	1674.8	5	1
1692[a]	1690.8	4	2
1708	1706.8	3	3
Heptamers			
1948	1947.1	6	1
1965[a]	1963.1	5	2
1982	1979.1	4	3
Octamers			
2237[a]	2235.4	6	2
Nonamers			
2510[a]	2507.7	7	2
Decamers			
2782[a]	2780.0	8	2
2798	2796.0	7	3

[a] Dominant oligomer.



The peak at 585 Da is also explained by the presence of a dimer according to the same equation above composed of a A-unit plus a B-unit plus 2H endgroups plus Na^+. The peak at 375 Da is obtained from the 585 Da dimer by elimination of a catecholic B-ring (585−110 = 375).

There is however an alternate, and more correct explanation for the 683 Da peak. Industrial quebracho tannin extract is sulphited/bisulphited, which introduces a sulphite or sodium sulphite group on the C1 of the flavonoid structure and causes the opening of the heterocycle ring. Thus, if one of the flavonoid units of a 857 Da trimer loses its catechol B-ring (−110) from a type A repeat unit as well as the $-SO_2^-$ group (−64), the 683 Da signal is obtained.



This is more likely as the introduction of the $-SO_2^-$ group should favour under certain conditions the elimination of the B-ring of the unit. The origin of the much smaller 665 Da peak is the same but by elimination of the $-SO_2H$ group (−65) and of a pyrogallol B-ring (−126) from a type B repeat unit. The fact that the intensity of the 665 Da peak is considerably lower than that of the 683 Da peak indicates the novel finding that as a consequence of sulphitation catechol rings appear to be much more easily detached than pyrogallol ones from a flavonoid unit. That the 683 peak is caused by the presence of the sulphonic group on C1 and the relative ease of decomposition indicated above is shown by the fact that in mimosa tannin which in general is not sulphited the 683 Da peak does not exist while presenting a very small peak at 687 Da which come from the first explanation (Fig. 2a).

Also of interest are the existence of peaks at 1965, 2237, 2510 and 2800 Da for commercial quebracho tannin, these representing, respectively, heptamers, octamers, nonamers and decamers (Fig. 1). Tannins are not easily water soluble at this higher molecular weight and thus it is of interest to find definite proof of the existence of such higher molecular weight oligomers in a commercial tannin extract. The sample in question had been found by ^{13}C NMR to have a number average degree of polymerization of 6.74 [19,20] which appears to confirm the existence of such higher molar mass oligomers in this commercial tannin extract. The same type of pattern is obtained for solvent purified commercial quebracho extract (MALDI spectrum not reported here), in which all the carbohydrates have been eliminated, confirming that the patterns observed are really due to the polyflavonoid components of the tannin extract. It is, however, of interest to note that the tannin extract which

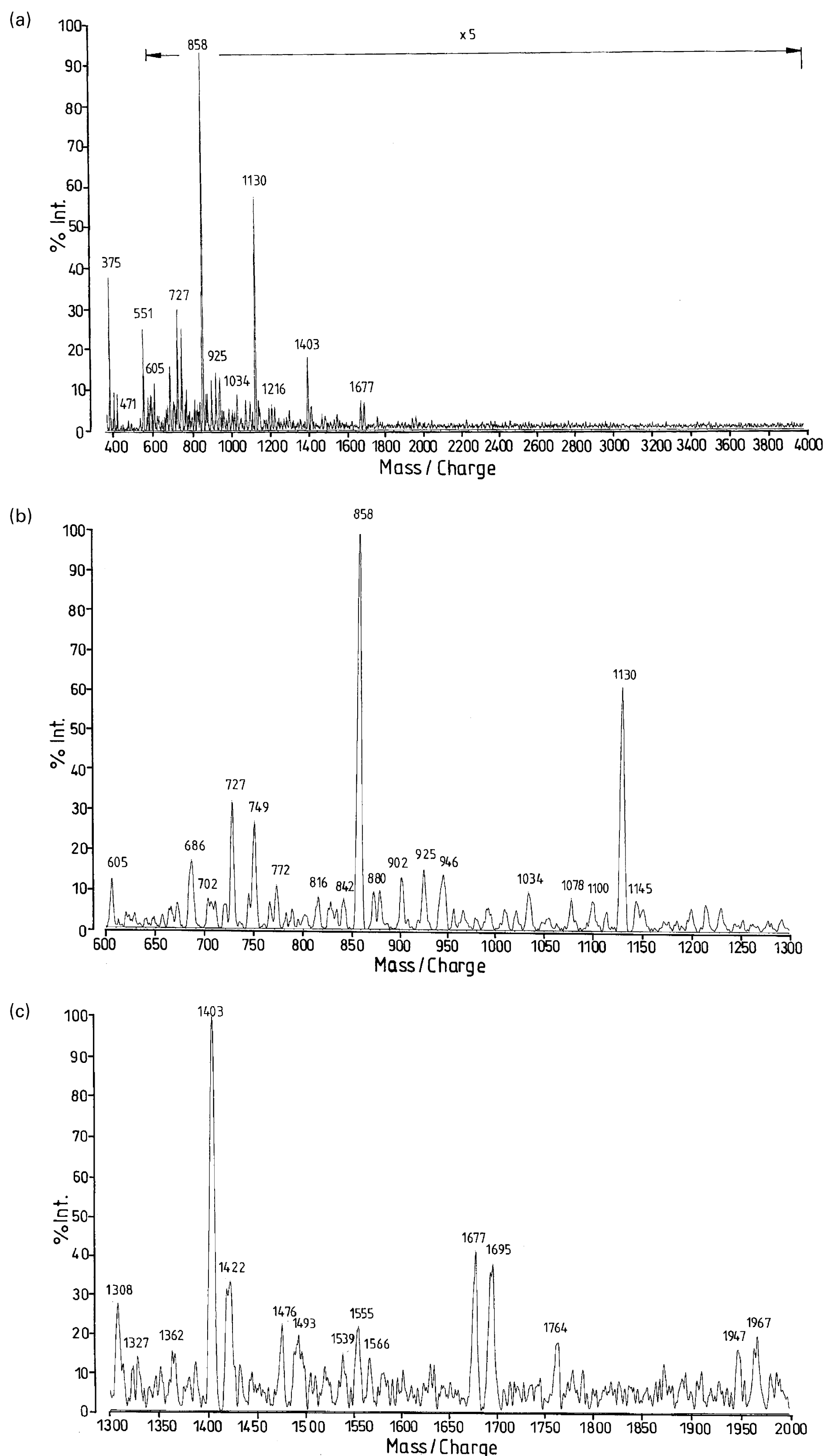
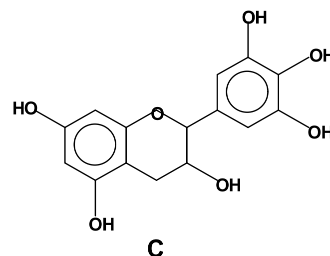


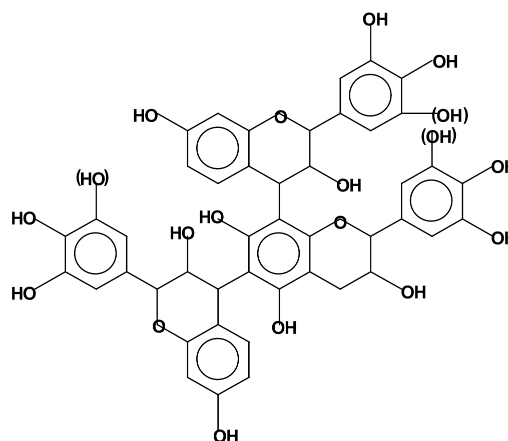
Fig. 2. MALDI mass spectrum of: (a) acid/base treated modified quebracho tannin extract; (b) details of the 600–1300 Da range; (c) details of the 1300–2000 Da range.

has undergone an acid/base treatment [18] to obtain an adhesive intermediate gives at best a pentamer at 1967 Da, see Fig. 2. This is accompanied by a considerable increase in the proportion of the 858 Da trimers, of the 727 degraded trimers (two flavonoid units + one A-ring + its C4), of degradation product composed of a single flavonoid unit linked to a single A-ring of another flavonoid unit (375 Da) and also an increase in the 1130 Da tetramers confirming that the treatment to yield a tannin adhesive intermediate clearly induces some level of hydrolysis of the interflavonoid bond and hence some level of depolymerization in quebracho tannin. This confirms previous findings [6–8,18] obtained by ^{13}C NMR that contrary to what widely thought the interflavonoid bond in the profisetinidins/prorobinetinidins of quebracho tannin are fairly labile and that this particular type of tannin can be subject to some depolymerization. It also confirms what has been up to now only a suspicion, namely that the decrease in viscosity [18] of tannin solutions as a consequence of acid/base treatments is not only due to hydrolysis of the hydrocolloid polymeric carbohydrates present in the extract, but also to the decrease in degree of polymerization of the tannin itself, this at least in the case of quebracho tannin. It is also interesting to observe a definite, clear peak at 605 Da (see Fig. 2) which can only belong to a pure robinetinidin dimer (289 + 289 + 25 = 605), MALDI–TOF analysis appearing to indicate here that it is the interflavonoid inter-fisetinidin link, or at least links in which fisetinidin units are involved which appear to be more sensitive to cleavage. The acid/base treatment to produce a tannin adhesive intermediate involves the use of acetic anhydride or maleic anhydride for the acid hydrolysis phase. As the treatment is done in water solution but being the tannin extract strongly colloidal in nature a question of interest is to know if some of the tannin –OH groups have been acetylated within the micelles present in the solution and before the induced hydrolysis has drastically decreased the level of colloidality of the system. Past investigations by ^{13}C NMR and by other techniques [18] indicate that a certain amount (small) of acetylation appears also to occur, this being of importance in accelerating subsequently, on application, the polycondensation of tannins with aldehydes. MALDI–TOF appears to confirm this by the presence of small but detected peaks at 772 Da (in theory 769) and 902 Da (see Fig. 2), respectively, a flavonoid dimer and a flavonoid trimer both mono-acetylated.

The MALDI–TOF analysis of mimosa tannin extract (Fig. 3) indicates the presence in the tannin of oligomers to the maximum of octamers (2333 Da) in line with the lower number average degree of polymerzation of 4.90 obtained by other means for this tannin [19,20], and the distribution obtained is shown in Table 2. The flavonoid repeating units present in this tannin extract are of type A and B as for quebracho but with a relatively important proportion of units of type C.



The correct equation to calculate the different possibilities does then become $M + Na^+ = 23.0(Na) + 2.0$(endgroups, $2 \times H$) + 272.3A + 288.3B + 304.3C (Table 2). Table 2 indicates that many valid combinations of different repeating units are possible. There are however some cases in which unequivocal assignment of the structure can indeed be done. This is the case of angular tannins, namely oligomers in which a repeating unit of type C is bound through both its 6 and its 8 A-ring sites to A and B type units, with its C4 sites equally bound and unbound.



These structures were discovered by high temperature ^{1}H NMR on rotational isomers [1,21,22]. The MALDI–TOF analysis also shows clearly the existence of fragments of angular tannins by the presence of definite peaks at 906, 1195 and 1211 Da. Their presence in mimosa tannin extract, where it is known that angular tannins exist, underlines their total absence in the otherwise similar quebracho tannin extract. It is not possible to say with the data available if angular tannins are naturally absent in quebracho tannin extract or if their absence is the result of the fairly heavy sulphitation this tannin has always to undergo for solubility reasons. The high relative intensity of the very marked peaks of the angular trimer at 906 Da and of the angular tetramer at 1195 Da in Fig. 3 indicate that the frequence of angular structures in mimosa tannin extract is rather high. The lower viscosity of solutions of mimosa extract, much lower than solutions of quebracho tannin extract at equal concentration and under the same conditions, is not only due then to mimosa tannin lower number average degree of

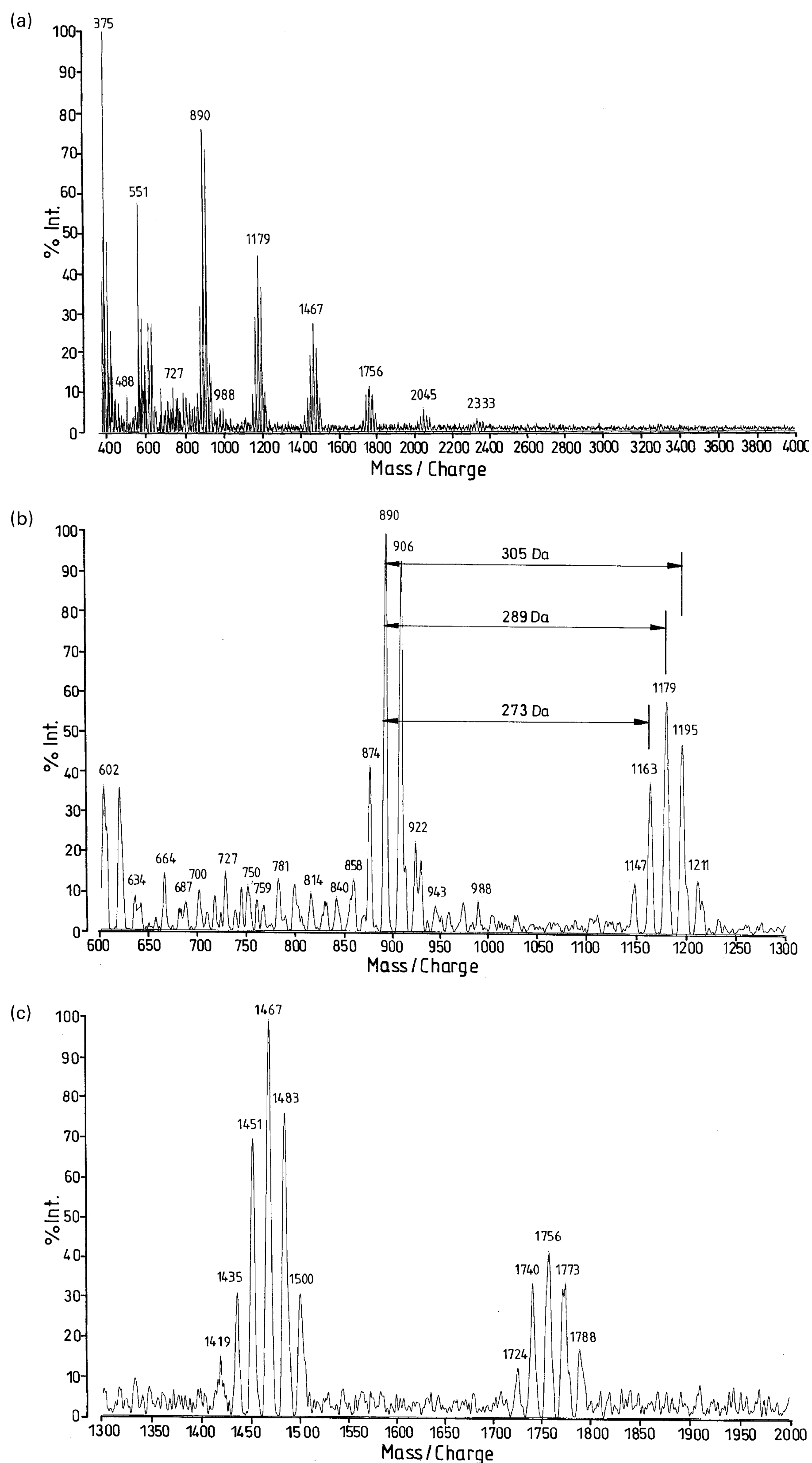


Fig. 3. MALDI mass spectrum of: (a) natural mimosa tannin extract; (b) details of the 600–1300 Da range with indication of the relevant 288 Da repeat unit; (c) details of the 1300–2000 Da range.

Table 2
MALDI peaks for industrial mimosa tannin extract. Note that the predominant repeat units in this tannin is 288 Da, indicating that this tannin is predominantly a prorobinetinidin

M + Na$^+$	M + Na$^+$		Unit type			
Experimental	Calculated		A	B	C	
Dimers						
602	601.6		–	2	–	
Trimers						
858	857.9		2	1	–	
874	873.9		1	2	–	
		or	2	–	1	Angular tannin
890[a]	889.9		1	1	1	
		or	–	3	–	
906[a]	905.9		–	2	1	Angular tannin
		or	1	–	2	Angular tannin
922	921.9		–	1	2	A diangular structure
Tetramers						
1147	1146.2		2	2	–	
		or	3	–	1	
1163	1162.2		1	3	–	
		or	2	1	1	
1179[a]	1178.2		–	4	–	
		or	1	2	1	
		or	2	–	2	
1195	1194.2		–	3	1	Angular tannin
		or	1	1	2	
1211	1210.2		–	2	2	Angular tannin
		or	1	–	3	A diangular structure
Pentamers						
1467	1466.5					
Hexamers						
1756	1754.8					
Heptamers						
2045	2043.1					
Octamers						
2333	2331.4					

[a] Dominant oligomer.

polymerization [19,20] but also to its more 'branched' structure as opposed to the fundamentally 'linear' structure of quebracho tannin. The susceptibility to hydrolysis of the interflavonoid bond of quebracho tannin remarked about above, in relation to the well-known total lack of it in mimosa tannin [2,18], could then be ascribed also to this conformational difference between the two otherwise similar profisetinidin/prorobinetinidin tannins. This observation is of importance indicating for the first time that the difference in spacial structure is one of the main contributing reasons why two tannins of fundamentally very similar chemical composition (they are both profisetinidins/prorobinetinidins) do behave rather differently under several aspects. In the case of mimosa tannin of even greater interest is the existence of a well definite and clear peak at 1211 Da: this is formed by 4 flavonoid repeating units two of which are of type C. If the sample was just a dimer one could claim that this was a procyanidin fragment, namely two C-type units linked 4,8 and conclude that a certain number of separate procyanidin units exists in mimosa tannin. That the fragment is instead a tetramer first of all negates that the phloroglucinol A-ring units can exist in mimosa tannin as separate procyanidins, but confirms that in this tannin such units are exclusively present reacted as an angular within the profisetinidin/prorobinetinidin predominant structures. Second, this fragment is clearly then a 'di-angular' unit never observed or isolated before, again confirming the high frequence of angular structures in mimosa tannin.

A further interesting difference between mimosa and quebracho tannins can be observed by comparing the results in Figs. 1 and 3 and Tables 1 and 2. In quebracho the predominant repeat unit has 272 Da (a type A unit), while in mimosa the predominant repeating unit is of 288 Da (a type B unit). This is particularly evident in the higher oligomers for the two tannins. Based on this, on the dominant fragments for different oligomers and on the relative intensities for the different peaks in Figs 1 and 3 it is possible to conclude that quebracho tannin is composed of between 20 and 30% of B units and of between 70 and 80% of A-type units. Quebracho is then predominantly a profisetinidin. Mimosa tannin instead is composed predominantly of between 50 and 70% of type B units and only of between 15 and 25% of type A units. Mimosa tannin is then predominantly a prorobinetinidin. It is also interesting to note that the number average degree of polymerization obtained from the MALDI-derived oligomer distributions yield values of 6.25 and 5.4 for quebracho and mimosa tannins respectively. Considering the variability of such natural materials, these values compare well with the DP_n values of 6.74 and 4.9 for the same tannins obtained by ^{13}C NMR and other techniques [19,20].

It is known from other MALDI–TOF experiments that mass discrimination may occur for higher molar mass oligomers. In this case, the observed intensity for these mass peaks is too low or they are not detected at all, thus lowering the calculated values of DP_n. To make sure that mass discrimination does not occur in the present experiments, a mixture of two polyethylene glycol calibration standards of M_w of 1,000 and 3,000 g/mol was measured under similar experimental conditions. For this mixture, mass discrimination of the higher molar mass oligomers was not observed. It was, therefore, assumed that for the present experimental set-up mass discrimination does not occur for oligomer masses up to about 4,500 Da.

A mainly prodelphinidin tannin, namely pecan nut tannin extract was also examined (Fig. 4). The main prodelphinidin repeat unit has a molar mass of 306 Da and the main fragments found arrived only up to trimers (304.3+304.3+288.3+2.0+23.0=921.9 Da). This unusual result leads to two consequences. In pecan nut tannin extract robinetinidin units are linked within the prodelphinidin main oligomers, and that the interflavonoid bond of

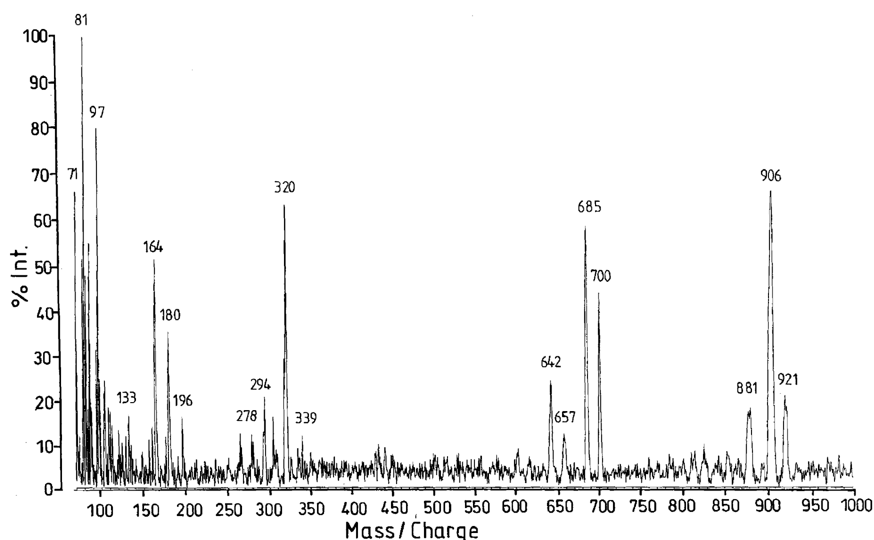


Fig. 4. MALDI mass spectrum of natural pecan nut tannin extract.

prodelphinidins is particularly prone to cleavage (this is known to be so). It means that the finding of trimers only, when the number average degree of polymeryzation of this tannin is known to be of 5.50 [19,20], indicates that the cleavage of the interflavonoid bond here is mainly a fabrication of the method of analysis used and if MALDI–TOF has to be used in this case much milder conditions need to be used.

4. Conclusions

In conclusion MALDI–TOF appears to be a suitable method for examining polyflavonoid tannin oligomers and it appears to be able to determine through this technique facts on polyflavonoid tannins which are already known by using other approaches. It also appears capable however to determine aspects of the structure and characteristics of the tannins which are too difficult to determine by other techniques. In the present investigation of the two major polyflavonoid tannins it has been possible to determine by MALDI–TOF that: (i) mimosa tannin is predominantly composed of prorobinetinidins while quebracho is predominantly composed of profisetinidins, that (ii) mimosa tannin is heavily branched due to the presence of considerable proportions of angular units in its structure while quebracho tannin is almost completely linear. These structural differences also contribute to the considerable differences in viscosity of water solutions of the two tannins, (iii) the interflavonoid link is more easily hydrolysable, and does appear to sometime hydrolyse in quebracho tannin and profisetinidins, partly due to the linear structure of this tannin, and confirming NMR findings that this tannin is subject to polymerisation/depolymerisation equilibrium. This is not the case for mimosa tannin in which the interflavonoid link is completely stable to hydrolysis, (iv) Sulphitation has been shown to influence the detachment of catechol B-rings much more than pyrogallol-type B-rings, (v) the distribution of tannin oligomers, and the tannins number average degree of polymerisation obtained by MALDI–TOF appear to compare well with the results obtained by other techniques.

References

[1] Pizzi A. Wood adhesives chemistry and technology, vol. 1. New York: Dekker, 1983.
[2] Meikleham N, Pizzi A, Stephanou A. J Appl Polym Sci 1994;54:1827.
[3] Pizzi A, Meikleham N, Stephanou A. J Appl Polym Sci 1995;55:929.
[4] Pizzi A, Meikleham N. J Appl Polym Sci 1995;55:1265.
[5] Merlin A, Pizzi A. J Appl Polym Sci 1996;59:945.
[6] Masson E, Merlin A, Pizzi A. J Appl Polym Sci 1996;60:263.
[7] Masson E, Pizzi A, Merlin A. J Appl Polym Sci 1996;60:1655.
[8] Masson E, Pizzi A, Merlin A. J Appl Polym Sci 1997;64:243.
[9] Pizzi A, Meikleham N, Dombo B, Roll W. Holz Roh Werkstoff 1995;53:201.
[10] Pizzi A. Advanced wood adhesives technology. New York: Dekker, 1994.
[11] Karas M, Bachmann D, Bahr U, Hillenkamp F. Int J Mass Spectrom Ion Processes 1987;78:53.
[12] Bahr U, Deppe A, Karas M, Hillenkamp F, Giessmann U. Anal Chem 1992;64:2866.
[13] Ehring H, Karas M, Hillenkamp F. Org Mass Spectrom 1992;27:472.
[14] Danis PO, Karr DE, Mayer F, Holle A, Watson CH. Org Mass Spectrom 1992;27:843.
[15] Danis PO, Karr DE. Org Mass Spectrom 1993;28:923.
[16] Pasch H, Resch M. GIT Fachz Lab 1996;40:90.
[17] Pasch H, Gores F. Polymer 1995;36:1999.
[18] Pizzi A, Stephanou A. J Appl Polym Sci 1994;51:2109.
[19] Thompson D, Pizzi A. J Appl Polym Sci 1995;55:107.
[20] Fechtal M, Riedl B. Holzforschung 1993;47:349.
[21] Botha JJ, Ferreira D, Roux DG. J Chem Soc, Chem Commun 1978:700.
[22] Pizzi A, Cameron FA, Eaton NJ. J Macromol Sci, Chem Ed 1986;A23(4):515.