Study on the Structure of Mangrove Polyflavonoid Tannins with MALDI-TOF Mass Spectrometry

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Received 16 November 2007; accepted 15 December 2007
DOI 10.1002/app.28135
Published online 11 April 2008 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry is a suitable method for examining polyflavonoid tannin oligomers as it is capable to determine aspects of their oligomeric structure and characteristics, which are otherwise too difficult to determine by other techniques. It has been possible to determine by MALDI-TOF for *Rhizophora apiculata* mangrove polyflvonoid tannins that: (i) procyanidins oligomers formed by catechin/epicatechin, epigallocatechin, and epicatechin gallate monomers are present in great proportions; (ii) oligomers, up to nonamers, in which the repeating unit at 528–529 Da is a catechin gallate dimer that has lost both the gallic acid residues and an hydroxy group which are the predominant species; (iii) oligomers of the two types covalently linked to each other also occur. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 109: 963–967, 2008

Key words: MALDI; mass spectrometry; polyflavonoids; tannins; structure; structural composition

INTRODUCTION

Polyflavonoid tannins are natural polyphenolic materials, which can be used for a variety of industrial applications.[1] Industrial polyflavonoid tannin extracts are mostly composed of flavan-3-ols repeating units, and smaller fractions of polysaccharides and simple sugars. Recently, mangrove polyflavonoid tannins have been promoted both as the basis of corrosion inhibiting varnishes on metals[2] as well as heavy metal complexating agents for pollution control in water[3] as they constitute an important, potential industrial resource in several countries in South East Asia. The structures of the main monomers constituting the tannin oligomers were identified, these being catechin, epicatechin, epigallocatechin, and epicatechin gallate.[2,4] However, different polyflavonoid tannins present different structures, different average molecular mass distribution, and different degrees of polymerization[5] and nothing of these is known in the case of mangrove tannins.

Since its introduction by Karas et al.[6] in 1987, matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) has greatly expanded the use of MS towards large molecules and has revealed itself to be a powerful method for the characterization of both synthetic and natural polymers.[7–12] 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. 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. Its usefulness in the elucidation of the distribution of different oligomers in the case of polyflavonoid tannins in general has already been demonstrated.[5] This study investigates the structure distribution and composition as well as the degree of polymerization of mangrove polyflavonoid tannins by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS.

EXPERIMENTAL

Samples

Mangrove (Bakau minyak: *Rhizophora apiculata*) bark from Malaysia was obtained as a waste from the Larut Matang, Taiping, malaysian charcoal manufactur of charcoal from mangrove wood. The bark was dried and ground to 250 mesh followed by further drying until a constant weight was obtained. The extraction of tannin from the mangrove bark was

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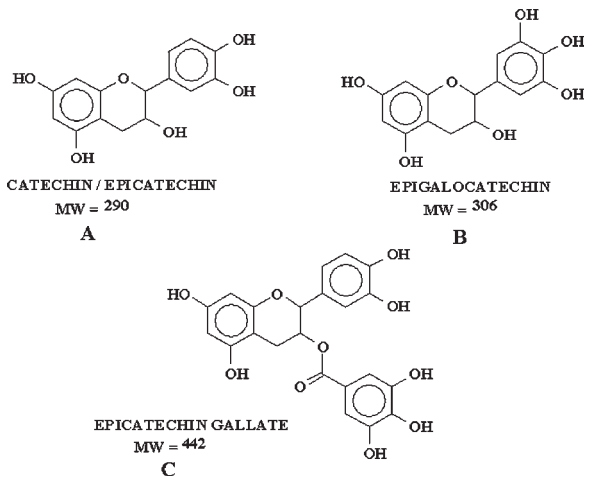

Figure 1 Catechin structures of *Rhizophora apiculata* mangrove tannins.

carried out in the laboratory by total immersion of the finely ground bark in 70% aqueous acetone (acetone/water 70/30 v/v) for 72 h at 30°C according to the procedure of Rahim.[2,4] The acetone was removed under pressure and the resulting aqueous fraction was freeze dried. The extract so obtained was used for analysis.

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.

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.

RESULTS AND DISCUSSION

Mangrove tannins can come from a variety of different *Rhizophora* species. In the case of *Rhizophora apiculata*, the tannins found are of the procyanidin type.[2] These are the most common tannins existing in nature. *Rhizophora apiculata* mangrove tannin most common monomer constituents are catechin, epicatechin, epigallocatechin, and epicatechin gallate with molecular weights (MW), respectively, of 290.3, 290.3, 306.3, and 442.4 Da (Fig. 1).

For *Rhizophora apiculata* mangrove tannins as in the present study, the MALDI-TOF spectra in Figure 2(a–c) indicates clearly that alternate repeating units with mass increments of 264–264.9 Da occur. These have not been identified by previous analysis by other methods[2,4] in mangrove tannins indicating possibly the presence of also other monomers than those shown in Figure 1 and/or different combinations of various structures.

Combination of the masses of the catechinin monomers shown in Figure 2 can be used to calculate the masses of the oligomer peaks in the spectra according to the expression $M + Na^+ = 23\ (Na) + 2$ (endgroups, 2xH) + 290.3 (−2H)A + 306.3 (−2H)B + 442.4 (−2H)C (Table I). The only problem about this is the presence of a repeating structure the MW of which is regular at 264.0–264.9 Da. This unit has not been identified before, and we will call it here structure D. Calculation of the MALDI masses indicate that certain peaks can only be explained by the presence of epicatechin gallate units, in which the gallic acid residue has being removed, of 274.3 Da (structure E, Fig. 3), these being related to the unknown structure. The equation than becomes $M + Na^+ = 23\ (Na) + 2$ (endgroups, 2xH) + 290.3 (−2H)A + 306.3 (−2H)B + 442.4 (−2H)C + 274.3 (−2H)E. In Table I are shown the results of the combination of monomer units forming the different oligomers observed by MALDI-TOF. It must be noticed that only very few of the dominant peaks [Fig. 2(a)] can be explained only on the basis of the catechinic structures in Figure 1. Some mass peaks however are not easily explained without the use of structure D. The majority of the dominant peaks are shown in Tables II and III.

Structure D cannot be inserted in the equation simply because no known flavonoid structure could be found with such a MW. However, this structure participates markedly, from peak intensities in Figure 1(a) its participation being predominant to the formation of the mangrove tannin oligomers. Two series of the most intense MALDI mass peaks rely on the repetition of this 264 Da structure. Thus, the oligomers of the series of peaks at 835, 1099, 1363, 1628, 1892, 2157, and 2422 Da are separated by the 264 Da motive recurring six times. Equally, the oligomers of the series of peaks at 967, 1231, 1495, 1760, 2024, 2288, and 2557 Da are separated by the 264 Da motive again recurring six times, which means that attached to the starting oligomer to an hexamer of the 264 Da unit is progressively linked

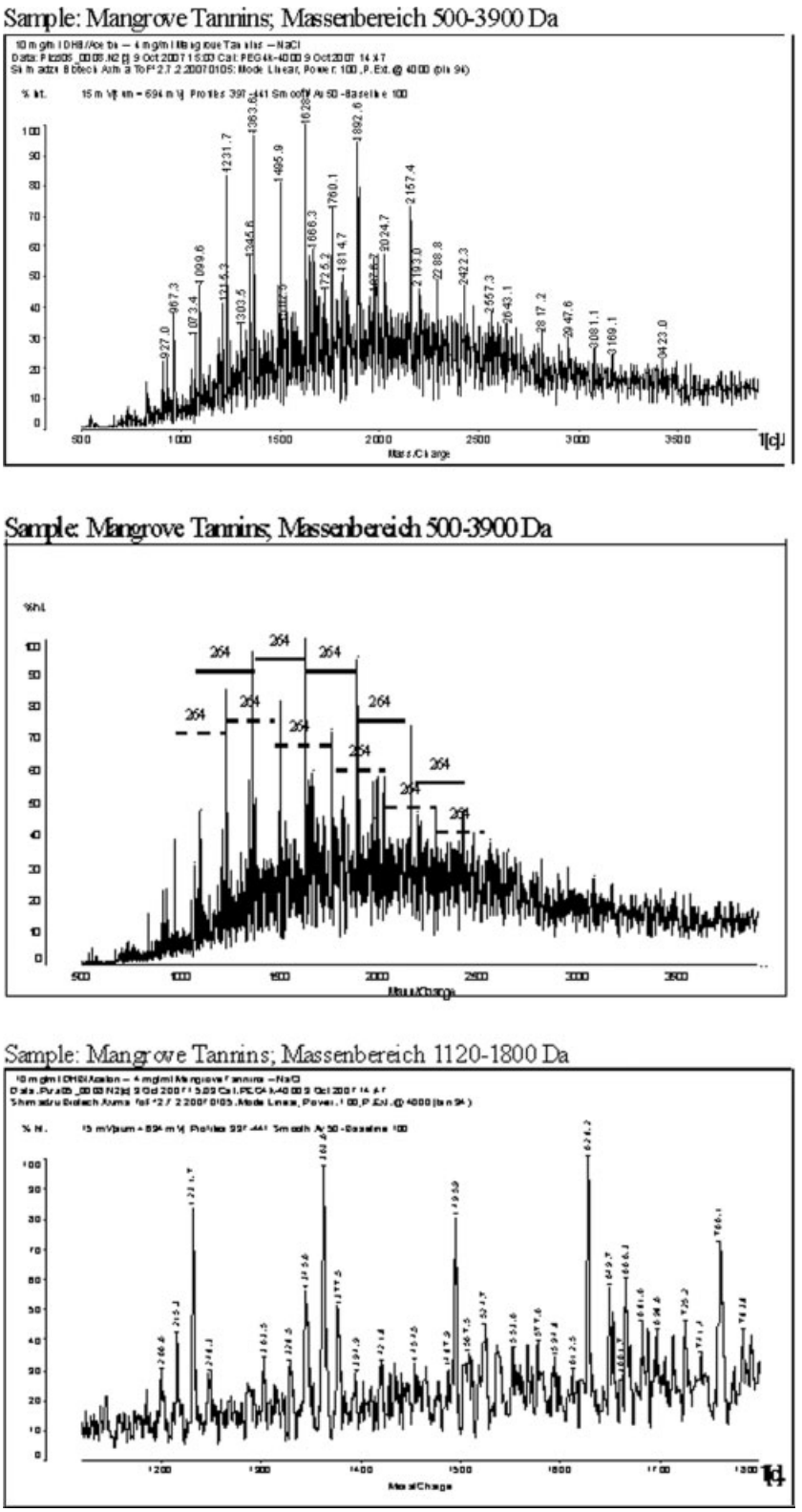

Figure 2 MALDI mass spectrum of (a) mixed *Rhizophora apiculata* mangrove tannin extract, 500–3900 Da range, (b) indication of the relevant 264 Da repeat unit, and (c) details of the 1120–1800 Da range.

to the starting oligomer, whatever this may be. Tables II and III show the interpretation of the two predominant series present.

It is of interest to find out which structure corresponds to 264 Da. No known monoflavonoid corresponds to such a structure. If one takes structure E

TABLE I
MALDI Fragmentation Peaks for Mixed *Rhizophora spp.* Mangrove Tannin Extract

		Unit type				
$M + Na^+$ (exp.) (Da)	$M + Na^+$ (calc.)	A 290.3	B 306.3	C 442.4	D 264/265	E (C-gallic) 274.3
835	841.9	–	–	–	–	3
927	921.9	1	2	–	–	–
967[a]	see Table III					
1073.4	1074	–	2	1	–	–
1099.6[a]	1094.1	1	–	2	–	–
or	1098.6	–	–	–	2	2
1200	1200		3		1	
1215.3	1210.2	–	3	–	–	1
or	1210.1	–	1	-2		
or	1210.2	2	2			
1231.7[a]	1226.2	1	3	–	–	–
or						
1248.3	1242.2	–	4			
1328.5	1330.3	1	1	1	–	1
1345.6	1346.2	–	–	3		
or	1346.3	2	1	1		
or	1346.3	–	2	1	–	1
1363[a]	1362.3	1	2	1		
1377.5	1378.3	–	3	1		
1454.5	1450.5	4	–	–	–	1
1487.9	1482.5	–	3	–	–	2
1507.5	1507.2	–	4	–	1	
1649.7	1650.5	–	1	3		
or	1650.6	2	2	1		
1666.3	1666.3	1	3	1		
1681.6	1682.6	–	4	1		
1725.2	1722.8	4	–	–	–	2
1741.4	1738.8	5	–	–	–	1
or	1738.7	1	–	2	–	2
	1738.8	3	1	–	–	2
1892.6[s]	1890.9	4	–	1	–	1
2193	2195.2	6	–	1		
2557.3	2556.2	–	6	1	1	
2817.2	2819.7	3	2	3		
or	2819.6	1	1	5		
2947.6	2940.0	–	6	–	–	4
or	2940.0	2	5	–	–	3
	2939.9	–	4	2	–	3
3081	3076.1	4	3	1	–	2
or	3076.0	2	2	3	–	2
	3076.0	–	3	3	–	3
3169.1	3164.2	5	–	2	–	3
or	3164.3	7	1			3

Note that the predominant repeat units in this tannin is Da, indicating that this tannin is predominantly a prorobinetinidin.
[s] Dominant fragment

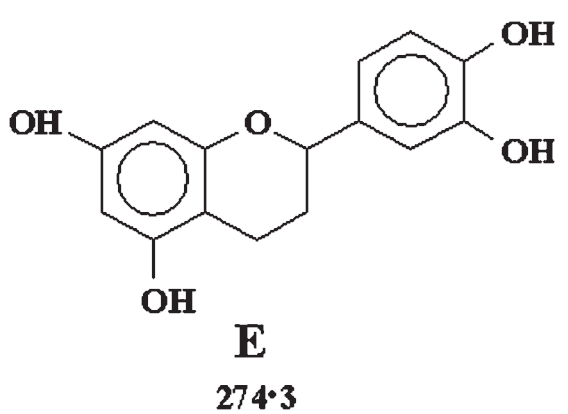

Figure 3 E monomer structure.

TABLE II
MALDI Fragmentation Peaks for Mixed *Rhizophora spp.* Mangrove Tannin Extract

		Unit type				
$M + Na^+$ (exp.) (Da)	$M + Na^+$ (calc.)	A 290.3	B 306.3	C 442.4	E 274.3	D 264–264.9
835	841.9	–	–	–	3	–
1099.6[a]	1105.6	–	–	–	3	1
1363.6[a]	1362.3	1	2	1		
1628.2[a]	1628.2	1	2	1	–	1
1892.6[a]	1892.6	1	2	1	–	2
2157.4[a]	2157.4	1	2	1	–	3
2422.3[a]	2422.3	1	2	1	–	4

Note that the predominant repeat units in this tannin is 528-530 Da.
[a] Dominant fragments.

TABLE III
MALDI Fragmentation Peaks for Mixed *Rhizophora spp.* Mangrove Tannin Extract

		Unit type						
		A	B	C	E (C-gallic)	D		
M + Na^+ (exp.) (Da)	M + Na^+ (calc.)	290.3	306.3	442.4	274.3	264–264.9	DPn Tot	DPn E unit
967.3	967.7			1	1	1 (−2xO)	3	1
1231.7[a]	1226.2	1	3				4	–
1495.9[a]	1497.7			1	1	3 (−2xO)	5	3
1760.1[a]	1756	1	3			2	6	2
2024.7[a]	2025.7			1	1	5 (−2xO)	7	5
2288.8[a]	2285.8	1	3			4	8	4
2557.3	2553.7			1	1	7 (−2xO)	9	7

Note that the predominant repeat units in this tannin is still 528–530 Da, indicating that in this MALDI series of peaks this tannin presents predominantly a profisetinidin component but linked to procyanidins units too.
[a] Dominant fragments.

however, for a E repeating unit at 272.3 Da, the difference with 264 Da is always of 8 Da, that does not correspond to the mass of any leaving functional group. However, if one considers that there is an —OH group less in a dimer formed by two joined E structures this will give the loss of 16 Da, hence of an oxygen. It means that the repeating unit of the system is not 264 Da but appears to be 264 × 2 = 528 Da. Thus, the repeating unit is a dimer of structure E whith a —OH group missing. The unit has a single phenolic —OH group that has been lost, as shown in Figure 4, the alcoholic —OH groups in C3 having already been lost at the separation of the gallic acid.

Table II shows the main series of dominant MALDI masses indicating that oligomers of this unit appear to occur in *apiculata* mangrove tannins. This is the most likely case showing the regular progression from trimer to octamer. Thus, mixed oligomers where a procyanidin oligomer formed by structures of type A, B and C (1363.6 Da) is linked to progressively increasing number of D structure oligomers are possible. The results in Table III of the second more important series of recurrent MALDI peaks clearly confirms that mixed procyanidin and D oligomers covalently linked do exist in such mangrove tannins because none of the masses of the series 835, 1099, 1363, 1628, 1892, 2157, and 2422 Da can be explained without having units of structures A, B, and C linked to the D oligomers. It appears most likely that both pure oligomers of the two types as well as linked mixed oligomers do coexist in this tannin.

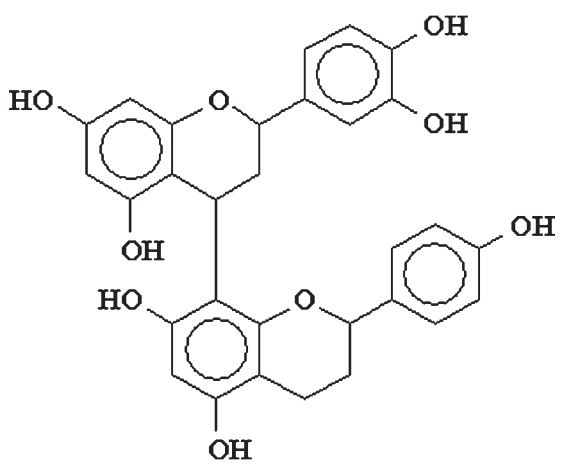

Figure 4 Dimer structure corresponding to the repeating unit of 264 Da (structure D). One of the phenolic —OH groups is absent in the 2 × 264 = 528/529 Da repeating unit.

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