Pergamon

PII: S0031-9422(97)01070-4

Phytochemistry, Vol. 48, No. 7, pp. 1251–1258, 1998

Printed in Great Britain
0031-9422/98 $19.00+0.00

A-TYPE PROANTHOCYANIDINS FROM PERICARP OF *LITCHI CHINENSIS*

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(*Received in revised form* 6 *November* 1997)

Key Word Index—*Litchi chinensis*; Sapindaceae; litchi; pericarp; proanthocyanidins; A-type linkages; thioacidolysis; ESI-mass spectrometry.

Abstract—A tannin extract was isolated from freeze-dried litchi pericarp (*Litchi chinensis*). HPLC analysis, after thioacidolysis reaction, revealed that this tannin fraction consisted of epicatechin units linked by both A- and B-type interflavonoid bonds. The calculated average degree of polymerization, considering the presence of A-type linkages, was 6.4. Characterization of oligomeric and polymeric procyanidins was performed using electrospray ionization mass spectrometry. Numerous oligomers containing one or more A-type interflavanoid linkages were detected. A regular repartition of the number of A-type linkages for each polymer length (e.g. from 2 to 7 for DP17 with mostly 4 A-type linkages) was observed. The average number of A-type linkages also increased with the DP, the predominant species containing one A-type linkage (denoted 1A) from DP2 to 5, 2A from DP6 to 10, 3A from DP11 to 15 and 4A from DP16 to 20. The highest DP was estimated at 22, with predominantly 5–6 A-type interflavanoid linkages.

INTRODUCTION

Litchi (*Litchi chinensis*) is a tropical fruit, originating from China, appreciated for its sweet taste. Exportation through international markets is limited by its postharvest conservation, the red colour of the pericarp turning brown early. It has been demonstrated that oxidative mechanisms involving phenolic compounds can lead to brown products resulting from polymerization of monomeric phenolics [1]. Except for anthocyanins [2], the phenolic composition of litchi is unknown. The purpose of the present work was to characterize the phenolic compounds present in the pericarp, in particular, flavanols, which are known to be the major precursors of brown pigments in numerous fruit-derived products [3].

RESULTS AND DISCUSSION

Polymeric proanthocyanidins extracted from freeze-dried litchi pericarps were separated from other phenolic compounds by chromatography on Toyopearl TSK HW-40(F), as described earlier [4]. The reverse-phase chromatographic profile at 280 nm of the crude tannin extract showed numerous compounds eluted as a hump between 15 and 50 min. The UV-visible spectra recorded along this hump showed a single absorbance band at 276.8 nm corresponding to the absorbance spectrum of flavan-3-ols.

Analysis of the tannin extract, after butanol-HCl degradation, led to the characterization of cyanidin and its butyl-ether derivative [5], indicating the presence of procyanidins. We also observed trace amounts of an unknown anthocyanidin; this additional peak may indicate either the presence of another proanthocyanidin type or that of A-type linkages in the procyanidin structure, as described by Bate-Smith [6].

The determination of the average composition of these polymeric procyanidins was performed using thiolytic degradation [7] (Table 1), followed by HPLC as described by Rigaud *et al.* [8]. The chromatogram recorded at 280 nm indicated the presence of five compounds corresponding to either terminal units or extension units, the latter being detected as benzylthioethers. In order to identify these compounds, their R_ts were compared with those of classical units obtained by degradation of B-type proanthocyanidins from grape [4]; the thioacidolysis solution was analysed by LC-ESI-mass spectrometry. The thioacidolysis reaction yielded three terminal units, including (+)-catechin (R_t 18.06) and (−)-epicatechin (R_t

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Table 1. Thioacidolysis degradation of A-type procyanidins from litchi pericarps

	% Terminal units	% Extension units
Catechin	2.45 (2.10)	nd
Epicatechin	10.43 (0.87)	51.33 (1.94)
Procyanidin A2	7.72 (2.48)	28.08 (1.81)
DP	6.4 (1.1)	

DP: average degree of polymerization; nd: not detected; ( ): standard deviation ($n = 3$).

24.41) ($[M-H]^-$ at m/z 289) and an additional compound (R_t 35.0) with a mass value at m/z 575. The extension units consisted of the benzylthioether derivatives of (−)-epicatechin (R_t 57.38, $[(\text{epicatechin}+C_6H_5CH_2S)-H]^-$ at m/z 411) and a compound (R_t 61.78) with a mass signal at m/z 697 ($[(575+C_6H_5CH_2S]^-$). The compound of m/z 575 gave a M_r corresponding to the loss of two protons from a B-type procyanidin dimer, suggesting that it was an A-type procyanidin dimer with a double linkage (C_2—O—C_7 and C_4—C_8 or C_4—C_6) [9, 10], the compound at m/z 697 thus corresponding to its benzylthioether derivative. In order to check the above structural hypotheses, these compounds were purified by HPLC on a semi-preparative scale and analysed by NMR. The ^1H and ^{13}C NMR spectral data of the first compound corresponded with those previously described for procyanidin A2 (Fig. 1) [11], with a typical shift of the (C-2) carbon signal at 100.18 ppm due to the linkage of the oxygen to this carbon which was thus shown to be involved in the ether C_2—O—C_7 linkage. The heteronuclear HMQC 2D NMR sequence showed the presence of a proton linked to the carbon (D-6). The HMBC 2D NMR sequence allowed us to distinguish cross-peaks between the proton H (D-6) and the close carbons (D-5) and (D-7), indicating that the position of the interflavanoid linkage is not C_4—C_6. Moreover, we also observed correlations between the proton (C-4) and the carbons (D-8) and (F-8a) confirming the C_4—C_8 interflavanoid linkage. This information indicated that the position of the linkages was C_2—O—C_7 and C_4—C_8. It should be noted that, although the NMR data are identical to that published for procyanidin A2, the $J_{2,3}$ and $J_{3,4}$ coupling constants do not allow identification of the upper unit as epicatechin or catechin. In contrast, the H_2 and H_3 of the F ring appear as broad singlets indicative of a relative 2,3-*cis*-configuration for the lower unit. Epicatechin was the only unit found in litchi procyanidins and the presence of catechin in small amounts (2.45% of total) can be interpreted as an artefact resulting from epimerization when heating in acidic medium [12]. This suggests that the A-type dimer is procyanidin A2 (epicatechin-(2 → O → 7, 4 → 8)-epicatechin). Structural analysis of the second compound showed the same correlations, meaning that it was the benzylthioether derivative of procyanidin A2, released during the thioacidolysis reaction.

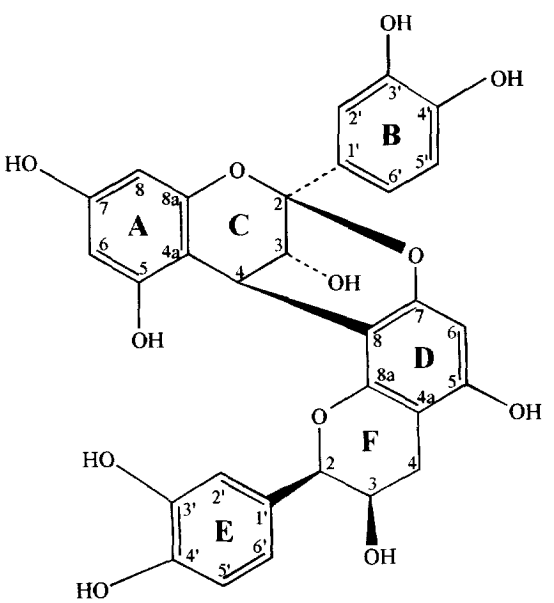

Fig. 1. Structure of procyanidin A2.

The average number of units in proanthocyanidin polymers (mDP) can be determined by calculating the ratio between total (terminal + extension) units and terminal units. In order to take into account the presence of procyanidin A2, which is not cleaved by the thiolytic degradation, we developed the following formula, including the A2 dimer as a double unit, to express the mDP for tannins containing A-type linkages.

$$\text{mDP} = \frac{(\text{Cat}+\text{Epi}+2\times\text{DimA}) + (\text{CatSR}+\text{EpiSR}+2\times\text{DimASR})}{(\text{Cat}+\text{Epi}+\text{DimA})}$$

Terminal units: Cat: catechin; Epi: epicatechin; DimA: Dimer A2.
Extension units: CatSR, EpiSR: benzylthioethers of (epi)-catechin; DimASR: benzylthioether of dimer A2.

Considering the calibration coefficients of each unit, the mDP evaluated for the tannin fraction was 6.4. The thioacidolysis reaction yield, calculated as the total concentration of units compared with the initial procyanidins concentration was 70% and of the same magnitude as that obtained by other authors [12, 13].

The characterization of highly polymerised procyanidins, using ESI-mass spectrometry, has already been achieved for the tannin fraction from cider apples [14]. The same method was applied to the crude extract of litchi tannins; ionization, performed in the negative mode, was increased by acidification with 0.5% HCO_2H. The ESI-mass spectrum, recorded in the m/z range 200 to 2400, showed numerous peaks with

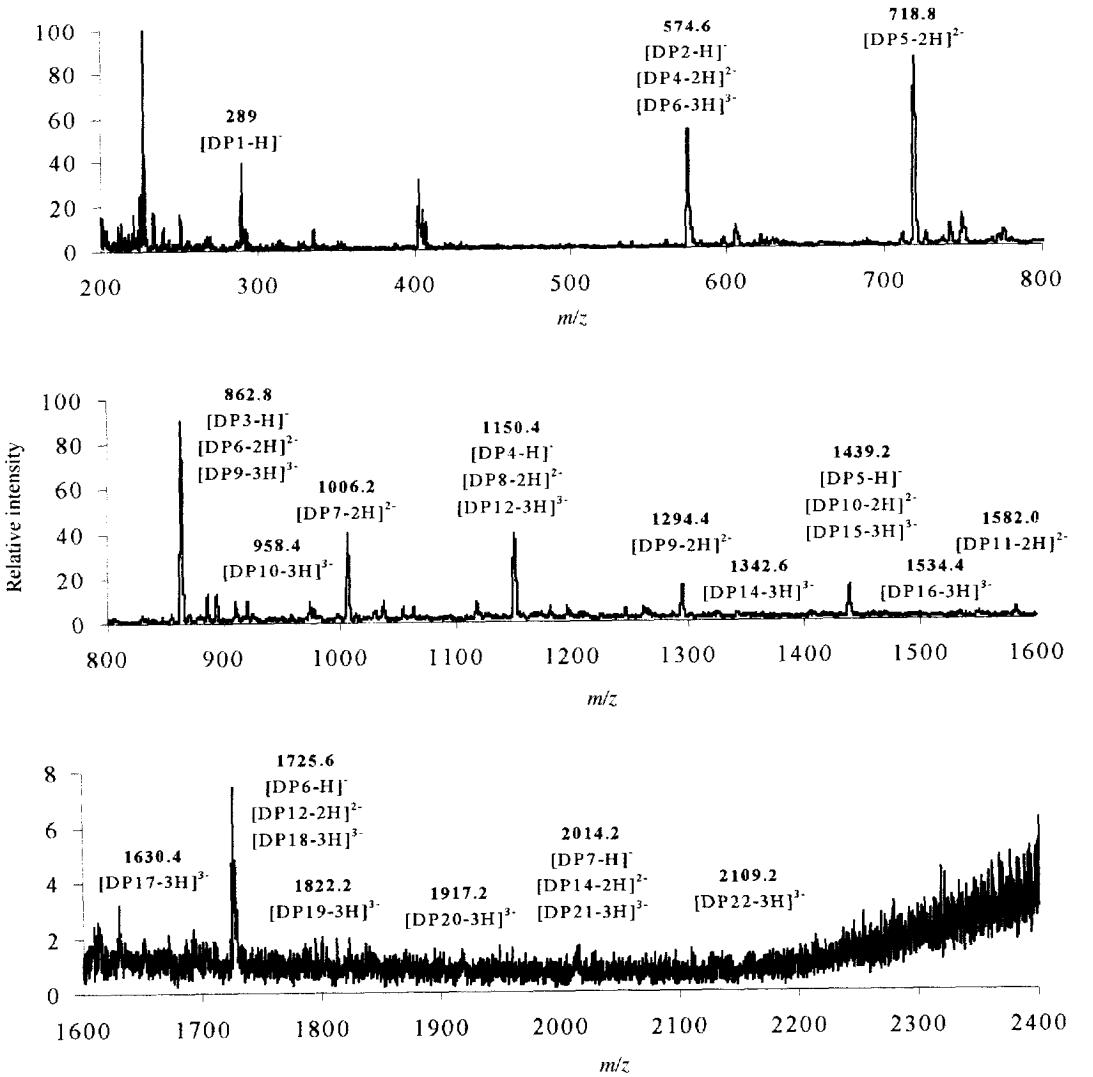

Fig. 2. ESI mass spectrum of crude extract of tannin with 0.5% HCO_2H.

$[M-H]^-$ at *m/z* 289, 574.8, 718.8, 862.8, 1006.2, 1150.4, 1294.4, 1439.2, 1582.0, 1725.6, 2014.2 and 2109.2 (Fig. 2). These peaks do not correspond to the typical masses found for B-type procyanidins but they can be explained by the presence of A-type linkages, as demonstrated by thioacidolysis. Thus, as the M_r of epicatechin is 290, the ions at *m/z* 574.8, 862.8, 1150.4, 1439.2, 1725.6 and 2014.2 may correspond, respectively, to the dimeric, trimeric, tetrameric, pentameric, hexameric and heptameric (noted, respectively DP 2, 3, 4, 5, 6 and 7) procyanidins containing one A-type interflavanoid linkage. In addition, polymers (e.g. DP 10) may contain one, two, three or more A-type interflavanoid linkages. We also had to take into account the presence of multi-charged species, the *m/z* value then being divided by the number of charges. Moreover, the isotopic ^{13}C is no longer negligible when the number of carbons exceeds 90 (natural abundance ~1%). Therefore, we constructed a database of expected *m/z* values taking into account the number of A-type linkages, the occurrence of multicharged species and the natural abundance of ^{13}C.

According to the calculated values, the above mentioned *m/z* peaks at 574.8, 862.8, 1150.4, 1439.2, 1725.6 and 2014.2, may also be interpreted as doubly-charged DP 4, 6, 8, 10, 12 and 14, respectively, or triply-charged DP 6, 9, 12, 15, 18 and 21, respectively, and thus cannot be attributed to specific polymeric compounds. However, other peaks corresponding to doubly-charged species were detected, with *m/z* values at 718.8, 1006.2, 1294.4, 1582.0 and 1872.0, corresponding to DP 5, 7, 9, 11 and 13. Peaks with *m/z* values 958.4, 1054.4, 1246.0, 1342.6, 1534.4, 1630.4, 1822.2, 1917.2 and 2109.2, corresponding to triply charged ions of DP 10, 11, 13, 14, 16, 17, 19, 20 and 22, respectively, were also detected. In fact, as the *m/z*

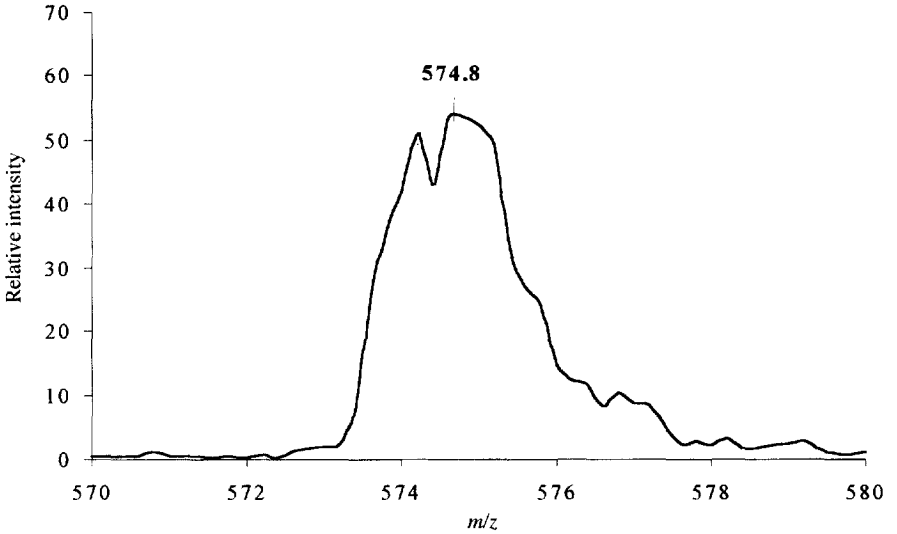

Fig. 3. Mass spectrum enlargement of signal ranging from *m/z* 573 to 578, with a maximum relative intensity at 574.8, representing the distribution of multicharged species.

scale ranges from 200 to 2400, the procyanidins, with a DP higher than DP 15, could only be detected as the triply-charged ions.

Some *m/z* peaks have to be considered as groups of molecules. For example, the peak at *m/z* 574.8 (Fig. 3) consists of an enlarged signal from *m/z* 573 to 578, which contains variously charged species of DP 2, 4 and 6 (Table 2). Thus, the predominant relative intensity at 574.8 theoretically corresponds to triply charged DP 6, containing two A-type linkages, with other species ranging from DP 2 to 6.

The above interpretations did not describe the number of A-type interflavanoid linkages present in the procyanidins. For example, the peak at *m/z* 1630.4, corresponding to a triply-charged DP 17, is detected as a broad signal (Fig. 4) with distinct peaks at 1628.8, 1629.8, 1630.4, 1630.8, 1631.4 and 1632.4, corresponding with decreasing numbers of A-type interflavanoid linkages from 6A to 1A. In this case, the predominant procyanidins, with a DP 17, contain four A-type linkages. Each peak in the mass spectrum was analysed in order to check the number of A-type linkages for different values of DP. We observed that the number of A-type linkages increased with the length of the polymeric chain. For example, the predominant species from DP 2 (*m/z* 575.2) to 5 (*m/z* 1439.2) contains a 1A linkage; between DP 6 (*m/z* 1725.6) and 10 (*m/z* 1439.0), the major species containing 2A linkages. At DP 11, we observe predominantly 3A (*m/z* 1582.0). We can also check these considerations for DP 15 (3A dominant) and DP 16 (4A dominant). For the highest DP detected (22 units, *m/z* 2109.2), the predominant species contain 5 and 6 A-type linkages.

Table 2. Distribution of DPs, multicharged species and numbers of A-type linkages for signal at *m/z* 574.8

DP	Ion charge	Calculated *m/z*	Number of A-type linkages
2	$[M-H]^-$	575.2	1A
4	$[M-2H]^{2-}$	575.2	1A
		574.2	2A
6	$[M-3H]^{3-}$	575.5	1A
		574.8	2A
		574.2	3A

The crude extract of tannins was also analysed by normal-phase HPLC in order to separate procyanidins according to their M_r [15]. The chromatogram at 280 nm (Fig. 5) showed a hump and a peak at the end of the gradient. The average degree of polymerization was estimated on each collected fraction using thiolytic degradation. Each fraction contained the same extension and terminal units as those found in the tannin crude extract. The average degree of polymerization, (Fig. 4) increased with R_t, starting from mDP 2 up to mDP 15, with an important quantity of polymeric compounds between mDP 5 and 7. However, we observed a decrease of mDP for the last two fractions collected at the end of the elution which may be attributed to the insolubility of highly polymerised compounds during the concentration step. Nevertheless, these results confirmed the ability of the silica column to separate procyanidins according to their M_r. This separation was further checked by analysing the most concentrated fractions, corresponding to mDP 5 and 7, by ESI mass spectrometry. The fraction corresponding to mean DP5 (Fig. 6A) showed three groups of masses at *m/z* (575.2, 593.2 and 611.2), (718.2, 736.2 and 754.6) and (862.2, 880.4 and 898.2). Considering the first mass of each group and according to the calculated masses, M_rs 575.2, 718.2 and 862.2, could be respectively attri-

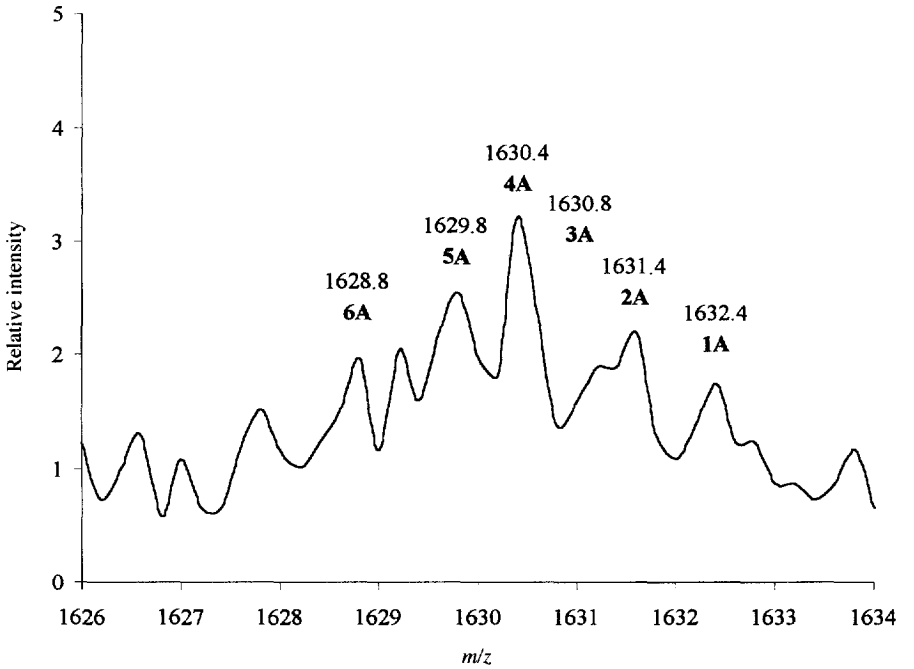

Fig. 4. Mass spectrum enlargement of signals at *m/z* 1630.4 representing triply-charged DP 17 procyanidins (bold characters indicate the number of A-type linkages in the molecule).

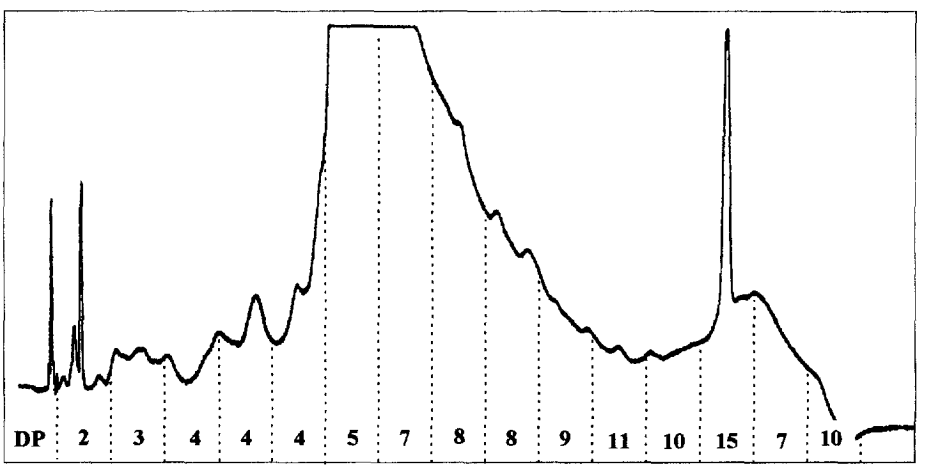

Fig. 5. Normal-phase HPLC profile of crude extract of tannins at 280 nm. The degrees of polymerization (DP) determined by thiolysis are given at the bottom of the chromatogram.

buted to doubly-charged compounds ($[M-2H]^{2-}/2$) corresponding to DP 4 with one A-type interflavonoid linkage (8.8% relative intensity), DP 5 and DP 6, both with two A-type linkages (46.4% and 16.5% relative intensity). The adjacent peaks at *m/z* 593.2, 736.2 and 880.4 corresponded to the addition of one chloride ($[M-H+Cl]^{2-}/2$) to these molecules; the masses at *m/z* 611.2, 754.6 and 898.2 corresponded to the addition of two chlorides ($[M+2Cl]^{2-}/2$). The fraction corresponding to mDP 7 (Fig. 6B) also showed three groups of masses (*m/z* 718.2, 736.2 and 754.2), (*m/z* 862.6, 880.8 and 898.2) and (*m/z* 1006.8, 1024.4 and 1042.8). These M_rs corresponded to doubly-charged ion peaks of DP 5 (*m/z* at 718.2, two A-type interflavonoid linkages), DP 6 (*m/z* at 862.6, two A-type interflavonoid linkages) and DP 7 (*m/z* at 1006.8, two A-type interflavonoid linkages) and traces corresponding to doubly charged DP 8 (*m/z* at 1150.8).

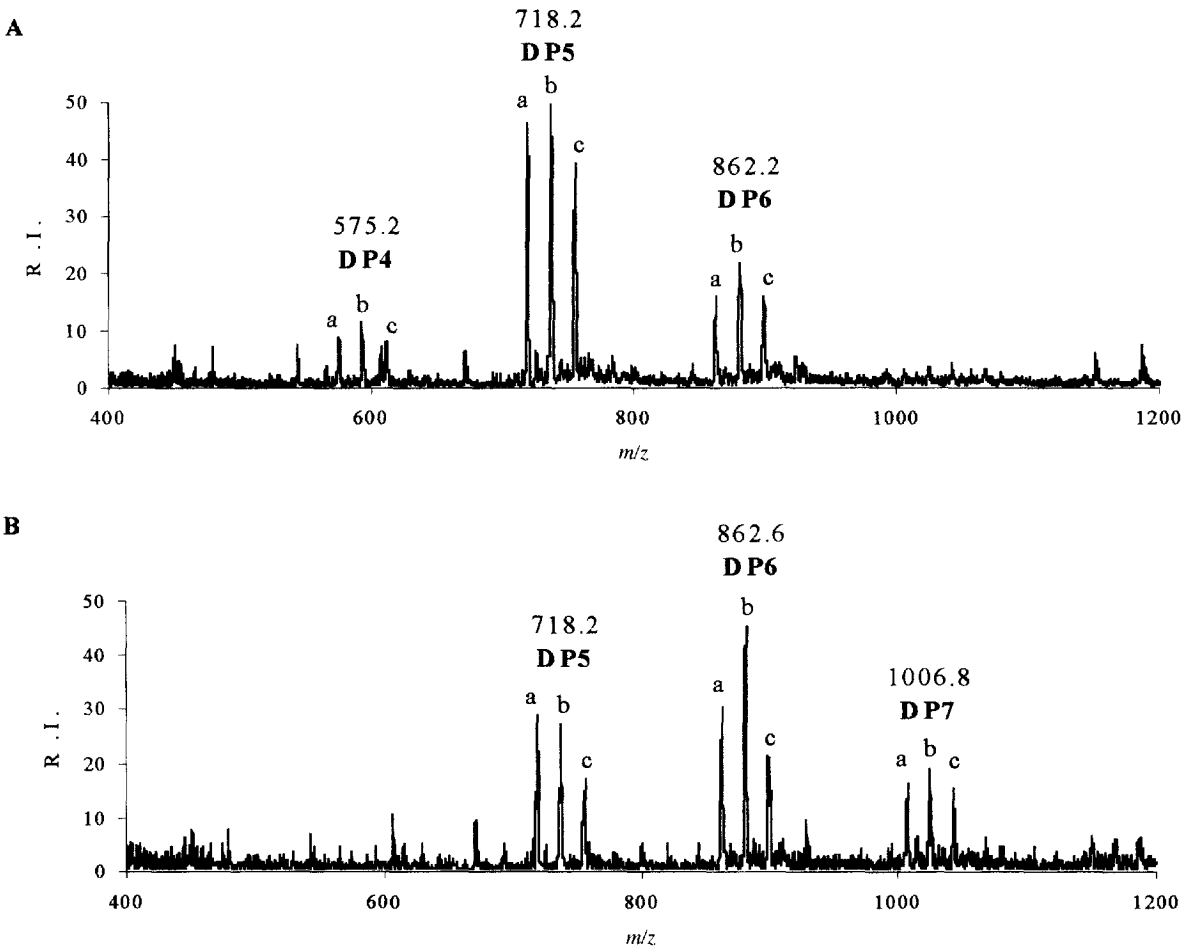

Fig. 6. ESI mass spectra of normal-phase fractions corresponding to mDP 5 and mDP 7. (a) $[M-2H]^{2-}$; (b) $[M-H+Cl]^{2-}$; (c) $[M+2Cl]^{2-}$.

Thus, the most important DP estimated by mass spectrometry is 5 in the first fraction (mDP 5 by thioacidolysis) and 6 for the second (mDP 7 by thiolytic degradation); the latter discrepancy may be due to the presence of DP 8 in trace amounts.

Thus, the mass spectrometric method is mostly qualitative and describes the repartition of DPs leading to a global fraction, whereas the average DP can be calculated quantitatively by thiolytic degradation.

ESI mass spectrometry and thiolytic degradation showed that litchi proanthocyanidins are constituted of epicatechin units, 52% of these being involved in A-type linkages. It has been demonstrated that the mass spectrometric technique is powerful enough to determine highly polymerised procyanidins containing A-type interflavanoid linkages. The largest procyanidin identified by this method in the pericarp of litchi, showed a DP 22 with predominantly 5 or 6 A-type linkages. Thiolysis degradation allowed us to estimate the mean degree of polymerization of the crude extract of tannins at 6.4. Using normal-phase HPLC, the predominant polymeric compounds showed a DP of 5 to 7, estimated by both thiolytic degradation and ESI-mass spectrometric analyses.

EXPERIMENTAL

Extraction and isolation of proanthocyanidins

Pericarps obtained from *L. chinensis* Sonn. var. Kwaï Mi fruits were freeze-dried. Dried material (10 g) was ground in 350 ml of MeOH–1.5 N HCl (17:3) mixed for 30 min and centrifuged (10,000 *g* for 15 min). The pellet was washed 2×30 min with 2×350 ml of MeOH–1.5 N HCl (17:3) and centrifuged. The supernatant frs were taken to dryness under vacuum and dissolved in EtOH–H_2O–TFA (11:9:0.001) before low pressure chromatography. Monomeric phenolics were eliminated by chromatography on Toyopearl HW40(F) (TOSOHAAS) (20 cm, 2.5 cm ID, 2 ml min^{-1}), using EtOH–H_2O–TFA (11:9:0.001) (1 l) as eluent. The crude tannin fr. was then eluted by

Me_2CO–H_2O (3:2) (200 ml) and then taken to dryness under vacuum. The powder obtained (75 mg) was stored at −20° until used.

BuOH–HCl hydrolysis

A 0.1 g l^{-1} crude tannin soln (500 μl) was taken to dryness under vacuum, dissolved in BuOH–12 N HCl (19:1) (2.5 ml) with 100 μl of iron reagent (2% w/v soln of $NH_4Fe(SO_4)_2 \cdot 12H_2O$ in 2 N HCl). The soln was heated at 90° for 30 min, taken to dryness and dissolved in 500 μl of MeOH for analysis by reverse-phase HPLC.

Thiolytic degradation

Tannins (100 μl of a 1 g l^{-1} sln) were added to an equal vol. of thiolytic reagent (toluene-α-thiol 5% in 0.2 N HCl in MeOH), stirred, heated for 2 min at 90° and analysed by HPLC [4]. Calibration curves were established using standards purified by semi-prep HPLC and identified by LC-ESI-MS.

Reverse-phase HPLC

Reverse-phase HPLC analysis was performed using a diode array detector. The column was a Lichrospher 100-RP18, 5 μm, 250 × 4 mm (Merck). The elution conditions were as follows: flow rate 1 ml min^{-1}, temp. 30°, solvent A: 2% aq. HCO_2H; solvent B: MeCN–H_2O–HCO_2H (40:19:1 v/v/v), elution with linear gradients from 5% to 30% B in 40 min, 30% to 50% B in 20 min, 50% to 80% B in 10 min, followed by washing and reconditioning of the column. Semi-prep. HPLC was performed using a Microsorb "Fast PCLC" C18, 3 μm, 50 × 21.4 mm (Rainin). The elution conditions were: flow rate 10 ml min^{-1}, solvent A: 2% aq. HCO_2H, solvent B: MeOH–H_2O–HOAc (40:19:1). Different linear gradients were used. For total thiolytic compounds, to remove excess acid and toluene-α-thiol prior to MS analysis: 15% B to 61% in 8 min, 61% B to 63% in 10 min, 63% B to 75% in 5 min, 75% B to 100% in 5 min. For procyanidin A2, 4-benzylthioproanthocyanidin A2 and 4-benzylthioepicatechin: 30% B to 61% in 5 min, 61% B to 63% in 10 min, 63% to 70% in 2 min. Each elution was followed by washing and column reconditioning.

Normal-phase HPLC

Normal-phase HPLC was performed using a Lichrospher Si 100, 5 μm, 250 × 4 mm column (Merck). Elution conditions were as follows: flow 1 ml min^{-1}, temp. 30°, injections: 25 μl; solvent A: HOAc–H_2O–MeOH–CH_2Cl_2 (11:143:5); solvent B: HOAc–H_2O–MeOH–CH_2Cl_2 (2:2:18:81), elution with linear gradients from 100% to 60% B in 50 min, 60% to 45% B in 5 min, 45% to 0% B in 5 min, followed by washing and column reconditioning. During elution, 5 ml frs were collected, taken to dryness under vacuum, dissolved in 200 μl MeOH and 50 μl aliquots used for thiolytic reaction.

ESI-MS analysis

ESI-MS analysis was carried out with a simple quadrupole mass spectrometer. The crude extract of tannins obtained by chromatography on Toyopearl was dissolved in MeOH containing 0.5% HCO_2H. Direct ESI-MS injection of the tannin fr. was performed using the following conditions: a −4 kV voltage was applied to the electrospray needle and −60 V to the orifice. The mass scale was defined from m/z 200 to 2400, in steps of 0.2 mu, with a dwell time of 1 msec.

Procyanidin A2. ^{1}H NMR (400 MHz, CD_3OD): δ 2.75/2.94 (2H, *dd*/*dd*, $J_{4\alpha,\beta} = 17.2$, $J_{4\alpha,3} = 4.9$, $J_{4\beta,3} = 2.0$, F-4), 4.05 (1H, *d*, $J_{3,4} = 3.4$, C-3), 4.23 (1H, *br s*, F-3), 4.40 (1H, *d*, $J_{4,3} = 3.4$, C-4), 4.92 (1H, *s*, F-2), 6.00 (1H, *d*, $J_{6,8} = 2.4$, A-6), 6.06 (1H, *d*, $J_{8,6} = 2.1$), 6.08 (1H, *s*, D-6), 6.79 (1H, *d*, $J_{5',6'} = 2.28$, B-5′), 6.8 (1H, *d*, $J_{5',6'} = 2.12$, E-5′), 6.98 (1H, *dd*, $J_{6',5} = 8.4$, $J_{6',2'} = 2$, E-6′), 7.01 (1H, *dd*, $J_{6',5'} = 8.3$, $J_{6',2'} = 2.1$, B-6′), 7.12 (1H, *d*, $J_{2',6'} = 2.1$, B-2′), 7.15 (1H, *d*, $J_{2',6'} = 2$, E-2′). ^{13}C NMR (100 MHz, CD_3OD): δ 29.3 (C-4), 29.9 (F-4), 67.0 (F-3), 68.1 (C-3), 81.8 (F-2), 96.5 (D-6), 96.6 (A-8), 98.3 (A-6), 100.2 (C-2), 102.4 (D-4a), 104.3 (A-4a), 107.5 (D-8), 115.6 (B-2′), 115.7 (B-5′), 115.9 (E-2′), 116.0 (E-5′), 119.8 (B-6′), 120.4 (E-6′), 131.2 (E-1′), 132.5 (B-1′), 145.7 (B-3′), 146.0 (E-3′), 146.3 (E-4′), 146.8 (B-4′), 152.1 (D-8a), 152.3 (D-7), 154.3 (A-8a), 156.6 (D-5), 158.1 (A-7).

4-Benzylthioproanthocyanidin **A2**. ^{1}H NMR (400 MHz, CD_3OD): δ 3.9 (1H, *d*, $J_{3,4} = 2.24$, F-3), 3.97 (2H, *s*, CH2-S), 4.04 (1H, *d*, $J_{4,3} = 2.36$, F-4), 4.37 (1H, *d*, $J_{4,3} = 3.2$, C-4), 5.28 (1H, *s*, F-2), 6.00 (1H, *d*, $J_{6,8} = 2.32$, A-6), 6.05 (1H, *d*, $J_{8,6} = 2.32$, A-8), 6.1 (1H, *s*, D-6), 6.79 (1H, *s*, B-5′), 6.81 (1H, *s*, E-5′), 6.95 (1H, *dd*, $j = 1.2$–8.8, E-6′), 7.00 (1H, *dd*, $J = 2.16$–8.32, B-6′), 7.11 (1H, *d*, $J_{2',6'} = 2.12$, B-2′), 7.15 (1H, *d*, $J_{2',6'} = 1.96$, E-2′), 7.20/7.29/7.4 (5H, *m*, Ar-CH_2-S). ^{13}C NMR (100 MHz, CD_3OD): δ 29.3 (C-4), 38.1 (CH_2-S), 44.4 (F-4), 66.1 (C-3), 71.3 (F-3), 77.5 (F-2), 96.6 (A-8), 97.0 (D-6), 98.3 (A-6), 100.1 (C-2), 102.5 (D-4a), 103.9 (C-4a), 107.1 (D-8), 115.6 (B-2′), 115.6 (B-5′), 116.1 (E-2′), 116.2 (E-5′), 119.7 (B-6′), 120.5 (E-6′), 128.0/129.5/130.1 (ArCH_2S-), 131.0 (E-1′), 133.0 (B-1′), 140.2 (ArCH_2S-), 145.7 (B-3′), 146.0 (E-3′), 146.4 (E-4′), 146.8 (B-4′), 152.1 (D-8a), 153.8 (D-7), 154.2 (A-8a), 157.5 (D-5), 158.2 (A-7).

Acknowledgements—This work was supported by the EU (contract TS3*CT94-0342). The authors thank Mrs Delbos for mass spectra and Mr Faure, Laboratoire de Phytochimie, URA CNRS 1411, Faculté des

Sciences et Techniques de Saint-Jérôme, Marseille, for NMR analysis.

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