Characterisation of highly polymerised prodelphinidins from skin and flesh of four cashew apple (Anacardium occidentale L.) genotypes

Laetitia Michodjehoun-Mestres a, Jean-Marc Souquet b, Hélène Fulcrand b, Emmanuelle Meudec b, Max Reynes a, Jean-Marc Brillouet a,b,*

a UMR «QUALISUD», Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), Département PERSYST, TA50/16, F-34398 Montpellier Cedex 5, France
b UMR1083 «Sciences pour l’Économie», INRA, Université Montpellier 1, F-34000 Montpellier, France

ARTICLE INFO

Article history:
Received 29 July 2008
Received in revised form 10 September 2008
Accepted 27 October 2008

Keywords:
Cashew apple
Pseudo fruit
Skin
Flesh
Tannins
Prodelphinidins
Phloroglucinol
Epigallocatechin
Epigallocatechin gallate
Size-exclusion chromatography
Tannase

1. Introduction

Cashew apple, the pseudo fruit of cashew (Anacardium occidentale L.), is a by-product of cashew nut production (Purseglove, 1974), and although 30 millions metric tons are produced yearly (FAOSTAT, 2007), its nutritional potential has not yet been fully utilised.

Aside from monomeric phenols (mainly flavonol glycosides) (Michodjehoun-Mestres et al., 2009), tannins are the major phenolics of the cashew apple (Satyanarayana, Mythirayee, Krishnamurty, & Madhavakrishna, 1978), giving rise to astringency in the derived products (juice, candy, preserves...), which limits their export (da Silveira Agostini-Costa et al., 2003; Satyanarayana et al., 1978). Gallic and protocatechuic acids were observed as products from alkaline fusions of tannins from an Indian cashew apple variety (Satyanarayana et al., 1978). However, to our knowledge, no detailed characterisation of cashew apple tannins is available.

Thus, the object of the present work was to describe more precisely the constitution of skin and flesh tannins from cashew apples originating from Brazil and Bénin (West Africa).

2. Experimental

2.1. Reagents and enzyme

(-)-Epicatechin (EC), (-)-epicatechin-3-O-gallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCG), gallic
2.4. Characterisation of tannins

2.4.1. Acid-catalysed degradation in presence of phloroglucinol

Tannins were dissolved in 400 μl of freshly prepared MeOH containing 0.2 N HCl, 50 g/l phloroglucinol, and 10 g/l ascorbic acid and heated for 60 min at 50 °C. Then, an equivalent volume of aqueous 200 mM sodium acetate was added to stop the reaction (Kennedy & Jones, 2001). Samples were immediately analysed by HPLC–DAD/ESI–MS.

2.4.2. Effects of tannase on phloroglucinol adducts

The above degradation medium (800 μl; pH ~5) was added with tannase (~1 mg), left at ambient temperature for 1 h, then analysed by HPLC–DAD/ESI–MS.

2.4.3. Treatment in acidic methanol with \((\text{NH}_4)_2\text{Fe}(\text{SO}_4)_{2}\)

Tannin samples were added to 200 μl of methanol/HCl (95:5, v/v) and 8 μl of 2 N HCl containing 2% of \((\text{NH}_4)_2\text{Fe}(\text{SO}_4)_{2}\)·12H₂O. The medium was heated at 100 or 120 °C for 60 min, evaporated to dryness and the residue was redissolved in methanol prior to HPLC analysis.

2.5. HPLC–DAD analysis

Anthocyanidins liberated by treatment of tannins with acidic methanol in presence of \((\text{NH}_4)_2\text{Fe}(\text{SO}_4)_{2}\) were separated using an Agilent 1100 separation system (Agilent Technologies) including a quaternary pump coupled to a diode array detector and controlled by Chemstation A.10.02 software. Separations were achieved using a \((250 \times 4.6 \text{ mm i.d.})\) Modulocart QL-Lichrophers 5 μm ODS2 column (Interchim, Montluçon, France) with a guard column, operated at 30 °C. The mobile phase consisted of water/formic acid (98:2, v/v) (elucent A) and water/acetonitrile/formic acid (18:80:2, v/v/v) (elucent B). The flow rate was 0.5 ml/min. The elution programme was as follows: 5–10% B (0–4 min); 10–16% B (4–8 min); 16–25% B (8–45 min); 25–35% B (45–55 min); 35–80% B (55–72 min); 80–100% B (72–75 min); 100–5% B (75–80 min). Duplicate samples were injected at a level of 10 μl. The column effluent was monitored at 280 and 520 nm. Quantification was achieved by injection of solutions of known concentrations of delphinidin and cyanidin.

Released terminal subunits and extension subunit-phloroglucinol adducts before and after action of tannase were analysed by HPLC (Fournand et al., 2006), using an Agilent 1100 separation system (Agilent Technologies). Separations were achieved on a reversed-phase Atlantis dC18 column (Waters, Milford, USA; 5 μm packing, 250 × 4.6 mm i.d.) protected by a guard column of the same material (20 × 4.6 mm i.d.; Waters) and a Security-guardTM cartridge C18 (Phenomenex, Torrance, USA; 4.0 × 3.0 mm i.d.). The mobile phase was a linear gradient of water/formic acid (98:2; solvent A) and water/acetonitrile/formic acid (80:18:2; solvent B) at a flow rate of 0.25 ml/min at 30 °C.

The elution programme was as follows: isocratic 0% B (5 min); 0–10% B (5–35 min); 10–20% B (35–65 min); 20–100% B (65–70 min); 100–0% B (70–75 min). The reaction products were detected by two successive detectors: the first one consisted of a photodiode array 1100 (Agilent Technologies) and was used at 280 nm for the quantitative determination of extension subunit-phloroglucinol adducts; the second one, a spectrophotometer (Shimadzu, Kyoto, Japan), was used to improve accuracy of the quantitative determination of terminal subunits. Excitation and emission wavelengths were 275 and 322 nm, respectively, as previously described (Carando, Teissedre, & Cabanis, 1999). Chromatograms were monitored on a Chemstation A.10.02 software (Agilent Technologies). Each analysis was performed in duplicate. Quantification was achieved by injection of solutions of known concentrations of EGC-ph, EC-ph, ECG-ph, and EGCG. Measurement of EGCG-ph was carried out by injection of EGCG solutions of known concentrations using the conversion factor (EGCG-ph = 1.1 × EGC). The mDP of tannins [total number of released units/number of terminal units] as well as all qualitative data was calculated on a molar basis. Tannins were expressed as mg/100 g fresh weight.
2.6. HPLC–DAD/ESI–MS analysis

Separations of phloroglucinol-adducts and terminal subunits were performed on a (250 × 4.6 mm i.d.) Modulocart QS-Lichrospher 5 μm ODS2 column (Interchim, Montluçon, France) with a guard column, operated at 30 °C. The mobile phase consisted of water/formic acid (99.9:0.1, v/v) (eluent A) and water/acetonitrile/formic acid (19.9:80.0:1.0, v/v/v) (eluent B). Flow rate was 0.5 ml/min. The elution programme was as follows: 5–10% B (0–4 min); 10–16% B (4–8 min); 16–25% B (8–45 min); 25–35% B (45–55 min); 35–80% B (55–72 min); 80–100% B (72–75 min); 100–5% B (75–80 min). The column eluate was then split and 0.25 ml/min was directed to an LCQ ion trap spectrometer fitted with an electrospray interface (Thermo Finnigan, San Jose, USA). Experiments were conducted in the negative mode. Scan range was 100–2000 a.m.u. and scan rate 1 scan/sec. The desolvation temperature was 300 °C. High spray voltage was set at 5000 V. Nitrogen was used as the dry gas at a flow rate of 75 ml/min. Identifications of compounds, but EGC–ph–, were achieved on the basis of co-injections of standards, ion molecular masses, and UV spectra.

2.7. High-performance size-exclusion chromatography (HP–SEC)

The molecular size distribution was studied by HP–SEC with a Shodex OHpak KB-804 column (0.8 × 30 cm, pore size 500 Å, Showa Denko, Japan) equipped with an OHpak KB-800P Guard column (0.6 × 5 cm), and equilibrated at 1 ml/min with N,N-dimethylformamide containing 1% (v/v) acetic acid, 5% water and 0.15 M LiNO₃ (Kennedy & Taylor, 2003). The eluent was monitored at 280 nm. Weight-average molecular weights (Mₐ) were estimated by using a calibration curve of the column established at 50 °C with a calibration kit of pullulans with known weight-average molecular weights (Mₐ) (Showa Denko).

3. Results and discussion

3.1. Extraction and purification of tannins

Tannins were extracted with acetone/water (60:40) and further purified by adsorption chromatography (Kennedy et al., 2001). However, we have previously reported an incomplete separation of grape skin tannins from monomeric phenols by adsorption chromatography on Toyopearl TSK HW 40-F. After the size-exclusion chromatography of their purified skin tannins and an extensive washing of the column after tannin adsorption, the presence of two low molecular weight materials eluting at the same times as malvidin-3-glucoside and (-)-epicatechin, respectively, were observed. Since we also noted an incomplete segregation, tannins were preliminarily flocculated with caffeine (Satyanarayana et al., 1978); under these conditions, monomeric phenols including anthocyanins, but not flavan-3-ols, are eliminated in the supernatant. However, the caffeine–tannins, although repeatedly treated with chloroform were still contaminated with residual caffeine, justifying the subsequent adsorption chromatography. At this stage, a point of methodology must be raised: although this two-step purification procedure allowed highly purified tannins (see further) to be obtained, some weak and variable losses (~5–15%) were noted after adsorption chromatography even using a new batch of gel for each purification. Indeed, we measured tannins by the acid degradation technique (phloroglucinol) alongside the purification procedure and observed that minor quantities of the caffeine–tannins were not released from the column by acetone/water (60:40), even after a stepwise increase in the percentage of acetone up to 100%. However, the composition of caffeine- and purified tannins did not basically differ. Thus, quantification of the tannin levels as mg/100 g fresh weight (Table 1), an important index in, e.g., plant breeding programmes (Sastry et al., 1962), was achieved on caffeine–tannins rather than on purified ones. Due to the tediousness of this double-step purification technique, it was applied only to tannins from skin and the flesh of the clone CCP 76 and molecular weight determination was carried out on purified fractions (see further).

3.2. Characterisation and quantification of tannins

Contrary to unfermented cocoa bean procyanidin oligomers which were nicely separated under the same conditions (Kelm, Johnson, Robbins, Hammerstone, & Schmitz, 2006), our attempts to fractionate tannins according to their degree of polymerisation by normal phase HPLC failed, only unresolved humps being eluted late (Fig. 1), suggesting high molecular weights. As already mentioned, we never detected (-)-epicatechin, (+)-catechin, and oligomeric tannins in cashew apples, either in extracts fated for monomeric phenolics analysis (Michodjehoun-Mestres et al., 2009), or in solutions of caffeine–tannins (present article). Indeed, caffeine renders insoluble, as polymeric tannins, monomeric flavan-3-ols (Satyanarayana et al., 1978), if they had been present, we would have detected them as well.

UV spectra of cashew apple skin tannins are shown in Fig. 2 in comparison with those of EGC and EGCG. Whilst EGC exhibited a λmax at 270 nm, a rapid decrease of absorbance between 280 and

![Fig. 1. HPLC chromatogram of cashew apple skin tannin (Parakou Rouge variety) on Develosil 100ODS-5 column (for chromatographic conditions, see Kelm et al., 2006).](image-url)
290 nm, and a zero absorbance at 295 nm, EGCG showed a $\lambda_{\text{max}}$ at 274 nm, symmetry around the maximum from 258 to 290 nm, then a “shoulder” from 290 to 325 nm, typical of galloyl groups. Cashew apple tannins displayed a spectrum in between EGC and EGCG ones ($\lambda_{\text{max}}$), with a spectrum shape around the maximum close to EGC, and the presence of a “shoulder” due to galloyl substituents; their spectrum was different from spectra of other galloylated or ungalloylated flavan-3-ols. Thus, these tannins might have EGC and EGCG as their main constituents (see further).

HPLC analysis of depolymerisation products of skin and flesh tannins in acidic methanol with the presence of (NH$_4$)$_2$Fe(SO$_4$)$_2$ revealed strongly dominant proportions of delphinidin along with lower amounts of cyanidin, in average $\sim$85% and 15%, respectively. This indicates that cashew apple condensed tannins are mainly built of (-)-epigallocatechin [and/or (+)-gallocatechin] associated with some (-)-epicatechin [and/or (+)-catechin] units. This technique did not allow quantification of tannins since a large amount of material precipitated during treatment which might have resulted from the progressive polymerisation to yield red–brown amorphous phlobaphen-like substances (Matsuo & Ito, 1978; Matsuo and Itoo1981). There was also very low erroneous tannin levels, e.g. $\sim$20 mg/100 g of Parakou Rouge skin. It must be noted that a Brazilian team (da Silveira Agostini-Costa et al., 2003), using the same technique for measuring cashew apple tannins, obtained an average of $\sim$400 mg/100 g (whole apple), a value consistent with tannin contents of Indian varieties (Sastry et al., 1962). However, they used, as a standard, tannins from immature green cashew apples which must undergo the same kind of insolubilisation; thus, they did not face the same problem of standardisation as ours. We standardised with soluble delphinidin and cyanidin. Finally, the (delphinidin/cyanidin) proportion agrees well with the [(tri-OH/di-OH) units] relative percentage observed after acid degradation (see below).

Tannins were initially submitted to acid degradation with the presence of phloroglucinol according to Kennedy and Jones (2001), i.e. 20 min at 50 °C; however, due to the low yields obtained, time of treatment at 50 °C was varied from 20 to 480 min (Fig. 3). It appeared that yields in different extension subunits [negative mode: EGC-ph (m/z 429), EC-ph (m/z 413), (-)-EGCG-ph (m/z 581), and ECG-ph (m/z 565)] were increased from 20 to 60 min; then, the galloylated units remained roughly constant up to 480 min whilst non-galloylated ones decreased. Degradations were also conducted for 60 min at 40 and 60 °C, giving lower yields. Skin and flesh tannins were thus submitted to acid-catalysed degradation in the presence of phloroglucinol for 1 h at 50 °C and the reaction products (flavanol–phloroglucinol adducts and terminal subunit) were analysed by HPLC–DAD/ESI–MS giving information on the polymeric structure (Fig. 4) (constitutive flavanols and mean degree of polymerisation) (Fig. 4A). EGCG-ph, EGC-ph, ECG-ph, and EC-ph were seen as extension subunits. Action of tannase helped in confirming identities of galloylated terminal and extension subunits (Fig. 4B). More precisely, the stereochemistry of EGCG-ph, which was not available as a standard, was ascertained as being derived from (-)-EGCG since it was quantitatively converted into EGC-ph; however, the site of galloylation was not determined, and this compound was therefore referred as (-)-epigallocatechin-O-gallate-phloroglucinol. Gallic acid (+ methyl gallate formed from gallic acid in methanol) was quantitatively liberated by the action of tannase on EGCG-ph, EGC-ph, and EGCG. In addition to the monomeric flavanol–phloroglucinol adducts, two
very minor dimer adducts were seen: EGC-EGC-ph (m/z 733) and EGC-EGCG-ph (m/z 885); they may result from incomplete cleavage and/or recombination of monomers in solution.

In tannins from skins, EGC and EGCG accounted for about 70–75% and 20–25% of total extension subunits, whilst EC and ECG represented 6% and 2%, respectively (Table 1). The percentage of trihydroxylated units (EGC, EGCG) averaged 92%, confirming the (85:15) (delphinidin/cyanidin) ratio observed after degradation in acidic methanol with the presence of (NH₄)₂Fe(SO₄)₂. The percentage of galloylated units was around 20%; EGC and EC units were galloylated at levels of 20–25% and 33%, respectively.

Cashew apple skins were richer in tannins than the corresponding flesh; this is a common fact that fruit epidermis are richer in tannins than the corresponding fleshes (Porter, 1986), e.g. grape (Kennedy, 2002) and apple (Takos, Ubi, Robinson, & Walker, 2006). Furthermore, skins from African genotypes were also richer in tannins than those from Brazil.

Tannins from fleshes exhibited somewhat different characteristics: EGC and EGCG accounted for about 50–60% and 35–40% of total extension subunits whilst EC and ECG represented 4–6% and 3–5%, respectively (Table 1). The percentage of trihydroxylated units (EGC, EGCG) averaged 91%. The percentage of galloylated units averaged 40%, i.e. twice that of skin tannins, EGC and EC units being esterified at levels of 40% and 45–50%, respectively.

Fleshes from African cashew apples and those from Brazil seemed equivalent with regards to their tannin contents and far poorer than skins.

In both caffeine–tannins from skins and fleshes and also in purified tannins from clone CCP 76, EGCG (m/z 457) was repeatedly and unambiguously detected in trace amounts as the sole terminal subunit; this was confirmed by addition of minute amounts of EGCG to phloroglucinol degradation media and it perfectly coeluted with endogenous EGCG. This finding was coherent with the predominance of trihydroxylated units in these tannins. Moreover, we failed in finding other possible terminal units, e.g. flavan O- and C-glycosides (Hatano et al., 2002), dimer A, sulphate and amino-conjugates (Schötz & Nöldner, 2007). Calculation of mean degrees of polymerisation (mDP) gave aberrant values of several thousands; this was due to the hardly measurable trace amounts of EGCG. Finally, one can only say that cashew apple tannins must have high number-average molecular weights (Mn) as usually obtained by acid degradation with the presence of excess nucleophile.

Finally, as previously observed on red grape berry skin tannins (Kennedy et al., 2001), caffeine–tannins and purified ones from the Parakou Rouge and CCP 76 genotypes showed red to deep orange color. Flesh and yellow pigmented skin tannins were light cream-colored.

**Fig. 4.** HPLC chromatograms of: (A) reactions products of Parakou Rouge skin tannins with presence of phloroglucinol and (B) of (A) treated with tannase. Peaks (1) ascorbic acid, (2) phloroglucinol; insert in (A): enlarged portion of the chromatogram showing terminal (-)-epigallocatechin 3-O-gallate (50.2–51.5 min).
Highly purified fractions from clone CCP 76 (92% purity for skin tannins, >100% for flesh tannins, based on flavanol conversion after acid phloroglucinol treatment), were submitted to HP-SEC. Tannins eluted as broad unresolved humps (Fig. 5), and part of these populations were excluded from the gel indicating the polydispersive nature of these polymers (void volume $V_v$ at $R_g$ 5.1 min; $M_w$ > 400,000 Da for pullulans). Little is known about the conformation of tannin chains in solution; it has been reported that an increase in C-3 galloylation results in a more extended conformation (Kennedy & Taylor, 2003). Taking into account the average structure and length of cashew apple tannin chains deduced from the compositional analysis (Table 1), one may assume a non-aggregated random coil conformation in $N,N$-dimethylformamide containing 0.15 M LiNO$_3$. Pulullan macromolecules which serve as weight-average molecular weight standards behave as random coils in solution (Nishinari et al., 1991); thus, an estimation of weight-average molecular weights will show exactly the same hydrodynamic volume $V_v$ as the pullulan standards P5 $M_w$ 5800; P10 12,200; P20 23,700; P50 48,000; P100 100,000; P200 186,000; P400 380,000.

Thus, cashew apple tannins are high-molecular weight class D tannins, i.e. prodigelinidins (>70%) (Macheix, Fleuriet, & Billot, 1990); they have been qualitatively but not quantitatively reported as such (da Silveira Agostini-Costa et al., 2003; Satyanarayana et al., 1978). These tannins may be of the B type, i.e. polymers composed of flavan-3-ol units linked mainly through carbon–carbon interflavan C4→C8 bonds, although there exists the possibility of C4→C6 linkages.

Such a constitution is not common in the plant kingdom: with 100% of constitutive units being in a 2,3-cis configuration, with (-)-epigallocatechin as the main extender unit (>90%), ~20–40% of which being 3-O-galloylated, and (-)-epigallocatechin-3-O-galloylated as the terminal unit, cashew apple tannins have strong similarities with prodigelinidins of Myrica esculenta Buch.-Ham. ex D. Don (= hairy bayberry) bark (Sun, Zhao, Wong, & Foo, 1998), and also to some extent with those of Ribes rubrum (= redcurrant) leaves (Foo & Porter, 1980). This has to be related to the fact that the cashew apple is not a fruit but a receptacle (Purseglove, 1974), its tannins being quite different from those of edible fruits (Foo & Porter, 1981).

**Acknowledgments**

Thanks are due to Empresa Brasileira de Pesquisa Agropecuaria (EMBRAPA, Brazil) for providing Brazilian cashew apples; thanks are also due to orchard owners from the Parakou District (Bénin). Thanks are also due to Dr. G. Mazerolles and C. Bouchut (UMR “Science pour l’Énologie”, Plate-forme Polyphenols, INRA, Montpellier, France) for their help. This work was financially supported by European Union (PAVUC project, INCO 015279).

**References**


