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CHARACTERIZATION OF HIGHLY POLYMERIZED PROCYANIDINS IN CIDER APPLE (*MALUS SYLVESTRIS* VAR. *KERMERRIEN*) SKIN AND PULP

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Key Word Index—*Malus sylvestris*; Rosaceae, apple; cider; thioacidolysis; procyanidins; condensed tannins; electrospray ionization.

Abstract—Freeze-dried materials of cider apple pulp and skin were submitted to three successive solid–liquid extractions. The water–acetone extracts contained significant amounts of condensed tannins; thioacidolysis revealed that they were a mixture of highly polymerized procyanidins mainly constituted of (−)-epicatechin units. Electrospray ionization mass spectrometry showed a complete series of polymeric procyanidins with a degree of polymerization up to 17. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Proanthocyanidins (condensed tannins) are oligomeric and polymeric flavanols widely distributed in the plant kingdom. In many fruit derived products they are essential phenolic constituents owing to their physicochemical properties. They can play a considerable role in browning phenomena of apple and apple juices [1, 2]. Moreover, in fruit juices and fermented beverages (wine, cider and beer) polymeric proanthocyanidins are partly responsible for some organoleptic criteria such as bitterness and astringency [3]. Owing to their ability to interact strongly with proteins, they are involved in the formation of hazes and precipitates in beverages [4–6]. For the same reason, they are able to strongly inhibit various enzymic systems [7]. Thus, they could be responsible of the partial inhibition of pectinase preparations commonly used in the fruit juice industry [8].

Some cider apple varieties are known to contain high concentrations of condensed tannins [9]. A recent study showed that all tissues of apple (skin, pulp and seeds) contained condensed tannins, the higher concentration being in the skin [10], but no indication was given about the structural characteristics of these compounds. Cider contains several polymerized proanthocyanidins essentially based on an epicatechin structure [11]. Molecules up to the heptamer were present in cider [12] but the author supposed that procyanidins of higher M_r should exist in the apple fruit itself [11].

The existing techniques of reversed [13] or normal [14] phase liquid chromatography allow separation of oligomeric proanthocyanidins, but in the case of polymeric proanthocyanidins the isolation of pure compounds appeared more difficult. In most cases, only fractions corresponding to mixtures of several polymers can be obtained [15]. Acid degradation in the presence of a nucleophilic agent such as phloroglucinol or toluene-α-thiol is usually used to determine the nature of the flavanol units and the average degree of polymerization of these mixtures [16]. Many studies on characterization of proanthocyanidins were performed by mass spectrometry. A hexameric procyanidin, as its methyl ether derivative, was detected by mass spectrometry using a field desorption ionization technique [17], but fast atom bombardment was more commonly used for the analysis of proanthocyanidins in their native state [18]. Thus, cider procyanidins of the epicatechin series up to the tetramer were characterized by this ionization method [19].

RESULTS AND DISCUSSION

Freeze-dried skin and pulp of the *Kermerrien* cider apple variety were submitted to three successive solid–liquid extractions. Water–acetone extraction yielded a residue containing the polymeric proanthocyanidins which were analysed.

The reversed-phase chromatograms at 280 nm of the water–acetone extract of the apple skin and pulp are shown in Fig. 1. Chlorogenic acid, (−)-epicatechin and phloridzin were identified in the pulp and skin extracts on the basis of their R_t and their UV–visible spectra which were compared to those of commercial standards. However, these compounds were minor in both fractions and the chromatograms were mainly characterized by the presence of a broad and unresolved hump eluting between 15 and 35 min. As observed in

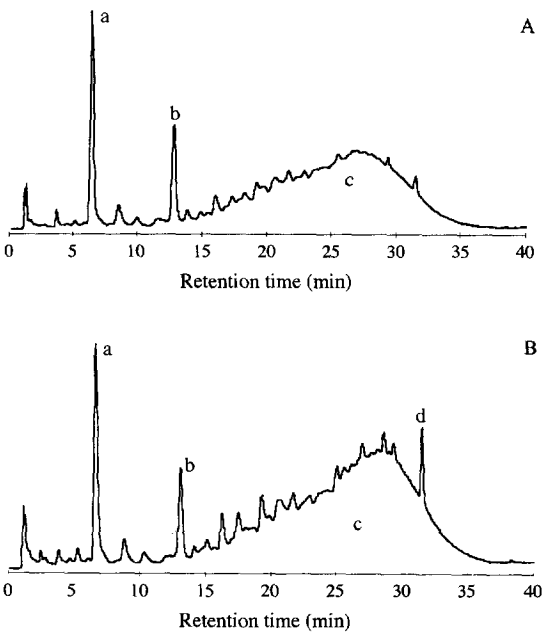

Fig. 1. Reversed-phase chromatograms at 280 nm of the water–acetone extract from apple pulp (A) and skin (B) (var. *Kermerrien*). (a) Chlorogenic acid; (b) (−)-epicatechin; (c) condensed tannins; (d) phloridzin.

previous chromatographic studies on phenolics from apple juices [20], such a hump in the 280 nm chromatographic profile could be attributed to polymeric polyphenols. A series of UV–visible spectra registered on the whole width of the hump showed a single absorption band at 278.3 nm and all these spectra were quite similar to that of (−)-epicatechin or (+)-catechin thus suggesting that the corresponding phenolic molecules presented structural analogies with these flavanols. The butanol–HCl hydrolysis of the water–acetone extracts gave a deep red coloration, which confirmed the presence of a significant amount of condensed tannins. Spectrometric quantifications showed a total polyphenol content of 37 and 25% (in (+)-catechin equivalent) in the skin and the pulp extracts, respectively. Thus, the polyphenol concentration was greater in the skin extract than in the pulp extract; nevertheless, non-phenolic components constituted a large proportion of both residues.

Thioacidolysis was performed according to the method already described by Prieur *et al.* [15] but the reaction was carried out for 24 hr at 25° instead of 2 min at 90° in order to minimize epimerization of (−)-epicatechin and (+)-catechin [16, 21]. HPLC analysis of the reaction medium allowed the assay of the major products resulting from the thiolytic degradation of the water–acetone fractions. The disappearance of the aforementioned hump in the chromatographic profiles after thioacidolysis suggested that the degradation was complete. Identifications and quantifications were performed on the basis of UV–visible spectra, R_t and calibration curves at 280 nm of standard compounds and results are shown in Table 1. (−)-Epicatechin and (+)-catechin, corresponding to terminal units of the proanthocyanidins, and the benzylthioether derivative of (−)-epicatechin, corresponding to the extension units of the proanthocyanidins, were present in the reaction medium. Neither (−)-epicatechin-3-*O*-gallate nor its benzylthioether derivative were found, whereas the benzylthioether derivatives of (+)-catechin were only present as traces. Thus, (−)-epicatechin was highly preponderant in the constitution of the proanthocyanidins since it represented 97% of the flavanol units for the pulp and skin extracts. Moreover, this value may be underestimated if we take into account the reaction of epimerization which principally affects (−)-epicatechin [15]. In order to estimate this reaction, (+)-catechin and (−)-epicatechin were separately incubated in our experimental conditions of thioacidolysis and the two compounds were then quantified by HPLC. For (+)-catechin, the rate of conversion was only 4.5%, whereas it was 17.5% for (−)-epicatechin. Thus, the conversion of (−)-epicatechin to (+)-catechin is preponderant in comparison to the reverse conversion. The average degree of polymerization of the fraction was estimated to be 12.5 and 11.3 for the pulp and the skin extracts, respectively. In comparison with previous studies, these results showed that the fruits might contain more polymeric proanthocyanidins than the derived products, since only the presence of polymers up to heptameric structures was previously shown in apple juices and ciders using methods based on R_f on TLC analysis [20] or on the elevation of boiling point [12] to determine M_r.

Mass spectral analyses were performed using an electrospray ionization mass spectrometry (ESI) technique. ESI-mass spectrometry is a soft ionization technique which usually gives molecular ion peaks, making it useful for M_r determination of large bio-

Table 1. Characterization of procyanidins in the skin and pulp water–acetone extracts of *Kermerrien* cider apple determined by HPLC following thioacidolysis degradation

	Terminal units (%)		Extension units (%)	DP
	CAT	EC	EC	
Skin extract	2.8 (0.22)	5.2 (0.68)	92.0 (7.8)	12.5
Pulp extract	2.7 (0.08)	6.1 (0.22)	91.2 (3.7)	11.3

CAT = (+)-catechin; EC = (−)-epicatechin; DP = average degree of polymerization; values in parentheses correspond to standard deviations ($n = 3$).

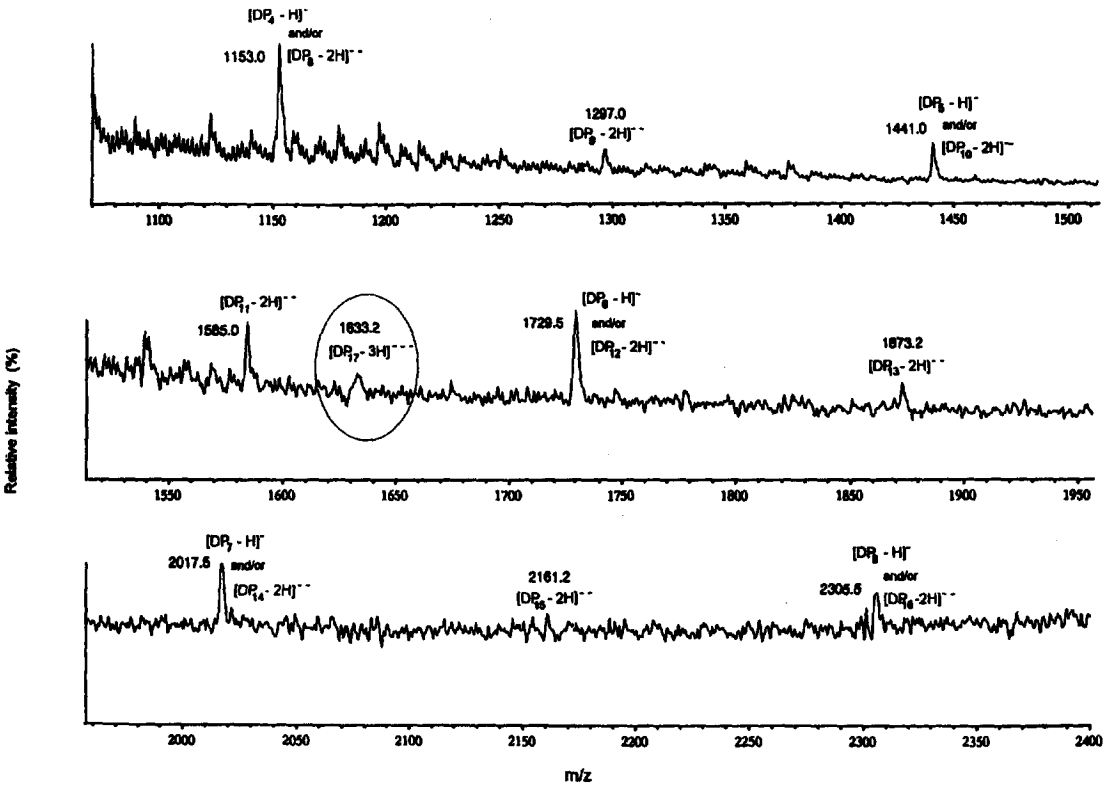

Fig. 2. ESI-MS of water–acetone extract of cider apple skin (var. *Kermerrien*).

logical compounds that are difficult to vaporize or ionize. Ionization was performed in the negative mode by the loss of one or several protons and formation of as many charges. The ESI-mass spectrum of the water–acetone extract of apple skin is shown in Fig. 2. For m/z ions above 1000, the spectrum was characterized by the distinct presence of several peaks at 1153.0, 1297.0, 1441.0, 1585.0, 1633.2, 1729.5, 1873.2, 2017.5, 2161.2 and 2305.5. All of these signals were consistent with molecular ion peaks of polymeric catechins of the procyanidin type.

Considering the M_r of a catechin unit (i.e. 290) and the loss of two protons for each interflavan linkage, m/z 1153.0, 1441.0, 1729.5, 2017.5 and 2305.5 were consistent with $[M-H]^-$ of tetrameric, pentameric, hexameric, heptameric and octameric procyanidins, respectively.

The interpretation had to be completed by considering the existence of multi-charged species. As a matter of fact, in the case of a multi-charged ion, we must observe as many proton losses as charge gains on the ionized polymer and the m/z value must be divided by the number of the charges according to the following formula:

$$m/z = \frac{[(\mathrm{DP} \times M_W C) + \varepsilon - 2 \times (\mathrm{DP} - M_H)] - z \times M_H}{z}$$

where m/z is the apex of the isotopic clump of the polymeric catechin, z is the number of charges, ε is the corrective term for the isotopic contribution*, DP is the degree of polymerization, $M_W C$ is the absolute M_r of the catechin unit (=290) and M_H is the mass of a hydrogen atom.

In respect of these considerations, signals at m/z = 1153.0, 1441.0, 1729, 2017.5 and 2305.5 could also correspond to the doubly charged DP_8, DP_{10}, DP_{12}, DP_{14} and DP_{16}. The existence of doubly charged species was proved by the presence of signals at m/z = 1297.0, 1585.0, 1873.2 and 2161.2, which could be attributed without any ambiguity to doubly charged DP_9, DP_{11}, DP_{13} and DP_{15}, respectively, according to the above formula. As 9, 11, 13 and 15 are uneven numbers, no smaller polymers could give molecular ion peaks with the same m/z and corresponding to monocharged ions. Moreover, according to the results of thiolyses, these ions appeared unlikely to correspond to tetra-charged DP_{18}, DP_{22}, DP_{26} or DP_{30} species, even if this opportunity could not be totally excluded.

Two examples of enlarged signals corresponding to mono- and doubly charged ions are given in Fig. 3. They showed evidence of the presence of the isotopic peaks whose existence was mainly due to the natural

*If the number of carbon atoms exceeds 90 (DP_6), the isotopic contribution of ^{13}C becomes significant. Thus, $\varepsilon = 0$ or 1 or 2 or ... according to the number of statistically present ^{13}C in the polymeric molecule.

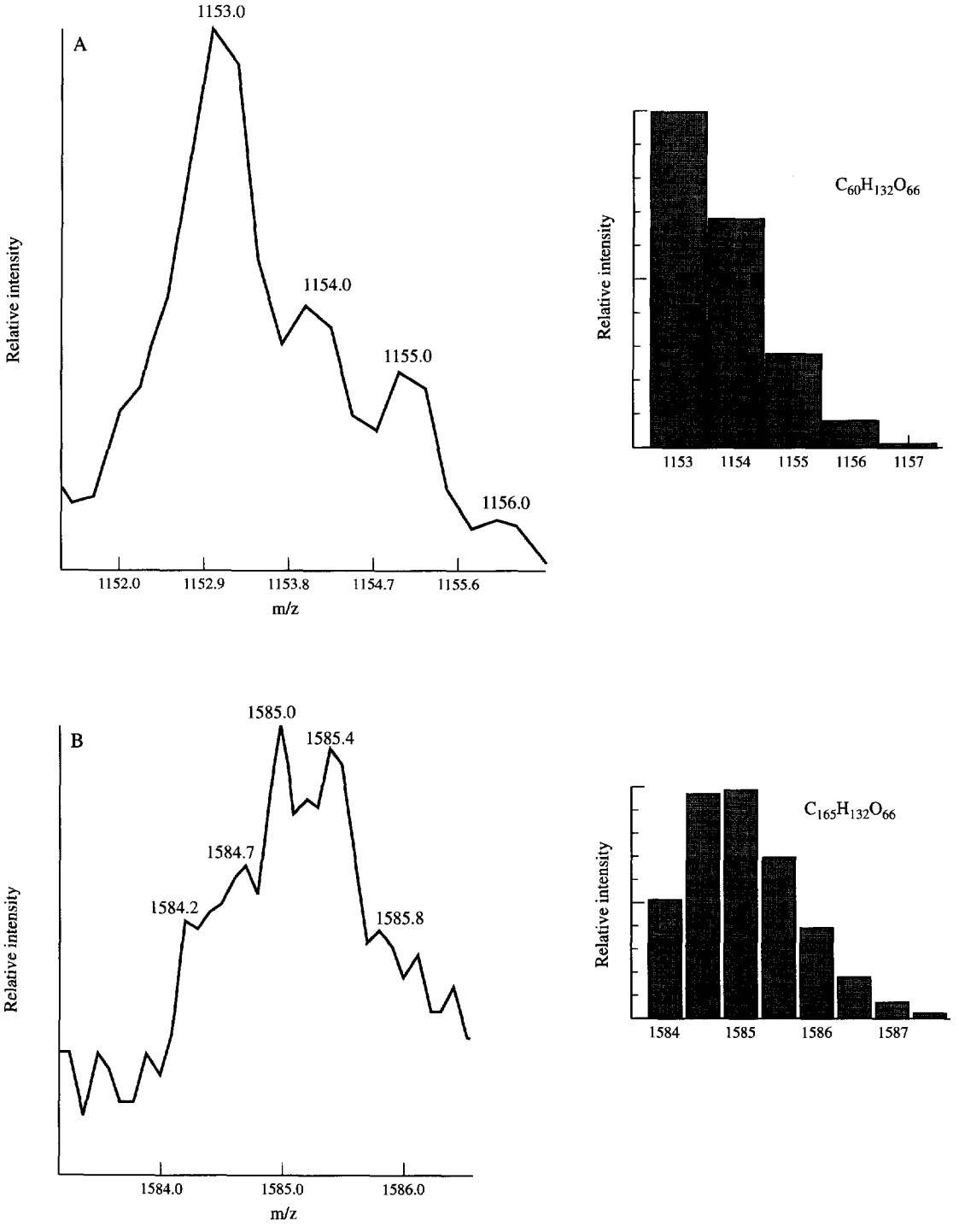

Fig. 3. Mass spectrum enlargement (skin water–acetone extract) of signals at $m/z = 1153$ (A) and $m/z = 1585$ (B); diagrams on the right present the calculated isotopic distribution expected for DP_4 mono-charged ions (A) and DP_{11} doubly charged ions (B).

occurrence of ^{13}C. For the signal at $m/z = 1153.0$ (Fig. 3(A)), the distance between the isotopic peaks was distinctly one mass unit, which provide another argument to show that this signal corresponded essentially to a mono-charged DP_4 species. The distance would have been 0.5 for doubly charged ions (see formula). Despite its weak intensity, the signal at $m/z = 1585.0$ (Fig. 3(B)) clearly indicated its correspondence to doubly charged DP_{11} species according to the distance of approximately 0.5 between the major isotopic peaks. Fig. 3 presented also the calculated isotopic distribution in front of the really observed signals. Thus, for signals at $m/z = 1153.0$ (Fig. 3(A)), the isotopic ion peak $[DP_4 - H]^-$ ($m/z = 1153.0$, no ^{13}C in the molecule)

was more intense than $[(DP_4 + 1 - H]^-$ ($m/z = 1154.0$, one ^{13}C in the molecule) which is consistent with the expected isotopic distribution for a tetrameric procyanidin containing only 60 carbon atoms. On the other hand, for the signals of the doubly charged DP_{11} procyanidin ion at $m/z = 1585.0$ (Fig. 3(B)), the isotopic peak $[(DP_{11} + 2) - 2H]^{2-}$ ($m/z = 1585.0$, two ^{13}C in the molecule) was more intense than the corresponding ions having none or one ^{13}C in the molecule ($m/z = 1584.2$ and $m/z = 1584.7$, respectively). In this later case, the distribution clearly indicated its correspondence to a polymeric procyanidin containing more than 90 carbon atoms which rendered more probable the possibility of the molecule containing ^{13}C atoms in its structure.

The signal at $m/z = 1633$ (Fig. 2) was attributed without any ambiguity to the largest polymer of procyanidin observable on the mass spectra. It corresponded to the triply charged DP_{17} procyanidin molecular ion peak; 17 is a prime number which makes it impossible to confuse with less polymerized and less charged species. Moreover, the correspondence to a DP_{34} tetra-charged procyanidin species appeared unlikely according to the average degree of polymerization for the fraction which was given by thioacidolysis (Table 1).

The above discussion which concerns the mass spectrum of a solution of the cider apple skin water–acetone extract could be applied to the pulp extract, since most of the signals observed in the apple skin spectrum could also be observed in the apple pulp spectrum. Thus, both the signals at $m/z = 1153$ and at $m/z = 1633$ corresponding, respectively, to monocharged tetrameric ions and the triply-charge DP_{17} ions, were present. Signals corresponding to intermediate polymerized structures were also observed. These results suggested that the composition of the polymeric procyanidins pool of the two tissues is quite similar, at least from a qualitative point of view.

As a conclusion, according to the results of thioacidolysis and ESI-mass spectral analyses, cider apple pulp and skin were shown to contain polymeric tannins having a structure based on the (−)-epicatechin unit (Fig. 4). Nevertheless, a lack of information still exists about the C_4–C_8 or C_4–C_6 position of the interflavan linkage since our experiments did not allow us to elucidate this structural feature.

As far as we known, ESI-mass spectra have provided evidence, for the first time, of a complete series of polymeric procyanidins from DP_4 to DP_{17} in their native state. Moreover, this analytical technique seemed to be applicable even on crude fractions containing also non-polyphenolic constituents. The interpretation, based on the observation of multi-charged ion signals, allowed the characterization of polymeric procyanidin structures having a M_r near to 5000 daltons, which is probably not a limiting value. Cider apple condensed tannins, which present a large homogeneity for their constitutive flavanol units [essentially (−)-epicatechin], undoubtedly provide a good model to develop the

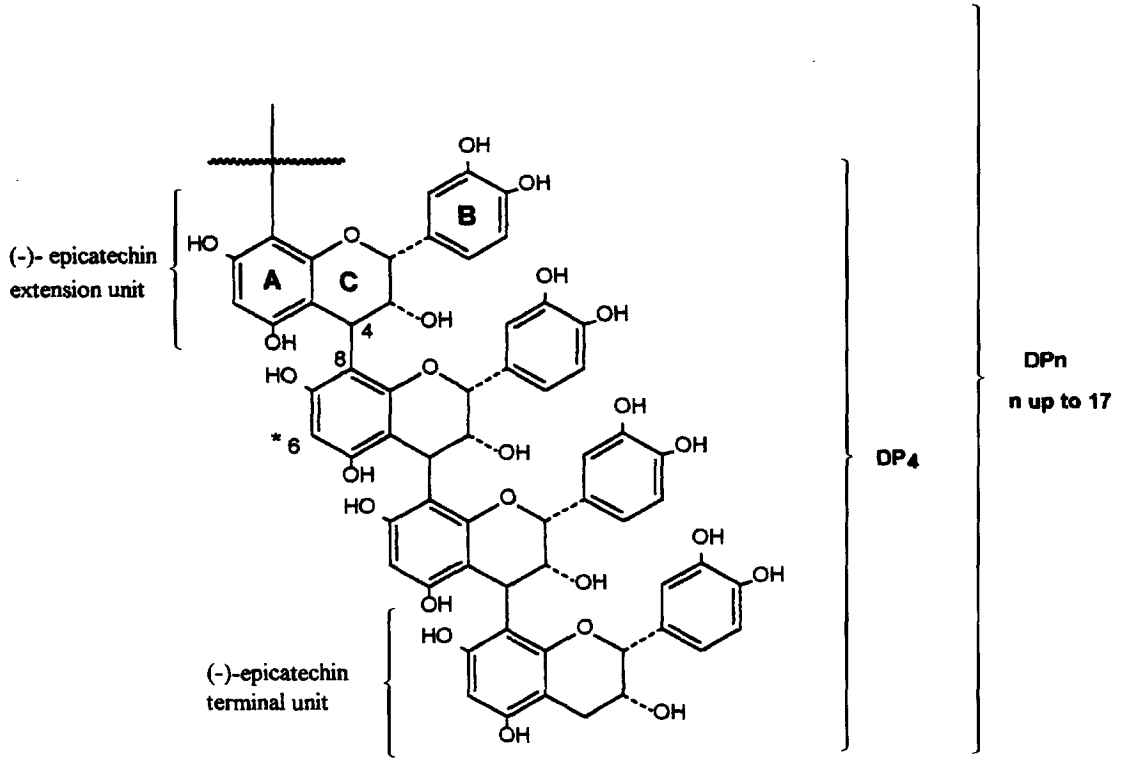

Fig. 4. Structures of cider apple pulp and skin polymeric procyanidins (*eventuality of C_4–C_6 linkages can not be excluded).

potential of the ESI-mass spectrum method for the characterization of highly polymerized procyanidins.

EXPERIMENTAL

Standard phenolics. (+)-Catechin, (−)-epicatechin, chlorogenic acid and phloridzin were provided by Sigma Chemicals Co. (St Louis, MO, U.S.A.). (−)-Epicatechin-3-*O*-gallate, (+)-catechin-benzylthioethers*, (−)-epicatechin-benzylthioether and (−)-epicatechin-3-*O*-gallate-benzylthioether were obtained and characterized as already described [15].

Plant materials. Cider apples of the *Kermerrien* variety, grown during the 1995 season in an orchard in the Department of Mayenne (France), were picked at maturity and stored at 4° until used. They were manually calibrated (50–55 mm) and mechanically peeled and cored at 4°. A soln of formic acid (3% v/v in water) was sprayed on the fresh material during these later operations to avoid oxidations (a preliminary experiment had shown that neither browning due to oxidation nor pink coloration due to proanthocyanidin degradation, appeared after 12 hr). Skins† and pulps were separately frozen and freeze-dried until used.

Extraction of polymeric proanthocyanidins. Dried materials (skin or pulp) (5 g) were homogenized by crushing in a mortar. The powder was first extracted by 3 × 50 ml hexane to remove lipids, carotenoids and chlorophylls. It was then extracted by 3 × 50 ml of MeOH to remove sugars, organic acids, and phenolic compounds of low M_r. Finally, it was treated by 3 × 50 ml Me_2CO–H_2O (60:40) to extract polymeric proanthocyanidins. Each solvent extraction was carried out by mixing the powder during 5 min with an Ultraturrax blender and the mixt. was filtered through a G3 sintered glass filter. H_2O–Me_2CO filtrates were combined, evapd under vacuum to an aq. soln (no smell of Me_2CO), frozen and freeze-dried to yield 83 mg and 142 mg of residue, from the pulp and the skin, respectively.

Reversed-phase HPLC analysis of the crude H_2O–Me_2CO *extracts.* About 2 mg of the extract were dissolved in 2.5% CH_3COOH–MeOH (75:25, v/v) at a concn of 1 mg ml^{-1} and 20 μl were injected onto the HPLC system. The HPLC apparatus was a Waters (Milford, MA, U.S.A.) system (717 plus autosampler, 600E multisolvent system, 996 Photodiode array detector and the Millenium 2010 Manager system). The column was a Nova-pak C18, 4 μm particle size, 100 × 3.9 mm length (Waters, Milford, U.S.A.). The elution conditions were as follows: Flow rate 1 ml min^{-1}, temp. 30°. The solvent system used was a gradient of solvent A (AcOH 2.5% v/v in H_2O) and solvent B (CH_3CN). The following gradient was applied: 0–3 min, 3% B isocratic; 3–13 min, 9% B linear; 13–18 min, 11% B linear; 18–25 min, 18% B linear; 25–30 min, 18% B isocratic; 30–45, 30% B linear, follow by washing and reconditioning the column.

BuOH–HCl *hydrolysis.* BuOH–conc. HCl (95:5, v/v) (2.5 ml) and 100 μl of iron reagent (2% w/v soln of $NH_4Fe(SO_4)_2$. 12 H_2O in 2 M HCl) were added to about 5 mg of H_2O–Me_2CO extract in a glass tube and sealed with a teflon-lined screw cap. After agitation, the tube was heated at 95° during 40 min.

Thiolytic degradation. A 2 mg ml^{-1} suspension was prepared by sonicating the residue in MeOH acidified by HCl (1.66%, v/v); 100 μl of the suspension was introduced into a glass bulb together with 100 μl of phenylmethane-thiol (5% in MeOH). After sealing, the reaction was carried out at 25° for 24 hr and then 10 μl were directly injected on to the HPLC system. All incubations were performed in triplicate.

HPLC analysis of the thiolytic media. The HPLC apparatus was the same as described above for the HPLC analyses of the crude extracts. Only gradient conditions were different: 0–1 min, 3% B isocratic; 1–3 min, 6% B linear; 3–5 min, 12% B linear; 5–8 min, 25% B linear; 8–12 min, 25% B isocratic; 12–16 min, 28% B linear; 16–20 min, 28% B isocratic; 20–23 min, 30% B linear; 23–26 min, 60% B linear follow by washing and reconditioning the column. Calibration curves (based on peak area at 280 nm) were established using flavan-3-ol and benzylthioether standards.

Spectrometric quantification. 2.5% CH_3COOH–MeOH (75:25, v/v) was used to prepare a soln of the extract at a concn of 0.5 mg ml^{-1} (both pulp and skin H_2O–Me_2CO extracts were soluble in this solvent). Absorbencies were measured at 280 nm on a Uvikon double-beam spectrophotometer Model 860 (Kontron, Milano, Italy) with the above solvent in the reference cell. The quantification was performed by reporting the average absorbance values (assays were in triplicate for each extract) onto a calibration curve of (+)-catechin.

ESI-MS analysis. Negative-ion ESI-MS spectra were recorded on a Sciex API I Plus (Sciex, Thornhill, Ontario, Canada) simple quadrupole mass spectrometer with a nominal mass range up to $m/z = 2400$, equipped with an ion spray source. The mass spectrometer was operated with a −4000 V voltage applied to the electrospray needle and −60 V to the orifice. The mass spectrometer was scanned from m/z 500 to 2400, in step of 0.1 msec unit and with a dwell time of 25 msec at each step. The instrumentation was calibrated using a soln of polypropylene glycol oligomers. Molecular masses are reported as isotopically averages values. H_2O–Me_2CO extracts were dissolved in 2.5% CH_3COOH (v/v in water)–MeOH (75:25) at the concn of 2 g l^{-1} and the soln was introduced into the ionspray source at a constant flow rate of 10 μl min^{-1} with a medical syringe infusion pump (Harvard Apparatus,

*(+)-Catechin gives two benzylthioether adducts [15].

†According to the mechanical peeling of the apple fruits, the 'skin material' also contained a significant part of pulp parenchyma so it would be better to consider it as the 'epidermic zone' of the apple fruits.

Model 22, Southnatick, U.S.A.) in combination with a microlitre syringe (100 μl).

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