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Isolation and identification of procyanidins in apple pomace

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Abstract

The chemical nature of the procyanidin components in apple pomace was investigated as part of a programme seeking alternative uses of the large quantity of pomace that is being generated from the apple juice industry. The homogeneous nature of the procyanidin fractions was demonstrated by the isolation and identification of a range of epicatechin oligomers, namely epicatechin-(4β-6) epicatechin, epicatechin (4β-8) epicatechin, epicatechin (4β-8) epicatechin (4β-8) epicatechin and epicatechin (4β-8) epicatechin (4β-8) epicatechin (4β-8)-epicatechin. The procyanidin polymers were also isolated and purified by a Sephadex LH20 column and shown to consist exclusively of epicatechin units. The chemical structures of these procyanidins were established by ^{13}C-NMR spectroscopy, by acid-catalysed degradation reaction with phloroglucinol and by electrospray mass spectrometry.

1. Introduction

Apple pomace is an heterogeneous mixture consisting of peel, core, seed, calyx, stem and soft tissue. Numerous studies have been devoted to finding practical uses for this vast waste resource estimated to be about several million tonnes per annum globally (Hang, 1985; Jewell & Cummings, 1984; Jarosz, 1988). Many of these studies were based on wholescale applications such as animal feed (Givens & Barber, 1987; Singh & Narang, 1992), compost or fertiliser (Chong, 1992; Schaub & Leonard, 1996), fuel (Hang, 1987; Sargent, Steffe, & Pierson, 1986) and some relied on varying degrees of treatments such as fermentation for production of ethanol (Jarosz, 1988; Gupta, Pathak, & Tiwari, 1990), citric acid (Hang, 1988; Hang & Woodams, 1989), pectin (Davies, 1982; Lopez, Coalia, De La Torre, & Diez, 1990), and aroma or flavouring compounds (Almosino & Belin, 1991; Almosino, Bensoussan, & Belin, 1996). Studies devoted to the utilisation of polyphenols have been conspicuously absent, probably because this group of compounds had been viewed as problematic, causing discoloration to apple and products (Coseteng & Lee, 1987). These compounds possess antioxidant activities and, as a result of a better understanding of the harmful effect of free radicals on human health (Ames, 1983; Steinberg, 1988, 1992), there is now growing interest in polyphenols as potential disease-preventing agents. As these compounds are predominantly found in the peel and pulp (Burda, Oleszek, & Lee, 1990; Lee, Wall, Beveridge, & Suttil, 1995) it would be worthwhile investigating the nature of polyphenols that are present in apple pomace. The flavonoid and related components were reported earlier (Lu & Foo, 1997) and the present report is the continuation of this study and deals with the chemical nature of the procyanidin components; both classes of compounds have been credited as possible factors in reducing risks of coronary disease in a cohort study in Finland (Knekt, Järvinen, Reunanen, & Maatela, 1996).

2. Materials and methods

2.1. Extraction

Freeze-dried gala apple pomace (200 g) from a juice operation, supplied by Frucor Processors, formerly Enza Processors (Hastings, New Zealand), was ground to powder (sieve 1 mm) and extracted at ambient temperatures with 70% aqueous acetone (3×500 ml). The combined extract was concentrated on a rotatory

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evaporator at 40°C under reduced pressure and the aqueous residue defatted with hexane (3×300 ml), concentrated and freeze-dried to afford 45 g of solid.

2.2. Chromatographic separation

Chromatographic separation of the freeze-dried extract (130 g) was performed on a Sephadex LH-20 column (60×4 cm ID) using aqueous methanol as solvent, the methanol content being increased from 0 to 100% in increments of 10%. The column was eluted with 1 litre of each solvent mixture to yield 11 fractions. Fractions eluted with 80% methanol or more contained the bulk of the procyanidin oligomers. Following this treatment, the column was eluted with 1 litre of 60% aqueous acetone, which yielded the polymeric procyanidin fraction. The various oligomeric procyanidin fractions were separately subjected to further chromatographic separations on Sephadex LH-20 using 50% aqueous ethanol. Fractions were collected using a fraction collector and monitored by thin-layer chromatography (TLC). Fractions of the same composition were combined and, if necessary, re-chromatographed until homogeneous products were obtained.

2.3. TLC analysis

TLC analysis of chromatographic fractions was performed on Schleicher and Schuell cellulose plates which were developed with 6% aqueous acetic acid solution. The plates were visualised by spraying with a 5% vanillin solution in 10% HCl in ethanol (v/v), followed by heating the plate with a hot air blower. The flavans and procyanidins were revealed as orange to reddish spots. In the 2D TLC analysis the plates were first developed with *tert*-butanol–acetic acid–water (3:1:1, v/v) (solvent A) in one direction and after drying with a hot air blower the plate was again developed with 6% aqueous acetic (solvent B) in the other direction.

2.4. BuOH–HCl hydrolysis

This reaction was performed with a small sample of procyanidin (1 mg) in a sealed vial containing 5% HCl in *tert*-BuOH (2 ml) and the mixture heated in a boiling water bath for 1 h. The resulting cyanidin was analysed by TLC using Forestal solvent (acetic acid–conc. hydrochloric acid–water 30:3:10 v/v).

2.5. Degradation with phloroglucinol

A sample of procyanidin (2 mg) was mixed with phloroglucinol (2 mg) and to this mixture was added 1 ml of 1% conc. HCl in EtOH. After standing for 1 h at ambient temperature the reaction products were examined by HPLC.

2.6. HPLC analysis

The HPLC analyses were performed on a Hewlett–Packard Series 1100 instrument equipped with a HP ChemStation using a reverse phase column [LiChroCART® 100 RP-18 (5 μm)] with column temperature set at 30°C. The following solvents: C [acetic acid/water (2/98 v/v)] and D [acetic acid/acetonitrile/water (2/20/78 v/v)] were used with a flowrate of 0.5 ml per min. The solvent gradient applied was 100 to 80%C at 20 min and then to 100%D at 60 min followed by washing and reconditioning for 5 min. A 50 μl of sample was injected and products were detected by UV absorption at 280 nm.

2.7. Identification

Purified procyanidin compounds were identified by ^{13}C-NMR on a Bruker AC300 instrument. Samples (50–100 mg) were dissolved in 0.5 ml deuterated methanol or fully deuterated aqueous acetone and chemical shifts were referenced to the ^{13}C chemical shifts of the deuterated solvent. Compounds were also identified by electrospray mass spectrometry (ESMS) using a negative ion probe with samples dissolved in acetonitrile–water (1:1 v/v).

2.7.1. Epicatechin (4β→6) epicatechin (2)

R_f 0.43 (A) and 0.43 (B), ESMS gave $[M-H]^-$ at m/z 577. ^{13}C-NMR (75 MHz, methanol-d_4): δ29.71, 37.80, 67.6, 72.8, 77.4, 79.8, 96.2, 96.9 (×2), 100.8, 115.4, 115.5, 116.1, 119.3, 119.6, 132.4, 145.8, 145.9, 146.1, 155.6, 156.0, 158.1, 159.5. Degradation with phloroglucinol in 1% HCl in EtOH gave epicatechin and epicatechin (4β-2)-phloroglucinol.

2.7.2. Epicatechin (4β-8) epicatechin (3)

R_f 0.43 (A) and 0.63 (B), ESMS gave [M–H] at m/z 577. ^{13}C-NMR (75 MHz, methanol-d_4): δ29.6, 37.1, 67.0, 73.5, 77.1, 79.7, 96.1, 96.5, 97.3, 100.5, 101.4, 115.3 (×2), 115.9 (2×), 119.3 (2×), 132.1, 132.6, 145.6 (2×), 145.8 (2×), 154.5, 156.4, 157.8 and 158.3. Degradation with phloroglucinol in 1% conc. HCl in EtOH gave epicatechin and epicatechin (4β→2) phloroglucinol.

2.7.3. Epicatechin (4β-8) epicatechin (4β-8)-epicatechin (4)

R_f 0.25 (A) and 0.60 (B). ESMS gave $[M-H]^-$ peak at m/z 865. ^{13}C-NMR (75 MHz, methanol-d_4): δ29.9, 37.6, 66.9, 73.0, 73.6, 77.2, 79.8, 96.4, 96.8, 97.9, 100.8, 101.6, 102.3, 107.8, 115.3, 115.5, 116.2, 119.3, 119.5, 132.3, 132.8, 145.7, 145.8, 145.9, 146.1, 154.7, 155.1, 156.7, 157.0, 157.4, 158.0, 158.5. Degradation with phloroglucinol gave epicatechin, epicatechin (4β-2) phloroglucinol, epicatechin (4β-8) epicatechin and epicatechin (4β-8) epicatechin (4β-2) phloroglucinol.

2.7.4. Epicatechin (4β-8) epicatechin (4β-8)-epicatechin (4β-8)-epicatechin (5)

R_f 0.27 (A) and 0.6 (B). ESMS gave $[M-H]^-$ peak at m/z 1153: ^{13}C-NMR (75 MHz, methanol-d_4): δ29.8, 37.4–37.6, 66.8, 72.9, 73.5, 77.1, 79.8, 96.3, 96.6, 97.8, 100.7, 101.5, 102.4, 107.3–107.7, 115.4, 116.1, 119.2–119.4, 132.2, 132.6, 145.6–146.0, 154.6–156.9. Degradation with phloroglucinol in 1% HCl in EtOH gave epicatechin, epicatechin (4β-2) phloroglucinol, epicatechin (4β-8) epicatechin, epicatechin (4β-8) epicatechin (4β-2) phloroglucinol, epicatechin (4β-8) epicatechin (4β-8) epicatechin and epicatechin (4β-8) epicatechin (4β-8) epicatechin (4β-2) phloroglucinol.

2.7.5. Epicatechin oligomer (6)

ESMS gave an $[M-H]^-$ at m/z 1441. ^{13}C-NMR (75 MHz, acetone-d_6): δ29.9 (obscured by acetone-d_6 peak), 37.0, 66.3, 73.1–73.7, 76.6, 79.1, 96.1–97.4, 100.6, 101.4–102.1, 107.2–107.5, 115.2, 116.2, 119.2, 131.9–132.2, 144.9–145.2, 154.1–157.8.

2.7.6. Epicatechin polymer (7)

ESMS gave an $[M-H]^-$ at m/z 1729. ^{13}C-NMR (75 MHz, acetone-d_6): δ29 (obscured by solvent peak), 36.6–37.2, 66.7–66.9, 72.3–72.8, 76.3–76.5, 79.5, 96.4–96.7, 99–103, 107.2–107.5, 115.2–115.3, 116.5, 119.3–119.4, 132.0–132.3, 144.7, 154.8–156.0.

3. Results and discussion

3.1. Isolation

The isolation of the low molecular weight polyphenols, namely epicatechin, phloretin-2′-xyloglycoside, phloridzin, caffeic acid, 3-hydroxyphloridzin and a range of quercetin-3-glycosides from apple pomace, was effected by column chromatography on Sephadex LH20 using aqueous methanol as solvent was described earlier (Lu & Foo, 1997). The procyanidin oligomers, which were less mobile under this condition, were found in later fractions eluted with 80% aqueous methanol. Fractions showing similar procyanidin composition on TLC were combined to give two fractions which were separately subjected to further chromatographic separation on Sephadex LH20 and on a reverse-phase matrix (Mitsubishi Dianion HP20) until chromatographically (TLC) homogeneous samples were obtained. In this manner the procyanidin dimers, trimer and tetramer were obtained. The higher procyanidin oligomers were obtained from the Sephadex LH20 columns by eluting the column with 60% aqueous acetone after the lower molecular weight procyanidins had been recovered.

3.2. Identification of procyanidins

The procyanidin dimers (2) and (3) in an approximate ratio of 1:4, respectively, were isolated from earlier fractions and were shown to be procyanidin by the production of cyanidin on heating with 5% HCl in *tert*-butanol. The dimeric procyanidin constitution of both compounds was apparent from the ESMS data which gave an $[M-H]^-$ peak at m/z 577 in both instances. The ^{13}C-NMR spectra of (2) and (3) were very similar with aromatic carbon resonances diagnostic of phloroglucinol A-ring and the catechol B-ring in addition to aliphatic carbon resonances of the heterocyclic pyran ring (Porter, Newman, Foo, Wong, & Hemingway, 1982). The upfield position of the C-2 resonances, δ72.8 and 77.4 for (2) and 73.5 and 77.1 for (3) clearly established the 2,3-*cis* configuration of all the flavanoid units in both compounds. This structural deduction was also corroborated by the acid-catalysed reaction of (2) and (3) with phloroglucinol. This reaction, which involved cleavage of the inter-flavanoid bonds of procyanidins to generate carbocations at C-4 of the flavan extender units were then captured by the nucleophilic phloroglucinol to give flavan–phloroglucinol addition products and releasing the bottom or terminal flavan-3-ol, is one of the principal reactions used to establish the identity of the constitutive units and the nature of the interflavanoid linkage in proanthocyanidin oligomers (Foo & Karchesy, 1989). The reaction of (2) and (3) with phloroglucinol both yielded epicatechin (1) and epicatechin (4β-2) phloroglucinol (8) as the only major degradation products, thus establishing both compounds were epicatechin dimers differing only in the position of the interflavanoid bonding. Compound (2), by virtue of its lower mobility on TLC (R_f 0.43 in 6% acetic acid), was assigned to epicatechin (4β-6)-epicatechin (commonly known as procyanidin B5) while (3) with its higher mobility (R_f 0.64 in 6% acetic acid) was assigned as epicatechin (4β-8) epicatechin (procyanidin B2). The assignments, based on relative mobility on TLC, were confirmed by HPLC comparison with authentic samples, the relative mobility on TLC of the two dimers being also observed on HPLC where (2) had a longer retention time of about 55 min while that of (3) was 36.3 min under the HPLC conditions used.

Compound (4) was shown to be a procyanidin by its reaction with 5% HCl in *tert*-butanol to yield cyanidin. The molecular weight derived from ECMS which gave $[M-H]^-$ at m/z 865 indicated it to have a triflavanoid constitution. This was also apparent from its ^{13}C-NMR spectrum (see Fig. 1) which showed two carbon peaks at δ73.0 and δ73.6 attributable to the two C-3 carbons of different extender flavan-3-ol units and a higher-field carbon signal at δ66.9 which was that of the C-3 carbon of the bottom or terminal flavan-3-ol unit. The trimeric flavanoid constitution was also corroborated by similar

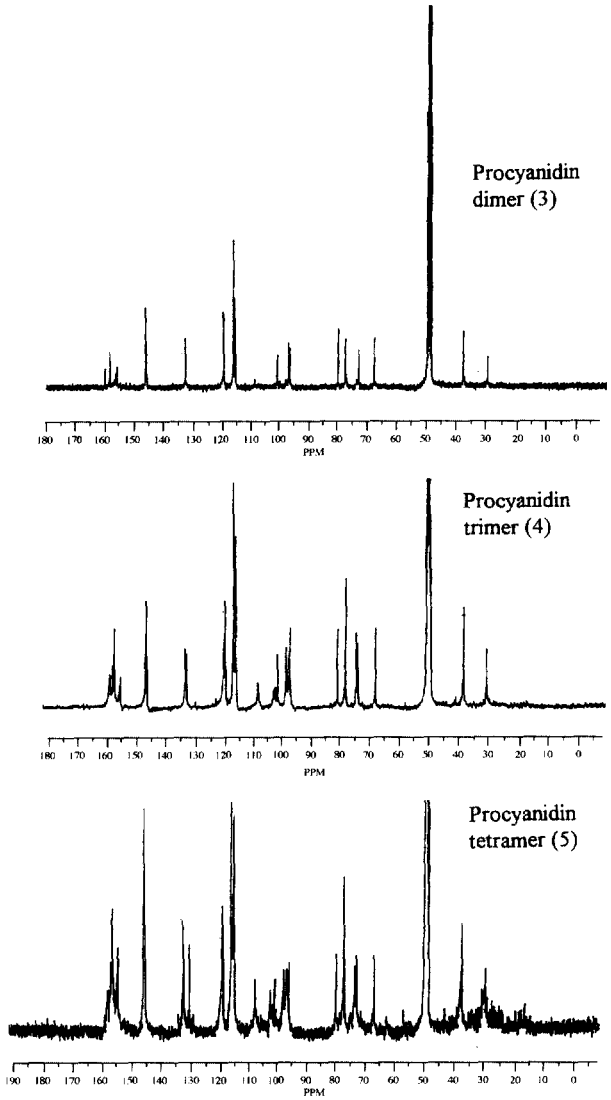


Fig. 1. ^{13}C-NMR of procyanidin dimer (3), trimer (4) and tetramer (5).

examination of the C-2 carbon resonances, the lower field resonance at δ79.8 being that of the terminal epicatechin unit and the larger signal at δ77.2 being the degenerate signals of the two corresponding extender units, their respective peak size being in the ratio of 1 to 2 for the terminal to extender C-2 signals, respectively. This ratio and those from the C-3 signals could be used to calculate the degree of polymerisation of a procyanidin sample. As observed for the dimers (2) and (3), the terminal and extender C-2 carbon resonances all occurred in the upfield region which indicates that all the flavanoid units in (4) had the 2,3-*cis* configuration and hence an epicatechin trimer. In order to establish the nature of the interflavanoid linkage, compound (4) was subjected to acid-catalysed cleavage in the presence of excess phloroglucinol and the products after 1 h of reaction were examined by HPLC (see Fig. 2). After this period, most of the trimer had already reacted to give rise to epicatechin, confirming that this moiety was the terminal unit. Epicatechin (4β-8) epicatechin and the phloroglucinol addition products, epicatechin (4β-2) phloroglucinol and epicatechin (4β-8) epicatechin (4β-2) phloroglucinol were also detected as the main reaction products. The identities of these products were confirmed by HPLC comparison with authentic reference materials which had been fully characterised (Foo, Newman, Waghorne, McNabb, & Ulyatt, 1996). The C-4 to C-8 interflavanoid linkages of all the major reaction products indicated that the procyanidin trimer (4) was similarly linked and hence the compound was epicatechin (4β-8) epicatechin (4β-8) epicatechin.

Compound (5) was similarly shown to be a procyanidin by its reaction with 5% HCl in *tert*-butanol and its ^{13}C-NMR spectrum (see Fig. 2) was very similar to (4); hence the constitutive flavanoid units were all epicatechin units. Estimation of the ratio of terminal unit to extender units, using the signal intensity of the terminal C-2 or C-3 to the combined C-2 or C-3 intensities, respectively, of the extender units gave a value of about 1 to 3, suggesting (5) was an epicatechin tetramer. This molecular size was also confirmed by ESMS which gave the $[M-H]^-$ peak a m/z 1153. This chemical constitution was also consistent with the acid-catalysed degradation studies with phloroglucinol (see Scheme 1), the reaction yielding all the expected degradation products as a result of random nucleophilic substitution by phloroglucinol at various interflavanoid bonds along the procyanidin oligomer chain. Degradation products, which include epicatechin (4β-8) epicatechin, epicatechin (4β-8) epicatechin (4β-2) phloroglucinol and epicatechin (4β-8) epicatechin (4β-8) epicatechin (4β-2) phloroglucinol, not only confirmed all epicatechin constitutive units in (5) as established from ^{13}C-NMR studies but also confirmed that these four epicatechin units were joined together by C4 to C8 interflavanoid bonds.

The higher procyanidin oligomers or polymers were obtained during purification of lower oligomers on Sephadex LH20 by subsequent washing of the column with 60% aqueous acetone. While it would appear that the methodology used for the structural elucidation of the lower oligomers could also be extrapolated to the study of the higher molecular weight procyanidins, the reality was somewhat different. The ESMS technique appeared to have limitations in establishing a molecular weight higher than that of a pentamer as mass ion peaks beyond this range were for some reasons very weak and hence could not be employed for confirmation of molecular size, which was estimated by ^{13}C-NMR.

A plausible explanation for this limitation could be due to the tendency for multiple ionization of the polyphenol giving rise to more than one charge on a polymer chain; this tendency increases with a longer chain as a result of better separation of like charges. A polymer carrying two charges therefore will be recorded as

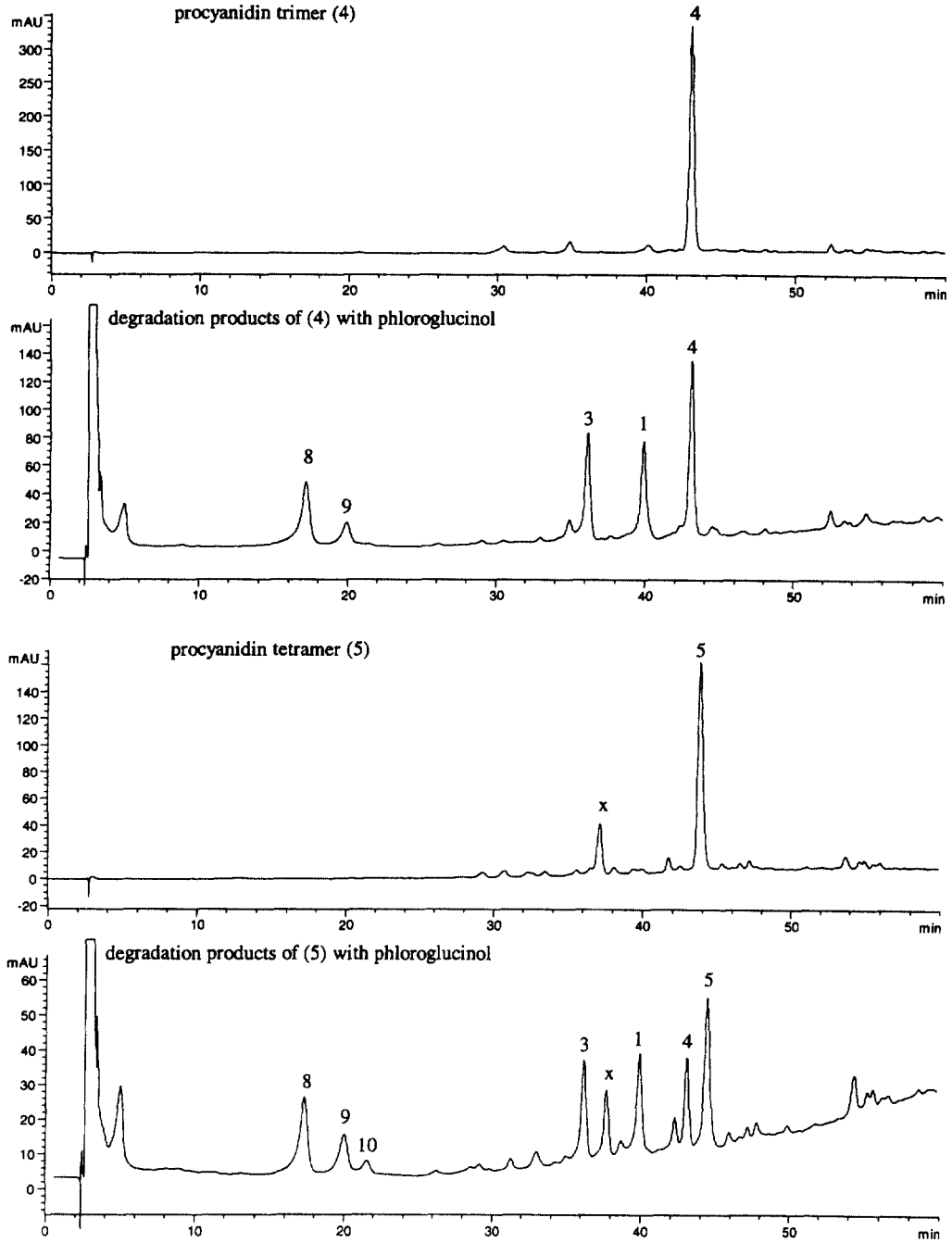
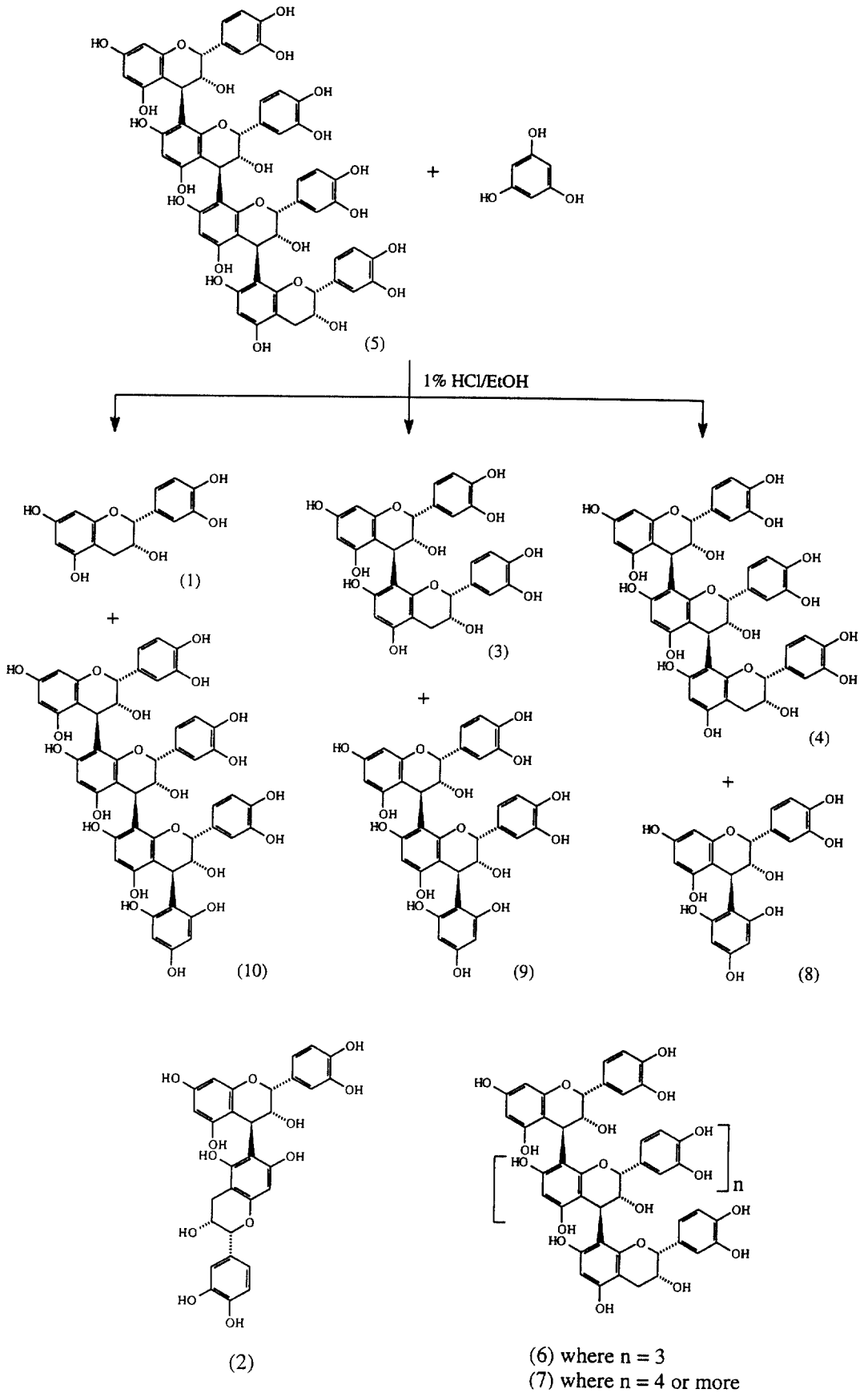


Fig. 2. HPLC of procyanidin (4) and (5) and their phloroglucinol degradation products. The numbering on each peak refers to the chemical compounds in the text.

having half its actual molecular weight. Evidence for this happening could be observed in the mass spectrum (Fig. 4(a)) for DP3 or procyanidin trimer (4) which showed the strong parent ion $[M-H]^-$ at m/z 865 as well as a peak at m/z 432 which could satisfactorily be rationalised as the parent trimer carrying two negative charges $[M-2H]^{2-}$. For higher oligomers (6) and (7), the ESMS spectra were dominated by more intense doubly charged molecules with m/z peaks located in the lower mass region while the $[M-H]^-$ peaks were distinctly weaker. Mass ion peaks at about m/z 720 and 1008 (see Fig. 4(b) and (c) could only be rationalised on the basis of double charged species as a result of loss of two protons for procyanidin pentamers (DP5) and heptamers (DP7), respectively. While the m/z 864 peak was more likely to be due to the doubly charged hexamers, the possibility that this could also arise from ionization of the trimer cannot be completely ruled out. Multiple ionization in ESMS for procyanidin has also been reported recently (Guyot, Doco, Souquet,



Scheme 1.

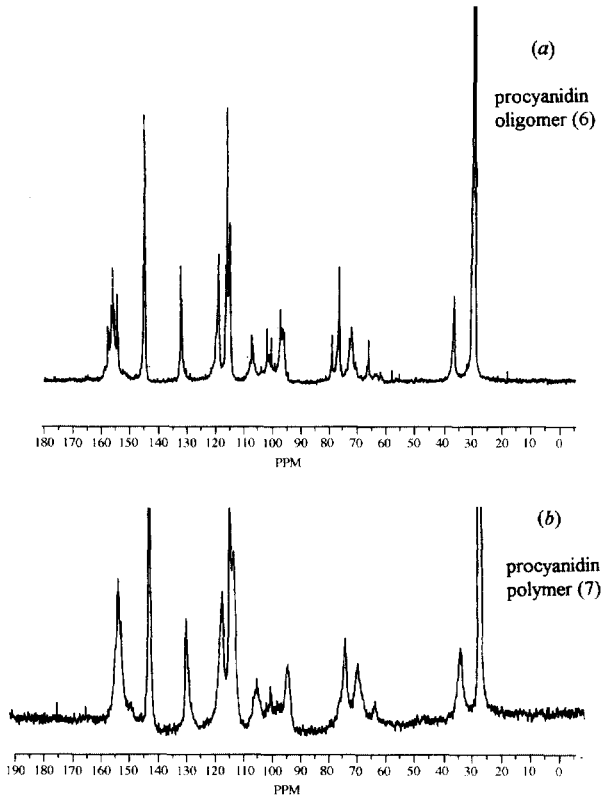


Fig. 3. ^{13}C-NMR of procyanidin oligomers, (a) sample (6) with average of about 5 DP and (b) sample (7) with average of about 8 DP.

Moutounet, & Drilleau, 1997) on the highly polymerised procyanidins in cider apple. In another study (Ohnishi-Kameyama, Yanagida, Kanda, & Nagata, 1997), the detection of procyanidin polymer larger than the decamers was also reported using matrix-assisted desorption/ionization time-of-flight mass spectrometry and fast-atom bombardment mass spectrometry. While higher molecular weight species were detected in both these studies, the higher mass ion peaks were relatively weak and declined rapidly with increasing molecular weight.

The HPLC of these higher oligomeric fractions appeared as multiple peaks in the case of sample (6) and as a broad hump for (7) at the region of higher retention time. These observations were due to the samples being complex mixtures of procyanidin oligomers which differed from one another in some structural details such as molecular weight and the position and nature of the interflavanoid linkages. The ^{13}C-NMR spectra of these samples showed that the procyanidin units were exclusively of the 2,3-*cis* configuration as the C-2 and C-3 chemical shifts were all observed in the upfield region like those of the lower oligomers. In contrast to the lower oligomers, where these ^{13}C-NMR signals were sharp, the carbon peaks of the higher oligomers were broad and unresolved (Fig. 3) reflecting the complex composition of the samples, which was also evident

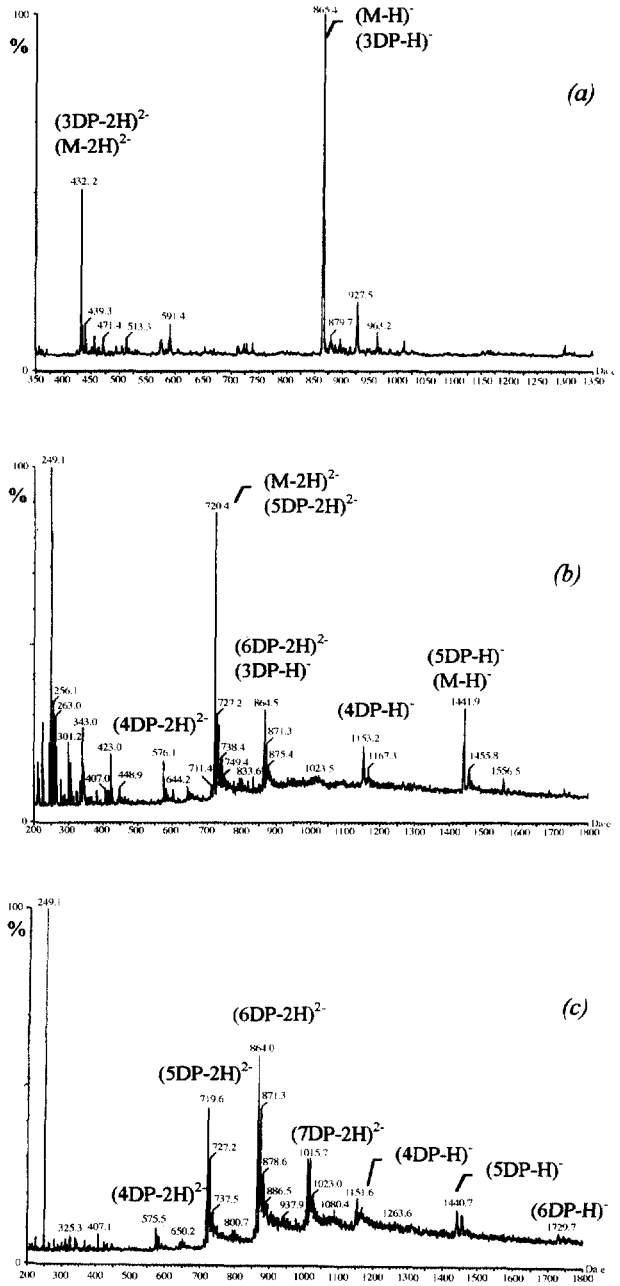


Fig. 4. ESMS of (a) procyanidin trimer (4), (b) oligomer (6) and (c) oligomer (7) where DP denotes degree of polymerisation.

from their mass spectra by the presence of ion peaks which could be attributable to different molecular species. Estimation of the average molecular weight of the samples using the signal intensity ratio of terminal C-2 or C-3 to the extenders C-2 or C-3, respectively, gave a value of about 1 to 4 or pentameric for sample (6), and 1 to about 7 or octomeric for (7). All these three samples yielded identical major degradation products with phloroglucinol after 1 h reaction as the tetramer (5), suggesting they were all made from epicatechin monomer units with predominantly C-4 to C-8 interflavanoid linkages.

4. Conclusion

Procyanidins from apple pomace could be separated by column chromatography using Sephadex LH20 to give discrete compounds up to the tetramer level. Higher oligomers were obtained as homogeneous procyanidin mixtures. The flavan monomeric units in these procyanidins were exclusively of the 2,3-*cis* configuration and the interflavanoid linkages were predominantly C-4 to C-8. These compounds, together with the related flavonoids which have potential as nutriceuticals, could serve as the basis for further downstream processing of apple pomace.

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