Thiolysis−HPLC Characterization of Apple Procyanidins Covering a Large Range of Polymerization States

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Procyanidins from the cortex of two cider apple varieties (*Malus domestica*; Kermerrien and Avrolles) were extracted by solvents. After a solid-phase extraction step, they were fractionated by normal- or reversed-phase HPLC at the semipreparative scale to obtain a series of purified fractions covering a wide range of polymerization states. Freeze-dried fractions were characterized by reversed-phase HPLC following thiolysis. Elution on normal-phase HPLC gave oligomeric procyanidins fractions with $\overline{DP}n$ (average degree of polymerization) values varying from 2 to 8, whereas polymeric fractions ($\overline{DP}n$ values varying from 7 to 190) were obtained by reversed-phase HPLC. Constitutive units were mainly (−)-epicatechin with a proportion above 95% for all fractions. Thiolysis yields were wholly homogeneous with an average value of 75%, which indicates that the efficiency of the reaction did not depend on the polymerization state of the procyanidin fractions.

Keywords: *Apple; chromatographic fractionation; HPLC; thiolysis; procyanidins*

INTRODUCTION

Proanthocyanidins are major phenolic constituents in many fruits and their derived products. In juices and fermented beverages they are involved in many quality criteria such as bitterness, astringency, and shelf life (Lea, 1990). Moreover, they contribute to the fruit product intake which is presumed to have a health-protective action. The structure of these compounds (Figure 1) is characterized by their hydroxylation pattern, by their stereochemistry, and by their proportions of their flavan-3-ol constitutive units, by the location and the nature of the interflavanyl linkages, and by their degree of polymerization (DP, number of flavanol units) (Hemingway, 1989). In apple and apple products (juices, ciders, etc.), proanthocyanidins are mainly procyanidins with (−)-epicatechin and (+)-catechin as constitutive units (Figure 1), the former being largely preponderant (Lea, 1990). Procyanidin structures up to heptamer have been characterized in cider (Lea, 1978), but more polymerized structures up to DP17 have been found in cider apple skin and parenchyma (Guyot et al., 1997) and up to DP 12 in unripe dessert apples (Ohnishi-Kameyama et al., 1997).

Considering their extraction, their biological activities, and their sensory effects, proanthocyanidins often behave according to their molecular weight and the nature of their constitutive flavanol units. For instance, Lea and Arnold (1978) pointed out the influence of the DP of procyanidins in relation to bitterness and astringency of cider. Procyanidins are also partly involved in haze formation in apple juices (Beveridge and Tait, 1993). On the whole, the DP and the nature of the constitutive units are important structural features that are related to the ability of proanthocyanidins to associate with proteins and polysaccharides (Haslam, 1974; Cheynier et al., 1992).

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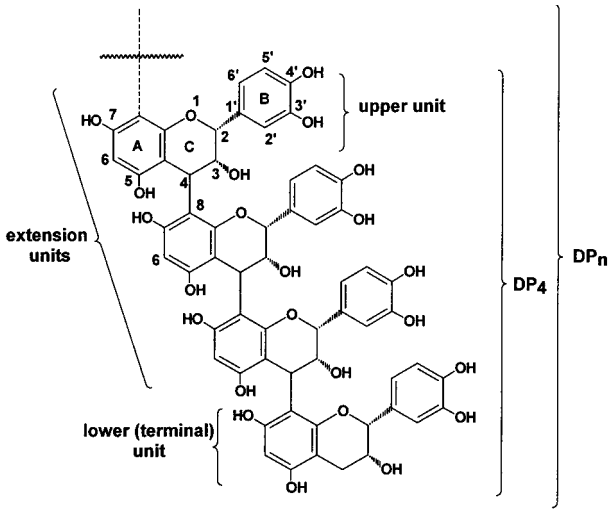

Figure 1. Structure of procyanidins − example of a (−)-epicatechin based structure (according to GUYOT et al., 1997).

The main difficulty in studies dealing with condensed tannins is probably to obtain them in an individual molecular form. The complete purification of a proanthocyanidin molecule with a DP above five is almost impossible. Therefore, only mixtures containing more or less polymerized structures are used when studying their structures and their properties. Several methods have been proposed to fractionate oligomeric and polymeric proanthocyanidins. The normal-phase size fractionation of cider proanthocyanidins was first proposed by Lea (1978) using a TLC method. The method was then adapted to HPLC and was applied to cocoa (Rigaud et al., 1991; Hammerstone et al., 1999), grape skins and seeds (Souquet et al., 1996), litchi pericarp (Leroux et al., 1998), and cider apple parenchyma proanthocyanidin extracts (Guyot et al., 1999). Other various techniques such as liquid chromatography on Sephadex LH-20 (Lea and Timberlake, 1974) or Fractogel (Toyopearl)

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(Derdelinckx and Jerumanis, 1984; Yanagida et al., 1999) have been used to separate proanthocyanidins according to their molecular weight. Recently, Labarbe et al. (1999) proposed a method of quantitative fractionation that corresponded to a sequential dissolution of proanthocyanidins on an inert glass powder column.

The structural characterization of proanthocyanidins fractions was generally performed by chemical or physical methods. ^{13}C NMR was shown to be a powerful tool for giving precise information on the average degree of polymerization, on the stereochemistry of the heterocycles of the constitutive units, and on the hydroxylation pattern of the B nuclei (procyanidins/prodelphinidins ratio) (Czochanska et al., 1980). However, polymers with molecular weight above 8000 cannot be precisely characterized according to this procedure (Czochanska et al., 1979). ^{1}H NMR was used to give information on the stereochemistry of the flavan-3-ol units of oligomeric fractions (Kolodziej, 1992), and recently a ^{1}H NMR method was proposed to estimate the $\overline{DP}n$ of homogeneous apple procyanidin fractions (Guyot et al., 1999) up to $\overline{DP}n$ 10. Chemical methods generally consisted of the acid-catalyzed cleavage of the interflavanyl linkages of proanthocyanidins in the presence of a nucleophile reagent such as phloroglucinol (Matsuo et al., 1984) or toluene-α-thiol (Thompson et al., 1972). Thiolysis allowed distinction between extension and terminal units of proanthocyanidins (Thompson et al., 1972), and, when coupled to reversed-phase HPLC (Rigaud et al., 1991), the method was used to calculate the average degree of polymerization ($\overline{DP}n$) and to determine the proportions of the constitutive units in proanthocyanidins fractions (Rigaud et al., 1993; Prieur et al., 1994; Souquet et al., 1996). Recent works have shown the benefit of this method for the quantification of partly purified or insoluble condensed tannins (Matthews et al., 1997; Guyot et al., 1998; Labarbe et al., 1999) in plant extracts. Nevertheless, some factors such as presence of impurities, occurrence of side reactions (Brown and Shaw, 1974; McGraw et al., 1993), and instability of some reaction products (Hemingway and McGraw, 1983) may alter the yields of the reaction.

In a recent paper (Sanoner et al., 1999), we showed evidence of the diversity of the French cider apple varieties on the basis of their polyphenol profiles. Some varieties showed significant amounts of highly polymerized procyanidins. In the present work, some of these varieties were chosen as a raw material for a fractionation procedure that permitted us to obtain a series of homogeneous apple procyanidin fractions covering a large range of $\overline{DP}n$ values. These fractions were used to show the pertinence of thiolysis−HPLC for characterizing procyanidins on a molecular weight basis giving information on the nature and the proportion of the constitutive units. A study of the thiolysis yields showed the benefits of the method for the quantification of procyanidins even in a highly polymerized state.

MATERIALS AND METHODS

Solvents and Phenolic Standards. Organic solvents of HPLC-grade were purchased from Biosolves LTD (Nertherlands). Toluene-α-thiol was purchased from Merck (Darmstadt, Germany). (+)-Catechin, (−)-epicatechin, chlorogenic acid, phloridzin, and benzylthioether derivatives have been obtained as described in a preceeding paper (Guyot et al., 1998).

Plant Materials. Apple fruits from the *Kermerrien* and *Avrolles* variety were harvested at maturity during the 1997 season in the experimental orchard of the CTPS (Centre Technique des Productions Cidricoles; Sées, Orne, France). Fruits of the predominant size were mechanically peeled and cored as already described (Guyot et al., 1997) and cortex tissues were freeze-dried.

Crude Extractions of Freeze-Dried Material. Freeze-dried apple powders (50 g) were extracted by successive solvent extraction (hexane, methanol, and, at last, aqueous acetone) according to a previously described procedure (Guyot et al., 1997). Crude methanolic fractions (CMF) and crude aqueous acetone fractions (CAF) were freeze-dried. *Kermerrien* (CMF-*Ke* and CAF-*Ke*) and *Avrolles* (CMF-*Av* and CAF-*Av*) crude extracts were available for further purification steps. However, the CMF-*Av* fraction was not considered for further purification (see result and discussion).

Solid-Phase Extraction of CMF-*Ke* (17g), CAF-*Ke* (225 mg), and CAF-*Av* (350 mg). Fractions were suspended in 30 mL of diluted acetic acid (2.5% v/v) and this volume was distributed onto ten C18 Sep-PaK (5 g, Waters, Milford, MA) cartridges (3 mL per cartridge) previously conditioned by diluted acetic acid (2.5%). Each cartridge was eluted with 20 mL of diluted acetic acid to remove sugar and other high-polarity compounds. Polyphenols extracts were then recovered by elution with 20 mL of aqueous acetonitrile (1:1 v/v, the water being acidified with acetic acid 2.5% v/v). For each extract, aqueous acetonitrile volumes were pooled, evaporated, and freeze-dried. These residues were noted CMFp-*Ke* (645 mg), CAFp-*Ke* (117 mg), and CAFp-*Av* (164 mg). Solutions (4 g L^{-1} in methanol, 3 mL) were prepared to perform thiolysis and reversed-phase HPLC analyses of the thiolysis media according to the procedure described below for procyanidin fractions.

Normal-Phase HPLC Fractionation of CMFp-*Ke*. The residue was fractionated by HPLC at a semipreparative scale with the following HPLC gradient system (Rainin Instrument Company Inc., Woburn, MA): two high-pressure pumps (Dynamax SD 300, Rainin) equipped with 100 WTI pump heads, a manual injection valve (Rheodyne L. P., Rohnert Park, CA) equipped with a 5 mL injection loop, and a UV detector (Dynamax UV1, Rainin). Data were acquired and processed by the EZ-chrom software (Scientific Software, Inc., Pleasanton, CA). The semipreparative column system (Waters) was a PrepLC 25-mm radial compression module equipped with a PrepNova-Pak HR Silica cartridge (25 × 100 mm, 6 μm, 60 Å) and a Guard Pak cartridge (25 × 10 mm). The flow rate was 40 mL min^{-1} and the gradient conditions were solvent A ($MeOH/CH_2Cl_2/HCOOH/H_2O$, 43:5:1:1); solvent B ($MeOH/CH_2Cl_2/HCOOH/H_2O$, 7:41:1:1); initial 0% A; 0−30 min, 20% A linear; 30−35 min, 25% A linear; 35−37 min, 100% A linear; 37−40 min 100% A isocratic; 40−42 min, 0% A linear. Four dilutions of CMFp-*Ke* (about 80 mg in 500 μL of MeOH) were successively injected, and 8 fractions were collected for each injection (Figure 2A).

Fractions which corresponded to the same collection were pooled, evaporated to remove organic solvents, and freeze-dried. They were labeled according to Figure 2.

Reversed-Phase HPLC Fractionation of CAFp-*Ke* and CAFp-*Av*. The semipreparative HPLC system was the same as described above for normal-phase fractionation except for the column and gradient conditions. The column (Waters, Milford, MA) was a PrepNova-Pak HR C18 cartridge (25 × 100 mm, 6 μm, 60 Å) and a Guard Pak cartridge (25 × 10 mm).The flow rate was 40 mL min^{-1} and the gradient conditions were solvent A (acetonitrile); solvent B (acetic acid, 2.5% in water); initial 3% A; 0−2 min, 3% A isocratic; 2−5 min, 9% A linear; 5−15 min, 15% A linear; 15−40 min, 44% A linear; 40−43 min 90% A linear; 43−48 min, 90% A isocratic; 48−50 min, 3% A linear. Successive injections of CAFp-*Ke* (2 × 50 mg in 2 mL of acetic acid 2.5%) and CAFp-*Av* (2 × 50 mg in 2 mL of acetic acid 2.5%) were performed and 5 fractions were collected for each injection (Figure 2A and B).

Thiolysis Conditions. Thiolysis was carried out on both global aqueous acetone extracts (CAFp-*Ke* and CAFp-*Av*) and their corresponding fractions (F*X*-M-*Ke*, F*X*-A-*Ke*, and F*X*-A-*Av*, where *X* corresponds to the number of the purified fraction

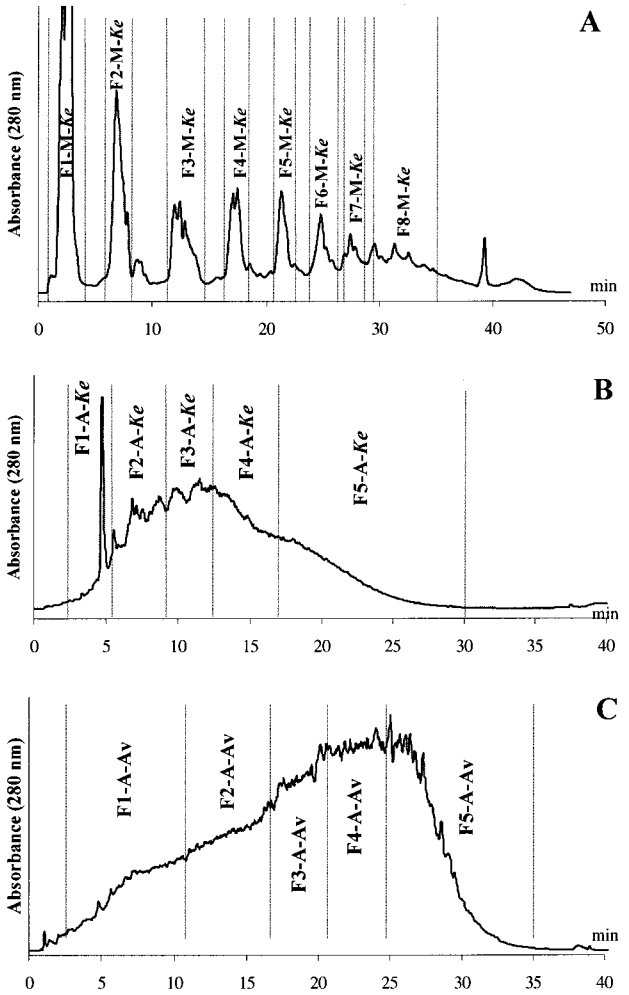

Figure 2. Semipreparative fractionation of oligomeric and polymeric procyanidins. A, CMFp-*Ke* on normal-phase; B, CAFp-*Ke* on reversed-phase; C, CAFp-*Av* on reversed-phase.

in the increasing elution time order) obtained by chromatographic fractionation. A 4-g L^{-1} solution of each dry fraction was prepared by sonicating the residue in methanol. In a 250-μL glass insert (02CTVG, Chromacol, Trumbull, CT), 50 μL of the solution (or suspension depending on the solubility of the residue in methanol) was mixed together with 50 μL of methanol acidified by concentrated HCl (3.3%, v/v), and 100 μL of toluene-α-thiol (5% v/v in methanol) was added. After sealing with an inert cap (8AC-CBT1, Chromacol), reactions were carried out at 40 °C for 30 min.

Analytical Reversed-Phase HPLC. Thiolysis reaction media (10 μL) were directly injected into the HPLC system. The HPLC apparatus was a Waters (Milford, MA) system (717 plus autosampler equipped with a cooling module set at 4 °C, 600E multisolvent system, a 996 Photodiode array detector, and a Millenium 2010 Manager system). The column was a Purospher RP18 endcapped, 5 μm (Merck, Darmstadt, Germany). The solvent system was a gradient of solvent A (aqueous acetic acid, 2.5% v/v) and solvent B (acetonitrile): initial, 3% B, 0−5 min, 9% B linear; 5−15 min, 16% B linear; 15−45 min, 50% B linear, followed by washing and reconditioning the column.

Processing of Thiolysis Chromatograms and Data. (+)-Catechin (RT, 14.5 min), (−)-epicatechin (RT, 18.8 min), and (−)-epicatechin benzylthioether (RT, 40.1 min) were identified on chromatograms according to their retention times and UV−visible spectra. Peaks were manually integrated, and quantification was performed by reporting the measured area into the calibration curve of the corresponding compound. Thiolysis yields were calculated as the ratio of total flavanols assayed by HPLC to the quantity that was injected. Another yield value was obtained by comparing to the Folin Ciocalteu assays of the fractions. The average degree of polymerization (DPn) was measured by calculating the molar ratio of all the flavan-3-ol units (thioether adducts plus terminal units) to (−)-epicatechin and (+)-catechin corresponding to terminal units.

Kinetic Study of Thiolysis Yields. Thiolysis was carried out at 40 °C at several incubation times (5, 10, 20, 30, 40, and 60 min) for several fractions (F5-M-*Ke*, F2-A-*Av*, F3-A-*Av*, and F5-A-*Av*) differing significantly by their polymerization states. The other thiolysis conditions, HPLC analysis conditions, and yields calculations were the same as describe above.

Folin-Ciocalteu Assays. The estimation of global polyphenol content in the fractions was performed by the Folin Ciocalteu method adapted from Singleton and Rossi (1965). The methanolic solutions used for thiolysis reactions (see paragraph above) were diluted 10-fold in acetic acid 2.5%/acetone (60:40, v/v). Folin Ciocalteu reagent (0.25 mL; Merck, Darmstadt, Germany) was added to the diluted solutions or suspensions (0.5 mL). Then, 1 mL of a 200 g L^{-1} solution of Na_2CO_3 was added, and the volume was adjusted to 5 mL with pure water. The mixture was then heated at 70 °C for 10 min and, after cooling, absorbance was measured at 700 nm (Uvikon 860 spectrophotometer, Kontron, Milano, Italy) with a blank sample (0.5 mL acetic acid 2.5%/acetone (60:40, v/v) plus reagents) in the reference cell. Quantifications were obtained by reporting the absorbances in the calibration curve of (−)-epicatechin used as standard phenol.

RESULTS AND DISCUSSION

Extraction of Cider Apple Polyphenols. Methanolic and aqueous acetone extracts of apple polyphenols were obtained by successive solvent extraction of the freeze-dried pulp material of two French cider apple varieties (*Malus domestica*, Kermerrien and Avrolles varieties). As previously shown (Guyot et al., 1998), oligomeric procyanidins were extracted by methanol together with other polyphenol classes (hydroxycinamic derivatives, dihydrochalcones, and monomeric catechins), whereas polymeric procyanidins were extracted by aqueous-acetone. The Kermerrien methanol extract and the Kermerrien and the Avrolles aqueous acetone extracts were considered for further purification steps. The Avrolles methanol extract was not used further in this study because its phenolic composition (mixture of hydroxycinnamic acids, dihydrochalcones, and fairly polymerized procyanidins with a DPn close to 35) make difficult the fractionation of procyanidins on both the normal- and reversed-phases. Moreover, the three other fractions were considered to be sufficient as crude materials to obtain procyanidins fractions that covered a large range of DPn values.

Solid-phase extraction allowed recovery of dried polyphenol extracts that were referred to as CMFp-*Ke*, CAFp-*Ke*, and CAFp-*Av* for Kermerrien methanol extract, Kermerrien aqueous-acetone extract, and Avrolles aqueous acetone extract, respectively. Reversed-phase HPLC analyses following thiolysis were performed on each extract to assay terminal and extension units of procyanidins. Chromatogram analyses allowed the calculation of the average degree of polymerization of the procyanidins (Rigaud et al., 1993; Prieur et al., 1994) and also gave quantitative results on the procyanidin concentration in each sample (Matthews et al., 1997; Guyot et al., 1998).

As shown in Table 1, cider apple procyanidins found in the 3 fractions corresponded to homogeneous structures mainly made up of (−)-epicatechin units, results which are consistent with previous works (Lea, 1990; Guyot et al., 1998). However, the fractions differed

Table 1. Thiolysis−Reversed-Phase HPLC Characterization of the Kermerrien and Avrolles Polyphenol Extracts Obtained by Solvent Extraction Followed by Solid-Phase C18-SEP-PAK Extraction

	CMFp-*Ke*[a]	CAFp-*Ke*	CAFp-*Av*
ECe (%)	66.67	91.92	99.02
ECt (%)	28.41	6.08	0.84
CTt (%)	4.92	2.70	0.13
$\overline{DP}n$	3.0	11.3	102.5
thiolysis yield (%)	43	72.2	78.2

[a] CMFp-*Ke*, methanolic extract of Kermerrien; CAFp-*Ke*, aqueous acetone extract of Kermerrien; CAFp-Av, aqueous acetone extract of Avrolles; ECe, (−)-epicatechin extension units of procyanidins; ECt, (−)-epicatechin terminal units of procyanidins; CTt, (+)-catechin terminal units of procyanidins; $\overline{DP}n$, average degree of polymerization. Yields were estimated with regard to the weighting of the fraction.

markedly according to their average degree of polymerization ($\overline{DP}n$). CMFp-*Ke* showed a $\overline{DP}n$ of 3.0 which corresponded to oligomeric procyanidins, whereas highly polymerized procyanidins ($\overline{DP}n$ 102.5) were present in the Avrolles aqueous acetone fraction. The Kermerrien aqueous acetone extract exhibited a $\overline{DP}n$ of 11.3, which agreed with previous works that showed this extract was mainly made up of a series of oligomeric and polymeric procyanidins from DP4 to DP17 (Guyot et al., 1997). Yields attested that the aqueous-acetone fractions were highly procyanidins concentrated. A lower yield was observed for CMFp-*Ke*; which might be explained by the large contamination of this fraction by other phenolic compounds such as chlorogenic acid and dihydrochalcones.

Fractionation of the Apple Polyphenol Extracts. Oligomeric procyanidins (CMFp-*Ke*) were fractionated on normal-phase semipreparative HPLC (Figure 2 A) to separate them according to their molecular weight as already described by Rigaud et al. (1993). Simple phenolics (hydroxycinnamic acids, dihydrochalcones, and monomeric catechins) were eluted at first (F1-M-Ke in Figure 2A), whereas oligomeric procyanidins were successively eluted according to their increasing degree of polymerization as already observed for cocoa and grape proanthocyanidins (Rigaud et al., 1993). Seven procyanidin fractions, numbered from F2-M-*Ke* to F8-M-*Ke*, were collected and evaporated to dryness.

Dry extracts of polymeric procyanidins (CAFp-*Ke* and CAFp-*Av*) were fractionated by reversed-phase HPLC at a semipreparative scale, and five fractions (numbered from F1-A-*Ke* to F5-A-*Ke* and F1-A-*Av* to F5-A-*Av*) were collected according to the increasing elution time for *Kermerrien* and *Avrolles* extracts, respectively (Figure 2B and C). Except the F1-A-*Ke* fraction, the retention time windows attributed to each of the 5 fractions were obtained by dividing the total area of the chromatogram into five equal areas in order to obtain approximately the same quantity for each fraction (Figure 2). The time range of the F1-A-*Ke* fraction was chosen in order to collect residual chlorogenic acid that appeared as a well-resolved peak on the chromatograms (Figure 2**B**). It was identified according to its retention time and its UV−visible spectrum. This fraction that represented 7% of the total area of the chromatogram was not retained for further investigation.

Thiolysis−Reversed-Phase HPLC Analysis of the Fractions. All dried fractions were submitted to reversed-phase HPLC analysis following thiolysis to characterize procyanidins and to estimate their procyanidin concentration.

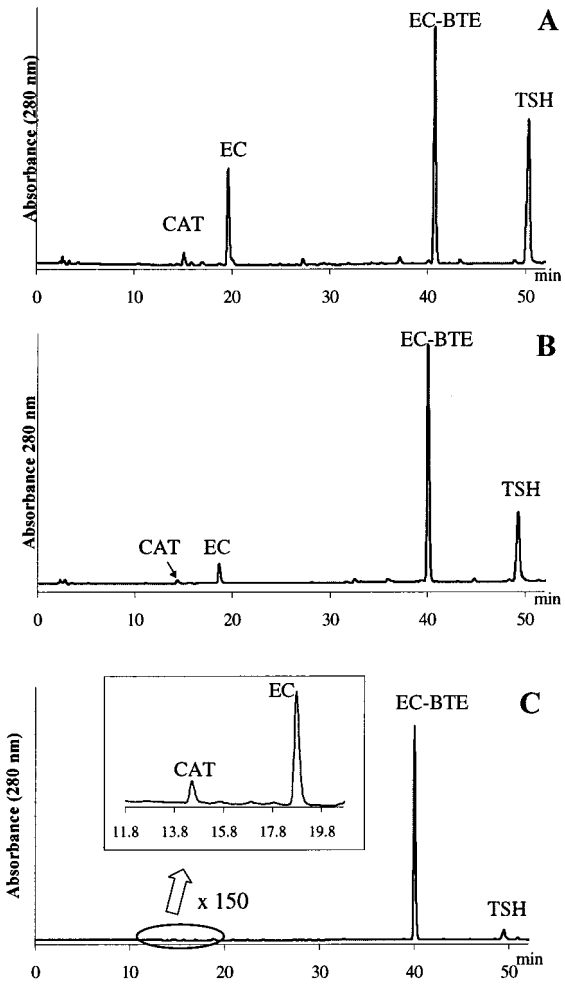

Figure 3. Reversed-phase HPLC chromatograms (280 nm) of thiolysis media. A, fraction F3-M-*Ke* ($\overline{DP}n$ = 3.1); B, fraction F3-A-*Ke* ($\overline{DP}n$ = 11.7); C, Fraction F3-A-*Av* ($\overline{DP}n$ = 112.1). Abbreviations: CAT, (+)-catechin; EC, (−)-epicatechin; EC-BTE, (−)-epicatechin benzyl thioether; TSH, toluene-α-thiol.

As observed on Figure 3, chromatograms of thiolysis media were quite simple: four peaks corresponding to (+)-catechin (R_T = 14.5 min), (−)-epicatechin (R_T = 18.8 min), (−)-epicatechin benzylthioether (R_T = 40.1 min), and the excess of toluene-α-thiol reagent (R_T = 50.0 min) were sufficient to explain more than 95% of the total area of the chromatograms. These chromatographic observations acted in favor of a high degree of purity for procyanidin fractions. At the same time, fractions were analyzed by the Folin-Ciocalteu method to measure the total polyphenol concentrations and to compare to thiolysis−HPLC quantification.

Thiolysis yields were in a range of 65 to 83% when yields were calculated according to the weight (Table 2). On average, the yield was close to 75%, which is consistent with previously published data (Labarbe et al., 1999); and no correlation was observed between the yield and the polymerization state of the procyanidins in the fraction, showing that, in our conditions, thiolysis yields do not depend on the polymerization state of the procyanidin molecules. When they were calculated according to the Folin-Ciocalteu assays, thiolysis yields showed very high values, with some of them being above 100%. One explanation may be that, in the Folin-

Table 2. Thiolysis Yields for the Procyanidin Fractions

	Oligomeric Procyanidin Fractions (Kermerrien): CMFp-*Ke*						
	F2-M-*Ke*	F3-M-*Ke*	F4-M-*Ke*	F5-M-*Ke*	F6-M-*Ke*	F7-M-*Ke*	F8-M-*Ke*
Y*folin*[a] (%)	105	92	95	91	94	89	110
Y*weight* (%)	71	77	83	77	78	78	78

	Polymeric Procyanidin Fractions (Kermerrien): CAFp-*Ke*			
	F2-A-*Ke*	F3-A-*Ke*	F4-A-*Ke*	F5-A-*Ke*
Y*folin* (%)	74	86	89	75
Y*weight* (%)	69	73	77	72

	Polymeric Procyanidin Fractions (Avrolles): CAFp-*Av*				
	F1-A-*Av*	F2-A-*Av*	F3-A-*Av*	F4-A-*Av*	F5-A-*Av*
Y*folin* (%)	82	87	86	86	88
Y*weight* (%)	65	78	75	77	68

	Average Yield Value
Y*folin*	89 (± 9)
Y*weight*	75 (± 5)

[a] Y*folin*, estimation of the thiolysis recovery by comparing to the Folin Ciacalteu estimation; Y*weight*, estimation of the thiolysis yields according to the weight.

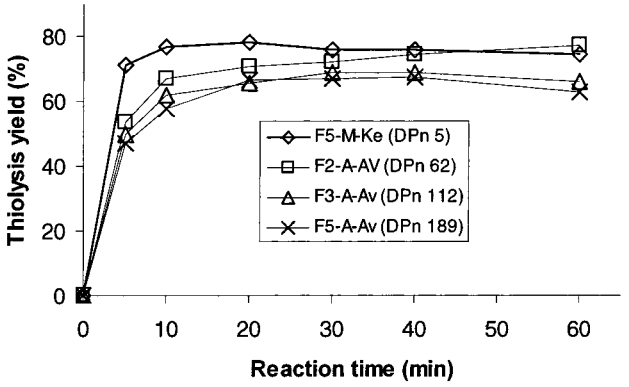

Figure 4. Kinetic study of the thiolysis yields.

Ciocalteu assay, procyanidins reacted in a slightly different manner than the standard, (−)-epicatechin, did. However, (−)-epicatechin seems to be the standard of choice to quantify apple procyanidins because it corresponds to the exact structure of the monomeric unit. Thus, another explanation may be that some nonphenolic plant components have not been eliminated by the solid-phase and chromatographic purification procedure or that some impurities coming from the chromatographic solvents have been concentrated during the purification process. According to literature, a third explanation can be seriously proposed: previous works dealing with polymeric procyanidins purification (Czochanska et al., 1980; Foo and Porter, 1980) have shown that freeze-dried purified fractions contained approximately three molecules of hydration water per flavanol monomer unit. Therefore, the yield differences which were observed for the two methods of yield calculation (Table 2) may be explained above all by the presence of hydration water in the fractions. Thus, data in Table 2 could be consistent with true thiolysis reaction yields that are superior to the presented values, and may be close to 100% with regard to Folin Ciocalteu data.

A kinetic study of the reaction was performed for several fractions with various polymerization states (Figure 4). Yields increased rapidly according to the reaction time, up to the optimum yield value between 67 and 79% depending on the considered fraction. Then, the yields decreased slowly for longer reaction times. The reaction time for obtaining the maximum yield was slightly longer for the highly polymerized fractions than it was for the less polymerized ones. Nevertheless, the yields were considered to be stable and close to the optimum for all fractions in the time period between 20 and 40 min. Therefore, a reaction time of 30 min was chosen for the standard conditions of thiolysis.

Characterization of the procyanidin fractions is summarized in Table 3. (−)-Epicatechin was always the predominant constitutive unit, accounting for more than 95% of total units for all fractions.

The estimation of $\overline{DP}n$ was based on the measurement of the chromatographic peak areas corresponding to extension and terminal units of procyanidins. Therefore, the more the procyanidins were polymerized, the more the peaks corresponding to terminal units were small. Nevertheless, Figure 3C showed that (+)-catechin and (−)-epicatechin peaks were well resolved even for highly polymerized procyanidin fractions. The fractions showed a very large range of $\overline{DP}n$ values varying from 2.0 for F2-M-*Ke* to 190 for F5-A-*Av*. From another source (Guyot et al., 1999), the $\overline{DP}n$ values of several oligomeric fractions have been calculated according to a ^{1}H NMR procedure and a good equivalency was obtained with the $\overline{DP}n$ values that have been calculated by thiolysis−HPLC.

As shown for fractions F2-M-*Ke* to F8-M-*Ke*, the normal-phase fractionation allowed a precise separation of oligomeric procyanidins according to their $\overline{DP}n$ as already observed for cacao (Rigaud et al., 1993; Hammerstone et al., 1999) or grape procyanidins (Souquet et *al*., 1996; Prieur et al., 1994). As shown in Table 3 for polymerized procyanidin fractions, reversed-phase elution also proceeds according to increasing $\overline{DP}n$. Although this behavior on reversed-phase does not constitute a rule for proanthocyanidins, it was already observed for *Sorghum* polymeric procyanidins (Putman and Butler, 1989). The elution according to the size may be due to the homogeneous structure of the tannins, the overall polarity of which may decrease with increasing molecular weight. The recalculation of the $\overline{DP}n$ of CAFp-*Ke* and CAFp-*AV* was performed by taking into account the area percentages of each fraction on the

Table 3. Characterization of the Procyanidin Fractions According to Their Constitutive Units and Their Average Degree of Polymerization ($\overline{DPn}$)

Oligomeric Procyanidin Fractions (Kermerrien): CMFp-*Ke*

	F2-M-*Ke*	F3-M-*Ke*	F4-M-*Ke*	F5-M-*Ke*	F6-M-*Ke*	F7-M-*Ke*	F8-M-*Ke*
ECe[a] (%)	51.20	67.32	75.41	80.18	83.40	85.31	88.56
ECt (%)	44.80	28.62	22.03	17.64	14.43	12.77	9.57
CTt (%)	4.00	4.07	2.56	2.18	2.18	1.92	1.87
$\overline{DPn}$	2.0	3.1	4.1	5.0	6.0	6.8	7.9

Polymeric Procyanidin Fractions (Kermerrien): CAFp-*Ke*

	F2-A-*Ke*	F3-A-*Ke*	F4-A-*Ke*	F5-A-*Ke*
ECe (%)	85.15	91.46	93.11	93.37
ECt (%)	12.13	7.31	5.98	5.63
CTt (%)	2.72	1.24	0.90	1.00
$\overline{DPn}$	6.7	11.8	14.9	15.1
$\overline{DPn}$***R***			11.8	

Polymeric Procyanidin Fractions (Avrolles): CAFp-*Av*

	F1-A-*Av*	F2-A-*Av*	F3-A-*Av*	F4-A-*Av*	F5-A-*Av*
ECe (%)	97.88	98.38	99.11	99.41	99.47
ECt (%)	1.72	1.42	0.77	0.49	0.41
CTt (%)	0.40	0.20	0.12	0.10	0.11
$\overline{DPn}$	47.2	61.6	112.1	168.1	189.5
$\overline{DPn}$***R***			120.8		

[a] ECe, (−)-epicatechin extension units; ECt, (−)-epicatechin terminal units; CTt, (+)-catechin terminal units; $\overline{DPn}$, average degree of polymerization; $\overline{DPn}R$, $\overline{DPn}$ of the global fraction which has been recalculated by taking into account the chromatogram areas of the fractions.

chromatograms (Figure 2) and the $\overline{DPn}$ of the corresponding fractions. It permitted us to check the consistency of the results (Table 3). The recalculated $\overline{DPn}$ values were 11.8 for CAFp-*Ke* and 120.8 for CAFp-*Av*. By comparing to the initial value ($\overline{DPn}$ = 11.3, Table 1), a good correspondence was obtained for the CAFp-*Ke*, whereas the recalculated $\overline{DPn}$ of CAFp-*Av* was slightly superior to the initial value ($\overline{DPn}$ = 102.5, Table 1). Therefore, $\overline{DPn}$ may have been slightly overestimated in the *Avrolles* aqueous acetone fractions.

When it was applied to homogeneous procyanidins, thiolysis followed by reversed-phase HPLC may serve as a powerful method to characterize and quantify procyanidin oligomers and polymers. In our conditions, thiolysis yields were shown to be close to 75%, even for highly polymerized forms, and this value might be underestimated if we consider that purified procyanidin fractions contained a significant proportion of hydration water. As far as we know, such highly polymerized procyanidins, with average molecular weight close to 55 000, have never been shown before in the cortex constitution of apple varieties. Cider apples may be used as an adapted raw material to obtain purified procyanidin fractions in a wide range of $\overline{DPn}$ values. These fractions may be suitable to study procyanidin properties knowing that their biological properties (bioavailability and anti-oxidant potential) and their contribution to astringency and bitterness in foodstuffs may be related to the polymerization state.

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