ARTICLES

Reversed-Phase HPLC following Thiolysis for Quantitative Estimation and Characterization of the Four Main Classes of Phenolic Compounds in Different Tissue Zones of a French Cider Apple Variety (*Malus domestica* Var. Kermerrien)

Sylvain Guyot,*,† Nathalie Marnet,† Djamel Laraba,‡ Philippe Sanoner,† and Jean-François Drilleau†

Laboratoire de Recherches Cidricoles, Biotransformation des Fruits et Légumes, Institut National de la Recherche Agronomique, B.P. 29, 35650 Le Rheu, France, and Laboratoire de Biotechnologies, Institut des Sciences de la Nature, Université de Constantine, Constantine, Algeria

Phenolic compounds, which are present in the epidermis zone, parenchyma zone, core zone, and seeds of fruit (*Malus domestica* var. Kermerrien), were extracted from freeze-dried material by three successive solvent extractions. The dry methanol extract and the dry aqueous acetone extracts were analyzed using reversed-phase HPLC coupled with diode array detection following thiolysis to quantify phenolic compounds according to their classes (hydroxycinnamic acid derivatives, flavan-3-ols, flavonols, and dihydrochalcones). The method is suitable for the determination of the relative proportions of the different classes of polyphenols and provided information on the constitutive units and the average degree of polymerization of oligomeric and polymeric procyanidin structures. Results showed that procyanidins are the predominant phenolic constituents in cider apple fruits, much of them corresponding to highly polymerized structures.

Keywords: *Apple; phenolics; procyanidins; cider; HPLC; thiolysis*

INTRODUCTION

Cider apple varieties contain large amounts of phenolic compounds that are greatly involved in the quality of manufactured ciders (Lea, 1990). Generally, total phenolic contents of apples are between 0.1 and 5 g/kg of fresh weight; however, concentrations >10 g/kg have been observed in some varieties (Van Buren, 1970; Herrmann, 1973). Four classes of phenolic compounds are present in apple fruit (Figure 1): hydroxycinnamic acid derivatives, monomeric and oligomeric flavan-3-ols (the latter corresponding to procyanidins), flavonols, and dihydrochalcones. Anthocyanins can be considered as an additional class because they are present in the skin of some red varieties (Nicolas et al., 1994).

In the cider apple industry, the two classes that are important because of their physicochemical properties are hydroxycinnamic acid derivatives and flavan-3-ols.

The flavan-3-ols are present in monomeric form, which is essentially (−)-epicatechin (**1**) (Nicolas et al., 1994), and in oligomeric and polymeric forms, named procyanidins (Figure 1A). Procyanidin B2 (**4**), a two-epicatechin unit with a C4−C8 interflavanic linkage, is the most frequently found procyanidin in apple and apple products. However, highly polymerized forms (**5**) have been shown recently for the first time in a cider apple variety (Guyot et al., 1997). Procyanidins contribute to the astringency of ciders (Lea, 1990) through their capacity to interact strongly with proteins and, in this case, salivary proteins (Murray et al., 1994). The ability to associate increases with the degree of polymerization (number of flavan-3-ol units) of procyanidins (Ricardo da Silva et al., 1991). In some cases, the instability of ciders or apple juices during storage is attributed to this property, because hazes and precipitates may result from associations of these tanning substances with other macromolecules such as proteins and polysaccharides (Van Buren, 1989).

The hydroxycinnamic acid derivatives (Figure 1B) are mainly represented as 5′-caffeoylquinic acid [i.e., chlorogenic acid (**6**)] in apple (Nicolas et al., 1994). Other compounds of the same class such as chlorogenic acid isomers and 5′-*p*-coumaroylquinic acid and its isomers may also be present in apple composition but are generally found in lower concentrations (Nicolas et al., 1994). Chlorogenic acid is one of the most important substrates of polyphenol oxidase, which is important in apple fruit processing. In the presence of oxygen and polyphenol oxidase, chlorogenic acid is converted into its *o*-quinone, which further reacts with other phenolic compounds, resulting in the formation of yellow and brown pigments (Oszmianski and Lee, 1990). In cider, the yellow or orange coloration may result from these oxidation and condensation processes (Brugirard and Tavernier, 1952). Moreover, these new phenolic struc-

* Author to whom correspondence should be addressed (telephone 02 99 28 52 12; fax 02 99 28 52 10; e-mail guyot@rennes.inra.fr).

† Laboratoire de Recherches Cidricoles.

‡ Laboratoire de Biotechnologies.

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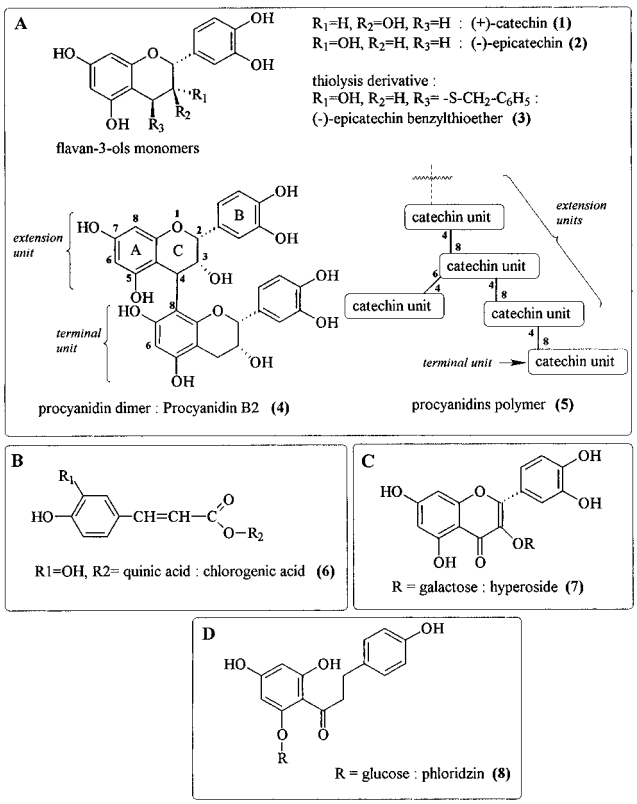

Figure 1. Main phenolic classes in apple: flavan-3-ols (A); hydroxycinnamic acid derivatives (B); flavonols (C); dihydrochalcones (D).

tures, resulting from oxidation, may exhibit tanning properties and, thus, may be involved in the sensory properties of cider as well as the native tannins themselves (Lea, 1990; Dietrich et al., 1990).

Although the concentrations of the other classes, flavonols (Figure 1C) and dihydrochalcones (Figure 1D), are not as high as those of flavan-3-ols and hydroxycinnamic acid derivatives, many different structures are present in apple (Oleszek et al., 1988). These compounds contribute to the pigmentation of apples but are not very good substrates for polyphenol oxidase (Nicolas et al., 1994). Flavonols are mainly located in apple skin and are responsible for the yellow coloration of the epidermis of some apples (Monties, 1966). These compounds may also be present in apple juice (Fernandez de Simon et al., 1992) and ciders (Whiting and Coggins, 1975); therefore, they might contribute to the coloration of these products (Brugirard and Tavernier, 1952). Moreover, some of these compounds, such as quercetin glycosides, show biological activities (Macheix et al., 1994).

In the past decade, significant progress has been made in the field of phenolic compound analysis in fruits and vegetables through the development of reversed-phase HPLC. However, precise determination of compounds remains difficult because of the great complexity of chromatograms due to the presence of a large number of different compounds in the same sample. To avoid these problems, several authors have developed techniques of sample preparation that allowed, for example, the separation of acidic phenolic compounds (hydroxycinnamic acid derivatives) from neutral compounds (flavonoids) (Jaworski and Lee, 1987; Delage et al., 1991; Mangas et al., 1994). Lea also proposed a technique based on the pH shift during HPLC elution (Lea, 1982). In all cases, separations were better, but some problems still existed, often due to the presence of oligomeric and polymeric proanthocyanidins in the extracts. In fact, proanthocyanidins exhibiting different degrees of polymerization or different constitutive units were often present in the extracts. However, these differences are too slight to expect a good HPLC separation, and chromatograms show unresolved clumps. Therefore, the quantification of oligomeric and polymeric proanthocyanidins is often underestimated because only dimers or trimers that gave resolved peaks are considered; moreover, the distorted baseline of chromatograms may interfere with the precise integration of the peaks corresponding to other phenolic compounds.

The depolymerization of proanthocyanidin based on thiolysis prior to HPLC analysis removed unresolved clumps from the chromatograms by converting these compounds into monomers corresponding to the terminal and to the extension units of the polymer (Thompson et al., 1972). Degradation products can be analyzed by reversed-phase HPLC, and the results will provide information on the nature of extension and terminal units and on the average degree of polymerization (Rigaud et al., 1991). Moreover, integration allows quantitative information when response factors of derived compounds are known and when the reaction was performed with a good yield (Matthews et al., 1997).

By using a method based on thiolysis in conjunction with reversed-phase HPLC analysis, this paper deals with the characterization, quantification, and distribution of compounds from different phenolic classes in a cider apple fruit (*Malus domestica* var. Kermerrien).

MATERIALS AND METHODS

Solvents and Standard Phenolics. Methanol, acetonitrile, and acetone (Merck, Darmstadt, Germany) were of chromatographic grade quality. Hexane was of analytic grade quality (Merck). Deionized water was obtained with a Milli-Q water system (Millipore, Bedford, MA). (+)-Catechin, (−)-epicatechin, chlorogenic acid, and phloridzin were provided by Sigma Chemical Co. (St. Louis, MO). Hyperoside (quercetin 3-galactoside) and cyanidin chloride were provided by Extrasynthese S.A. (Lyon, France). (+)-Catechin benzylthioethers [(+)-catechin gives two benzylthioether adducts after thiolysis when located in extension unit in procyanidins (Thompson et al., 1972)], (−)-epicatechin benzylthioether and (−)-epicatechin 3-*O*-gallate benzylthioether, were kindly furnished by J. M. Souquet (Unité de Recherche des Polymères et des Techniques Physico-Chimiques, INRA, Montpellier, France) and were previously characterized (Prieur et al., 1994).

Plant Materials. Cider apples of the Kermerrien variety, grown during the 1995 season in an orchard in the Department of Mayenne (France), were harvested at maturity and stored at 4 °C until use. They were manually calibrated (50−55 mm) and mechanically peeled and cored (20 mm core diameter) using a KALI apparatus (KALI, France) at 4 °C. The seeds were manually separated from each core, and tips of each core (8% of the fresh weight of the fruit) were manually eliminated. The weight of each tissue fraction was measured to determine its proportion in fresh apple. Four plant tissue materials were obtained including the parenchyma zone, the epidermis zone, the core zone, and the seeds. The parenchyma zone constituted the most important part of apple tissues (62%) and, in our conditions of mechanical apple peeling (due to the mechanical peeling of the apple fruits, this material contained also a non-negligible part of parenchyma), the epidermis zone represented ∼18%. The core and the seed corresponded to 11% and 1% of the fresh fruit, respectively.

Aqueous formic acid (3% v/v) was sprayed on the fresh material during these latter operations to avoid oxidation.

Preliminary experiments had shown that neither browning due to oxidation nor pink coloration due to procyanidins degradation appeared on the fresh tissues after 12 h at ambient temperature. Plant materials were frozen and freeze-dried until used.

Extraction Procedure. Five grams of freeze-dried material of each apple tissue zone was homogenized by crushing in a mortar. The powder was first extracted with 3 × 50 mL of hexane to remove lipids, carotenoids, and chlorophyll. It was then extracted with 3 × 50 mL of methanol to dissolve sugars, organic acids, and phenolic compounds of low molecular weight. Last, the resulting residue was extracted with 3 × 50 mL of aqueous acetone (4:6) to extract polymerized polyphenols. Each solvent extraction was carried out by blending the powder with each solvent for 5 min using an Ultra-turrax blender, and the mixture was filtered through a G3 sintered glass filter. Hexane filtrates were combined, evaporated to dryness and stored under vacuum in a desiccator. Methanol filtrates were combined and concentrated to ~30 mL by evaparation under vacuum at 40 °C. About 50 mL of aqueous acetic acid (2.5% v/v) was then added, and evaporation was continued until methanol was eliminated. The aqueous suspensions were frozen and freeze-dried. The aqueous acetone filtrates were combined, concentrated under vacuum to an aqueous solution, frozen, and freeze-dried. For each apple tissue zone, the insoluble residue of extraction was frozen and freeze-dried. Each dry hexane, methanol, and aqueous acetone extract and insoluble residue was weighed to obtain the distribution of the extractable residues.

Spectrometric Evidence of Phenolic Compounds in the Extracts. The dry hexane extracts and the dry methanol extracts were dissolved in methanol at a concentration of 0.5 g L^{-1}, whereas aqueous acetone extracts were dissolved in aqueous acetic acid (2.5% v/v)/methanol (75:25) at the same concentration. Absorbances were measured at 280 nm on a Uvikon double-beam spectrophotometer (model 860, Kontron, Milano, Italy) with the corresponding solvent in the reference cell.

BuOH−HCl Hydrolysis. Butanol/concentrated HCl (95:5, 2.5 mL) and iron reagent [2% w/v solution of $NH_4Fe(SO_4)_2 \cdot 12H_2O$ in 2 M HCl, 100 μL] were added to about ~2 mg of each dry hexane, methanol, or aqueous acetone extracts in a glass tube sealed with a Teflon-lined screw cap. After agitation, the tube was heated at 95 °C for 40 min. Absorbances of the reaction media were measured at 540 nm in the same way as previously described for 280 nm measurements. The quantification was performed by placing the average absorbance values (assays were performed in triplicate) onto a calibration curve of cyanidin chloride.

Thiolytic Degradation. A 100 g L^{-1} solution for dry methanol extracts and a 4 g L^{-1} suspension for dry aqueous acetone extracts were prepared by sonicating the residue in methanol. In a glass bulb, 50 μL of the solution or 50 μL of the suspension 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, reactions were carried out at 40 °C for 30 min and at 50 °C for 10 min for thiolysis of methanol and aqueous acetone extracts, respectively. Thiolyses were performed on triplicate samples for each methanol and aqueous acetone extracts. The media were then injected onto the HPLC system.

HPLC Conditions. For direct HPLC analyses without prior thiolysis, a solution of each dry methanol and dry aqueous acetone extract was prepared in acetic acid 2.5%/methanol (75:25) (all methanol and aqueous acetone extracts were soluble in this solvent) at concentrations of 10 and 1 g L^{-1}, respectively. All samples were filtered through a 0.45 μm filter (HV, Millipore, Bedford, MA), and 20 μL of each filtrate was injected onto the HPLC system. For the reaction mixtures of thiolysis, only 10 μL of the reaction mixture was injected without any prior filtration. The HPLC apparatus was a Waters (Milford, MA) system (717 plus autosampler, 600E multisolvent system, 996 photodiode array detector, and the Millenium 2010 Manager system). The column was a Nova-Pak C_{18} (4 μm, 100 × 3.9 mm i.d.; Waters). The elution conditions were as follows: flow rate, 1 mL min^{-1}; temperature, 30 °C. The solvent system used was a gradient of solvent A (aqueous acetic acid, 2.5% v/v) and solvent B (acetonitrile). 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 min, 30% B linear, followed by washing and reconditioning the column. HPLC analyses were performed in triplicate for each extracts.

For HPLC analyses after thiolysis, the HPLC apparatus and procedure were the same as described above for the analyses of the crude extracts. Only the injection volume (10 μL instead of 20 μL) was different.

Thiolysis of Standards. To estimate the influence of the conditions of thiolysis on compounds other than flavan-3-ols, 0.5 g L^{-1} methanol solutions of chlorogenic acid, hyperoside, and phloridzin were prepared, and each solution was submitted to thiolysis in the conditions described above for methanol extracts. The reaction mixtures were analyzed by HPLC using the same conditions as described above.

Characterization of Phenolic Compounds on Chromatograms Registered at 280 nm. (All phenolic compounds presented an absorbance at this wavelength.) Phenolic compounds, for which reference standards were available, were identified on chromatograms according to their retention times and UV−visible spectra: (+)-catechin, (−)-epicatechin, chlorogenic acid, phloridzin, and toluenethiol derivative of (−)-epicatechin (no other derivatives were detected when compared to the available standards). Although the other compounds could not be wholly identified, they were characterized according to their class on the basis of their UV−visible spectra (Delage et al., 1991): flavan-3-ols (monomers and procyanidins) presented a single symmetric band with a maximum absorbance at 278 nm, hydroxycinnamic acid derivatives presented a maximum absorbance between 300 and 330 nm, flavonols were characterized by an absorption band with a maximum above 340 nm, whereas dihydrochalcones presented a single broad and asymmetric band with a maximum of 285 nm.

HPLC Quantification on Chromatograms after Thiolysis. Phloridzin was used as an external standard for quantification of the dihydrochalcones at 280 nm, chlorogenic acid was used for that of the hydroxycinnamic acid derivatives at 320 nm, and hyperoside was used as an external standard for quantification of the flavonols at 350 nm. Calibration curves were available for each of these compounds, and response factors were applied to the sum of areas of the chromatographic peaks corresponding to compounds that were at first characterized and classified according to their UV−visible spectrum.

For total flavan-3-ols (monomers + proanthocyanidins), the estimation was performed on chromatograms at 280 nm by the sum of the terminal units [i.e., (−)-epicatechin and (+)-catechin] and the extension units [(−)-epicatechin benzylthioether]. Calibration curves were available for each of these compounds.

The average degree of polymerization was measured by calculating the molar ratio of all the flavan-3-ol units (thioether adducts + terminal units) to (−)-epicatechin and (+)-catechin corresponding to terminal units.

To take into account their proportion in their respective class, the flavan-3-ol monomers [(+)-catechin and (−)-epicatechin] and the main hydroxycinnamic acid derivative (chlorogenic acid) were individually assayed at 280 and 320 nm, respectively, in every methanol and aqueous acetone extracts on chromatograms obtained without thiolysis.

RESULTS AND DISCUSSION

Four tissue zones (the epidermis zone, the parenchyma zone, the core zone, and the seeds) of cider apple fruit (*M. domestica* var. Kermerrien) were considered for the analysis of their polyphenolic content.

The freeze-dried tissues were successively fractionated into hexane (HE), methanol (ME), and aqueous

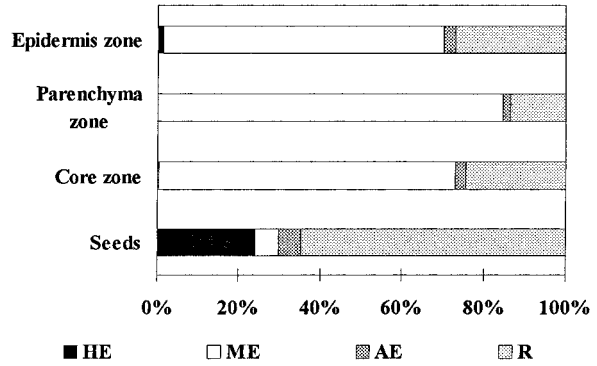

Figure 2. Yields (percent of freeze-dried material, w/w) of the extraction procedure for the four apple tissue zones (HE, dry hexane extracts; ME, dry methanol extracts; AE, dry aqueous acetone extracts; R, dry insoluble residues).

acetone extracts (AE) (Figure 2). Most of the tissue constituents were extracted by methanol from all three tissues except from the seeds. The majority of seeds residues were either insoluble or extracted by hexane. Simple sugars, oligosaccharides, and organic acids, which are abundant in ripe apples, were considered to be extracted by methanol. Methanol extracts also contained low molecular weight phenolic compounds. The direct spectrometric measurement of absorbance at 280 nm was used to show evidence of the presence of phenolic compounds in the extracts. Only methanol and aqueous acetone fractions gave positive absorbances. Therefore, no further study was performed with hexane extracts.

Reversed-Phase HPLC Analysis of Phenolic Compounds following Thiolysis (Figure 3). Chromatograms of methanol (A) and aqueous acetone (B) extracts from the epidermis zone before (a) and after (b) thiolysis are presented in Figure 3. The thiolytic degradation resulted in obvious alterations of the chromatograms. Without thiolysis, compounds of the proanthocyanidin type appeared as more or less unresolved peaks (Figure 3Aa) or as broad clumps (Figure 3Ba) for methanol and aqueous acetone extracts, respectively. After thiolytic depolymerization (Figure 3Ab,Bb), these compounds were converted into monomer units (flavan-3-ols or flavan-3-ol benzylthioethers according to the position in the native proanthocyanidins). The corresponding peaks were well resolved, which permitted a good estimation of procyanidins in the extracts. The chromatograms also showed alterations of the peaks corresponding to compounds of the other phenolic classes. Some of them were reduced and partly converted into secondary peaks, but no significant consequence was observed for the quantification of these products in the extracts as shown later. In addition, some of these compounds were not coeluted with proanthocyanidins after thiolysis any more, which might be favorable to the accuracy of their quantification.

Optimization of Thiolysis (Figure 4). The optimization of thiolysis was separately performed on methanol and aqueous acetone extracts of parenchyma at 40 and 50 °C, respectively. The best conditions were estimated by following the formation of (−)-epicatechin benzylthioether versus time in HPLC (Figure 4) at 280 nm. For methanol and aqueous acetone extracts, 30 min at 40 °C and 10 min at 50 °C, respectively, were considered to give the best results.

Yields of thiolysis were determined by performing thiolysis on several pure cider apple procyanidin fractions with an average degree of polymerization between 1 and 10. These fractions were obtained from a crude methanol extract of cider apple parenchyma by separation on normal-phase semipreparative HPLC (unpublished results) according to a method already described by Rigaud et al. (1993) for grape seed procyanidins. The yields (~90% whatever the considered fraction) were significantly higher than those presented recently by Matthews et al. (1997). These authors have analyzed bark samples differing from apples as the proanthocyanidins present in the tissues have aged during several years and may have undergone secondary reactions that should affect the yields of depolymerization.

Effect of Thiolysis Conditions on Hydroxycinnamic Derivatives, Flavonols, and Dihydrochalcones (Table 1). The conditions of thiolysis caused a partial degradation of non-procyanidin phenolic compounds. The degradation was estimated by comparing HPLC analyses of pure solutions of standards (i.e., chlorogenic acid, hyperoside, and phloridzin) before and after thiolysis. Thiolyses were performed in the conditions used for methanol extracts. For each product, the degradation was shown by a novel peak on chromatograms. The partial conversion is clearly observed on chromatograms (Figure 3) for chlorogenic acid (**6**), which was largely transform into product **6′** after thiolysis. The UV−visible spectrum of each degradation product was similar to that of the native compound, which indicates that the integrity of the phenolic moiety has not been altered. The degradation might correspond to the loss of the nonphenolic moiety under the acidic conditions of thiolysis. By summing the calculated amounts of the residual standards to their respective derived products (Table 1), recovery was estimated as 97.6, 94.8, and 96.5% for chlorogenic acid, phloridzin, and hyperoside, respectively. Thus, quantification of hydroxycinnamic acid derivatives, flavonols, and dihydrochacones was considered to be correct even after thiolysis.

Phenolic Composition of Cider Apple Tissues (Table 2). Quantitative data (Table 2) are presented in two different ways: the concentrations in apple tissues are reported in column A, whereas column B deals with the quantity in the entire apple fruits. Moreover, procyanidin contents estimated by two different methods (HPLC following thiolysis and BuOH/HCl assay) are also reported in Table 2.

The total phenolic concentration in each tissue zone did not show any significant differences as it was ~5 g/kg of fresh weight. The value, however, was greater in the epidermis zone (>6.5 g kg^{-1}) as already observed in previous works (Burda et al., 1990). By considering the entire apple, most of phenolic compounds (65%) were located in the parenchyma, whereas skin, core, and seeds represented 24, 10, and 1%, respectively.

Except for the seeds, flavan-3-ol oligo- and polymers (i.e., procyanidins) were the most concentrated polyphenols (at least 75% in all tissues) and represented 65% of total phenolic compounds in apple fruits when estimated by HPLC following thiolysis. On the whole, these concentrations are noticeably higher than previous published data concerning flavan-3-ol content in apple tissues (Nicolas et al., 1994). These results might be due to the fact that cider apples contained a large amount of tannins in comparison to dessert apples (Lea, 1990). Moreover, the extraction procedure used in this work involved an aqueous acetone extraction of the apple tissues that permitted us to take into account the

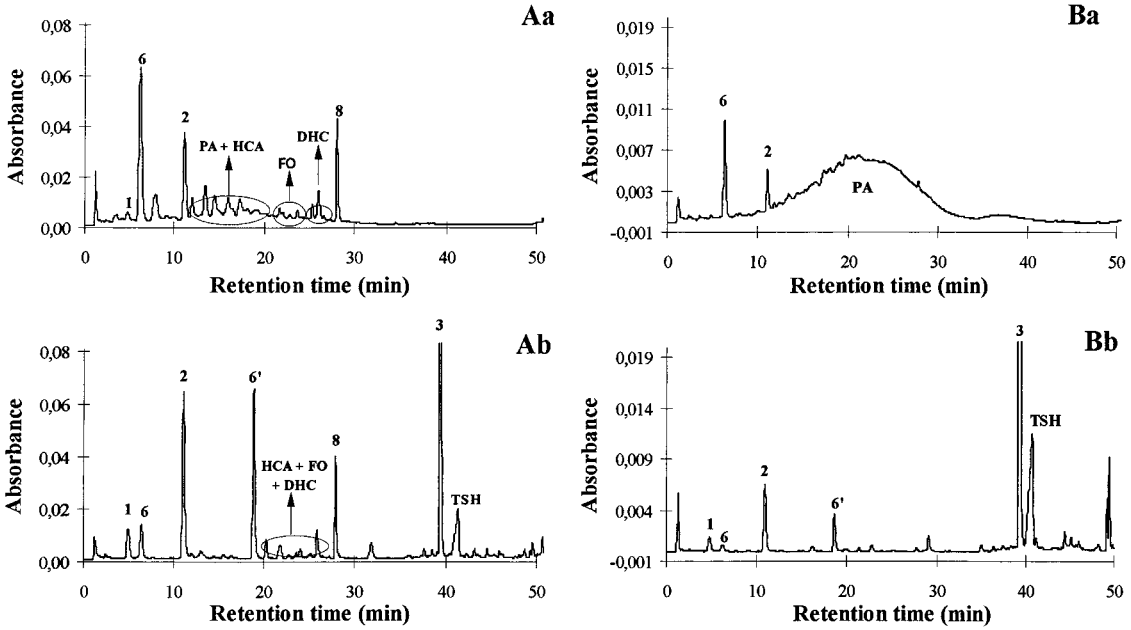

Figure 3. Reversed-phase HPLC chromatograms of methanol (A) and aqueous acetone (B) extracts from epidermis zone before (a) and after (b) thioacidolysis. Peaks corresponding to available standards are numbered according to Figure 1 (6′ corresponds to the degradation product of chlorogenic acid after thiolysis); the other peaks have been characterized according to their UV−visible spectra (HCA, hydroxycinnamic derivatives; FO, flavonols; PA, procyanidins).

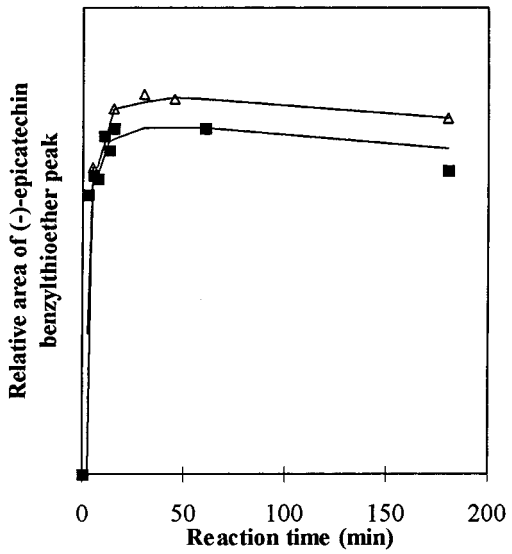

Figure 4. Kinetics of thiolysis of methanol extract at 40 °C (△) and aqueous acetone extract at 50 °C (■) of Kermerrien parenchyma.

Table 1. Reversed-Phase HPLC Estimation of the Effect of Thiolysis Conditions on Non-Flavan-3-ol Phenolic Standards

	chlorogenic acid		hyperoside		phloridzin	
	BT[a]	AT[a]	BT	AT	BT	AT
rel peak area of std compd	100	5.0	100	81.5	100	89.9
rel peak area of degradn prod		92.6		15.0		4.9
recovery (%)	97.6		96.5		94.8	

[a] BT, before thiolysis; AT, after thiolysis.

proportion of polymeric procyanidins; these compounds may not be extracted from the fruits by pressing or when only methanol or ethanol fruit extractions are carried out (Delage et al., 1991; Amiot et al., 1992). At last, thiolysis prior to HPLC analysis may give a more representative estimation of polymeric procyanidins in the extracts than direct HPLC does; in the latter method, these compounds are analyzed in their native state, leading to unresolved chromatographic peaks that make difficult an accurate integration.

HPLC analyses of the extracts after thiolysis allowed the characterization of the procyanidins by their constitutive units and their average degree of polymerization (Prieur et al., 1994). Results, presented in Table 3, indicate that the two different solvents used successively for extraction permitted a selective separation of two populations of flavan-3-ols. The average degree of polymerization in the extracts (DPe) was ∼3 for methanol extractable procyanidins, whereas it was ∼11 for aqueous acetone extractable procyanidins. The estimation of the latter in the entire apple fruits (Table 2) showed that they represented >26% of total procyanidins of the fruits. Globally in the tissue zones, the average degree of polymerization in the entire tissus (DPt) (calculated by taking into account the relative quantity of flavan-3-ols in methanol and aqueous acetone extracts) was higher in the epidermis than in parenchyma and core, the highest value (DPt = 11.2) being observed for the seeds (Table 3). Moreover, it is noteworthy to observe an increasing gradient of concentrations from the core to the periphery of the fruit for procyanidins (Table 2). These findings may be in relation with a protective role of flavan-3-ols against attacks by fungi as a correlation between flavan-3-ols contents of apple fruits and fungi resistance that has been already observed (Feucht et al., 1992). Moreover, the ability of procyanidins to inhibit enzymatic attacks by fungi might be more efficient when their molecular weight increases (Scalbert, 1991).

When compared to values obtained by HPLC following thiolysis, the estimations of procyanidins according to the BuOH/HCl assay (bottom of Table 2) were much lower. The BuOH/HCl assay is commonly used for the estimation of condensed tannins and allows the quantification of procyanidins on the basis of the extension units of these compounds, which are transformed into

Table 2. Phenolic Composition of the Kermerrien Cider Apple

	epidermis zone		parenchyma zone		core zone		seeds		total in apple fruits
	A	B	A	B	A	B	A	B	
Data Obtained by RP-HPLC following Thiolysis									
procyanidins	4964	892	3379	2104	2121	229	1232	11	3236
	125	*22*	*116*	*72*	*131*	*14*	*36*	*0*	*77*
flavan-3-ol monomers	728	131	858	533	673	73	128	1	739
	3	*0*	*32*	*20*	*64*	*7*	*6*	*0*	*24*
hydroxycinnamic acids	687	123	908	565	1364	146	365	3	837
	5	*1*	*30*	*19*	*77*	*8*	*10*		*27*
flavonols	129	23	nd	nd	nd	nd	nd	nd	23
	2	*0*							*0*
dihydrochalcones	137	24	74	46	625	67	3416	30	169
	5	*0*	*3*	*2*	*42*		*120*	*1*	*6*
total	6644	1193	5219	3250	4782	516	5141	45	5004
	120	*22*	*117*	*73*	*112*	*12*	*118*	*1*	
Data Obtained by BuOH/HCl Assay									
procyanidins	1231	221	804	501	743	80	669	6	808
	59	*11*	*22*	*14*	*41*	*4*	*58*	*1*	*14*

[a] A: data are given in mg/kg of fresh tissue. B: data are given in mg/kg of fresh apple fruits. All values are given by taking into account the relative quantity of each class of phenolic compounds in methanol and aqueous acetone extracts. Flavan-3-ols are expressed in catechin equiv, hydroxycinnamic acids in chlorogenic acid equiv, flavonols in hyperoside equiv, and dihydrochalcones in phloridzin equiv. Italic values correspond to standard deviations ($n = 3$). nd, nondetected.

Table 3. Characterization of Procyanidins according to Their Constitutive Units and Their Average Degrees of Polymerization[a]

		epidermis zone		parenchyma zone		core zone		seeds	
		ME	AE	ME	AE	ME	AE	ME	AE
terminal units (%)	CAT	5.1 (0.06)	2.8 (0.02)	3.8 (0.04)	2.7 (0.02)	4.9 (0.13)	1.8 (0.08)	0 (0)	0.7 (0.10)
	EC	23.8 (0.10)	5.2 (0.26)	29.6 (0.44)	6.1 (0.03)	33.3 (0.98)	8.4 (0.05)	23.1 (1.89)	7.0 (0.22)
extension units (%)	EC	71.1 (0.05)	92.0 (0.26)	66.5 (0.40)	91.2 (0.04)	61.8 (0.86)	89.8 (0.12)	76.9 (1.89)	92.2 (0.32)
DPe		3.5 (0.01)	12.5 (0.41)	3.0 (0.004)	11.3 (0.05)	2.6 (0.06)	9.8 (0.12)	4.3 (0.34)	12.9 (0.85)
DPt		6.0 (0.21)		4.5 (0.05)		4.4 (0.09)		11.2 (1.07)	

[a] ME, dry methanol extract; AE, dry aqueous acetone extract; Dpe, average degree of polymerization in the extracts; DPt, average degree of polymerization in the tissue zones; CAT, (+)-catechin; EC, (−)-epicatechin; values in parentheses correspond to standard deviations ($n = 3$).

anthocyanidins when placed in acidic media (Porter et al., 1986). Terminal units are not taken into account in this test, and the lower the degree of polymerization of procyanidin is, the more important the underestimation is (Scalbert, 1992). This explanation is supported by comparing the underestimation for seed procyanidins (2 times lower; DPt = 11) with that of other tissue zones (4 times lower; DPt = 4−6). Moreover, the yield of the BuOH/HCl reaction may be lowered because of secondary reactions leading to the formation of phlobaphenes (Scalbert, 1992).

The nature of the constitutive units was similar for all tissue zones because (−)-epicatechin represented >95% of constitutive units in all extracts (Table 3). About 5% was identified as (+)-catechin (**1**) in the terminal position, which partly results from epimerization of (−)-epicatechin, a secondary reaction that cannot be completely avoided in the conditions of thiolysis. As far as we know, this preponderance of (−)-epicatechin as constitutive unit of procyanidin has always been observed in apples and apple-derived products (Nicolas et al., 1994; Lea, 1990).

The flavan-3-ol monomers were detected only as (−)-epicatechin and (+)-catechin, the former being highly preponderant (Table 2). The relative proportion of flavan-3-ols monomers and procyanidins may be of great importance in the phenolic composition of raw materials. The former, which are good substrates for polyphenol oxidase, are directly involved in enzymatic oxidation occurring when apples are crushed. On the other hand, procyanidins are strongly implicated in astringency or bitterness of ciders (Lea, 1990), although they may be also involved in the oxidation reactions (Cheynier and Ricardo da Silva, 1991).

Hydroxycinnamic acid derivatives, which constituted another important class of phenolic compounds in the three main tissue zones (epidermis, parenchyma, and core), were mainly obtained in the methanol extracts. In entire apple fruits, they constituted 18% of total phenolic compounds, most of them being located in the parenchyma, and they accounted for 7−28% of total phenolic content depending on the considered tissue zone. Except for the seeds, the concentration of hydroxycinnamic acid derivatives increased from the epidermis to the core in the same way as already observed for polyphenol oxidase (Murata et al., 1995) in apple fruits. This gradient of concentration was the inverse of that of procyanidins. In each tissue zone, chlorogenic acid was the most abundant hydroxycinnamic acid derivative, representing 79, 76, 79, and 87% of this class in epidermis, parenchyma, core, and seeds zones, respectively.

As previously shown (Durkee and Poapst, 1965), the dihydrochalcones were abundant in seeds, where they contribute to 66% of the total phenolic compounds, but they represented <3% in epidermis and parenchyma zones. Compared to other polyphenols, flavonols were found in the epidermis zone at a level of ∼2% of total polyphenols in the zone. Finally, flavonols and dihydrochalcones could be considered quantitatively as minor classes, because they represented less than 0.5 and 3.5% of total phenolic compounds in entire apple

fruits, respectively. The former were located only in the epidermis zone, whereas the latter were present in all tissues.

CONCLUSION

Thiolysis followed by reversed-phase HPLC may serve as an efficient method to characterize and quantitate the phenolic compounds in apple fruits without any prior purification. Depolymerization of proanthocyanidins yielded well-resolved chromatograms allowing accurate determination of the proportion of each class of phenolic compounds. The method described here permitted us to show the real proportion of procyanidins in the raw material even when present in highly polymerized forms. It also allowed the characterization of procyanidins by their constitutive units and by their average degree of polymerization. This information may be of great interest in the understanding of the role of phenolic compounds of the raw materials in derived products such as apple juices or ciders. Results obtained on the phenolic composition of different tissue zones of a cider apple fruit showed that flavan-3-ols and especially procyanidins were preponderant in apple, a significant proportion being highly polymerized. Further works are in progress to compare the compositions of different cider apple varieties and to evaluate the change of the phenolic composition when apples are processed into juices and ciders.

ABBREVIATIONS USED

HE, hexane extracts; ME, methanol extracts; AE, aqueous acetone extracts; CG, chlorogenic acid; EC, (−)-epicatechin; CAT, (+)-catechin; PA, procyanidins; HCA, hydroxycinnamic acid derivatives; FO, flavonols; DHC, dihydrochalcones; TSH, toluene-α-thiol; DPe, average degree of polymerization in the considered extract; DPt, average degree of polymerization in the considered tissue zone; RP-HPLC, reversed-phase high-performance liquid chromatography.

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