Polyphenol Profiles of French Cider Apple Varieties (*Malus domestica* sp.)

Philippe Sanoner, Sylvain Guyot,* Nathalie Marnet, Daniel Molle,† and J.-F. Drilleau

Laboratoire de Recherches Cidricoles, Biotransformation des Fruits et Légumes, Institut National de la Recherche Agronomique, BP 29, 35650 Le Rheu, France

The cortex of 14 French apple varieties (12 cider and 2 juice varieties), one English cider variety, and one dessert apple (i.e., Golden Delicious) were studied for their polyphenol composition. Total polyphenols were assayed by the Folin-Ciocalteu method, and the precise polyphenolic composition (monomeric catechins, proanthocyanidins, hydroxycinnamic acids, and dihydrochalcones) was obtained by HPLC following thiolysis. ESI-MS and ESI-MS/MS analyses showed that chlorogenic acid and *p*-coumaroylquinic acid were methylated under the conditions of thiolysis. Depending on the variety, the global polyphenol concentration varied from 1 to 7 g per kilogram of fresh cortex. Cider varieties globally showed a higher polyphenol concentration than the dessert apple Golden Delicious, bitter varieties being the more concentrated. The proportion of the polyphenol classes varied greatly from one cultivar to another. For all varieties, procyanidins were always the predominant class. They were mainly constituted of (−)-epicatechin units with a small proportion of (+)-catechin as a terminal unit. The average degree of polymerization ranged between 4.2 and 7.5 depending upon the variety with an exception for the sharp varieties Guillevic and Avrolles which showed significant concentrations of procyanidins with DP*n* of 40 and 50, respectively.

Keywords: *Apple; cider; HPLC; thiolysis; mass spectrometry; polyphenols; procyanidins*

INTRODUCTION

Polyphenols of apple are largely implicated in cider quality. Besides, the global tannin content of the must in combination with the must acidity has been used to classify French (Tavernier and Jacquin, 1949) and English (Barker and Burroughs, 1953) cider apple varieties. Many reasons can be produced to justify the importance of phenolic compounds in cider apple transformation. Polyphenols are involved in astringent and bitter tastes which contribute to "the overall mouthfeel" of ciders (Lea, 1990b). Moreover, the color of cider is essentially due to phenolic compounds which are implicated in oxidation reactions. Some polyphenols such as hydroxycinnamic acids are also precursors of volatile constituents that contribute to cider aroma (Whiting, 1975). Last, phenolic compounds of ciders might contribute to antioxidant intake, presumed to have a health protective action.

French ciders are traditionally made with particular apple varieties with a specific taste different from that of dessert apples. The latter are generally slightly acidulate and poorly concentrated in phenolic compounds, whereas some cider varieties contain 10-fold more polyphenols (Van Buren, 1970).

Although the "polyphenol" criterion is important for cider quality, data on the phenolic composition of French cider cultivars are still poor. They correspond essentially to the global estimation of the polyphenol concentration in apple musts. In some cases, the distinction between tannin and non-tannin polyphenols was made by using the cinchonin sulfate precipitation method (Brugirard and Tavernier, 1952). These global estimations do not make a precise distinction between the different classes of polyphenols and their different properties. Moreover, when performed in the musts, the assay does not give complete information on the "polyphenolic potential" of the fruits because an important part of the native compounds are oxidized and adsorbed on the apple cell wall when fruits are processed into juices. Much progress has been made in the field of polyphenol analysis with the development of HPLC. Thus, the reversed-phase HPLC analysis of several cider apple musts clarified their composition, differentiating between the main phenolic compounds (Lea, 1982; Delage et al., 1991; Picinelli et al., 1997). In apple fruits, most data concern dessert varieties (Nicolas et al., 1994; Mayr et al., 1995; Perez-Ilzarbe et al., 1991; Burda et al., 1990; McRae et al., 1990; Amiot et al., 1992). Nevertheless, comparisons of cider varieties on the basis of their phenolic profiles have been performed in the corresponding must (Delage et al., 1991) or cider (Whiting and Coggins, 1975) by use of HPLC methods. When associated with HPLC analyses, thiolysis may be a useful method for the characterization of polyphenols of the proanthocyanidin class (Rigaud et al., 1991; Prieur et al., 1994). When the reaction is performed with a good yield, the method is convenient for quantitative analyses (Matthews et al., 1997; Guyot et al., 1998). In recent work, the different polyphenol classes have been assayed and characterized by HPLC following thiolysis in the different tissue zones of Kermerrien cider apple fruits (Guyot et al., 1998). Five polyphenol classes have been distinguished (proanthocyanidins, monomeric catechins, hydroxycinnamic acids, dihydrochalcones, and flavonols). The method was par-

* To whom correspondence should be addressed (telephone 33 (0)2 99 28 52 12; fax 33 (0)2 99 28 52 10; e-mail guyot@rennes.inra.fr).

† Laboratoire de Technologie Laitière, INRA, 65 rue de St Brieuc, 35042 Rennes cedex.

10.1021/jf990563y CCC: $18.00
Published on Web 10/29/1999

ticularly well adapted to the characterization of procyanidins, giving access to their average degree of polymerization (DP*n*) and the nature of their constitutive units. By using the same analytical method, the purpose of our present work is to compare cider apple varieties which are representative of the French orchards. Knowledge of the precise composition of the apple varieties may contribute to a better understanding of their implication in cider quality and diversity.

MATERIALS AND METHODS

Standard Phenolics. (+)-Catechin, (−)-epicatechin, chlorogenic acid, phloridzin, and benzylthioether derivatives have been obtained as described by Guyot et al. (1998). *p*-Coumaroylquinique acid was purified from a commercial cider by liquid−liquid extraction followed by reversed-phase HPLC at the semipreparative scale (unpublished work), and its identification by ESI-MS is described in the present paper.

Plant Materials. About 30 kg of apple fruits of each variety were harvested at maturity during the 1997 season in the experimental orchard of the Comité des Fruits à Cidre (Sées, Orne, France). Guillevic variety apple was harvested in the experimental orchard of the "Association Pomme 56". Fruits were manually calibrated, and the predominant size was used for the analysis of phenolic compounds. For each variety, three batches of 10 fruits were constituted. Fruits were mechanically peeled and cored as already described (Guyot et al., 1998), but only the cortex zone, which constituted the main part of the fruit, was used for polyphenol analysis. Tissues were frozen and freeze-dried, and an aliquot of each batch was used for the determination of the fresh/dry matter ratio.

Extraction of Phenolic Compounds. The procedure was the same as that already described (Guyot et al., 1998). First, dried cortex was successively extracted with hexane, methanol, and finally aqueous acetone. Organic filtrates were eliminated, and residues were freeze-dried. Methanol and aqueous acetone dry extracts were used for the analysis of their polyphenol content.

A 100 g L^{-1} methanolic solution of each dry methanol extract and a 4 g L^{-1} sonicated suspension of each dry aqueous acetone extract was prepared and used for both Folin−Ciocalteu and thiolysis−HPLC assays.

Total Polyphenols Assay. Estimation of the global polyphenol content in the extracts was performed by the Folin Ciocalteu method adapted from Singleton and Rossi (1965). Solutions and suspensions of methanol and aqueous acetone extracts were diluted 50-fold and 16-fold in an acetic acid solution (2.5% v/v), respectively. Folin Ciocalteu reagent (0.25 mL; Merck, Darmstadt, Germany) was added to the diluted solutions (0.5 mL). The methanolic suspensions of the aqueous acetone extracts were soluble when diluted in aqueous acetic acid. 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, the absorbance was measured at 700 nm (Uvikon 860 spectrophotometer, Kontron, Milano, Italy) with a blank sample (water plus reagents) in the reference cell. Quantification was obtained by reporting the absorbances in the calibration curve of (−)-epicatechin used as standard phenol.

Thiolysis and Reversed-Phase (RP) HPLC Conditions. Thiolysis and HPLC conditions were the same as those previously described (Guyot et al., 1998) with some minor changes and improvements: The waters HPLC system was equipped with a cooling system for the autosampler 717 to avoid the degradation of some thiolysis derivative, thus allowing larger series injections. The column was a Purospher RP18 endcapped, 250 × 4 mm, 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), and the following gradient was applied: 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.

HPLC Characterization and Quantification of Phenolic Compounds. Phenolic compounds, for which standards were available, were identified by chromatograms according to their retention times and their UV−vis spectra as already described by Guyot et al. (1998). Flavan-3-ols and dihydrochalcones were assayed at 280 nm, whereas hydroxycinnamic acids were assayed at 320 nm. Moreover, the identification of two hydroxycinnamic derivatives formed under thiolysis conditions was performed by ESI-MS analysis of the corresponding collected RP-HPLC peaks (see details below).

Quantification was performed by reporting the measured integration area in the calibration equation of the corresponding standard. Flavan-3-ols (i.e., (−)-epicatechin, (+)-catechin, and proanthocyanidins) were assayed as (−)-epicatechin equivalent. Dihydrochalcones (i.e., phloridzin and phloretin xyloglucoside) were assayed as phloridzin equivalent. Chlorogenic acid and *p*-coumaroylquinic acid were assayed according to their own response factors.

Electrospray Infusion Mass Spectrometry and Tandem Mass Spectrometry. The mass spectrometry system was a API III^+ triple quadrupole (Perkin-Elmer Sciex Instruments, Tornhill, Canada) equipped with an atmospheric pressure ionization source (electrospray). Infusion analyses were performed in the negative mode with a −4100 kV ion spray voltage (ISV) and a −80 V orifice voltage (OR). For tandem analyses, parameters (voltages, collision energy) were optimized for each compound to show the parent ion together with the main product ions. More concretely, the resolution was set to pass a 2 Da window around the parent ion on Q1 and was adjusted about 1 unit *m*/*z* for the analysis of the product ions. The molecular masses were determined from these data using the package software supplied by Sciex (Tune 2.5, Multiview 1.2).

RESULTS AND DISCUSSION

Thiolysis−HPLC was used for characterization of the polyphenolic profiles in the cortex of 14 French apple varieties (12 cider and 2 juice varieties), one English cider variety (Dabinett), and one dessert apple (Golden Delicious).

Acid-catalyzed degradation of proanthocyanidins in the presence of toluene-α-thiols (Thompson et al., 1972) allows the distinction between terminal units (released as catechin units) and extension units (released as benzylthioether derivatives). When it was associated with reversed-phase HPLC, the method was used for the calculation of the average degree of polymerization of the proanthocyanidins in the corresponding sample (Rigaud et al., 1991; Prieur et al., 1994). Moreover, integration of the HPLC peaks allows quantitative information on the proanthocyanidin content when the reaction was performed with a good yield (Matthews et al., 1997; Guyot et al., 1998). In a recent paper (Guyot et al., 1998), we showed the benefit of HPLC following thiolysis to characterize and quantify proanthocyanidins in the different tissue zones of a cider apple variety. The procedure also allowed assay of the other main polyphenol classes such as hydroxycinnamic acids, flavonols, and dihydrochalcones. Nevertheless, under thiolysis conditions, some of these non-flavan-3-ol compounds, in particular hydroxycinnamic derivatives, were partly converted into new products (Guyot et al., 1998). The UV−vis spectra of these products were similar to those of the corresponding native compounds. Therefore, they were quantified as such but their precise structure remained unknown. To elucidate their structure, the main chromatographic peaks which were observed on chromatograms (Figure 1) were collected and analyzed onto the direct ESI-MS infusion system in the negative mode.

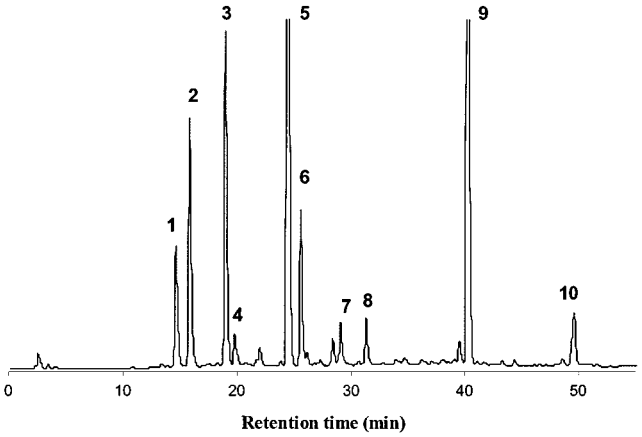

Figure 1. Example of a reversed-phase HPLC chromatogram (280 nm) of an apple (Dous Moen variety) methanol extract after thiolysis reaction. **1**: (+)-catechin; **2**: chlorogenic acid; **3**: (−)-epicatechin; **4**: *p*-coumaroylquinic acid; **5**: methyl ester of chlorogenic acid; **6**: methyl ester of *p*-coumaroylquinic acid; **7**: phloretin xyloglucoside; **8**: phloridzin; **9**: (−)-epicatechin benzylthioether; **10**: toluene-α-thiol.

The observation of the pseudo-molecular ions, $[M - H]^-$, on the spectra confirmed the nature of the following compounds, which were available as standards: (+)-catechin (**1**, R_t 14.5 min; $[M - H]^-$ *m/z* 289), (−)-epicatechin (**3**, R_t 18.7 min; $[M - H]^-$ *m/z* 289), (−)-epicatechin-benzylthioether (**9**, R_t 40.2 min; $[M - H]^-$ *m/z* 411), chlorogenic acid (**2**, R_t 15.5 min; $[M - H]^-$ *m/z* 353), phloridzin (**8**, R_t 31.2 min; $[M - H]^-$ *m/z* 435), and phloretin (R_t 39.5, $[M - H]^-$ *m/z* 273). Two nonstandard native compounds were presumed to be *p*-coumaroylquinic acid and phloretin xyloglucoside on the basis of their retention time (**4**, R_t 19.5 min, and **7**, R_t 28.9 min, respectively), their UV−vis spectra, and literature values. Their structures were confirmed by the observation of their respective $[M - H]^-$ ions at *m/z* 337 and 567 and also by the product ion mass spectra which show typical fragment ions: *p*-coumaroylquinic acid MS-MS spectrum ($[M - H]^-$ *m/z* 337) displayed fragment ions at *m/z* 191, 173, and 163 corresponding to quinic acid, trihydroxycyclohexane, and *p*-coumaric moieties, respectively, as already observed in previous work (Poon, 1998). The product ion spectrum of *m/z* 567 showed fragment ions at *m/z* 419 and 273 which correspond to the sequential losses of xylose and glucose moieties, respectively, from the structure of phloretin xyloglucoside.

Thiolysis-derived products of chlorogenic acid (**5**, R_t 24.2 min, $[M - H]^-$ *m/z* 367) and *p*-coumaroylquinic acid (**6**, R_t 25.4 min, $[M - H]^-$ *m/z* 351) showed a mass difference of +14 in comparison with their respective genuine compounds. They were then identified as the corresponding methylated molecules. The thiolysis conditions (methanol and hydrochloric acid) are favorable to the methanolic esterification of the free carboxylic group of the quinic acid moiety. The location of the methyl group on the quinic acid moiety was confirmed by the observation of the product ions spectra of *m/z* 367 and 351 containing fragment ions at *m/z* 179 and 163, respectively, which correspond to the loss of the methylated quinic acid moiety.

Under thiolysis conditions, phloridzin and phloretin xyloglycoside are slightly converted into a single derived product which was identified as phloretin according to its retention time (39.5 min), UV−vis spectrum (λ_{max} 285 nm), and infusion mass spectra ($[M - H]^-$ *m/z* 273).

The total polyphenol contents for each variety are presented in Table 1. Two methods were compared: the Folin−Ciocalteu assays and the sums of all phenolic compound concentrations obtained by thiolysis−HPLC. A statistical paired *t*-test (SigmaStat software, Jandel scientific, Germany) was performed to compare the two sets of data. It showed that the global difference between data obtained by the two methods was not great enough to exclude the possibility that it was due to chance. Therefore, under our conditions, no significant difference was found between the two methods. This observation is an indication of the reliability of the thiolysis−HPLC method because the Folin−Ciocalteu assay is generally considered as the method of choice to estimate total phenol contents in plant extracts (Singleton and Rossi, 1965; Scalbert, 1992).

Total polyphenol concentrations (Table 1) varied from 7-fold depending on the varieties, Golden variety presenting the lowest content close to 1 g/kg of fresh cortex whereas Jeanne Renard variety contained 7 g/kg. A large difference in polyphenol contents has been also shown between dessert and cider apple juices of English varieties (Lea, 1990a). On the whole, our results are in good agreement with the classification of cider apple varieties in taste categories partly based on the total polyphenol content of the juices (Tavernier and Jacquin, 1949; Barker and Burroughs, 1953). Bitter varieties (Kermerrien, Chevalier Jaune, and Jeanne Renard) had the highest polyphenol concentrations, whereas sharp (Avrolles and Guillevic) and acidulous (Petit Jaune, Juliana, and Judor) varieties showed lower concentrations. On the whole, sweet (Antoinette, Bedan, and Douce Coët Ligné) and bittersweet (Binet Rouge, Clozette, Douce Moen, and Dabinett) varieties showed an intermediate polyphenol concentration.

For each variety, Table 2 presents quantitative data of the phenolic compounds assayed by the thiolysis−HPLC method except for the monomeric catechins which were assayed by HPLC without prior thiolysis.

In all varieties, proanthocyanidins corresponded to the predominant class (Table 2). They accounted for 75% of total polyphenols in the Golden Delicious variety. On the whole, this proportion is noticeably higher than previously published data for this variety (Mosel and Herrmann, 1974; Perez-Ilzarbe et al., 1991; Amiot et al., 1992). It may be due to the extraction procedure and also to the thiolysis−HPLC method allowing a better estimation of polymerized proanthocyanidins. These compounds may be underestimated when alcoholic or hydro-alcoholic extractions are used because most of them are not extracted and remained in the insoluble part of the cortex. Moreover, after extraction, their estimation by direct HPLC remained incomplete because polymeric forms do not give well-resolved peaks on chromatograms. Therefore, in most cases, only oligomeric forms have been quantified by direct HPLC analyses. Nevertheless, the thiolysis−HPLC procedure has a drawback as it is not possible to assay individual oligomeric procyanidins.

Depending on the variety, the proportion of proanthocyanidins varied from 44% to 89% for Douce Coët Ligné and Avrolles, respectively, and concentrations varied from 0.5 to 4.7 g/kg of fresh cortex for Judor and Jeanne Renard, respectively. When compared to the literature, concentrations of cider apple proanthocyanidins in the cortex are close to those observed in red grape berries used for wine making (Bourzeix et al.,

Table 1. Total Polyphenol Content in the Cortex of Apple Varieties[a]

	GD	JD	GU	PJ	BR	JU	CL	AV	DM	AN	BD	DA	DC	CJ	KE	JR
total polyphenols by thiolysis−HPLC	1.04	1.04	1.70	2.02	2.32	2.42	2.45	2.80	3.04	3.10	3.26	3.41	3.43	3.83	4.50	6.99
standard deviation (n = 3)	*0.13*	*0.09*	*0.16*	*0.20*	*0.14*	*0.11*	*0.40*	*0.30*	*0.63*	*0.23*	*0.15*	*0.66*	*0.45*	*0.34*	*0.47*	*0.34*
total polyphenols by Folin Ciocalteu	1.28	1.10	1.74	2.22	2.50	2.25	2.40	2.56	3.13	3.35	3.21	3.33	3.38	3.83	3.57	6.00
standard deviation (n = 3)	*0.13*	*0.17*	*0.18*	*0.31*	*0.04*	*0.26*	*0.98*	*0.31*	*0.51*	*0.18*	*0.31*	*0.28*	*0.25*	*0.04*	*0.15*	*0.26*
paired *t*-test	$t = 1.34$ with 44.0 degrees of freedom ($P = 0.1876$)															

[a] Values are given in grams per kilograms of fresh weight. GD: Golden Delicious; JD: Judor; GU: Guillevic; PJ: Petit Jaune; BR: Binet Rouge; JL: Juliana; CL: Clozette; AV: Avrolles; DM: Dous Moen; AN: Antoinette; BD: Bedan; DA: Dabinett; DC: Douce Coët Ligné; CJ: Chevalier Jaune; KE: Kermerrien; JR: Jeanne Renard.

Table 2. Concentration (mg/kg of Fresh Matter) of Phenolic Compounds in the Cortex of Apple Varieties[a]

	CAT		EC		PC		CA		CQ		XPL		PL	
GD	14	*0*	88	*9*	761	*133*	132	*9*	20	*0*	11	*2*	15	*2*
JD	20	*2*	86	*12*	515	*38*	338	*45*	59	*4*	10	*0*	16	*0*
GU	*t*		*t*		1066	*170*	465	*3*	134	*1*	19	*0*	19	*0*
PJ	27	*3*	150	*8*	1372	*96*	415	*100*	29	*8*	12	*3*	16	*4*
BR	39	*1*	202	*12*	1254	*104*	601	*70*	176	*12*	14	*1*	37	*0*
JL	23	*3*	131	*6*	1551	*78*	522	*47*	72	*3*	97	*11*	25	*3*
CL	44	*5*	307	*41*	1144	*189*	832	*145*	84	*12*	12	*2*	28	*5*
AV	*t*		*t*		2424	*250*	154	*22*	104	*11*	55	*14*	25	*4*
DM	95	*8*	313	*26*	1528	*301*	967	*277*	100	*23*	21	*4*	18	*4*
AN	61	*4*	405	*34*	1902	*137*	641	*77*	36	*4*	13	*1*	42	*2*
BD	154	*6*	473	*16*	1796	*107*	649	*34*	147	*6*	12	*0*	30	*3*
DA	33	*3*	393	*49*	2417	*555*	390	*57*	53	*8*	22	*2*	102	*6*
DC	117	*33*	376	*59*	1499	*218*	1195	*121*	80	*10*	98	*7*	68	*8*
CJ	41	*2*	556	*28*	2370	*303*	767	*61*	37	*2*	31	*8*	30	*4*
KE	45	*2*	434	*13*	2932	*350*	917	*110*	95	*17*	35	*4*	48	*6*
JR	54	*11*	1410	*37*	4731	*289*	666	*57*	50	*15*	39	*3*	43	*6*

[a] GD: Golden Delicious; JD: Judor; GU: Guillevic; PJ: Petit Jaune; BR: Binet Rouge; JL: Juliana; CL: Clozette; AV: Avrolles; DM: Dous Moen; AN: Antoinette; BD: Bedan; DA: Dabinett; DC: Douce Coët Ligné; CJ: Chevalier Jaune; KE: Kermerrien; JR: Jeanne Renard; CAT: (+)-catechin; EC: (−)-epicatechin; PC: procyanidins; CA: chlorogenic acid; CQ: *p*-coumaroylquinic acid; XPL: phloretin xyloglucoside; PL: phloridzin. *t*: traces. Italic values correspond to standard deviation ($n = 3$).

Table 3. Characterization of Procyanidins According to Their Constitutive Units and Their Average Degrees of Polymerization (DP*n*)[a]

	GD	JD	GU	PJ	BR	JL	CL	AV	DM	AN	BD	DA	DC	CJ	KE	JR
ECe (%)	85.2	77.6	97.5	84.4	82.9	86.6	78.8	98.0	78.7	79.2	81.8	80.2	76.4	79.2	82.6	78.1
standard deviation (n = 3)	*1.7*	*0.9*	*0.3*	*1.4*	*2.0*	*1.1*	*1.6*	*0.2*	*3.1*	*0.8*	*2.3*	*2.0*	*1.5*	*2.3*	*0.6*	*1.4*
ECt (%)	11.7	18.4	2.3	11.6	12.1	12.7	15.8	1.8	13.9	14.7	9.8	16.6	14.7	16.9	13.9	18.5
standard deviation (n = 3)	*1.3*	*0.2*	*0.3*	*1.1*	*1.3*	*0.9*	*1.6*	*0.2*	*2.1*	*0.4*	*1.5*	*2.0*	*1.1*	*2.3*	*0.6*	*1.3*
CTt (%)	3.1	4.0	0.2	4.0	5.0	0.7	5.4	0.2	7.4	6.1	8.4	3.1	8.9	3.9	3.5	3.4
standard deviation (n = 3)	*0.6*	*0.8*	*0.0*	*0.3*	*0.7*	*0.2*	*0.1*	*0.0*	*1.0*	*0.5*	*0.8*	*0.1*	*0.5*	*0.2*	*0.2*	*0.1*
DP*n*	6.8	4.5	40.7	6.5	5.9	7.5	4.7	50.3	4.8	4.8	5.6	5.1	4.2	4.8	5.7	4.6
standard deviation (n = 3)	*0.8*	*0.2*	*5.4*	*0.6*	*0.7*	*0.6*	*0.3*	*5.0*	*0.6*	*0.2*	*0.8*	*0.5*	*0.3*	*0.5*	*0.2*	*0.3*

[a] GD: Golden Delicious; JD: Judor; GU: Guillevic; PJ: Petit Jaune; BR: Binet Rouge; JL: Juliana; CL: Clozette; AV: Avrolles; DM: Dous Moen; AN: Antoinette; BD: Bedan; DA: Dabinett; DC: Douce Coët Ligné; CJ: Chevalier Jaune; KE: Kermerrien; JR: Jeanne Renard ECe: (−)-epicatechin as extension unit; ECt: (−)-epicatechin as terminal unit; CTt: (+)-catechin as terminal unit; DP*n*: average degree of polymerization.

1986). Moreover, it is noteworthy that grape proanthocyanidins are mainly located in seeds and skins whereas apple proanthocyanidins are distributed in the entire fruit (Mayr et al., 1995; Guyot et al., 1998), a large proportion being in the cortex. Bitter varieties (Kermerrien, Chevalier Jaune, and Jeanne Renard) showed higher contents in proanthocyanidins than sharp varieties, which fits with previous work dealing with the implication of these compounds in the bitterness and astringency of ciders (Lea, 1990b). However, the sharp Avrolles variety is an exception considering its relatively high proanthocyanidin concentration.

The structure of apple proanthocyanidins showed a great homogeneity for all varieties on the basis of the nature of the constitutive flavan-3-ol units (Table 3). They corresponded mainly to (−)-epicatechin (>90% of total units) with a small proportion of (+)-catechin as terminal units. So, proanthocyanidins of cider apple are essentially procyanidins, as largely mentioned in the literature (Spanos et al., 1990; Lea, 1990b; Nicolas et al., 1994). The proportions of (−)-epicatechin and (+)-catechin as terminal units are noticeably variable from one variety to another. Terminal (−)-epicatechin is always predominant. However, some varieties (Dous Coët Ligné, Dous Moen, and Bedan) showed relatively high proportions of terminal (+)-catechin (7−9% of total units). These percentages may be slightly overestimated because of the epimerization reaction occurring under the conditions of thiolysis (Prieur et al., 1994). These structural differences may be important in the phenolic composition of raw materials because the properties of proanthocyanidins largely depend on the nature of their constitutive units (Haslam, 1974). With the exception of Avrolles and Guillevic varieties, the procyanidins average degrees of polymerization (DP*n*) ranged between 4.2 and 7.5 (Table 3). On this criterion, Guillevic and Avrolles differed markedly from the other varieties showing an exceptional DP*n* of 40 and 50, respectively, which corresponded to a number average molecular weight (M_n) close to 11−15 kDa. The evidence of highly

polymerized proanthocyanidins has been already mentioned in apples (Guyot et al., 1997, 1998; Ohnishi-Kameyama et al., 1997), in grape seeds (Prieur et al., 1994) and skin (Souquet et al., 1996), and other plants (Foo and Porter, 1980). Nevertheless, as far as we know, such a high DP*n* has never been observed for apple procyanidins. These polymerized compounds may not be quantitatively dissolved in apple juices because of their associations with the insoluble part of the fruits when fruits are processed. However, when purified, polymeric procyanidin fractions are soluble at ambient temperature in slightly acidic aqueous media at a concentration close to 0.5 g L^{-1} (unpublished results). Therefore, it cannot be excluded that a small proportion of these compounds can be dissolved in apple musts when fruits are processed. The DP*n* of procyanidins may be an important structural feature in relation with cider taste and quality. The degree of polymerization is directly involved in the balance of bitterness to astringency which defines the "mouthfeel" and the "body" of ciders (Lea, 1990b). Oligomeric procyanidins (DP 2−5) contribute to bitterness, whereas more polymerized structures (DP 6−10) are more involved in astringency (Lea and Arnold, 1978). Moreover, the presence of polymerized procyanidins in apple-based beverages may be prejudicial because these compounds can interact with proteins and polysaccharides and, therefore, may contribute to the formation of hazes and precipitate during the storage (Siebert, 1996; Beveridge, 1997).

Monomeric catechins were assayed in methanol and aqueous acetone extracts of the cortex by HPLC without prior thiolysis to differentiate them from the flavan-3-ol terminal units of procyanidins. Except for Avrolles and Guillevic which were almost devoid of compounds of this class, monomeric catechins accounted for 6−21% of total polyphenol content depending on the variety (Table 2). The lowest concentration was observed for Golden Delicious (0.1 g/kg of fresh matter) and the highest for Jeanne Renard (1.4 g/kg of fresh matter). Data obtained for monomeric catechins in the Golden variety were consistent with previous reports (Mosel and Herrmann, 1974; McRae et al., 1990). Together with hydroxycinnamic acids, monomeric catechins are the preferential substrates of polyphenoloxidase. Moreover, catechins are largely implicated in the formation of brown oxidation products (Nicolas et al., 1994). Therefore, it was not surprising to observe low catechin concentrations in dessert varieties, partly selected on the basis of their poor sensibility to enzymatic browning affecting the commercial value of these fruits. This is different for cider varieties because a yellow-orange color may be beneficial to cider quality. Considering individual compounds of this class, only (−)-epicatechin and (+)-catechin were found in the polyphenolic constitution of the cider apple varieties, the latter always being in lower concentration as already mentioned in the literature (Lea, 1990a). However, the (−)-epicatechin/(+)-catechin ratio varied from 3 to 26 depending on the considered variety. On the whole, bitter varieties (Kermerrien, Chevalier Jaune, and Jeanne Renard) showed a high (−)-epicatechin/(+)-catechin ratio.

Hydroxycinnamic acids corresponded to the second polyphenol class (the first being procyanidins) in apple cortex with the exception of Jeanne Renard variety which showed a higher monomeric catechin content. They accounted for 9−38% depending on the variety, the highest concentration being observed for Dous Coët Ligné with a value close to 1.2 g/kg of fresh cortex. Considering all varieties, the mean value was about 0.7 g/kg, which is relatively high in comparison with the hydroxycinnamic acid levels previously mentioned in the literature for dessert apples (Nicolas et al., 1994). Besides, the hydroxycinnamic acid concentration for Golden Delicious was close to 0.15 g/kg of fresh cortex, which was consistent with previous data concerning this variety at maturity (Mosel and Herrmann, 1974; McRae et al., 1990; Amiot et al., 1992). As a comparison, a conversion of the values (expressed relative to dry matter by these authors) was made using our own fresh/dry matter ratio for this variety. This relatively low concentration may contribute to limiting the browning sensitivity of this variety because hydroxycinnamic acids are largely involved in enzymatic oxidation reactions. Two compounds of this class were observed on chromatograms: chlorogenic acid and *p*-coumaroylquinic acid. No distinction was made between the different isomeric forms of these compounds. Chlorogenic acid was predominant for all varieties. However, the chlorogenic acid/*p*-coumaroylquinic acid ratio varied greatly from one variety to another (1.5 for Avrolles and 21 for Chevalier Jaune). This ratio may be important when apple fruits are processed into juices and ciders because chlorogenic acid is considered as the preferential natural substrate of the catecholase activity of polyphenoloxidase, whereas *p*-coumaroylquinic acid might act as a competitive inhibitor of this activity as previously shown for its aglycone (i.e., *p*-coumaric acid) (Janovitz-Klapp et al., 1990). Therefore, the relative concentration of these compounds may have an effect on the oxidation process and color development during cider making. Moreover, these compounds are the precursors of the volatile phenols which may positively contribute to cider flavor when they are present at low concentrations (Lea, 1992). During fermentation, ethylphenol and ethylcatechol may arise from the metabolization of *p*-coumaroylquinic acid and chlorogenic acid, respectively (Beech and Carr, 1977).

For all varieties, dihydrochalcones corresponded to a minor class which accounted for 1−5% of total polyphenols. The Douce Coët Ligné variety showed the highest content (166 mg/kg of fresh cortex), the lowest level being observed for Golden Delicious (26 mg/kg of fresh cortex). This value was in good agreement with previous data obtained for Golden Delicious variety (Burda et al., 1990; McRae et al., 1990; Amiot et al., 1992). As a whole, bittersweet and bitter apples showed high concentration levels, as already observed for English varieties (Lea, 1990a). Phloridzin and phloretin xyloglucoside were the major constituents of this class. Although they are present at low concentrations in raw material, dihydrochalcones might contribute significantly to apple juice and cider quality. When they are present in apple juice together with epicatechin, phloridzin may be involved in the formation of orange oxidation products which account for about one-half of the juice color (Lea, 1984; Oszmianski and Lee, 1991). Moreover, phloridzin and particularly some of its oxidation derived products may contribute to the antioxidant potential of apple products (Ridgway et al., 1996).

For all varieties, no compound of the flavonol class was found in the cortex. In apples, flavonols, which are mainly quercetin esters (Teuber et al., 1978), are essentially located in the peel (Burda et al., 1990; Oleszek et al., 1988; Guyot et al., 1998). Nevertheless,

they may be present in the flesh of some varieties (Perez-Ilzarbe et al., 1991; Mayr et al., 1995). Under particular processing conditions (i.e., diffusion extraction), these compounds may be present in a significant concentration in apple juices (Spanos et al., 1990).

CONCLUSION

Thiolysis−HPLC results which are presented confirm previous works (Matthews et al., 1997; Guyot et al., 1998), showing that this method is well-adapted to quantitative and qualitative analyses of polyphenols of the proanthocyanidin class. The accuracy of the thiolysis−HPLC assays was reinforced by the comparison with the Folin−Ciocalteu data. Moreover, the concentrations of catechins, hydroxycinnamic acid, and dihydrochalcones for the common Golden variety were in good agreement with those previously mentioned in the literature. Therefore, under our conditions, the procedure may be considered as well adapted to the assay of these compounds. The method also allows one to quantify hydroxycinnamic acids and dihydrochalcones. ESI-MS was used to show that, after thiolysis, hydroxycinnamic acids are converted into their corresponding methylated forms. For the 16 apple varieties, results showed a variety of polyphenol cortex composition. Globally, cider varieties showed a higher polyphenol content than the dessert apple Golden Delicious. For all varieties, procyanidins corresponded to the main class of polyphenols, their constitutive units being essentially (−)-epicatechin. For the first time, results permitted evidence of the presence of exceptionally highly polymerized procyanidins as the main polyphenolic constituents of some cider apple varieties.

ABBREVIATIONS USED

CA, chlorogenic acid; EC, (−)-epicatechin; CAT, (+)-catechin; PA, procyanidins; DP*n*, average degree of polymerization; RP-HPLC, reversed-phase high-performance liquid chromatography; ESI-MS, electrospray infusion mass spectrometry.

LITERATURE CITED

Amiot, M. J.; Tacchini, M.; Aubert, S.; Nicolas, J. Phenolic composition and browning susceptibility of various apple cultivars at maturity. *J. Food Sci.* **1992**, *57*, 958−962.

Barker, T. P.; Burroughs, L. F. Cider apple varieties then and now: a survey of vintage-quality trials. In *Science and Fruit*; Wallace, T., Marsh, R. W., Eds.; J. W. Arrowsmith Ltd.: Bristol, 1953; pp 45−67.

Beech, F. W.; Carr, J. G. Cider making. In *Economic Microbiology, Vol. 1—Alcoholic Beverages*; Rose, A. H., Harrison, J. S., Eds.; Academic Press: London, U.K., 1977; pp 139−313.

Beveridge, T. Haze and Cloud in apple juices. *Crit. Rev. Food Sci.* **1997**, *37*, 75−91.

Bourzeix, M.; Weyland, D.; Heredia, N. Etude des catéchines et des procyanidols de la grappe de raisin, du vin et d'autres dérivés de la vigne. *Bull. O.I.V.* **1986**, *669*, 1171−1253.

Brugirard, A.; Tavernier J. Les matières tannoïdes dans les cidres et les poirés. *Ann. Technol. Agric.* **1952**, *3*, 311−343.

Burda, S.; Oleszek, W.; Lee, C. Y. Phenolic compounds and their changes in apples during maturation and storage. *J. Agric. Food Chem.* **1990**, *38*, 945−948.

Delage, E.; Bohuon, G.; Baron, A.; Drilleau, J. F. High-performance liquid chromatography of the phenolic compounds in the juice of some french cider apple varieties. *J. Chromatogr.* **1991**, *555*, 125−136.

Foo, L. Y.; Porter, L. J. The phytochemistry of proanthocyanidin polymers. *Phytochemistry* **1980**, *19*, 1747−1754.

Guyot, S.; Doco, T.; Souquet, J. M.; Moutounet, M.; Drilleau, J. F. Characterisation of highly polymerised procyanidins in cider apple (*Malus silvestris* var. Kermerrien) skin and pulp. *Phytochemistry* **1997**, *44*, 351−357.

Guyot, S.; Marnet, N.; Laraba, D.; Sanoner, P.; Drilleau J.-F. 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*). *J. Agric. Food Chem.* **1998**, *46*, 1698−1705.

Haslam, E. Polyphenol-protein interactions. *Biochem. J.* **1974**, *139*, 285−288.

Janovitz-Klapp, A. H.; Richard, F. C.; Goupy, P. M.; Nicolas, J. J. Inhibition studies on apple polyphenol oxidase. *J. Agric. Food Chem.* **1990**, *38*, 926−931.

Lea, A. G. H. Reversed-phase high-performance liquid chromatography of procyanidins and other phenolics in fresh and oxidising apple juices using a pH shift technique. *J. Chromatogr.* **1982**, *238*, 253−257.

Lea, A. G. H. Farb- und gerbstoffe in englischen mostapfeln. *Flüssiges Obst* **1984**, *8*, 356−361.

Lea A. G. H. Apple juice. In *Production and packaging of non-carbonated fruit juices and fruit beverages*; Hicks, D., Ed.; Van Nostrand Reinhold: New York, 1990a; pp 182−225.

Lea, A. G. H. Bitterness and astringency: the procyanidins of fermented apple ciders. In *Bitterness in food and beverages*; Roussef, R. L., Ed.; Elsevier: Oxford, New York, 1990b; Chapter 7, pp 123−143.

Lea, A. G. H. Flavor, color, and stability in fruit products: the effect of polyphenols. In *Plant Polyphenols. Synthesis, Properties, Significance*; Hemingway, R. W., Laks, P. E., Eds.; Plenum Press: London, U.K., 1992; pp 827−848.

Lea, A. G.; Arnold, G. M. The phenolics of ciders: bitterness and astringency. *J. Sci. Food Agric.* **1978**, *29*, 478−483.

McRae, K. B.; Lidster, P. D.; DeMarco, A. C.; Dick, A. Comparison of the polyphenol profiles of apple fruits cultivars by correspondence analysis. *J. Sci. Food Agric.* **1990**, *50*, 329−342.

Matthews, S.; Mila, I.; Scalbert, A.; Pollet, C.; Lapierre, C.; Hervé du Penhoat, C. L. M.; Rolando, C.; Donnelly, D. M. X. Method for estimation of proanthocyanidins based on their acid depolymerization in the presence of nucleophiles. *J. Agric. Food Chem.* **1997**, *45*, 1195−1201.

Mayr, U.; Treutter, D., Santos-Buelga, C.; Bauer, H.; Feucht, W. Developmental changes in the phenol concentrations of Golden delicious apple fruits and leaves. *Phytochemistry* **1995**, *38*, 1151−1155.

Mosel, H. D.; Herrmann, K. Changes in catechins and hydroxycinnamic acid derivatives during development of apples and pears. *J. Sci. Food Agric.* **1974**, *425*, 251−256.

Nicolas, J.-J.; Richard-Forget, F. C.; Goupy, P. M.; Amiot, M.-J.; Aubert, S. Y. Enzymatic browning reaction in apple and apple products. *Crit. Rev. Food Sci. Nutr.* **1994**, *34*, 109−157.

Ohnishi-Kameyama, M.; Yanagida, A.; Kanda, T.; Nagata, T. Identification of catechin oligomers from apple (*Malus pumila* cv. Fuji) in matrix-assisted laser desorption/ionization time of flight mass spectrometry and fast-atom bombardment mass spectrometry. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 31−36.

Oleszek, W.; Lee, C. Y.; Jaworsky, A.; Price, K. R. Identification of some phenolic compounds in apples. *J. Agric. Food Chem.* **1988**, *29*, 277.

Oszmianski, J.; Lee, C. Y. Enzymatic oxidation of phloretin glucoside in model system. *J. Agric. Food Chem.* **1991**, *39*, 1050−1052.

Perez-Ilzarbe, J.; Hernandez, T.; Estrella, I. Phenolic Compounds in Apples—Varietal Differences. *Z. Lebensm. Unters. Forsch.* **1991**, *192*, 551−554.

Picinelli, A.; Suarez, B.; Mangas, J. J. Analysis of polyphenols in apple products. *Z. Lebensm. Unters. Forsch.* **1997**, *204*, 48−51.

Poon, G. K. Analysis of catechins in tea extracts by liquid chromatography-electrospray ionization mass spectrometry. *J. Chromatogr. A* **1998**, *794*, 63−74.

Prieur, C.; Rigaud, J.; Cheynier, V.; Moutounet, M. Oligomeric and polymeric procyanidins from grape seeds. *Phytochemistry* **1994**, *36*, 781−784.

Ridgway, T.; Oreilly, J.; West, G.; Tucker, G.; Wiseman, H. Potent antioxidant properties of novel apple-derived flavonoids with commercial potential as food-additives. *Biochem. Soc. Trans.* **1996**, *24*, 391.

Rigaud, J.; Perez-Ilzarbe, J.; Ricardo da Silva, J. M.; Cheynier, V. Micromethod for identification of proanthocyanidin using thiolysis monitored by high-performance liquid chromatography. *J. Chromatogr.* **1991**, *40*, 401−405.

Scalbert, A. Quantitative methods for the estimation of tannins in plant tissues. In *Plant polyphenols. synthesis, properties and significance*; Hemingway, R. W., Laks, P. E., Eds.; Plenum Press: London, 1992; p 269.

Siebert, K. J. Formation of protein-polyphenol haze in beverages. *J. Agric. Food Chem.* **1996**, *44*, 1997−2005.

Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144−158.

Souquet, J. M.; Cheynier, V.; Brossaud, F.; Moutounet, M. Polymeric proanthocyanidins from grape skins. *Phytochemistry* **1996**, *43*, 509−512.

Spanos, G. A.; Wrolstad, R. E.; Heatherbell, D. A. Influence of storage on the composition of apple juice. *J. Agric. Food Chem.* **1990**, *38*, 1572−1579.

Tavernier, J.; Jacquin, P. Etude technologique de variétés de pomme à cidre. *Revue interne du Groupement National Interprofessionnel des fruit à cidre et dérivés*, 1949; Paris, 1; p 36.

Teuber, H.; Wünscher, G.; Herrmann, K. Flavonolglykoside der apfel (Malus silvestris Mill). *Z. Lebensm. Unters. Forsch.* **1978**, *166*, 81−84.

Thompson, R. S.; Jacques, D.; Haslam, E.; Tanner, R. N. J. Plant proanthocyanidins. Part I. Introduction; the isolation, structure, and distribution in nature of plant procyanidins. *J. Chem. Soc., Perkin Trans. 1* **1972**, 1387−1399.

Van Buren, J. Fruit phenolics. In *The biochemistry of fruits and their products*; Hulmes, A. C. Ed.; Academic Press: London, 1970; Vol. 1, pp 269−304.

Whiting, G. C. Some biological and flavour aspects of lactic acid bacteria in ciders and other alcoholic bevereages. In *Lactic acid bacteria in bevereages and food*; Carr, J. G., Cutting, C. V., Whiting, G. C., Eds.; Academic Press: London, 1975; pp 69−85.

Whiting, G. C.; Coggins, R. A. Estimation of the monomeric phenolics of ciders. *J. Sci. Food Agric.* **1975**, *26*, 1833−1838.

Received for review May 27, 1999. Revised manuscript received September 2, 1999. Accepted September 10, 1999. We are grateful to the “Comité des Fruits à Cidre”, the “Region Basse-Normandie”, and the “Association Pomme 56” for the provision of apple fruits and financial support.

JF990563Y