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ScienceDirect
Phytochemistry 68 (2007) 1128–1138
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Concentrations and characteristics of procyanidins and other phenolics in apples during fruit growth

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Received 10 November 2006; received in revised form 12 February 2007

Abstract

Apples (*Malus domestica* Borkh.) of two table and two cider cultivars were collected during fruit growth and maturation from the end of cell proliferation. Concentrations of flavonoids (flavan-3-ols, dihydrochalcones and flavonols) in the fruit flesh decreased sharply between circa 35 and circa 100 days after flowering. For hydroxycinnamic acids, the decrease appeared slower. In a second experiments apples of the cider cultivars Kermerrien and Avrolles were sampled every 2 weeks from 40 days after flowering to overripeness for a detailed characterisation of polyphenol accumulation kinetics in the fruit flesh. Most polyphenol synthesis had occurred at 40 days after full bloom, though it persisted at a low (Kermerrien) to very low (Avrolles) level during all the fruit growth. All qualitative characteristics of the polyphenols were remarkably stable. The degree of polymerisation of the procyanidins increased slightly in Avrolles and decreased in Kermerrien. This was accompanied by a relative increase in procyanidin B2, while size-exclusion chromatography of Kermerrien polyphenol extracts showed the disappearance of a highly polymerised fraction.

Keywords: *Malus domestica* Borkh.; Flavonoid; Condensed tannins; Molecular weight; Catechin; Dihydrochalcone; Hydroxycinnamic acid

1. Introduction

Apple polyphenols are of interest due to their health potential as anti-oxidants but also to their organoleptic and functional properties in ciders. There is a fair body of data on their occurrence and concentrations in ripe apple fruits (a.o. Amiot et al., 1992; Sanoner et al., 1999; Podsedek et al., 2000; Alonso-Salces et al., 2001, 2004; Napolitano et al., 2004; Vrhovsek et al., 2004; Thielen et al., 2004; Kahle et al., 2005). Cider apples contain the same polyphenols as table apples, but the concentrations are generally much higher (Guyot et al., 1998, 2003; Sanoner et al., 1999; Alonso-Salces et al., 2001, 2004; Thielen et al., 2004). The main classes of polyphenols in apple flesh are procyanidins, hydroxycinnamic acids, monomeric flavan-3-ols and dihydrochalcones. Flavonols and anthocyanins are found almost exclusively in the peels. Procyanidins of apple fruits are of the B-type i.e. the flavan-3-ols are joined by C4–C6 or C4–C8 bonds (Shoji et al., 2003). They are mostly constituted of (−)-epicatechin, which can be present as extension units or as terminal units; (+)-catechin is exclusively present as terminal units. The average size of these oligomers or polymers at maturity is a varietal characteristic (Sanoner et al., 1999; Guyot et al., 2002; Alonso-Salces et al., 2004) which varies little with maturity, mode of cultivation, harvest year or storage (Guyot et al., 2002, 2003). The degree of polymerisation of procyanidins is closely related to their organoleptic properties: procyanidins with degrees of polymerisation higher than 6–8 are predominantly astringent while the smaller oligomers are predominantly bitter (Lea and Arnold,

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doi:10.1016/j.phytochem.2007.02.012

1978; Vidal et al., 2003). The hydroxycinnamic acids are represented mostly by caffeoylquinic acid and *p*-coumaroylquinic acid, and the dihydrochalcones by phloretin glucoside (phloridzin) or xyloglucoside.

Less is known on the variation of polyphenol concentrations during apple growth. Mosel and Herrmann (1974) found large quantities of hydroxycinnamic acids and catechins in the early fruit, and a decrease of their concentrations during growth. Mayr et al. (1996; reported by Treutter, 2001) observed that changes in the concentrations of individual polyphenols in the flesh and skin of apples (up to 120 days after full bloom) do not run parallel. Hamauzu et al. (1999) showed a sharp decline in phenolic concentrations during the early phases of fruit growth, while the evolution of the contents per fruit of individual compounds differed. Kondo et al. (2002), studying both skin and flesh of table and cider apples, found the same decrease of concentrations in the flesh during fruit growth. Recently, most interest has been devoted to the polyphenols in apple skin, and in particular to the anthocyanins as the red colour is an important factor of "outer quality" of red and bicoloured fruits (Lister et al., 1994, 1996; Awad et al., 2000; Treutter, 2001; Honda et al., 2002; Kim et al., 2003; Takos et al., 2006; Ubi et al., 2006). Takos et al. (2006) showed two stages of accumulation of anthocyanins, early in the fruit life development then at maturation. They also found the highest concentration for flavonols in their earliest samples (32 days after full bloom), declining throughout fruit growth and maturation. In contrast, little is known about procyanidins, though they are quantitatively the main class of polyphenols in apples. Takos et al. (2006) found a decrease in procyanidin concentrations and degree of polymerisation in the fruit skin during growth and maturation.

Recent advances on the biosynthesis of polyphenols have led to the identification of most relevant enzymes. Treutter (2001) indicated that activity of selected enzymes of flavonoid biosynthesis is the highest during the cell division phase of apples. Particular attention was paid to genes of anthocyanin synthesis in the skin (Honda et al., 2002; Kim et al., 2003; Takos et al., 2006). Transcripts of the common flavonoid genes are abundant at two phases, early in development (synthesis of anthocyanins and procyanidins), and at fruit ripening (synthesis of anthocyanin). The procyanidins themselves can derive from two pathways (Marles et al., 2003): action of leucoanthocyanidin reductase (LAR) (Tanner et al., 2003), or of anthocyanidin reductase (ANR) (Xie et al., 2003). Genes encoding for one ANR and 2 LARs have been identified in apple fruit skin (Takos et al., 2006). Transcripts for these genes are differently expressed both in time and location (sunny versus shady side). However, the enzyme which catalizes the polymerisation reaction remains unknown and control of the degree of polymerisation is still obscure.

The evolution of polyphenol concentrations were compared in the skin and flesh of two cider and two table apples, taking into account the stages of fruit development (cell proliferation versus cell expansion). Thereafter, in order to clarify the respective importance of dilution and *de novo* synthesis in the evolution of polyphenol concentrations, a detailed time course was determined on two cultivars of cider apples, which have high concentrations of procyanidins and differ by their degrees of polymerisation. As it is generally recognised that fruits become less astringent upon maturation, we were interested to see if this could be linked to a change in procyanidin concentration or size.

2. Results and discussion

2.1. Growth patterns of cider and table apples

During the first series of experiments, apples from cultivars Elstar, Gala (table apples), Dous Moen and Kermerrien (cider apples) were sampled at 4 or 5 stages of growth for biochemical analysis: end of cell multiplication (circa 35 days after full bloom), two points during the cell growth phase and at maturity (two maturity stages for table apples, corresponding to harvest for conservation and harvest for short term storage). The global growth patterns of the fruits are described in Table 1: the apples were in the linear growth phase from the second sampling, and average fruit weight showed no plateau, except for cv Dous Moen. The average fruit weight of both cider apples was much lower. The dry matter content was measured on the flesh only and thus reflects the actual water content of the fruit, not differences in skin/flesh ratios due to differences in fruit sizes. It showed a slight increase during growth and was on average higher in the cider apples. Starch contents and their evolutions were also comparable in all four cvs, with accumulation during the first 2–3 months followed by disappearance close to maturity. The cell walls concentration (related to dry matter) decreased continuously during fruit growth, they were slightly higher in cider apples. The most marked difference occurred between circa 40 days and 60 days, when there was a marked decrease only for table apples.

Percy et al. (1997) showed a stable amount of cell wall material during the second and third months after full bloom and then a decrease during the two following months. The authors suggested that this decrease during the second growth phase of the fruits may be due to the large expansion of the cells and intercellular air spaces at this stage. However, Harada et al. (2005), showed recently that, besides differences in cell size, great differences in cell numbers 30 days after full bloom, explained the variability of fruit size among 5 *Malus* species.

The observation of cellular structures (Fig. 1, results shown only for cvs Elstar and Dous Moen) suggested that the main differences in cell numbers and sizes between cider and table apples occurred during the first stage of development. At 22 days after full bloom, parenchyma of cvs Dous Moen (and Kermerrien) had noticeably smaller cells. At the same date, a great number of table apple cells seemed to have begin their enlargement phase, whereas a similar

Table 1
Growth patterns and evolution of dry matter, starch and cell wall contents of apples of cv Elstar, Gala, Dous Moen and Kermerrien

Cultivar and DAFB	Average fruit weight (g)	Dry matter concentration (g/g fresh weight)	Starch concentration (mg/g dry matter)	Cell wall concentration (mg/g dry matter)
Elstar				
42		0.131[a]	3[a]	295[a]
70	54[a]	0.132[b]	119[b]	173[b]
93	95[a]	0.151[b]	138[b]	142[b]
126	168[a]	0.165[b]	86[b]	101[b]
135	208[a]	0.161[b]	22[b]	92[b]
Gala				
44		0.136[a]	4[a]	309[a]
72	48[a]	0.136[b]	114[b]	235[b]
95	91[a]	0.148[b]	139[b]	170[b]
137	160[a]	0.152[b]	60[b]	110[b]
146	213[a]	0.156[b]	8[b]	97[b]
Dous Moen				
35	2[a]	0.161[a]	2[a]	283[a]
70	19[a]	0.149[b]	53[b]	296[b]
112	51[a]	0.175[b]	90[b]	213[b]
147	57[a]	0.178[b]	2[b]	150[b]
Kermerrien				
44	5[a]	0.165[a]	2[a]	255[a]
72	33[a]	0.179[b]	112[b]	234[b]
121	91[a]	0.209[b]	175[b]	211[b]
142	113[a]	0.221[b]	92[b]	163[b]

DAFB: days after full bloom.
[a] Data on whole fruit.
[b] Data on fruit flesh.

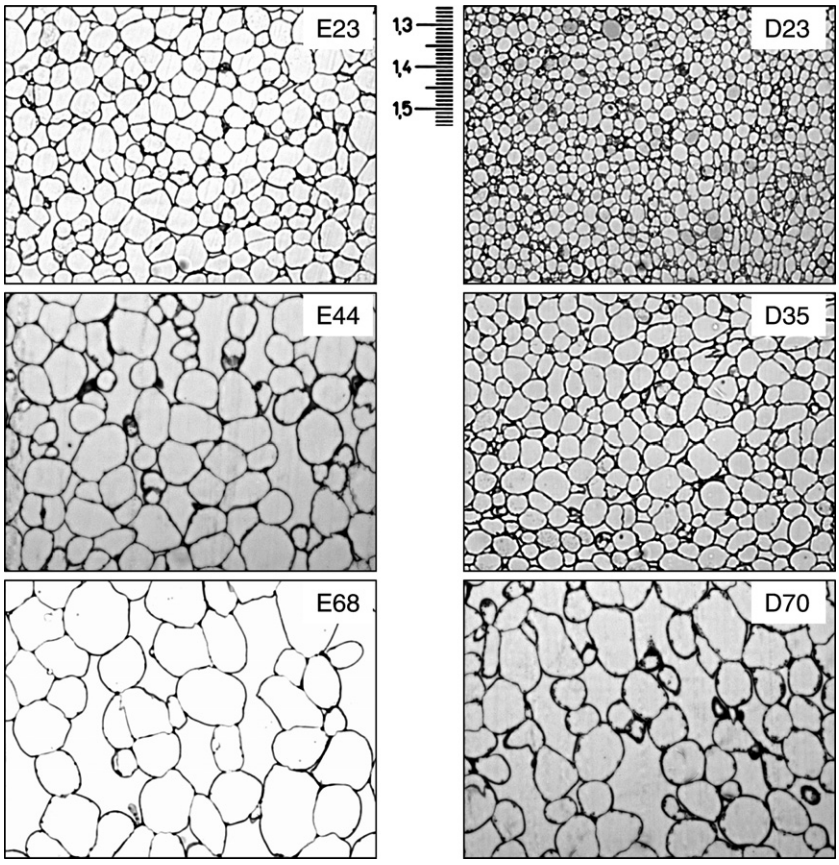

Fig. 1. Histological images of Elstar and Dous Moen apple tissues at different days after full bloom indicated by the number at right top of each slide. E: Elstar, D: Dous Moen. Scale bar: the finest division equal 0.01 mm.

structure, with larger cells, was reached only 10 to 15 days later for Dous Moen and Kermerrien. At 70 days after full bloom, differences were reduced though cider apples seemed to have greater heterogeneity of cellular sizes. Formation of intercellular spaces was precocious and quite similar for both groups of cultivars. This suggests that cell enlargement begins later in cider apples, and the differences in cell wall content between cider and table apples seem to be linked to different cell sizes.

During 2001 we focused on the evolution of the polyphenols in the flesh of two cider apple cultivars, Kermerrien and Avrolles, the last being chosen because it presents procyanidins of very high number average degree of polymerisation. Fruits were collected every two weeks during the linear growth phase (Fig. 2). For cv Avrolles the collection was continued for another 2 months while the fruit increase had slowed. During this period, the dry matter content was stable for cv Avrolles, and increased slightly at the beginning for cv Kermerrien.

2.2. Evolution of polyphenols in flesh and skin of cider and table apples

Table 2 shows the evolution of the concentrations of the different polyphenol classes. At the first stage (end of cell division) whole fruits were analysed and afterwards the skin and flesh were treated separately. Kermerrien and Dous Moen apples had generally much higher polyphenol contents.

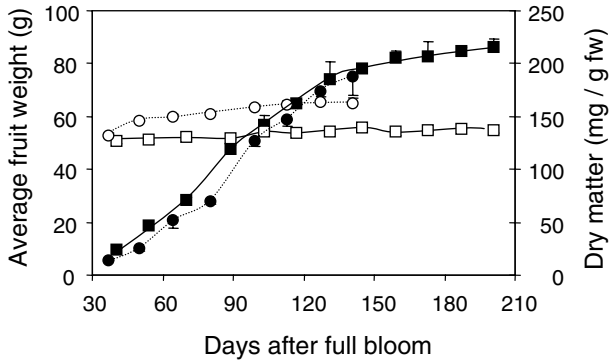

Fig. 2. Growth kinetics of the Kermerrien and Avrolles apples: evolution of average fruit weight and dry matter contents. ■: Avrolles, fruit weight; □: Avrolles, fruit dry matter content; ●: Kermerrien, fruit weight; ○: Kermerrien, fruit dry matter content.

The concentrations were highest at the first stage of collection except for flavonols in the skins of table apples. The concentrations of flavan-3-ols were higher in the skin than in the flesh, as expected (Guyot et al., 1998; Thielen et al., 2004). They had reached a stable low level in cv Elstar and in the flesh of cv Gala at 70 days after full bloom. In the skin and flesh of cvs Kermerrien and Dous Moen the concentrations also decreased between the second and third stages of collection. The degrees of polymerisation were

Table 2
Concentrations of phenolic compounds (in mg/g dry matter) of skin and flesh of apples of cv Elstar, Gala, Dous Moen and Kermerrien during growth

Cultivar and DAFB	Flavan-3-ols (DP)		Caffeoylquinic acid		*p*-Coumaroylquinic acid		Dihydrochalcones		Flavonols	
	Flesh	Skin	Flesh	Skin	Flesh	Skin	Flesh	Skin	Flesh	Skin
Elstar										
42	33.1 (4)[a]		2.4[a]		0.63[a]		2.1[a]		nd	
70	8.1 (3)	16.3 (4)	0.7	0.20	0.30	0.21	0.24	0.7	0.04	0.9
93	4.2 (3)	21.7 (4)	0.4	0.17	0.19	0.19	0.17	0.9	0.02	1.8
126	4.5 (3)	18.4 (5)	0.4	0.04	0.16	0.09	0.09	0.6	0.09	1.1
135	3.0 (3)	12.9 (4)	0.3	0.00	0.12	0.04	0.11	0.4	0.01	1.4
Gala										
44	64.5 (5)[a]		10.1[a]		2.4[a]		2.9[a]		0.8[a]	
72	6.0 (4)	38.9 (4)	1.5	0.10	0.3	0.05	0.3	1.0	0.11	2.5
95	7.6 (3)	28.3 (4)	2.1	0.88	0.5	0.19	0.3	0.8	0.02	2.8
137	6.6 (3)	15.2 (4)	2.0	0.33	0.3	0.06	0.3	0.4	0.04	1.3
146	6.5 (4)	19.8 (4)	2.6	0.40	0.3	0.05	0.3	0.7	0.02	3.2
Dous Moen										
35	145.0 (5)[a]		37.0[a]		3.6[a]		26.2[a]		7.7[a]	
70	51.2 (3)	82.2 (4)	19.4	13.7	2.9	3.0	0.9	3.6	0.08	3.0
112	14.5 (3)	46.9 (4)	5.6	5.6	0.7	1.2	0.09	2.6	0.02	2.1
147	13.7 (3)	32.1 (4)	5.4	2.8	0.6	0.6	0.03	1.6	0.00	1.4
Kermerrien										
44	215.0 (7)[a]		41.0[a]		4.0[a]		9.6[a]		2.9[a]	
72	91.4 (4)	180.2 (7)	16.2	13.8	1.8	1.8	1.2	1.7	0.14	5.1
121	34.4 (4)	65.3 (5)	6.2	3.6	0.5	0.3	0.3	1.0	0.07	3.3
142	32.7 (3)	50.4 (5)	6.9	3.9	0.5	0.3	0.3	0.7	0.06	2.4
SEM	*3.2 (0.3)*		*1.5*		*0.2*		*0.2*		*0.04*	

DAFB: days after full bloom. DP: number average degree of polymerisation (all flavan-3-ols); SEM: standard error of the mean; nd: not done.
[a] Data on whole fruit.

higher in the skin than in the flesh; they were stable in the flesh and decreased slightly in the skin and for cv Kermerrien during the fruit development. For caffeoylquinic acid and *p*-coumaroylquinic acid the concentrations in the skin and flesh were similar in the cider cultivars, but lower in the skin for the table cultivars. Here also a sharp decrease of the concentrations was seen between the first and second stages of sampling for cvs Elstar and Gala, while a substantial concentration was still present in the cider apples and the decrease continued until the third stage of sampling for cvs Kermerrien and Dous Moen. This can be related to the delay observed in cell expansion (Fig. 1) for cider apples, and the higher expression of enzymes of flavonoid biosynthesis during cell division (Treutter, 2001). For dihydrochalcones, there was a decrease between the first and second stages of collection followed by a plateau. The concentrations were higher in the cider apples, and in the skins. Flavonols were absent or only at the trace level in the flesh of all apples, but in much higher concentrations in the skins. Their concentrations decreased in the cider apples but increased in the table apples. This was also reported by Mayr et al. (1995) in the skin of Golden Delicious apples: maximal flavonol concentrations were obtained during the third month after full bloom.

2.3. Evolution of hydroxycinnamic acids and dihydrochalcones in the flesh of Avrolles and Kermerrien apples

The evolution of hydroxycinnamic acids concentrations (Fig. 3) were similar to those observed above: there was a continuous decrease in the concentration, faster at the earlier stages then a relative stabilisation. For Kermerrien the amount per fruit increased little between 37 and 140 days after full bloom; in Avrolles contents per fruit were stable throughout the period of measurement. The ratios of caffeoylquinic acid to *p*-coumaroylquinic acid were remarkably stable (6 in Kermerrien, 1.5 in Avrolles). The dihydrochalcones (Fig. 4) showed a sharper decrease in concentration, and in Avrolles there actually was a decrease in the amount per fruit between 40 and 70 days after full bloom. The amount per fruit was stable for Kermerrien. The phloridzin to phloretin xyloglucoside ratio was stable during the whole period for Avrolles (close to 0.6), but showed a sharp decrease at the initial stages for Kermerrien (from 12 to 2). There might thus have been interconversion from the glucoside to the xyloglucoside in those early stages.

2.4. Evolution of flavan-3-ols in the flesh of Avrolles and Kermerrien apples

The evolution of procyanidins (Fig. 5) showed clearly a dilution effect linked to fruit growth, visible when the polyphenol concentrations were expressed relative to dry matter or fresh weight. However, expressed per fruit, the procyanidin content did not vary much: each Avrolles apple contained on average 210 mg of procyanidins at 40 days after full bloom, and 240 mg at 200 days after full bloom. For Kermerrien the biosynthesis was active longer: the amount per fruit doubled between 40 and 60 days after full bloom, but there were no significant quantitative evolutions later. The evolution of the degrees of polymerisa-

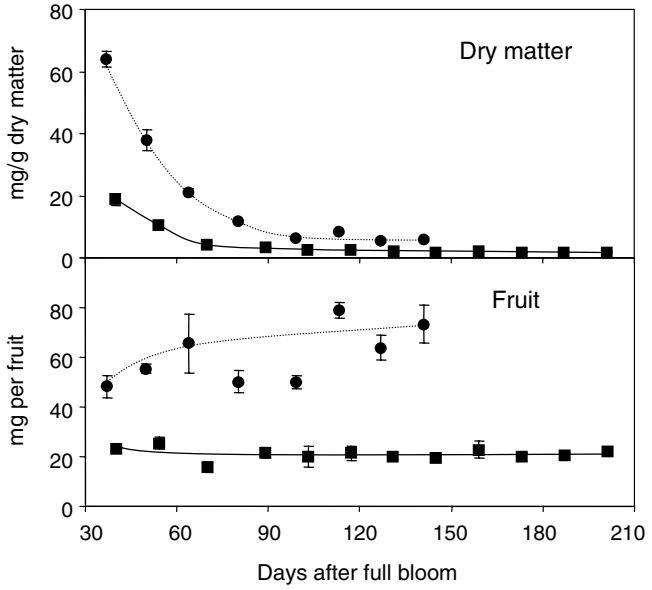

Fig. 3. Evolution of the hydroxycinnamic acids in the apple flesh during the growth of Kermerrien and Avrolles apples: concentrations relative to dry matter (upper graph) and content per fruit (lower graph). ■: Avrolles; ●: Kermerrien.

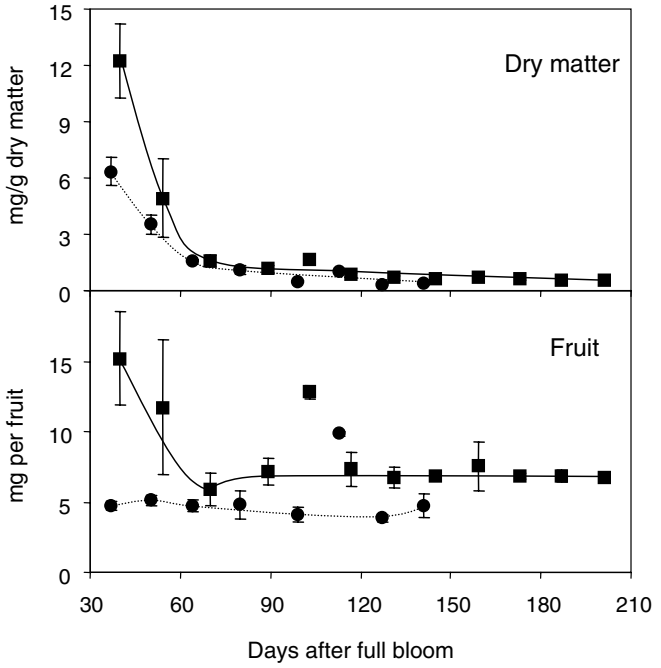

Fig. 4. Evolution of the dihydrochalcones in the apple flesh during the growth of Kermerrien and Avrolles apples: concentrations relative to dry matter (upper graph) and content per fruit (lower graph). ■: Avrolles; ●: Kermerrien.

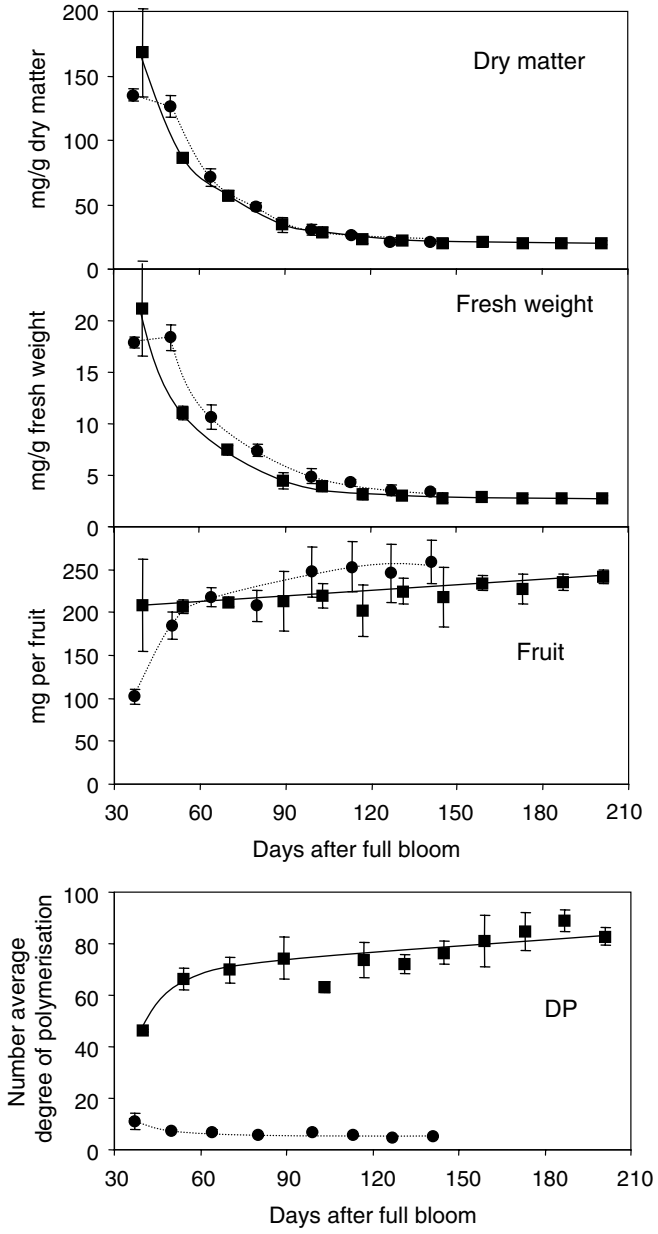

Fig. 5. Evolution of the procyanidins in the apple flesh during the growth of Kermerrien and Avrolles apples: concentrations relative to dry matter, to fresh weight, and content per fruit, and evolution of the number average degree of polymerisation. ■: Avrolles; ●: Kermerrien.

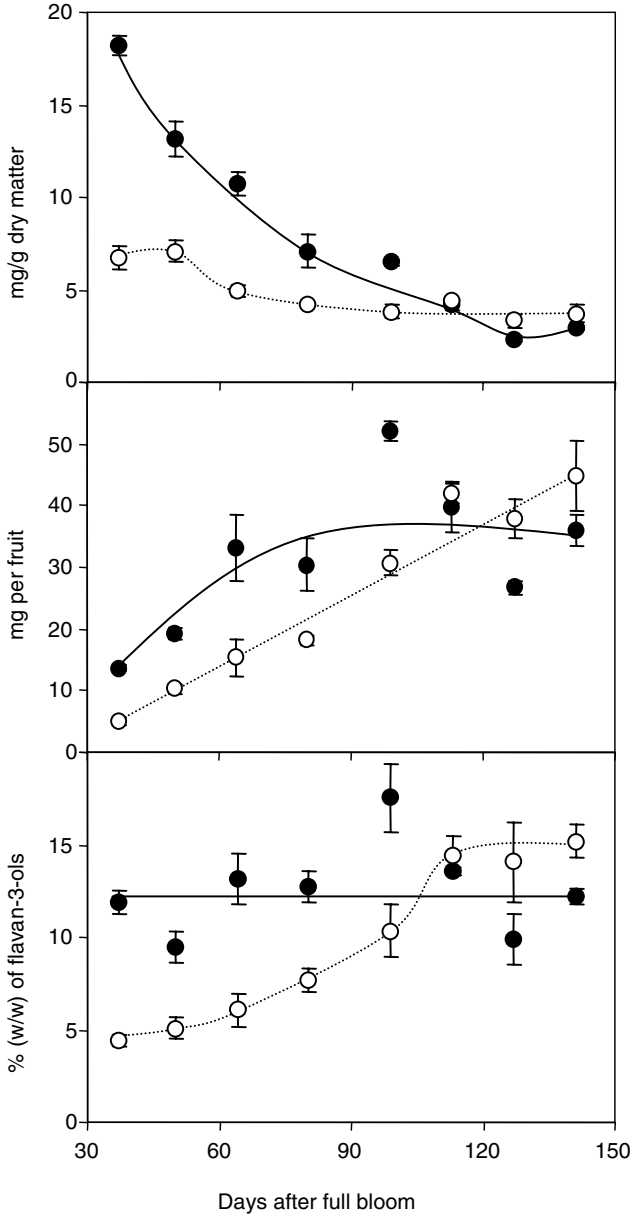

Fig. 6. Evolution of the concentrations and the relative abundance of flavan-3-ols monomers ((−)-epicatechin and (+)-catechin) and procyanidin B2 in the apple flesh during the growth phase of Kermerrien apples. ●: monomers; ○: procyanidin B2.

tion was contrasted: an increase was noted for Avrolles, at a very high level (circa 50–80), and a decrease for Kermerrien, from 11 to 5. Takos et al. (2006) also report a decrease in degree of polymerisation in apple skin between early development and maturation.

The evolution of monomeric (catechins) and oligomeric flavan-3-ols was only followed for Kermerrien (Figs. 6 and 7). These polyphenols were absent in Avrolles throughout the sampling period: we could only detect traces of procyanidin B2 at the earliest date. Absence of catechin is known for this cultivar at maturity (Sanoner et al., 1999; Guyot et al., 2003).

The concentration of monomeric catechins in Kermerrien decreased during growth; there was a net biosynthesis up to 60 days after full bloom but for later stages no clear trend can be seen due to the higher variability of this data (estimated from the standard deviation of the triplicates), linked to difficulties in integrating the (+)-catechin and (−)-epicatechins peaks in the non-thiolysed samples. In contrast, the concentration of procyanidin B2 decreased much less, its content per fruit increased until 120 days after full bloom and it represented a growing proportion of flavan-3-ols during fruit growth, from <5% at 37 days after full bloom to 15% at maturity. The increase in procyanidin B2 was also noted by Mayr et al. (1995) in apple

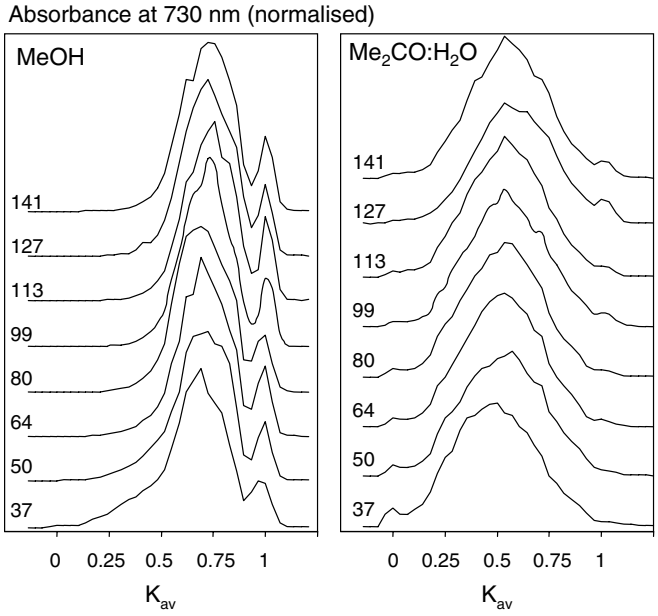

Fig. 7. Elution profiles of polyphenols in purified extracts of Kermerrien apples on Toyopearl TSK-gel HW-40S (MeOH extracts, left panel) or Toyopearl TSK-gel HW-50S (Ac:H_2O extracts, right panel) eluted by acetone : 6 M urea: acetic acid at 20 ml/min. The numbers on the graphs are the days after full bloom for the corresponding extracts.

skin; however they did not analyse the whole procyanidin fraction and attributed this increase to a shift from monomeric to oligomeric flavan-3-ols. Here the increase of procyanidin B2 is concommittant with an increase of the contents of monomers per fruit, a limited evolution of the total procyanidin content, a decrease of the number average degree of polymerisation and changes in the size-exclusion chromatograms of the procyanidin extracts (see below and Fig. 7). It appeared that there was either a net biosynthesis of the smaller procyanidins (with some loss of the larger molecules) or more likely a cleavage of larger procyanidins into lower oligomers.

2.5. Extraction and size-exclusion chromatography of Kermerrien polyphenols

To investigate the evolution of the procyanidins in Kermerrien, the polyphenols were sequentially extracted from the freeze-dried apple powders (Table 3). The two extraction steps were acidic MeOH, extracting most low molecular weight polyphenols (together with sugars and organic acids), and aq. Me_2CO (Me_2CO–H_2O–AcOH 60:39:1), extracting the higher molecular weight procyanidins. The polyphenols were further purified from the extracts by

Table 3
Concentrations of the polyphenols in the apple powders and their purified, freeze-dried extracts

DAFB		Purified extracts (in mg/g apple powder)	Composition of the apple powder or extract (mg/g freeze-dried apple or extract), mg/g					
			Flavan-3-ols				Hydroxycinnamic acids, mg/g	Dihydrochalcones, mg/g
			Monomers, mg/g	Procyanidins, mg/g	Out of which B2, mg/g	DP		
37	Apple		18	132	6	12.1	61	7.2
	MeOH	161	84 (74%)	434 (53%)	20 (54%)	6.5	289 (76%)	36.0 (81%)
	Acetone	36	–	657 (18%)	–	15.6	16 (1%)	1.9 (1%)
50	Apple		13	117	7	7.2	36	3.1
	MeOH	132	63 (62%)	459 (52%)	35 (61%)	5.0	210 (78%)	18.2 (77%)
	Acetone	74	–	668 (43%)	–	11.2	23 (5%)	–
64	Apple		10	72	5	6.3	20	1.5
	MeOH	77	99 (76%)	525 (56%)	45 (71%)	4.5	216 (83%)	16.9 (84%)
	Acetone	24	6 (1%)	676 (22%)	–	12.0	18 (2%)	–
80	Apple		7	46	4	6.2	12	1.0
	MeOH	45	87 (61%)	558 (55%)	73 (76%)	4.2	206 (81%)	16.2 (75%)
	Acetone	19	7 (2%)	602 (25%)	4 (2%)	10.8	24 (4%)	–
99	Apple		6	33	4	6.6	7	0.5
	MeOH	36	100 (56%)	516 (56%)	108 (93%)	4.4	193 (102%)	12.9 (97%)
	Acetone	12	21 (4%)	549 (19%)	7 (2%)	10.8	54 (9%)	–
113	Apple		4	25	4	5.6	8	1.1
	MeOH	32	92 (73%)	482 (62%)	105 (76%)	4.4	208 (80%)	28.7 (83%)
	Acetone	6	20 (3%)	524 (13%)	7 (7%)	13.7	53 (4%)	1.9 (1%)
127	Apple		2	18	3	5.0	6	0.3
	MeOH	27	86 (100%)	556 (83%)	109 (88%)	4.0	175 (85%)	11.5 (100%)
	Acetone	7	nd	nd	nd	nd	nd	nd
141	Apple		3	21	4	5.4	6	0.4
	MeOH	28	84 (78%)	639 (89%)	112 (86%)	3.0	181 (81%)	11.6 (88%)
	Acetone	6	25 (5%)	586 (16%)	–	13.0	47 (4%)	–

The values between parentheses are the recoveries relative to the amount originally present in the apple powder.
DAFB: days after full bloom. MeOH: methanol extract; Acetone: aqueous acetone extract; DP: number average degree of polymerisation of the procyanidins; –: not detected; nd: not done.

adsorption on C18 cartridges prior to concentration, freeze-drying, weighing and analysis. Recoveries were calculated from the weights of purified extracts (i.e. after C18) and their composition determined by thioacidolysis, compared to the composition of the initial freeze-dried apple powder. The many steps might explain why the global recoveries were variable and not always very good (e.g. only 77% for hydroxycinnamic acids for the sample at 37 days after full bloom). As this was very time consuming it was only carried out for a single sample (out of a triplicate) at each collection date.

The composition of the original apple powders showed the trends described in Figs. 3–6. Catechins, dihydrochalcones and hydroxycinnamic acids were concentrated in the MeOH extract. Most of the procyanidins were also extracted with MeOH, the proportion actually increasing during the last stages of growth. The number average degree of polymerisation of procyanidins in the aq. Me_2CO extract was more than double that of the procyanidins extracted by MeOH. It was stable or even increased during the last stages of growth: there was not so much a shift in the molecular weight in each fraction than an increase in the MeOH-extractable fraction.

Size-exclusion chromatography of the extract (Fig. 7) was carried out as described in Le Bourvellec et al. (2006). Two different resins were used so that an adequate fractionation range was obtained for both the methanol and the acetone extracts. In the methanol extracts, the main peak at Kav 0.75 was due to hydroxycinnamic acids and dihydrochalcones, covering the dimeric and trimeric flavan-3-ols. However, a fraction at Kav 0.25–0.5, clearly visible in the extracts from the early growth stages, disappeared during growth. At the last stages, the main peak could clearly be seen to lose material at low Kav values, corresponding to the elution volumes of tetrameric and trimeric flavan-3-ols. In the acetone extracts, the most noticeable changes were a disappearance of a minor fraction at the exclusion volume i.e. at very high number average molecular weights while the main peak became more symmetrical. It must however be kept in mind that this whole fraction tended to represent less and less of the procyanidins over the growth phase.

3. Conclusions

Polyphenol accumulation in the apple flesh occurred early during fruit life. For cider apples most of the fruit polyphenol content was present 50 days after flowering though there was still some increase between 40 and 60 days after full bloom for cv Kermerrien, and a low level of biosynthesis throughout fruit growth for cvs Kermerrien and Avrolles. The evolution of concentrations during fruit growth and maturation was due essentially to dilution of an initially accumulated store. There was in particular no noticeable evolution close to maturity. The stage, around 50 days after full bloom corresponds, for cider apple, to definitive transition between cell proliferation and cell expansion.

Similar results were obtained for table apples, in accordance with literature results. Kondo et al. (2002) for example had noted some accumulation process between 17 and 97 days after full bloom (Kondo et al., 2002), and Takos et al. (2006) found the highest concentrations at 32 days after full bloom for flavonols and procyanidins. Our results indicate an early stop, as suggested by the marked decrease of polyphenol concentration between 30 and 60 days, for cvs Elstar and Gala. After that point, polyphenol concentration would evolve mostly by dilution, which could also explain the dramatic decrease of total phenolics (expressed in $mg\,kg^{-1}$ FW) observed by Kondo et al. (2002) with a division by ten between 17 and 97 days after full bloom in Fuji. In Takos et al. (2006), the concentrations of flavonols and procyanidins in apple skin also appear to run antiparallel to the growth curve. This earlier beginning of cells enlargement in table apples also probably explained the larger decrease of cell wall concentration between 30 and 60 days after full bloom compared to measurements on cvs Dous Moen and Kermerrien.

Treutter (2001) had suggested a possible linkage between fruit growth phases and phenol accumulation via modifications of enzymes activities. Our results also indicate a correlation between the end of active biosynthesis and the beginning of cells enlargement. The possible mechanisms implied are still not known, but the metabolic shift between cell proliferation and cell expansion clearly appears to include the polyphenol biosynthesis pathway.

There were differences in kinetics of accumulation of procyanidins and evolution of their degrees of polymerisation between Kermerrien and Avrolles cultivars. For Avrolles, most procyanidin biosynthesis took place before the sampling period, and degrees of polymerisation actually increased during growth. The biosynthesis appeared to continue longer in Kermerrien and there was a decrease of degree of polymerisation during fruit growth. This was due to a redistribution of the molecular weight distribution inside the procyanidin class, with increase in low molecular weight species such as procyanidin B2 and disappearance of a high molecular weight fraction on the size-exclusion chromatograms. Both of these factors resulted in higher concentrations of low-molecular weight procyanidins, which can be related to the ''bitter'' character of this variety.

4. Experimental

4.1. Plant material

Apple fruits (*Malus domestica* Borkh.) were harvested in the experimental orchard of the Centre Technique des Production Cidricoles (Sées, Orne, France) in the 2000 or 2001 season (Avrolles, Dous Moen and Kermerrien cultivars) or in the experimental orchard of INRA (Angers) during the

2000 season (Elstar and Gala cultivars). Fruits were peeled manually (except the first stage of growth, used whole) and then cut in 4–32 (vertically in 16, horizontally in 2) depending on their sizes (Renard, 2005). Four pieces were kept for each apple, chosen to represent each quarter of the fruit and both top and bottom hemispheres. The whole skin was collected for apples of 2000 season. In 2001 each harvest date and cultivar was represented by three times 10 apples; in 2000 only 20 to 14 apples were collected per set, giving duplicates for flesh and a single sample for skin. All apple samples were frozen in liquid nitrogen and freeze-dried. Freeze-dried apple tissues were ball-milled for 3 min in a paint mixer (Idex Corp. Northbrook, Ilinois, USA).

4.2. Reagents

MeOH, acetonitrile, Me_2CO of chromatographic quality were provided by Biosolve (Distribio, Evry, France). Hexane (Merck™, Darmstadt, Allemagne) was of analytical quality.

4.3. Extraction and purification of apple procyanidins

MeOH and aq. Me_2CO extracts of apple polyphenols were obtained by successive solvent extractions of freeze-dried pulp material according to Guyot et al., 1997 as described in Le Bourvellec et al. (2006). Fractions of approximately 1 g of apple powders were treated with 12.5 ml hexane. After 10 min of incubation under vigorous stirring, the hexane was filtered off on a G3 sintered glass filter, and discarted. The remaining powder was extracted five times with 12.5 ml of MeOH (acidified with AcOH at 5 ml/l) with the same procedure except that methanol extracts were kept and pooled. The remaining powder was then extracted three times with 12.5 ml aq. Me_2CO (Me_2CO–H_2O–AcOH 60:39:1), and all three aq. Me_2CO extracts were pooled. MeOH and aq. Me_2CO extracts were concentrated on a rotary evaporator prior to removal of sugars on Sep-Pack Vac 20cc C-18 cartridges (WatersCorp., Milford, MA). The cartridges were first conditioned by elution with twice 20 ml of aq. Me_2CO followed by twice H_2O. The samples were injected and the cartridges were rinsed with 110 mL of H_2O. This volume was checked to be sufficient by absence of sugars in the eluate, as monitored by the PhOH-H_2SO_4 reaction (Dubois et al., 1956). The polyphenols were eluted with three times 10 ml of aq. Me_2CO and concentrated on a rotary evaporator then freeze-dried.

4.4. Size-exclusion chromatography

The size-exclusion chromatography method was carried out as described in Le Bourvellec et al. (2006). The method was based on that of Yanagida et al. (1999). TSK HW 40S (methanol extracts) or TSK HW 50S (acetone extracts) resins (Tosohaas, Stutgartt, Germany) of superfine grade were equilibrated in Me_2CO-6M aq. Urea-AcOH (60:39:1) and packed in a thermostated SR 25/45 column (Amersham Biosciences, Amersham, UK), equipped with a 500 µl injection loop and a Pharmacia P500 pump (Amersham Biosciences). The elution rate was of approximately 20 ml/h. V_o and V_t were determined by elution of blue dextran (Amersham Biosciences) and (−)-epicatechin, respectively. All data will be presented as a function of the partition parameter K_{av}, in order to standardize the variations in column volume.

4.5. Analytical

Polyphenol contents of SEC fractions were measured spectrophotometrically after reaction with the Folin-Ciocalteu reagent (Merck) as described by Yanagida et al. (1999).

Polyphenols were analysed by HPLC after thioacidolysis as described by Guyot et al. (2001a,b). The average degree of polymerisation was measured by calculating the molar ratio of all the flavan-3-ols units (thioether adducts plus terminal units) to (−)-epicatechin and (+)-catechin corresponding to terminal units. The HPLC apparatus was a Waters (Milford, MA, USA) system 717 plus autosampler equipped with a cooling module set at 4 °C, a 600 E 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 (H_2O-AcOH, 975:25) and solvent B (acetonitrile): initial, 3% B; 0–5 min, 9% B linear; 5–15 min, 16% B linear; 15–45, 50% B linear, followed by washing and reconditioning the column.

Cell walls were prepared as the PhOH-buffer insoluble material as described in Renard (2005): the freeze-dried apple slices were ground as above and suspended in a PhOH-H_2O (4:1 vol) mixture (100 ml for 10 g of dried powder, i.e. presence of salts and electrolites (from the apple) at concentrations close to those in the fruit) and incubated for 1 h at room temperature, after which the phenol was filtered off on a G3 sintered glass filter, and the suspensions washed with cold buffer solution until the phenol smell disappeared. A buffer simulating the ionic conditions in apple juice (1.2 mM $CaCl_2$, 2.0 mM $MgCl_2$, 0.5 g/l KCl, 60 mg/l ascorbic acid and 4 g/l malic acid, plus sodium disulfite at 1 g/l, adjusted to pH 3.5 with 5 M NaOH) was used throughout the procedure. The cell wall material was then washed twice with Me_2CO–H_2O (60:40) and dried by increasing concentrations of Me_2CO (80:20, three times, pure Me_2CO three times) then overnight in an oven at 40 °C.

4.6. Histology

Histological slides were performed from apple pieces ($l = 5$ mm, $L = 10$ mm, $H = 5$ mm) sampled on the parenchyma of the fruits, fixed on glutaraldehyde solution, dehydrated and included in methylacrylate resin.

Semi-fine (5 μm) sections were cut using a microtome and colored with toluidin blue. Images were shot under light microscope (LEICA DMR) equipped with a black and white camera (CoolSNAP HQ) drived by the Metaview system (Roper Scientific-Universal-Imaging Corporation).

4.7. Statistics

For the samples of 2000 (Figs. 1,2, and 4) standard deviations were calculated for each series of duplicates using the sum of individual variances pondered by the individual degrees of freedom (Box et al., 1978). The standard deviations shown on Figs. 5–7 are the actual standard deviations for each series of triplicates.

Acknowledgements

We thank Ms. M. Picot and A. Gacel and Mr. Ch. Massacrier for their technical help. This work was supported by Ministère de l'Agriculture et de la Pêche (program CTPS C9907Phenolpom ''Variabilité et évolution de la texture et de la composition phénolique des pommes à cidre et à couteau: conséquence sur la qualité'').

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