Carbohydrate Polymers 75 (2009) 251–261

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Interactions between apple (*Malus x domestica Borkh.*) polyphenols and cell walls modulate the extractability of polysaccharides

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ARTICLE INFO

Article history:
Received 4 June 2008
Received in revised form 4 July 2008
Accepted 5 July 2008
Available online 11 July 2008

Keywords:
Tannin
Pectin
Hemicellulose
Sequential extraction

ABSTRACT

Apple polyphenol (procyanidin)–cell wall interactions were investigated and their impact on polysaccharide extractability were determined. Native and oxidised procyanidins with average degrees of polymerisation of 13 and 55 were incubated with cell walls. The effect of polyphenol oxidation was evaluated according to two designs: polyphenols were chemically oxidised either before or during interaction. The extent of procyanidin binding to cell walls was assessed by the weight increase of procyanidin–cell wall complexes as compared to weights of cell walls alone. Pectins and hemicelluloses were subsequently extracted from cell walls and from cell wall–procyanidin adducts using a chelating agent (ammonium oxalate), a pectin lyase treatment and NaOH.

Weight increases of complexes ranged from 20% to 29%. Weight gains increased in the following order: native, pre-oxidised, simultaneously oxidised and bound procyanidins, these different fractions were, respectively, bound to cell walls. In presence of native procyanidins, oxalate extracted less pectins, and those pectins had lower degrees of methylation, as compared to cell walls alone. When cell walls were incubated with oxidised and oxidising procyanidins, even less pectins with lower degree of methylation were extracted. Major findings indicated that procyanidins mainly bound to pectins as compared to other cell wall compounds: (1) the procyanidin adsorption to cell walls limited the depolymerisation of pectins supposedly induced by pectin lyase. Thus less pectins were extracted but their degree of methylation increased, indicative of products of lysis of pectin lyase. (2) Hemicelluloses extracted using NaOH (4 M) were more abundant in pectins when oxidised or oxidising procyanidins were complexed rather than non complexed to cell walls.

1. Introduction

Although some polyphenols can be constitutive of cell walls, such as lignin and ferulic acid in Poaceae or Chenopodiaceae, polyphenolics are mainly located in vacuoles. Apple processing often involves tissue disruptions and brings compounds contained within the cellular organites and cytoplasm together with extracellular molecules. During such a process, polyphenols breach the vacuolar frontiers and bind to extracellular cell walls. Simultaneously, as the destructuration compromises the protection and the regulation provided by cellular and ultracellular membranes, intracellular molecules, including polyphenols become exposed to the air oxygen and to other oxidising compounds. Note that polyphenol–cell wall complexes can also be formed after an environmental stress (such as pathogen attack or injury) has disorganized the cell or the tissues and initiated decompartimentalization and destructuration.

While the affinity of tannins for proteins has largely been studied, their interactions with cell walls have received less attention (de Freitas, Carvalho, & Mateus, 2003; Renard, Baron, Guyot, & Drilleau, 2001). Procyanidins (i.e. condensed tannins) are the main polyphenol class in apples, accounting for more than 50% of total polyphenols depending on the variety (Guyot, Marnet, Sanoner, & Drilleau, 2003). They are oligomers and polymers of flavan-3-ols units such as (+)-catechin or (−)-epicatechin, with >95% (−)-epicatechin, linked to one another by either C4 → C8 and/or C4 → C6 interflavan bond (B-type) (Fig. 1). Their molecular weight differ depending on the varieties; thus $\overline{DP}n$ between 2 and 50 can be observed (Guyot, Marnet, Laraba, Sanoner, & Drilleau, 1998; Guyot et al., 2003; Sanoner, Guyot, Marnet, Molle, & Drilleau, 1999). Tannins contribute

Abbreviations: AV, procyanidins from Avrolles apple variety; $\overline{DP}n$, number average degree of polymerisation; DM, degree of methylation; MM, procyanidins from Marie Menard apple variety; CWM, cell wall material; CQAq, caffeoylquinic acid *o*-quinone

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doi:10.1016/j.carbpol.2008.07.010

to the organoleptic properties of cider, especially by their astringency and bitter taste (Lea & Arnold, 1978). The other three main classes of polyphenols in apples are phenolic acids, mostly caffeoylquinic acid, monomeric flavan-3-ols, again mainly (−)-epicatechin, and dihydrochalcones (phloretin glycosides) (Guyot et al., 1998, 2003; Sanoner et al., 1999). Previous studies have shown a discrepancy between procyanidin concentrations and $\overline{DP}n$ in apple fruits and processed apple products, including juices and ciders (Guyot et al., 2003; Hubert, Baron, Le Quéré, & Renard, 2007), and have shown that is mainly during apple pressing that procyanidins are transferred from fruit vacuoles to juice (Le Bourvellec, Guyot, & Renard, 2004; Renard et al., 2001). Two mechanisms operate concomitantly: retention of procyanidins by cell wall and oxidation by polyphenoloxidase (PPO).

Previous studies have shown that oligomeric and polymeric procyanidins bind non-covalently to apple cell walls during simple incubation in aqueous buffer (Le Bourvellec, Bouchet, & Renard, 2005; Le Bourvellec & Renard, 2005; Le Bourvellec, Guyot, et al., 2004; Renard et al., 2001). As this binding is fast and spontaneous, it is indicative of tissue degradation. The cell wall–procyanidin adsorption mechanism involves weak associations, more precisely a combination of hydrogen bonds and hydrophobic interactions (Le Bourvellec, Guyot, et al., 2004; Renard et al., 2001). The amounts of bound procyanidins and their affinity constants for cell walls are strongly influenced by compositional and structural parameters, such as stereochemistry, conformational flexibility, molecular weight, and concentration (Le Bourvellec, Guyot, et al., 2004; Le Bourvellec et al., 2005; Renard et al., 2001). It is the modification of the physical state of cell walls (i.e. harsh drying) that affects the most their capacity to bind procyanidins (Le Bourvellec & Renard, 2005) as compared to their chemical modifications, including elimination of pectins and xyloglucans, or to their varietal origin (Renard et al., 2001). The apparent affinity constants between procyanidins and pure polysaccharides decrease as follows: pectins $\gg$ xyloglucan > starch > cellulose (Le Bourvellec et al., 2005).

Oxidation is one of the consequences of plant tissue destructuration. It allows the enzyme, polyphenoloxidase (PPO), originally in the plasts and the substrates (polyphenols and oxygen) to come in contact. In apple, the main substrate of PPO is caffeoylquinic acid, an hydroxycinnamic acid (Janovitz-Klapp, Richard, & Nicolas, 1989). It is first enzymatically oxidised to its highly reactive *o*-quinones, with the simultaneous consumption of oxygen, the second substrate of PPO (Mayer 1987; Mayer & Harel 1979). Then, *o*-quinones react following different pathways according to their electrophilic and/or oxidant character with nucleophilic compounds such as polyphenols – or proteins, to produce secondary products by covalent interactions (Jervis & Pierpoint, 1989). Procyanidins are not substrates of PPO (Goodenough & Lea, 1979), but they can be converted into corresponding *o*-quinones by coupled oxidation/reduction involving primary *o*-quinones formed by PPO catalysis. During this reaction the primary *o*-quinones are reduced back to their *o*-diphenol form (Cheynier & Ricardo da Silva, 1991). Procyanidin oxidations may have two distinct consequences.

- Firstly, oxidation products of procyanidins are formed and these molecules could have different affinities toward cell walls compared to the native molecules.
- Secondly, procyanidin *o*-quinone intermediates could form covalent bonds with cell wall polymers.

Indeed, some have reported the existence of polyphenol–polysaccharide complexes in cell walls (Coimbra, Waldron, & Selvendran, 1995; Femenia, Rigby, Selvendran, & Waldron, 1999; Femenia, Waldron, Robertson, & Selvendran, 1999; Selvendran 1985; Stevens & Selvendran, 1984), notably in apple, others extracted cell walls devoid of polyphenols (Le Bourvellec, Guyot, et al., 2004; Renard et al., 2001).

In this study, our aim is to characterise apple tannin–cell wall interactions *ex vivo*. To better reconstitute what occurs during pressing of apple, we introduced the effect of oxidation according to two designs: cell walls were in contact with chemically oxidised procyanidins and cell walls were in contact with native tannins under oxidising conditions. We used chemically rather than *in vivo* oxidised procyanidins as the determination of binding isotherms of isolated oxidised procyanidin–cell wall complexes was not an applicable strategy in this study.

2. Material and methods

2.1. Plant material

Apple fruits (*Malus x domestica Borkh.*) from the "Avrolles" and "Marie Menard" varieties were harvested at maturity during the 2002 season in the experimental orchard of the Centre Technique des Productions Cidricoles (Sées, Orne, France). Fruits were mechanically peeled and cored as previously described by Guyot, Doco, Souquet, Moutounet, and Drilleau (1997) and cortex tissues were freeze-dried.

2.2. Chemicals

Methanol, acetonitrile, acetone of chromatographic quality were provided by Biosolve (Distribio, Evry, France), toluene-α-thiol was provided by Merck (Darmstadt, Germany). Hexane (Merck, Darmstadt, Germany) was of analytical quality.

2.3. Isolation of cell walls

Cell walls were isolated from "Petit Jaune" apples, devoid of starch (visual iodine test), by the phenol: buffer method according to Renard et al. (2001).

2.4. Extraction of procyanidins

Hexane, methanol and aqueous acetone extracts of apple polyphenols were obtained by successive solvent extractions of Avrolles" and "Marie Ménard" freeze-dried pulps (Guyot, Marnet, & Drilleau, 2001). Hexane and methanol extracts were discarded. Aqueous acetone extracts containing procyanidins were pooled and concentrated on a rotary evaporator prior to freeze-drying.

Procyanidin aqueous acetone extracts named AV and MM were obtained from "Avrolles" and "Marie Ménard" varieties, respectively.

2.5. Procyanidins oxidation

AV and MM procyanidins (5 g/l) were oxidised with a caffeoylquinic acid *o*-quinone (1.25 g/l) at a molar ratio of 1 for 4 (−)-epicatechin units, in citrate/phosphate buffer pH 3.8. Caffeoylquinic acid *o*-quinone was generated by oxidation of caffeoylquinic acid with resin-bound periodate (Le Bourvellec, Le Quéré, Sanoner, Guyot, & Drilleau, 2004). The fractions were incubated at 30 °C for 2 h under agitation.

Oxidised procyanidins were named AVOx and MMOx.

2.6. Adsorption experiments

2.6.1. Complexes of native and oxidized procyanidins with cell walls

Native and oxidised AV and MM procyanidins (fractions AV, MM, AVox and MMox) (5 g/l) were incubated for 1 h at 25 °C with

a suspension of cell walls (20 g/l, in citrate/phosphate buffer pH 3.8) according to the method described by Renard et al. (2001) and Le Bourvellec, Guyot, et al. (2004). All cell wall/tannin complexes were collected by filtration on G3 sintered glass filters, rinsed with double-distilled water and freeze-dried.

The various complexes obtained were named as follows: complex of cell wall material and native procyanidins: CAV and CMM, complex of cell wall material and oxidized procyanidins: CAVOx and CMMOx.

2.6.2. Oxidation of procyanidins in the presence of cell wall material

Native (fractions AV and MM) procyanidins (5 g/l) and caffeoylquinic acid *o*-quinone (1.25 g/l, molar ratio of 1 *o*-quinone caffeoylquinic acid for four (−)-epicatechin units) were incubated for 1 h with a suspension of cell wall material (20 g/l, in citrate/phosphate buffer pH 3.8) (Le Bourvellec, Guyot, et al., 2004; Renard et al., 2001). All cell wall/tannin complexes were collected by filtration on G3 sintered glass filters, rinsed with double-distilled water and freeze-dried.

A blank (quinone with cell wall suspension) was carried out.

The various complexes obtained were named as follows: the blank was named OxCWM and complex of oxidized cell wall material and procyanidins: OxCAV and OxCMM.

2.7. Sequential extraction

Pectins were extracted from cell wall material using a method adapted from Renard (2005). Cell walls and cell wall/procyanidin complexes were sequentially extracted by ammonium oxalate, pectin lyase, and NaOH 4 M. Sequential extractions were carried out in triplicate.

One gram of cell wall preparation and 30 ml of ammonium oxalate (50 mM, pH 5) were incubated three times 1 h at 40 °C in a 35 ml empty Sep-pack prep column (Interchim, France) equipped with a sinter of porosity 20 μm under slow planetary agitation. Then, the extract and the cell walls were separated by filtration under vacuum. The three oxalate extracts were pooled and dialysed three times 8 h against deionised water in a dialysis tube of theoretical porosity of 12 kDa (Sigma Chemical Co., St. Louis, MO, USA) and freeze-dried.

The cell walls and 20 ml of sodium acetate buffer (1 mM, pH 5) supplemented with the pectin lyase (Megazyme, Bray, Ireland, 11 U/g of cell wall material) were incubated at 40 °C during 24 h under slow planetary agitation. The pectin-lyase extracts were freeze-dried without dialysis.

The residue and 20 ml of NaOH (4 M) were incubated one night at ambient temperature under slow planetary agitation. NaOH was supplemented by $NaBH_4$ 1 g/l to prevent the β-elimination and peeling reactions of the polysaccharides. The NaOH extract was neutralised by acetic acid and dialysed as above during 96 h against deionised water.

2.8. Analytical

Polyphenols were measured by HPLC-DAD (Waters, Milford, MA, USA) after thioacidolysis as described by Guyot et al. (2001). The average degree of polymerisation was evaluated by calculating the molar ratio of all the flavan-3-ol units (thioether adducts plus terminal units) to (−)-epicatechin and (+)-catechin corresponding to terminal units. 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 (aqueous acetic acid, 2.5% v/v) and solvent B (acetonitrile): initial composition 3% B; linear gradient to 9% B from 0 to 5 min; linear gradient to 16% B from 5 to 15 min; linear gradient to 50% B from 15 to 45 min; followed by washing and reconditioning the column.

Galacturonic acid was determined according to the method describe by Thibault (1979) on an Alliance instruments (Mery/Oise, France).

Neutral sugars were determined after sulfuric acid hydrolysis (Seaman, Moore, Mitchell, & Millet, 1954) and derivation to alditol acetates (Englyst, Wiggins, & Cummings, 1984). Alditol acetates were analysed by gas chromatography-FID detection (capillary column of 30 m × 0.25 mm i.d. coated with DB225, 0.15 μm film thickness, JW scientific, Folson) at 215 °C, using hydrogen as carrier gas. Myo-inositol was used as internal standard.

To determine the degree of methylation, methanol was determined according to Klavons and Bennet (1986). The degree of methylation (DM) was calculated as molar ratio of methanol to galacturonic acid.

Nitrogen was analysed by the Kjeldahl method. Protein contents were calculated using the equation $N \times 6.25$ (Hach, Brayton, & Kopelove, 1985).

Pectin lyase activity in presence of procyanidins was measured spectrophotometrically by apparition of OD at 235 nm using citrus pectin (pectin "A" Unipectine, Sanofi-Bio-Industries, France) as substrate in a sodium acetate buffer, pH 4.5.

2.9. Statistical analyses

Yields and data were compared using the one-way ANOVA at a P level of <.05.

3. Results and discussion

3.1. Polyphenol composition

The composition and the characterisation of the polyphenol fractions are summarised in Table 1.

The two water–acetone extracts, fractions MM and AV, obtained after methanol elimination of sugars, acids and polyphenols of low molecular weight, contained 22–27% of flavan-3-ols composed mainly of highly polymerized procyanidins (Guyot et al., 1997, 1998, 2001). Procyanidins represented 95% and 99% of polyphenols present in fractions MM and AV, respectively. Their respective $\overline{DP}ns$ were 13 and 55. Therefore, as among the different apple polyphenol classes only procyanidins bind to cell wall (Renard et al.,

Table 1
Composition (mg/g) of the procyanidin fractions before and after oxidation by caffeoylquinic acid *o*-quinone

Fraction	Flavan-3-ols				Phenolic acids		Dihydrochalcones	
	CAT	EPI	PCA	$\overline{DP}n$	CQA	pCoQA	PLZ	XPL
MM	nd	3.4	259.4	2	8.3	0.2	0.7	nd
		0.4	*7.2*	*0.2*	*0.1*	*0.0*	*0.1*	
MMox	nd	nd	176.6	9.9	19.5	0.2	0.7	nd
			7.2	*0.2*	*0.1*	*0.0*	*0.1*	
AV	nd	nd	221.7	55.1	0.7	0.4	0.1	0.4
			16.9	*2.7*	*0.2*	*0.1*	*0.0*	*0.0*
AVox	nd	nd	119.7	46.1	37.3	0.4	0.1	0.4
			16.9	*2.7*	*0.2*	*0.1*	*0.0*	*0.0*

The measurements were carried out in triplicate. The average and the corresponding error (standard deviation) of measurement were then calculated. Values in italic correspond to the standard deviation. CAT, (+)-catechin; EPI, (−)-epicatechin; PCA, procyanidins; CQA, caffeoylquinic acid; pCoQA, para-coumaroylquinic acid; PLZ, phloridzin; XPL: phloretin xyloglucoside; nd, not detected.

2001) these extracts were estimated suitable for use without further purification.

Fraction MM also contained traces of monomeric flavan-3-ols ((−)-epicatechin), hydroxycinnamic acids (caffeoylquinic acid and *p*-coumaroylquinic acid) and dihydrochalcones (phloridzin) and fraction AV also contained traces of hydroxycinnamic acids (caffeoylquinic acid and *p*-coumaroylquinic acid) and dihydrochalcones (phloridzin and phloretin xyloglucoside).

MMox and AVox fractions were obtained by coupled oxidation in the presence of caffeoylquinic acid *o*-quinone produced chemically to mime the oxidation phenomena that take place during the crushing of apples. Caffeoylquinic acid *o*-quinone was produced chemically so as to better control the reaction and to avoid the introduction of an enzyme protein. The stoichiometry of 1 *o*-quinone caffeoylquinic acid for 4 (−)-epicatechin units used corresponds to ratios commonly found in cider apple (Sanoner et al., 1999). Coupled oxidation is not the only mechanism occurring: in our system, some oxidation products may be the result of additions between procyanidins and caffeoylquinic acid or also dimers of caffeoylquinic acids (Bernillon, Guyot, & Renard, 2004).

Oxidation resulted in a 50% and 68% decrease of the concentration of procyanidins from AV and MM fractions, respectively, that could be measured by thioacidolysis and HPLC, and a decrease of the apparent $\overline{DP}n$ compared to the initial $\overline{DP}n$. The same phenomenon has already been reported by Le Bourvellec, Le Quéré, et al. (2004) (Table 1). The "loss" in procyanidins was probably due to formation of new bonds, resistant to thioacidolysis, with two possible mechanisms:

- Formation of adduct by reaction of caffeoylquinic acid *o*-quinone with (−)-epicatechin unit of the procyanidin structure.
- Electron transfer reaction with caffeoylquinic acid *o*-quinone to form procyanidin *o*-quinones or semi-quinones which further react by intermolecular or intramolecular additions. For example this reaction could result in the conversion of the native B-type procyanidins to A-type (Burger et al., 1990; Kondo et al., 2000). These linkages are resistant to thioacidolysis.

Monomeric (−)-epicatechin, initially present in the fraction MM, was not detected after oxidation while *p*-coumaroylquinic acid and dihydrochalcones seemed little affected (Table 1). (−)-Epicatechin is largely oxidized by the coupled oxidation mechanism (Cheynier, Basire, & Rigaud, 1989; Oszmianski & Lee, 1990) and is also involved in the formation of addition products with *o*-quinone (Weinges & Muller, 1972). *p*-Coumaroylquinic acid and phloretin xyloglucoside, which are monophenols with redox potentials around 1V (Jovanovic, Steenken, & Hara, 1998), do not react with caffeoylquinic acid *o*-quinone. Caffeoylquinic acid concentrations increased after oxidation (Table 1). This is due to reduction of caffeoylquinic acid *o*-quinone, used as oxidant, back to caffeoylquinic acid by coupled oxidation/reduction mechanisms between caffeoylquinic acid *o*-quinone and other phenols (Cheynier & Ricardo da Silva, 1991). These results were in agreement with those observed earlier (Guyot et al., 2003; Le Bourvellec, Le Quéré, et al., 2004). The increase in caffeoylquinic acid concentration had no impact on call wall/procyanidin complexes as this molecule does not bind to cell walls (Renard et al., 2001).

3.2. Cell wall material composition

Cell walls isolated from fresh ripe apples were devoid of polyphenols as checked by thioacidolysis and contained <30 mg/g proteins.

Apple cell walls (Table 2) were composed of cellulose, highly methylated pectins relatively rich in xylogalacturonans, fucogalactoxyloglucan and mannan, and their concentration were within in the ranges of the literature values (de Vries, Rombouts, Voragen, & Pilnik, 1982; Le Bourvellec, Guyot, et al., 2004; Massiot & Renard, 1997; Renard, 2005; Renard, Voragen, Thibault, & Pilnik, 1990, 1991a, 1991b; Renard et al., 2001; Voragen, Heutink, & Pilnik, 1980).

3.3. Characterisation of cell wall/procyanidin complexes

After incubation of procyanidins with cell walls, the extent of binding was assessed by the both weight increase compared to the blank and by the measurement of tannins detectable by thioacidolysis (Table 2).

The blank (oxCWM) decrease possibly because of an elimination of either residual organic acids or/and polysaccharides during extraction procedure. The first suggestion seemed prevalent as oxCWM was more abundant in sugars as compared to CWM. Increase in weight were observed for the different complexes, and were as follows: native procyanidins (weight increase of 22% and

Table 2
Yields and composition (mg/g) of the cell wall material and the different cell wall material/procyanidin complexes

Fraction	Yields[a]	Composition (mg/g)									
		Procyanidins		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Gal A
		mg/g	$\overline{DP}n$								
CWM	–	nd	–	12	12	93	57	19	65	293	210
				0	*0*	*3*	*3*	*0*	*4*	*17*	*10*
OxCWM	0.85	tr	–	9	10	108	68	26	71	327	283
				3	*2*	*2*	*5*	*5*	*5*	*8*	*12*
CMM	1.04	100	17	6	8	86	62	19	58	276	226
		8	*0*	*1*	*1*	*11*	*9*	*2*	*7*	*11*	*1*
CMMox	1.05	84	16	8	8	78	59	21	52	267	232
		3	*0*	*1*	*2*	*3*	*15*	*5*	*1*	*14*	*7*
OxCMM	1.09	105	15	7	8	79	51	19	56	262	214
		5	*1*	*1*	*0*	*9*	*6*	*1*	*5*	*15*	*1*
CAV	1.02	137	148	7	8	81	55	18	58	273	214
		10	*4*	*1*	*1*	*7*	*6*	*1*	*3*	*12*	*22*
CAVox	1.06	119	120	8	9	83	55	18	58	244	208
		8	*2*	*1*	*1*	*14*	*5*	*3*	*7*	*34*	*22*
OxCAV	1.10	139	142	7	8	73	50	18	52	258	202
		9	*3*	*1*	*0*	*3*	*2*	*0*	*4*	*12*	*9*

The measurements were carried out in triplicate. The average and the corresponding error (standard deviation) of measurement were then calculated. Values in italic correspond to the standard deviation. nd, not detected.

[a] Yields calculated using the initial weight of the CWM. tr, trace.

Table 3
Yields and composition (mg/g) of the sequential extraction of the native and oxidized cell wall material

Fraction	Yields[a]		Composition (mg/g)									
	PS yields[b]		PP	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Gal A	MeOH (DM)
CWM												
Ammonium oxalate	4.3	*0.1*	nd	ND	ND	ND	ND	ND	ND	ND	524	71 *3*
											26	(73 *5*)
Pectin lyase	23.3	*2.3*	nd	9	nd	47	4	nd	15	nd	442	53 *2*
				1		*6*	*0*		*1*		*34*	(67 *3*)
NaOH 4 M	23.9	*3.1*	nd	13	24	155	153	53	124	201	78	nd
	20.5	*3.1*		*1*	*3*	*9*	*17*	*5*	*10*	*11*	*17*	
OxCWM												
Ammonium oxalate	3.1	*0.6*	23	ND	ND	ND	ND	ND	ND	ND	435	44 *16*
			2								*125*	(56 *8*)
Pectin lyase	21.9	*5.8*	8	17	nd	70	5	nd	25	nd	591	79 *3*
			1	*3*		*1*	*0*		*1*		*25*	(74 *3*)
NaOH 4M	20.4	*1.1*	25	10	26	119	130	65	101	214	100	nd
	15.6	*0.6*	*4*	*0*	*4*	*4*	*15*	*9*	*7*	*25*	*19*	

All extractions were carried out in triplicate. The average and the corresponding error (standard deviation) of measurement were then calculated. Values in italic correspond to standard deviation. nd, not detected. ND, not determined.
[a] Yields calculated using the initial weight of the CWM.
[b] Yields for polysaccharide only. PP, polyphenols (detection at 280 nm).

20% with $\overline{DP}n$ 13 and 55, respectively) < oxidised polyphenols (weight increase of 24% and 25%) < polyphenols oxidised and bound (weight gains of 28% and 29%).

Procyanidin contents of the complexes varied between 84 and 139 mg/g. The amount of bound procyanidins increased significantly with the $\overline{DP}n$: 137 mg/g for fraction AV (initial $\overline{DP}n$ 55) versus only 100 mg/g for fraction MM. The $\overline{DP}n$ of the bound procyanidins were higher than those of the initial material. This confirms that cell walls bind to high rather than to low molecular weight polymers (Le Bourvellec, Guyot, et al., 2004; Renard et al., 2001). The $\overline{DP}n$ of the procyanidins retained was higher than those that had been observed previously (Le Bourvellec, Guyot, et al., 2004; Renard et al., 2001). This could be explained by successive washings during extraction that have selectively eliminated procyanidins of low $\overline{DP}n$, which were less retained by cell walls (Renard et al., 2001).

After thioacidolysis of native and oxidised polyphenols, less procyanidins were detected by thioacidolysis for the oxidised polyphenols than for native procyanidins while the increases in weight were higher. Note that after oxidation only 50% and 32% of the phenolic material present in AV and MM fractions could be accounted for by thioacidolysis (Table 1). Oxidation increased significantly the ability of procyanidins to bind to apple cell wall material; however the quantitative difference with native polyphenols was slight.

Relative proportions of the different sugars remained the same. This observation was expected insofar as procyanidins were only added to the cell wall material. The concentrations of the various sugar thus decreased proportionally.

Table 4
Yields and composition (mg/g) of the sequential extraction of the Marie Ménard procyanidin/cell wall material complexes

Fraction	Yields[a]		Composition (mg/g)									
	PS yields[b]		PP	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Gal A	MeOH (DM)
CMM												
Ammonium oxalate	3.5	*0.8*	264	ND	ND	ND	ND	ND	ND	ND	415	41 *6*
			14								*30*	(57 *6*)
Pectin lyase	16.2	*1.6*	51	13	nd	52	4	nd	10	nd	603	78 *2*
			6	*1*		*2*	*0*		*0*		*87*	73
NaOH 4 M	21.4	*1.1*	155	9	20	127	107	50	92	179	102	–
	14.8	*3.1*	*11*	*2*	*1*	*22*	*10*	*5*	*15*	*25*	*33*	
Ammonium oxalate	3.5	*1.1*	186	ND	ND	ND	ND	ND	ND	ND	495	47 *10*
			7								*51*	(51 *12*)
CMMox												
Pectin lyase	15.6	*0.1*	53	12	nd	50	34	nd	12	nd	581	79 *2*
			8	*1*		*1*	*0*		*1*		*17*	(76 *7*)
NaOH 4M	21.7	*0.2*	167	8	16	117	105	47	86	173	107	–
	14.3	*0.1*	*10*	*1*	*7*	*10*	*2*	*2*	*4*	*5*	*5*	
OxCMM												
Ammonium oxalate	3.1	*1.1*	163	ND	ND	ND	ND	ND	ND	ND	421	33 *13*
			36								*179*	(46 *13*)
Pectin lyase	12.1	*1.0*	64	9	nd	24	2	nd	8	nd	578	76 *1*
			6	*1*		*3*	*0*		*1*		*20*	(72 *2*)
NaOH 4M	19.7	*1.5*	297	7	20	77	97	50	71	172	107	–
	11.8	*0.5*	*25*	*1*	*1*	*7*	*6*	*6*	*4*	*10*	*16*	

All extractions were carried out in triplicate. The average and the corresponding error (standard deviation) of measurement were then calculated. Values in italic correspond to standard deviation. nd, not detected. ND, not determined.
[a] Yields calculated using the initial weight of the CWM.
[b] Yields for polysaccharide only. PP, polyphenols (detection at 280 nm).

Table 5
Yields and composition (mg/g) of the sequential extraction of the Avrolles procyanidin/cell wall material complexes

Fraction	Yields[a]		Composition (mg/g)									
	PS yields[b]		PP	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Gal A	MeOH
CAV												
Ammonium oxalate	3.0	*0.9*	204	ND	ND	ND	ND	ND	ND	ND	418	32 *13*
			13								*175*	(44 *5*)
Pectin lyase	13.8	*1.1*	39	8	nd	35	2	nd	6	nd	480	84 *4*
			12	*1*		*2*	*0*		*0*		*107*	(97 *14*)
NaOH 4 M	19.4	*1.5*	192	9	19	125	113	50	86	159	106	-
	12.9	*1.4*	*14*	*1*	*1*	*6*	*5*	*2*	*4*	*5*	*20*	
CAVox												
Ammonium oxalate	2.4	*0.9*	129	ND	ND	ND	ND	ND	ND	ND	427	30 *5*
			2								*8*	(39 *8*)
Pectin lyase	16.3	*0.7*	33	9	nd	34	3	nd	8	nd	526	77 *6*
			5	*0*		*1*	*1*		*1*		*40*	(81 *3*)
NaOH 4 M	23.8	*1.3*	203	9	21	117	107	46	84	159	101	–
	15.3	*1.4*	*8*	*1*	*1*	*3*	*1*	*1*	*2*	*3*	*18*	
OxCAV												
Ammonium oxalate	2.2	*0.9*	136	ND	ND	ND	ND	ND	ND	ND	347	26 *0*
			1								*94*	(44 *11*)
Pectin lyase	14.0	*0.8*	33	10	nd	40	3	nd	8	nd	579	82 *3*
			3	*1*		*4*	*0*		*1*		*93*	(77 *3*)
NaOH 4 M	24.2	*0.1*	329	9	20	130	104	49	90	155	117	nd
	16.4	*1.3*	*30*	*1*	*6*	*1*	*25*	*6*	*6*	*27*	*14*	

All extractions were carried out in triplicate. The average and the corresponding error (standard deviation) of measurement were then calculated. Values in italic correspond to standard deviation. nd, not detected. ND, not determined.
[a] Yields calculated using the initial weight of the CWM.
[b] Yields for polysaccharide only. PP, polyphenols (detection at 280 nm).

Table 6
Neutral sugar/galacturonic acid (NS/GalA) and glucose/galacturonic acid (Glc/GalA) ratios of the pectin lyase and the NaOH 4 M extracts of the different procyanidin/cell wall material complexes

Fraction	NS/GalA Pectin lyase extract	Glc/GalA NaOH extract
CWM	0.17	2.9
	0.027	*0.4*
OxCWM	0.20	2.5
	0.001	*0.5*
CMM	0.13	1.9
	0.015	*0.3*
CMMox	0.14	1.6
	0.002	*0*
OxCMM	0.07	1.7
	0.007	*0.3*
CAV	0.12	1.6
	0.055	*0.3*
CAVox	0.10	1.6
	0.009	*0.3*
OxCAV	0.10	1.4
	0.007	*0.4*

Values in italic correspond to standard deviation.

3.4. Sequential extraction

Data were analysed for significance by ANOVA (Fisher *t*-test) for comparisons of means. When referring to the results presented in Tables 3–6, the words "increase" and "decrease" are used throughout the text for differences that are significant at a level of $P < .05$.

3.4.1. Activity of pectin lyase in the presence of procyanidins

Procyanidins bind to proteins and inhibit enzymes such as trypsin, β-glucosidase, xanthine oxidase, or polyphenoloxidase (Guyot, Pellerin, Brillouet, & Cheynier, 1996; Helsper, Kolodziej, Hoogendijk, & Van Norel, 1993; Le Bourvellec, Le Quéré, et al., 2004; Moini, Guo, & Packer, 2000). Here, we first verified that pectin lyase was not inhibited by procyanidins.

In the range of procyanidin concentrations used in the procedure pectin lyase was not inhibited by AV and MM fractions.

3.4.2. Extraction using ammonium oxalate

3.4.2.1. Extraction Yields. The extraction yield of CWM using ammonium oxalate was approximately 4% (Table 3). These results were low compared with those reported by Stevens and Selvendran (1984), Selvendran (1985) and Massiot, Baron, and Drilleau (1994). Stevens and Selvendran (1984) reported yields of 13.2% for the extraction of soluble pectins using hot water (80 °C) and ammonium oxalate in the case of the variety of apple Cox's Orange Pippin. Selvendran (1985) reported yields of 20.9% for the soluble pectins extraction with hot ammonium oxalate. Massiot et al. (1994) obtained yields of 16.9% for soluble pectins extraction with CDTA from the variety of Judeline apple. The differences observed could be due:

- To a lower effectiveness of ammonium oxalate compared to CDTA.
- To the lower temperature used. In our study the extractions were carried out at 40 °C whereas they were done at 80 °C in the other studies (Massiot et al., 1994; Selvendran, 1985; Stevens & Selvendran, 1984). Low temperature was chosen to avoid the decomposition of polysaccharides, in particular the β-elimination of pectins (Renard, 2005), and the formation of phlobaphens from tannins (Bate-Smith, 1954).

Although ammonium oxalate gives yields lower than the CDTA we have used it for two reasons:

- The manipulation of ammonium oxalate is easier than CDTA.
- And the use of CTDA does not provide reliable results as it is extremely difficult to dialyse and can result in artificial weight gain (Mort, Moerschbacher, Pierce, & Marness, 1991).

The low extraction yields and high standard deviation of the yields between repetition, as already reported by Renard (2005),

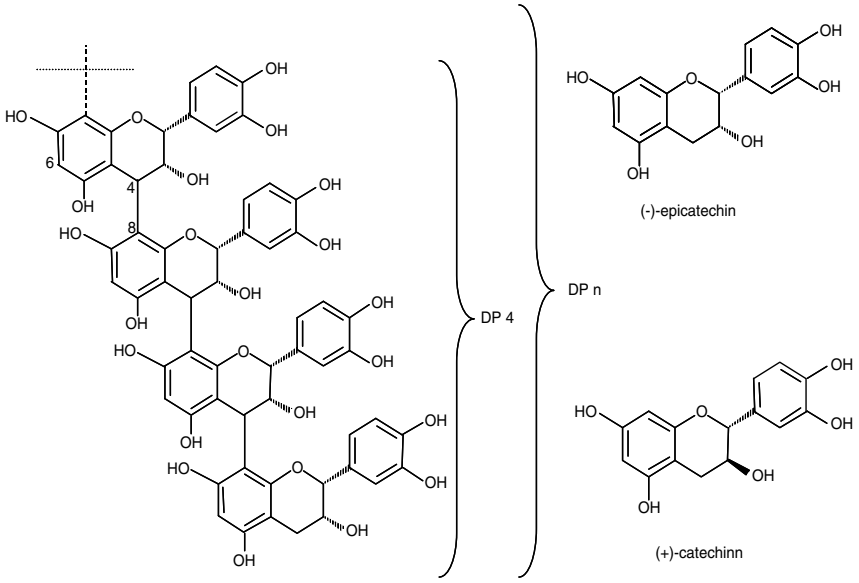

Fig. 1. Structure of procyanidins – example of a (−)-epicatechin based structure adapted from Guyot et al. (1997) and flavan-3-ols constitutive units.

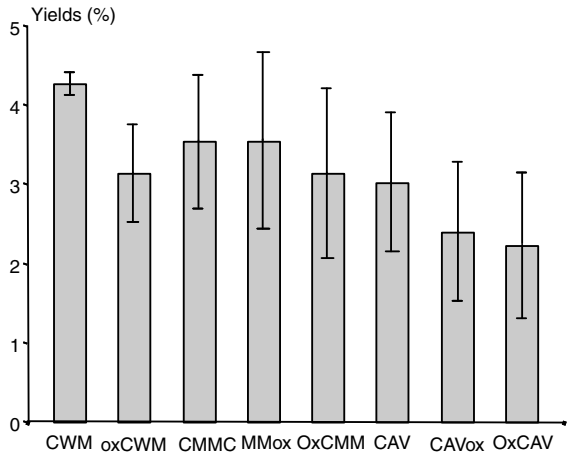

Fig. 2. Polymeric material (weight yield and standard deviation) extracted by ammonium oxalate from apple cell walls and apple cell wall–procyanidin complexes. All extractions were carried out in triplicate. The average and the corresponding error (standard deviation) of measurement were then calculated.

prevented detection of significant differences. However, there was a clear trend for lower extraction yields with procyanidins binding (Fig. 2 and Tables 3–5). There was also a trend for slightly lower yields for high $\overline{DP}n$ and oxidised procyanidins, but it was not statistically significant. Moreover, polyphenols could represent up to 25% of the extract weight. These results and trend were in agreement with Ella-Missang, Massiot, Baron, and Drilleau (1993) and Massiot and Renard (1997) who had shown a reduction in the quantity of pectins extracted by water, oxalate ammonium and buffer after oxidation.

3.4.2.2. Extract composition. Pectins extracted by chelating agents possibly correspond to pectins of the middle lamella (Selvendran, 1985). They consist of highly esterified (degree of methylation (DM > 50) pectins with few rhamnogalacturonic regions and neutral sugars (Massiot et al., 1994; Renard et al., 1990; Stevens & Selvendran, 1984).

There was a trend to lower galacturonic acid concentration in the ammonium oxalate extracts after procyanidin binding, but this was not statistically significant (Tables 3–5). The degree of methylation (DM) of pectins decreased after procyanidin binding, all the more so as the procyanidins had high $\overline{DP}n$. There was a tendency to lower pectin DM of ammonium oxalate extract after procyanidins oxidation, but this was not statistically significant.

Moreover, the quantity of procyanidins extracted using ammonium oxalate treatment decreased when $\overline{DP}n$ increased and with the level of oxidation (Tables 4 and 5).

3.4.2.3. Discussion. There is a tendency to lower ammonium oxalate extraction yields with procyanidins binding, as seen by Ella-Missang et al. (1993), and also a DM reduction of released pectins.

In the case of the initial cell wall CWM, ammonium oxalate, a chelating agent, extracted a low amount of highly methylated pectins, DM 72, as observed by Stevens and Selvendran (1984), Selvendran (1985) and Massiot et al. (1994). Highly methylated pectins were co-extracted with the slightly methylated pectins released by ammonium oxalate, resulting in a high degree of methylation.

In the case of the different procyanidin–cell wall complexes, these highly methylated pectins, were retained in the cell wall by the procyanidins binding. There was, with the procyanidins condensation on highly methylated pectins and the formation of "bridges" between soluble pectins and protopectin, an increased in the specificity of the extraction of the ionically linked pectins, leading to a DM reduction.

The trend to the yields reduction, the diminution of polyphenols extracted by the ammonium oxalate treatment and the DM reduction with $\overline{DP}n$ and oxidation may be related to:

- The strong affinity of the high $\overline{DP}n$ procyanidins for highly methylated pectins because of presence of structures able to bind pectins.
- And with the possibility of formation of covalent bonds (Le Bourvellec et al., 2005; Renard et al., 2001).

This high affinity also resulted in a relatively high polyphenols content in the ammonium oxalate extracts, from 13% to 26%.

3.4.3. Extraction using pectin lyase

The residual pectic material, "insoluble" pectins or protopectin, more strongly retained in the cell wall, can be partly released by

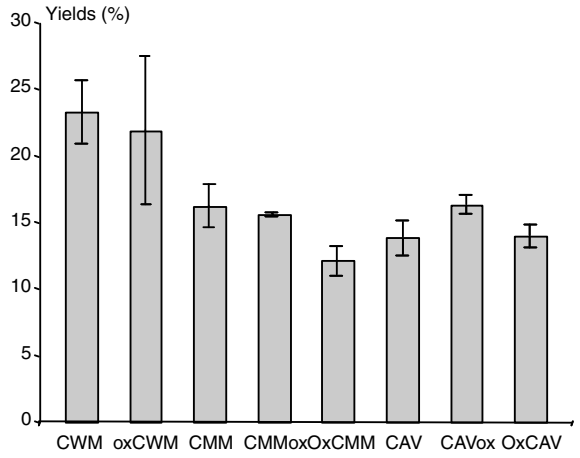

Fig. 3. Polymeric material (pondered yield and standard deviation) extracted by pectin lyase from apple cell walls and apple cell wall–procyanidin complexes. All extractions were carried out in triplicate. The average and the corresponding error (standard deviation) of measurement were then calculated.

the use of methods that degrade cell wall polymers. One of these methods is the use of pectin lyase (Renard, Searle van Leeuwen, Voragen, Thibault, & Pilnik, 1991). Pectin lyase acts by β-elimination between two esterified galacturonic acid residues. Its depolymerizing action degrades the smooth regions of highly methylated pectins and releases galacturonic acid both as oligomers and as a constituent of polymers. These polymers are rich in neutral sugars and correspond to the hairy regions of pectins (Renard, Searle van Leeuwen, et al., 1991).

3.4.3.1. Extraction yields. The yield using pectin lyase was approximately 23% in the case of CWM (Table 3). This was close to those reported by Renard (2005) and Renard et al. (1991a, 1991b), which obtained extraction yields using pectin lyase of 22% or 18.5% and 17.6%, respectively, after preliminary treatment with CDTA of cell walls prepared from Golden Delicious apples.

The extraction yields of sugars by pectin lyase decreased after procyanidins binding and oxidation (Fig. 3 and Tables 3–5). There is a tendency to lower yields of pectin lyase extract with high $\overline{DP}n$ procyanidins, but this was not statistically significant.

3.4.3.2. Extract composition. The material extracted by pectin lyase was mainly composed of galacturonic acid. The main neutral sugar was arabinose followed by galactose, rhamnose and xylose (Table 3). The results obtained were in agreement with those reported by Renard et al. (1991a, 1991b), Renard, Searle van Leeuwen, et al. (1991) and Renard (2005) with however a more marked prevalence of galacturonic acid in the extracts.

The neutral sugar/galacturonic acid ratios of the pectin lyase extracts decreased after procyanidins binding and oxidation (Table 6). There was a tendency to lower neutral sugar/galacturonic acid ratio of pectin lyase extract with high $\overline{DP}n$ procyanidins, but this was not statistically significant. This reduction represented an enrichment in "smooth regions" and oligomers. The DM of the pectin lyase extracts increased when procyanidins $\overline{DP}n$ increased (Tables 4 and 5).

The amount of procyanidins extracted by pectin lyase were low and there was a tendency to lower procyanidin concentration in the pectin lyase extract with higher $\overline{DP}n$. Moreover, it appeared a trend for higher procyanidin concentrations in the pectin lyase extract with oxidation in the case of the Marie Ménard variety (Tables 4 and 5). However, these results were not statistically significant.

3.4.3.3. Discussion. Pectin lyase was used to degrade pectin homogalacturonan and extract "hairy regions" of pectins, with yields >20% (Renard, Searle van Leeuwen, et al., 1991), resulting in a DM of 53 and a high ratio neutral sugar/galacturonic acid of 0.17 in the case of CWM.

The pectin lyase extraction yields also decreased with procyanidins binding. Few polyphenols were extracted at this stage. We had verified before that the pectin lyase was not inhibited by the procyanidins in solution. The yields reduction observed after formation of complexes were thus not due to an enzyme activity reduction but to a reduction in the polysaccharides released. It could be due to the reduction in the accessibility of pectins to pectin lyase (Ella-Missang et al., 1993), in particular by their association with the hairy regions of pectins, which were initially "loose" in the cell wall material, but also with the formation of new bonds. This phenomenon resulted then in:

- An enrichment in smooth regions resulting in a decrease of the neutral sugar/galacturonic acid ratio because procyanidins bound on the initially "loose" polymers.
- And an increase in the DM of released pectins, high DM homogalacturonans being the preferential substrate of pectin lyase. It has recently been demonstrated that pectin neutral sugar side chains, arabinan and galactan, are able to bind to cellulose microfibrils under in vitro and in muro conditions (Zykwinska, Ralet, Garnier, & Thibault, 2005; Zykwinska, Rondeau-Mouro, Garnier, Thibault, & Ralet, 2006; Zykwinska et al., 2007). Procyanidins also have the ability to bind to pectins and cellulose microfibrils (Le Bourvellec & Renard, 2005) and could thus reinforce the non-covalent interactions between pectin neutral sugar side chains, i.e. arabinans and galactans, and the xyloglucan/cellulose network, thus reducing their extraction.

The accessibility limitation of the enzyme was all the more important as procyanidins $\overline{DP}n$ were high and thus that the steric hindrance and/or the possibility of forming new bond were high. The amount of procyanidins extracted by the pectin lyase treatment increased with oxidation in the case of the Marie Ménard variety but decreased with $\overline{DP}n$. This phenomenon could be explained by a weaker affinity of the native procyanidins for oligomers and pectin hairy regions released during the pectin lyase treatment than for the fractions retained in the cell wall.

3.4.4. Extraction using NaOH

3.4.4.1. Extraction yields. The hemicelluloses, which are strongly associated with cellulose by hydrogen bonds can be extracted by concentrated alkali using the residue of pectin extraction (Renard, Lemeunier, & Thibault, 1995; Renard et al., 1991a; Selvendran & O'Neill, 1987; Stevens & Selvendran, 1984). In our case, they were extracted by NaOH 4 M. The yields obtained were ~ 24% (Table 3) higher than those reported by Stevens and Selvendran (1984) (18.2%) for an extraction with KOH 4 M after treatment of the cell wall by successively hot water and ammonium oxalate.

A trend to lower global NaOH 4 M yield was observed with procyanidins binding and high $\overline{DP}n$, but it was not statistically significant (Tables 3–5). However, polyphenols represented a large part of these extracts. In the case of the Marie Ménard variety, the extracts were made, respectively, up to 15%, 17% and 30% of procyanidins, and the polysaccharide yields were only of 15%, 14% and 12%. CAV, CAVox and oxCAV NaOH 4 M extracts were made respectively up of 19%, 20% and 33% of procyanidins, hence the polysaccharide yields were therefore only of 13%, 15% and 16%, respectively. The polysaccharide extraction yields by NaOH 4 M decreased after procyanidins binding (Fig. 4 and Tables 3–5). There is a tendency to lower yields with high $\overline{DP}n$, but this was not statistically significant. A trend to higher NaOH yields was observed with the state of oxidation of the Avrolles fractions whereas a trend to lower NaOH yields was observed with the state of oxida-

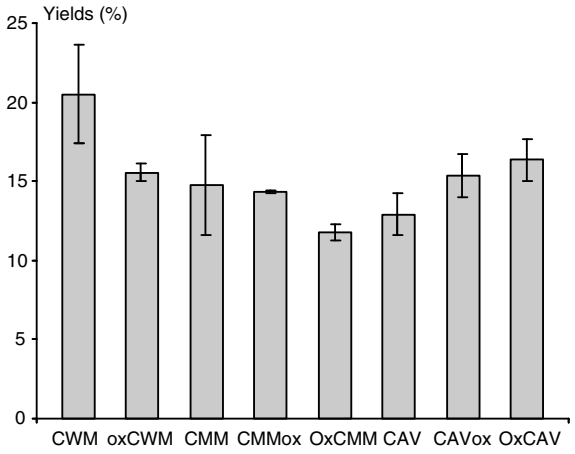

Fig. 4. Polysaccharides material (pondered yield and standard deviation) extracted by NaOH 4 M from apple cell walls and apple cell wall–procyanidin complexes. All extractions were carried out in triplicate. The average and the corresponding error (standard deviation) of measurement were then calculated.

tion of the Marie Ménard fractions. The high standard deviation of the yields between repetition prevented detection of significant differences.

3.4.4.2. Extract composition. The NaOH 4 M extract were mostly composed of fucogalactoxyloglucan, the main hemicellulose of apple fruit (Renard et al., 1991a; Renard, Searle van Leeuwen, et al., 1991; Stevens & Selvendran, 1984) as shown by the typical fucose, xylose, galactose and glucose composition (Tables 3–5). The hemicellulosic fraction also contained mannose, galacturonic acid and rhamnose. The high concentration of mannose translates the presence of mannans (Renard et al., 1991a). Pectins were also extracted by the NaOH 4 M treatment as proved by the presence of galacturonic acid, rhamnose and arabinose (Renard et al., 1991a; Stevens & Selvendran 1984). Femenia, Rigby, et al. (1999) and Femenia, Waldron, et al. (1999) have detected covalently bound pectic–xylan–xyloglucan complexes in the cell walls of cauliflower.

The glucose/galacturonic acid ratio of the CWM NaOH 4 M extract was 2.9 (Table 6) indicating a strong prevalence of hemicelluloses whereas for procyanidin/cell wall material complexes, the ratios decreased with the procyanidins binding. The reduction represented an impoverishment into hemicelluloses relative to pectins. A trend to lower NaOH 4 M yield was observed with high $\overline{DP}n$ and oxidation, but it was not statistically significant.

The amount of polyphenols extracted by NaOH 4 M treatment, higher than those extracted during the two other treatments, increased with procyanidins binding and oxidation (Tables 3–5). A trend to higher procyanidin concentrations with high $\overline{DP}n$ was observed but this was not statistically significant.

3.4.4.3. Discussion. In the case of the initial CWM, concentrated NaOH extracted apple hemicelluloses, a fucogalactoxyloglucan, a mannan as well as pectic material (Renard et al., 1991a). This later was retained in the cell wall after the pectin lyase treatment, either because of a limited diffusion, or because of the existence of hydrogen bonds with other components of the cell wall (Renard et al., 1991a).

In the case of the different complexes, the rupture of the hydrogen bonds during the NaOH 4 M treatment (Selvendran & O'Neill, 1987) induced the release, in addition to hemicelluloses, of hemicelluloses-associated pectins and procyanidins. This phenomenon resulted in an increase in the quantity of extracted polyphenols. The reduction in the ratio glucose/galacturonic acid was connected to affinity of procyanidins for pectins and the procyanidins effect on the former stages as illustrated in Fig. 5. The NaOH 4 M treatment also caused a auto-oxidation of polyphenols and thus the possibility of covalent bonds. Balance between the two phenomena of extraction (rupture of the hydrogen bonds) and reticulation (auto-oxidation) thus seemed to vary between the two methods. The covalently bound pectic–xylan–xyloglucan complexes detected by Femenia, Rigby, et al. (1999) and Femenia, Waldron, et

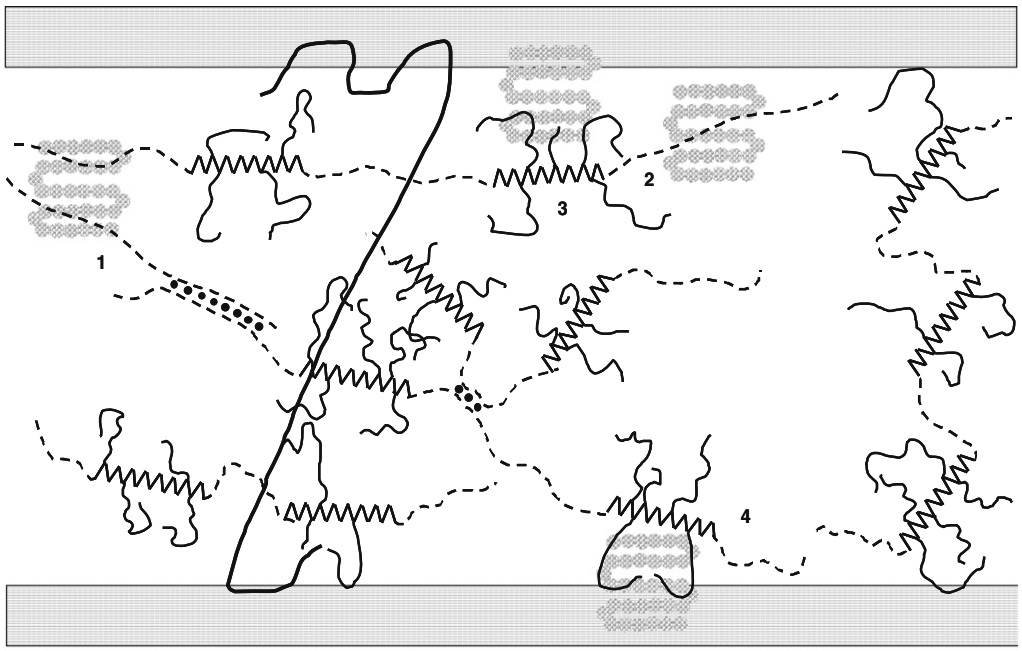

Fig. 5. Potential binding sites of procyanidins and their impact on the postulated extraction mechanisms. (Redrawn from a figure of Zykwinska et al. (2005) illustrating the mode of binding of pectins in the cell wall.) Shaded solid bands, cellulose microfibrils; jagged line, rhamnogalacturonan; dotted line, homogalacturonan; thin solid line, neutral sugar side chain; thick solid line, xyloglucan; grey shaded chain, procyanidin. (1) Highly methylated pectins initially soluble in chelating agent with calcium-mediated cross-link pectins. (2) Pectin smooth regions initially degraded by pectin lyase treatment. (3) Pectin hairy regions initially loose in the cell wall material and liberated by pectin lyase treatment. (4) Reinforced interactions between pectin neutral side chains and cellulose (NaOH 4 M).

al. (1999) in the cell walls of cauliflower could be formed through polyphenol bridges caused by polyphenol auto-oxidation.

4. Conclusions

By interactions between cell walls and procyanidins, one of the main classes of intracellular plant polyphenols, with or without oxidation, we created a new material. The presence of these polyphenols modified the extractability of cell wall polysaccharides:

- Highly methylated pectins became less readily soluble.
- And degradation of pectins by pectin lyase released less "hairy regions".

Binding of intracellular polyphenols thus appears to be an hitherto neglected phenomenon which might have marked consequences on the extractability of cell wall polysaccharides and the picture we build from these results. It is an essential factor to take into account for studies on the cell wall structure. In addition, high amount of polyphenols could be present in the polysaccharide extracts. Thus, the complexes polysaccharides/polyphenols described by Selvendran (1985) are, at least in the case of apple cell wall, an artefact of the isolation procedure related to the adsorption of intracellular polyphenols. This study also confirmed what had been supposed previously, namely a more marked affinity of the procyanidins for pectins than for hemicelluloses and in particular for highly methylated pectins (Le Bourvellec et al., 2005; Renard et al., 2001). The various mechanisms through which polyphenols can influence cell wall polysaccharide extractability are illustrated in Fig. 5. Procyanidins can form bridges between readily soluble pectins (originally soluble after removal of calcium) and protopectin (1). They can also protect pectin homogalacturonans (2) thus preventing their degradation by pectin lyase. By forming bridges (3) between pectin neutral side chain and cellulose (or any cell wall polymers) they decrease the proportion of "loose" hairy regions and thus the amount of rhamnogalacturonan I that can be released from the cell wall by pectolytic enzymes. Last by reinforcing existing interactions between pectic galactan and cellulose (4), they change the effect of concentrated alkalis.

Oxidation increased the ability of procyanidins to bind to apple cell wall material; however the quantitative difference with native polyphenols was slight. The effect of the procyanidins $\overline{\mathrm{DP}}n$ appears to be more important or hides the oxidation effect.

The selectivity of the cell wall material for high molecular weight procyanidins could also have consequences on nutritional and functional properties. A study was undertaken in rats moderately hypercholesterolemic and receiving feeds rich in various fractions of apple: apple pectins, a polyphenol-rich apple concentrate and these two fractions at the same time. Apple pectin and a polyphenol-rich apple concentrate are more effective together than separatively on fermentations of the large intestine and the lipidic metabolism, in particular by reducing the cholesterol and triglycerides of plasma (Aprikian et al., 2003). The effectiveness observed would be due to the interaction between polyphenols and pectins and protection in the gut.

Moreover, when one consumes a fresh apple, enzymatic oxidation may take place already in the mouth when chewing and grinding, we will then be in the case oxCAV and oxCMM with perhaps consequences on the bioavailability of these molecules and their functional activity. Such a material would anyway be more relevant for nutritional studies than separated polyphenol and polysaccharide fractions.

Acknowledgements

The authors thank Agnès Gacel for her excellent technical assistance and Simona Birtic for her critical reading. Part of this work was supported by la Région Bretagne et la Région Pays-de-La Loire (programme inter-régional "Cidre et Polyphénols").

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