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-Interactions between polyphenols and polysaccharides: mechanisms and consequences in food processing and digestion

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ABSTRACT

Interactions between intracellular polyphenols and plant cell-walls have received little attention. They are becoming recognized as a factor to understand extractability, functional and health effects of polyphenols. The most common polyphenols are phenolic acids and oligo or polymeric flavanols (proanthocyanidins), located inside the vacuole in intact plant cells. The proanthocyanidins bind spontaneously to the plant cell-wall polysaccharides through plant tissue disruption, for example during grinding, mastication or thermal treatments, etc. This has profound consequences on the extractability and bioavailability of the polyphenols, on the functional characteristics of extracted polysaccharides, and on the fermentation kinetics of dietary fibers and polyphenols. This presentation will highlight our results on this topic since 2001.

Keywords: procyanidin; pectin; adsorption; isothermal titration calorimetry; colonic fermentation

INTRODUCTION

In intact plant tissues, cell walls, polyphenols and polyphenoloxidase (PPO) are present in distinct compartments. When cells are ruptured, e.g. by grinding and pressing, these three elements come in contact. Polyphenols react with cell wall polysaccharides and can be oxidised by PPO. Winemakers, those who treat external timber with creosote, or laboratory workers using any form of chromatography all know this. The literature however when we started on this investigation in 1999 was sparse and disperse, in contrast to the abundance of data for protein/polyphenol interactions, concentrated on tannin and astringency perception. Three mechanisms can be jointly responsible for formation of polyphenol – cell walls complexes: in addition to adsorption of native and oxidised polyphenols to the cell wall matrix, which will be detailed in the Results, covalent bonds may be formed by reaction between quinones (resulting primarily from action of polyphenoloxidase) or carbocations (resulting from procyanidin cleavage under acidic conditions, Beart et al., 1985) and cell walls polymers. We have taken advantage of the development in polyphenol analysis and purification to set up simple systems that allow to quantify these interactions, to modify the conditions and investigate structure / affinity relationships for polyphenols and for polysaccharides (Renard et al., 2001; Le Bourvellec et al., 2004, 2005a,b, 2013). More recently physical methods such as isothermal titration calorimetry were used to gain insight in the mechanisms (Le Bourvellec et al., 2012 ; Watrelot et al., 2013, 2014). In the last few years we have turned our attention to the impact of thermal treatments (Le Bourvellec et al., 2011; 2013), with formation of covalent adducts between polyphenols and cell walls, and to consequences in terms of polyphenol bioavailability.

Polyphenols are for the most part present in cell vacuoles. Apple and pear fruits were chosen because of their polyphenol composition and high concentrations, notably in cider apples and perry pears (up to 7 g/kg fresh weight in the parenchyma of ripe fruit). These polyphenols are relatively simple, compared to e.g. grapes. Procyanidins (flavan-3-ol oligomers and polymers) composed essentially of (-)-epicatechin are the main class in the fruit flesh of both apple and pear; their degree of polymerisation vary between the cultivars and can be very high (> 100). The other classes are phenolic acids, mostly chlorogenic acid, and (in apple only) dihydrochalcones (phloretine glycosides), and monomeric flavan-3-ols, again mainly (-)-epicatechin. All of this makes them eminently suitable for isolation of well-defined fractions. Plant cell walls are a complex, porous polysaccharidic material. In fruits and vegetables, they can be described by the type I model of Carpita & Gibeaut (1993) as composed of three interpenetrating but not interconnected network:

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a cellulose / xyloglucan framework (> 50% dry weight) is embedded in a pectin matrix (25-40% dw), locked into shape by cross-linked glycoproteins (extensine, about 1% dw). The cell wall composition of apple is well studied, and corresponds well to this model.

Consequences of polyphenol – polysaccharide interactions are far reaching in food processing. For example, they contribute to the selective extraction of polyphenols from apple to apple juice (Le Bourvellec et al., 2007; Renard et al., 2011), and even more important from grape to must, as has been clearly shown by Bindon, Kennedy or Gomez-Plaza's works (e.g. Ruiz-Garcia et al., 2014; Revelette et al., 2014; Bautista-Ortin et al., 2015). They also result in the major part of the so-called "unextractable polyphenols" (Perez-Jimenez et al., 2013) or formation of pomaces, where cell walls and (oxidised) polyphenols form a single material, with antioxidant capacity but also difficult reextraction of the polyphenols and colours which may be detrimental for their valorisation as dietary fibers. It also modifies the extractability of the cell wall polymers (Le Bourvellec et al., 2009), decreases their enzyme susceptibility and affects their fermentability (Bazzocco et al., 2008; Aura et al., 2013). This has also nutritional impacts: polyphenol- cell wall interactions limit bioavailability of polyphenols, but they may contribute to the formation of bioactive phenolic metabolites in the gut.

Our main results on mechanisms, affinities, and consequences of polyphenol (primarily procyanidins) – cell wall interactions will be presented.

MATERIALS & METHODS

Preparation of cell walls and polyphenols: Preparation of cell walls was carried out using a phenol-buffer method detailed in Renard (2005a) in order to obtain cell wall devoid of procyanidins and with low protein content. Polyphenols were extracted from apple or pears using basically the procedure detailed in Le Bourvellec & Renard (2004). In summary, freeze-dried fruit powders are extracted first with hexane (discarded), then by acidified methanol (25 mL acetic acid per liter), then by acetone-water (600mL:400mL); polyphenols are first purified by preparative chromatography on a C18 column and for purification of individual oligomers by normal phase. Methanol-acetic acid extracts were used for purification of procyanidins of low degree of polymerisation, and acetone-water-acetic acid extracts for high molecular weight procyanidins, the exact degree of polymerization depending on the chosen apple or pear cultivar. All polyphenol analyses are carried out after thioacidolysis (Guyot et al. 2001), and all polysaccharide analyses as described in Renard (2005a) and Renard & Ginies (2009).

Adsorption experiments: Cell wall suspensions (25 mg in 4 ml) and polyphenol solutions (1 mL, varying concentrations) were incubated in an 8 mL empty Sep-pack prep column (InterchimTM) equipped with a sinter of porosity 20 µm under planetary agitation at 25°C. After incubation, the solution and the cell wall/polyphenol complexes were separated by filtration under vacuum. Polyphenol contents were measured by absorbance at 280 nm and/or thioacidolysis after freeze-drying as described in Analytical. Sugar composition was determined after freeze-drying and hydrolysis. The conditions which were varied were solvent composition, time (5 to 120 min), pH (phosphate: citrate buffer pH 2.5, 4 and 6) and polyphenol nature and concentration (up to 10 g/L). The cell wall polyphenol complexes thus prepared were used for desorption experiments and enzyme degradation.

Isothermal titration calorimetry: VP-ITC microcalorimeter (Microcal[®], GE Healthcare, Chalfont St. Giles, United Kingdom) or a TAM III microcalorimeter (TA instruments, New Castle, USA) were used at 25°C. Procyanidins and pectin fractions were dissolved in the same phosphate: citrate buffer pH 3.8, ionic strength 0.1 mol/L, filtered on 0.45µm membrane. All solutions were degassed prior to measurements. The reference cell was filled by water. In a typical experiment, a pectin solution (30mM) was placed in the sample cell of the calorimeter and a procyanidin solution (30mM) was loaded into the injection syringe. Procyanidin solution was titrated into the sample cell as a sequence of 30 injections of 10 µL aliquots. The duration of each injection was 20 sec, and the time delay between successive injections was 5-10 min. The contents of the sample cell were stirred throughout the experiment to ensure mixing.

Fermentation: *in vitro* fermentation experiments were carried out using purified polyphenols, purified cell walls, or apple purees produced with different heat treatments. They were fermented by microbiota from

healthy human volunteers. Extent of fermentation was followed by production of short chain fatty acids by RMN, production of colonic polyphenol metabolites by GC-MS and degradation of polyphenols by HPLC after thioacidolysis.

Theoretical considerations: Isotherms were fitted using the Langmuir equation, generalized to the binding of a solute to a solid.

$$PPb = N_{max} K_L [PP]f / (1 + K_L [PP]f) \quad \text{Eq. 1}$$

Where PPb is the amount of bound polyphenols in g per g of cell wall, N_{max} is the total amount of available binding sites, [PP]f the concentration of free procyanidins after interaction and K_L an affinity constant.

For ITC, the thermodynamic parameters were calculated from the van't Hoff equation using the manufacturers' software:

$$\Delta G = -RT \ln K_a = \Delta H - T\Delta S \quad \text{Eq. 2}$$

Where ΔG is free enthalpy, K_a is the association constant, ΔH is the enthalpy and ΔS is the entropy of reaction.

RESULTS & DISCUSSION

The first observation that led us to investigate this phenomenon was the observation of a marked discrepancy in polyphenol composition between apples and their juice (Fig.1). While in apples the main polyphenol class is that of procyanidins (condensed tannins), in apple juice phenolic acids become much more important. This was especially obvious for apples like GU (Guillevic) variety, containing primarily procyanidins of high degree of polymerisation.

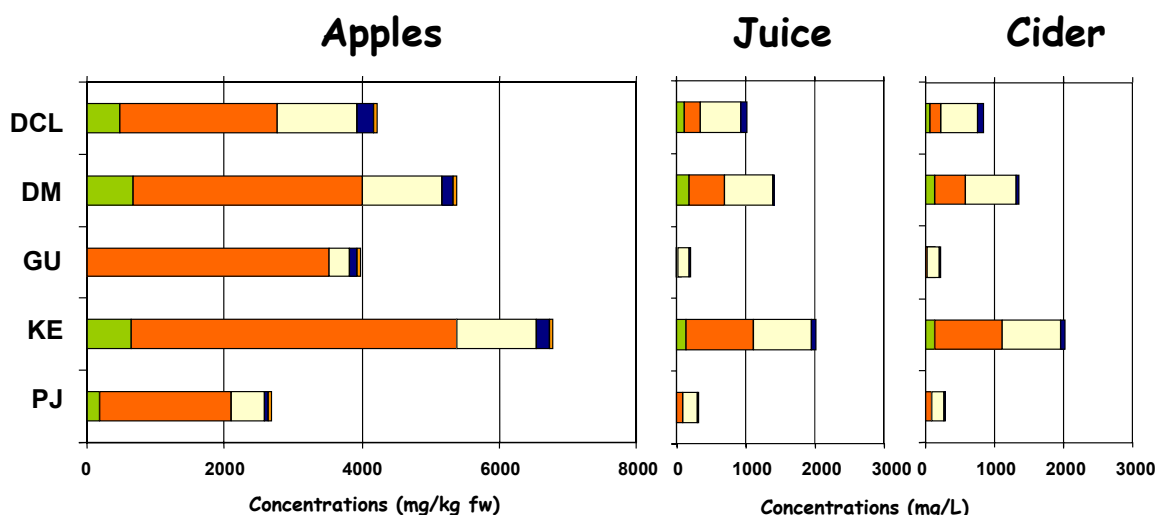


Fig 1: Polyphenol composition of apples, their juices obtained in oxidation-limited conditions and the ciders. Apple varieties: DCL: 'Douce Coët Ligné' ; DM 'Douce Moens'; GU: 'Guillevic'; KE: 'Kerrmerien' and 'PJ 'Petit Jaune'. Polyphenol classes (from left to right): in green monomeric catechins; in orange procyanidins; in pale yellow phenolic acid; in dark blue dihydrochalcones, and in orange flavonols.

To better investigate the phenomena leading to retention of polyphenols, and particularly procyanidins, in apples during pressing, we devised a simple system in which procyanidins and cell walls, previously isolated from apples, were put in contact, and after incubation the free procyanidins were separated by filtration and analysed quantitatively and qualitatively (for their degree of polymerisation). A fast absorption of procyanidins to the cell walls was observed, with most absorption occurring in the first 5 min, and maximum absorption levels being reached in 20 min, whatever the degree of polymerisation. Similar levels were observed for pHs from 2 to 7. Varying procyanidin concentrations were used to obtain binding isotherms, such as the ones pictured in Fig 2, with procyanidins varying from 3 to 70 monomeric (-)-epicatechin units. At low polyphenol concentrations, very little procyanidins remain free in solution, particularly for the higher degrees of polymerisation. As the procyanidin concentrations increased, the

proportion of free procyanidins increased, until a plateau was reached, particularly for the larger procyanidins. The plateau levels were surprisingly high, as the weight of bound procyanidins reached 80% of the initial cell wall weight. The data were adequately fitted by Langmuir isotherm formulation, allowing calculation of affinity constants and saturation levels.

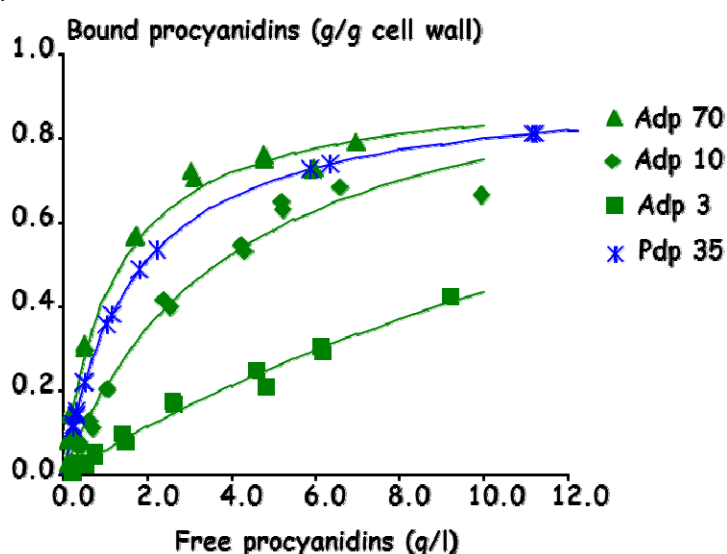


Fig 2: Binding isotherms obtained with apple cell walls and procyanidins from different degrees of polymerisation. Redrawn from Le Bourvellec et al., 2005a.

Impact of cell wall physical and chemical features was less dramatic than for procyanidins, however drying impacted the binding affinities and maximum levels, while pectin extraction decreased affinity (Le Bourvellec et al., 2005a, 2012; Renard et al., 2001). Binding isotherms carried out on purified polysaccharides clearly indicated highest affinities for pectins, compared to cellulose, starch and xyloglucans (Le Bourvellec et al., 2005b). Temperature also modified the binding of the procyanidins, with decreased levels for higher temperatures. This was validated in apple pressing, for temperatures from 4°C to 25°C: while, in absence of oxidation, extraction was almost quantitative for hydroxycinnamic acids at all temperatures, there was an impact on transfer for all flavan-3-ols, particularly procyanidins (Renard et al., 2011), with about 30% higher extraction at 25°C than at 4°C.

Table 1: Procyanidins and chlorogenic acid concentrations (mg/L) in apple juices prepared under anaerobic conditions as a function of temperature. CQA: chlorogenic acid; Procy: procyanidins.

	Douce Moens		Guillevic		Kermerrien	
	CQA	Procy	CQA	Procy	CQA	Procy
4°C	1 099	853	80	121	862	1 349
11°C	1 158	986	82	107	1 000	1 520
18°C	1 213	1 067	85	132	950	1 821
25°C	1 311	1 396	96	146	990	1 842

Isothermal titration calorimetry was carried out using pectins and pectin fractions, namely homogalacturonans with different degrees of methylation (0, 30 and 70), rhamnogalacturonan II and type I rhamnogalacturonans with different side chain contents. ITC confirmed that affinities increase with procyanidin degree of polymerisation and with homogalacturonan degree of methylation. For type I rhamnogalacturonans, there was a strong impact of the precise nature of the side chains, while no affinity was obtained with rhamnogalacturonan II (Watrelet et al., 2013, 2014). The thermodynamic parameters further confirmed a predominant role of hydrophobic interactions.

Impact of thermal treatments: Retention of procyanidins occurs also after plant tissue disruption induced by thermal treatments. This was evidenced in analysis of apple purees and pear wedges, where high

concentrations of procyanidins are detected in the solid products (apple puree particles, pear wedges) (Renard, 2005b; Le Bourvellec et al., 2011, 2013). In pears submitted to prolonged thermal treatment a further phenomenon was observed with apparition of a pink discoloration. This pink discoloration could be replicated in model solutions or in cell wall - procyanidin adducts, and was not re-extractable with solvent or with enzymes, leading us to suspect formation of covalent adducts (Fig. 3). Mass spectrometry identification of thyoacidolysis products allowed us to identify formation of cyanidin and (-)-epicatechin-cyanidin dimer, probably through acid cleavage of the interflavanic bond followed by oxidation, as described by Beart et al. (1985).

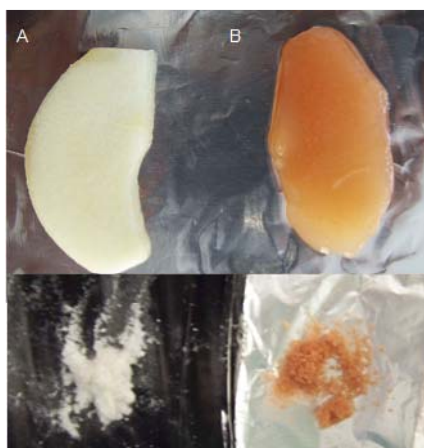


Fig 3: Pink discoloration in pear after prolonged heat treatment in acidic conditions: colour effects

Fermentation experiments: Pure procyanidins led to slow formation of volatile metabolites, and their addition to cell walls decreased the speed and extent of fermentation. This effect was all the more marked for larger procyanidins. Fermentation of cell-wall – procyanidin adducts and cell-wall procyanidin complexes isolated from thermally treated fruits indicated an effect of heat-treatment, with increased fermentation extents.

CONCLUSION

Interactions between polyphenols, and particularly tannins, and polysaccharides are fast and spontaneous, which means that they occur systematically in fruit and vegetable processing. The binding is due to a combination of hydrogen bonds and hydrophobic interactions, and is favored by increased ionic strength and decreased temperature. Higher affinities are found for proanthocyanidins of high degree of polymerization. Within the cell walls, higher affinities for pectins were confirmed in solution with various structural pectin parts using isothermal titration calorimetry. Another parameter is the physical state of the cell-walls, with a major impact of drying.

As the interactions are susceptible to temperature, we could use them to manipulate the phenolic composition of ciders: lowering the temperature and increasing duration of maceration leads to lower bitterness and astringency. In fruit purees, proanthocyanidins are concentrated in the particles, bound to the insoluble cell walls, contributing to their very low bioavailability. In the lower gut, polyphenols decrease the fermentability of the polysaccharides, while the cell-walls act as substrate for bacteria that are able to degrade the polyphenols in bioavailable metabolites.

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