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Isolation and characterisation of the two main cell-wall types from guava (*Psidium guajava* L.) pulp *

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ABSTRACT

Cell-wall materials (CWM) were extracted separately from the endocarp and mesocarp portions of guava fruit (cv. Suprême) by the sodium deoxycholate–(phenol–acetic acid–water) extraction procedure, and further fractionated by enzymatic and physical means into fragile parenchymatous (PAR) and hard stone (STO) cell walls. Mesocarp contained ~ 90% of the total CWM of the edible part (excluding skin and seeds) of guava, ~ 74% of which were stone cells, while endocarp was relatively richer in parenchymatous tissue. PAR cell walls had similar composition in both endocarp and mesocarp, and were made up of ~ 55–60% of neutral polysaccharides (mainly cellulose, xyloglucan, xylan, arabinan, and arabinogalactans of both types I and II) associated with weakly methyl-esterified galacturonan. STO cell walls were strongly lignified, secondarised elements, ~ 50% consisting of equivalent amounts of cellulose and acetylated xylan.

INTRODUCTION

Guava (*Psidium guajava* L.), a popular fruit throughout the tropics, is eaten fresh or made into juice, nectar, puree, jam, or jelly after elimination of the seeds[1]. Fruit pulps are nowadays increasingly treated with exogenous enzymes[2,3], e.g., to improve juice extraction, but few data are available on guava enzymatic processing[4–6]. However, enzymes are still often applied on empirical grounds without considering the specificity and variability of the soluble and cell-wall polysaccharides to be degraded.

Guava pericarp, like pear[7,8], exhibit heterogeneous tissues, two main cellular types being encountered[5,9,10] in pulp: parenchyma (PAR) and stone (STO) cells

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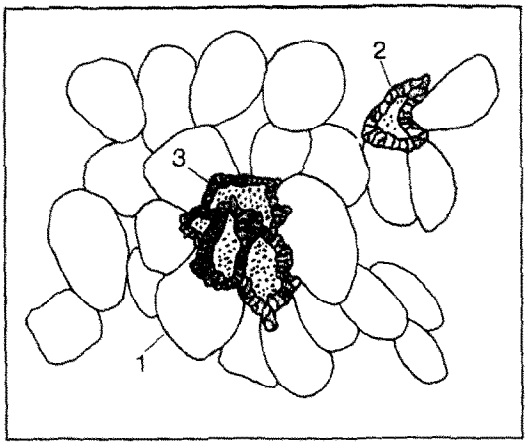

Fig. 1. 1, Parenchymatous cells; 2, single stone cell; 3, stone cell nugget.

(Fig. 1) which give a characteristic sandy or gritty feeling in the mouth when guava is eaten. Since stone cells are strongly lignified woody material[8,9], they are resistant to enzymatic degradation[8], thus, hydrolases probably act on parenchymatous cell walls only. Estimation of the effects of enzyme mixtures on total cell-wall material[6] from guava pulp might be obscured because of the unknown proportions of both cell types. Moreover, guava fruit is made up of a fleshy mesocarp of varying thickness and of a softer endocarp that embeds the seeds. We now report the isolation, quantitation, and characterisation of the two main cell-wall types from guava seed-free endocarp and mesocarp.

EXPERIMENTAL

Plant material.—Sound white guavas (cv. Suprême) belonging to the same batch were harvested at the "turning" stage (mature–ripe transition) in Cameroon during the summer of 1989[11,12]. Fruits were frozen in liquid nitrogen and stored at −30 °C until used.

Preparation of the cell-wall materials (CWM).—Frozen guavas were peeled, and the mesocarp and endocarp (including the central core and the seeds) were carefully hand-separated[11], cut into small pieces, and dipped in cold 1% sodium deoxycholate (SDC) containing 6 mM sodium metabisulfite (liquid–solid w/w ratio, 2)[13]. The slurry was gently homogenised with a Polytron® (Kinematica GmbH, Switzerland) and stirred for 16 h in the cold (4°C). Seeds were eliminated from the endocarp mixture by screening on a 2-mm sieve. Both suspensions were then centrifuged at 5000*g* for 10 min (0°C), and the residues were washed (3 ×) with excess aq 6 mM sodium metabisulfite with intermittent centrifugations. Wet

residues were then extracted (3 ×) with 2 : 1 : 1 phenol–acetic acid–water (PAW)[13], centrifuged, and washed once with 6 mM sodium metabisulfite and twice with water. Residues [$CWM_{(PAW)}$-meso, $CWM_{(PAW)}$-endo] were then dried on glass crucibles by solvent exchange through EtOH, acetone, and ether, and stored for 24 h in a vacuum oven (50°C, 9 kPa) over phosphorus pentaoxide (20°C).

Alcohol-insoluble residues (AIR) were also prepared for comparison purposes from portions (mesocarp, endocarp) of guavas from the same batch[11]. Water-soluble pectic substances were then extracted[11] with 0.2 M sodium acetate buffer (pH 5) containing pronase at 25°C, and the residues were dried by solvent exchange as above [$CWM_{(AIR)}$-meso, $CWM_{(AIR)}$-endo].

Preparation of parenchyma and stone cell walls.—Stone cell walls were quantitatively obtained as follows: cell wall materials [$CWM_{(PAW)}$-meso, $CWM_{(PAW)}$-endo] (1 g) were suspended in 0.1 M acetate buffer (200 mL, pH 4.2), and Pectinex Ultra SP-L (1.85 mL, batch BA 016; Novo-Nordisk Ferments AG, Switzerland) was added. After 48 h at 30°C, with stirring, the medium was centrifuged at variable decreasing speeds ($2000g \rightarrow 25g$) for various times (1 → 5 min), the upper layer of residual parenchymatous cell-wall ghosts being carefully eliminated with a Pasteur pipette while avoiding removal of denser stone cell walls. The purity of stone cells was checked under a light microscope by the absence of Congo Red stainable parenchyma cell walls, then the pellet was extensively washed with distilled water and dried by solvent exchange [$STO_{(PAW)}$-meso, $STO_{(PAW)}$-endo].

Parenchyma cell walls were cleanly separated, non-quantitatively, from stone cells as follows: cell-wall materials [$CWM_{(PAW)}$-meso, $CWM_{(PAW)}$-endo] (0.2 g) were suspended in aqueous 6 mM sodium metabisulfite (50 mL) and treated in an Elvehjem Potter (Wheaton, USA) for dissociating both types of cells. Parenchyma cell walls were recovered in the upper aqueous phase above the rotating pestle, while stone cells tended to settle at the bottom. Partly purified parenchyma cell walls (still contaminated by some stone cells) were then centrifuged at variable speeds, and the upper layer was carefully recovered. These operations were repeated until parenchyma cell walls were free from stone cells as checked under a light microscope by the absence of phloroglucinol–HCl stainable elements. Walls were then washed and dried as described above [$PAR_{(PAW)}$-meso, $PAR_{(PAW)}$-endo].

Analytical methods.—Moisture contents were determined by drying over phosphorus pentaoxide at 50°C for 24 h under diminished pressure (9 kPa); all data are given on a moisture-free basis. Prior to analyses, cell walls were ultra-milled in liquid nitrogen (−196°C) in a Spex 6700 Freezer-mill for 3 min (top impact frequency)[14].

Neutral monosaccharides were released from cell walls (5 mg) by hydrolysis with 2 M trifluoroacetic acid (0.5 mL, 1.25 h, 120°C)[15] or following an optimised Saeman procedure (0.15 mL of 72% H_2SO_4, 30 min, 25°C; then dilution to M, 2 h, 100°C)[16], derivatised as their alditol acetates, and then analysed by GLC at 210°C on a DB-225 capillary column (30 m × 0.32 mm i.d., 0.25-μm film; J&W Scientific) with H_2 as the carrier gas[17]. Ultra-milled cell walls were also methylated with

methyl iodide according to Hakomori[18]. After hydrolysis with 2 M trifluoroacetic acid (1.25 h, 120°C), the partially methylated sugars were converted into alditol acetates and analysed on DB-1 and DB-225 capillary columns[17]. Identifications were based on retention times and confirmed by GLC–MS using the DB-225 column (on-column injection at 50°C, injector 50 → 220°C at 60°C/min, oven 90 → 170°C at 10°C/min then 5°C/min to 210°C; He as carrier gas at 2 mL/min) coupled to a Finnigan Mat ITD 400 mass spectrometer.

Uronic acids were measured by the *m*-phenylphenol method according to Ahmed and Labavitch[19], galacturonic (methyl-esterified or not) and glucuronic acids giving the same responses. Starch was determined after hydrolysis by amyloglucosidase[20].

Methyl groups were measured after de-esterification, by enzymatic oxidation of MeOH by alcohol oxidase followed by colorimetric determination of formaldehyde[21] as follows: cell walls (1 mg) were treated under continuous stirring by 0.5 M KOH (2 mL) at ambient temperature. After adjustment to pH 7.5 with 85% orthophosphoric acid (~ 30 μL), 0.5 M phosphate buffer (pH 7.5, 2 mL) was added, and then MeOH was determined as described.

Acetyl groups were determined by an enzymatic UV method[22] as follows: cell walls (10 mg) were treated under continuous stirring with 0.4 M NaOH (1 mL). After 2.5 h at ambient temperature, samples were neutralised by addition of 85% orthophosphoric acid (20 μL), and then centrifuged for 5 min at 10000*g*. Determination was carried out on the supernatant solution as follows: acetic acid is converted into acetyl-CoA in the presence of acetyl-CoA synthetase (ACS) with adenosine-5′-triphosphate (ATP) and co-enzyme A (CoA) (reaction *1*); acetyl-CoA reacts with oxaloacetate to form citrate in the presence of citrate synthase (CS) (reaction *2*); the oxaloacetate required for reaction *2* is formed from malate and nicotinamide adenine dinucleotide (NAD) in the presence of malate dehydrogenase (MDH) (reaction *3*), determination being based on formation of NADH measured by the increase in absorbance at 340 nm. Enzymes, coenzymes, and substrates were obtained from Boehringer Mannheim (Meylan, France). Absence of possible interfering or inhibiting substances was checked by increasing the volume of supernatant solution to be assayed or adding known amounts of sodium acetate.

Methanol and acetic acid were also measured[23] after treatment of cell walls (20 mg) with 0.4 M NaOH in 1:1 water–2-propanol (1 mL) for 2.5 h at ambient temperature. A column (30 × 0.78 cm i.d.) of Aminex HPX 87H (Bio-Rad), thermostated at 30°C, was used and eluted with 0.03 M H_2SO_4 at 0.7 mL/min; detection was achieved with an ERMA-ERC 7512 refractive index detector set at 40°C.

Proteins were measured by the Kjeldahl procedure as described by Moll et al.[24]. Lignin was determined according to Klason[25], as adapted by Effland[26], and was not corrected for protein. Proanthocyanidins were measured according to ref. 27, a grape-seed proanthocyanidin trimer[28] being used for standardisation.

RESULTS AND DISCUSSION

Separation of parenchyma and stone cell walls.—Cell-wall materials [$CWM_{(AIR)}$-meso, $CWM_{(AIR)}$-endo] were initially prepared from guava mesocarp and endocarp, respectively, by inactivation of endogenous enzymes in boiling 80% ethanol and subsequent extraction of water-soluble pectic substances in 0.2 M acetate buffer (pH 5) containing pronase[11]. Parenchyma and stone cell walls were then separated as indicated above. Nitrogen determination gave "protein" contents ($N \times 6.25$) of ~ 15–20% for both mesocarp and endocarp parenchyma cell walls [$PAR_{(AIR)}$-meso, $PAR_{(AIR)}$-endo], which was indicative of contamination by alcohol-coprecipitated cytoplasmic proteins[13] resistant to the pronase treatment. Moreover, attempts to "liquefy" these purified parenchymatous cell walls with a commercial enzyme mixture (Pectinex Ultra SP-L from Novo-Nordisk Ferments AG, Switzerland) were only partly successful, an average maximum ~ 50% hydrolysis being observed, which led to the hypothesis that access of enzymes to wall polysaccharides was partly hindered by coprecipitated proteins.

Thus, the SDC–PAW extraction procedure[13] was preferred, providing parenchyma cell walls [$PAR_{(PAW)}$-meso, $PAR_{(PAW)}$-endo] with lower "protein" contents, typically ~ 5–6%. It must be noted that blending of guava pulp in deoxycholate with a Polytron was efficient enough to disrupt fragile thin parenchyma cells, while light microscope examination of purified hard stone cells revealed single or overlapping, heavily pitted, intact cells (Fig. 1). However, stone cells appeared under electron transmission microscopy[29] to have virtually no cytoplasmic content. Purified cell walls were starch-free.

Distribution of parenchyma and stone cell walls in guava is given in Table I. Cell walls from stone cells [$STO_{(PAW)}$-meso, $STO_{(PAW)}$-endo] (including minute proportions of vascular elements) represented by far the major portion of total CWM in guava pulp (~ 74%) and, since they are resistant to enzymatic hydrolysis[8], calculation of a percentage of hydrolysis on a total CWM basis is not possible without preliminary separation of both cellular types. The mesocarp portion contains ~ 90% of total CWM of guava pulp (excluding epicarp and seeds), ~ 77% of which are stone cells. This must be related to the harder consistency of mesocarp as compared to the soft endocarp which is poorer in CWM but relatively richer in parenchyma cells. Thus, the "sandy" feeling in the mouth[10] is mainly due to the mesocarp flesh. The relative percentage weight of mesocarp and endocarp in fresh guava (Table I), which depends on maturity stage and cultivar, could be an important parameter in juice extraction technology due to this unequal cell-wall distribution.

The composition of parenchyma and stony cell walls is given in Table II. Half of the stone cell walls was made up of neutral polysaccharides, ~ 94% of which are cellulosic glucose and xylose. Some uronic acids (presumably glucuronic acid and/or its 4-methyl ether) were also present, and proteins were negligible. Conversely, *O*-acetyl groups were important and could be related to xylose-con-

TABLE I

Distribution of parenchymatous (PAR) and stone (STO) cell walls in guava fruit [a] and its main edible portions (endocarp and mesocarp)

	Endocarp			Mesocarp		
	CWM	PAR	STO	CWM	PAR	STO
Fresh fruit [b] (%)	0.3	0.2	0.1	3.1	0.7	2.4
Fresh endocarp or mesocarp [c] (%)	1.5	0.9	0.6	4.9	1.1	3.8
CWM [d] (%)	100	63.3	36.7	100	21.9	78.1

[a] Relative fresh weight distribution of constitutive portions of guava fruit (as %/whole fresh fruit): mesocarp (65%), endocarp (+central core) (22%), seeds (2%), epicarp (skin) (11%). [b] Expressed as weight percent of fresh guava fruit (excluding epicarp and seeds, i.e., 87% by weight of the fruit = guava pulp). [c] Expressed as weight percent of each fresh portion of the fruit, endocarp and mesocarp, respectively. [d] Expressed as weight percent of total cell-wall material (CWM) from each portion of the fruit, endocarp and mesocarp, respectively.

taining polymers[30]. Stone cell walls were highly lignified, as measured by the Klason procedure, and stained deep fuschia with phloroglucinol–HCl reagent, as did very scarce xylem elements. The Klason lignin was not corrected for sulfuric acid-insoluble proteins due to their negligible amount in native cell walls (Table II). Stone cell walls, also encountered in pear[7,8], plum[7], and olive[31] mesocarps, appear under electron transmission microscopy as very thick, dense, multilayered walls surrounding an almost empty lumen. These observations are corroborated by our data which characterise these elements as woody secondarised "dead" cells.

Parenchymatous cell walls, apart from their galactose content, had virtually the same composition in mesocarp and endocarp tissues. They were very different from stone cell walls by being virtually unlignified. Although it has not been possible to measure lignin in parenchyma cell walls due to the large amounts required, they exhibit negative staining with phloroglucinol–HCl. Cellulosic glucose constituted ~50% of neutral polysaccharides, other sugars being, in decreasing order of importance, arabinose, xylose, galactose, mannose, rhamnose, and fucose. Acidic polysaccharides were also present in appreciable amounts, and were partially methyl-esterified. *O*-Acetyl groups were also detected, but could not be assigned to galacturonans only since they could also esterify xylose- and/or non-cellulosic glucose-containing polymers which were found in the walls. The presence of contaminating cytoplasmic proteins in parenchymatous cell walls purified from alcohol-insoluble residues [$PAR_{(AIR)}$-meso, $PAR_{(AIR)}$-endo] which were further resistant to pronase led us to think of the presence of tannins, as previously observed in cell-wall materials from tannin-containing plants[32]. Indeed, proanthocyanidins were found in similar amounts in both parenchymatous tissues, and to a lesser extent in stone cell walls. However, values appearing in Table II must be considered as minima, since it is known that the recovery of anthocyanidins from the parent proanthocyanidins is not quantitative for highly condensed tannins. Their actual location in guava fruit is not known, since they could either be an integral part of walls or might have been insolubilised from the liquid phase

TABLE II

Composition [a] of parenchymatous (PAR) and stone (STO) cell walls from guava endocarp and mesocarp and of total cell-wall material (CWM) from guava pulp

	Endocarp	Mesocarp		Pulp
	PAR	PAR	STO	CWM
Uronic acids [b]	17.3	16.4	5.2	8.6
Total neutral sugars [c]	55.6	58.3	50.0	53.3
Protein (N×6.25)	6.4	5.3	1.1	1.9
Methanol	1.0 (32.8) [d]	0.9 (31.2) [d]	0.1	0.5
Acetic acid	2.5	2.7	7.3 (72.6) [e]	6.5
Proanthocyanidins	>1.1	>1.4	>0.5	>0.8
Lignin	nd	nd	22.3	20.2
Rhamnose [f,g]	2.4	1.4	0.6	0.8
Fucose [f]	1.5	1.6	–	0.3
Arabinose [f]	14.5	16.6	1.3	5.8
Xylose [f]	14.8	14.4	50.0	41.9
Mannose [f]	3.4	4.4	0.7	1.1
Galactose [f]	10.2	5.5	0.7	1.9
Glucose (non-cellulosic) [f,g]	6.6	2.4	2.7	2.6
Glucose (cellulosic) [f,h]	46.6	53.7	44.0	45.6

[a] %(w/w). [b] Expressed as "anhydrogalacturonic acid". [c] Neutral sugars determined by GLC of the alditol acetates, and expressed as "anhydrosugars". [d] Values in parentheses are the degrees of methylation (dm) calculated as the molar ratios of methanol to "anhydrogalacturonic acid"×100. [e] The value in parentheses is the degree of acetylation (da) calculated as the molar ratio of acetic acid to "anhydroxylose"×100. [f] Mol% of the constituent monosaccharides. [g] Obtained by CF_3CO_2H hydrolysis. [h] Obtained by difference between Saeman and CF_3CO_2H hydrolyses.

during the purification treatment. However, this uncertainty must be kept in mind for further interpretations of enzymatic treatments of guava puree.

Analysis of the neutral and acidic sugar composition of total CWM obtained by SDC–PAW extraction of the whole pulp (parenchyma and stone cells from both mesocarp and endocarp in admixture) from guava has also been performed and compared favourably (~90–100% recovery) (Table II) with analyses of each cell-wall fraction and their respective proportions in fruit (Table I). Our data were very different from the compositions of mature and ripe guava AIRs published by Askar et al.[5]. Since guava water-soluble pectic substances, still present in AIRs, contain only traces of mannose[11], the dominant proportion of mannose (higher than cellulose and acidic polysaccharide contents of AIRs) found by these authors might arise from cultivar variability, alcohol-coprecipitated cytoplasmic proteins, residual epicarp or seed fragments, and/or epimerisation of glucose into mannose under Saeman hydrolysis conditions[33]. In fact, except in seed endosperms from *Leguminosae* species[24], mannose is a very minor constituent of dicotyledonous cell-wall polysaccharides.

Structure of the cell-wall polysaccharides.—Ultra-milled parenchyma and stone cell walls from the mesocarp have been methylated once according to Hakomori, and the results are displayed in Table III. Ultra-milling in liquid nitrogen is a

TABLE III

Methylation analysis of parenchymatous (PAR) and stone (STO) cell-wall polysaccharides from guava mesocarp

Methyl ether	PAR	STO
2,3,4-Me_3-Rha [a]	0.2 [b]	
3,4-Me_2-Rha	0.7	0.1
3-Me-Rha	0.7	0.1
Rha	0.4	0.4
Total	2.0	0.6
	(1.4) [c]	(0.6)
2,3,4-Me_3-Fuc	1.5	
	(1.6)	(0.1)
2,3,5-Me_3-Ara	6.0	0.5
2,5-Me_2-Ara	2.6	0.2
3,5-Me_2-Ara	0.9	0.2
2,3-Me_2-Ara	3.4	0.3
2-Me-Ara	2.0	
Ara	1.0	0.8
Total	15.9	2.0
	(16.6)	(1.3)
2,3,4-Me_3-Xyl	3.4	0.4
2,3-Me_2-Xyl	9.3	32.4
2-Me-Xyl	0.2	4.8
3-Me-Xyl	1.2	5.0
Xyl	0.5	16.7
Total	14.6	59.3
	(14.4)	(50.0)
2,3,4,6-Me_4-Gal	0.9	
2,4,6-Me_3-Gal	2.7	
2,3,6-Me_3-Gal	1.0	
2,6-Me_2-Gal	0.8	1.5
2,4-Me_2-Gal	0.3	0.8
Total	5.7	2.3
	(5.5)	(0.7)
2,3,4,6-Me_4-Glc	0.9	0.3
2,3,6-Me_3Glc	47.6	31.9
2,3-Me_2-Glc	6.7	1.8
Total	55.2	34.0
	(56.1)	(46.7)
2,3,6-Me_3-Man	2.5	0.6
2,3-Me_2-Man	1.5	0.1
3,6-Me_2-Man	1.1	1.1
Total	5.1	1.8
	(4.4)	(0.7)

[a] 2,3,4-Me_3-Rha denotes 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-rhamnitol, etc. [b] Relative mole ratio. [c] Values in parentheses are based on analysis of alditol acetates.

prerequisite for cell-wall fragments to swell properly in dimethyl sulfoxide and undergo a complete methylation. A good agreement was observed in the case of parenchyma between the relative sugar mole ratios obtained from compositional (alditol acetates) and structural (partially methylated alditol acetates) analyses. Conversely, methylation of finely milled stone cell walls led to a lower glucose recovery than by direct compositional analysis contrary to methylation data for secondary-thickened fibre cell walls from rye grass[35]. Although cryo-milling reduced dramatically the particle size[36], the very compact and strongly lignified nature of stone cell walls might have limited the swelling and thus the penetration of methylating reagents.

The dominant structural feature in parenchyma cell walls is cellulose, 6-substituted (1 → 4)-linked glucans being also present, possibly as xyloglucans due to the presence of terminal fucose, galactose, and xylose. Arabinose was found, in the furanose form, mainly as non-reducing terminal units and also as 5-, 3-, 3,5-, and 2,3,5-linked units. Galactose, a minor constituent, exhibited moieties from both type II (3- and 3,6-linked residues) and type I (4-linked galactose) (arabino)-galactans. Rhamnose had a distribution typical of rhamnogalacturonans. Xylose, which was found in proportion equivalent to arabinose, had a methyl ether distribution characteristic of a (1 → 4)-xylan carrying substituents on *O*-2, *O*-3, and *O*-2,3. Since purity of parenchyma cell walls has been carefully and repeatedly checked, this xylan structure can be unambiguously assigned to this unlignified cell type. Xylans are usually present in secondarised lignified cell walls[37], but have seldom been found in primary[38] and secondary[39,40] unlignified cell walls. A xylan has been isolated from the mesocarp cell walls of pear[41], but parenchyma and stone cell walls, which are also present in pear flesh, were not separated, thus preventing this xylan being ascribed to the parenchyma tissue.

Guava stone cell walls contain large amounts of (1 → 4)-xylan as the main constituent. The high proportion of 2,3,4-linked xylose might reflect an undermethylation although methylation of cellulose seemed to be complete as seen by the small proportion of 4,6-linked glucose and the absence of other glucose dimethyl and monomethyl ethers. Reis et al.[42] extracted glucuronoxylans with potassium hydroxide from delignified stony endocarps of cherry, prune, and walnut, having (GlcA–Xyl) molar ratios in the range 0.12–0.17, which is close to the 0.18 ratio observed in guava stone cell walls (Table II). The calculated degree of branching (% of *O*-substitution) of stone cell-wall xylan was ~ 37%, of which only ~ 18% would be attributable to terminal glucuronic acid. So, ~ 80% of branching points were not identified but, due to heavy lignification, the possibility of ether linkages between polyphenolics and polysaccharides cannot be ruled out. Indeed, alkali-resistant *p*-hydroxy and *p*-alkoxy benzyl ether bonds between lignin and saccharidic moieties have been observed in a lignin–saccharidic complex from spruce wood[43]; in our case, such linkages would resist the strong base used in methylation, and thus constitute branching points without corresponding terminal non-reducing carbohydrate residues.

The (terminal–substituted) ratio was reasonably good for parenchymatous cell-wall polysaccharides (0.7), and one must bear in mind that terminal uronic acids (presumably glucuronic), which were not accounted for in this study, might substitute xylans. Conversely, this ratio was almost nil in the case of stone cell walls for the above-mentioned reasons.

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