The Relationship between Acetyl Content and Sugar Composition in Expanding Leaves of Beech (*Fagus sylvatica* L.)

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Samples of beech (*Fagus sylvatica*) leaves were taken from the time of leaf emergence until leaf fall. They were extracted with boiling aqueous ethanol and the residues analysed for their acetyl-group content and their content of sugars released by acid hydrolysis. The accumulation of acetyl groups closely paralleled that of xylose, and the two were present in approximately equimolecular proportions, except during the period of leaf expansion, when the acetyl-group/xylose ratio exceeded 3:2. The galactose present in each leaf reached a maximum as leaf expansion ceased and then declined during the period of rapid xylose accumulation. The results are discussed in relation to the known association of acetyl groups with xylans and pectic substances, and to the disappearance of constituents during cell-wall maturation.

Acetyl groups have been shown to be present, as substituents on various polysaccharides, notably hemicelluloses and pectins, but their location has been established with certainty in only a few cases (Bouveng, 1961; Kenne *et al.*, 1975). We are interested in the possibility that their presence could impede enzymic digestion of plant cell walls and so be a factor influencing the utilization of forages by ruminants (cf. Morris & Bacon, 1977).

Pectic substances are considered to be major constituents of primary cell walls (Albersheim, 1976), whereas hemicelluloses predominate in the secondary wall (Whistler & Richards, 1970; Northcote, 1972). In the present paper we examine the relationship of acetyl-group content to sugar composition while plant tissue is passing from the stage of cell expansion to that of secondary thickening. The expanding leaves of deciduous trees offer an opportunity to collect large quantities of such material over a relatively short period of time.

The only chemical treatment applied was extraction with boiling 80% ethanol, because we considered that any other treatment would carry with it the risk of hydrolysis of the acetyl esters or loss of macromolecular material carrying acetyl groups. The analyses refer to acetyl content and to the cell-wall sugars xylose, galactose and arabinose in expanding leaves of beech. Some preliminary results were the subject of a brief comment by Bacon *et al.* (1976), and the results were presented in part at the 27th Congress of the International Union of Pure

and Applied Chemistry (IUPAC) at Helsinki in August 1979.

Materials and Methods

Leaf samples

These were taken in the summer of 1977 from a single copper beech (*Fagus sylvatica* 'Purpurea') tree growing in a garden on the outskirts of Aberdeen. Undamaged leaves with the minimum of petiole attached were picked at random from branches near the ground. The samples (100 leaves at first, 50 later) were taken daily in the forenoon from the side of the tree that faced east. They were weighed at once, wrapped in aluminium foil and immersed in liquid nitrogen. The frozen material was broken by manipulating the foil wrapping and dropped into 200 ml of boiling ethanol containing sufficient water to give a final concentration of ethanol of 80% (v/v), and boiling continued for 10 min. The extract and residue were blended and the supernatant decanted through a sintered-glass funnel. The residue was extracted several times with boiling 80% ethanol and washed on the funnel until the washings were almost colourless, the total extract used being about 1 litre. The residue was dried by solvent exchange with ethanol, acetone and light petroleum (b.p. 40–60°C). Fibrous particles were evident, especially in the later samples, and before analysis all the samples were milled for 1 min in a Janke and Kunkel (Staufen i. Breisgau, Germany) IKA analytical mill.

Acetyl determination (Bethge & Lindstrom, 1973)

Acetic acid liberated by treatment with sodium ethoxide was converted into the benzyl ester and separated by g.l.c.; pivalic acid (trimethylacetic acid) was used as internal standard instead of hexanoic acid (A. H. Gordon, personal communication). The benzyl pivalate was eluted shortly after benzyl acetate and before benzyl alcohol, under isothermal conditions (in the range 105–115°C); the temperature was then increased to 165°C for 20 min.

Sugar analysis

The procedure of Sloneker (1972) was used, involving hydrolysis, reduction, acetylation of the resulting alditols and separation by g.l.c. on Supelco SP-2340 (Chromatography Services, Hoylelake, Merseyside). The output from a Pye–Unicam GCD gas chromatograph was integrated with a Infotronics CRS 309 microprocessor, or fed into an on-line data-logging system for analysis by an IBM 1130 computer, a program written in this Institute by Mr. G. McPherson being used. A typical g.l.c. chart showed, after the solvent peak, peaks corresponding to the alditol acetates derived from rhamnose, fucose, ribose, arabinose, xylose, mannose, galactose, glucose and the internal standard, inositol; if any other peaks were seen above threshold (which corresponded to about 0.1% sugar) the results were discarded. Some samples were analysed four or more times, in duplicate. Agreement between duplicate g.l.c. runs was within $\pm 1\%$, and for duplicate hydrolyses better than $\pm 5\%$.

Considerable difficulty was experienced with interfering substances. Although these substances had very reproducible retention times relative to the inositol acetate standard (0.15, 0.26, 0.35, 0.45, 0.64) and hence interfered with the measurement of ribose (0.29), arabinose (0.32), xylose (0.44) and mannose (0.66), they did not appear with any regularity; this led us to many false conclusions about their origin. Eventually they were found by preparative g.l.c. and mass spectrometry to be phthalate esters. Dudman & Whittle (1976) have drawn attention to the danger presented by these substances, which are used extensively as plasticizers and behave chromatographically very like the alditol acetates. In our procedure they were not present as contaminants of the reagents used, but came from synthetic(buna)-rubber connectors in an apparatus used for multiple evaporations (Buchler Rotary Evapomix; Arnold R. Horwell, London NW6 2BP, U.K.), the degree of contamination apparently depending on how much refluxing of methanol took place during the removal of boric acid as the methyl ester. They did not appear in noticeable amounts during the first 2 years in which this apparatus was used in the method.

Other procedures

Uronic acids were determined in the unneutralized sulphuric acid hydrolysate by the method of Blumenkrantz & Asboe-Hansen (1973). Starch was extracted from the alcohol-insoluble residues with aqueous 90% (v/v) dimethyl sulphoxide (Ring & Selvendran, 1978). Pectic substances were extracted with 0.5% ammonium oxalate at 80°C and neutral pH.

Results

Leaf expansion, as indicated by increasing fresh weight, ceased around 29/30 May (Table 1); thereafter the average weight of 100 leaves was 31 g (12 samples). The fresh weight and the weight of alcohol-insoluble residue per 100 leaves were subject to variation because the leaves on a shoot vary in ultimate size. A more elaborate sampling programme would be needed to take account of this.

The alcohol-insoluble residue as a proportion of fresh weight remained below 20% until 31 May, the average value for the preceding 11 samples being 16.4%. It then increased, and in mid-June exceeded 25%, remaining at this value until autumn. Starch was present in the residue in variable amounts, judging by the reaction with iodine. The last samples taken, of green and brown leaves on 20 October, had rather less residue than the samples in June, July and September that preceded them. Even at this most mature state the cell-wall polysaccharides were richer in galactose and arabinose relative to xylose than the woods of *Fagus* species, more closely resembling bark (Dietrichs *et al.*, 1978).

Discussion

The main intention was to obtain numerous samples during a period of deposition of cell-wall material and to analyse them with the least possibility that cell-wall sugars or acetyl groups would be lost. For this reason no attempt was made to separate cell contents from cell walls, and consequently the absolute concentrations of constituents are subject to fluctuations, caused particularly by starch accumulation. The total neutral sugar did not exceed 40% of the alcohol-insoluble residue in any sample analysed. Despite the large amount of non-carbohydrate material present, the method of Sloneker (1972) often yielded chromatograms of the alditol acetates in which there were negligible signs of interference. Unfortunately as the work proceeded there was increasing evidence of interference from (as it proved) plasticizers. In attempting to explain this we investigated the behaviour of pectin samples and various aldonic and uronic acids (cf. Blake & Richards, 1970). Reexamining these results with the knowledge that most of the interfering peaks were due to phthalate esters, and knowing the likely pro-

Table 1. Leaf samples and analyses of the ethanol-insoluble residues derived from them

Month	Day	Fresh weight (g/100 leaves)	Ethanol-insoluble residues (g/100 leaves)	Composition (mmol/g air-dry material)					Total neutral sugar (% of air-dry material)
				Acetyl	Xylose	Arabinose	Galactose	Mannose	
May	15	7.4	1.40	0.24	0.18	0.15	0.33	0.05	30.5
	16	8.0	1.47	0.28	0.20	0.14	0.28	0.05	27.4
	18	10.0	1.81	0.29	0.17	0.16	0.33	0.04	32.3
	24	26.8	3.68	0.27	0.17	0.18	0.39	0.08	28.3
	25	25.4	3.82	0.26	0.15	0.18	0.38	0.06	31.0
	27	27.0	4.36	0.28	0.17	0.17	0.34	0.06	32.5
	28	30.2	4.92	0.33	0.33	0.20	0.29	0.08	34.9
	29	30.6	5.38	0.37	0.35	0.16	0.28	0.08	34.8
	30	34.6	6.22	0.31	0.26	0.15	0.26	0.06	34.6
	31	36.2	7.14	0.35	0.31	0.14	0.26	0.07	36.7
June	1	31.2	6.18	0.43	0.34	0.14	0.24	0.07	38.6
	3	30.6	6.14	0.52	0.52	0.15	0.17	0.07	35.7
	4	31.8	7.08	0.61	0.57	0.14	0.14	0.05	36.6
	5	32.2	7.36	0.55	0.48	0.13	0.13	0.06	30.9
	8	28.6	6.78	0.52	0.52	0.15	0.13	0.06	35.3
	16	33.0	8.82	0.61	0.57	0.14	0.13	0.06	36.7
	24	29.8	9.08	0.54	0.51	0.13	0.12	0.06	35.8
	8	29.8	9.34	0.58	0.55	0.16	0.13	0.06	35.1
	7	28.2	8.50	0.64	0.62	0.17	0.14	0.07	33.1
	20*	31.2	7.90	0.67	0.58	0.17	0.13	0.06	30.6
October	20†	28.2	7.86	0.71	0.60	0.18	0.15	0.06	30.1

* Leaves wholly green.

† Leaves wholly brown.

ducts of intervention of uronic acids in the procedure, we concluded that the uronic acid content of the samples (6–8%) was not a significant source of error. The method of Bethge & Lindstrom (1973) for acetyl determination showed little interference in the isothermal region of the chromatogram, and raising the temperature to 165°C for 20 min expelled benzyl esters of higher acids and eliminated any interference with later runs.

Table 1 shows, as expected, increases in the amounts of xylose and arabinose per leaf throughout the period of leaf expansion and maturation. The changes in galactose were unexpected; the amount present increased at first but during the period of most rapid increase of xylose content it showed a decline well beyond the range of experimental error. Thus the average value for eight samples taken from 24 May until 1 June was 270 mg/100 leaves, and for the four samples taken from 3 June until 8 June, 157 mg; thereafter the amounts increased slowly to just short of 200 mg.

Galactose is a constituent both of pectic materials and of the hemicelluloses of beech (Timell, 1964). I interpret these changes as being due to the disappearance of a galactose-containing polysaccharide during the period of secondary thickening. Thornber & Northcote (1961) observed a disappearance of galactose, both absolute and also relative to arabinose, during the maturing of cambial cells of dicotyledonous trees, and Knee (1973) found a loss of galactose (with a slight increase in xylose) from the cell-wall fraction of apple fruit during ripening. In the present study, the loss of galactose cannot be due to changes in the raffinose series of oligosaccharides, because these would have been extracted during treatment with hot 80% ethanol.

The accumulation of arabinose is gradual and does not show an acceleration at the time of rapid xylose accumulation. Acetyl content, on the other hand, closely parallels xylose content, as would be the case if it were mainly associated with the hemicellulose fraction.

When acetyl content was plotted against xylose content (Fig. 1) the points fell into three groups, around xylose contents of 2.0–2.5%, 4.0–4.5% and 6.5–8.0%. In Fig. 1 all points lie on or above a line representing equimolecular proportions of the two constituents, and those in the last two groups are close to a straight line through the origin, indicating an acetyl/xylose ratio of 1.1:1. Taking into account the under-estimation of xylose in the alditol acetate procedure, this would suggest a 1:1 ratio in the secondary cell wall. The changes in leaf residue weight are such that the three groups of points correspond respectively to 33–99 mg, 213–296 mg and 419–695 mg of xylose/100 leaves; that is, the second two groups belong to the period of rapid xylose accumulation and of maturity.

Fig. 1. Relationship between the contents of xylose and acetyl groups in the ethanol-insoluble residues listed in Table 1

The line represents equimolecular proportions of these two constituents.

The first group, which incidentally falls in the period of most rapid galactose accumulation, has a ratio of acetyl groups to xylose of 1.54:1. During this period (15 May until 27 May) only one-seventh of the ultimate xylose content has been accumulated and that sugar is still one of the minor constituents of the neutral sugars present. The relative proportions of xylose, galactose and arabinose remain more or less constant, probably corresponding to a period of growth of primary cell walls.

In exploratory experiments, residues from these early samples were extracted with dimethyl sulphoxide to remove starch and then with ammonium oxalate solution. Materials were obtained with about 1% of acetyl groups, a barely detectable xylose content and much uronic acid; their i.r. spectra resembled those of pectic fractions from roots of beet (*Beta vulgaris*) and radish (*Raphanus sativus*), and incidentally confirmed the presence of acetyl groups (Fillipov *et al.*, 1978). Although the yields were low (around 5%), this points to the possibility that the pectic fraction of the leaf cell walls is acetylated and that in young leaves, where it is a higher proportion of the total wall constituents, it contributes acetyl groups without contributing xylose, and so raises the acetyl/xylose ratio above that characteristic of the hemicelluloses.

The relationship between acetyl groups and xylose is so close throughout the life of the leaf that it is reasonable to assume that it is mainly xylose residues that are acetylated, a conclusion reached in a number of studies of wood hemicelluloses of angiosperms (Timell, 1964). The contents of arabinose and galactose in the leaf residue are higher relative to xylose than in woods, including beech wood, but neither sugar has sufficient vacant hydroxy groups to accommodate all the acetyl groups present in the later stages of leaf growth.

The closeness of the acetyl/xylose molar ratio to unity throughout the expansion and maturation of the leaf could mean that every xylose residue is acetylated, and that the lower proportions found in woods and in hemicelluloses extracted from them are due to hydrolysis of the ester linkages by extraction procedures and, perhaps more important, by natural processes; Stewart *et al.* (1961) found that the *O*-acetyl content of woods decreased as the trees aged. Even if the acetyl groups are not disposed in a regular way on the xylan chain, it would seem that there will be on average only one position per xylose residue free for other substituents. An alternative, but less likely, hypothesis is that acetyl groups are also present on a wall constituent that is laid down simultaneously with the xylan.

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