The Institute of Paper Chemistry

Appleton, Wisconsin

Doctor's Dissertation

An Investigation of Pectic Substance in the Developing Xylem of *Populus Tremuloides*

Kenneth C. Larson

June, 1967

AN INVESTIGATION OF PECTIC SUBSTANCE IN THE DEVELOPING XYLEM OF POPULUS TREMULOIDES

A thesis submitted by

Kenneth C. Larson

B.S. (Ch.E.) 1962, California Institute of Technology
M.S. 1964, Lawrence University

in partial fulfillment of the requirements
of The Institute of Paper Chemistry
for the degree of Doctor of Philosophy
from Lawrence University
Appleton, Wisconsin

June, 1967

TABLE OF CONTENTS

SUMMARY

Six Populus tremulöides (quaking aspen) trees were felled in June, 1965, and developing xylem was collected by previously established methods at four stages of growth: soft xylem – the youngest, stringy xylem, new xylem, and one-year-old xylem. Upon collection, the year-old and new xylems were air-dried and the stringy and soft xylems were frozen; the latter were subsequently freeze-dried.

Before analysis, the xylem was extracted with water at ambient temperature for 24 hours. The water extracts from all four growth stages contained, among other components, sucrose, glucose, and fructose. Those from the new, stringy, and soft xylems contained myo-inositol and a water-soluble xylan. Polygalacturonic acid and free galacturonic acid were found in small amounts only in the extracts from stringy xylem.

The water-extractive-free soft xylem contained 18.6% uronic anhydride and only 26.1% glucan. Nitrobenzene oxidation yielded only 0.4% of syringaldehyde (S) plus vanillin (V), and the ratio S/V was only 1/1. At the other extreme, year-old xylem contained 3.8% uronic anhydride, 47% glucan, and 8.0% of S plus V, and S/V was 2.9/1. The year-old xylem values approximate those found for mature aspenwood.

The preceding techniques and results were duplicated with xylem collected from eight trees in July, 1965.

Assuming that all of the galacturonic acid in the water-extractive-free xylem originated from protopectin, and that citrus pectin and aspen protopectin had essentially similar structures, enzymatic assays for determining galacturonic acid and methyl ester in citrus pectin were applied to the xylem.

The assays were applicable directly to soft xylem ground to pass an 80-mesh screen but not to the other three growth stages. Grinding the year-old xylem to pass a 200-mesh screen did not increase the accessibility of its protopectin to the enzymes. Partial delignification with acidic sodium chlorite at room temperature did provide this accessibility for the stringy, new, and year-old xylems.

Optimization of the enzymatic assays showed that less than 66, 53, and 69% of the lignin needed to be removed from stringy, new, and year-old xylem, respectively, to attain maximum galacturonic acid yields.

The galacturonic acid and ester methanol values obtained required correction for (1) removal of galacturonic acid, methanol, and other components during chloriting, (2) the approximately 93% degree of hydrolysis attained by the enzymes (Pectinol 10M) at equilibrium, (3) small amounts of galacturonic acid and methanol in the Pectinol 10M, and (4) methanol from sources other than the protopectin.

The corrected galacturonic acid and ester methanol values were reproducible and also accurate, judging from (1) statistical agreement of the enzymatic galacturonic acid values with values estimated by uronic acid and tissue balances, and (2) statistical agreement of the enzymatic methanol values with values derived by saponification.

Galacturonic acid and methanol were identified as products of the enzymatic hydrolyses by preparation of sodium calcium D-galacturonate and methyl-3,5-dinitrobenzoate.

The average degree of esterification of the protopectin was calculated by dividing total moles of ester methanol by total moles of galacturonic acid.

It did not exceed 0.68 $\pm$ 0.11 in any growth stage and, therefore, is below that of citrus protopectin. It remained constant or may have increased slightly as the xylem matured.

The year-old and soft xylems were extracted with the pickling process, which extracts a jelly-grade pectin from citrus albedo. These growth stages yielded no jelly-grade pectin. Extraction of soft xylem at 25°C. with oxalic acid followed by sodium bicarbonate likewise yielded no jelly-grade pectin or pectate. The crude pectic substances extracted had average degrees of esterification ranging from 0.07 to 0.79.

INTRODUCTION

Protopectin has some economic importance to the pulp industry, because its degradation consumes chemical in current pulping processes, and it may have much greater importance in the rational development of better pulping methods.

The first assertion is based on the fact that polygalacturonic acid is not found in the pulps or liquors from commercial pulping processes (1). The second assertion is based on the observation that protopectin may be an intercellular cement, as it appears to be confined to the compound middle lamella (2, 3). Maceration experiments with wood (3, 4) and other plants (5) have produced evidence favoring this view but have not proved it. The cementing mechanism and its importance to pulping might be elucidated by studying the state of wood protopectin.

The percentage of protopectin decreases as cellulose, hemicellulose, and lignin (in woody plants) are deposited. This was once interpreted to mean that protopectin was transformed into these other components. However, the current interpretation favors dilution by, not transformation into, these components. Moreover, the relative amounts of dilution by each component during the transformation from a cambial cell to a year-old tracheid have been calculated by employing pectic acid as an internal index (6, 7).

Pectic substances have been obtained from several species of trees (2, 8-11). The uronic acid, specific optical rotation, and ash contents of several of these preparations were similar to those of commercial citrus pectins, which led to the conclusion that "wood pectins are closely related to citrus pectins" (11). This investigation was undertaken to test this conclusion.

The work was divided into five sections: (1) collect and characterize developing woody tissue, (2) develop methods to measure the galacturonic acid (GUA) and ester methanol contents of the protopectin in the tissue, (3) calculate the average degree of esterification with methanol (DM) of the protopectin, (4) postulate, then test rational methods for extraction of pectinic acid from the tissue, and (5) prepare derivatives from the GUA and methanol to prove their existence.

HISTORICAL REVIEW

GENERAL PROPERTIES OF PECTIN

A very important commercial application of pectin lies in the preparation of jams and jellies. It can form a resilient gel with a large quantity of sugar at room temperature and the proper pH. This property is a combination of its GUA content, degree of polymerization (DP), and average DM[1]. Generally, a high GUA content and DP are desired. For many years a high DM was also required. Technologists are now learning to prepare useful gels from low DM pectins with divalent cations (12).

The albedo of citrus fruits has become a prime source for high DM pectins because it has a high protopectin content, it is a cheap by-product of the juice industry, and the pectin is easily extracted with acid.

The composition of a particular citrus pectin depends upon its method of extraction and purification and the state of the protopectin, which in turn depends upon the variety and age of the fruit. Commercial production conditions have been refined to the extent that high quality citrus pectins of reproducible composition are available (13).

"The citrus pectins, though they contain the highest galacturonic acid content of any pectins, have not been prepared free of sugars" (14). A citrus pectin for jellymaking may contain over 8% neutral sugars and ash (15).

---

[1] By definition, DM = moles of methanol per mole of anhydrogalacturonic acid. Therefore, $0 \leq DM \leq 1.0$.

Acid hydrolysis of pectin yields, in addition to GUA, L-arabinose, D-galactose, L-rhamnose, and sometimes other monosaccharides. The arabinose and galactose may originate in the polygalacturonic acid backbone, in side groups or sidechains, or from separate polymers (14). A branched α-L-arabinan has been isolated from the pectic substance from peanuts (16), and a β-1,4-galactan has been isolated from the pectic substance in Lupinus albus (16a).

A linkage between GUA and a neutral sugar has been proven in only two cases: (1) a 2-O-(O-galactopyranosyluronic acid)-L-rhamnose has been isolated from the pectic substance of several plants (17-20), and (2) a 2-O-(O-galactopyranosyluronic acid)-D-xylopyranose has been isolated from maritime pinewood (19).

Therefore, from a structural standpoint, it can be said that pectin is essentially a partially esterified polymer of D-galacturonic acid which has a specific optical rotation over +200°.

The average DM of pectin depends upon its source. Apple pectin has been isolated with a DM over 0.9 (21). Pectins from flax, ramie, jute, and hemp may have a DM below 0.25 (22). Commercial high DM citrus pectins for jellymaking have a DM around 0.7 (15).

The average DM of pectin can change during maturation of the plant tissue. The DM of apple (21) and peach (23, 24) pectins increases until the fruit ripens, then decreases. In one study the DM of pectin from the albedo of Valencia oranges decreased from 0.78 to 0.68 as the fruit ripened, while the amount increased (25). This trend is expected for citrus fruits in general (26).

GENERAL PROPERTIES OF PROTOPECTIN

By analogy to the known structure of pectins, protopectin is assumed to be polygalacturonic acid esterified with methanol. Its DM is assumed to be at least 0.8, because pectin derived from it has a DM between 0.7 and 0.8 and the acid extraction process causes some deesterification (15).

Protopectin is insoluble in water but soluble in acid and hot ammonium oxalate (27). Several reasons have been suggested for its water insolubility: a DM close to either zero or one, a very high DP, covalent, ionic, or hydrogen bonding with other cell wall components, or physical entrapment (15).

The DM of protopectin could be very important. Protopectin with a high DM displays strong swelling characteristics (28). It could be a factor in water retention in the cell and in providing elasticity for the growing cell wall (14). Protopectin with a low DM would have more carboxyl groups free to form ester bonds or cation bridges with other cell wall components. Practically every cell wall component has been mentioned as possibly linked to protopectin, but no direct evidence exists.

Unlike the DM of citrus pectin, that of the protopectin may remain constant during maturation of the plant tissue. The DM of the acid-extracted pectic substance from three citrus fruits remained fairly constant during maturation, decreasing only after the fruit reached maturity. The total of pectin plus protopectin also remained constant until maturity (25).

WOOD PECTIC SUBSTANCE

The isolation of pectic substance from wood was first reported in 1925 (29). About 0.4% of a pectic acid having a specific optical rotation of +264° was isolated from beechwood. In 1931 about 0.4% pectic substance was isolated from boxwood with hot ammonium oxalate (30).

The most extensive investigations of wood pectic substances were conducted by Anderson and coworkers between 1936 and 1954 (2, 8-11, 31, 32). They typically obtained 0.2-1.1% anhydrouronic acid from the wood and 5-10% from the "cambial zone"[2] of many species of trees. They used the decarboxylation method (33) to analyze for uronic acid, which is not specific for GUA. However, they did prepare mucic acid from their pectic substances by oxidation with bromine, and this is specific for GUA. In addition, the specific optical rotations of their purified products were always over +200°, so most, if not all, of the reported uronic acid was probably GUA.

They did not measure the DM of their products, except for those from black locustwood (2). Pectic substance isolated from the "cambial zone" with hot water had a DM of 0.22, and with hot 0.05N hydrochloric acid, a DM of 0.37. Pectic substance isolated from the sapwood with 5% ammonium hydroxide had a DM of 0.15.

They drew several conclusions regarding wood pectic substance from their studies. These are listed below in the order in which they appeared in the literature.

---

[2] In one case (2), the "cambial zone" material contained both phloem and xylem elements (13).

TABLE I

COMPARISON OF PROPERTIES OF CITRUS AND WOOD PECTIN AND PROTOPECTIN

Property	Citrus Pectin	Wood "Pectin"
Basic structure	Contains α-1,4-poly-D-galacturonic acid	Proved (9,10,37)
	Purified, has $[\alpha]_D^{20} > +200°$	Proved (2,8,29,37)
	Contains 2-O-(O-galactopyranosyluronic acid)-L-rhamnose	Probably (19,20,37)
	Contains arabinose and galactose	?
D-galacturonic acid present	Proved	Proved (9,10,37)
Ester methanol present	Proved	Not proved
Upper limit on average DM	About 0.75	?
Average DM vs. age of tissue	Decreases	?
Amount vs. age of tissue	Increases	?
Procedure for isolating high DM pectin	Standardized	?
Jelling ability	Excellent	?
Property	**Citrus Protopectin**	**Wood Protopectin**
Average DM vs. age of tissue	Probably remains constant to maturity	?
Amount vs. age of tissue	Probably decreases to maturity	?
Method to measure GUA in situ	Enzymatic method	?
Reasons for its water-insolubility	Many suggested, none proved	?

approximately constant while the tissue matures. There should also be some pectin present in developing xylem. Its amount should increase, and its average DM decrease, while the tissue matures.

Such analogous changes may not occur in wood due to the phenomenon of lignification (6). An investigation of the chemical properties of wood protopectin and pectin versus the age of wood tissue is needed to elucidate these changes.

Several investigators have measured the amount of uronic acid versus the age of wood tissue (6, 31, 34-36), but none was able to differentiate between GUA and other uronic acids in a quantitative manner. This should no longer be a problem, as preliminary results indicate that an enzymatic method is specific for GUA (7).

Another drawback of previous work was the inability to collect several discrete samples of developing wood tissue of reproducible composition. Recently, however, methods have been developed for obtaining such samples at five distinct stages of growth within one annual cycle (6, 38). The earliest stage, called "xylem scrapings," often contains no lignin and is high in uronic acid content, while the "year-old xylem" has a chemical composition closely approximating that of mature wood.

In a study of aspen tissue with these methods, it was found that uronic acid, arabinan, and galactan comprised 65% of the carbohydrate material of the xylem scrapings (6). Assuming that the uronic acid was entirely GUA, and assuming the truth of the conclusion that "wood pectic substance is laid down early ... and is not transformed into other components" (8), three hypotheses were presented: (1) xylem scrapings represented this "early" stage, (2) those

three components were deposited completely at this stage, and (3) they were diluted by deposition of other components in later growth stages. Thus, any or all of the three components could serve as an internal index to cell growth. Calculations based on arabinan content indicated that the weight of a cambial cell should increase by a factor of 4.8 from the slightly lignified, soft xylem stage to the mature, year-old xylem stage[3].

In a later study (7), dilution factors of 15, 7.2, and 6.6, based on GUA, galactan, and arabinan, respectively, were obtained for aspen. Dilution factors based on GUA are generally the highest of the three (6). Assuming that these differences are real, there are at least two explanations: (1) not all of the GUA was being detected in the year-old xylem, or (2) some arabinan and galactan were deposited later than the soft xylem stage.

It has been claimed that GUA is deposited during secondary thickening (36). Judging from the experimental methods employed, however, the claim cannot be accepted without further proof. For example, it was assumed that all pectic substance was extracted by 0.2M ethylenediaminetetraacetic acid (EDTA) in four hours at room temperature and that all uronic acid so extracted was GUA. Furthermore, possible changes in protopectin accessibility due to delignification at 70°C. were not taken into account. Finally, only four samples from each of four trees were analyzed.

[3]The data also showed that the average DM of the GUA in the xylem scrapings was 0.54, assuming that the methoxyl content originated solely from GUA methyl ester groups.

There is only one report in which GUA and methanol measurements were explicitly combined to give an average DM for "wood pectin" (39). A DM of 1.0 was found for pectic substance extracted with hot water and EDTA from the cell walls of callus tissue of English sycamore. The total amount of GUA was only 4%, however, which casts doubt on the similarity of callus tissue and true cambial tissue.

PRESENTATION OF THE PROBLEM

The objective of this investigation is to examine the implications of the statement that "wood pectins are closely related to citrus pectins" (11).

From our extensive knowledge of citrus pectins, the following characteristics of wood pectic substance can be postulated:

1. Wood pectin is an essentially linear polymer of α-1,4-linked D-galacturonic acid units with carboxyl groups partially esterified with methanol.
2. Jelly-grade pectin can be obtained from wood by mild acid hydrolysis.
3. Polygalacturonase hydrolyzes over 90% of wood pectin to GUA after deesterification.
4. The amount of pectin increases as the xylem matures.
5. Wood pectin is derived from wood protopectin.
6. Wood protopectin has an average DM of at least 0.8.
7. The average DM of the protopectin remains essentially constant as the xylem matures.
8. Dilute sodium hydroxide quantitatively saponifies protopectin methyl ester groups.
9. Polygalacturonase hydrolyzes over 90% of the protopectin to GUA after deesterification.

COLLECTION AND CHARACTERIZATION OF ASPEN XYLEM

COLLECTION

All experiments were carried out on developing xylem from Populus tremuloides (hereafter called "aspen"), because much was already known about it (6, 7, 39a), it was commercially important, and it was readily available.

Materials were collected at two intervals during the 1965 growing season. Six trees (12-14 years old) were felled during June 1-5 at the Ford Forestry Center, Michigan Technological University, L'Anse, Mich. Eight trees (17-25 years old) were felled during July 13-16 at the tree farm of The Institute of Paper Chemistry in the Town of Greenville, Outagamie County, Wisconsin.

Materials were collected from the bole of each tree at four stages of growth: soft xylem (SX), stringy xylem (StX), new xylem (NX), and one-year-old xylem (YOX). The material remaining after the YOX was removed was designated "mature wood" (MW).

The collection methods have been described previously (6, 38). The usual method of preserving the material in absolute methanol was not used, because any adsorbed methanol might have interfered in subsequent assays for pectin ester methanol. Instead, the ribbons of SX and StX were immersed immediately in cold tap water. After about four grams (airdry basis) had been collected, the water[4] was drained off and the tissue was frozen in an insulated chest containing solid carbon dioxide.

[4] Samples of this water were frozen and later analyzed. Paper chromatography indicated sucrose, fructose, and glucose, but no free or combined GUA.

The ribbons of NX and YOX were placed in wire cages which permitted the free circulation of air. They appeared to be dry within four hours.

SAMPLE PREPARATION

The frozen SX and StX were freeze-dried and stored at 5°C. Trouble was encountered in freeze-drying the SX collected in June, but its composition did not appear to be affected (see Table III). The NX and YOX were ground in a Wiley mill to pass a 20-mesh screen and also stored at 5°C. The MW was cut into disks two inches thick and stored at room temperature.

Composite samples of SX, StX, NX, YOX, and MW were prepared from equal weights of materials from each tree. The material collected in June was labeled SX-1, StX-1, etc.; that collected in July was labeled SX-2, StX-2, etc.

Two samples of tissue were prepared for later use in the extraction experiments. Sample YOX-P was prepared by mixing 459 g. of YOX-1 and 92 g. of YOX-2 (both airdry basis), extracting with water to remove pectin (see next page), and air drying. Sample SX-0 was prepared by mixing 56 g. of SX-1 and 42 g. of SX-2 (both airdry basis), extracting with water, and drying *in vacuo* at 70°C. overnight.

DETERMINATION OF PARTICLE SIZE

Previous experiments had shown that the yield of GUA from aspen NX and YOX was similar whether the material was ground to pass an 80- or 200-mesh screen (40). My experiments confirmed that result and showed that it was also true for methanol yield. Consequently, all material was ground in a micro-Wiley mill with an 80-mesh screen attached. About 10% of the material did not pass through but was combined with the finer material.

Later experiments (reported under "Experimental Results") showed that lignin content of the tissue is much more important than particle size in enzymatic assays for GUA and methanol. Thus, the use of a larger particle size might be possible but might result in longer delignification times.

WATER EXTRACTION

To remove pectin and, thus, to strengthen the premise that the remaining GUA would originate only from the protopectin, the ground material was extracted with water at room temperature [a standard technique (15)].

The xylem was stirred in about 15 volumes of distilled water for 24 hours at 25-28°C., recovered by filtration, washed with 1-2 volumes of water, and air dried overnight, except for the SX, which was dried *in vacuo* at 30°C. overnight. The filtrate was evaporated and the weight of the water-soluble extractives determined.

COMPOSITION OF WATER EXTRACTIVES

The extractives were analyzed qualitatively for carbohydrate materials before and after enzymatic and acid hydrolysis. The paper chromatographic techniques were standard ones and are described in Appendix III. They can detect carbohydrates whose concentration exceeds about 0.2% of the dry weight of dissolved solids.

The results are shown in Table II. Note that GUA was indicated only in the water extractives from StX, indicating that no appreciable amount of pectin is present in developing aspen xylem.

TABLE II

YIELD AND COMPOSITION OF THE WATER-SOLUBLE EXTRACTIVES[a]

Tissue	Weight of Extractives, %[b]	GUA	Arabinose	Galactose	Myo-Inositol	Xylose	Aldobiuronic Acid[c]	Glucose, Fructose, Sucrose[d]
SX-1	34.0	-	+	++	++	+	-	++
SX-2	n.t.	-	++	++	++	+	-	++
StX-1	7.0	++	++	++	++	+	n.t.	++
StX-2	n.t.	++	++	++	++	+	n.t.	++
NX-1	10.4	-	+	++	n.t.	+	n.t.	++
NX-2	5.5	-	+	++	++	+	n.t.	++
YOX-1	1.8	-	+	+	-	+	+	++
YOX-2	1.6	-	-	+	-	-	+	++

[a] Symbols: ++, compound present before and after hydrolysis; +, compound present only after hydrolysis; -, compound absent; n.t., not tested.

[b] Calculated as percentage of ovendry, unextracted xylem.

[c] 2-O-(4-O-methyl-α-D-glucopyranosyluronic acid)-D-xylopyranose.

[d] Sucrose did not survive hydrolysis.

COMPOSITION OF WATER-EXTRACTIVE-FREE XYLEM

The water-extractive-free xylem was analyzed quantitatively to determine the composition of each growth stage and to compare it with material examined by other investigators. The analytical methods employed were standard and are described in Appendix IV.

The results are shown in Table III. Note that YOX-1 was analyzed before and after water extraction.

The lignin determination deserves special mention. "It is known that proteins and some carbohydrates condense with lignin when the Klason method is used" (6). This would be particularly likely for SX and StX. Therefore, the "lignin" in these growth stages was taken to be the sum of the syringaldehyde and vanillin yields obtained upon oxidation with the alkaline nitrobenzene method of Stone and Blundell (41). This sum was 8.0 and 8.9% for YOX-1 and YOX-2, respectively, or about 40% of the Klason plus acid-soluble lignin.

All future experiments utilized the materials shown in Table III.

DISCUSSION OF RESULTS

The data in Table II indicate that free GUA and polygalacturonic acid were present only in the water extractives from StX. A possible explanation is that, analogous to citrus fruits, degradation of protopectin to pectin does begin. However, lignification in the NX and YOX stages prevents further degradation.

Note that the extractives from seven of the eight tissues contained xylose after, but not before, hydrolysis. A water-soluble xylan must have been present.

TABLE III

COMPOSITION OF WATER-EXTRACTIVE-FREE XYLEM[a]

Component	Tissue								
	SX-1	StX-1	NX-1	YOX-1	YOX-1[b]	SX-2	StX-2	NX-2	YOX-2
Uronic anhydride, %	18.6	8.0	5.4	3.8	4.9	15.8	8.2	5.2	5.2
Galactan, %	6.8	1.2	0.6	0.8	0.8	4.8	1.6	0.7	0.9
Araban, %	4.6	1.2	0.7	0.7	0.7	3.7	1.3	0.5	0.6
Rhamnan, %	1.1	0.4	0.2	0.5	0.4	0.8	0.4	0.2	0.4
Glucan, %	26.1	46.6	50.2	47.0	49.7	34.4	46.6	48.8	42.6
Xylan, %	8.9	20.8	24.0	19.2	17.8	18.4	22.9	22.7	20.1
Lignin, %[c]	0.4	3.2	17.1	19.6	20.4	1.0	3.5	20.1	21.4
Total, %	66.5	81.4	98.2	91.6	94.7	78.9	84.5	98.2	91.2
Syringaldehyde / Vanillin	1.0	2.7	--	2.9	--	2.1	3.5	--	3.3

[a] All values are based on the o.d., water-extractive-free xylem, are averages of duplicate determinations, and have 95% confidence limits of ± 5% of the value.

[b] Composition before water extraction.

[c] For SX and StX, Lignin = sum of syringaldehyde and vanillin from alkaline nitrobenzene oxidation. For NX and YOX, Lignin = sum of Klason and acid-soluble lignin.

Also note that 2-O-(4-O-methyl-α-D-glucopyranosyluronic acid)-D-xylopyranose was found in the acid-hydrolyzed extractives from YOX but not in those from SX. A possible explanation is that the glucuronic acid-to-xylose ratio increases as the xylem matures, as suggested previously (36).

Table III shows that SX-1 and SX-2 have high uronic anhydride and low lignin, glucan, and xylan contents compared to YOX. By these criteria they are more immature than either the "cambial tissue" of Thornber and Northcote (36) or the "ML + P" fraction of Meier (43).

At the other extreme, YOX-1 and YOX-2 have compositions similar to mature wood, as Klason plus acid-soluble lignin contents of MW-1 and MW-2 were 20.4 and 21.0%, respectively. The carbohydrate composition of the water extractives from YOX-1 is also similar to that from mature aspenwood (44).

The assays in Table III do not add to 100% for any tissue because protein, ash, mannan, and acetyl assays were not run. Protein and ash combined make up 23-31% of the ovendry weight of aspen SX, about 10% of StX, but less than 3% of NX and YOX (7). Mannan and acetyl make up 3.6-6.0% of mature aspenwood (45, 46). The percentage of mannan is less in younger hardwood tissue (7), but there are no data concerning acetyl content *versus* age.

The decrease in "total" of all assays from NX to YOX probably indicates deposition of components not assayed. Acetyl could be added to the 4-O-methylglucuronoxylan during this period, and water-insoluble extractives might also be formed.

Finally, syringaldehyde-to-vanillin ratios were calculated and are shown at the bottom of Table III. Ratios of 2.5-4.0 have been observed for practically all mature dicotyledonous plants (47), whereas ratios close to 1.0 are typical for monocotyledonous plants (48, 49).

If these aldehydes originated only from ligninlike materials, then the ratios of 1.0 and 2.1 found for SX-1 and SX-2, respectively, have at least two explanations: (1) the first lignin deposited is typical of monocotyledonous lignin, but the bulk of it yet to be deposited has the usual ratio; or (2) syringaldehyde units are formed by methoxylation of vanillin units.

DEVELOPMENT OF THE ANALYTICAL METHODS FOR GALACTURONIC ACID AND ESTER METHANOL

The goal was to determine the average degree of methylation of aspen protopectin. By definition this required measuring both the galacturonic acid and methyl ester contents of the protopectin.

CHOICE OF A CONTROL

New adaptations for analytical methods should be tested on a substance of known composition (the control) to define their capability. Citrus pectin is the logical control for the proposed methods because citrus and wood pectins have been assumed to be related (11) and because citrus pectin of reproducible composition is readily available.

DETERMINATION OF GALACTURONIC ACID

Five different methods have been used to determine the GUA content of pectic substances: (1) decarboxylation (33), (2) titration (50), (3) the sulfuric acid-carbazole color reaction (51), (4) precipitation as calcium pectate (25), and (5) enzymatic hyrolysis to GUA (52).

Only the enzymatic method is sufficiently specific for use with mixtures of uronic acids. It was originally proposed for use with citrus pectic substance (52), but preliminary studies (40) have shown that it can also be applied to wood.

The mechanism (53, 54) and initial rate (55) of enzymatic hydrolysis of pectin have been studied as well as the specificity of commercial enzyme preparations, notably the Pectinol products from Rohm and Haas (54, 56). It was found that pectin must be demethylated before it can be depolymerized by these enzymes (53).

The following conditions were proposed for the quantitative measurement of GUA in citrus pectic substance (52). They were adapted to aspen xylem by optimizing the reaction times marked with an asterisk (*).

1. Stir the sample one-half hour* with disodium ethylene-diamine-tetraacetate at pH 11.5 to deesterify the protopectin and chelate divalent cations.

2. Stir it two hours* with Pectinol 10M at pH 4.0-5.5 to hydrolyze the protopectin to GUA.

Experiments with woody tissue have shown that the GUA can be separated from the hydrolyzate by paper chromatography, eluted into aqueous solution, and measured colorimetrically with Somogyi and Nelson reagents (40).

DETERMINATION OF ESTER METHANOL

Three methods have been used to determine the methyl ester groups in pectic substances: (1) liberation as methyl iodide (57), (2) saponification and titration (50, 57), and (3) enzymatic hydrolysis (57).

The first two methods are not specific for the methyl ester groups of protopectin, whereas the enzymatic method should be. Moreover, saponification followed by distillation should serve as a check on the enzymatic methanol, because the only known methyl ester group in extractive-free wood is that on the protopectin.

Methanol in an aqueous distillate can be measured in several ways, including gas chromatography, isotope dilution, and colorimetry. Colorimetry was used because it probably is the fastest technique and is at least as precise as the others.

The following conditions were proposed for quantitative measurement of ester methanol: Stir the tissue x hours (time x to be optimized) with Pectinol 10M at pH 4.0-5.5 to demethylate the protopectin. Distill the methanol and determine its concentration colorimetrically with chromotropic acid.

ASSUMPTIONS IMPLICIT IN THE PROPOSED METHODS

Five assumptions are implicit in the proposed application of the enzymatic methods to aspen protopectin:

1. The structures of aspen protopectin and citrus pectin are similar.
2. Pectinol 10M hydrolyzes aspen protopectin to GUA to the same extent that it hydrolyzes citrus pectin.
3. The only source of GUA in aspen is the protopectin.
4. Pectinol 10M hydrolyzes the methyl ester groups in aspen protopectin to methanol to the same extent that it hydrolyzes them in citrus pectin.
5. The only source of ester methanol in aspen is the protopectin.

EXPERIMENTS TO TEST THE ASSUMPTIONS

ASSUMPTION 1. THE STRUCTURES OF ASPEN PROTOPECTIN AND CITRUS PECTIN ARE SIMILAR

This assumption should be valid if Pectinol 10M is to hydrolyze both aspen protopectin and citrus pectin. There is already good evidence that both substances contain α-1,4-poly-D-galacturonic acid (9, 10, 37). Further evidence for their similarity is that hydrolysis of woody tissue with Pectinol 10M yields GUA (40). Additional evidence, subject to the limits of the enzyme's specificity,

was obtained when the proposed enzymatic method for hydrolysis of ester methanol gave satisfactory results with both aspen protopectin and citrus pectin.

ASSUMPTION 2. PECTINOL 10M HYDROLYZES ASPEN PROTOPECTIN TO THE SAME EXTENT THAT IT HYDROLYZES CITRUS PECTIN

This assumption cannot be tested directly but should be valid if the protopectin is completely accessible to the enzyme. Lignin might shield the protopectin from the enzyme, in which case the shielding should be least in SX (lowest lignin content) and greatest in YOX. It was assumed that the protopectin was completely accessible when the maximum GUA value for each growth stage was attained by a combination of delignification and optimization of enzymatic hydrolysis conditions.

The reasonableness of the GUA values was checked by two independent methods. A uronic acid balance can be made because aspen tissue contains significant amounts of only two uronic acids, 4-O-methylglucuronic and galacturonic (57a). The maximum GUA content can be estimated by subtracting the 4-O-methylglucuronic acid from the total uronic acid.

The second method is also an estimate of the maximum GUA content. It involved a tissue balance before and after delignification.

ASSUMPTION 3. THE ONLY SOURCE OF GUA IN ASPEN IS THE PROTOPECTIN

Pectin should have been eliminated as a source by the water extraction preceding analysis.

Another source might be 2-O-(O-galactopyranosyluronic acid)-D-xylose, which was tentatively identified in an acid hydrolyzate from aspen SX by paper chromatography (37). Pectinol probably does not hydrolyze this

to GUA, as it does not hydrolyze methyl-α-D-galacturonide (54). Nevertheless, the amount of the aldobiuronic acid is so small in aspen that it can be assumed negligible as a source of GUA.

Since the independent methods for estimating GUA agreed with the enzymatic values, this was further evidence for the validity of Assumption 3.

ASSUMPTION 4. PECTINOL 10M HYDROLYZES THE METHYL ESTER GROUPS IN ASPEN PROTOPECTIN TO METHANOL TO THE SAME EXTENT THAT IT HYDROLYZES THEM IN CITRUS PECTIN

The same reasoning applies here as for Assumption 2.

The reasonableness of the enzymatic methanol values was checked by a saponification technique which gives quantitative hydrolysis of citrus pectin methyl ester groups. The saponification and enzymatic values should agree because there are no known methyl ester groups in aspen other than those on the protopectin.

ASSUMPTION 5. THE ONLY SOURCE OF ESTER METHANOL IN ASPEN IS THE PROTOPECTIN

Four potential sources of methanol exist in aspen besides protopectin: (1) pectin, (2) the ether methoxyl of 4-O-methylglucuronoxylan, (3) the ether methoxyls of lignin, and (4) labile methyl groups of proteins.

Pectin should have been eliminated by the water extraction preceding analysis. The other three possibilities were tested by applying the Pectinol 10M to models: O-[4-O-methyl-α-D-glucopyranosyluronic acid-(1 → 2)-β-D-xylopyranosyl-(1 → 4)]-β-D-xylopyranosyl-(1 → 4)-β-D-xylopyranose (aldotriuronic acid) for glucuronoxylan; Brauns' native lignin (BNL) from aspen NX for lignin; and methionine, an amino acid, for protein.

In addition, all distillates were tested for formaldehyde, which would interfere in the colorimetric test for methanol.

EXPERIMENTAL METHODS

The enzymatic method for determination of GUA is described in detail in Appendix I. The enzymatic and saponification methods for determination of ester methanol are described in Appendix II.

Standard methods for uronic anhydride, methoxyl, and neutral sugars are described in Appendix IV.

The delignification method, an adaptation of the room temperature acid chlorite method (58), is described in Appendix V.

EXPERIMENTAL RESULTS

The purpose of the experimental program was to test the five assumptions listed on page 26. Therefore, the majority of experimental data is organized according to the assumption to which it chiefly pertained. However, certain data pertained to more than one assumption and are presented first: GUA and methanol from Pectinol 10M, composition of the control, and analysis of the control with the enzymatic methods.

GALACTURONIC ACID AND METHANOL FROM PECTINOL 10M CONCENTRATE

Blank enzymatic hydrolyses were carried out to test the Pectinol 10M Concentrate for GUA and methanol. The results are shown in Table IV.

TABLE IV

GALACTURONIC ACID AND METHANOL FROM PECTINOL 10M CONCENTRATE

Time Stirred in Water, hr.	Anhydrogalacturonic Acid, mg./10 mg. pectinol	Methanol, mg./10 mg. pectinol
0	0.50 ± 0.04	--
2	0.49 ± 0.03	--
4	0.51 ± 0.02[a]	0.033 ± 0.002[b]
24	0.55 ± 0.02[a]	0.031 ± 0.002[a]
240[c]	0.73 ± 0.03	--

[a] Average of two determinations.
[b] Average of five determinations.
[c] This was the "zero hour" sample after standing 10 days at 40°F.

The results show that this Pectinol 10M preparation released reproducible amounts of GUA and methanol under the conditions of the assays. Consequently, all GUA and methanol assays employing Pectinol 10M were appropriately corrected.

The GUA correction was overlooked in previously published data with Pectinol 10M (7, 38).

COMPOSITION OF THE CONTROL

Citrus pectin (Sample 3642-S) for use as a control was obtained from the Hollywood pectin plant of the Atlantic Gelatin Division, General Foods Corporation. Its composition was established with several different techniques and is shown in Table V. Its average DM was 0.72, typical for a high DM, jelly-grade pectin.

TABLE V

COMPOSITION OF THE CITRUS PECTIN CONTROL

Component	Composition, % of o.d. wt.	
	Actual Values	Av. Value
Galacturonic anhydride		83.2 ± 1.0[a]
Decarboxylation	82.4, 81.8	
Titration	(87.1)[b], 83.6, 81.7, 81.1	
Methyl ester (as methanol)		11.4 ± 0.2
CH_3I evolution	11.6, 11.9	
Titration	(11.4), 11.2, 11.1, 11.1	
Sapon.	11.0, 11.6	
Neutral sugars		4.9 ± 0.2
Galactan	2.5, 2.6	
Rhamnan	1.1, 1.2	
Araban	0.8, 0.8	
Xylan	0.3, 0.4	
Ash[c]		3.4

[a] 95% Confidence limits.
[b] Values in parentheses are averages of duplicate tests performed by General Foods Corp. Others were obtained by the Anal. Dept. of The Institute of Paper Chemistry.
[c] The conditions of this determination are not known.

ANALYSIS OF THE CONTROL WITH PECTINOL 10M

Table VI shows the anhydrogalacturonic acid (AGUA) and methanol contents of the citrus pectin control according to the enzymatic methods described in Appendices I and II. The pectinwas dissolved and completely accessible before the Pectinol 10M was added. Therefore, the figures represent the maximum degree of hydrolysis attainable with this enzyme.

The short and long hydrolysis times both gave the same results at the 95% confidence level. Therefore, the values were combined to give averages of 78.5 ± 1.7% and 10.5 ± 0.1% for AGUA and methanol contents, respectively.

TABLE VI

ANALYSIS OF THE CONTROL WITH PECTINOL 10M[a]

Component	Hydrolysis Time, hr.	Found, %	Average, %
AGUA	2	81.5 ± 4.0[b]	
		80.1 ± 3.2	
		77.6 ± 3.2	
		75.4 ± 2.4	78.6 ± 2.4
	24	79.1 ± 3.2	
		77.7 ± 3.2	78.4 ± 2.3
Methanol	4	11.2 ± 0.3	
		10.5 ± 0.3	
		10.3 ± 0.3	10.7 ± 0.2
	24	10.3 ± 0.3	
		10.3 ± 0.3	10.3 ± 0.2

[a] Based on the o.d. pectin wt.
[b] 95% Confidence limits.

Comparison of these averages with those in Table V shows that hydrolysis with Pectinol 10M gave 94.3 ± 2.2% (= 78.5/83.2) and 92.1 ± 1.8% (= 10.5/11.4) of the yields of AGUA and methanol, respectively, obtained by standard methods. These percentages were assumed to apply to the hydrolysis of aspen protopectin, as discussed previously, and were applied as correction factors to all AGUA and methanol yields from aspen unless otherwise noted.

ASSUMPTION 1. THE STRUCTURES OF ASPEN PROTOPECTIN AND CITRUS PECTIN ARE SIMILAR

It will be shown that both the GUA and ester methanol contents of aspen protopectin can be determined with Pectinol 10M. This is evidence, limited by the enzymes' specificity, that aspen protopectin and citrus pectin have similar structures.

When this evidence is added to that in Table I, it appears that both aspen protopectin and citrus pectin are essentially linear polymers of α-1,4-linked D-galacturonic acid units partially esterified with methanol.

ASSUMPTION 2. PECTINOL 10M HYDROLYZES ASPEN PROTOPECTIN TO THE SAME EXTENT THAT IT HYDROLYZES CITRUS PECTIN

As discussed previously, Pectinol 10M should hydrolyze the protopectin to the same extent that it hydrolyzes citrus pectin (94.3%) if the protopectin is completely accessible to the enzyme. Accessibility was to be assured by finding reaction conditions which maximized the yield of GUA.

The first step in this maximization had been taken while preparing the material for analysis, namely, it was shown that reduction of particle size from 40- to 200-mesh did not change the yield of GUA. Consequently, the tissue was ground so that 90% passed an 80-mesh screen. It was then analyzed enzymatically for GUA as described in Appendix I. The results are in Table VII. All values are averages of duplicate assays unless otherwise noted.

Note that a one-half hour saponification followed by a two-hour enzymatic hydrolysis consistently gave maximum yields within the 95% confidence limits of the assay. These are the identical conditions employed for citrus pectin (50).

However, it was believed that the AGUA yields from YOX were too low because more than 0.5% AGUA has been isolated from many species of trees (15).

TABLE VII

ANHYDROGALACTURONIC ACID YIELDS VERSUS REACTION CONDITIONS[a]

Tissue	AGUA, % of o.d. tissue	0.035N NaOH, hr.	Pectinol 10M, hr.
YOX-1	0.16 ± 0.03	0.5	4
"	0.15 ± 0.02	0.0	4
"	0.13 ± 0.03	0.5	2
"	0.13 ± 0.04[b]	0.0	6
"	0.13 ± 0.04[b]	0.0	23
"	0.03 ± 0.04	0.5	1
"	0.02 ± 0.04[b]	0.0	2
NX-1	0.82 ± 0.06	0.5	2
"	0.71 ± 0.08[b]	0.0	4
StX-1	3.3 ± 0.1	0.5	2
"	3.0 ± 0.2[b]	0.0	4
SX-1	14.1 ± 0.3	0.5	2
YOX-2	0.08 ± 0.03	0.5	2
NX-2	0.23 ± 0.05	0.5	2
StX-2	2.9 ± 0.1	0.5	2
"	2.8 ± 0.2[b]	0	24
"	2.4 ± 0.1	0	6
"	2.4 ± 0.1	0	2
SX-2	9.1 ± 0.3	0.5	2
"	8.3 ± 0.3[b]	0	4

[a] Only SX-1 was corrected for the 94.3% degree of hydrolysis of AGUA by Pectinol 10M.

[b] Single assay.

As discussed previously, there was reason to assume that the lignin might shield the protopectin from the enzyme. Therefore, the June tissues were partially delignified with acid chlorite at room temperature before enzymatic hydrolysis to see if higher AGUA yields could be obtained. A one-half hour saponification followed by a two-hour enzymatic hydrolysis was employed initially because it had given the highest yields from the unchlorited tissue

in the shortest time. To show that the maximum AGUA yield had been achieved, longer hydrolysis times were also employed. The results are shown in Table VIII. All AGUA values are single assays uncorrected for losses due to chloriting.

If the chloriting did not cause loss of protopectin, then a plot of AGUA content[5] versus chloriting time would increase to the correct value and remain there, reflecting the increasing, and finally the complete, accessibility of the protopectin to the enzyme.

Some loss of protopectin does occur, however, by solution in the chlorite liquor (see Table VIII) and probably by degradation similar to, but to a much lower degree than, that which occurs at 75°C. (59). Consequently, a plot of AGUA content versus chloriting time increases to a maximum and then decreases as shown in Fig. 1, which is a plot of the data in Table VIII. The curve for SX-1 shows only the decrease because its maximum was obtained at zero chloriting time.

Three significant points are shown in Fig. 1. First, higher AGUA contents were obtained after chloriting YOX-1, NX-1, and StX-1 but not SX-1. Second, a definite maximum AGUA content was found for each growth stage. Third, the chloriting time required to give that maximum increased from SX to YOX, as does the lignin content. These facts support the premises that lignin shields the protopectin from the Pectinol 10M even when the tissue is ground to pass a 200-mesh screen and that chloriting makes the protopectin accessible to the Pectinol.

---

[5] "AGUA content" is the percentage of AGUA based on the o.d. weight of the tissue before chloriting. It is calculated by multiplying the percentage of AGUA in the chlorited tissue by the amount of tissue recovered (expressed as a fraction).

TABLE VIII

ANHYDROGALACTURONIC ACID YIELDS VERSUS CHLORITING AND ENZYMATIC HYDROLYSIS TIMES

Tissue	Chloriting Time, days	Pectinol 10M, hr.	AGUA Yield, %[a]	AGUA in Chlorite Liquor, %[a]	Tissue Recovered, %[b]
YOX-1	3	2	0.50 ± 0.04	--	90.5
"	8	2	0.86 ± 0.04	0.050 ± 0.005	89.6
"	10	2	1.00 ± 0.04	0.004 ± 0.001	87.1
"	10	12	1.75 ± 0.09	--	87.1
"	10	24	1.08 ± 0.09	--	87.1
"	10	64	1.04 ± 0.09	--	87.1
"	12	2	0.93 ± 0.04	--	87.3
"	14	2	0.97 ± 0.04	0.0	86.0
"	14	24	1.33 ± 0.09	--	84.8
NX-1	2	2	1.2 ± 0.1	--	96.1
"	3	2	1.7 ± 0.1	0.090 ± 0.009	92.8
"	3	24	1.9 ± 0.1	--	92.8
"	5	2	1.7 ± 0.1	0.094 ± 0.009	91.8
"	7	2	1.5 ± 0.1	0.20 ± 0.02	90.1
"	7	24	2.1 ± 0.1	--	88.5
StX-1	1	2	5.1 ± 0.2	--	96.1
"	1	24	4.2 ± 0.2	--	96.1
"	3	2	4.1 ± 0.2	0.16 ± 0.01	96.3
"	5	2	3.9 ± 0.2	0.30 ± 0.01	90.9
"	5	24	4.2 ± 0.2	--	90.9
SX-1	1	2	14.0 ± 0.5	0.066 ± 0.006	95.7
"	1	24	13.8 ± 0.5	--	95.7
"	3	2	12.5 ± 0.7	--	95.2
"	3	24	17.0 ± 0.7	--	95.2

[a] Based on the o.d. wt. of tissue after chloriting.
[b] Based on the o.d. wt. of tissue before chloriting.

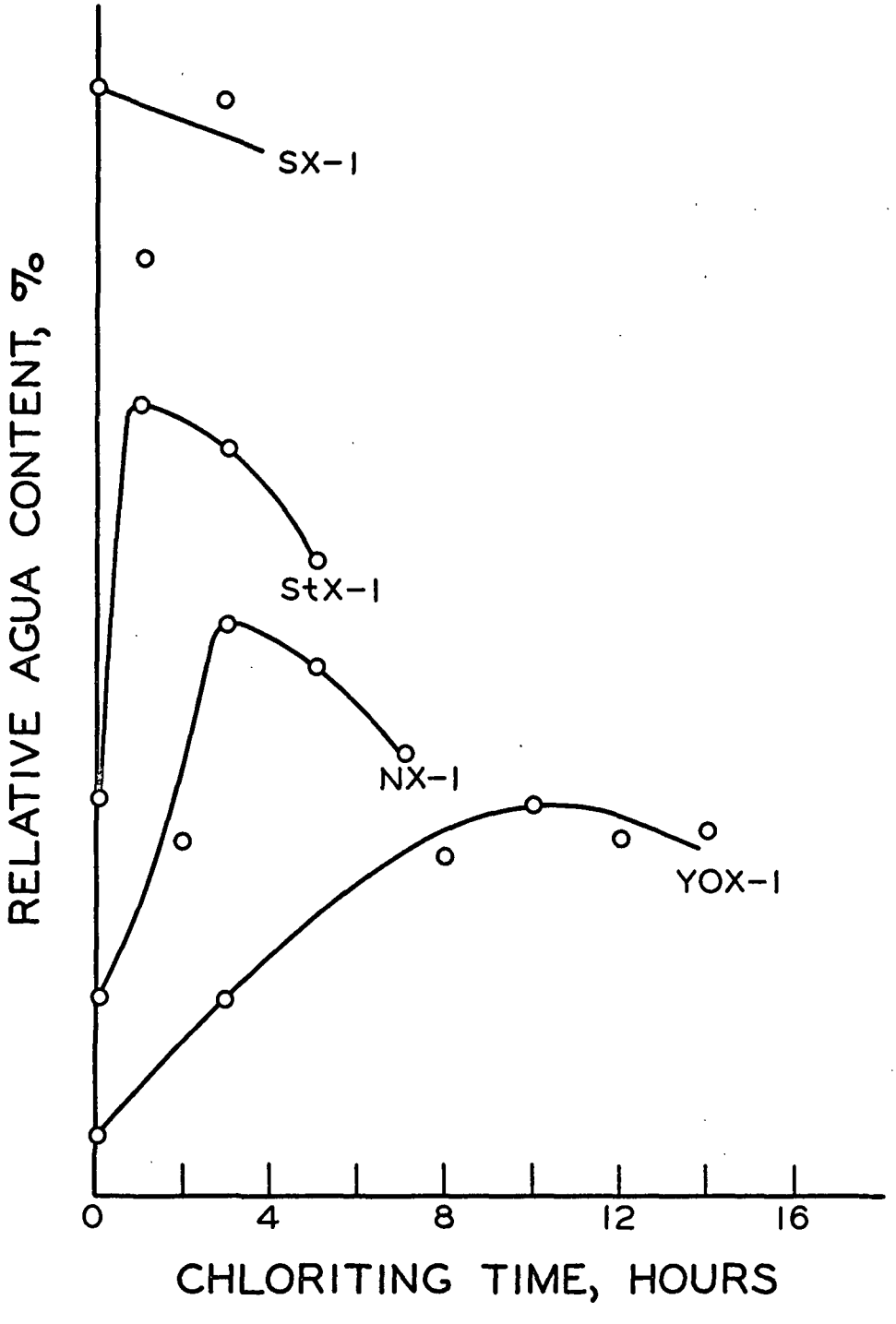


Figure 1. AGUA Content Versus Chloriting Time

Two estimates were made of the AGUA losses during chloriting: (1) the rate of loss was assumed linear with time, and (2) the AGUA found in the chlorite liquor was added to that remaining in the chlorited tissue.

Concerning the first method, there are two excellent reasons for choosing a simple, linear expression for the loss of AGUA with time: (1) only a small amount of data are available, and (2) the system sodium chlorite-acid-woody tissue has not been studied sufficiently to justify a more elaborate expression. An additional reason is that in a study of the intrinsic viscosity of apple pectic acid versus chloriting time at 75°C. (59), the plots obtained were initially concave upward but approached a straight line after only three hours of chloriting.

A separate rate expression was calculated for each growth stage, except SX-1, by regression analysis. Details are given in Appendix VI. The results are in Table IX, Column 2.

TABLE IX

CORRECTED AND ESTIMATED MAXIMUM AGUA CONTENTS OF ASPEN XYLEM

Growth Stage	Corrected AGUA, %[a]		Est. Max. AGUA, %[a]	
	Linear Loss Method	Chlorite Liquor Method	Uronic Acid Balance	Tissue Balance
YOX-1	1.3 ± 0.2	1.1 ± 0.1	1.0 ± 0.1	1.3 ± 0.1
NX-1	1.7 ± 0.4	1.8 ± 0.1	2.0 ± 0.1	1.7 ± 0.1
StX-1	4.6 ± 0.3	4.1 ± 0.2	5.0 ± 0.3	8.2 ± 0.3
SX-1	14.1 ± 0.3	13.4 ± 0.5	17.3 ± 0.9	17.6 ± 0.9

[a]Based on the unchlorited, water-extractive-free, o.d. xylem.

The second method for estimating the AGUA losses was to add the AGUA recovered from the spent chlorite liquor (the recovery procedure is in Appendix I) to that remaining in the chlorited tissue. This gave two estimates for YOX-1; three for NX-1; two for StX-1; and one for SX-1. The maximum estimate for each growth stage was then chosen and is shown in Table IX, Column 3.

In addition to the above estimates, maximum AGUA contents were estimated by two independent material balances. One was a uronic acid balance described by Equation (1):

$$\text{GUA} = (\text{total uronic acid}) - (4\text{-}\underline{O}\text{-methylglucuronic acid}). \qquad (1)$$

The estimated GUA values were maximums because the "Total Uronic Acid" values were determined by a decarboxylation method which can degrade proteinaceous material and thereby inflate the total yield of carbon dioxide (60). Details of the material balance are in Appendix VII. The results are in Column 4, Table IX.

Maximum AGUA contents were also estimated by calculating maximum AGUA losses from a tissue balance applied during chloriting. Details are in Appendix VIII. The results are in Column 5, Table IX.

All of the corrected AGUA values in Table IX are below or statistically equal to the estimated maximums, which strengthens the assumptions involved in calculating the corrected values. The "Linear Loss" values were considered more reliable than the "Chlorite Liquor" values, because the latter would be low if any GUA was lost during dialysis of the spent liquor. Note that the "Linear Loss" values show an AGUA dilution from SX to YOX of 10.8.

The major reason for the differences between the corrected values in Column 2 and the maximum values in Column 4 was probably inclusion of amino acid carbon dioxide in the total uronic acid term of the material balance. Note that the difference is significant only for SX-1, which contains the most protein (7). In particular, serine and threonine have been found in immature aspen xylem (39a) and could be degraded to carbon dioxide under the conditions of the decarboxylation procedure (60). Calculations described in Appendix IX show that their degradation could account for all of the difference for SX-1.

The values for SX-1 and StX-1 in Column 5 are probably high because more carbohydrate material was removed from these tissues than from NX-1 and YOX-1 by the acid chlorite.

In summary, AGUA values have been established for each growth stage in the June collection. They agree very well with values estimated by two independent methods and they agree in order of magnitude with values previously published (7, 38).

Consequently, the data strongly favor the premise that the protopectin has been made accessible to the enzyme. This in turn strengthens the assumption that Pectinol 10M hydrolyzes aspen protopectin to the same extent that it hydrolyzes citrus pectin.

ASSUMPTION 3. THE ONLY SOURCE OF GUA IN ASPEN IS THE PROTOPECTIN

Pectin should have been eliminated as a source by the water extraction.

The data shown in Table IX strongly support Assumption 3, especially that for NX-1 and YOX-1 for which the corrected and maximum values agreed so well. The data for SX-1 and StX-1, while not agreeing as well, do not contradict this assumption because they were explained satisfactorily in Paragraph 2 above.

Therefore, this assumption was accepted as valid under the mild hydrolysis conditions employed.

ASSUMPTION 4. PECTINOL 10M HYDROLYZES METHYL ESTER GROUPS IN ASPEN PROTOPECTIN TO THE SAME EXTENT THAT IT HYDROLYZES THEM IN CITRUS PECTIN

Pectinol 10M should hydrolyze methyl ester groups in aspen protopectin to the same extent that it hydrolyzes them in citrus pectin (92.1%) if the protopectin is accessible to the enzyme. The criterion for accessibility was the attainment of maximum methanol yields.

As with the AGUA assays, a reduction of particle size from 40- to 200-mesh did not change the yield of methanol. Consequently, all tissue was ground so that 90% passed an 80-mesh screen.

Enzymatic hydrolysis time was then investigated. A series of enzymatic assays for methanol were carried out with YOX-1 and StX-2 as described in Appendix II. The data, plotted in Fig. 2, showed that methanol yields from both tissues increased rapidly up to four hours but very slowly thereafter. Since enzymatic hydrolysis for four hours was sufficient to reach a "plateau" in methanol yield, it was accepted as the standard hydrolysis time for the remaining, unchlorited tissues.

Table X shows the methanol contents obtained for all tissues with the four-hour enzymatic hydrolysis. All values are averages of duplicate determinations. The data are very consistent in showing a sequential increase from YOX to SX.

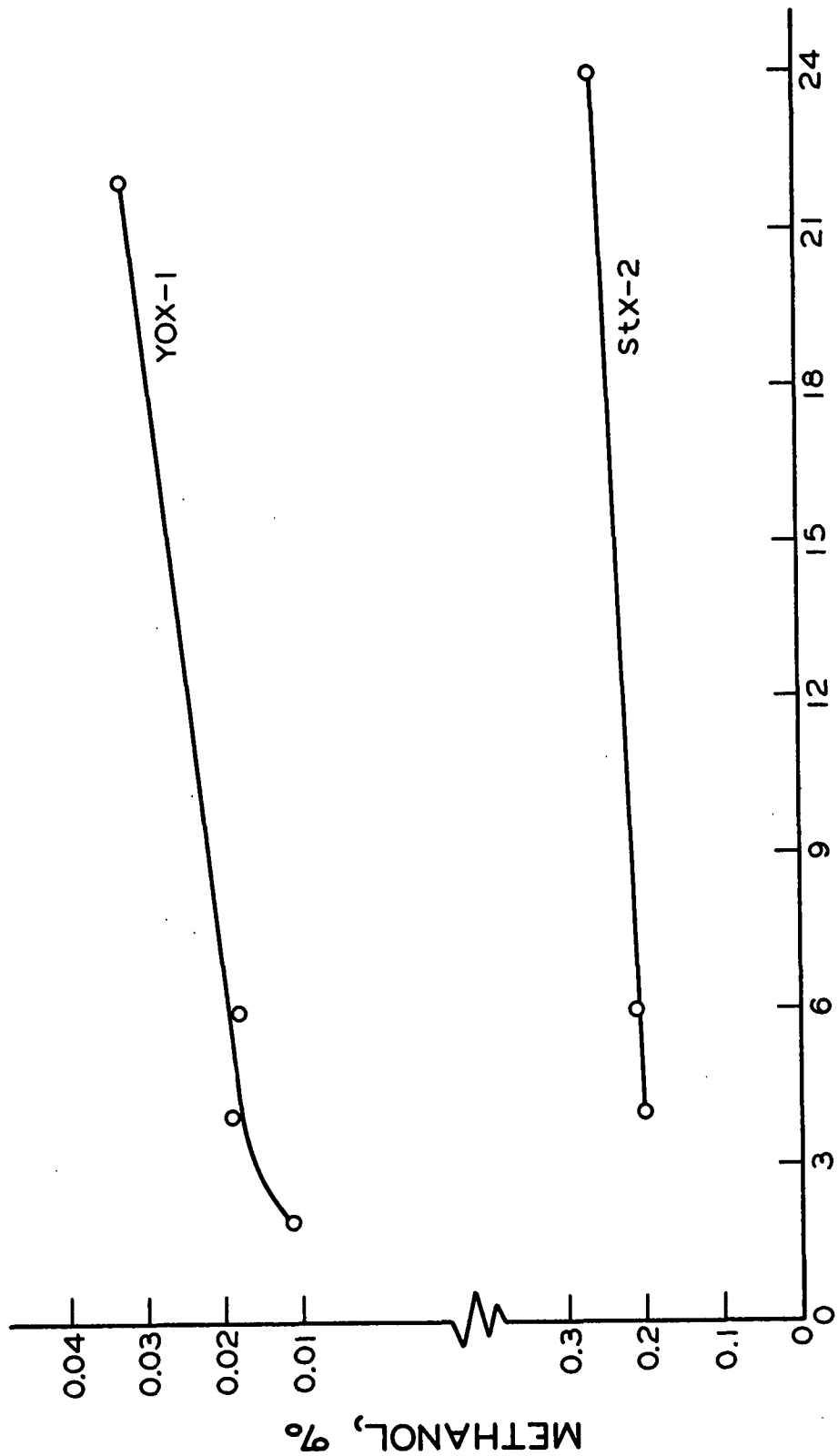


Figure 2. Methanol Content Versus Enzymatic Hydrolysis Time for YOX-1 and StX-2

TABLE X

METHANOL CONTENTS OF UNCHLORITED TISSUES BY ENZYMATIC HYDROLYSIS[a]

Growth Stage	Pectinol 10M, hr.	Methanol, % of o.d. tissue
YOX-1	22	0.032 ± 0.014[b]
"	6	0.018 ± 0.008
"	4	0.019 ± 0.006
"	2	0.011 ± 0.008
NX-1	4	0.088 ± 0.008
StX-1	4	0.28 ± 0.008
SX-1	4	1.2 ± 0.1
YOX-2	4	0.028 ± 0.008
NX-2	4	0.033 ± 0.008
StX-2	24	0.26 ± 0.01[c]
"	6	0.21 ± 0.01[b]
"	4	0.20 ± 0.01
SX-2	4	0.64 ± 0.01

[a]Only SX-1 was corrected for the 92.1% degree of hydrolysis of methanol by Pectinol 10M.

[b]Corrected for HCHO in the distillate. See Appendix II.

[c]Single assay.

The reasonableness of these values was checked by assaying YOX-1 for methanol by saponification, time again being the independent variable. The procedure is described in Appendix II. The results, plotted in Fig. 3, showed a rapid increase in methanol content up to about one hour but only a very slow increase thereafter. Accordingly, the one-hour saponification time was considered optimum for YOX tissue. However, a one-half hour saponification time was applied to the younger tissues, because it gave the standard methanol value for citrus pectin and because they contained less lignin. The results are shown in Table XI and are averages of duplicate determinations.

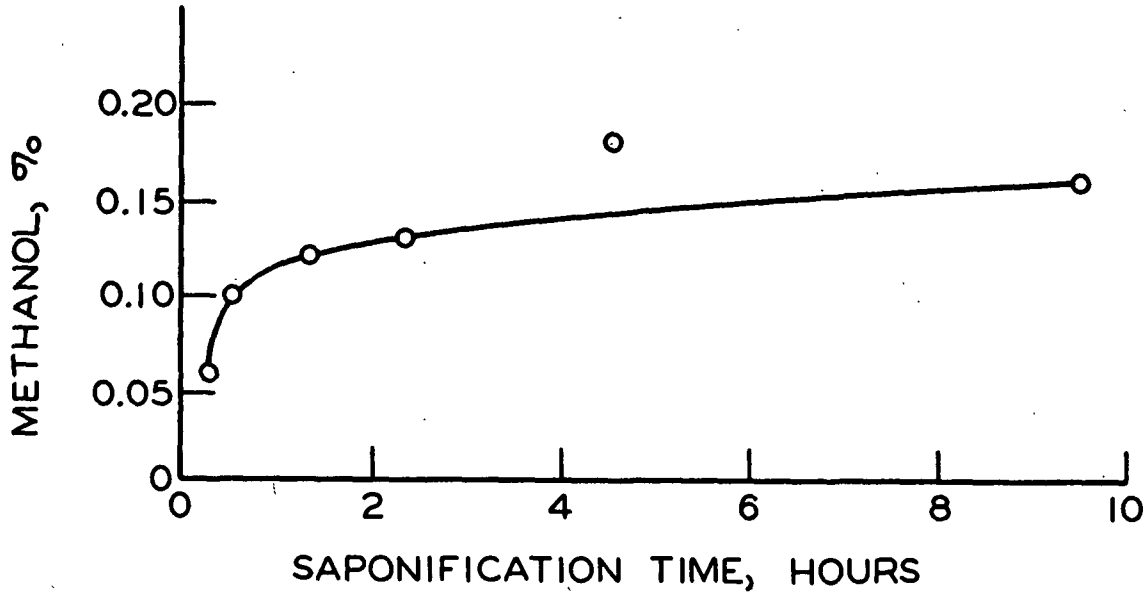


Figure 3. Methanol Content Versus Saponification Time for YOX-1

Comparison of Tables X and XI shows that for YOX, NX, and StX, saponification gave methanol contents which were higher than those given by enzymatic hydrolysis. For SX, the tissue with the lowest lignin content, the methanol values by the two methods agreed reasonably well.

Therefore, remembering that AGUA yield had increased after delignification, experiments were run to measure the methanol yield versus time of delignification by acid chlorite. The four-hour enzymatic hydrolysis was employed because of its reproducibility with the unchlorited tissues. The results are shown in Table XII.

TABLE XI

METHANOL CONTENTS OF UNCHLORITED TISSUES BY SAPONIFICATION

Growth Stage	0.035N NaOH, hr.	Methanol, % of o.d. tissue
YOX-1	9.5	0.16 ± 0.004[a]
"	4.5	0.18 ± 0.004[b]
"	2.3	0.13 ± 0.004
"	1.3	0.12 ± 0.004
"	0.5	0.10 ± 0.003
"	0.5	0.09 ± 0.01[c]
"	0.25	0.060 ± 0.004
"	0.25	0.061 ± 0.008[c]
NX-1	0.5	0.21 ± 0.01
StX-1	0.5	0.39 ± 0.01
SX-1	0.5	0.97 ± 0.02
YOX-2	0.5	0.08 ± 0.01
NX-2	0.5	0.10 ± 0.01
StX-2	0.5	0.29 ± 0.01[c]
"	0.5	0.28 ± 0.01
SX-2	0.5	0.57 ± 0.02

[a] Corrected for 17% HCHO.

[b] Probably contained HCHO.

[c] Sapon., then a 2-hr. enzymatic hydrolysis.

Methanol content increased to a maximum and then decreased for YOX-1, analogous to the situation found for AGUA content. Maximum values for NX-1 and StX-1 were obtained after only two days and one day of chloriting, respectively, and did not decline significantly even after seven days of chloriting. The maximum for SX-1 was obtained without chloriting (see Table X) and did not decline during three days of chloriting. For the latter three growth stages, the loss of ester methanol in chloriting was probably being offset by a gain from other sources in the tissue.

TABLE XII

METHANOL CONTENTS VERSUS CHLORITING TIMES

Growth Stage	Chloriting Time, days	Methanol Content, [a,b] %
YOX-1	1	0.10 ± 0.01
"	3	0.13 ± 0.01
"	6	0.11 ± 0.01[c]
"	10	0.095 ± 0.008[d]
"	14	0.065 ± 0.005[d]
NX-1	2	0.13 ± 0.01
"	3	0.13 ± 0.01
"	5	0.15 ± 0.01
"	7	0.12 ± 0.01
StX-1	1	0.33 ± 0.01
"	3	0.32 ± 0.01
"	5	0.32 ± 0.01
SX-1	1	1.2 ± 0.1
"	3	1.2 ± 0.1

[a] Based on o.d. wt. of tissue before chloriting. See footnote 5 in the text.

[b] Values for tissue recovered were given in Table VIII, except for YOX-1 chlorited 1 day (94.2%) and 6 days (89.4%).

[c] Sapon. gave 0.28 ± 0.02% methanol after correction for about 10% HCHO.

[d] Sapon. gave 0.29 ± 0.02% methanol after correction for about 14% HCHO.

To verify that these other sources were still present after chloriting, StX-1 chlorited three days was hydrolyzed with Pectinol 10M for 12, 24, and 64 hours and gave methanol yields of 0.41, 0.42, and 0.48%, respectively. The results, plotted in Fig. 4, showed that the sources were still present and that the rate of gain of methanol with enzymatic hydrolysis time was 0.0012 ± 0.0001%/hr., calculated from the plateau of the curve in Fig. 4 by regression analysis.

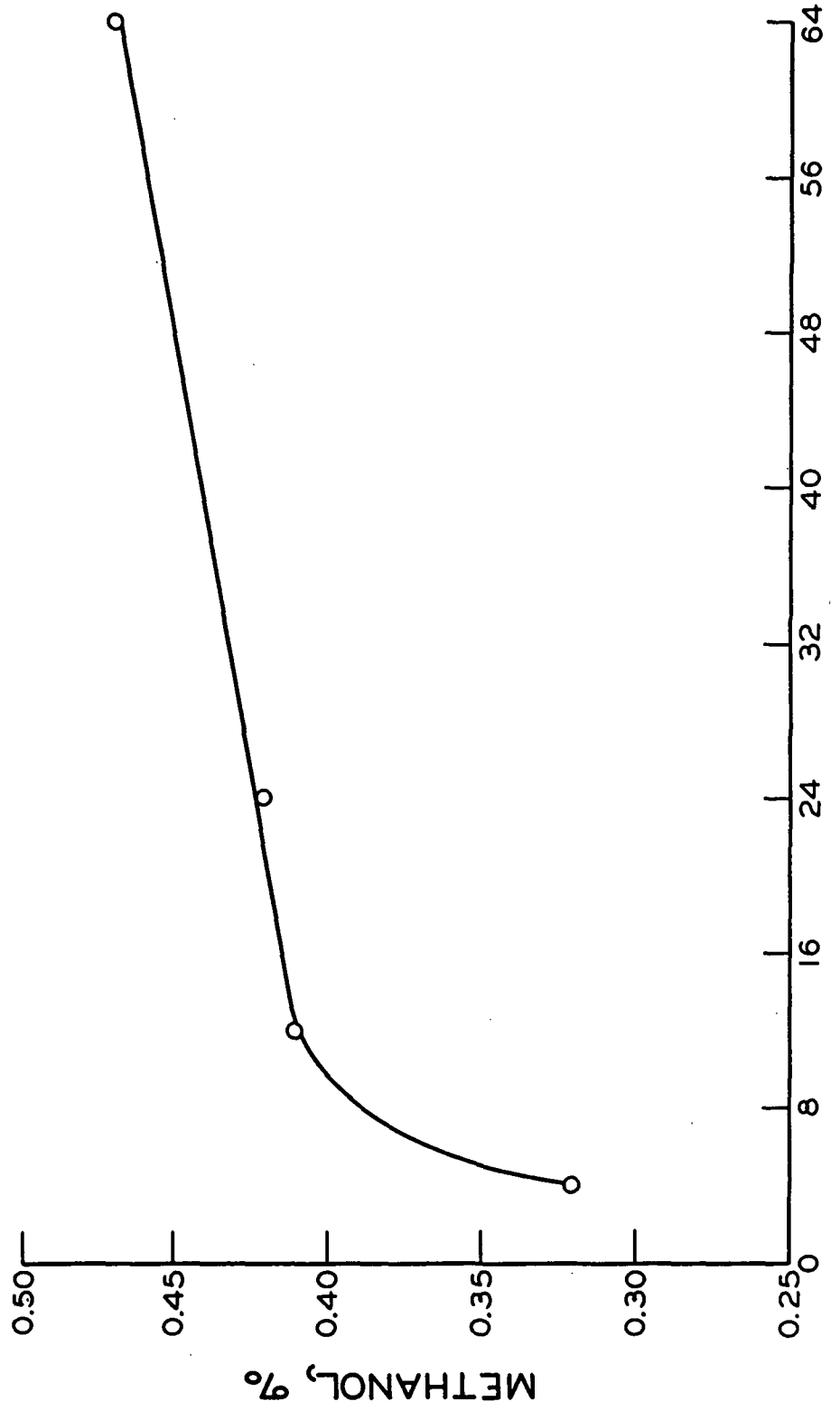


Figure 4. Methanol Yield Versus Enzymatic Hydrolysis Time for Chlorited StX-1

Figure 4 also shows that an enzymatic hydrolysis time of about 12 hours was required to achieve the plateau in methanol content. This implied that a four-hour hydrolysis was insufficient and that the values in Table XII were low, except for SX-1, for which the same values were found with and without chloriting.

Consequently, a safe hydrolysis time of 24 hours was chosen and two samples of each tissue were reassayed. The results are in Table XIII. The new values were all significantly higher than corresponding values in Table XII, except for SX-1.

TABLE XIII

METHANOL CONTENTS OF CHLORITED TISSUES WITH A 24-HOUR ENZYMATIC HYDROLYSIS TIME

Growth Stage	Time Chlorited, days	Methanol, %
YOX-1	3	0.16 ± 0.01
"	14	0.10 ± 0.01
NX-1	3	0.21 ± 0.01
"	7	0.17 ± 0.01
StX-1	1	0.41 ± 0.02
"	5	0.48 ± 0.02
SX-1	1	1.3 ± 0.1
"	3	1.4 ± 0.1

It was then obvious that two corrections would be necessary for YOX, NX, and StX, one for the loss of methanol during chloriting, a second for the gain of methanol from other sources. Furthermore, the values from the 24-hour hydrolyses should be the base values to be corrected. The correction for loss due to chloriting was calculated from the four-hour values in Table XII, exactly as was done for AGUA yield, and was added to the values in Table XIII. Details are given in Appendix VI, and results are shown in Table XIV, Column 3.

TABLE XIV

CORRECTED ESTER METHANOL CONTENTS OF ASPEN XYLEM[a]

Tissue	Time Chlorited, days	Methanol, % Corrected for Loss During Chloriting	Methanol, % Corrected for Other Sources
YOX-1	3	0.18 ± 0.01	0.15 ± 0.01
"	14	0.18 ± 0.02	0.15 ± 0.02
NX-1	3	0.21 ± 0.01	0.18 ± 0.01
"	7	0.17 ± 0.01	0.14 ± 0.01
StX-1	1	0.41 ± 0.02	0.38 ± 0.02
"	5	0.49 ± 0.02	0.46 ± 0.02
SX-1	1	1.2 ± 0.1	1.2 ± 0.1
"	3	1.2 ± 0.1	1.2 ± 0.1

[a]Uncorrected data given in Table XIII, except for SX-1 (Table XII).

The correction for other sources of methanol was assumed to be equal for all growth stages and was taken to be the value calculated for StX-1 − 0.0012 ± 0.0001%/hr. This was multiplied by 24 hours and subtracted from the values in Column 3, Table XIV, to give those in Column 4. The agreement within each growth stage, except for StX-1, was quite good.

The latter values were averaged to obtain one corrected value for each growth stage. Table XV shows the final values, which represent the best estimates of the ester methanol contents of aspen xylem by the enzymatic hydrolysis method. The independent values obtained by saponification are also shown.

TABLE XV

CORRECTED ESTER METHANOL CONTENTS OF ASPEN XYLEM

Growth Stage	Methanol, %[a] By Enzymatic Hydrolysis[b]	Methanol, %[a] By Sapon.[c]
YOX-1	0.15 ± 0.01	0.12 ± 0.004
NX-1	0.16 ± 0.01	0.21 ± 0.01
StX-1	0.42 ± 0.01	0.39 ± 0.01
SX-1	1.2 ± 0.1	1.0 ± 0.02

[a] Based on the unchlorited, water-extractive-free, o.d. xylem.
[b] From Table XIV. [c] From Table XI.

The agreement between the two methods was quite satisfactory. When the average DM of the protopectin is calculated, it will be shown that both sets of data give the same overall results.

In summary, methanol values for each growth stage have been estimated by an enzymatic method, and they agree with values obtained by saponification. The fact that the methanol values always reached a plateau indicated that the protopectin was accessible to the Pectinol 10M. This in turn strengthened the assumption that Pectinol 10M hydrolyzed the methyl ester groups in aspen protopectin to the same extent that it hydrolyzed them in citrus pectin.

ASSUMPTION 5. THE ONLY SOURCE OF ESTER METHANOL IN ASPEN IS THE PROTOPECTIN

It was shown in Fig. 3 and 4 that methanol yield increased rapidly to a plateau, then increased very slowly as reaction time was increased with both the saponification and enzymatic methods. The initial, rapid increase was attributed to rapid hydrolysis of ester methanol from the protopectin, while the later, slower increase was attributed to hydrolysis of methanol from nonpectic sources.

Such sources might be the lignin, 4-O-methylglucuronoxylan, or proteins with labile N- or S-methyl groups. Each of these possible sources was represented by a model and tested for release of methanol under the usual conditions for saponification and enzymatic hydrolysis.

The model for lignin was Brauns' native lignin (BNL) from aspen NX (39a), because the mild extraction procedure for BNL minimizes changes during isolation. Coniferin and syringin were also tested because of their possible presence and importance to lignin synthesis.

The model for protein was L-methionine, because its methyl group can serve as a source for the methyl ester group in pectinic acid (62). Choline and betaine were also tested because they were readily available and can serve as methyl donors in transmethylation reactions (63).

The model for 4-O-methylglucuronoxylan was the aldotriuronic acid originating from it.

The conditions and results of these experiments are shown in Table XVI. The data show that the nonpectic methanol might originate from lignin or ligninlike material in the tissue. It probably does not originate from L-methionine, betaine, choline, or 4-O-methylglucuronoxylan under the conditions of the assays.

The 40-minute distillation at 100°C. probably hydrolyzes the aromatic methoxyl groups to the slight extent required. About 0.18% of methanol has been obtained from veratraldehyde in three hours at 190°C. (64), and methanol has been obtained from BNL upon treating it with 1.25N sodium hydroxide at 23°C. for 48 hours (65).

TABLE XVI

METHANOL FROM MODEL COMPOUNDS

Substrate	Model For:	Test Conditions	Methanol, % of substrate wt.
Brauns' native lignin from aspen NX	Lignin	Blank	0.076 ± 0.052
		Blank, after two humidity cycles[a].	0.059 ± 0.040
		4-Hr. enzymatic hydrolysis.	0.029 ± 0.022[b]
		1/2-Hr. sapon.	0.020 ± 0.060
Syringin monohydrate	Ligninlike material	1/2-Hr. sapon., then 2-hr. enzymatic hydrolysis.	0.13 ± 0.12
		Blank, 2-1/2-hr. hydrolysis.	0.0
Coniferin dihydrate	Ligninlike material	1/2-Hr. sapon., then 2-hr. enzymatic hydrolysis.	0.062 ± 0.072
		Blank, 2-1/2 hr. hydrolysis.	0.0
L-methionine	Protein	4-Hr. enzymatic hydrolysis.	0.0
Betaine hydrochloride		1/2-Hr. sapon.	0.0
Choline chloride			
Aldotriuronic acid[c]	4-O-methylglucurono-xylan	4-Hr. enzymatic hydrolysis.	0.0

[a] To remove any methanol retained during isolation.
[b] After correction for 0.110 ± 0.040% HCHO.
[c] Aldotriuronic acid = O-[4-O-methyl-α-D-glucopyranosyluronic acid-(1 → 2)]-β-D-xylopyranosyl-(1 → 4)-β-D-xylopyranose.

In summary, Assumption 5 must be qualified as follows: There are, even under the mild hydrolysis conditions employed, other sources of methanol in the tissue. These are probably guaiacyl or syringyl derivatives but may also be heretofore unknown methyl ester groups.

However, corrections for these nonpectic sources were made, so that the corrected methanol values in Table XV should represent only ester methanol from the protopectin.

CALCULATION OF DEGREE OF ESTERIFICATION OF THE PROTOPECTIN

The average degree of esterification of the protopectin with methanol (average DM) was calculated by Equation (2):

$$\text{Average DM} = (M/32) \,/\, (G/176), \qquad (2)$$

where $\underline{M}$ = percentage of methanol in the tissue, and

$\underline{G}$ = percentage of AGUA in the tissue.

Two sets of methanol values were employed, one from enzymatic hydrolysis and the other from saponification. The results, shown in Table XVII, illustrate two points: (1) the average DM of the protopectin is below 0.8 at the 95% confidence level in seven of eight cases, and (2) the average DM remains constant or may increase slightly as the tissue matures. It definitely does not decrease. The same trends are shown by both sets of methanol values.

TABLE XVII

AVERAGE DM OF ASPEN PROTOPECTIN[a]

Growth Stage	Average DM	
	Enzymatic Hydrolysis	Sapon.
YOX-1	0.68 ± 0.11	0.51 ± 0.08
NX-1	0.52 ± 0.13	0.68 ± 0.16
StX-1	0.50 ± 0.04	0.47 ± 0.03
SX-1	0.47 ± 0.04	0.39 ± 0.04

[a]AGUA data from Table IX, Column 2; methanol data from Table XV.

DISCUSSION OF RESULTS

Tables VII-XVII show the iterative steps by which the enzymatic assays for GUA and ester methanol were developed to their present stage of reproducibility and accuracy. For example, it was assumed that grinding the tissue to pass an 80-mesh screen would make the protopectin accessible to Pectinol 10M. Subsequent experiments (Table VII) proved this assumption untrue. Then it was assumed that removal of lignin was necessary, and subsequent experiments (Table VIII) indicated that this was true.

Proceeding in this manner, the following assay requirements were established:

1. The protopectin must be made accessible to the enzymes. If delignification is employed, the protopectin will probably be degraded somewhat, and the GUA and ester methanol values must be corrected appropriately.

2. The degree of hydrolysis attained by the enzymes must be established with a control and, if significantly less than 100%, the GUA and methanol values should be corrected.
3. Blank assays must be run and corrections applied if needed.
4. Methanol may be obtained from sources other than the protopectin, requiring an appropriate correction.

These assays yielded information which, in some cases, supports and, in other cases, limits the analogy between citrus and wood pectins. For example, the successful use of Pectinol 10M to determine GUA and methyl ester groups both in citrus pectin and aspen tissue is evidence that the structures of citrus and aspen protopectin are similar. They are not identical, however, because the average DM of aspen protopectin is below 0.68 ± 0.11 and, therefore, lower than that of citrus protopectin.

A possible reason for this difference is that the function of protopectin in aspen requires more intermolecular, ionic, or ester bonds than it does in citrus fruits. Partially esterified polygalacturonic acid has a high degree of flexibility in this respect.

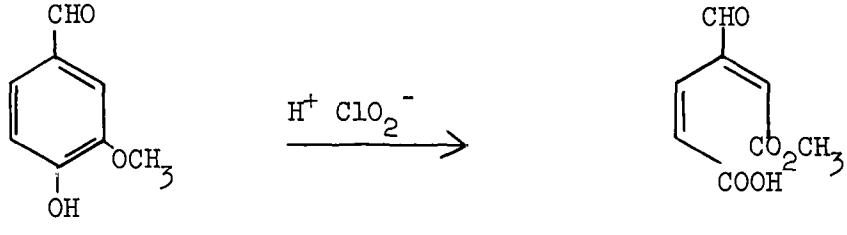
Another reason for the lower average DM in aspen may be that the lignin prevents the enzymatic attainment of higher DM's, just as it shields over 90% of the protopectin in YOX from Pectinol 10M.

At least three mechanisms can be postulated for this shielding: (1) the density of the cell wall due to lignification may physically prevent diffusion of the Pectinol enzymes; (2) lignin-protopectin bonds may exist; and (3) the lignin may be a biochemical inhibitor of enzymatic activity.

The available evidence does not favor any particular one of these mechanisms. The lignin-protopectin relationship is probably a very intimate one, because grinding the tissue to pass a 200-mesh screen (74 micron openings) did not increase the accessibility of the protopectin to the enzymes.

If the protopectin is confined to the compound middle lamella, then only a portion of the lignin can be associated with it. Comparison of Table XXXI (Appendix X) with Table III shows that a maximum of 69, 53, and 66% of the lignin in YOX-1, NX-1, and StX-1, respectively, needed to be removed to render the protopectin accessible to the enzymes. Moreover, over 96% of the material removed from YOX-1 and NX-1 was lignin. Of equal importance, however, may be that over 60% of the lignin not removed from YOX-1 and NX-1 was rendered acid-soluble (see Table XXXI).

One way in which the lignin was probably altered by the chloriting is shown by the following reaction (61):



(3).

The methyl ester groups produced would be a source of methanol in saponification assays and, in fact, saponification values were significantly higher after chloriting (Table XII, footnotes c and d) than before chloriting (Table XI). Such methyl ester groups should not be hydrolyzed by the pectinesterase in Pectinol 10M, but they might be partially hydrolyzed by heat during the 40-minute distillation in the enzymatic methanol assay. This could explain how the enzymatic methanol yields from NX-1, StX-1, and SX-1 remained constant

during chloriting (Table XII) while the protopectin, i.e., the AGUA, was being slowly dissolved in the liquor.

One other experimental result deserves comment. Pectinol 10M hydrolyzed about 93% of the AGUA and methyl ester groups available in citrus pectin. This may indicate that about 7% of the nonesterified AGUA units are atypical.

For example, it has been shown (53) that the hydrolysis of a pectin methyl ester group by pectinesterase requires that at least one of the carboxyl groups on the two adjacent AGUA units be unesterified. If both of the adjacent carboxyl groups were, e.g., esterified with neutral sugars, then that methyl ester group might not be measured by the enzymatic methanol assay, and the three AGUA units probably would not be measured by the enzymatic GUA assay.

The same situation could arise for aspen protopectin, where the possibilities for attachment also include lignin.

EXTRACTION OF PECTINIC ACIDS

GENERAL CONSIDERATIONS

One purpose of this investigation was to suggest a rational method for extraction of pectinic acids from aspen tissue. The average DM of aspen protopectin lies between 0.47 ± 0.04 and 0.68 ± 0.11 (Table XVII). If the DM's of the individual protopectin molecules are heterogeneous, then the water-insolubility of the protopectin could result from some molecules having a DM over 0.8 while the rest have a DM below 0.43 and qualify as low DM pectinic acids. The latter are water-insoluble if present as calcium pectinates and acid-insoluble if present as free acids (15). If the DM's of the individual molecules are uniform, then the water-insolubility of the protopectin must be due to factors other than DM or formation of calcium pectinate. It may be due to an extremely high DP, to covalent, ionic, or hydrogen bonding with other cell wall components (also producing, in effect, a high DP), or to physical entrapment (15).

If aspen protopectin is analogous to citrus protopectin, then pectinic acid should be obtained upon treating aspen tissue with N HCl at 35-40°C. for 48 hours – the "pickling" process (68). The merits of this process are that it causes very little depolymerization (66), it frees pectinic acids from their calcium salts (15), and it deesterifies very gradually (66). Therefore, it should solubilize aspen protopectin molecules with DM's from 1.0 down to about 0.43.

The existence of insoluble calcium pectinates in wood is indicated by the successful extraction of up to 0.4% AGUA from beechwood (29) and boxwood (30) with hot ammonium oxalate, which implies the following reaction:

$$\begin{pmatrix}\text{calcium}\\ \text{pectinate}\end{pmatrix}(\underline{i}) + \begin{pmatrix}\text{ammonium}\\ \text{oxalate}\end{pmatrix} = \begin{pmatrix}\text{ammonium}\\ \text{pectinate}\end{pmatrix}(\underline{s}) + \begin{pmatrix}\text{calcium}\\ \text{oxalate}\end{pmatrix}(\underline{i}) \qquad (4)$$

This solvent is objectionable because it can cause decarboxylation (67) and some amidation (13).

Theoretically, a room temperature process employing oxalic acid to decalcify and sodium bicarbonate to form soluble sodium pectinates would be more appropriate. Like the pickling process, it should not depolymerize the pectinic acids, nor should it deesterify them.

This process was applied to SX, because that growth stage may have a lower average DM, hence more calcium pectinate, than YOX. As controls, the oxalic acid and sodium bicarbonate were added in stoichiometric amounts based on the assumption that the alkalinity of the ash in SX is calcium carbonate, and the extraction was evaluated by a material balance.

SAMPLE PREPARATION

The preparation of YOX-P to be extracted with the pickling process and of SX-O to be extracted with oxalic acid-sodium bicarbonate was described on page 17.

Soft xylem (coded SX-P) had already been subjected to the pickling process, and the extracted products had been analyzed for total uronic acid and ash (69) but not for GUA or ester methanol. The SX-P had been extracted with – and air-dried from – methanol in accordance with previously described procedures (35, 39). No analysis of its composition was available, but it appeared to be normal SX (13) and, therefore, similar in composition to that shown in Table III.

EXPERIMENTAL METHODS

The analytical methods for uronic anhydride, ash, and specific optical rotation are described in Appendix IV. The methods for GUA and ester methanol are described in Appendices I and II, respectively. The pickling and oxalic acid-sodium bicarbonate extraction processes are shown schematically in Fig. 5, 6, and 7.

RESULTS OF THE EXTRACTIONS

The properties of the products extracted with the pickling process are shown in Table XVIII. The YOX probably contained about 1% AGUA before extraction, so only about 1% (= 0.01/1 x 100) of that was extracted. Fraction I from YOX did contain sufficient ester methanol to give an average DM of 0.45 for the AGUA.

Each of the three fractions extracted from SX-P contained more AGUA than did those from YOX, but they still did not exhibit the fibrous qualities typical of citrus pectin. If SX-P contained 10% AGUA before extraction, then 25% (= 2.5/10 x 100) of the AGUA was extracted, a yield much higher than for YOX.

Table XVIII also shows that the pectic substances had a wide range of DM. The DM of 0.79 for the AGUA in Fraction III is the highest reported for woody tissue, excluding callus tissue (39). It is interesting that the weighted average DM[6] for all three fractions from SX-P was 0.42.

Also note that all of the uronic anhydride in Fractions I and II was probably AGUA.

---

[6] Weighted average DM = $[(0.39)(0.348)(0.019) + (0.28)(0.561)(0.023) + (0.79)(0.090)(0.061)]/(0.025)$.

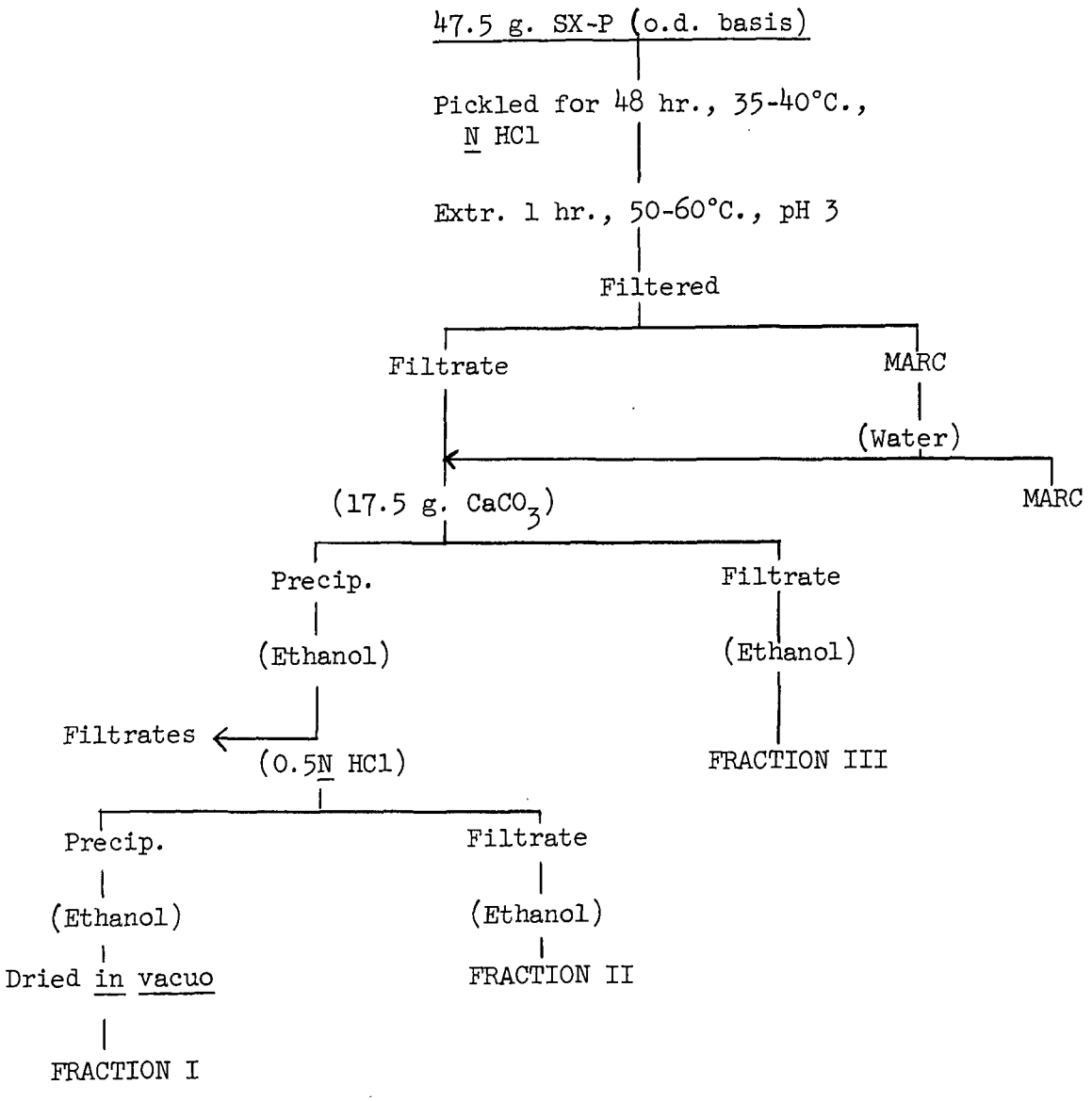


Figure 5. Extraction of Aspen SX-P with the Pickling Process (68). Work Done by Haas and Kremers (69)

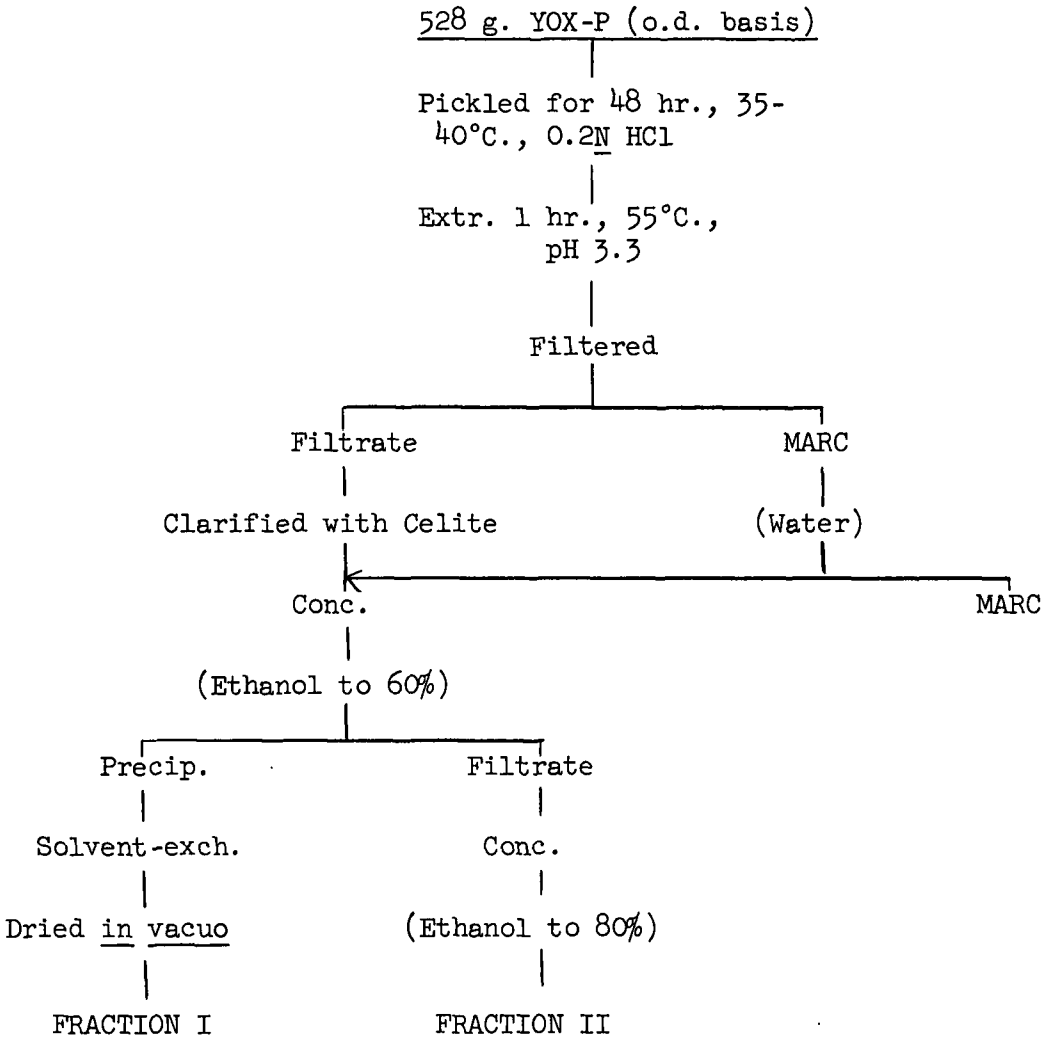
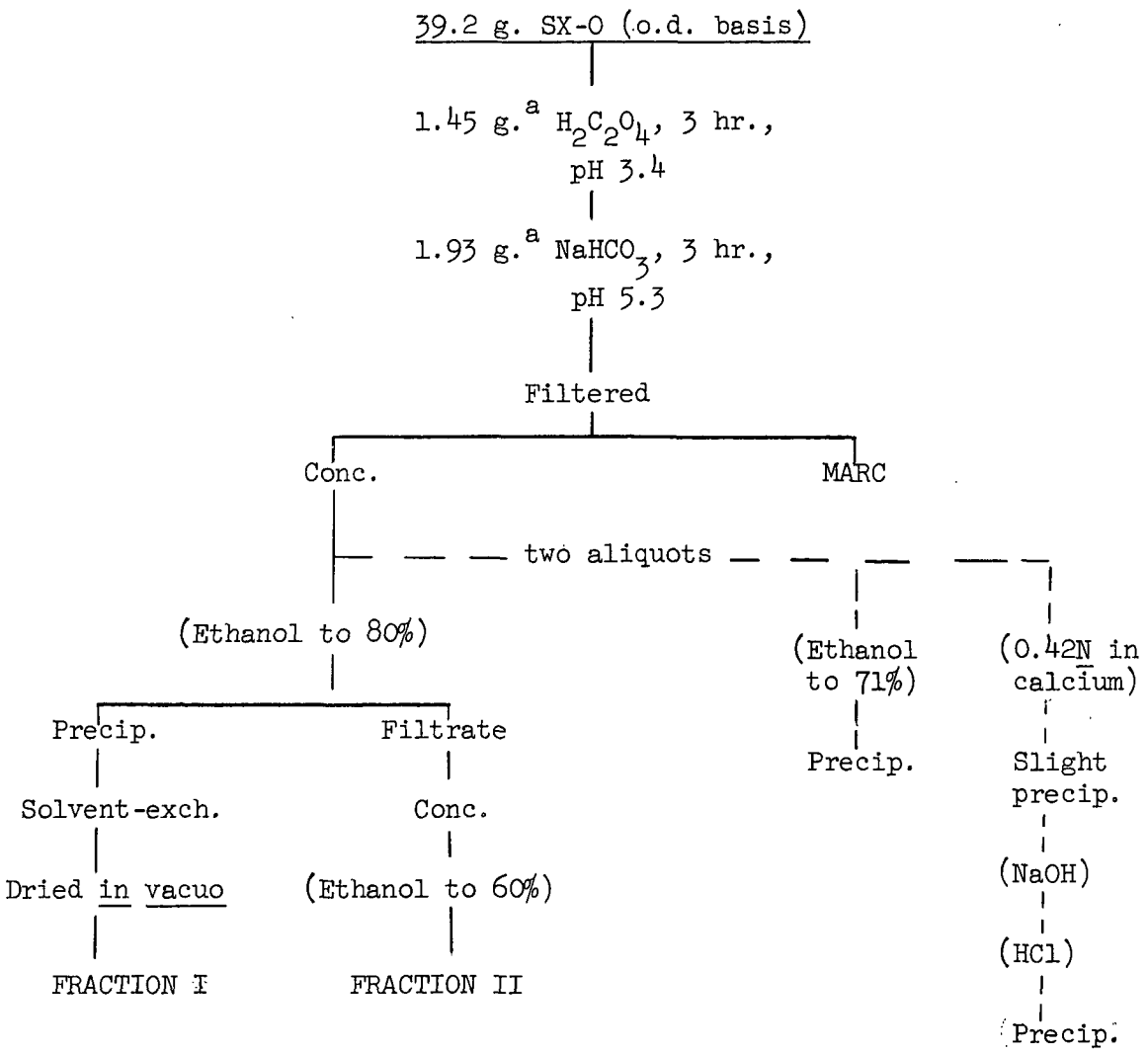


Figure 6. Extraction of Aspen YOX-P with the Pickling Process (68)



[a]Stoichiometric amount, assuming alkalinity of ash in SX-O is $CaCO_3$.

Figure 7. Extraction of Aspen SX-O with Oxalic Acid-Sodium Bicarbonate

TABLE XVIII

PROPERTIES OF PRODUCTS EXTRACTED WITH PICKLING PROCESS

Fraction	SX-P I	SX-P II	SX-P III	YOX-P I	YOX-P II
Uronic anhydride, %[a,b]	34.7	55.3	--	--	--
AGUA, %[a]	34.8	56.1	9.0	2.6	0.0
Average DM	0.39	0.28	0.79	0.45	--
Ash, %[a,b]	28.8	10.9	--	--	--
Yield of fraction, %[b,c]	1.9	2.3	6.1	0.53	0.07
Yield of AGUA, %[c]	0.7	1.3	0.5	0.01	0.0
		2.5			

[a] Calculated as percentage of the o.d. fraction.
[b] Values supplied by Haas and Kremers (69).
[c] Calculated as percentage of o.d. xylem before extraction.

The extraction of pectic substance from SX-O with oxalic acid-sodium bicarbonate was followed by means of AGUA and methanol balances. The results are shown in Table XIX. About 27% (= 1170/4370) of the theoretically available AGUA was extracted. About 93% (= 36.6/39.2) of the total SX-O, but only 50% (= 114/228) of the ester methanol, were recovered.

The reason for the loss of methanol is not known, but one possible explanation is that the tissue contained pectin methylesterase which hydrolyzed the methyl ester groups during the three-hour extraction with sodium bicarbonate at pH 5.3 – the ideal pH for optimum functioning of methylesterases of the type found in Pectinol 10M (70). Calculations showed that if 80% of the "unrecovered" methanol were assigned to Fraction I, its AGUA would have an average DM of 0.5 – the upper limit on formation of insoluble calcium pectinate (15). Therefore, at least 20% of the "unrecovered" methanol was probably hydrolyzed from the AGUA remaining in the marc.

TABLE XIX

AGUA AND METHANOL BALANCE FOR SOFT XYLEM EXTRACTED WITH OXALIC ACID-SODIUM BICARBONATE

	Fraction Wt., g.	AGUA Wt., mg.	Methanol Wt., mg.	Average DM
SX-O before extraction[a]	39.2	4370	228	0.29
SX-O after extraction (marc)	31.4	3180	99	0.17
Extracted fractions:				
I	3.7	1170	15	0.07
II	1.5	0	--	--
Unrecovered	2.6	20	114	--

[a]Ash content was 2.93%.

Table XX shows the properties of the extracted products.

TABLE XX

PROPERTIES OF THE PRODUCTS EXTRACTED WITH OXALIC ACID-SODIUM BICARBONATE

Fraction	I	II
Ester methanol, %[a]	0.41	--
AGUA, %[a]	31.6	0.0
Average DM	0.07	--
Ash, %[a]	17.7	--
Specific optical rotation, $[\alpha]_D^{23}$	+97.3°	--
Yield of fraction, %[b]	9.4	3.8
Yield of AGUA, %[b]	3.0	0.0

[a]Calculated as percentage of the o.d. fraction.
[b]Calculated as percentage of the o.d. SX-O before extraction.

The positive specific rotation, low average DM, and presence of ash of Fraction I are evidence that sodium pectinate is present. However, the product did not exhibit any qualities typical of sodium pectinate, and it contained twice as much ash (assumed Na_2CO_3) as the 8.8% required stoichiometrically by a sodium pectinate with an average DM of 0.07. The excess could easily have been adsorbed by the product as it was precipitated with ethanol.

Another interesting feature of this product was shown in Fig. 7: it was not precipitable with 0.42N calcium and required 70% ethanol when it finally did precipitate. True sodium pectinate with an average DM of 0.07 should be precipitable with calcium and 60% ethanol.

There are at least three possible explanations for its behavior: (1) protective action by other colloids in solution, (2) a low molecular weight, and (3) a branched structure. The first possibility is considered the most probable, because the mild extraction conditions should not have caused depolymerization and because a linear structure was implied by its susceptibility to hydrolysis with Pectinol 10M.

DISCUSSION OF RESULTS

The yields and properties of the extracted products were disappointing from the standpoint of extraction of citrus-type pectin from wood, but they were meaningful with regard to elucidation of the state of aspen protopectin. For example, the fact that the pickling process extracts pectin from citrus albedo but not from aspen tissue indicates that the state of the protopectin differs in the two, either chemically and/or physically.

Moreover, all of the data in Tables XVIII and XX corroborated the intermediate DM's shown in Table XVII for aspen YOX and SX.

The extraction by pickling of 25% of the AGUA from SX but only about 1% from YOX indicates that a change occurred (probably lignification) which decreased the accessibility of the protopectin and probably also indicates that the water-insolubility of the protopectin molecules with DM's over 0.43 cannot be due solely to bonding to other cell wall components through divalent cations.

The extractions also showed that about 20% of the AGUA in XS-P had an average DM below 0.5 and that the 27% of AGUA extracted from SX-O had an average DM of 0.07. This may indicate that at least 20% of the protopectin in SX is present as calcium pectinate.

The fact that a wide range of average DM was found for the pectinic acids extracted from both YOX and SX indicates that aspen protopectin molecules probably also have a wide range of DM.

From these results and from the many previous attempts at extraction of pectinic acids from wood (2, 8-10, 29, 30, 37, 66), the following statements can be made regarding future extraction attempts:

1. The solvents cannot be alkaline.
2. Wood should be partially delignified before extraction (8).
3. More than one solvent will probably be needed.
4. Hot acid will probably be more effective than cold acid.
5. Divalent cations should be removed.

IDENTIFICATION OF GALACTURONIC ACID AND METHANOL

To prove that GUA and methanol were the products of enzymatic hydrolysis of aspen tissue, both were isolated and identified – the GUA in the form of its sodium calcium double salt and the methanol as its 3,5-dinitrobenzoate.

PREPARATION OF SODIUM CALCIUM D-GALACTURONATE

The starting material was Fraction I extracted from SX-O (see Table XX for composition of Fraction I). About 1.95 g., dissolved in 85 ml. of water, were treated according to the directions of Isbell and Frush (71), except that:

1. Quantities were scaled down proportionately.
2. The pH was brought to 4.2 with 70% nitric acid added dropwise.
3. The solution was stirred at ambient temperature for 4.5 hours.

Table XXI compares the properties of the derivative from aspen with derivatives from two other sources. From these data it was concluded that GUA was a product of hydrolysis with Pectinol 10M of aspen tissue.

TABLE XXI

PROPERTIES OF SODIUM CALCIUM D-GALACTURONATES

Property	Source		
	Authentic GUA (71)	Aspen SX-O	Black Spruce Bark (11)
Purity of source, %	100	31.6	10
Yield, % of theory	>99	52	28
Equil. $[\alpha]_D^{20}$	+32.4 (c,1.4%; H_2O)	+31.4 (c,2.3%; H_2O)	+43.4[a] (c,2%; 0.1N HNO_3)
Calcium, %	5.4	--	5.3
Ash, %	20.4	22.5	--

[a]Should be +44.0 (71).

Note that the percentage yields of derivative decrease as the AGUA content of the source decreases, a trend that had been observed previously (71).

PREPARATION OF METHYL-3,5-DINITROBENZOATE

The starting material was 197 g. (o.d.) of 20-mesh soft xylem, which assayed 0.69% (=1360 mg.) methanol by the enzymatic method.

This SX was divided into ten batches and hydrolyzed with Pectinol 10M as usual (Appendix II), except that:

1. Only 110 mg. of EDTA and two drops of glacial acetic acid were added per gram of SX.
2. The concentration of SX in water was 3% (o.d. basis).
3. On the average, 205 ml. of distillate were collected from each batch.

The combined volume of the distillates was 2050 ml. This was cohobated to 780 ml. (Solution A) containing 1320 mg. of methanol. The 1270 ml. of residual solution (B) containing 40 mg. of methanol were cohobated to 700 ml. (C). Solutions A and C were combined and cohobated to 585 ml. (D), which contained the entire 1360 mg. of methanol and represented 100% recovery of ester methanol from the SX.

Solution D was concentrated to 1.3 ml. of about 70% methanol (E) by distillation in two batches with a 60-cm. spinning band distillation column. The temperature at the top of the column at the beginning of collection of distillate was 66°C., the boiling point of methanol (71a).

Methyl-3,5-dinitrobenzoate was prepared from E by reaction with 3,5-dinitrobenzoyl chloride (71a). The yields and melting points of the crude

and recrystallized products are compared with those prepared from authentic methanol in Table XXII.

TABLE XXII

PROPERTIES OF METHYL-3,5-DINITROBENZOATES

Property	Source: Authentic Methanol	Source: Aspen SX
Wt. of 3,5-dinitrobenzoyl chloride, g.	0.54	0.50
Yield, crude, %	87.0	88.0
M.p., crude, °C.	105-106	105-107
Yield, recryst'd., %	77.8	76.0
M.p., recryst'd., °C.	106-108	101-103
Mixed m.p., °C.	--	102-104

The data compare favorably except for the melting points of the recrystallized derivatives. It was postulated that transesterification occurred in the aspen derivative during recrystallization from hot, 80% ethanol, resulting in a mixture of the ethyl and methyl derivatives. A 9:1 mixture of authentic methyl- and ethyl-3,5-dinitrobenzoates had a melting point of 101-104°C., while a 1:1 mixture melted at 79-81°C. Thus, about 10% of the ethyl derivative as an impurity would be sufficient to cause the observed melting point. Conclusive proof was obtained from the NMR spectra of authentic ethyl-3,5-dinitrobenzoate and the aspen derivative. The δ-values of the peaks are shown in Table XXIII.

TABLE XXIII

DELTA-VALUES FROM NMR SPECTRA OF
ETHYL-3,5-DINITROBENZOATE AND ASPEN DERIVATIVE[a]

Peak Identity	Source	
	Ethyl-3,5-dinitrobenzoate	Aspen Derivative
$-CH_3$ of $-OCH_2CH_3$	1.45	1.28
$-CH_3$ of OCH_3	--	3.90
$-CH_2$ of $-OCH_2CH_3$	4.51	4.34
-CH= of benzene ring	9.20	9.20

[a]A Varian Model A-60A NMR Spectrometer was employed.

The data show that the aspen derivative contained both ethyl- and methyl-3,5-dinitrobenzoates. Integration of the spectrum of the aspen derivative showed that the ethyl derivative represented about 13% of the mixture, which agreed very well with the 10% figure predicted from melting point data. Therefore, it was concluded that 87% of the mixture was methyl-3,5-dinitrobenzoate and that methanol was a product of enzymatic hydrolysis of aspen tissue.

SUMMARY OF EXPERIMENTAL RESULTS

Developing tissue was collected from 14 aspen trees in 1965. Upon collection, the YOX and NX were air dried and the StX and SX were frozen; the latter were subsequently freeze-dried. Experiments with this tissue gave the following results.

1. Enzymatic assays for determining AGUA and methyl ester in citrus pectin were equally successful with SX-1:
   a. Saponification for one-half hour followed by Pectinol 10M for two hours gave an AGUA content of 14.1% for SX-1;
   b. Pectinol 10M alone for four hours gave an ester methanol content of 1.2% for SX-1.
2. Partial delignification was required before these enzymatic assays were equally successful with StX-1, NX-1, and YOX-1.
3. Certain of the tissues were analyzed for lignin before and after chloriting with acidic sodium chlorite at room temperature:
   a. Less than 69, 53, and 66% of the lignin needed to be removed from YOX-1, NX-1, and StX-1, respectively, to attain maximum AGUA yields with the enzymatic assays;
   b. Over 96% of the material removed from YOX-1 and NX-1 during chloriting was lignin; and
   c. Of the lignin remaining in YOX-1 and NX-1, over 60% was rendered acid-soluble.
4. Decreasing the particle size of YOX-1 from 80- to 200-mesh did not increase the accessibility of the protopectin to Pectinol.

5. Maximum AGUA contents were estimated for each growth stage by a uronic acid balance:
   a. The maximums estimated for YOX-1, NX-1, and StX-1 agreed statistically with the enzymatic values;
   b. The maximum estimated for SX-1 was higher than the enzymatic value. The known instability of serine and threonine to the decarboxylation conditions employed in determining uronic acid could account for the difference.
6. Maximum AGUA values were also estimated for each growth stage by a tissue balance applied during chloriting:
   a. The maximums estimated for YOX-1 and NX-1 agreed statistically with the enzymatic values;
   b. The maximums estimated for StX-1 and SX-1 were higher than the enzymatic values.
7. Both the enzymatic and saponification methods yielded methanol values which, as a function of reaction time, showed an initial, rapid increase--attributed to hydrolysis of ester methanol from the protopectin--followed by a much slower increase--attributed to sources other than the protopectin.
8. Brauns' native lignin from aspen NX, coniferin, and syringin yielded about 0.03-0.13% of their weights of methanol under the conditions of the assays.
9. Saponification for one-half hour gave ester methanol contents of 0.21, 0.39, and 1.0% for NX-1, StX-1, and SX-1, respectively.
10. Saponification for one hour gave 0.12% of methanol from YOX-1.
11. The Pectinol 10M itself released GUA and methanol:
   a. The correction for GUA amounted to about 11% of the GUA content of YOX-1 but less than 1% of the GUA in SX-1;
   b. The methanol correction amounted to about 6% of the methanol content of YOX-1 but less than 1% of the methanol in SX-1.

12. Methyl-3,5-dinitrobenzoate was prepared from the methanol hydrolyzed from SX with Pectinol 10M..

13. Sodium calcium D-galacturonate was prepared from GUA hydrolyzed with Pectinol 10M from pectic substance extracted from YOX.

14. The average DM of the protopectin, as determined by the enzymatic methods, was 0.68 ± 0.11 in YOX-1, 0.52 ± 0.13 in NX-1, 0.50 ± 0.04 in StX-1, and 0.47 ± 0.04 in SX-1.

15. The pickling process extracted about 25% of the total AGUA from SX; about 80% of it had an average DM below 0.5.

16. The pickling process extracted about 1% of the total AGUA from YOX; all of it had an average DM below 0.5.

17. A citrus pectinlike product was not obtained from aspen with the pickling process.

18. Extraction of SX with oxalic acid and then sodium bicarbonate, each for three hours at 25°C., removed 27% of the total AGUA. This AGUA had an average DM of only 0.07.

19. The average DM's of the polygalacturonic acids in the extracted products ranged from 0.07 to 0.79.

20. Cold-water-soluble polygalacturonic acid and free GUA were found in small amounts only in StX.

21. Xylose was found in the water extractives from SX, StX, and NX only after acid or enzymatic hydrolysis.

22. The syringaldehyde-to-vanillin ratio was 1.0 in SX-1 and 2.1 in SX-2 but approached the usual 3.0 in subsequent growth stages.

Experiments with a citrus pectin control gave these results:

1. Saponification for one-half hour followed by Pectinol 10M for two hours hydrolyzed about 94% of the AGUA.

2. Pectinol 10M alone hydrolyzed about 92% of the methyl ester groups in four hours, while saponification for one-half hour hydrolyzed 100% of these groups.

CONCLUSIONS

Enzymatic techniques have been developed which give reproducible and accurate values for galacturonic acid and methyl ester contents of the protopectin in developing aspen xylem.

Since the same enzymes and techniques reproducibly determined the galacturonic acid and methyl ester contents of a citrus pectin control, it follows that aspen protopectin and citrus pectin have the same structures, within the limits of the enzymes' specificity.

The application of these techniques to aspen xylem has shown that the statement "wood pectins are closely related to citrus pectins" must be qualified. For example, aspen "pectin" differs from citrus pectin in that:

1. It has not been obtained as a jelly-grade pectin with the pickling process.
2. It is not present as a cold-water-soluble product to any appreciable extent, even in stringy xylem.

Moreover, aspen protopectin differs from citrus protopectin in that:

1. It has an average degree of esterification with methanol below 0.68 ± 0.11.
2. Its average degree of esterification cannot, alone, account for its water-insolubility.
3. It is intimately associated with the lignin in the compound middle lamella. Any analytical method relying on diffusion of large molecules to the protopectin must allow for this fact.

Aspen "pectin" is similar to citrus pectin in that:

1. It is probably a linear polymer of α-1,4-linked D-galacturonic acid units partially esterified with methanol.

2. It yields galacturonic acid and methanol upon hydrolysis with Pectinol 10M.

Aspen protopectin is similar to citrus protopectin in that:

1. It is essentially a linear polymer of α-1,4-linked D-galacturonic acid units partially esterified with methanol.
2. It yields galacturonic acid and methanol upon hydrolysis with Pectinol 10M.
3. It is not present entirely as water-insoluble calcium pectinate or pectate.
4. Dilute sodium hydroxide quantitatively saponifies its methyl ester groups to methanol.
5. Its average degree of esterification remains constant or may increase slightly.

Possibly 20% or more of the individual protopectin molecules in soft xylem are present as pectinic acids made water-insoluble by the presence of multivalent cations.

Significant numbers of protopectin molecules probably have degrees of esterification differing from the average for that growth stage.

The validity of the enzymatic galacturonic acid values is supported by a uronic acid balance and, for year-old xylem and new xylem, by a tissue balance.

The validity of the enzymatic methanol values is supported by saponification values. A one-half hour reaction time suffices in the saponification assay for soft xylem, stringy xylem, and new xylem, but a one-hour reaction time is required for year-old xylem.

Methanol and galacturonic acid have been identified as products of the enzymatic hydrolysis of aspen protopectin.

Methanol is obtained from sources in the aspen xylem other than the protopectin under the conditions of the enzymatic and saponification assays. These sources may be guaiacyl or syringyl derivatives or unknown methyl esters.

The high protein content of soft xylem is a source of error in the determination of its total uronic acid content by decarboxylation.

A water-soluble xylan is present in soft xylem, stringy xylem, and new xylem. It may have fewer 4-O-methylglucuronic acid groups attached than does the xylan extracted with alkali from mature aspenwood.

GLOSSARY

AGUA – Anhydrogalacturonic acid.

DM – Degree of esterification with methanol.

GUA – Galacturonic acid.

marc – The insoluble residue after a substance is extracted with a solvent.

pectic substances – A group designation for those colloidal carbohydrate derivatives which occur in, or are prepared from, plants and contain a large proportion of anhydrogalacturonic acid units which are thought to exist in a chainlike combination (71b).

pectic acid – Colloidal polygalacturonic acid free from methyl ester groups.

pectinic acids – Colloidal polygalacturonic acids containing more than a negligible proportion of methyl ester groups (71b).

pectin – "Those water-soluble pectinic acids of varying methyl ester content and degree of neutralization which are capable of forming gels with sugar and acid under suitable conditions" (71b).

protopectin – "The water-insoluble parent pectic substance which occurs in plants and which, upon restricted hydrolysis, yields pectinic acids" (71b).

Developing xylem

SX – Soft xylem: the youngest xylem collected for this investigation.

StX – Stringy xylem: the second stage in the current year's growth.

NX – New xylem: the first xylem in the current year's growth.

YOX – One-year-old xylem: representative of the previous year's growth.

MW – Mature wood: the wood remaining after the previous four growth stages have been removed.

ACKNOWLEDGMENTS

I am especially indebted to Dr. R. E. Kremers, chairman of the thesis advisory committee, for his encouragement and counsel throughout this investigation. I am also sincerely grateful to Dr. N. S. Thompson and Dr. J. A. Van den Akker, members of the advisory committee, for their suggestions and interest.

I would also like to thank Mr. Carl Piper, Mr. E. E. Dickey, and Dr. I. A. Pearl for helpful suggestions and Mr. L. Borchardt, Mr. H. Hanel, Miss M. Hollenback, Mr. L. Sell, and Mr. A. Wegner for performing necessary analytical tests. I also wish to acknowledge the help of Dr. D. Johnson in preparing and identifying the methyl-3,5-dinitrobenzoate.

Finally, I would like to thank my wife, Barbara, for encouragement throughout this investigation and for help in preparing this manuscript.

LITERATURE CITED

1. Thompson, N. S., Personal communication, 1966..
2. Anderson, E., J. Biol. Chem. 112:531-9(1936).
3. Kerr, T., and Bailey, I. W., J. Arnold Arbor. 15:327-49(1934).
4. Thompson, N. S. Unpublished work, 1965.
5. Payen, A., Ann. Chim. Phys. 26:329(1824).
6. Sultze, R. F., Jr. A study of phenolic and carbohydrate materials in the newly formed tissues of aspenwood. Doctor's Dissertation. Appleton, Wis., The Institute of Paper Chemistry, 1956. 120 p.
7. Haas, B. R., and Kremers, R. E., Tappi 47:568-73(1964).
8. Anderson, E., Seigle, L. W., Krznarich, P. W., Richards, L., and Marteny, W. W., J. Biol. Chem. 121:165-74(1937).
9. Anderson, E., J. Biol. Chem. 165:233-40(1946).
10. Anderson, E., Kaster, R. B., and Seeley, M. G., J. Biol. Chem. 144:767-72 (1942).
11. Pigman, W. W., Anderson, E., and Leaf, R. L., J. Am. Chem. Soc. 70:432 (1948).
12. Bender, W. A. In Whistler and BeMiller's Industrial gums. p. 377. New York, Academic Press, 1959.
13. Kremers, R. E. Personal communication, 1965.
14. Joslyn, M. A. The chemistry of protopectin. Advan. Food Res. 11:1-107 (1962).
15. Kertesz, Z. I. The pectic substances. New York, Interscience, 1951. 628 p.
16. Hirst, E. L., and Jones, J. K. N., J. Chem. Soc. 1938:496-505.

16a. Hirst, E. L., J. Chem. Soc. 1942:70-4.

17. Bhattacharjee, S. S., and Timell, T. E., Can. J. Chem. 43:758-65(1965).
18. Aspinall, G. O., and Fanshawe, R. S., J. Chem. Soc. 1961:4215-25.
19. Roudier, A. J., and Eberhard, L., Bull. Soc. Chim. France 1960:2074.

20. Meier, H., Acta Chem. Scand. 16:2275-83(1962).

21. Schlubach, H. H., and Hoffmann-Walbeck, H. P., Makromol. Chemie 4:5-14 (1949).

22. Lüdtke, M., Holzforschung 19:13-30(1965).

23. McCready, R. M., and Reeve, R. M., J. Agr. Food Chem. 3:260-2(1955).

24. McCready, R. M., and McComb, E. A., Food Research 19:530-5(1954).

25. Gaddum, L. W. Bulletin 268. Gainesville, Florida, University of Florida Agricultural Experiment Station, 1934. 23 p.

26. McCready, R. M. Personal communication to R. E. Kremers, The Institute of Paper Chemistry, 1965.

27. Roelofsen, P. A. The plant cell wall. Berlin, Gebrüder Borntraeger, 1959. 335 p.

28. Henglein, F. A., Makromol. Chemie 3:121(1943).

29. O'Dwyer, M. H., Biochem. J. 19:694(1925).

30. Preece, I. A., Biochem. J. 25:1304(1931).

31. Anderson, E., and Pigman, W. W., Science 105:601(1947).

32. Anderson, E., Wise, L. E., and Ratliff, E. K., Tappi 37:422-4(1954).

33. Anderson, E., and Sands, L. In Advances in Carbohydrate Chemistry. Vol. 1. p. 329. New York, Academic Press, 1945.

34. Allsopp, A., and Misra, P., Biochem. J. 34:1078-84(1940).

35. Meier, H., and Wilkie, K. C. B., Holzforschung 13:177-82(1959).

36. Thornber, J. P., and Northcote, D. H., Biochem. J. 81:449-55; 455-64 (1961); Biochem. J. 82:340-6(1962).

37. Banerji, N. Unpublished work, 1966.

38. Kremers, R. E. In Browning's The chemistry of wood. p. 369. New York, Interscience, 1963.

39. Becker, G. E., Hui, P. A., and Albersheim, P., Plant Physiol. 39:913-20 (1964).

39a. Mugg, J. B. The methanol-extractable aromatic materials in newly formed aspenwood. Doctor's Dissertation. Appleton, Wis., The Institute of Paper Chemistry, 1958. 92 p.

40. The Institute of Paper Chemistry. Unpublished work, 1962.

41. Stone, J. E., and Blundell, M. J., Anal. Chem. 23:771-4(1951).

42. Institute Method 25. Uronic acids. Appleton, Wis., The Institute of Paper Chemistry, 1951.

43. Meier, H., J. Polymer Sci. 51:11-18(1961).

44. Quick, R. H., Tappi 39:357-61(1956).

45. Timell, T. E., Tappi 40:568-72(1957).

46. Browning, B. L. In Browning's The chemistry of wood. p. 57. New York, Interscience, 1963.

47. Kavanagh, K. R., and Pepper, J. M., Can. J. Chem. 33:24-30(1955).

48. Stone, J. E., Blundell, M. J., and Tanner, K. G., Can. J. Chem. 29: 734-45(1951).

49. Higuchi, T., Kawamura, I., Morimoto, I., and Kanda, T., J. Japan Forest Soc. 36:101-6(1954); C.A. 49:1324.

50. Agricultural Research Administration of the United States Department of Agriculture. Methods used at Western Regional Research Laboratory for extraction and analysis of pectic materials. Albany, Cal., Western Regional Research Laboratory, 1952. 24 p.

51. McComb, E. A., and McCready, R. M., Anal. Chem. 24:1630-2(1952).

52. McCready, R. M., and McComb, E. A., Anal. Chem. 24:1986-8(1952).

53. Jansen, E. F., and MacDonnell, L. R., Arch. Biochem. 8:97-112(1945).

54. Lineweaver, H., Jang, R., and Jansen, E. F., Arch. Biochem. 20:137-52 (1949).

55. Joslyn, M. A., Mist, S., and Lambert, E., Food Technol. 6:133-9(1952).

56. Fish, V. B., and Dustman, R. B., J. Am. Chem. Soc. 67:115-7(1945).

57. Hills, C. H., Ogg, C. L., and Speiser, R., Ind. Eng. Chem. Anal. Ed. 17:507-10(1945).

57a. Timell, T. E. In Advances in carbohydrate chemistry. Vol. 19. p. 247. New York, Academic Press, 1964.

58. Thompson, N. S., and Kaustinen, O. A., Tappi 47:157-62(1964).

59. Zienius, R. H., and Purves, C. B., Tappi 43:27-35(1960).

60. Dillingham, E. O. Personal communication, 1966.

61. Kaheki, K., Murphy, R. A., White, H., and Sarkanen, K. V., Tappi 45: 24-8(1962).

62. Dashek, W. V., and Rosen, W. G., Plant Physiol. 40 (suppl.):xxxix(1965).

63. Fruton, J. S., and Simmonds, S. General biochemistry. 2nd ed. New York, Wiley and Sons, 1961. 612 p.

64. Sarkanen, K. V., Chirkin, G., and Hrutfiord, B. F., Tappi 46:375-9(1963).

65. Wacek, A. V., and Limontschew, W., Ind. Eng. Chem. 49:1389-90(1957).

66. Olsen, A. G. Personal communication to R. E. Kremers, The Institute of Paper Chemistry, 1966.

67. Anderson, D. M. W., Bews, A. M., Garbutt, S., and King, N. J., J. Chem. Soc. 1961:5230-4.

68. Olsen, A. G., and Stuewer, R. F., U. S. pat. 2,132,577(1938).

69. Haas, B. R., and Kremers, R. E. Unpublished work, 1961.

70. Technical Bulletin No. 10. Pectinol products. Philadelphia, Pa., Rohm and Haas, 1964.

71. Isbell, H. S., and Frush, H. L., J. Res. Natl. Bur. Stds. 32:77-94 (1944).

71a. Shriner, R. L., Fuson, R. C., and Curtin, D. Y. The systematic identification of organic compounds. 4th ed. New York, Wiley and Sons, 1962. 426 p.

71b. Baker, G. L., Joseph, G. H., Kertesz, Z. I., Mottern, H. H., and Olsen, A. G., Chem. Eng. News 22:105(1944).

72. Saeman, J. F., Moore, W. E., Mitchell, R. L., and Millett, M. A., Tappi 37:336-43(1954).

73. Somogyi, M., J. Biol. Chem. 195:19-23(1952).

74. Nelson, N., J. Biol. Chem. 153:375-80(1944).

75. Davies, O. L. Statistical methods in research and production. 3rd ed. New York, Hafner, 1961. 396 p.

76. Natrella, M. G. Experimental statistics. National Bureau of Standards Handbook No. 91. Washington, D. C., U. S. Department of Commerce, 1963. 210 p.

77. Boos, R. N., Anal. Chem. 20:964-5(1948).

78. MacFadyen, D. A., J. Biol. Chem. 158:107-33(1945).

79. Bricker, C. E., and Johnson, H. R., Ind. Eng. Chem. Anal. Ed. 17:400-2 (1945).

80. Cox, R. E. General Foods Corp. Personal communication to R. E. Kremers, 1965.

81. Institute Method 18. Methoxyl groups. Appleton, Wis., The Institute of Paper Chemistry, 1951.

82. Institute Method 13. Lignin in wood. Appleton, Wis., The Institute of Paper Chemistry, 1951.

83. Buchanan, M. A., Brauns, F. E., and Leaf, R. L., J. Am. Chem. Soc. 71: 1297-9(1949).

APPENDIX I

ENZYMATIC DETERMINATION OF GALACTURONIC ACID

Determination of the GUA content of aspen tissue consisted of five operations: (1) deesterification, (2) enzymatic hydrolysis, (3) chromatographic separation, (4) elution, and (5) colorimetric measurement. The deesterification and hydrolysis conditions resulted from the optimization experiments described on pages 29-54.

DEESTERIFICATION

One-half gram (o.d.) of ground tissue was weighed into a 250-ml. F.B. flask. About one-half gram of disodium ethylenediaminetetraacetate, 85 ml. of distilled water, and 14 ml. of 1% sodium hydroxide were added, and the mixture was stirred with a magnet for one-half hour.

ENZYMATIC HYDROLYSIS

Fourteen drops of glacial acetic acid and 10.0 mg. of Pectinol 10M Concentrate were added. The mixture was stirred for two hours, then filtered through Whatman Number 40 filter paper; the filtrate was concentrated to an appropriate volume V_1 (depending upon the GUA content of the tissue) with a Rinco rotary film evaporator. The concentrated hydrolyzate was chromatographed within two hours.

CHROMATOGRAPHIC SEPARATION

Whatman No. 1 chromatography paper (24 by 57 cm.) was used for the separation of GUA from the hydrolyzate. Three, 3-cm. guide strips, one on each side and one in the middle, were used to locate the GUA on the chromatogram.

A known volume V_2 of hydrolyzate was spotted on each of the two, 7.5-cm. sample strips. Volume V_2 always contained between 30 and 200 micrograms of GUA – well within the optimum range for absorbency measurements.

Three sample strips were usually prepared from each hydrolyzate. Chromatograms were developed with ethyl acetate–pyridine–acetic acid–water (5:5:1:3). The sheets were equilibrated with vapor from the developer for about one hour, then developed for 20 hours, all at 27°C.

A blank chromatogram was also developed to serve as a reference in the colorimetric measurement.

The developed chromatograms were air dried, the sample strips cut out, and the guide strips sprayed with aniline hydrogen phthalate (preparation described in Appendix III). The 5 by 7.5 cm. tabs containing the GUA were then cut from the sample strips and air dried for 24 hours to ensure complete removal of developer.

ELUTION

The GUA was eluted from each tab with distilled water into an 0.375 ± 0.005 ml. pipet as described by Saeman, et al. (72).

The recovery of GUA in the chromatography and elution steps was 97.1 ± 2.0% with pure GUA.

COLORIMETRIC MEASUREMENT

The colorimetric method of Somogyi (73) was modified (40) to give the following procedure:

1. Transfer the 0.375 ml. of solution into a 10-ml. volumetric flask (the test flask).
2. Add 1.0 ml. of Somogyi reagent (73).
3. Stopper the flask, using a rubber band to keep the stopper in place, and suspend it in boiling water for 20 minutes.
4. After cooling in air, slowly add 1.0 ml. of Nelson reagent (74). Swirl the flask to allow the gas to escape.
5. Dilute the contents to 10.0 ml.
6. Measure the optical density at 520 nm. (74).

The concentration of GUA [GUA] was calculated from the average optical density (OD) of the three test solutions by Equation (5):

$$[GUA]\ (\text{in } \mu g./ml.) = 601.65\ OD + 7.9 \qquad (5).$$

The weight of GUA in the original hydrolyzate was then calculated from Equation (6):

$$\text{Wt. of GUA} = (0.375)[GUA](V_1)/(V_2) \qquad (6).$$

Equation (5) was obtained by calculating the regression of optical density on GUA from the data in Table XXIV with the usual methods of regression analysis (75).

The 95% confidence limits were calculated from an equation specifically derived for use with calibration data (76).

The GUA solutions of known concentration were prepared from galacturonic acid monohydrate (Pfanstiehl, Reagent Grade). Its GUA content was 87.8%, established with the standard decarboxylation (42) and titration (see Appendix V) techniques. The pure monohydrate should have a GUA content of 91.5%.

TABLE XXIV

CALIBRATION DATA FOR COLORIMETRIC MEASUREMENT OF GALACTURONIC ACID

GUA, µg./ml.	Av. Optical Density[a]	Standard Error	Number of Measurements
34.7	0.039	0.003	3
40.0	0.056	0.001	6
47.2	0.069	0.004	3
58.9	0.084	0.001	3
69.4	0.104	0.005	3
79.9	0.118	0.002	6
94.4	0.142	0.006	3
118.0	0.185	0.003	2
139.0	0.220	0.008	2
160.0	0.250	0.001	6
189.0	0.300	0.006	3
235.0	0.383	0.010	3

[a] Optical density = Log $(1/\underline{T})$.

APPENDIX II

DETERMINATION OF ESTER METHANOL

Determination of the methyl ester content of aspen protopectin consisted of three operations: (1) hydrolysis, (2) distillation, and (3) colorimetric measurement. The hydrolysis conditions resulted from the optimization experiments described on pages 29-54.

HYDROLYSIS AND DISTILLATION

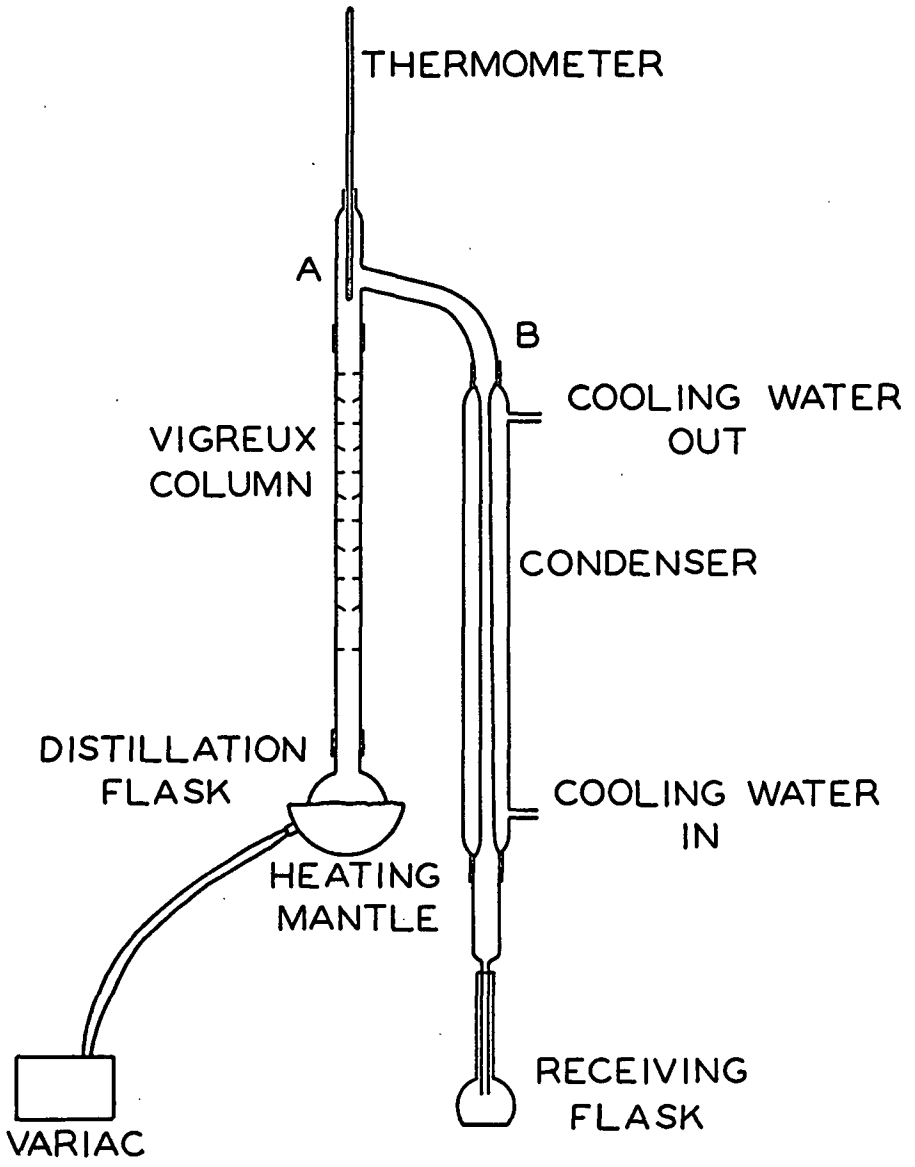
Two methods were employed for hydrolysis of the methyl ester groups to methanol: (1) enzymatic hydrolysis, the primary method, and (2) saponification.

ENZYMATIC HYDROLYSIS

About one-half gram (o.d.) of ground tissue was weighed into a 250-ml. F.B. flask; 100 ml. of distilled water, one-half gram of disodium ethylenediaminetetraacetate, and 10.0 mg. of Pectinol 10M Concentrate were added. The flask was attached to the distillation apparatus (Fig. 8) and the contents stirred with a magnet for 24 hours[7].

The flask was heated for about 40 minutes to distill the methanol at atmospheric pressure and 99-100°C. The distillate entered the receiver (a volumetric flask of appropriate size depending on the amount of methyl ester in the tissue) below the liquid level. About 35-40 ml. were collected. The distillation was stopped by opening the apparatus at Points A and B (see Fig. 8), draining section AB into the condenser, then washing down the condenser

[7]Enzymatic hydrolysis of SX required only four hours.



ALL JOINTS WERE 24/40 AND HAD A LIGHT COATING OF SILICONE STOPCOCK GREASE

Figure 8. Distillation Apparatus

with distilled water. These procedures always gave 100% recovery of methanol from solutions of known methanol concentration.

SAPONIFICATION

The procedure for enzymatic hydrolysis was followed except:

1. Eighty-six milliliters of distilled water were added;
2. Fourteen milliliters of 1% sodium hydroxide were substituted for the Pectinol 10M, giving a pH of 11.5;
3. The contents were stirred for one hour (YOX) or one-half hour (NX, StX, SX, and citrus pectin);
4. The pH was brought to about 5 with 14 drops of glacial acetic acid before distillation.

COLORIMETRIC MEASUREMENT

The methanol concentration in the distillate was measured with a modification of the chromotropic acid method of Boos (77). Other versions of this method were tried (78, 79), but they were unsatisfactory.

1. Transfer 1.0 ml. of the distillate to a 10-ml. volumetric flask (the test flask).
2. Add 4 drops of 5% phosphoric acid.
3. Add 5 drops of 5% potassium permanganate.
4. Let the stoppered flask stand for 10 minutes, swirling occasionally.
5. Add a 20% solution of sodium bisulfite dropwise until the solution is colorless.

6. Cool in an ice bath.
7. Add 4.0 ml. of concentrated sulfuric acid.
8. Add 4 drops of 2% aqueous chromotropic acid (1,8-dihydroxy-naphthalene-3,6-disulfonic acid).
9. Place in a boiling water bath for 15 minutes.
10. Cool, dilute to the mark, and read the optical density at 570 nm. within 24 hours against a blank containing distilled water.

Three test flasks were prepared from each distillate. Their average optical density OD was substituted into Equation (7) to calculate the methanol concentration (in μg./ml.) in the distillate.

$$\text{Log(methanol conc.)} = 1.01575 \log (10\ OD) + 1.22539 \qquad (7)$$

Equation (7) was obtained by calculating the regression of optical density on methanol concentration from the calibration data in Table XXV.

The 95% confidence limits were calculated from an equation specifically derived for use with calibration data (76).

The methanol solutions of known concentration were prepared from re-distilled, Reagent Grade methanol.

TEST FOR FORMALDEHYDE

In the colorimetric methanol assay, the permanganate oxidizes the methanol to formaldehyde, which then reacts with chromotropic acid to give the characteristic purple color. Therefore, formaldehyde in the distillate was detected by omitting the oxidation steps (Steps 2-5).

TABLE XXV

CALIBRATION DATA FOR COLORIMETRIC MEASUREMENT OF METHANOL

Methanol, μg./ml.	Av. Optical Density[a]	Standard Error	Number of Measurements
6.3	0.037	0.002	6
19.8	0.119	0.007	9
39.5	0.244	0.003	11
63.2	0.374	0.002	10
79.0	0.466	0.005	11
98.8	0.570	0.002	17
126.4	0.706	0.003	14

[a]Optical density = Log $(1/\underline{T})$.

This procedure could not be used to measure formaldehyde, quantitatively, however. Table XXVI shows that 35-56% of the formaldehyde in the test flask is oxidized by the permanganate during the methanol assay.

TABLE XXVI

CHANGE IN FORMALDEHYDE CONCENTRATION UPON OXIDATION WITH PERMANGANATE

Conditions: Same as for Colorimetric Measurement of Methanol

Before Oxidation, μg./ml.	After Oxidation, μg./ml.	Decrease, %
14.1 ± 1.1	9.2 ± 1.1	35
8.1 ± 1.1	3.6 ± 1.1	56
3.4 ± 1.1	2.1 ± 1.1	38

An advantage of this partial loss was that up to 1.6 μg./ml. of formaldehyde could be tolerated in a distillate without changing the precision of the methanol measurement (± 1.1 μg./ml.).

APPENDIX III

QUALITATIVE PAPER CHROMATOGRAPHIC TECHNIQUES

DEVELOPERS

1. Ethyl acetate–pyridine–water (8:2:1) (24 hr.).
2. Ethyl acetate–acetic acid–water (9:2:2) (36 hr.).
3. Butanol–pyridine–water (10:3:3) (72 hr.).
4. Ethyl acetate–pyridine–acetic acid–water (5:5:1:3) (20 hr.).
5. iso-Propanol–pyridine–acetic acid–water (8:8:1:4) (36 hr.).
6. Butanol–acetic acid–water (4:1:5, upper phase) (48 hr.).

Developers 1 and 2 separate GUA, galactose, glucose, mannose, arabinose, and xylose; Developer 3 separates all of these except mannose and arabinose. All three developers separate fructose from sucrose.

Developer 4 separates GUA from the sugars, 4-O-methylglucuronic acid, and 2-O-(4-O-methyl-α-D-glucopyranosyluronic acid)-D-xylopyranose.

Developer 5 separates GUA from D-glucuronic acid.

Developer 6 separates myo-inositol from the sugars and uronic acids.

SPRAY REAGENTS

1. Aniline hydrogen phthalate – for aldoses and uronic acids: About 8.3 g. of o-phthalic acid were dissolved in 500 ml. of n-butanol saturated with water. Before each use, 1 ml. of aniline was added per 100 ml. of solution. After spraying, the chromatogram was heated at 105°C. for five minutes.

2. Para-anisidine – for aldoses and uronic acids: One gram of p-anisidine hydrochloride was dissolved in a solution containing 21.5 ml. of 95% ethanol, 8.5 ml. of distilled water, and 170 ml. of n-butanol. It was stored at 5°C. After spraying, the chromatogram was heated at 105°C. for five minutes.

3. Urea phosphate – for sucrose and fructose: 1.5 g. of urea were mixed with 5.7 g. of 87% phosphoric acid, 10 ml. of water, 10 ml. of absolute ethanol, and 75 ml. of n-butanol. After spraying, the chromatogram was heated at 105°C. for 5-10 minutes.

4. Permanganate – periodate – for myo-inositol: (A) 1% potassium permanganate in 2% aqueous sodium carbonate and (B) 2% aqueous sodium metaperiodate were prepared. Before each use, one volume of A was mixed with four volumes of B.

APPENDIX IV

STANDARD QUANTITATIVE METHODS

URONIC ANHYDRIDE

1. Decarboxylation method (42): The sample is digested with 12% hydrochloric acid at an oil bath temperature of 137-140°C. for four hours.

2. Titration method (80): Step A – About two grams (o.d.) of pectic substance in 400 ml. of distilled water are treated with MB1 (mixed bed cation and anion) resin batchwise. The mixture is filtered, and the filtrate is divided equally for titration in duplicate. The free carboxyl groups are titrated with 0.1N NaOH to a phenolphthalein end point.

   Step B – An excess of alkali is added to the above titrated solution to saponify the methyl ester groups during one hour at room temperature. Hydrochloric acid is added in an amount equal to the saponification alkali. Finally, the excess acid is titrated with 0.1N NaOH.

   The total titer of 0.1N NaOH from Steps A and B represents the total uronic acid.

METHYL ESTER GROUPS

3. Saponification and titration method (80): The equivalents of 0.1N NaOH from Step B described above represents the equivalents of methyl ester groups.

4. Methyl iodide evolution method (81): The sample is boiled with hydriodic acid for 30-45 minutes.

NEUTRAL SUGARS

5. Method of Saeman, et al. (72): The sample is digested in 72% sulfuric acid at 30°C. for one hour, then in 2.5% acid at 15 p.s.i.g. for one hour. It is neutralized, concentrated, chromatographed, eluted, and measured colorimetrically with Somogyi (73) and Nelson (74) reagents.

LIGNIN

6. Klason lignin (82): The extractive-free sample is digested with cold, 72% sulfuric acid for three hours, then with 3% acid at 100°C. for four hours. It is filtered, dried, and weighed.

7. Acid-soluble lignin (83): The soluble lignin in the filtrate from the Klason procedure was determined by its optical density at 230 nm.

8. Vanillin and syringaldehyde (41): The sample is digested with sodium hydroxide and nitrobenzene at 160°C. for about two hours. The aldehydes are then separated chromatographically, eluted, and measured colorimetrically.

ASH

9. The sample is alternately heated with a microburner and treated with 30% hydrogen peroxide dropwise until white.

SPECIFIC OPTICAL ROTATION

10. The specific optical rotation was measured with a Zeiss-Winkel polarimeter and a 2.0-dm. tube.

APPENDIX V

DELIGNIFICATION OF ASPEN TISSUE WITH ACIDIC SODIUM CHLORITE

A room temperature acid chlorite procedure (58) was modified as follows:

1. To four grams (o.d.) of ground tissue in a 125-ml. Erlenmeyer flask were added 28-33 ml. of deionized water. Soft xylem required more water to form a slurry than did year-old xylem.

2. The slurry was aspirated for 15 minutes.

3. Ten milliliters of 5% acetic acid were added.

4. Five milliliters of 40% aqueous sodium chlorite (Tech. Grade) were added.

5. The flask was covered and placed in a hood.

6. On the fifth and tenth days, 0.5 ml. of glacial acetic acid and 2.0 g. of sodium chlorite were added.

7. The tissue was filtered through medium-pore sintered glass and washed thoroughly.

8. The chlorited tissue was air-dried, except for SX, which was dried *in vacuo* at 35°C. overnight. Its yield was determined as a percentage (o.d. basis) of the starting material.

The GUA content of the chlorite liquor (filtrate from Step 7) was determined as follows:

1. Residual chlorine dioxide was removed by bubbling air through it until colorless (1-4 hours).

2. It was dialyzed against deionized water until the dialyzate gave a negative test for chloride (1-7 days).

3. It was concentrated at reduced pressure, then tested for GUA as usual.

APPENDIX VI

CORRECTION OF GALACTURONIC ACID AND ESTER METHANOL VALUES

Corrections for loss of GUA and ester methanol during chloriting were calculated by assuming that the rate of loss was linear. Only that data representing the maximum and decreasing segments of the curves in Fig. 1 were used.

CORRECTION OF GUA VALUES

First, AGUA contents were calculated (see footnote 5, page 35). The results are in Table XXVII, Column 3.

Then linear expressions relating AGUA content to chloriting time t were calculated by regression analysis (75); they are shown in Table XXVII, Column 4.

The AGUA content at time $t = 0$, except for SX-1, represents the corrected AGUA value in Table IX, Column 2. The AGUA content for SX-1 was taken to be the value actually obtained (14.1%) rather than the value calculated by regression analysis (14.0%). This choice made no difference in the calculation of average DM.

CORRECTION OF ESTER METHANOL VALUES

The calculations were analogous to those for GUA. The resulting linear expressions relating ester methanol content to chloriting time t are in Table XXVII, Column 6.

The slopes of the linear expressions in Column 6 were used to correct the values in Table XIII to give those in Table XIV, Column 3.

TABLE XXVII

LINEAR EXPRESSIONS FOR RATE OF LOSS OF GUA AND ESTER METHANOL DURING CHLORITING

Growth Stage	Chloriting Time $\underline{t}$, days	AGUA Content, %	Linear Expression for AGUA, %	Methanol Content, %	Linear Expression for Methanol, %
YOX-1	3	--		0.13	
"	6	--		0.11	
"	10	0.87		0.095	
"	10	1.52		--	
"	10	0.94		--	
"	10	0.91		--	
"	12	0.81		--	
"	14	0.83		0.065	
"	14	1.13	$(1.31 \pm 0.16) - 0.027\underline{t}$	--	$(0.15 \pm 0.01) - 0.0057\underline{t}$
NX-1	2	--		0.13	
"	3	1.6		0.13	
"	3	1.8		--	
"	5	1.6		0.15	
"	7	1.4		0.12	
"	7	1.9	$(1.71 \pm 0.37) - 0.013\underline{t}$	--	$(0.14 \pm 0.02) - 0.0009\underline{t}$
StX-1	1	4.9		0.33	
"	1	4.0		--	
"	3	3.9		0.32	
"	5	3.5		0.32	
"	5	3.8	$(4.6 \pm 0.26) - 0.20\underline{t}$	--	$(0.33 \pm 0.04) - 0.0025\underline{t}$
SX-1	0	14.1		1.2	
"	1	13.4		1.2	
"	1	13.2		--	
"	3	11.9		1.2	
"	3	16.2	$(14.0 \pm 0.25) - 0.11\underline{t}$	--	$(1.2 \pm 0.1) - 0.0\underline{t}$

APPENDIX VII

ESTIMATED MAXIMUM GALACTURONIC ACID VALUES BY A URONIC ACID BALANCE

Maximum AGUA contents were estimated from the following uronic acid balance:

GUA = (total uronic acid) - (4-O-methylglucuronic acid) (1).

The total uronic acid was determined by the standard decarboxylation method (42). The glucuronic acid was estimated by assuming that it originated from 4-O-methylglucuronoxylan having the usual 1-to-10 mole ratio of acid-to-xylose (57a).

The calculations are shown step-by-step in Table XXVIII. The estimated maximum AGUA contents are in Row 6. These are the values that appear in Table IX, Column 4. The 95% confidence limits were calculated from the fact that the confidence limits on the total uronic anhydride and xylan values were ± 5%.

TABLE XXVIII

ESTIMATED MAXIMUM GUA VALUES

Row No.	Component	SX-1	StX-1	NX-1	YOX-1
1.	Total uronic anhydride, %[a]	18.6	8.0	5.4	3.8
2.	Xylan, %[a]	8.9	20.8	24.0	19.2
3.	Xylan, mole, %	0.0674	0.158	0.182	0.145
4.	AMGLUA[b], mole, %	0.0067	0.016	0.018	0.015
5.	AMGLUA, %	1.3	3.0	3.4	2.8
6.	AGUA, % (Row 1 - Row 5)	17.3	5.0	2.0	1.8

[a] From Table III.
[b] AMGLUA = Anhydro-4-O-methylglucuronic acid.

APPENDIX VIII

ESTIMATED MAXIMUM GALACTURONIC ACID VALUES BY A TISSUE BALANCE

The tissue balance applied during chloriting is given by Equation (8):

$$\begin{pmatrix}\text{Original}\\ \text{Tissue}\end{pmatrix} - \begin{pmatrix}\text{Tissue}\\ \text{Recovered}\end{pmatrix} - \begin{pmatrix}\text{Lignin}\\ \text{Removed}\end{pmatrix} = \begin{pmatrix}\text{Other Components}\\ \text{Removed}\end{pmatrix}, \quad (8)$$

where

$$\begin{pmatrix}\text{Other Components}\\ \text{Removed}\end{pmatrix} = \begin{pmatrix}\text{Maximum AGUA}\\ \text{Removed}\end{pmatrix}. \quad (9)$$

The lignin contents were determined before (Table III) and after (Table XXXI) chloriting, and the difference was called "Lignin Removed" and is shown in Table XXIX, Column 3. "Maximum AGUA Removed" was calculated by difference and is shown in Column 5. These values were added to the "AGUA Content of the Chlorited Tissue" (Column 6) to obtain the "Estimated Maximum AGUA Content" in Column 7. Where two values were obtained per growth stage, the average was calculated. The final values are shown in Table IX, Column 5. The 95% confidence limits were calculated from the confidence limits for the lignin determination ($\pm$ 5%) and the confidence limits for "AGUA Content" given in Table VIII.

TABLE XXIX

ESTIMATED MAXIMUM GALACTURONIC ACID CONTENTS

Growth Stage	Chloriting Time, days	Lignin Removed, %	Tissue Recovered, %[a]	Max. AGUA Removed, %	AGUA Content of Chlorited Tissue, %[b]	Est. Max. AGUA, %
YOX-1	14	13.5	86.0	0.5	0.8	1.3
NX-1	5	9.1	91.8	0.1	1.6	1.7
"	7	9.7	90.1	0.2	1.4	1.6
StX-1	1	2.1	96.1	1.8	4.5	6.3
"	5	2.8	90.9	6.3	3.7	10.0
SX-1	1	0.0	95.7	4.3	13.3	17.6

[a] From Table VIII.

[b] From Appendix VI, Table XXVII.

APPENDIX IX

ESTIMATED CARBON DIOXIDE YIELDS FROM SERINE AND THREONINE

Estimation of the yields of carbon dioxide from serine $CH_2(OH)CH(NH_2)$-COOH and threonine $CH_3CH(OH)CH(NH_2)COOH$ under the acidic conditions employed in the determination of uronic anhydride involves several assumptions. Serine and threonine were found in aspen YOX, but only serine was found in NX (61). Their concentrations were 10.3 and 9.3%, respectively, of the total amino acids in YOX and 17% of the total amino acids in NX. Therefore,

1. Assume that serine and threonine are also present in my tissues (SX-1 through YOX-1);
2. Assume that the concentration of serine is 17% in SX-1, StX-1, and NX-1;
3. Assume that the concentrations of serine and threonine are 10.3 and 9.3%, respectively, in YOX-1; and
4. Assume that serine and threonine each liberate one mole of carbon dioxide during their degradation. Then the yields of carbon dioxide will be 41.9 and 37.0% of the weights of serine and threonine, respectively.

The only set of data for the protein content (nitrogen multiplied by 6.25) of all four growth stages of aspen (7) showed that the protein content exceeded the galactan and arabinan contents by factors of 3.8 and 4.9, respectively, in SX; 3.9 and 4.4 in StX; 2.2 and 1.0 in NX; and 1.0 and 1.0 in YOX. To estimate the protein contents of my tissue, these factors were multiplied by the galactan and arabinan contents in Table III. The resulting estimates were averaged and are shown in Table XXX, Column 2.

TABLE XXX

ESTIMATED CARBON DIOXIDE YIELDS

Growth Stage	Est. Protein Content, %	Estimated CO_2 Yields	
		As CO_2, %	As Uronic Anhydride, %
YOX-1	0.7	0.054	0.2
NX-1	1.0	0.071	0.3
StX-1	5.0	0.36	1.4
SX-1	24.2	1.7	6.8

The yields of carbon dioxide from each growth stage were then calculated and are shown as equivalent uronic anhydride in Column 4. These values show that carbon dioxide from serine and threonine could account for the differences between the results in Columns 2 and 4, Table IX.

APPENDIX X

LIGNIN CONTENTS AFTER CHLORITING

Certain of the tissues were analyzed for Klason and acid-soluble lignin and vanillin and syringaldehyde after chloriting. The results are in Table XXXI.

TABLE XXXI

LIGNIN CONTENTS[a]

Growth Stage	Time Chlorited, days	Acid-Soluble Lignin, %	Klason Lignin, %	Syring-aldehyde, %	Vanillin, %
YOX-1	3	7.1	2.6	0.2	0.8
"	14	5.6	0.5	--	--
NX-1	5	5.9	2.1	--	--
"	7	6.1	1.3	--	--
StX-1	1	--	--	0.4	0.7
"	5	--	--	0.0	0.4
SX-1	1	--	--	0.1	0.3

[a] "Lignin content" is the percentage of lignin based on the o.d. weight of the tissue before chloriting. It is calculated by multiplying the percentage of lignin in the chlorited tissue by the amount of tissue recovered (expressed as a fraction).

The lignin contents of these tissues before chloriting were given in Table III.