# Characterization of Pectic Substances in Tobacco Stems

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The increased importance of reconstituted tobacco leaf in cigar and cigarette manufacturing practice has increased the utilization of tobacco stems. This paper describes a method of extracting and fractionating the pectic substances in a number of cigar-type tobacco stems and shows differences in the amount and kind of pectic substances found in cigarette and cigar tobacco stems. The monosaccharide components of the isolated pectic-substance fractions were identified by thin-layer chromatography, and the galacturonic acid in each fraction was determined quantitatively by a colorimetric method. Infrared spectra of films prepared from the isolated pectic fractions showed differences in the degree of esterification of the pectic materials. The isolation procedure and the qualitative and quantitative data obtained are discussed.

Pectic substances have long been recognized as an important constituent of tobacco. Numerous reports may be found on their extraction and determination in tobacco leaf (Neuberg and Schever, 1931; Wahl, 1950; Bacon *et al.*, 1951; Philips *et al.*, 1953; Pyriki and Moldenhauer, 1962, 1963). However, few specific reports deal with their occurrence and properties in tobacco stems (Philips and Bacot, 1958; Richards, 1962).

The increased importance of reconstituted tobacco flea in cigar and cigarette manufacturing practice has resulted in an increase in the utilization of tobacco stems. Thus a more complete and quantitative study of the pectic substances in tobacco stems appeared warranted.

The objective was to develop a more detailed fractionation scheme for the quantitative separation and characterization of the pectic substances, and to apply this scheme to the study of cigar and cigarette tobacco stems as well as individual members within these two types of stems.

## EXPERIMENTAL

**Sample Preparation.** The tobacco stem samples were ground in a Wiley mill, and the fraction passing through a standard U.S. 120 screen was used for the investigation. Moisture contents were determined (in triplicate) by drying the samples at 105° C. for 2 hours in a forced draft oven. All results are reported on a dry basis unless otherwise indicated.

**Fractionation and Analyses.** The fractionation scheme used in the separation and characterization of the pectic substances in tobacco stems is shown in Figure 1.

Ten grams (as is) of stem powder was suspended in 90 ml. of 80% methanol at room temperature. The suspension was shaken vigorously for a few minutes and then filtered through a Büchner funnel under house vacuum using Whatman No. 1 filter paper. The wet filter cake was resuspended in 90 ml. of 80% methanol, shaken, and refiltered as before; the process was repeated for a total of five extractions. The combined filtrates (fraction A) were analyzed for free carbohydrates by thin-layer chromatography (Jacin and Mishkin, 1965). The final filter cake was dried to constant weight in a forced draft oven at 105° C., and the loss in weight was taken as the 80% methanol extractables.

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The dried solids from the preceding step were suspended in 100 ml. of boiling 90% 2-propanol acidified to 0.1*N* with hydrochloric acid. The mixture was agitated for a few minutes, filtered as before, and the wet filter cake was resuspended in a fresh portion of acidified alcohol and refiltered. This process was repeated for a total of five extractions. The combined filtrates (fraction B) were analyzed for calcium content by a chloranilic acid method (Glick, 1963).

The extracted filter cake was suspended in about 40 ml. of boiling water, agitated for a few minutes, and filtered as before. This extraction process was repeated for a total of 10 extractions. The combined filtrates (fraction C) were made up to 500 ml. with distilled water. The galacturonic acid content of this fraction was determined on an aliquot using a modified carbazole method (Bitter and Muir, 1962). Another aliquot was hydrolyzed with 0.25*N* hydrochloric acid and the hydrolyzate chromatographed on a thin-layer plate (Jacin and Mishkin, 1965).

The remaining solids were suspended in 100 ml. of distilled water and titrated with constant stirring to pH 8.0 with 0.1*N* sodium hydroxide. The volume of alkali used was recorded, and the milliequivalents of acidity per gram of starting material (stem) was calculated. This neutralized suspension was then transferred to a round-bottomed flask equipped with a reflux condenser and refluxed for 16 hours. The mixture was filtered, as before, and the filter cake was washed with 10 50-ml. portions of distilled water. The combined filtrate and washings (fraction D) were made up to a 1-liter volume with distilled water and subjected to the same analyses as fraction C.

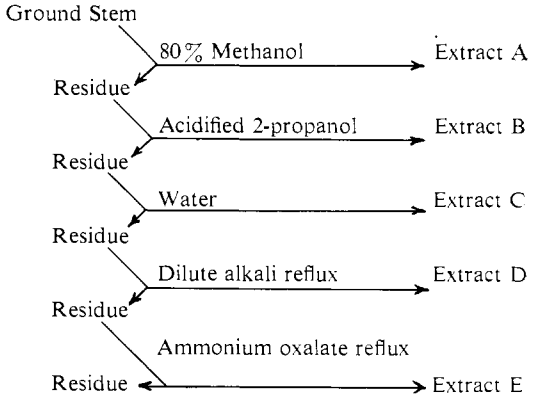


**Figure 1. Fractionation scheme for the separation and characterization of pectic substances**

The washed filter cake from the preceding step was suspended in 200 ml. of a 0.6% ammonium oxalate solution and refluxed for 16 hours. The mixture was then filtered, as before, and the filter cake was washed with ten 50-ml. portions of boiling distilled water. The combined filtrates and washing (fraction E) were made up to a 1-liter volume with distilled water and subjected to the same analyses as fraction C.

**Calcium Determination.** The calcium determinations by the chloranilic acid method (Glick, 1963) were reproducible with an accuracy of ±4%, and the calibration curve was a straight line passing through the origin for the concentration range used.

**Galacturonic Acid Determination.** The determination of galacturonic acid by the modified carbazole method of Bitter and Muir, (1962) was reproducible with an accuracy of ±5%, the calibration curve was a straight line through the origin.

Two potential interfering substances in these materials could affect the accuracy of the galacturonic acid determination: other uronic acids—e.g., glucuronic—and sugars. In fact, Bitter and Muir (1962) reported that 60 μg. per ml. of glucose will give a carbazole absorbance equivalent to 8 μg. per ml. of galacturonic acid.

Thin-layer chromatograms of the fractions containing galacturonic acid showed no evidence of glucuronic or any other uronic acid. The $R_f$ of glucuronic acid in the thin-layer system used in this investigation was sufficiently different from that of galacturonic acid to be certain of the absence of glucuronic acid in any of these fractions. The effect of monosaccharides on the determination of galacturonic acid by the carbazole method has been examined (Table I). Most of the carbohydrate interference is eliminated at concentrations below 20 μg. per ml. Kutz (1959) has reported that galacturonic acid results may be in error as much as 7% relative, owing to interfering sugars, and has recommended the use of the absorption measurements at 430 mμ with an appropriate correction of the results. The results reported here have not been corrected, but rather all of the quantitative determinations have been made at concentrations below 20 μg. per ml. of galacturonic acid. This minimizes, or eliminates, any possible interferences by the simple monosaccharides. The justification for this conclusion is based upon the fact that the sum total of the interfering sugars did not exceed the concentration of galacturonic acid. In making the measurements at these low concentrations, the potentially interfering sugars were at levels which do not contribute significantly to the absorbance.

**Thin-Layer Separation of Sugars.** The composition of the free carbohydrates and the carbohydrate hydrolyzates were determined using plates made with silica gel G impregnated with a 0.02*M* borate buffer of pH 8.0 (Jacin and Mishkin, 1965). The sugars were applied to the plates and the latter were subjected to ascending chromatography using a 1-butanol–acetic acid–water (5:4:1) solution. After double development in the same solvent, the plates were dried, sprayed with 1-naphtholsulfuric acid reagent, and heated at 100° C. for 3 to 6 minutes to make the spots visible.

**Table I. Absorbance of Carbohydrates by the Carbazole Method**

| Carbohydrate | Absorbance (530 mμ) at Concentration of 40 μg./ml. | 20 μg./ml. | 8 μg./ml. |
|---|---|---|---|
| Arabinose | 0.027 | None | None |
| Galactose | 0.087 | 0.045 | None |
| Glucose | 0.123 | 0.062 | None |
| Xylose | 0.027 | None | None |
| Rhamnose | 0.002 | None | None |
| Galacturonic acid | 0.509 | 0.250 | 0.100 |

## RESULTS AND DISCUSSION

**Fraction A.** These fractions contained, among other constituents, the free carbohydrates. The thin-layer chromatograms showed that most of the free sugars were removed after three extractions. In no instance was there any evidence of galacturonic acid, xylose, or rhamnose in this fraction. Figure 2 shows a typical thin-layer chromatography plate of the 80% MeOH washings from Turkish and Maryland stems.

Table II summarizes the amount of 80% methanol extractables in the various tobacco stem types and also the relative amounts of free sugars. There were considerable differences among the four cigarette tobacco types (Bright, Burley, Maryland, and Turkish) in the quantity of the extractables, with a large amount of extractables being an indication of a large amount of free sugars. On the other hand, the four cigar tobacco types (Pennsylvania, Puerto Rican, German, and Java-Brazil) were uniformly low in extractables and free sugars.

**Fraction B.** The calcium ion in the pectates, which was readily exchanged with hydrogen ion from the acidified alcohol, was present in these fractions; they gave a negative test for carbohydrates. The results are tabulated in Table III along with the total calcium determined on ashed samples of the same tobaccos. Positive and negative controls were used in all the quantitative calcium determinations. Unacidified alcohol was ineffective in removing calcium.

The extractable and total calcium contents varied considerably between the cigarette types and the cigar types. Pennsylvania and German stems contained the greatest amount of both extractable and total calcium. On the other hand, the ratio of extractable to total calcium was variable for the four cigarette types (varying from a low of 0.38 for Maryland to a high of 0.62 for Turkish) but uniform for the four cigar types (ranging from 0.51 to 0.57).

**Fractions C, D, and E.** Thin-layer chromatography of these fractions showed they consisted of polymeric material in all cases. However, on hydrolysis, galacturonic acid, galactose, arabinose, xylose, rhamnose, and probably glucose were found. The presence of glucose was not definitely established, since it has a similar $R_f$ and nearly the same spot color as arabinose in the chromatographic system used for these analyses. Glucose probably was present, since it was found in fraction A and in other stem investigations. Its presence was also reported by Richards (1962), who examined some of these same types of tobacco stems. A photograph of a thin-layer chromatography plate containing unhydrolyzed and hydrolyzed stems is shown in Figure 3.

A summary of the chromatographic results in Figure

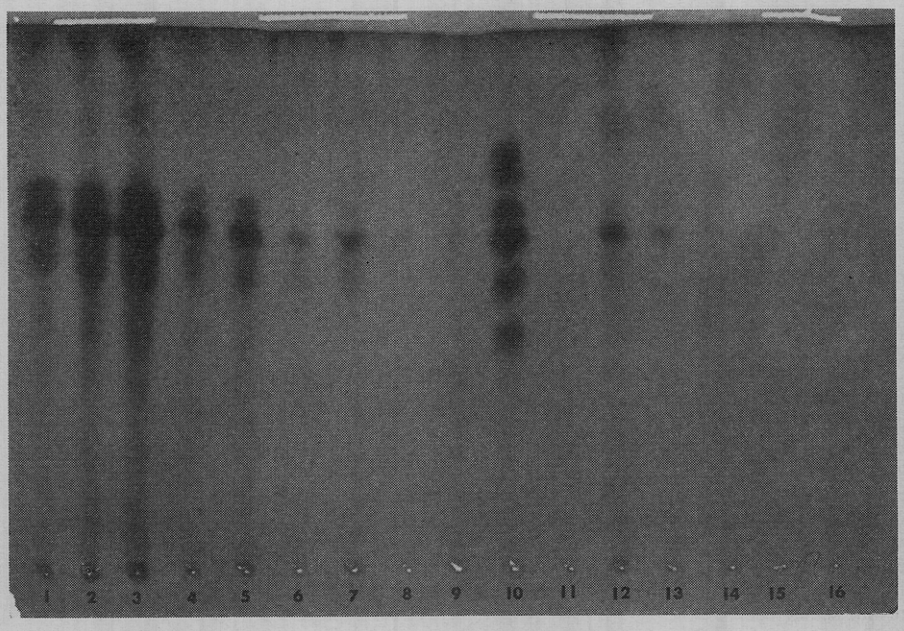


**Figure 2. Thin-layer chromatogram of 80% methanol washings of tobacco stems**

**Table II. 80% Methanol Extractables in Tobacco Stems**

| Stem Types | Extractables, % | Free Sugars |
|---|---|---|
| Bright | 42.8 | Large amounts of sucrose, fructose, and glucose |
| Burley | 28.2 | Trace of fructose and glucose |
| Maryland | 22.1 | Trace of sucrose, fructose, and glucose |
| Turkish | 36.8 | Large amounts of sucrose, fructose, and glucose |
| Pennsylvania | 17.7 | Trace of sucrose, fructose, and glucose |
| Puerto Rican | 19.5 | None |
| German | 16.6 | Trace of fructose |
| Java-Brazil | 21.8 | Trace of sucrose, fructose, and glucose |

**Table III. Calcium Content of Tobacco Stems**

| Stem | Extractable Calcium, % | Total Calcium, % | Ratio of Extractables/ Total |
|---|---|---|---|
| Bright | 1.00 | 2.02 | 0.48 |
| Burley | 1.70 | 2.92 | 0.58 |
| Maryland | 0.96 | 2.56 | 0.38 |
| Turkish | 1.88 | 3.04 | 0.61 |
| Pennsylvania | 2.22 | 3.90 | 0.57 |
| Puerto Rican | 1.18 | 2.14 | 0.55 |
| German | 2.26 | 4.30 | 0.53 |
| Java-Brazil | 1.58 | 2.80 | 0.56 |
| Havana seed leaf | 1.04 | 4.20 | 0.25 |

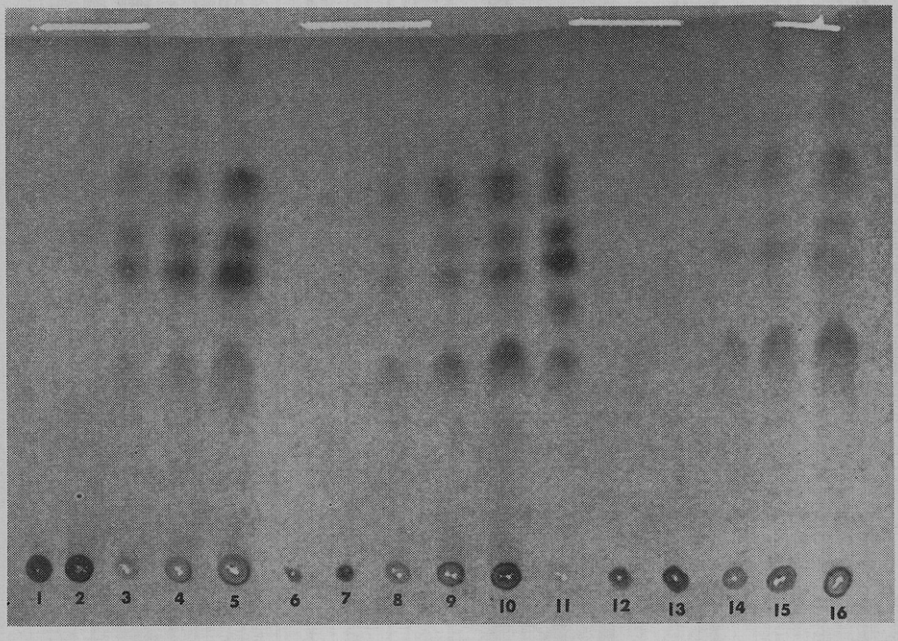


**Figure 3. Carbohydrate composition of fractions C, D, and E from Pennsylvania stems**

Table IV. Thin-Layer Chromatography of Pennsylvania Stem Extracts

| Sample | Volume, Ml. | |
|---|---|---|
| Fraction C | 0.005 | No migration; only polymeric material |
| | 0.010 | No migration; only polymeric material |
| Hydrolyzate of C | 0.005 | Galacturonic, galactose, arabinose, xylose, rhamnose |
| Fraction D | 0.001 | No migration; only polymeric material |
| | 0.003 | No migration; only polymeric material |
| Hydrolyzate of D | 0.005 | Galacturonic, galactose, arabinose, xylose, rhamnose |
| Fraction E | 0.005 | No migration; only polymeric material |
| | 0.010 | No migration; only polymeric material |
| Hydrolyzate of E | 0.005 | Galacturonic, galactose, rhamnose |
| | 0.010 | Galacturonic, galactose, arabinose, rhamnose |
| | 0.025 | Galacturonic, galactose, arabinose, xylose, rhamnose |

Table V. Galacturonic Acid Distribution in Tobacco Stems[a]

| Stem Type | NaOH Titer Meq./Gram | Fraction C, % | Fraction D, % | Fraction E, % | Total, % |
|---|---|---|---|---|---|
| Bright | 0.31 | 3.46 | 7.34 | 1.44 | 12.24 |
| Burley | 0.45 | 4.14 | 9.60 | 3.52 | 17.26 |
| Maryland | 0.47 | 3.44 | 9.15 | 4.66 | 17.25 |
| Turkish | 0.21 | 4.24 | 3.90 | 1.78 | 9.92 |
| Pennsylvania | 0.50 | 2.52 | 7.25 | 7.85 | 17.62 |
| Puerto Rican | 0.57 | 2.07 | 10.08 | 2.69 | 14.84 |
| German | 0.50 | 2.74 | 5.44 | 8.50 | 16.68 |
| Java-Brazil | 0.46 | 1.74 | 5.60 | 5.02 | 12.36 |
| Havana seed leaf | 0.52 | 1.59 | 3.64 | 8.10 | 13.20 |

[a] Per cent based on starting material.

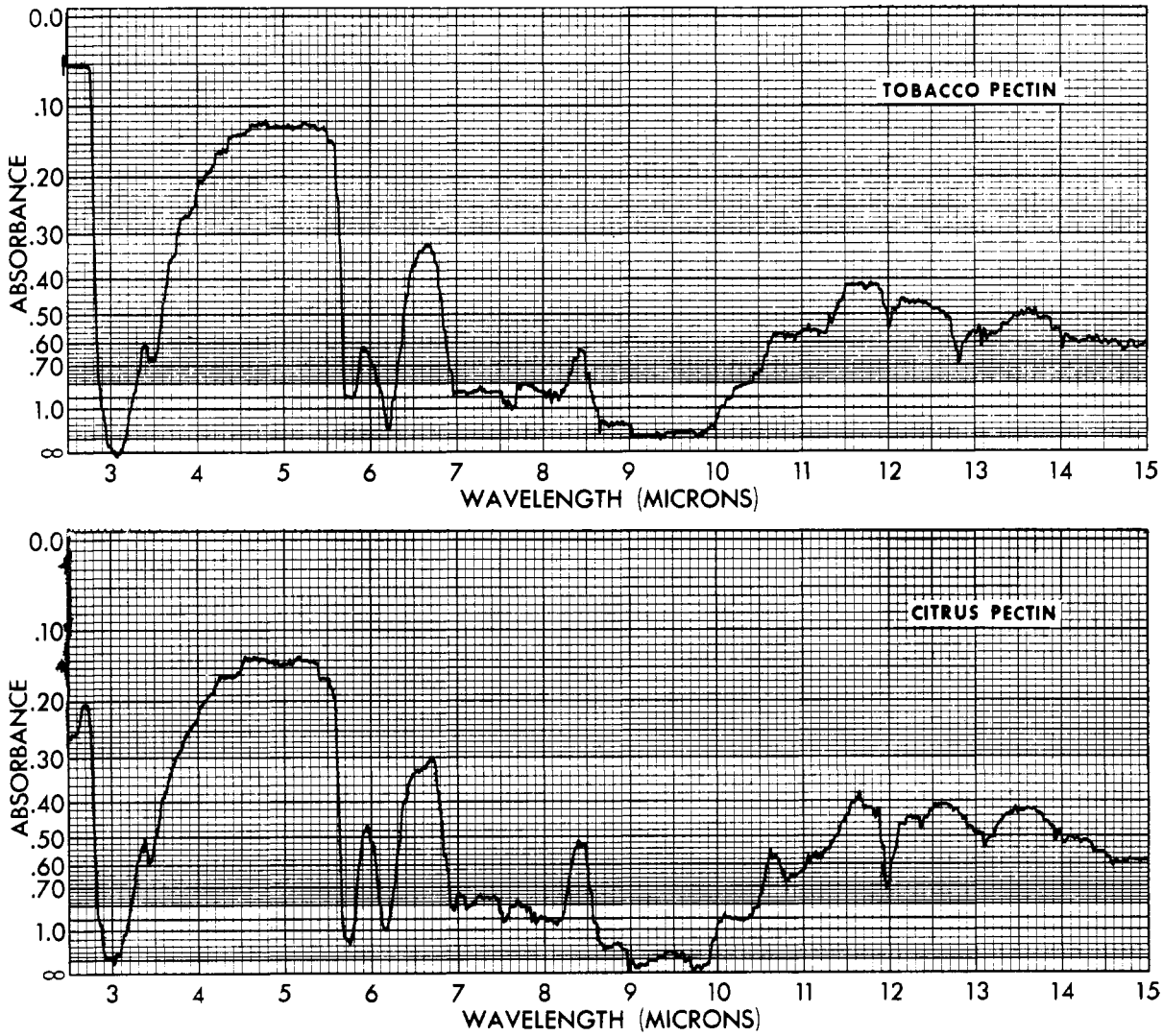


Figure 4. Infrared spectra of tobacco and citrus pectins

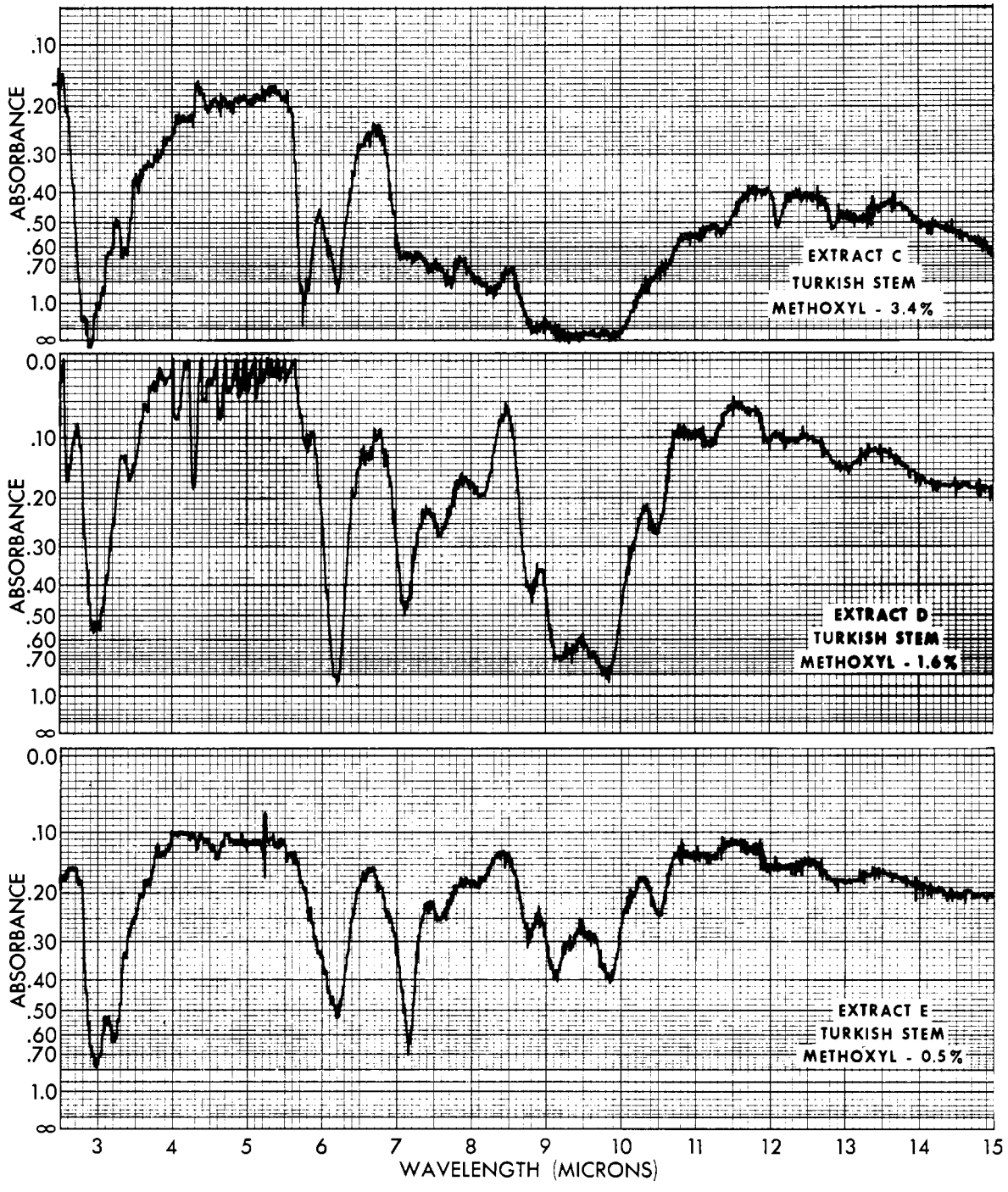


**Figure 5. Comparison of the pectic substances in extracts C, D, and E from Turkish stems**

3 is given in Table IV, which shows that there are differences in the amount of the carbohydrates in the hydrolyzates of the various fractions. No further effort was made to investigate this distribution.

The galacturonic acid contents found in these fractions are given in Table V. The sodium hydroxide titer of each sample after the extraction of calcium is also included for subsequent comparison. Galacturonic acid determinations on the successive extractions and washing comprising each fraction were carried out to make sure that each fraction was completely extracted.

To confirm that the materials isolated in fractions C, D, and E were pectins, the solutions were concentrated under vacuum, and the concentrates were poured on watch glasses and dried slowly to yield films. These films had infrared spectra similar to those of commercial pectins, and a comparison of a commercial pectic material with one of the tobacco fractions is shown in Figure 4. The general similarity in the absorbance curves is apparent. In addition, when the concentrate was mixed with equal quantities of either ethanol or acetone, a gel resulted. These characteristics plus the behavior on thin-layer chromatograms leave little doubt that these isolated fractions are all pectic materials.

Table VI. Methoxyl Content of Pectic Materials Isolated from Tobaccos[a]

| Tobacco | Extracts C | D | E |
|---|---|---|---|
| Turkish stem | 3.42 | 1.58 | 0.45 |
| Bright stems | 2.03 | 1.04 | 1.13 |
| Pennsylvania stems | ... | 1.20 | 0.45 |
| Bulgarian whole leaf | 2.76 | 2.32 | 0.66 |

[a] Per cent based on isolated pectic substances.

Table VII. Comparison of Results with Published Data

| Stem | Results of This Work, % | Literature Results, % | References |
|---|---|---|---|
| Bright | 11–12 | 10–13 | Richards (1962) |
| Burley | 16–17 | 14–17 | Philips and Bacot (1958) |
| Pennsylvania | 16–17 | 14–17 | Richards (1962) |
| German | 16–17 | 13–16 | Richards (1962) |
| Havana leaf | 13–14 | 12–13 | Pyriki and Moldenhauer (1963) |

The infrared spectra of the pectic substances in fractions C, D, and E of Turkish tobacco stems were compared (Figure 5). The infrared spectra show that fraction C has a prominent peak at 5.75 microns, indicating an ester group carbonyl, while the other two spectra seem to lack this prominent peak. Since other pectic fractions from tobacco stems showed similar results, the methoxyl content of the films used in the infrared work was determined (Table VI). The table shows that the isolated pectic substances differ in the degree of esterification. Thus, the fractionation procedure used separates the pectic substances into distinctive groups as distinguished from the gross extraction that is achieved by the classical ammonium oxalate treatment.

While no data could be found in the literature to compare with the galacturonic acid distribution reported in this paper, there were published values of total galacturonic acid content. The results in this investigation compare favorably with the ones reported in the literature (Table VII).

## DISCUSSION

The sodium hydroxide titer of the cigarette type stems is more variable (ranging from 0.21 to 0.47 meq. per gram) and generally lower than that of the cigar type stems (ranging from 0.46 to 0.57 meq. per gram). There appears to be some relationship between this titer and the quantity of fraction D in the case of cigarette stems but not cigar stems.

Fraction C is larger in the cigarette than in the cigar type stems. There does not seem to be any general relationship between the quantity of fraction C and the ratio of extractable calcium to total calcium. One could expect the release of a relatively large amount of calcium from the pectates to result in pectic acids which should be extracted in fraction C. The opposite is nearer the truth, particularly in the case of cigar type stems. The reason may be that although a large number of calcium bridges in the pectates may be broken, the breaking of these bridges and the conversion of the pectates to the acid form does not necessarily make them easily extractable. If so, samples with large amounts of extractable calcium should have a relatively high sodium hydroxide titer. This is the case for cigar type stems. The individual factors contributing to the ease of extraction of the pectins include availability to the solvent, molecular weight, etc. A study of the molecular weight distribution in the various fractions would, no doubt, throw considerable light on these different results among the tobacco stem types.

Both cigarette and cigar types of stems show considerable variation in the total galacturonic acid in the individual fractions. Also, the relative amount of fraction E and the total do not follow the pattern. For example, in the cigarette types the relative amount of fraction E ranges from a low of 10% for Bright to a high of 27% for Maryland, while in the cigar types they range from a low of 18% for Puerto Rican to a high of 50% for German.

No simple generalizations can be made with regard to the pectin distribution in cigar and cigarette types. Each individual tobacco type (whether cigar or cigarette) seems to have characteristics which are no doubt related to a complex interaction of variety, agricultural practice, climate, soil, curing, etc. Even though this study did not deal with the fractionation and characterization of pectins in different lots of the same tobacco—e.g., Maryland *vs.* Maryland, Turkish *vs.* Turkish, etc.—sectional, and other natural variations (including time of harvest) would probably result in differences in their pectin content and distribution. This is the very nature of the dynamic biological system.

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