conducting the nitrosamine analysis, J. G. Phillips, Consulting Statistician, Northeastern Region, Agricultural Research, Science and Education Administration, U.S. Department of Agriculture, for statistical information and advice, and the National Cancer Institute for the loan of a thermal energy analyzer under contract No. NO 1 CP 55715.

LITERATURE CITED

Barnes, J. M., Magee, P. N., *Br. J. Ind. Med.* **11**, 167 (1954).
Bharucha, K. R., Cross, C. K., Rubin, L. L., *J. Agric. Food Chem.* **27**, 63 (1979).
Bills, D. D., Hildrum, K. I., Scanlon, R. A., Libbey, L. M., *J. Agric. Food Chem.* **21**, 876 (1973).
Coleman, M. H., *J. Food Technol.* **13**, 55 (1978).
Ender, F., Ceh, L., *L. Lebensm.-Unters.-Forsch.* **145**, 133 (1971).
Fazio, T., Damico, J. N., Howard, J. W., White, R. H., Watts, J. O., *J. Agric. Food Chem.* **19**, 250 (1971).
Fazio, T., White, R. H., Dusold, L. R., Howard, J. W., *J. Assoc. Off. Anal. Chem.* **56**, 916 (1973).
Fiddler, W., Pensabene, J. W., Fagan, J. C., Thorne, E. J., Piotrowski, E. G., Wasserman, A. E., *J. Food Sci.* **39**, 1070 (1974).
Fine, D. H., Rounbehler, D. P., Dettinger, P. E., *Anal. Chim. Acta* **78**, 383 (1975).
Gray, J. I., Dugan, L. R., Jr., *J. Food Sci.* **40**, 484 (1973).
Gray, J. I., *J. Milk Food Technol.* **39**, 686 (1976).
Havery, D. C., Kline, D. A., Miletta, E. M., Joe, F. L., Jr., Fazio, T., *J. Assoc. Off. Anal. Chem.* **59**, 540 (1976).
Huxel, E. T., Scanlon, R. A., Libbey, L. M., *J. Agric. Food Chem.* **22**, 698 (1974).
Hwang, L. S., Rosen, J. D., *J. Agric. Food Chem.* **24**, 1152 (1976).
Lakritz, L., Spinelli, A. M., Wasserman, A. E., *J. Food Sci.* **41**, 879 (1974).
Lijinsky, W., Keefer, L., Loo, J., *Tetrahedron* **26**, 5137 (1970).
Mottram, D. S., Patterson, R. L. S., Edwards, R. A., Gough, T. A., *J. Sci. Food Agric.* **28**, 1025 (1977).
Patterson, R. L. S., Taylor, A. A., Mottram, D. S., Gough, T. A., *J. Sci. Food Agric.* **27**, 257 (1976).
Pensabene, J. W., Fiddler, W., Gates, R. A., Fagan, J. C., Wasserman, A. E., *J. Food Sci.* **39** 314 (1974).
Sen, N. P., Donaldson, B., Iyengar, J. R., Panalakas, T., *Nature (London)* **241**, 473 (1973).
Spackman, D. H., Stein, W. H., Moore, S., *Anal. Chem.* **30**, 1190 (1958).

Received for review July 5, 1979. Accepted November 28, 1979. Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Coniferyl and Sinapyl Alcohols: Major Phenylpropanoids Released in Hot Water Extracts of Tobacco and Alfalfa

Roger A. Andersen,* Thomas H. Vaughn, and Michael J. Kasperbauer

Coniferyl alcohol and sinapyl alcohol were identified in water and methanol extracts of tobacco stalk and alfalfa stem at molar concentration ratios near unity, but they were not detected in chloroform or acetone extracts. Water extracts contained higher levels of these alcohols than methanol extracts. Our interpretation was that these differential solvent effects were caused by different capacities of the solvents to hydrolyze covalently bound coniferyl and sinapyl alcohols in the plant lignin or carbohydrate matrix. The amount of each alcohol extracted from tobacco stalk was dependent upon the extraction time. The identification of coniferyl and sinapyl alcohols was based on high-performance liquid chromatography capacity factor (k') values and gas chromatography retention times of Me_3Si derivatives. Further confirmation was obtained by gas chromatography–mass spectrometry. Synthesis of coniferyl and sinapyl alcohols was accomplished by reduction of the corresponding acid chlorides to aldehydes and alcohols with lithium tri-*tert*-butoxyaluminohydride.

Metabolites of *p*-coumaric acid in higher plants are the source of most phenols, which include flavanoids, polyphenols, coumarins, tannin and lignin precursors, tannins, and lignin (Neish, 1964). Most tobacco plant parts contain soluble phenols as chlorogenic acid isomers, rutin, scopoletin, scopolin, and esculetin (Sheen, 1969; Vaughn and Andersen, 1973) in addition to insoluble phenols such as lignin (Andersen and Litton, 1975). Quantities of total soluble phenols determined in tobacco were not completely accounted for by the summations of individual phenolic constituents (DHEW Publication No. (NIH) 77-1280, 1977). Unidentified soluble tannins or phenylpropanoids may account for some of this discrepancy. There has been no report of the presence in tobacco of any of the postulated monomeric lignin precursors (Table I), namely, *p*-coumaryl, coniferyl, and sinapyl alcohols, the aldehyde and acid congeners, or their respective phenolic glycosides (Freudenberg, 1965; Freudenberg, 1966). Coniferyl alcohol, however, was recently identified in the phenolic fraction of condensate derived from the smoke of cigarettes made with either flue-cured tobacco leaf midrib or lamina; its concentrations were twice as high in midrib cigarette tobacco condensate as in lamina condensate (Ishiguro et al., 1976).

Recent statistical evidence based on correlations of the chemical composition of experimental cigarettes and the health-related biological activity of their derived smoke suggested that high levels of soluble phenols in tobacco leaf are undesirable (DHEW Publication No. (NIH) 76-1111, 1976;, DHEW Publication No. (NIH) 77-1280, 1977; USDA Technical Bulletin No. 1551, 1977). New smoking materials may contain greater proportions of tobacco from plant tissues that have more lignified cell walls than the conventional materials of the past (Atkinson, 1961; Andersen et al., 1979). Therefore, these materials may contain more phenolic lignin-precursor compounds. In this paper we

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Table I. Structural Relationships among Phenylpropanoid Precursors of Lignin[a]

(General structure: HO–C6H2(R1)(R2)–CH=CHR3)

compound	R_1	R_2	R_3
1. *p*-coumaryl alcohol [3-(4-hydroxyphenyl)-2-propen-1-ol]	H	H	CHOH
2. coniferyl alcohol [3-(4-hydroxy-3-methoxyphenyl)-2-propen-1-ol]	OCH_3	H	CHOH
3. sinapyl alcohol [3-(4-hydroxy-3,5-dimethoxyphenyl)-2-propen-1-ol]	OCH_3	OCH_3	CHOH
4. *p*-coumaraldehyde [3-(4-hydroxyphenyl)propenal]	H	H	CHO
5. coniferaldehyde [3-(4-hydroxy-3-methoxyphenyl)propenal]	OCH_3	H	CHO
6. sinapaldehyde [3-(4-hydroxy-3,5-dimethoxyphenyl)propenal]	OCH_3	OCH_3	CHO
7. *p*-coumaric acid [3-(4-hydroxyphenyl)propenoic acid]	H	H	CO_2
8. ferulic acid [3-(4-hydroxy-3-methoxyphenyl)propenoic acid]	OCH_3	H	CO_2
9. sinapic acid [3-(4-hydroxy-3,5-dimethoxyphenyl)propenoic acid]	OCH_3	OCH_3	CO_2

[a] The compounds may be present as aglycons as shown or as glycosides linked to saccharides in the respective 4-*O*-phenyl positions (Freudenberg, 1965, 1966).

report on our investigations of the presence and nature of three lignin monomers in burley tobacco stalk and leaf midvein and in genetically divergent alfalfa stem used for comparison.

MATERIAL AND METHODS

Reference *p*-Hydroxycinnamyl Acids, Aldehydes, and Alcohols. The structural relationships and nomenclature of the compounds appear in Table I. *p*-Coumaric acid, ferulic acid, and sinapic acid were obtained from Aldrich, Milwaukee, WI. *p*-Coumaraldehyde, coniferaldehyde, and sinapaldehyde were synthesized by reduction of their corresponding acetylated acid chlorides with lithium tri-*tert*-butoxyaluminohydride (Brown and McFarlin, 1956; Pearl and Darling, 1957). *p*-Coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol were each synthesized from their corresponding aldehyde by reduction with sodium borohydride at -5 °C (Nakamura and Higuchi, 1976). The final aldehyde and alcohol products were purified by low-pressure reverse-phase chromatography on a prepacked Lobar column (31 × 2.5 cm) containing 40–63 μm Lichroprep RP8 (EM Laboratories, Inc., Elmsford, NY). Isocratic methanol–water solvent systems (3:2 for the aldehydes and 2:3 for the alcohols) were used at a flow rate of 1.0 mL/min. Five-milliliter fractions of eluate were collected and their absorbancies and absorbance maxima between 200 and 450 nm were determined with a Beckman Acta III recording spectrophotometer. Chromatographically purified zones of the phenylpropanoid compounds were further purified if necessary on thin-layer plates coated with 2000-μm thickness of silica gel using the solvent system: *n*-hexane–diethyl ether–dichloromethane–formic acid (4:3:2:1). Aldehydes were located on the plates by observation of their appropriate color reactions (Gibbard and Schoental, 1969), and alcohols were located by their fluorescence under ultraviolet light. They were eluted from thin-layer plate scrapings with methanol and their identities were confirmed by GC–MS. Absorbance maxima and molar extinction coefficients from 200–450 nm were determined for purified coniferyl and sinapyl alcohol in methanol. Conditions were established for the chromatographic analysis of nine phenylpropanoid (C_6–C_3) acids, aldehydes, and alcohols that correspond to postulated precursors of lignin in plant cells derived from carbohydrate via the shikimic acid–phenylalanine–cinnamic acid pathway (Neish, 1964; Freudenberg, 1966).

High-Performance Liquid Chromatography (LC). Chromatography was performed with an ALC/GPC 204 Waters Associates system which included two 6000A pumps, a 660 solvent flow programmer, a U6K injector, a 440 UV absorbance detector adapted for 254 nm, a μBondapak C_{18} column (30 cm × 3.9 mm i.d.; 10-μm particles), and a Houston Instrument Series B-5000 Omniscribe recorder.

Underivatized reference phenylpropanoids were separated with an isocratic methanol–water (4:1) mobile phase containing 1% acetic acid at a flow rate of 1.0 mL/min. Calibration factors were obtained by dividing the quantity of reference compound injected (0.1–1.0 μg) by the peak height obtained. Acetic acid was omitted from the mobile phase when the LC-separated alcohols were collected off the column for purposes of GC or GC–MS analysis.

Gas Chromatography (GC) and Gas Chromatography–Mass Spectrometry (GC–MS). A Packard Model 7821 gas chromatograph was used with a Model 811 dual-flame ionization detector. The operating temperatures for inlet and detector were 200 and 210 °C, respectively. Argon was used as the carrier gas at a flow rate of 60 mL/min. A 1.83 m × 4 mm (i.d.) coiled glass column with 5% (w/w) silicone stationary phase (OV-101) on 80–90 mesh Anakrom AS at 150–190 °C was found useful for GC separations of the trimethylsilyl (Me_3Si) derivatives of the reference *p*-coumaric, ferulic, and sinapic acids and the corresponding Me_3Si-derivatized aldehyde and alcohol congeners.

The GC–MS analyses were performed in either electron impact or chemical ionization mode with a Finnegan Model 3300-6100 mass spectrometer–gas chromatograph with a computer-controlled data acquisition system. A 1.52 m × 2 mm (i.d.) glass column packed with either 3% (w/w) silicone stationary phase (OV-101) on 80–100 mesh Chromosorb WHP or 3% (w/w) silicone stationary phase (OV-1) on 100–120 mesh Supelcoport was used for gas chromatographic separations. For electron impact analyses the column was either programmed from 120 to 180 °C at 6 °C/min or used isothermally at 160 °C with a helium flow rate of 35 mL/min. The mass spectra were recorded at 70 eV. Chemical ionization analyses were carried out under the same temperature conditions as electron impact analyses, with methane at a flow rate of 30 mL/min and an ion source pressure of 1.0 torr. The mass spectra were recorded at 150 eV.

Plant Materials. Cigar-filler tobacco (*Nicotiana tabacum* L. cv. Wis 38) plants were grown in soil in 3-L pots in the greenhouse. The plants were watered by subirrigation with Hoagland's Nutrient Solution No. 1 (Hoagland and Arnon, 1950). When the plants reached the floral bud stage, transverse segments of stalk (about 1 cm in height) were taken from three adjacent midstalk leaf internode positions. Whole leaves were also harvested from these

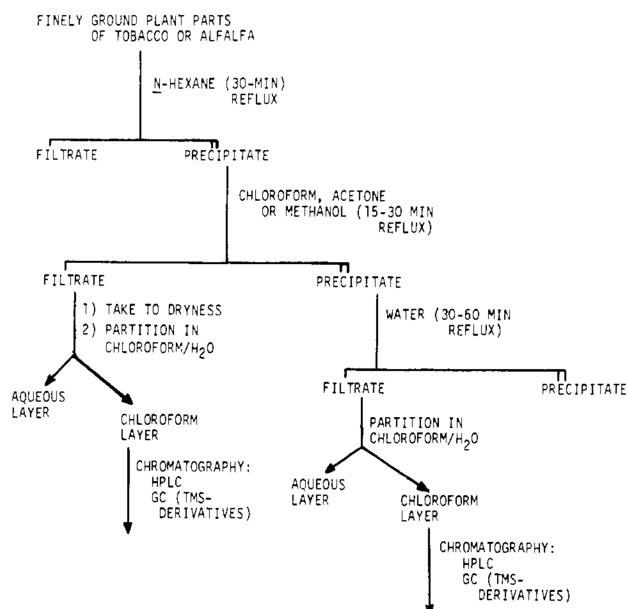

Figure 1. Scheme for analysis of plant materials.

internode positions at the same time. The stalk segments and leaves were freeze-dried, ground to 80–100 mesh, and stored in darkness in a desiccator until assayed.

Burley tobacco (cv. Ky 14) plants were grown at the Kentucky Agricultural Experiment Station South Farm at Lexington in 1977. Three plant spacings were used: "close", 30 × 30 cm; "normal", 45 × 100 cm; and "wide", 125 × 125 cm. Culture, harvest, and air-curing practices were similar to those normally used for burley tobacco. Leaf samples were collected from the middle positions on the stalk 6 weeks after transplanting to the field, at date of harvest and after air-curing. Leaves were separated into lamina and midvein components. At the same sampling times, stalk segments were taken from three adjacent midstalk leaf-internode positions as described for Wis 38 stalk samples. Samples were dried, ground, and stored as described for Wis 38 samples.

Alfalfa (*Medicago sativa* L. subsp. *sativa*) plants were grown at the Kentucky Agricultural Experiment Station Farm at Lexington. In 1978 the topgrowth was harvested in August after 36 days of regrowth following the second cutting of the season. Leaves and blooms were removed and discarded. The stems were dried, ground, and stored for analysis as in the case of the tobacco.

Extraction and Chromatography of Tobacco and Alfalfa. The following steps outlined in Figure 1 were generally applicable to all the extraction procedures used in the plant analyses. A 0.5–2.0-g sample of freeze-dried tobacco or alfalfa was refluxed with *n*-hexane (50 mL) for 30 min in an Erlenmeyer flask. The mixture was filtered through a medium porosity glass filter. The air-dried residue was transferred back to a flask with either chloroform, acetone, or methanol (50 mL). These solvents were used either as a principal extractant or more commonly one was used as a preextractant prior to extraction with hot water. The flask contents were refluxed for a specified period (10–240 min) prior to filtration. In cases where a final extraction with hot water was omitted, the filtrates were evaporated to dryness, taken up in water (50 mL) and extracted with chloroform as described below for the posttreatment of hot water extracts for LC, GC, or GC–MS analyses.

For final hot-water extractions, the insoluble residues obtained after preextraction with chloroform, acetone, or methanol were transferred to a beaker with water (50 mL).

Table II. LC Separation of Phenylpropanoid Reference Compounds on Reverse-Phase μBondapak C_{18} Columns

compound	no. of methoxyl groups/molecule	k' (retention)[a,b] A[c]	B[c]
p-coumaryl alcohol	0	3.16	2.85
coniferyl alcohol	1	3.92	3.42
sinapyl alcohol	2	5.03	4.22
p-coumaric acid	0	2.60	4.53
ferulic acid	1	2.78	6.08
sinapic acid	2	3.20	7.66
p-coumaraldehyde	0	5.52	4.67
coniferaldehyde	1	7.18	6.60
sinapaldehyde	2	9.54	7.94

[a] Mean value of three experiments. [b] Flow rate 1.0 mL/min. [c] Mobile phase: (A) 20:80 methanol/water, (B) 20:80 methanol/water + 1% acetic acid.

The mixture was heated to a gentle boil for a specified period of 15–180 min. If excess foaming occurred, the beaker contents were occasionally stirred and the sides of the beaker were washed down with a fine stream of water. The mixture was cooled and then filtered through a coarse sintered-glass filter. The flask and residue were then washed with chloroform (10 mL). The combined water–chloroform filtrates were transferred to a separatory funnel with chloroform (ca. 10 mL). The aqueous phase was extracted three times with chloroform (total volume = 150 mL). The chloroform phases were combined and taken to near dryness on a rotary vacuum evaporator. The remaining solvent was evaporated and dried with a stream of nitrogen. The residue was either dissolved in methanol and filtered through a 0.45-μm Millipore filter for LC, or it was dissolved in 200 μL of ethyl acetate and 50 μL of bis(trimethylsilyl)trifluoroacetamide (BSTFA) for silylation prior to GC and GC–MS analysis. The silylation solution was heated at 100 °C in a sealed tube for 15 min before injection.

RESULTS AND DISCUSSION

Chromatography of Reference Compounds. The k' (retention) values obtained for the nine phenylpropanoid compounds analyzed by LC are given in Table II. The resolution of the compounds was improved by the addition of 1% acetic acid to the mobile phase, which suppressed the ionization of phenolic hydroxyl groups. In general, retention among the phenylpropanoid compounds increased with the presence and increased number of methoxyl group substitutions. The order of increasing elution times with the neutral mobile phase for analogues with the same number of methoxyl groups was acids, alcohols, and aldehydes; however, the order with the acidified mobile phase was alcohols, acids and aldehydes.

Relative retention times (t_R) for GC analysis of the reference compounds are given in Table III. The order of increasing retention on the OV-101 or OV-1 columns was aldehyde, alcohol, and acid among the phenylpropanoids with the same substitutions on the benzene ring. The presence and total number of methoxyl and Me_3Si group substitutions on a phenylpropanoid molecule affected retention times (increased substitutions increased retention times). Peak areas of known amounts of the reference compounds were determined and standard curves were plotted for the quantification of phenylpropanoids confirmed to be in extracts of the plant materials by GC–MS analyses.

LC Analysis of Plant Extracts. Peaks corresponding to coniferyl alcohol, sinapyl alcohol, and a trace of sinapaldehyde were found in methanol extracts (30-min periods) of the following plant materials: (a) stalk samples of Wis 38 and Ky 14 plants of each plant spacing at har-

Table III. GC Relative Retentions (t_R) of Silylated Phenylpropanoid Reference Compounds on 5% OV-101 at 170 °C

compound	sum of methoxyl and trimethylsilyl groups per molecule	t_R[a,b]
p-coumaraldehyde	1	0.19
coniferaldehyde	2	0.25
p-coumaryl alcohol	2	0.29
p-coumaric acid	2	0.56
coniferyl alcohol	3	0.58
sinapaldehyde	3	0.69
ferulic acid	3	1.00
sinapyl alcohol	4	1.04
sinapic acid	4	1.87

[a] Relative retention time calculated by considering ferulic acid as 1.00. [b] Mean value of three experiments.

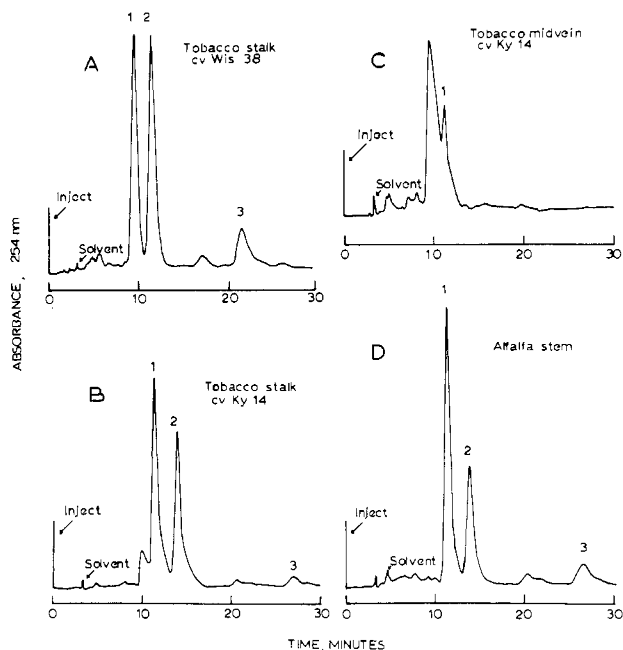

Figure 2. LC chromatograms of hot-water extracts of plant samples showing peaks corresponding to coniferyl alcohol (1), sinapyl alcohol (2), and sinapaldehyde (3). (A) Wis 38 tobacco stalk at floral bud stage, (B) Ky 14 tobacco stalk at harvest, (C) Ky 14 tobacco leaf midvein at 6-weeks posttransplant stage, and (D) alfalfa stem. A is developed with mobile phase of methanol/water (1.2:3.8), and B–D are developed with mobile phase of methanol/water (1.0:4.0).

vest, (b) leaf midveins from Ky 14 tobacco harvested 6 weeks after transplanting, and (c) alfalfa stem. These same peaks were not present, however, in 30- or 45-min chloroform or acetone extracts of these same plant materials. The yields of coniferyl alcohol and sinapyl alcohol in methanol extracts appeared to increase with a longer extraction period (45 min). Methanol-extractable sinapaldehyde and tyrosine as phenylpropanoids in a green plant tissue were previously reported (Mugg, 1959).

Hot water extracts (30-min periods) of the methanol preextracted (30 min) tissue insolubles yielded from one to three prominent, nonoverlapping peaks that were obtained under the same LC analytical conditions as described for the phenylpropanoid reference compounds (Figures 2A–D). Their elution times corresponded to the k' (retention) values of coniferyl alcohol, sinapyl alcohol, and sinapaldehyde. The k' values were obtained with the two mobile phases used in Table II, or in the case of Wis

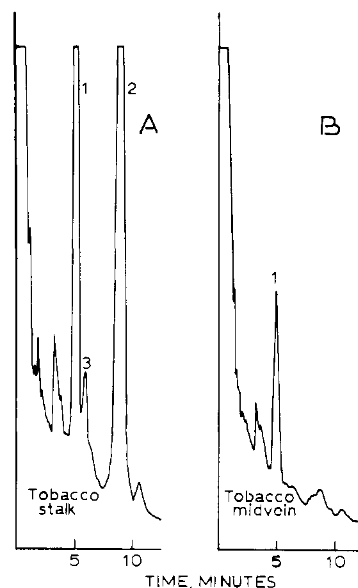

Figure 3. GC chromatograms of hot-water extracts of "normal"-spaced Ky 14 tobacco samples at 6-weeks posttransplant stage. Peaks correspond to Me_3Si–coniferyl alcohol (1), Me_3Si–sinapyl alcohol (2), and Me_3Si–sinapaldehyde (3). Amounts injected were 9.1 mg equivalent of stalk (A) and 30.3 mg equivalent of leaf midvein (B).

38 tobacco stalk (Figure 2A) a similar isocratic mobile phase of methanol–water (1.2:3.8) at 1.0 mL/min. The peaks corresponding to coniferyl and sinapyl alcohols in the tobacco stalk and alfalfa stem samples (Figures 2A, 2B, and 2D) and coniferyl alcohol in the tobacco midvein sample (Figure 2C) were more prominent than those corresponding to the k' values of sinapaldehyde. The peaks for coniferyl and sinapyl alcohols from hot water extracts were several times larger than those obtained from methanol extracts when extraction times were equal.

Since tobacco stalks contain about three times the concentration of total lignin present in leaf lamina or leaf midvein (Andersen and Litton, 1975), it is possible that the gross differences observed in the monolignol contents of tobacco leaf lamina, leaf midvein, and stalk were related to the lignin concentrations. The results showed that coniferyl and sinapyl alcohols (>10 μg/g dry wt) as well as sinapaldehyde (2–4 μg/g dry wt) were probably present in similarly extracted stalk samples of the two cultivars of tobacco (Figures 2A and 2B) and in alfalfa (Figure 2D).

GC and GC–MS Analysis of Lignin Monomers in Plant Extracts. At least three lignin monomer compounds were characterized in a plant part of tobacco or alfalfa. Gas chromatograms of trimethylsilylated hot water extracts (60-min periods) of Ky 14 tobacco preextracted for 30 min with methanol are shown in Figure 3. Peaks with retention times corresponding to Me_3Si derivatives of coniferyl alcohol and sinapyl alcohol and a minor peak corresponding to Me_3Si–sinapaldehyde were observed for the stalk sample (Figure 3A). By contrast, the chromatogram for a leaf midvein sample (Figure 3B) had a single peak corresponding to Me_3Si–coniferyl alcohol. The estimated concentration of coniferyl alcohol in the extract of midvein was about 10% of that of the stalk. The GC results of additional samples of Ky 14 tobacco stalk from plants at harvest and after completion of air curing were similar to those obtained with the less mature tobacco. Thus, the compounds were stable under the growth and curing conditions.

Structural identifications were based in part upon the electron impact and chemical ionization mass spectra of

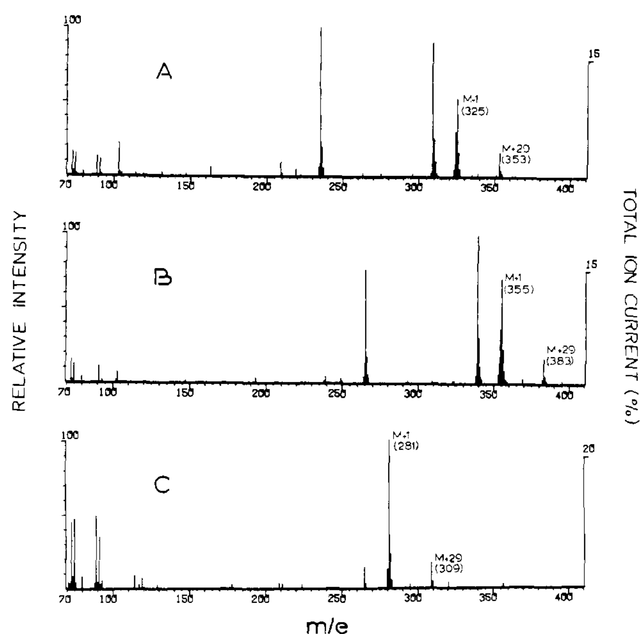

Figure 4. GC–mass spectra of components from hot-water extracts of Ky 14 tobacco stalk. Methane used as reactant gas at ion source pressure of 1.0 torr. Spectra correspond to (A) Me_3Si–coniferyl alcohol, (B) Me_3Si–sinapyl alcohol, and (C) Me_3Si–sinapaldehyde.

the chromatographically purified sample components (GC or LC), the relative intensities of the MS charged ions and the comparison of specific spectra with those of available standards. Mass spectra of phenylpropanoids were compared with literature values where available. Fragmentation patterns produced by electron impact have been tabulated by Nakamura et al. (1974) for the three underivatized *p*-hydroxycinnamoyl aldehydes and by Nakamura and Higuchi (1976) for coniferyl alcohol and coniferaldehyde. Although we found no specific literature values for chemical ionization spectra of the phenylpropanoids, we predicted the MS product ions formed with methane according to the generalized principles of chemical ionization mass spectra as reviewed by Munson (1971). Our electron impact MS data pertaining to confirmations of chemical structure for free or Me_3Si derivatives of coniferyl alcohol, sinapyl alcohol, and sinapaldehyde along with probable fragment assignments follow. Coniferyl alcohol, MS *m/e*: 180 (M^+), 162 ($M^+ - H_2O$), 147 ($M^+ - CH_5O$), 137 ($M^+ - C_2H_3O$, base ion), 124 ($M^+ - C_3H_4O$). Sinapyl alcohol, MS *m/e*: 210 (M^+), 182 ($M^+ - CO$), 181 ($M^+ - CHO$), 167 ($M^+ - C_3H_7$). Me_3Si–sinapyl alcohol, MS *m/e*: 354 (M^+), 339 ($M^+ - CH_3$), 324 ($M^+ - 2CH_3$), 309 ($M^+ - 3CH_3$), 294 ($M^+ - 4CH_3$). Sinapaldehyde, MS *m/e*: 208 (M^+, base ion), 207 ($M^+ - H$), 193 ($M^+ - CH_3$), 180 ($M^+ - CO$), 177 ($M^+ - H - CH_2O$), 165 (193 − CO), 137. Chemical ionization mass spectra are given for Me_3Si derivatives of coniferyl alcohol (Figure 4A), sinapyl alcohol (Figure 4B), and sinapaldehyde (Figure 4C) obtained by GC–MS from hot water extracted (methanol preextracted) Ky 14 stalk harvested from tobacco 6 weeks after transplant. The M + 1, M + 29, and M − 15 ($-CH_3$) peaks in these mass spectra were prominent and easily distinguished.

Quantitative Analysis of Extracted Plant Material. For measurement of the principal lignin monomers, ultraviolet-visible spectra were obtained for methanol solutions of synthetic coniferyl and sinapyl alcohols [coniferyl alcohol λ_{max} 266, ϵ_{max} 11420, ϵ_{254} 7820; sinapyl alcohol λ_{max} 271, ϵ_{max} 11070, ϵ_{254} 5870]. A calibration curve was plotted for the absorbance values at 254 nm corresponding to the peak heights obtained with different levels of authentic coniferyl and sinapyl alcohols during LC analysis. There was a linear relationship between the amounts of either monomer (0–1.0 μg) and the corresponding peak heights.

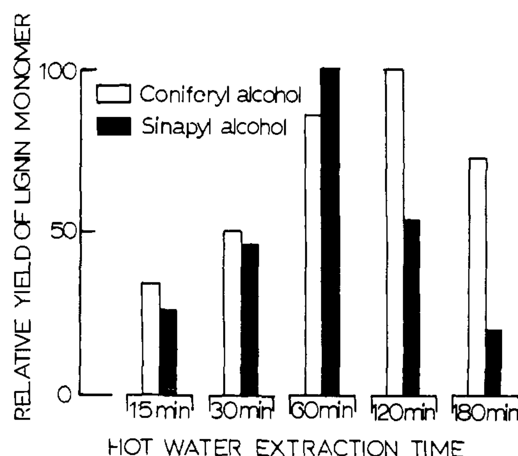

Figure 5. Effect of hot-water extraction time on yield of lignin monomers from Ky 14 burley tobacco stalk.

Ky 14 tobacco stalk and alfalfa stem were analyzed by LC for concentrations of coniferyl and sinapyl alcohol obtained during 30-min hot water extractions (methanol preextracted for 10 min). Concentrations were determined in six samples of burley tobacco grown at three different plant spacings and sampled at harvest or after air curing. Coniferyl alcohol ranged from 3.3 to 6.4 mg/100 g with a mean value of 4.7 mg/100 g ± 0.9 mg/100 g standard deviation. Sinapyl alcohol ranged from 3.2 to 7.8 mg/100 g with a mean value of 4.9 mg/100 g ± 1.6 mg/100 g standard deviation. The amounts of coniferyl and sinapyl alcohol in alfalfa stem were 6.5 and 5.4 mg/100 g, respectively. Molar ratios of the soluble lignin monomers approached unity in these samples (a ratio of 1.13 coniferyl alcohol/sinapyl alcohol was obtained when averaged over the tobacco stalk samples and 1.41 was found for the alfalfa stem sample).

Effect of Extraction Time on Release of Lignin Monomers. Experiments were carried out to determine the hot-water extraction time needed for maximum yields of coniferyl and sinapyl alcohols from methanol preextracted (for 10 min) tissue. Ky 14 stalk samples from 6-week posttransplant tobacco were extracted with hot water for 15-, 30-, 60-, 120-, and 180-min periods. The extracts were quantitatively analyzed for coniferyl and sinapyl alcohols by LC. The results (Figure 5) showed that yields of coniferyl and sinapyl alcohols became greater as the length of hot water extraction time increased from 15 to 60 min. Extraction periods of 60–120 min provided maximum levels of both alcohols, but yields decreased with the longer 180-min period. Losses may have been caused by oxidation of the phenolics.

The low levels or absence of coniferyl and sinapyl alcohols in the chloroform, acetone, and methanol extracts of the plant samples contrasted with the higher yields obtained with hot water. Solubility tests with all the reference phenolic phenylpropanoids demonstrated their ready solubility in either chloroform, acetone, methanol, or water at higher concentrations than those subsequently determined as extractives from plant tissue. We interpreted these results to mean that the differential solvent and extraction time effects on the yield of hot-water extractives (Figure 5) were caused by the hydrolytic action of water on covalently bound coniferyl and sinapyl alcohols in tobacco and alfalfa lignin or carbohydrate complexes. Freudenberg (1966) observed that mild hydrolysis with hot

water of sprucewood meal (preextracted with acetone) yielded chemically unaltered lignin degradation products such as coniferyl alcohol. He suggested that these products were released from the periphery of highly branched lignin where they were covalently linked to the main lignin matrix via benzyl-aryl ether bonds. Presumably much larger amounts of coniferyl alcohol and sinapyl alcohol were present and not released under these mild conditions.

Our data does not account for the composition of the major fraction of lignin which was not extracted by hot water because the yields of extractable lignin monomers were very low and *p*-coumaryl alcohol was not found in the extracts. Approximately 5 mg/100 g each of coniferyl alcohol and sinapyl alcohol were released in our hot water extracts of the plant materials. The sum of these alcohols is only about $^1/_{1000}$ of the approximately 5–25 g/100 g total lignin contents estimated as present in either tobacco stalk or alfalfa stem (Freudenberg and Sindhu, 1961; Andersen and Litton, 1975). No evidence for the release of *p*-coumaryl alcohol from these plant materials was obtained in our analyses by LC, GC, and GC–MS. It is thought that lignin from most plant sources contains at least 5% *p*-coumaryl alcohol (Freudenberg, 1966).

We consider it highly probable that certain cigarette tobacco components undergo hydrolytic reactions during burning in the presence of water or water vapor at elevated temperatures. Moisture is present in tobacco and is also released during pyrolysis. The release and transfer of coniferyl and sinapyl alcohols from tobacco lignin by this process may account for the recent identification of coniferyl alcohol in cigarette smoke condensate (Ishiguro et al., 1976). While these phenolic lignin monomers might contribute to flavor, aroma, and other desirable organoleptic properties of tobacco and tobacco smoke, lignin might also be a precursor of specific phenols in smoke that have undesirable implications with regard to the health hazards of smoking (Van Duuren et al., 1973).

The release of coniferyl and sinapyl alcohols in vitro by water extraction or hydrolysis as demonstrated by this investigation suggested that these or related phenylpropanoids might be released from feed and foodstuffs such as alfalfa during digestive processes in animals. This possibility is of interest because of the current speculation concerning the beneficial role of fiber in the diet of humans. The contribution of lignin monomers to animal nutrition is not known at the present time.

ACKNOWLEDGMENT

We thank R. Laine for the mass spectral analyses, H. Burton for total lignin analyses, R. Sigafus for the alfalfa sample, and the following persons for samples of reference chemicals for comparison purposes: G. Still and H. Balba for coniferyl alcohol, W. Conners for coniferaldehyde, and Y. Nakamura for *p*-coumaraldehyde and coniferaldehyde.

LITERATURE CITED

Andersen, R. A., Litton, C. C., *Tobacco Sci.* **19**, 69 (1975).
Andersen, R. A., Tso, T. C., Chaplin, J. F., *J. Agric. Food Chem.* **27**, 891 (1979).
Atkinson, W. O., *Tobacco* **152**, 28 (1961).
Brown, H. C., McFarlin, R. F., *J. Am. Chem. Soc.* **78**, 252 (1956).
DHEW Publication No. (NIH) 76-111, National Cancer Institute Smoking and Health Program, Report No. 2, Toward Less Hazardous Cigarettes, The Second Set of Experimental Cigarettes, 1976.
DHEW Publication No. (NIH) 77-1280, National Cancer Institute Smoking and Health Program, Report No. 3, Toward Less Hazardous Cigarettes, The Third Set of Experimental Cigarettes, 1977.
Freudenberg, K., *Science* **148**, 595 (1965).
Freudenberg, K., in "Symposium on Lignin Structure and Reactions", Gould, R. F., Ed., American Chemical Society Publications, Washington, DC, 1966, pp 1–21.
Freudenburg, K., Sindhu, G. S., *Holzforschung* **15**, 33 (1961).
Gibbard, S., Schoental, R., *J. Chromatogr.* **44**, 396 (1969).
Hoagland, D. R., Arnon, D. I., *Calif. Agric. Exp. Sta. Cir.* **347** (1950).
Ishiguro, S., Sato, S., Sugawara, S., Kaburaki, Y., *Agric. Biol. Chem.* **40**, 977 (1976).
Mugg, J. B., *Tappi* **42**, 289 (1959).
Munson, B., *Anal. Chem.* **43**, 28 (1971).
Nakamura, Y., Higuchi, T., *Wood Res.* **59**, 101 (1976).
Nakamura, Y., Nakatsubo, F., Higuchi, T., *Wood Res.* **56**, 1 (1974).
Neish, A. C., in "Biochemistry of Phenolic Compounds", Harborne, J. B., Ed., Acadernic Press, New York, 1964, p 295.
Pearl, I. A., Darling, S. F., *J. Org. Chem.* **22**, 1266 (1957).
Sheen, S. J., *Phytochemistry* **8**, 1839 (1969).
U.S. Department of Agriculture Technical Bulletin No. 1551, Simple Correlation and Multiple Regression Among Leaf Characteristics, Smoke Components, and Biological Responses of Bright Tobaccos, 1977.
Van Duuren, B. L., Katz, C., Goldschmidt, B. M., *J. Natl. Cancer Inst.* **51**, 703 (1973).
Vaughn, T. H., Andersen, R. A., *Anal. Biochem.* **56**, 626 (1973).

Received for review July 13, 1979. Accepted October 31, 1979. Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may be suitable. Presented in part at the 177th National Meeting of the American Chemical Society, Division of Agricultural and Food Chemistry, Honolulu, Hawaii, April 1979. This investigation is in connection with a project at the Kentucky Agricultural Experiment Station, and the results are published as Kentucky Agricultural Experimental Station Journal Article 79-3-73.