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IMPROVED METHODS FOR THE ESTIMATION BY GAS–LIQUID CHROMATOGRAPHY OF LIGNIN DEGRADATION PRODUCTS FROM PLANTS

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SUMMARY

A method is described for the gas–liquid chromatographic separation and estimation of the following compounds obtained by the saponification of ester linkages present in plant lignins: *p*-coumaric acid, ferulic acid and vanillin. A similar method is described for the separation and quantitative estimation of the following compounds produced by alkaline nitrobenzene or alkaline cupric oxide oxidation of plant lignin: *p*-hydroxybenzaldehyde, vanillin, syringaldehyde, *p*-hydroxyacetophenone, acetovanillone and acetosyringone. *p*-Coumaric and ferulic acids were identified in the oxidation products of grass lignin. As little as 1 μg of a compound could be quantitatively estimated by these methods.

INTRODUCTION

The presence of lignin in the cell-walls of plant materials is of considerable interest in studies concerned with ruminant nutrition as lignin is known to affect detrimentally the nutritional quality of herbages[1]. As a herbage ages, the changes which occur in the cell-walls in relation to nutritional quality are not well defined, and for this reason further characterisation of plant lignins is required. HIGUCHI *et al.*[2,3] have recently established the presence of ester linkages between the main portion of the lignin (the lignin core) of grasses and *p*-coumaric acid (PCA) and ferulic acid (FA), using gas–liquid chromatography (GLC) with Apiezon N as stationary phase. Several workers[4–6] have also used GLC with Apiezon N or SE-30 stationary phases for the estimation of some or all of the following six lignin oxidation products: *p*-hydroxybenzaldehyde (PHB), vanillin (V), syringaldehyde (S), *p*-hydroxyacetophenone (PHAP), acetovanillone (AV) and acetosyringone (AS). For our purposes, methods were required which would separate mixtures of the six lignin oxidation products and also resolve mixtures of PCA, FA and sinapic acid (SA) and enable as little as 1 μg of a compound to be estimated. SA was included in the mixture of acids as it is closely related to PCA and FA and could be present in lignin degradation products. Apiezon N and SE-30 stationary phases gave poor resolution of these

mixtures due to the production of broad peaks and of tailing. In agreement with BRAND[6], peak areas for the six oxidation products could not be estimated due to poor separation.

This paper describes improved GLC methods using cyclohexane dimethanol succinate (CDMS) as stationary phase both for the estimation of compounds obtained by saponification of the ester linkages present in lignins of plants and for the estimation of major degradation products produced by oxidation of lignin core materials.

EXPERIMENTAL

GLC conditions

Separation of PCA, FA, SA and V. A Pye Series 104 Chromatograph with a flame ionisation detector was employed with a 2.75 m glass column (I.D. 4 mm) containing 10% CDMS on 80–100 mesh Diatomite C'Q'. The rate of flow of argon carrier gas was 100 ml/min and the inlet pressure 4.1×10^5 Nm^{-2} (gauge). The column oven temperature was maintained at 190° and the detector oven at 210°. An inlet heater was not employed, the samples being injected directly into the column packing using an 11 cm needle. A Kent Chromalog 3 integrator was employed to determine peak areas for quantitative work.

Separation of PHB, V, S, PHAP, AV and AS. The above conditions were repeated using a flow rate of argon carrier gas of 110 ml/min, a column oven temperature maintained at 220° and a detector oven temperature of 230°.

Quantitative calibration

A reference solution containing 1.00 mg/ml each of chromatographically pure PHB (Hopkin & Williams), V (Hopkin & Williams), S (Koch–Light), PHAP (Koch–Light), AV (Koch–Light) and AS (Ralph N. Emanuel) was prepared using acetone and a calibration graph of peak area against weight was constructed for each compound in the range 1–8 μg. A linear relationship was found in each case. The method was found to be highly reproducible, for example, four consecutive samples of reference V (4 μg each time) gave integrator readings in the ratio of 1.00:1.02:1.05:1.01.

The quantitative estimation of PCA, FA and SA was complicated by variation in peak area from run to run due to differing levels of absorption on the column. To minimise these effects, a similar sample containing chromatographically pure PCA (Koch–Light), FA (Koch–Light) and SA (Koch–Light) was injected both before and after a plant extract. The amount of a particular acid in the plant extract was estimated from a comparison of the integrated value with the integrated values of the reference samples. The analysis was repeated if the results from the two reference samples differed by more than 10%. The reproducibility of the method and the absorption effect can be seen in the following example where four consecutive samples of FA (4 μg each time) were chromatographed. The integrator readings were in the ratio of 1.00:1.03:1.13:1.16.

Material used for degradation studies

The following materials were prepared by freeze-drying:

Sample 1. Mature leaf blade fraction of Italian ryegrass (*Lolium multiflorum*), var. RVP, harvested after tillering had occurred but before ear emergence.

Sample 2. Whole shoot fraction of perennial ryegrass (*Lolium perenne*), var. S. 24, harvested at the end of the flowering stage.

Their ash-free lignin contents, determined by the VAN SOEST procedure[7], were: sample 1, 0.79%; sample 2, 3.45%.

Preparation of cell-wall material

This fraction was prepared from dry grass material (0.5–1.0 g) by the method of VAN SOEST AND WINE[8] using boiling neutral detergent solution to remove cell contents; no sodium sulphite was used and the filtered neutral detergent fibre (NDF) fraction was thoroughly washed with hot water and air-dried.

Sodium hydroxide treatment of cell-wall material

The NDF fractions (0.2–0.5 g) were treated with 1 *N* NaOH (20 ml per sample) in stainless-steel tubes (25 ml capacity) fitted with screw caps. The mixtures were thoroughly shaken at 20 ± 2° for 24 h. Each reaction mixture was filtered (No. 1 porosity glass sinter), washed with water (20 ml) and the filtrate acidified to pH 2.5 with concentrated HCl. Solid NaCl was added to saturate the solution, and the mixture extracted with peroxide-free ether (3 × 60 ml). The combined ethereal extracts were dried (anhydrous Na_2SO_4) and the solvent evaporated at 30° *in vacuo*. The residue was dissolved in acetone (1.0–2.0 ml) and analysed by GLC (5 μl samples injected).

A second fraction was obtained by a further extraction of the acidified solution with ether (50 ml). GLC analysis of the residue showed the presence of only trace amounts of saponification products.

A further saponification was carried out on plant material which had been alkali-treated; no further PCA, FA or V could be detected.

With each series of plant samples being analysed by the saponification method, a blank experiment was carried out using reagents without plant material to ensure that there was no interference from reagent impurities.

Oxidation of the lignin "cores" of grasses

Two methods were employed; in both cases blank experiments were again carried out.

1. Alkaline nitrobenzene oxidation. The alkali-extracted NDF fractions were oxidised with nitrobenzene (1.0 ml) and 2 *N* NaOH solution (10 ml) in screw-capped stainless-steel tubes (25 ml capacity) at 160 ± 2° for 3 h; the tubes were heated and thoroughly shaken in a rotating aluminium block. Each reaction mixture was filtered (No. 1 porosity glass sinter), washed with water (10 ml) and then with peroxide-free ether (20 ml). The filtrate and washings were extracted with ether (2 × 50 ml) and the ethereal layers rejected. The aqueous layer was acidified to pH 2.5 with concentrated HCl and solid NaCl was added to saturate the solution which was then extracted with ether (2 × 60 ml). The combined ethereal solutions were dried (anhydrous Na_2SO_4) and the solvent evaporated. The residue was dissolved in acetone (0.5 ml) and PHB, V, S, PHAP, AV, AS, PCA and FA estimated by the GLC techniques previously described (5 μl injected).

2. Alkaline cupric oxide oxidation. The alkali-extracted NDF fractions, $CuSO_4 \cdot 5H_2O$ (1.7 g) and 3*N* NaOH (10 ml) were heated at 180 ± 2° for 2.5 h with constant

shaking by the above technique. The reaction mixtures were centrifuged at 10,000 *g* for 10 min in a refrigerated centrifuge. The supernatant solution and centrifuged washings (2 × 10 ml water) were combined, acidified, extracted and analysed as described above.

In both of the above oxidations, a further ether extraction gave only negligible quantities of the eight compounds being analysed. Similar results were obtained when cell-wall residues from each oxidation were re-oxidised.

After several GLC determinations of degradation products from the saponification or oxidation procedures, it was necessary to remove the non-volatile material which accumulated at the injection point. This was carried out by the replacement of the top 12 cm of column packing.

Thin-layer chromatographic (TLC) separation of PCA, FA and V present in lignin saponification and oxidation products

The following solvent systems were employed using Silica Gel G[9] with Fast Blue Salt B[9], alkaline potassium permanganate[9], and 2,4-dinitrophenylhydrazine[10] as reagents:

Toluene–methanol–acetic acid (79:14:7). R_F values: PCA, 0.52; FA, 0.58; V, 0.64.

Ethyl acetate. R_F values: PCA, 0.16; FA, 0.07; V, 0.79.

Hexane–amyl alcohol–acetic acid (100:16:0.25)[9]. R_F values: PCA, 0.59; FA, 0.40; V, 0.40.

Combined GLC–mass spectrometric (MS) analysis of compounds present in lignin saponification and oxidation products

For the identification of PCA, FA and V in lignin saponification products, the above GLC conditions were employed except that the column oven was maintained at 210° and the carrier gas (helium) flow rate reduced to 50 ml/min. Similar conditions were employed for the GLC–MS identification of the oxidation products of lignin core materials, except that the column oven temperature was increased to 225°.

Mass spectra were recorded at 70 eV with the source at 160° (A.E.I. MS9 Instrument).

RESULTS AND DISCUSSION

In order to carry out studies on lignins present in plant cell-walls, it was first necessary to remove the cell contents. Various solvents have been used for this purpose. In exploratory work, we have compared the neutral detergent method[8] with the ethanol–benzene method which has been frequently employed[2–6]. Both methods gave similar results but the former was preferred as it was more rapid and did not involve the use of a highly toxic solvent.

Saponification of the ester linkages present in the lignins of plant cell-walls was carried out at 20° by a method similar to that of HIGUCHI *et al.*[2]. Continuous ether-extraction after saponification and acidification was however found to be unnecessary.

The GLC method described in this paper was used to examine the saponification products. The results for grass sample 1, together with a reference chromatogram, are shown in Figs. 1 and 2. The PCA, FA and V identified were the only com-

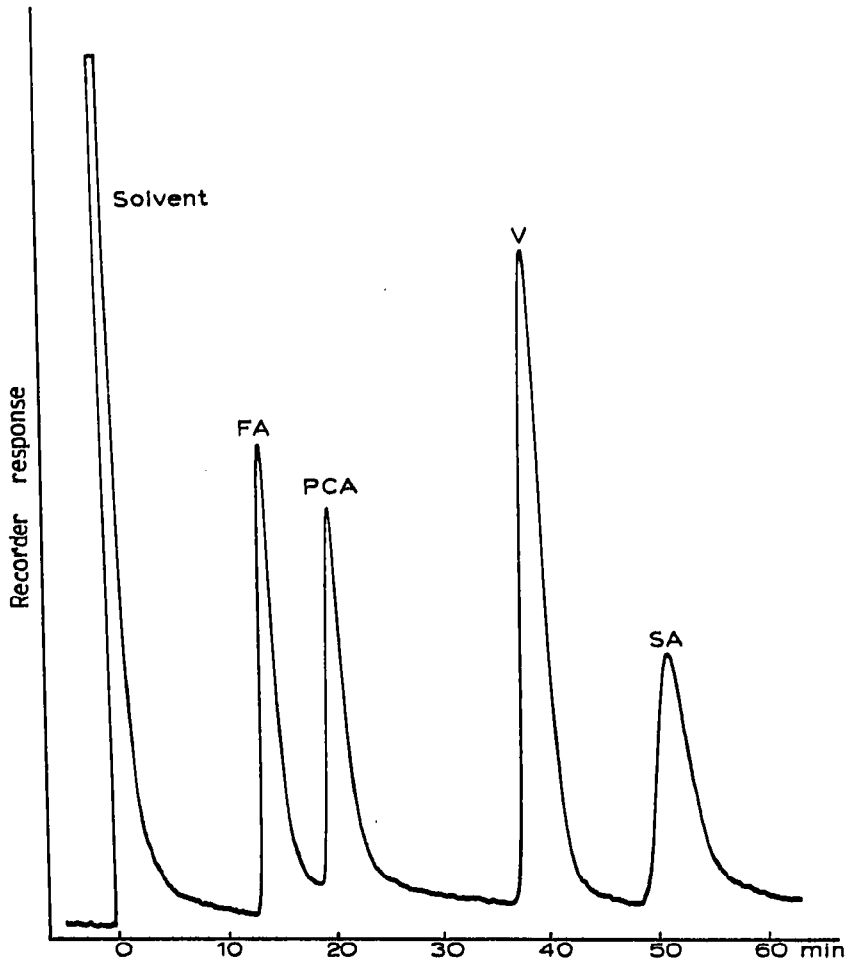

Fig. 1. Separation (3.0 μg of each) of *p*-coumaric acid (PCA), ferulic acid (FA), sinapic acid (SA) and vanillin (V) by GLC (conditions in text).

pounds detected in the saponification products from either of the two grass samples. Further evidence for the presence of these three compounds was obtained by TLC using three different solvent systems. Confirmation of the presence of PCA, FA and V was obtained by comparison of the GLC–MS analysis of the saponification products with a similar analysis of a reference mixture of the three compounds. The mass spectrum of V was characterised by strong parent (M) and M—1 peaks. PCA and FA did not give parent peaks but the base peak in each case was M—44, corresponding to loss of carbon dioxide. A strong peak (M—59) was also present in the spectrum of FA corresponding to further loss of a methyl group.

The two grass samples yielded only trace amounts of V, representing less than 3% of the total yield of the three compounds. The total amount of these substances obtained from grass sample 1 represented 34.9% of ash-free lignin as determined by the VAN SOEST procedure[7], or 0.28% of the original freeze-dried grass. The percentage of ester groupings was very high in this leaf blade material compared with the amounts obtained by HIGUCHI *et al.*[2] from stem-materials of various grasses. The ratio of FA/PCA was 6.5 in the blade material in contrast to ratios of between 0.31 and 0.74

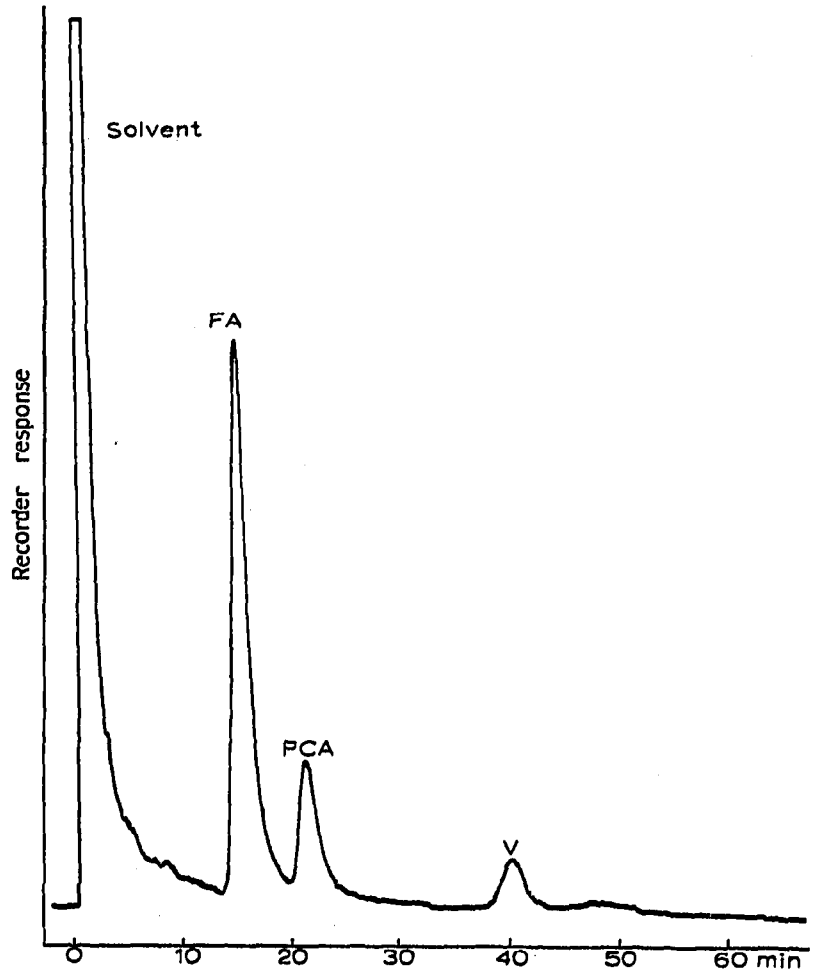

Fig. 2. Separation of the saponification products from cell-wall material from grass sample 1 by GLC (conditions in text).

TABLE I

YIELDS OF ALKALINE NITROBENZENE AND CUPRIC OXIDE OXIDATION PRODUCTS OF ALKALI-EXTRACTED CELL-WALL MATERIAL FROM GRASS SAMPLES 1 AND 2

Compound	*Yield*[a]		
	Alkaline nitrobenzene oxidation		*Alkaline cupric oxide oxidation*
	Sample 1	*Sample 2*	*Sample 2*
p-Hydroxybenzaldehyde (PHB)	zero	0.15	0.17
Vanillin (V)	1.15	4.50	2.85
Syringaldehyde (S)	0.16	3.02	1.51
p-Hydroxyacetophenone (PHAP)	zero	0.05	0.03
Acetovanillone (AV)	0.03	0.09	0.47
Acetosyringone (AS)	zero	0.58	0.58
p-Coumaric acid (PCA)	0.03	0.30	0.15
Ferulic acid (FA)	0.71	0.60	0.30
Total	2.08	9.29	6.06

[a] Per cent of ash-free lignin (determined by the VAN SOEST method[7]).

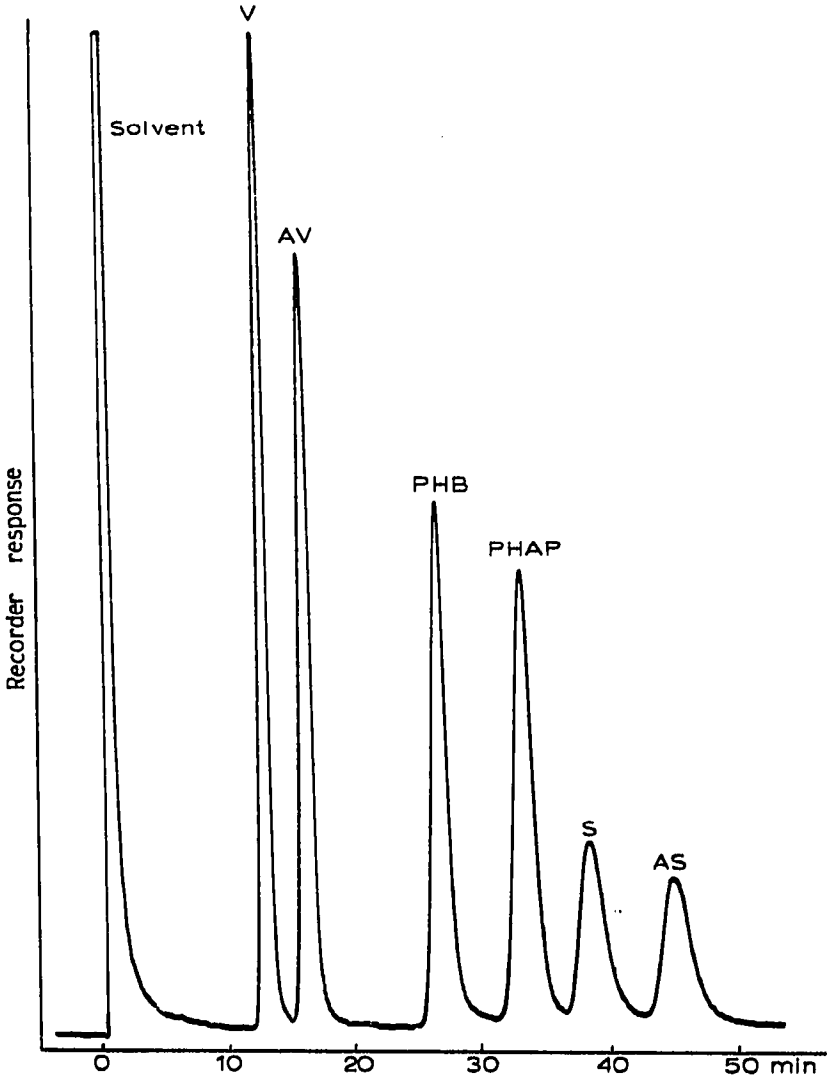

Fig. 3. Separation (1.0 μg of each) of *p*-hydroxybenzaldehyde (PHB), vanillin (V), syringaldehyde (S), *p*-hydroxyacetophenone (PHAP), acetovanillone (AV) and acetosyringone (AS) by GLC (conditions in text).

in HIGUCHI's stem materials. Grass sample 2, which was composed of stem, leaf and senescent material, gave only 11.7% (based on ash-free lignin) of saponification products and the FA/PCA ratio was 1.0.

The absence of SA in the saponification products, even though S was obtained by lignin oxidation, was in agreement with other results from grasses[2,11], wheat[12], maize[13] and sugar cane[14].

The possibility that V obtained by sodium hydroxide-treatment of lignins was being produced from liberated FA by air oxidation was investigated by submitting FA to the usual saponification procedure. No V was detected. Hence it seems likely that V is linked as an ester grouping either to the lignin core or to other peripheral groups.

Although there is good evidence[2,3] that, in some cell-wall fractions of grasses, all the PCA and at least some of the FA are present as lignin ester groups, further

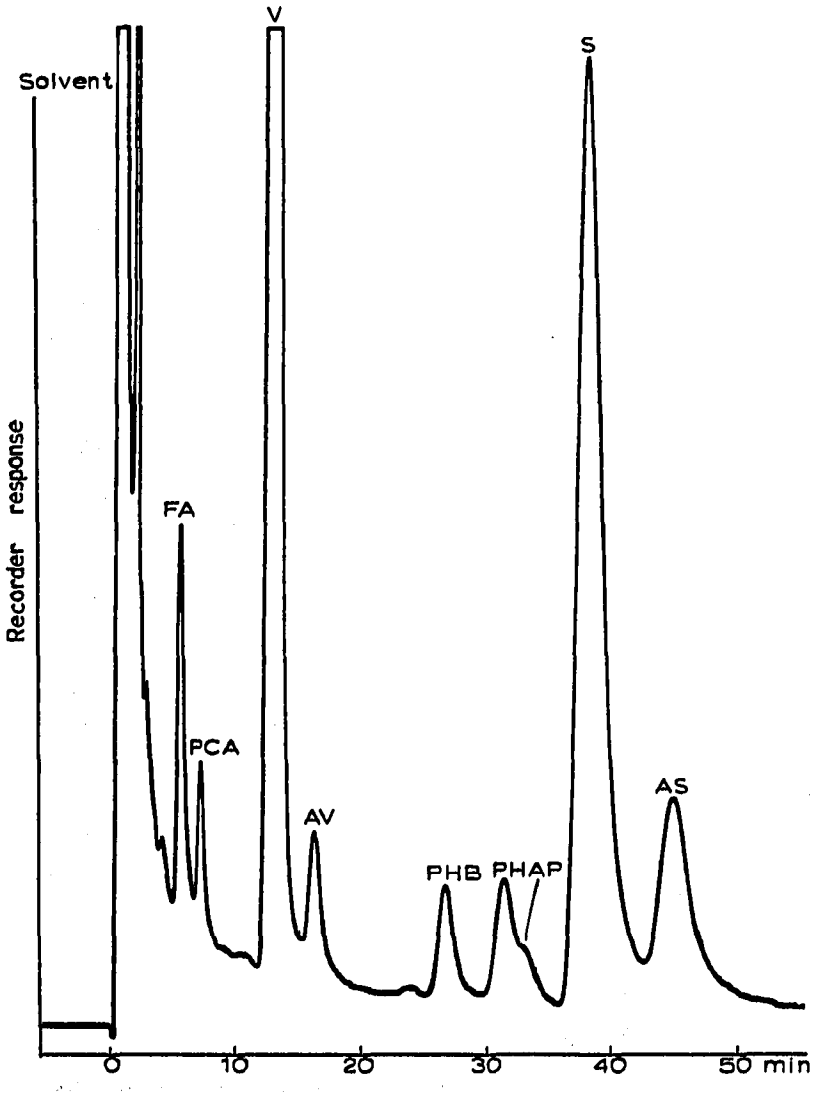

Fig. 4. Separation of the alkaline nitrobenzene oxidation products from alkali-extracted cell-wall material from grass sample 2 by GLC (conditions in text).

work is required to find out if, in some circumstances, PCA, FA and V can also arise from other sources in cell-wall materials.

The lignin core materials left after removal of the lignin ester groupings by sodium hydroxide-treatment, were examined by degradative oxidation followed by GLC determination of the products. The alkaline nitrobenzene[5] and alkaline cupric oxide[6] methods were employed with certain modifications. The reaction mixtures were continuously shaken and the oxidation products extracted with ether. Continuous solvent extraction was found to be unnecessary. The results for grass sample 2, together with a reference GLC separation of PHB, V, S, PHAP, AV and AS, are illustrated in Figs. 3–5 and summarised in Table I. Both methods of oxidation gave rise to two compounds which had the same retention times as PCA and FA on the CDMS column at either 190° or 220°. Further evidence for the presence of PCA and FA was obtained by TLC using Silica Gel G and three different solvent systems.

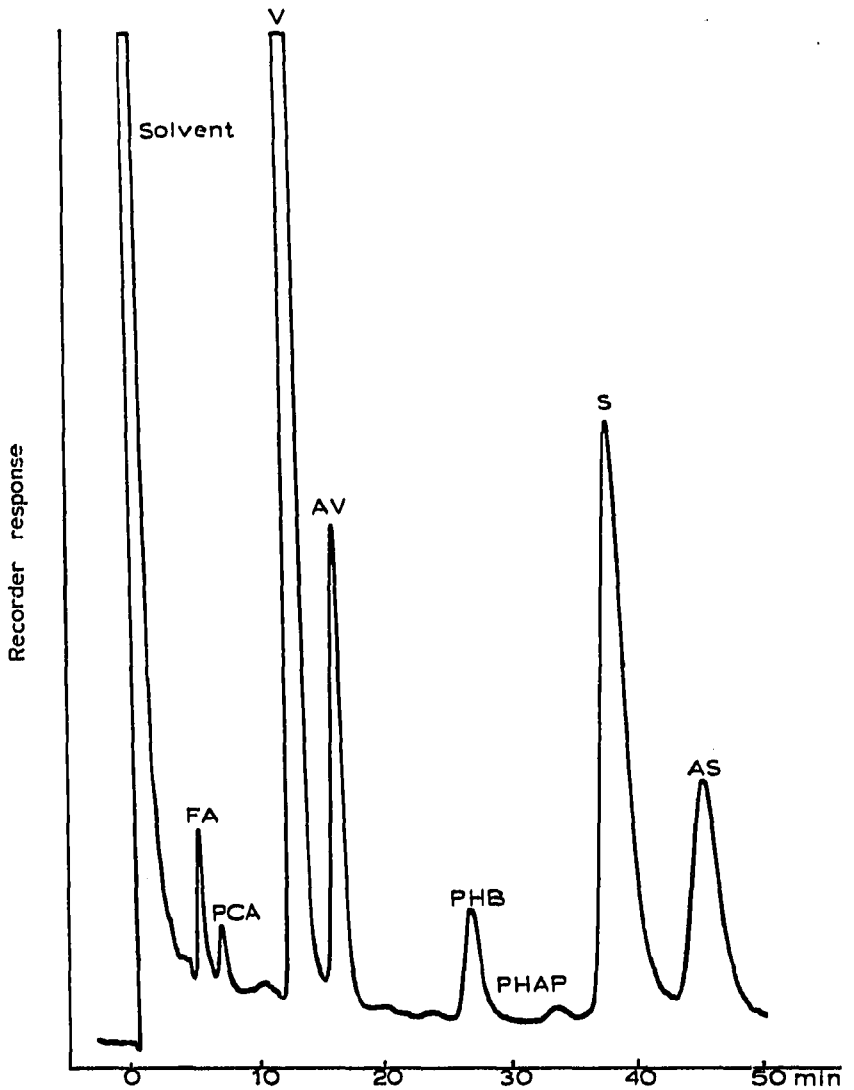

Fig. 5. Separation of the alkaline cupric oxide oxidation products from alkali-extracted cell-wall material from grass sample 2 by GLC (conditions in text).

Confirmation of the presence of all the major oxidation products was obtained by GLC–MS analysis and comparison with a similar analysis of a reference mixture containing the suspected compounds. PCA, FA and V gave similar mass spectra to those reported above. PHB and S were identified by their strong parent and M—1 peaks. AS and AV also gave strong parent peaks but the base peaks were M—15 due to loss of CH_3. The presence of PCA and FA in lignin oxidation products from maize and wood has been reported earlier[13,15] but this is apparently the first report of their occurrence in the products from grass lignin.

As shown in Table I, V and S were the main oxidation products, and the V/S ratio for the nitrobenzene oxidation was 1.49 compared with 1.89 for the cupric oxide reaction. Appreciable amounts of the corresponding ketones were obtained by both methods except that nitrobenzene gave only small quantities of AV. When alkali-treated grass sample 1 was subjected to alkaline nitrobenzene oxidation, V was again

the major product and, as expected, the V/S ratio (7.2) was considerably higher than for sample 2 which contained older plant material.

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