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A QUANTITATIVE GAS–LIQUID CHROMATOGRAPHIC DETERMINATION OF AROMATIC ALDEHYDES AND ACIDS FROM NITROBENZENE OXIDATION OF LIGNIN

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SUMMARY

A simplified gas–liquid chromatographic technique for the separation and quantitation of the major aromatic aldehydes and acids produced by the alkaline nitrobenzene oxidation of lignin has been described. Separation of *p*-hydroxybenzaldehyde, vanillin, syringealdehyde, *p*-coumaric acid, ferulic acid and four aromatic compounds appearing in low concentration has been achieved by a temperature programmed analysis using 5% OV-25 on Gas-Chrom Q as column packing. It was shown that the method can be applied to monitoring changes in the yield of phenolic compounds from the oxidation of isolated (Kraft) and *in situ* (corn plant) lignin which had been subjected to heat and digestive treatments.

INTRODUCTION

Alkaline nitrobenzene oxidation has been used in the elucidation of chemical composition of plant lignin[1]. Paper chromatography of the aromatic aldehydes and acids produced by the oxidation of plant lignin facilitated their quantitative determination[2]. REALE[3] introduced thin-layer chromatography to expediate this analysis. In our experience, however, both paper chromatography and thin-layer chromatography proved lengthy and were inconvenient for application to the routine analyses of the chemical composition of lignin oxidation products. Instead, it was decided to adapt the gas chromatographic methods of phenols and phenolic compounds[4–6] to the analysis of the alkaline nitrobenzene oxidation products of lignin. The use of the method has been extended to monitoring the changes which occur in lignin as a result of heat treatment and biodegradation of plant material.

EXPERIMENTAL

All compounds used were obtained commercially. Reference material included *p*-hydroxybenzaldehyde, vanillin, syringealdehyde, vanillic acid, *p*-hydroxybenzoic

acid, ferulic acid, *p*-coumaric acid, syringic acid and caffeic acid. Octyl ether was used as an internal standard since it was stable and had a retention time which did not interfere with the phenolic compounds. Tri-Sil (TMS) was purchased from Chromatographic Specialties Ltd. All solvents were redistilled and particular care was exercised in obtaining pure nitrobenzene.

Trimethylsilylation

The method of PELLIZZARI *et al.*[5] was used. Samples once prepared were immediately chromatographed.

Gas–liquid chromatography

Analyses were performed on a Hewlett-Packard Instrument (F & M 402) equipped with dual glass columns and a flame ionization detector. Glass columns were 4.0 ft. (3 mm I.D., 5 mm O.D.), packed with Gas-Chrom Q (80–100 mesh), coated with 5% OV-25 (Applied Science Co.). Various other packings were investigated in an attempt to obtain complete resolution of the aromatic compounds from lignin oxidation which were to be examined. 1–10% Carbowax 20 M/Chromosorb W and 2.5% OV-1/Chromosorb W packings gave serious peak tailing. 3% OV-1/Gas-Chrom Q and 3–5% OV-17/Gas-Chrom Q did not give complete resolution of peaks.

The flow rate of the carrier gas (nitrogen) was 80 ml/min. The injector port and detector temperatures were 240° and 225°. The column temperature was programmed between 120–210° at 2°/min. Peak areas were calculated by use of an integrator (Infotronics, Digital Readout System).

In order to facilitate the identification of the individual phenolic compounds in samples of unknown composition, the relative retention times of reference standards were used. Quantitative analysis of samples was performed by measuring peak areas of accurately weighed samples (2–6 mg) of reference standards using octyl ether as an internal standard.

In our experience the liquid phase maintained its efficiency for seventy-five injections. For this number of analyses the relationship of peak area to weight was not altered, and only a minimal decrease in retention times was observed.

Alkaline nitrobenzene oxidation of lignins

The procedure of GEE *et al.*[7] was used except the oxidation was carried out in sealed glass tubes instead of the stainless steel oxidation bombs because oxidation in the bombs led to the formation of extraneous compounds. Lignin (25–50 mg), nitrobenzene and 2.0 *N* NaOH were added to a 15 ml pyrex test tube; the test tube was sealed and placed in an oven at 160° for 3 h.

The oxidation products were purified by extracting nitrobenzene and its derivatives at a high pH (~13) with one washing of CH_2Cl_2. The solvent fraction was washed with 50 ml of 1 *N* NaOH. The combined sodium hydroxide layer and the original aqueous layer were acidified to pH 1 with 6 *N* HCl followed by extraction with 3 × 50 ml washings of CH_2Cl_2 and 2 × 50 ml washings of diethyl ether. The combined solvent fractions were dried over anhydrous sodium sulfate, reduced to a small volume on a rotary evaporator, then transferred to a 1 dr. vial and taken to near dryness (0.1–0.3 ml) on a sand bath (95°) under nitrogen. Care was taken to maintain an anhydrous sample. 1 ml of TMS was added. The sample was allowed to stand for

5 min, then immediately chromatographed. Recovery of reference standards was monitored for all extraction steps.

In vitro incubation

Ground corn stalk and leaf were extracted for 6 h each in ether and acetone; 10 g of the mixture were sterilized at 120° at 15 p.s.i. for 30 min in a 150 ml fermentation flask (construction of flasks is described elsewhere[8]) in the presence of 40 ml of culture medium[9]. To ascertain differences, if any, brought about in the composition of lignin oxidation products due to sterilization, another sample of the same corn material plus 40 ml of culture medium was gas sterilized at 40 ± 5° for 18 h. The sterile medium was inoculated with 80 ml of rumen fluid.

The standard incubation procedure included the bubbling of sterile carbon dioxide into the fermentation medium for 10 min. Subsequently, the head space was flushed continuously with sterile oxygen-free nitrogen. The gaseous effluent was passed through 0.1 *N* NaOH solution. The viability of microbial activity was monitored by recording electrical conductivity produced in an 0.1% NaOH solution. The pH of the incubation was maintained at 6.8 ± 0.5 during the entire 36 h incubation with appropriate base or acid addition. At the conclusion of the incubation, the medium was heated to 100° and the residue recovered by centrifugation and freeze-dried. The dry residue was subsequently extracted with DMF to obtain DMF lignin.

The Kraft lignin incubation medium consisted of 1 g of Kraft lignin, 20 ml of culture medium, and 40 ml of rumen fluid. To ascertain the effect of the sterilizations on the lignin, control incubations were carried out using heat deactivated rumen fluid. The rumen fluid was obtained from a fistulated steer, fed a maintenance diet of concentrate and forage.

In vivo incubation

The corn material was treated as above and 15 g were placed in each of 2 nylon bags made of 20 × 20 cm nylon. The bags were suspended for 36 h in the rumen of the above described animal. The residue was freeze-dried and extracted as before. Statistical analyses were performed according to STEEL AND TORRIE[10].

RESULTS

It has been shown that the major phenolic products of lignin oxidation with nitrobenzene in an alkali medium are *p*-hydroxybenzaldehyde, vanillin, syringealdehyde, *p*-coumaric acid, ferulic acid, and syringic acid[2]. Caffeic, *p*-hydroxybenzoic, and vanillic acid appear as minor products[2]. In addition some phenolic compounds accumulate in trace amounts[11].

The gas chromatographic method described has facilitated the resolution of all major and minor phenolic components of lignin oxidation by a single analysis (Fig. 1A). The recovery of pure standards was complete (Table I). Although results are only shown for one phenolic aldehyde and one phenolic acid, the observations were typical of all major compounds. The partial overlap of *p*-coumaric acid and syringic acid, and caffeic acid and ferulic acid did not appear to disturb the quantitative aspect of the analysis.

Changes in yield of aromatic compounds of 100 μg per g of lignin were detect-

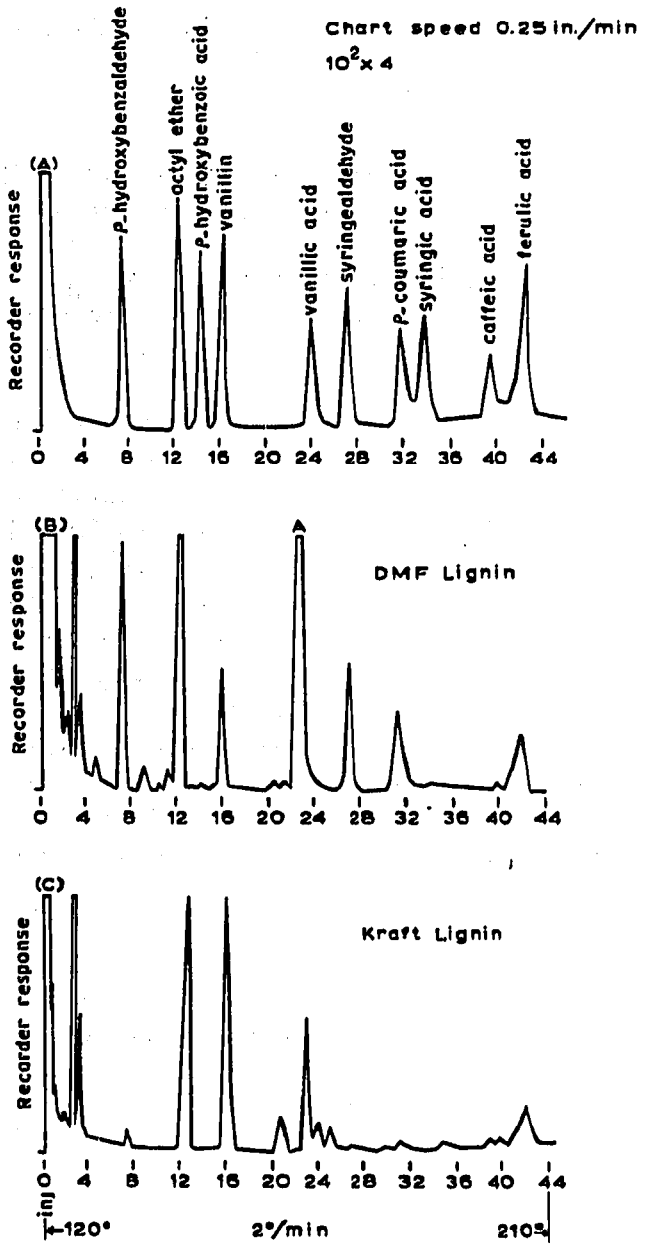

Fig. 1. (A) Chromatograms of an equimolar mixture of standard aromatic aldehydes and acids. (B) Typical chromatogram of alkaline nitrobenzene oxidation products of DMF lignin from a composite mixture of ground corn plant material. (C) Typical chromatograms of alkaline nitrobenzene oxidation products of Kraft lignin.

able by this method. The reproducibility of values between samples from the same lignin for individual components varied between 1–12.5%. Maximum accuracy was achieved when yield of components from the starting material was greater than 5 mg/g of lignin.

The chromatograms of the oxidation products of DMF and Kraft lignins are shown in Fig. 1B and C. Two unidentified peaks, one major (A) and one minor, were observed. The minor peak eluted between syringic acid and caffeic acid. Its concentration was decreased to trace levels by careful purification of the nitrobenzene. How-

TABLE I

RECOVERY OF VANILLIN AND *p*-COUMARIC ACID

Compound	*Amount added (mg)*	*Amount recovered (mg)*	*% recovery*
Vanillin	2.40	2.37	98.9
	3.54	3.59	101.4
	2.75	2.69	97.8
		Av.	99.3
p-Coumaric acid	4.22	4.19	99.3
	4.41	4.38	99.3
	3.55	3.555	100.1
	3.18	3.13	98.4
		Av.	99.3

ever, peak A was always present and probably is a reaction product of nitrobenzene. Neither compound interferes with the analysis of the major phenolic compounds from the oxidation of lignin. Only at high concentrations of compound A was interference in resolution of the vanillic acid observed.

Preliminary observations showed that the recovery of Kraft lignin was reduced by sterilization treatment. However, little if any additional lignin solution occurred during *in vitro* incubation in rumen fluid. The yield of DMF lignin from high temperature sterilized corn was marginally increased. Low temperature sterilization did not appear to affect the yield of DMF lignin. Marginal decreases in DMF lignin are associated with *in vitro* digestion. The recovery of DMF lignin from the *in vivo* digested corn material was reduced. The complete results will be presented in a subsequent paper.

Concentrations of the major phenolic products from the oxidation of the DMF lignins are shown in Table II. Quantitative comparison was not valid with results obtained by others due to the variability of the lignin in the starting material[12–14]. However the orders of magnitude and concentration for the phenolic aldehydes of DMF lignin from the composite mixture of corn leaf and stalk were similar to those reported by GEE *et al.*[7] for corn stalk. The higher absolute amounts of phenolic aldehydes in corn stalk probably reflect the greater concentration of lignin in this material.

The composition of the oxidation products from Kraft lignin was altered by treatment (Fig. 2A). The sensitivity of the method renders the changes in vanillin, syringealdehyde, *p*-coumaric acid and ferulic acid yield between original and treated

TABLE II

YIELDS OF MAJOR PHENOLIC OXIDATION PRODUCTS OF UNTREATED KRAFT AND DMF LIGNIN

Lignin	*Aromatic reaction products*				
	p-Hydroxy-benzaldehyde	*Vanillin*	*Syringe-aldehyde*	*p-Coumaric acid*	*Ferulic acid*
Kraft	1.0 ± 0.12	69.8 ± 2.34	5.8 ± 0.69	1.6 ± 0.20	3.0 ± 0.31
DMF	22.4 ± 2.68	27.8 ± 2.57	33.7 ± 2.11	21.6 ± 2.83	10.6 ± 0.46

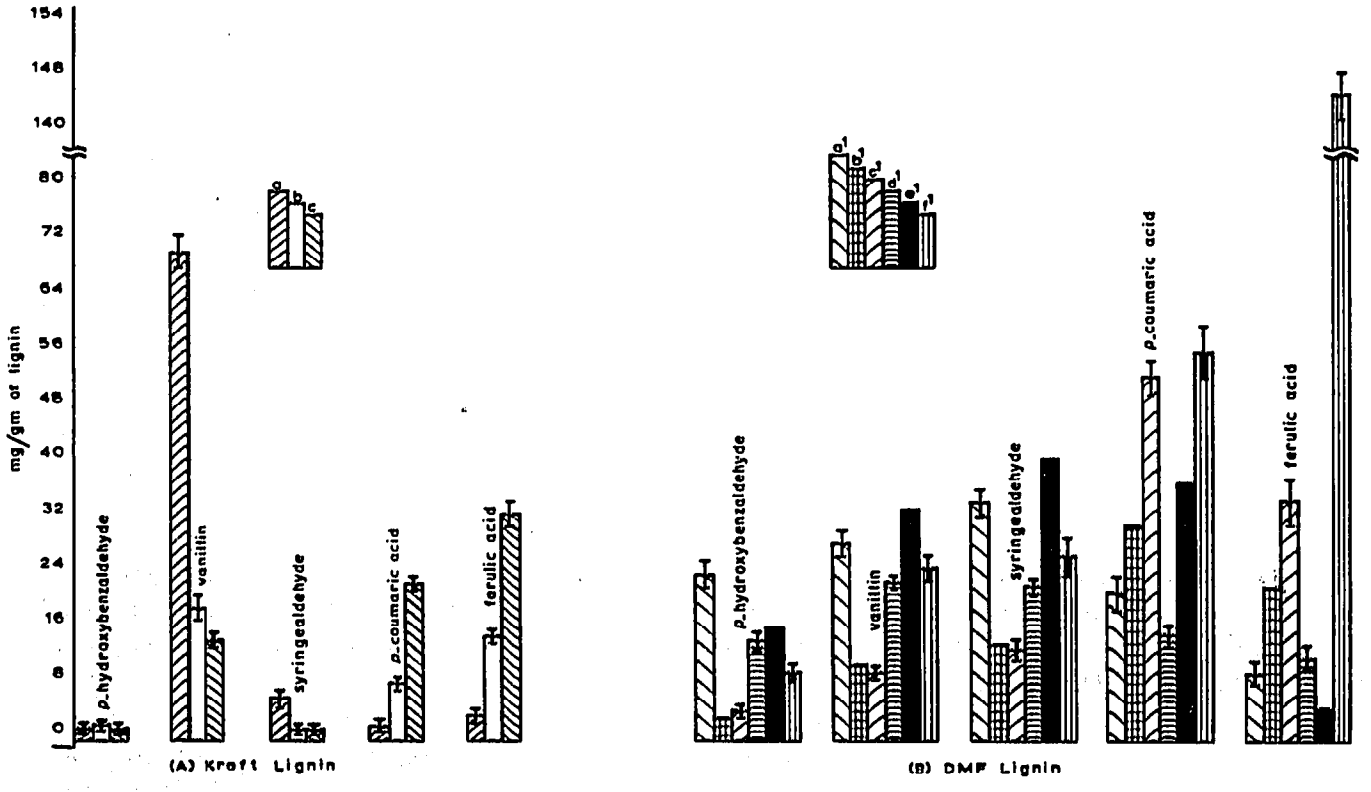

Fig. 2. (A) Effect of treatment on the yield of the five major aromatic oxidation products from Kraft lignin. Explanation of key: a, untreated; b, sterilized at 120°, 15 p.s.i.; c, *in vitro* digestion of high temperature sterilized Kraft lignin in rumen fluid for 36 h. (B) Effect of treatment on the yield of five major aromatic oxidation products from DMF lignin from corn plant. Explanation of key: a^1, untreated; b^1, sterilized at 120°, 15 p.s.i.; c^1, *in vitro* digestion of b^1 in rumen fluid for 36 h; d^1, gas sterilized at 40 ± 5° for 18 h; e^1, *in vitro* digestion of d^1 in rumen fluid for 36 h; f^1, *in vivo* suspension in the rumen for 36 h. I = standard deviation; where not shown insufficient sample was obtained to ascertain significance.

material highly significant. The differences in yield between treatments (b) and (c) are significant only for *p*-coumaric acid and ferulic acid. The most prominent change was a loss in yield of vanillin of 73.4% as a result of sterilization and 79.2% in consequence of sterilization and digestion. Both phenolic acids increased in concentration.

The analysis of the DMF lignin from corn plant which had been subjected to high and low heat sterilization, *in vitro* incubation in rumen fluid, and *in vivo* suspension in the rumen are given in Fig. 2B. Insufficient material was available to establish the significance of the changes in yield of phenolic compounds due to *in vitro* treatments. High temperature sterilization resulted in the reduction of phenolic aldehydes and an increase in phenolic acids. Low temperature sterilization brought about the same pattern of changes in phenolic products but the magnitude of change was smaller. *In vitro* digestion of high temperature sterilized corn material resulted in only minor, insignificant changes in yield of phenolic compounds. *In vitro* digestion of low temperature sterilized corn material was associated with an increase in all phenolic aldehydes and *p*-coumaric acid relative to sterilized material. The yield of vanillin, syringealdehyde and *p*-coumaric acid was also increased relative to the starting material. Comparison of the changes in the yield of phenolic products induced by indi-

vidual treatments suggests that the more severe the pre-digestive treatment, the lower the effects of digestion in an *in vitro* system.

Large differences in yield of phenolic compounds were observed as a result of the *in vivo* treatment of corn material. The sensitivity of the method has rendered the changes in *p*-hydroxybenzaldehyde, syringealdehyde, *p*-coumaric acid and ferulic acid yield between starting material and treated material highly significant. The changes were manifested by a decrease in phenolic aldehydes and increases in both phenolic acids. The ferulic acid yield increased by an order of magnitude.

DISCUSSION

Although lignin is believed to be resistant to the digestive process of the ruminant animal there is evidence that changes occur in the lignin during digestion of forages[15] and under some circumstances lignin itself is digested[16–17]. These studies on the effects of digestion on lignin have largely been based on data obtained by proximate analyses[18,19]. These methods define lignin as a residue resistant to certain chemical treatments and furthermore these methods do not permit the examination of the polymer itself. Therefore, although it is well established that the lignin polymer from different sources varies in composition[2,12], no systematic study of the relationship between lignin structure and digestive alteration has been possible. Most attempts to correlate lignin structure with breakdown have been conducted using model lignin compounds[20,21]. This method, however, has obvious limitations.

The method described here lends itself to an evaluation of chemical composition of lignin possibly irrespective of its method of preparation and permits the evaluation of chemical changes within the lignin polymer brought about by treatments. It is applicable to the characterization of treatment effects on the chemical composition of either isolated lignin or *in situ* lignin.

The application of the method in evaluating the digestion of forages with lignin of varying chemical composition will be reported in a subsequent paper.

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