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ANALYSIS OF THE PRODUCTS OF THE OXIDATION OF LIGNIN BY CuO IN BIOLOGICAL AND GEOLOGICAL SAMPLES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

A reversed-phase high-performance liquid chromatographic procedure is described for the resolution and quantitation of the phenolic aldehydes, ketones and acids produced from the alkaline oxidation of lignin with CuO. This technique was applied successfully to the detection of lignin degradation products in a variety of plant and geological samples. The results are in excellent agreement with those of the capillary gas chromatographic method of Hedges and Mann [*Geochim. Cosmochim. Acta*, 43 (1979) 1803].

INTRODUCTION

The alkaline oxidation of the tissues of vascular plants with CuO or nitrobenzene produces a series of phenolic aldehydes, ketones and acids (Fig. 1[1–3]). The distribution of the compounds serves as a taxonomic indicator of vascular plant class. The oxidation products of lignin can be used to characterize organic matter in recent geological deposits[4–7]. Apparently, the highly cross-linked phenolic network of lignin may survive for long periods of geological history[8,9]. The alkaline oxidation of sediments that contain detrital material originating from vascular land plants with CuO results in the release of the phenolic compounds. The distribution pattern of these compounds can be used as an index of the vascular plant types in the sedimentary record and as a measure of the relative contribution of vascular plant detritus to the total organic carbon of the sediment. Gas chromatography has been applied to the determination of several aldehydes and ketones released by oxidation of lignin with CuO or nitrobenzene[4,8–11]. Gas chromatography of trimethylsilyl derivatives has also

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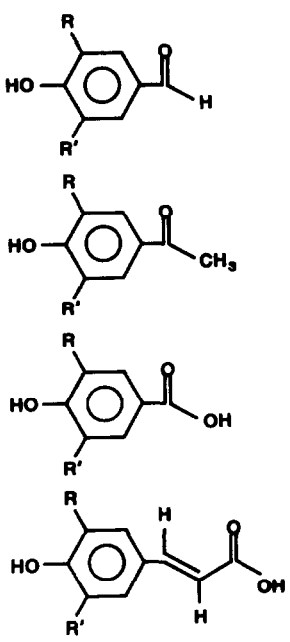

R = H, R' = H	R = H, R' = OCH_3	R = OCH_3, R' = OCH_3
p-OH-Benzaldehyde	Vanillin	Syringaldehyde
p-OH-Acetophenone	Acetovanillone	Acetosyringone
p-OH-Benzoic Acid	Vanillic Acid	Syringic Acid
(p-Coumaric Acid)	Ferulic Acid	----

Fig. 1. Eleven index phenols resulting from the oxidation of lignin with CuO.

been used for the quantitation of aldehydes, acids and ketones produced by alkaline oxidation of lignin with nitrobenzene or CuO[12,13].

High-performance liquid chromatography (HPLC) is also a powerful tool for the separation and identification of phenolic compounds[14]. Reversed-phase chromatography has been used for the analysis of phenolic acids and aldehydes in plant tissue extracts[15–18] and soil extracts[18]. Villeneuve *et al.*[17] described a procedure for the determination of the phenolic aldehydes produced by the oxidation of lignin with nitrobenzene. The reversed-phase procedure we introduce here differs from previous work in that the aldehydes, acids and ketones produced by alkaline oxidation of lignin with CuO are separated in a single chromatogram. This procedure is rapid and requires little sample purification prior to chromatography. The introduction of liquid chromatography constitutes a considerable simplification of the lignin analytical procedure. Using this technique, the rapid analysis of the eleven index phenols, defined by Hedges and Parker[5] (Fig. 1) in the lignin of vascular plant tissues and geological materials such as soils, peats, coals and sediments, has been achieved.

EXPERIMENTAL

Materials

Dichloromethane and methanol were of analytical-reagent grade (Fisher) and distilled in an all-glass still before use. Analytical-reagent grade diethyl ether (Burdick & Jackson) was stored over iron(II) ammonium sulfate [$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$] in order to minimize peroxide formation, and then used without further purification. Distilled water was purchased from Burdick & Jackson. HCl (12 *M*) (Baker) was

diluted to 6 *M* and then distilled twice in an all-glass still before use. Phenol standards were obtained from Aldrich and were of 98% purity or better. All other chemicals used were of analytical-reagent grade.

Samples

Marine and terrestrial sediment samples, coal and peat were extracted with methanol and then CH_2Cl_2 (or a mixture of CH_2Cl_2 and *n*-hexane) in order to remove lipid material. The extracted sediments were then air-dried and processed as described below. Kerogen was isolated by the method of Stuermer *et al.*[19]. Wood samples were processed as follows. After the bark had been removed, wood samples were extracted with *n*-hexane–CH_2Cl_2 (6:1) using ultrasound and then dried at room temperature overnight and finally in a sand-bath at approximately 45°C for 15 min.

Oxidation

All oxidation reactions are carried out in custom-made screw-cap stainless-steel vessels of total volume approximately 20 ml. A water vapor-tight seal is achieved with a PTFE O-ring.

The reaction conditions used are similar to those in previous work[1,5,7]. Sediment ($\leqslant$ 2 g) or wood ($\leqslant$ 30 mg) is mixed with 1.0 g of CuO and 0.1 g of $Fe(NH_4)_4(SO_4)_2 \cdot 6H_2O$ in the stainless-steel reaction vessel. NaOH (2 *M*) is stripped with N_2, while sonicating, to remove O_2. An 8-ml volume of this NaOH is added to the reaction vessel and the ultrasonication and N_2 bubbling are continued for an additional 10 min. Methanol (approximately 0.5 ml) can be added in order to minimize sample foaming. Following this, the vessel is sealed and heated at 170°C, in a convective air oven, for 3 h.

After heating, the sample is allowed to cool to room temperature before the reaction vessel is opened. The entire liquid phase is transferred to a Buchner funnel with a coarse glass frit. The sample is filtered under vacuum. An additional 8 ml of 2 *M* NaOH are added to the solid phase, which is then homogenized with ultrasound. The entire contents of the reaction vessel are then transferred to the Buchner funnel using a stainless-steel scoop and several 1-ml water washes.

The filtrate is transferred into a 40-ml conical centrifuge tube and the still alkaline sample is extracted by shaking with 20 ml of CH_2Cl_2. The sample is centrifuged at 2000 rpm to aid phase separation. The CH_2Cl_2 layer is discarded and the sample is acidified with 5–6 ml of 6 *M* HCl. The sample is then extracted three times by shaking with 15 ml of diethyl ether in a centrifuge tube and the centrifugation is used to separate the phases. The ether extracts are combined and rotary evaporated to dryness. The residue is dissolved in methanol to give a total volume of 2 ml. The sample is stored at −20°C until taken for analysis by HPLC. Samples should be analyzed within 3 days as prolonged storage results in the loss of aldehydes.

HPLC procedure

All separations were performed on an instrument consisting of two Model 6000A solvent delivery systems, a Model 660 solvent programmer and a UK6 sample injection valve, all from Waters Assoc. A C_{18} (5 μm) column (25 × 0.5 cm I.D.) from Alltech was used. The column effluent was monitored at 280 nm using an Altex Model 155-01 UV–visible detector with the sensitivity set at 0.02 absorption units. The peak areas were recorded on a Waters Assoc. Model 720 data module.

The mobile phase was a gradient mixture of 0.086% H_3PO_4 (A) and methanol–acetonitrile (1:1) (B). The mobile phase was saturated with silica gel by inserting an 25 × 0.5 cm I.D. column of Whatman pre-column silica gel between the pumps and the sample injection valve.

The C_{18} column was equilibrated with an A–B mixture (95:5). A 5–25-μl aliquot of the sample was injected and the solvent program was immediately initiated. The 40-min curvilinear gradient from 95:5 A–B to 80:20 A–B is illustrated in Fig. 2.

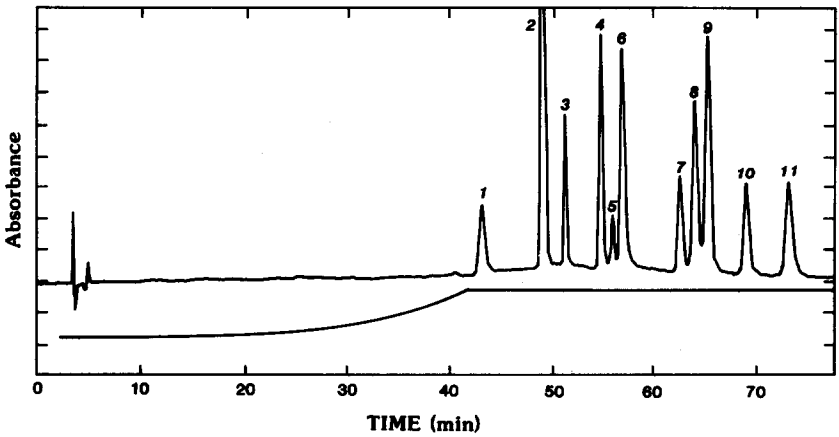

Fig. 2. Reversed-phase chromatogram showing the separation of eleven index phenols. Peaks: 1 = *p*-hydroxybenzoic acid; 2 = *p*-hydroxybenzaldehyde; 3 = vanillic acid; 4 = syringic acid; 5 = *p*-hydroxyacetophenone; 6 = acetovanillone; 7 = syringaldehyde; 8 = acetovanillone; 9 = *p*-coumaric acid; 10 = acetosyringone; 11 = ferulic acid. The solvent program is shown underneath the chromatogram; this curve is offset by 2.6 min from the chromatogram trace.

RESULTS

A chromatogram illustrating the resolution of all eleven index phenols is presented in Fig. 2. The phenols were quantified by peak area integration, the peak area being linearly related to concentration. Response factors were determined by analysis of standard mixtures and were reproducible to within 5%. The accuracy of this technique is maintained by frequent standardization, standard injections being made each day. Phenol quantitation by this technique has a maximum error of 10%.

Fig. 3 is a representative chromatogram resulting from the analysis of a marine sediment sample from the continental shelf off the state of Washington. The sediment sample was generously provided by Dr. J. I. Hedges, and was previously analyzed for lignin products in his laboratory.

Table I gives the resulting concentration of index phenols from several samples. Duplicate analyses of the sediment sample from Washington are presented together with the analytical data provided by Dr. J. I. Hedges.

Recovery experiments were conducted by spiking 1 g of a lignin-free sediment from the Antarctic region with 3–10-mg amounts of aldehydes, ketones and acids. The sediments were then oxidized and processed as described above. The average recoveries for the three aldehydes and three ketones are reported in Table II. Recoveries for the aldehydes, ketones and acids were generally higher than 85%, with

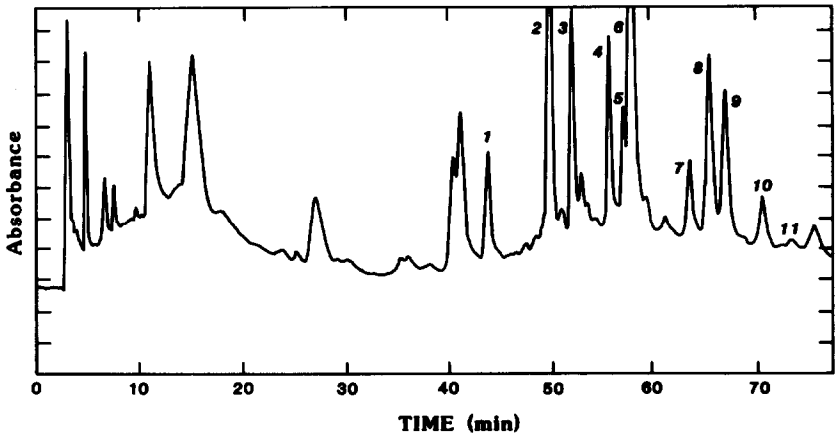

Fig. 3. Chromatogram of the products of the alkaline oxidation of lignin from Washington coastal sediment. Peaks as in Fig. 2.

the exception of acetosyringone, syringic acid, *p*-hydroxycinnamic acid and ferulic acid, for which the recoveries were 72, 67, 57 and 25%, respectively. The detection limits for the index phenols are given in Table III.

DISCUSSION

All eleven index phenols produced by CuO oxidation are resolved in less than 80 min using this chromatographic procedure. The acidic aqueous phase (A) was required in order to prevent severe tailing of these compounds on the Alltech reversed-phase column. In phosphate buffers of pH 4.5 and 6.8, most of the compounds analyzed produced highly asymmetric peaks. The pre-column used to saturate the mobile phase with silica prevents deterioration of the reversed-phase column from exposure to the low pH eluent. The organic modifier (B) was selected by trial and error. Methanol, added to the organic modifier, improved the selectivity of the reversed-phase column. The sensitivity was also enhanced by methanol. However, the use of 100% methanol as an organic modifier resulted in an excessive column pressure and greatly increased retention of the compounds. A 1:1 mixture of methanol and acetonitrile appeared to be optimal for this Alltech column. The optimal composition may vary with the stationary-phase manufacturer.

The results in Table I demonstrate that this procedure is applicable to a wide variety of sample types. Plant tissue, soil, recent marine sediments, peats and coals gave similar quality chromatograms. The procedure is extremely sensitive and, for example, can detect as little as 1 ng/g of vanillin.

Index phenol concentrations for several plant tissues are presented in Table I. Wherever the same measurements had also made by Hedges and co-workers[1,7], their results are presented in parentheses below our data. For *Pinus caribeae* wood and *Thuja plicata* leaves, the sum of the values of vanillin, acetovanillone and vanillic acid and that of syringaldehyde, acetosyringone and syringic acid are lower, but are present in the same relative proportions as given by Hedges and co-workers[1,7]. The

TABLE I

CONCENTRATIONS OF LIGNIN INDEX PHENOLS IN VARIOUS PLANT AND GEOLOGICAL SAMPLES (mg/100 mg ORGANIC CARBON)

P-ALD = *p*-hydroxybenzaldehyde; P-KET = *p*-hydroxyacetophenone; P-ACD = *p*-hydroxybenzoic acid; V-ALD = vanillin; V-KET = acetovanillone; V-ACD = vanillic acid; S-ALD = syringaldehyde; S-KET = acetosyringone; S-ACD = syringic acid; C = *p*-coumaric acid; F = ferulic acid; P-TOT = *p*-hydroxybenzaldehyde + *p*-hydroxyacetophenone + *p*-hydroxybenzoic acid; V-TOT = vanillin + acetovanillone + vanillic acid; S-TOT = syringaldehyde + acetosyringone + syringic acid.

Sample	*P-ALD*	*P-KET*	*P-ACD*	*V-ALD*	*V-KET*	*V-ACD*	*S-ALD*	*S-KET*	*S-ACD*	*C*	*F*	*P-TOT*	*V-TOT*	*S-TOT*
Pinus caribeae (Slash pine) wood	0.830	0.087	0.259	7.386	1.273	1.273	0.004	–	–	0.011 (0)*	0.052 (0)*	1.176	9.932 (13.0)*	0.004 (0)*
Thuja plicata (Red Cedar) leaves	0.129	0.175	0.207	1.067	0.113	0.215	0.027	–	0.007	0.195 (0.35)*	0.061 (0.70)*	0.511	1.395 (2.0)*	0.027 (0.04)*
Pheonix roebelenii (Pigmy Date Palm) wood	0.695	0.122	1.350	3.687	0.540	1.024	1.509	0.695	0.375	0.261	0	2.167	5.251	2.579
Kashmir (India) lignitic coal	0.104	0.022	0.088	0.054	0.048	0.075	0.084	0.071	0.044	0.027	0.012	0.214	0.177	0.199
Hula peat 154 (Israel)	0.462	0.206	0.358	0.989	0.280	0.892	0.442	0.892	0.196	0.236	0.363	0.157	1.026	1.711
Staten Island (California) deltaic peat	0.145	0.014	0.196	0.251	0.121	0.272	0.217	0.106	0.099	0.157	0.114	0.355	0.644	0.422
Tanner Basin (California) marine kerogen	0.006	0.001	0.023	0.006	0.001	0.019	0.001	–	0.002	0.004	–	0.029	0.026	0.002
Washington coastal sediment (1)	0.094	0.023	0.146	0.219	0.098	0.194	0.103	0.058	0.071	0.061	0.033	0.273	0.571	0.232
Washington coastal sediment (2)	0.115	0.030	0.133	0.244	0.144	0.198	0.125	0.056	0.064	0.063	0.031	0.278	0.556	0.245
Washington coastal sediment (Hedges lab.)	0.108 ± 0.039	0.036 ± 0.023	0.115 ± 0.024	0.373 ± 0.084	0.119 ± 0.028	0.150 ± 0.034	0.169 ± 0.045	0.060 ± 0.015	0.049 ± 0.014	0.060 ± 0.009	0.059 ± 0.013	0.259 ± 0.051	0.642 ± 0.096	0.278 ± 0.049

* Data in parentheses from ref. 1.

TABLE II

RECOVERY OF INDEX PHENOLS FROM A SPIKED LIGNIN-FREE SEDIMENT SAMPLE

Compound	*Recovery (%)*
p-Hydroxybenzaldehyde	86 ± 5
p-Hydroxyacetophenone	85 ± 3
p-Hydroxybenzoic acid	94 ± 11
Vanillin	91 ± 6
Acetovanillone	101 ± 10
Vanillic acid	92 ± 9
Syringaldehyde	97 ± 11
Acetosyringone	72 ± 6
Syringic acid	67 ± 7
p-Coumaric acid	57 ± 7
Ferulic acid	27 ± 2

TABLE III

DETECTION LIMITS FOR INDEX PHENOLS

Compound	*Detection limit (ng/g)*
p-Hydroxybenzaldehyde	0.5
p-Hydroxyacetophenone	0.7
p-Hydroxybenzoic acid	2.6
Vanillin	1.0
Acetovanillone	1.0
Vanillic acid	2.4
Syringaldehyde	2.6
Acetosyringone	1.9
Syringic acid	1.1
p-Coumaric acid	1.0
Ferulic acid	1.6

very low levels of *p*-coumaric acid and ferulic acid found in *Pinus caribeae* are consistent with the data of Hedges and Parker[5]. In *Thuja plicata* leaves, our *p*-coumaric acid value is in similar proportion, relative to the sum of vanillin, acetovanillone and vanillic acid, to that reported by Hedges and Mann[1]. However, our ferulic acid value for *Thuja plicata* is considerably lower. Hedges and co-workers[1,7] corrected their concentrations for non-quantitative recovery. This correction should account for their higher values for the sum of syringaldehyde, acetosyringone and syringic acid, the sum of vanillin, acetovanillone and vanillic acid, and *p*-coumaric acid. Our low ferulic acid value for *Thuja plicata* is due to our low recoveries of ferulic acid.

Comparison of our results for the Washington coastal sediment provided by Dr. J. I. Hedges is favorable. Many of the observed differences in the proportions of the aldehydes, ketones and acids can probably be ascribed to differences in the oxidation conditions used in the analytical procedures. Hedges and co-workers[1,7] employed constant agitation during heating whereas we did not. Hedges and co-workers[1,7] used a single ^{14}C-labeled tracer (*p*-hydroxyacetophenone) to correct for non-

quantitative recoveries. Because our recoveries varied for each compound, we believe that a uniform correction is not justified. In addition, because compound recoveries may also vary with the sample type, we have not used the results of our spiking experiments to correct for non-quantitative recoveries. We suggest that spiking experiments be performed for each sample type analyzed, before the measured concentrations of phenolic compounds are corrected for incomplete recovery.

The recovery experiments reported here demonstrate that the phenolic compounds are stable under the above experimental conditions and that our extraction procedure efficiently separates many of the important phenols from the oxidation products of the reaction mixture.

CONCLUSIONS

A reversed-phase HPLC procedure has been presented for the analysis of the phenolic aldehydes, ketones and acids produced by oxidation of lignin with CuO. The procedure has been applied successfully to a variety of sample types. This procedure is sensitive, rapid, and eliminates the necessity of preparing the volatile derivatives required for gas chromatography.

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