6-1982

Liquid Chromatographic Studies of Polar Aromatic Compounds in Soil

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LIQUID CHROMATOGRAPHIC STUDIES OF POLAR AROMATIC COMPOUNDS IN SOIL

by

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Submitted in partial fulfillment
of the requirements for
Honors in the Department of Chemistry

UNION COLLEGE

March, 1982

ABSTRACT

GREEN, JONATHAN Liquid Chromatographic Studies of Polar Aromatic Compounds in Soil. Department of Chemistry, March, 1982.

The purpose of this project is to identify a component or components in red pine tree soil which may be associated with fungus growth inhibition.

Seven similar aromatic acids, purchased in their "pure" form from a chemical supplier, were dissolved in 95% ethanol for chromatographic separation. Using reversed-phase High Performance Liquid Chromatography (HPLC) and linear gradient elution, the acids were separated while monitoring UV absorbance at 280 nm and 254 nm, and where possible, at 320 nm and 254 nm. Peak absorbance, absorbance ratios, peak areas, column selectivities, and column capacity factors were measured or calculated from the chromatograms.

The gradient program devised for the standard solution provides excellent resolution in a relatively short time, thereby fulfilling the desired requirements for an optimum chromatographic separation. Hopefully, this separation scheme can later be applied to a soil sample extract of unknown composition.

An extraction of phenolic acids from red pine tree soil was carried out, yielding minimal quantities of the desired compounds.

ACKNOWLEDGEMENT

First and foremost I would like to thank the person who is most responsible for my decision to become a chemist -- Richard M. Whitney. He taught me to think (very much against my will), and he turned me on to chemistry. I owe this to him. The undying encouragement and assistance provided by Professors Werner and Schaefer are also gratefully acknowledged and appreciated.

Jonathan S. Green

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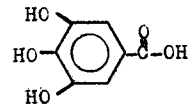
STRUCTURES OF COMPOUNDS USED



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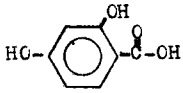

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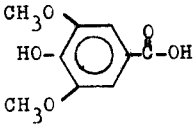

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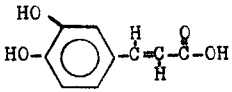

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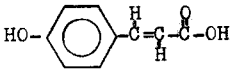

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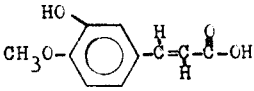

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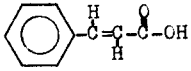
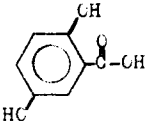


Cinnamic Acid



Gentisic Acid

INTRODUCTION

In the 1920's and 1930's, nearly half a million acres of pine plantations were planted in New York State, mainly to reclaim abandoned and eroding farmland.[1]

In examination of one such plantation, the E. N. Huyck Preserve in Rensselaerville, N. Y., differences were noted in the rate of hardwood seedling establishment beneath Scotch pine, Pinus sylvestris, and red pine, Pinus resinosa.[1] The seedling concentration beneath the red pines is far exceeded by that beneath the Scotch pines, so various analyses have been used to determine the cause of the seedling suppression.[1]

Light intensity was measured at assorted points beneath both types of pine, litter samples were collected, and soil samples were collected for chemical analysis of pH, organic matter, magnesium, phosphorous, potassium, nitrates, and nitrogen. Chemical analyses were also carried out on leaves of field grown seedlings, checking for chlorophyll content as well as the abovementioned chemicals.[1] In each case, the evidence suggested the reverse of the existing situation; for example, the light intensity was greater under tne red pines than under the Scotch, suggesting greater seedling growing ability beneath the red pines.[1]

However, upon analysis of the concentrations of magnesium, phosphorous, potassium, nitrates, and nitrogen

in the leaves of seedlings from each side of the plantation, it was found that those beneath the red pines had very low leaf concentrations of the essential chemicals.[1] This is surprising, considering the high soil concentrations of the same chemicals. The obvious implication is that something is limiting the ability of the plants to absorb these chemicals from the soil.

The fungus Endomycorrhiza has long been associated with nitrogen and phosphorous uptake for hardwood seedlings.[2-5] The mycorrhizal was found to colonize with the roots of seedlings beneath the Scotch pines, thereby aiding in the conversion of nitrogen and phosphorous to forms which are usable by the plants. However, there was no colonization observed with first-year seedlings beneath the red pines. This would explain the fact that even with sufficient concentrations of N and P in the soil, the plant leaves are deficient in these chemicals.

The lack of colonization does explain the lack of plant growth, but an explanation must be found for the lack of colonization to begin with. The assumption on which this project is based is that the lack of mycorrhizal colonization can be attributed to some type of chemical growth inhibition associated with the red pines.

Several aromatic acids (see Experimental) have long been associated with fungus growth inhibition,[6-11]

and a group of eight of the most common ones have been selected for analysis. The method to be used for analysis of these acids is reversed-phase High Performance Liquid Chromatography (HPLC).

The initial objective of the project is to use reversed-phase HPLC to obtain a separation of a mixture of the eight acids in water solution. In order to devise a separation scheme, the optimum elution and detection conditions must be established for solutions of each acid individually. Once conditions have been optimized for each individual acid, a mixture of all eight can be made, and the technique of gradient elution used to obtain maximum resolution in a minimum amount of time.

By first carrying out a separation of known components of known concentrations, a move to analysis of real soil samples should be fairly straightforward. A literature search has turned up a proven method for extraction of the acids from soil, and this method has been used with success.[9] Once an extraction has been completed, the previously developed HPLC separation will be used to look for significant differences in the levels of these acids in red pine and Scotch pine soil. It is hoped that, eventually, a component or components can be isolated and identified, and studied for mychorrhizal growth inhibition.

EXPERIMENTAL

A. Instrumentation:

The following instruments were used in this project: A Varian Cary 118 Spectrophotometer, Varian Model 5020 High Performance Liquid Chromatograph (HPLC) with a 30 cm MicroPak MCH-10 reversed-phase column, Tracor 970A Variable Wavelength Detector, and a Linear chart recorder.

B. Chemicals:

1. See Tables 1 and 2.

2. Purification of Caffeic Acid:

In early chromatograms, caffeic acid yielded a very broad, asymmetric peak. This was assumed to be the result of some impurities present in the orginal stock of caffeic acid. In an attempt to remove the impurities, the following procedure was used:
Dissolve impure acid in 1F $NaHCO_3$. Filter solution and discard residue. Acidify the filtrate with HCl. Filter the solution and discard filtrate. Dissolve the residue in hot water. Filter, save residue and put it aside. Recrystallize from the filtrate, in an ice bath. Filter, save residue - these are pure caffeic acid crystals.

C. Extraction of Phenolic Acids from Soil:

In order to extract the phenolic acids from soil for HPLC analysis, the following procedure was adapted from that used by M. A. K. Lodhi[9] for extraction of a

TABLE 1

Chemicals, Source, and Purity

Chemical	Source	Purity
Caffeic Acid	Aldrich	99+%
Cinnamic Acid	Aldrich	97%
P-Coumaric Acid	Aldrich	98%
Ferrulic Acid	Aldrich	99%
Gallic Acid	Aldrich	-
Gentisic Acid	Aldrich	99%
β-Resorcylic Acid	Aldrich	97%
Syringic Acid	Aldrich	97%
1-Heptanesulfonic Acid, Sodium Salt	Aldrich	98%
Acetic Acid	Alfa Products	Ultrapure
HPLC-grade Water	Burdick and Jackson	-
Acetonitrile	Burdick and Jackson	-

* * * * *

TABLE 2

Acids, Molecular Weights, and IUPAC names

Acid	Mol. Wt. (g/mole)	IUPAC name
Gallic	170.12	3,4,5-trihydroxybenzoic acid (b1919)[a]
Gentisic	154.12	2,5-dihydroxybenzoic acid (b1551)
β-Resorcylic	194.19	4-hydroxy-3-methoxy cinnamic acid (c400)
Syringic	198.17	3,5-dimethoxy-4-hydroxy benzoic acid (b1577)
Caffeic	180.17	3,4-dihydroxycinnamic acid (c386)
p-Coumaric	164.16	p-4-hydroxy-trans-cinnamic acid (c399)
Ferulic	194.19	4-hydroxy-3-methoxy cinnamic acid (c400)
Cinnamic	148.16	cinnamic acid (c350)

* * * * *

a. The number in parentheses which follows the IUPAC name is the reference number used in the 60th Edition CRC Handbook of Chemistry and Physics.

similar set of acids:

Place 75 g of an oven-dried soil sample in an autoclave with 150 ml of 2N NaOH for 45 minutes. Cool, centrifuge at 3000 rpm for 10 minutes, and decant (and save) supernatant. Reextract soil pellet twice with 75 ml portions of fresh NaOH by shaking on a wrist shaker for 15 minutes and recentrifuging. Suction filter the extracts, and acidify filtrate to pH 2.0 with HCl. Extract with three consecutive 100 ml portions of diethyl ether. Shake the ether fractions with three consecutive 150 ml portions of 5% $NaHCO_3$. Reacidify the alkaline portions with HCl to pH 2.0 and reextract with three 225 ml portions of ether. Evaporate the ether to dryness and take up the residue in a known amount of 95% ethanol.

1. Procedure for Checking Diethyl Ether for Peroxides:

When ether is evaporated to dryness, as in the last step of the above procedure, an explosion hazard exists if the peroxide concentration in the ether is much above .01%. Following are procedures for testing the peroxide level, and for removal of the peroxides:

Add 1 ml of ether to an equal volume of glacial acetic acid to which approximately 100 mg of NaI or KI has been added. A yellow color indicates a low peroxide concentration, and brown a high concentration of peroxide in the sample. A blank versus cyclohexane, KI, and acetic acid should be run for comparison. In the

event that the test indicates the presence of peroxides, see procedure (2) for a clean-up procedure.

2. Procedure for Peroxide Removal from Ether:

The ether should be shaken in a bottle with a solution of 60 g $FeSO_4$, 6 ml concentrated H_2SO_4, and 110 ml of H_2O. The ether layer should then be decanted and rechecked for peroxides.

D. Standard Procedure for Making Solutions:

Reservoir A: Weigh out 11.013 g of 1-heptanesulfonic acid (1-HSA). Add 1-HSA to 1 liter of High Purity water which has been poisoned with 1% acetonitrile. Adjust pH with glacial acetic acid to pH 3.0.

Solutions for Injection:

Weigh out proper quantity of acid. add to 10 ml of 95% non-denatured ethanol.

E. Preparation of Solutions for HPLC Analysis:

1. Making Solutions:

All acids except p-coumaric and cinnamic should be dissolved in 95% ethanol at concentrations of 200 ppm. Cinnamic and p-coumaric should be in concentrations of 50 ppm and 100 ppm, respectively.

2. Filtering Solutions:

All solutions should be filtered with .50 μm Millipore filters prior to injection.

F. Procedure for Purging Reversed-Phase HPLC Column After Use:

After working on the instrument, the following program should be run with water as Reservoir A.

Time(min)	Flow(ml/min)	%B
0	1.5	0
30	1.5	0
34	1.5	100
40	1.5	100
42	1.5	60
42.1	0.0	60

This will clean the column of any accumulated salts, which if left on the column would rapidly deteriorate the stainless steel tubing and the column packing. In addition, by leaving the final acetonitrile concentration at 60%, bacteria growth in the column is inhibited.

RESULTS AND DISCUSSION

A. Choice of Monitoring Wavelength:

In order to establish experimental λ_{max} values for each of the eight acids, the samples were made up to 200 ppm in water (2 mg/10 ml) from which they were diluted to 20 ppm. Ultraviolet (UV) scans were run on the Cary 118 from 400-220 nm. Each solution was acidified with acetic acid to a pH < 3 to maintain the acids in their protonated forms.

In running the UV scans, it was found that at the concentration of 20 ppm, caffeic, p-coumaric, and cinnamic acids all absorbed so strongly on the 0 to 2 absorbance range that the pen went off scale. In order to bring the pen back on scale, the solutions required substantial dilution. Since all of the originally weighed 2 mg of caffeic acid had dissolved in the 10 ml of water, dilution to 4 ppm was quite simple. However, both p-coumaric and cinnamic acids have sufficiently low solubilities in water that there was undissolved residue in each of the "200 ppm" solutions. In other words, when these solutions were diluted to 20 ppm, and later when they were diluted to 2 ppm, the concentrations were only approximate because the starting solution was not 200 ppm. Using values for molar absorptivity as listed in the Handbook of Chemistry and Physics, these two solutions were run on the Cary

118 at their λ_{max} and their concentrations were calculated from Beer's law, $A = \varepsilon bc$. The saturation point (in water) of p-coumaric is experimentally 157 ppm, while that of cinnamic acid is 69.8 ppm. The λ_{max} values are listed in Table 3. It should be noted that the λ_{max} values fall between 270 nm and 323 nm, but seem to cluster around 280 nm and 320 nm. As a result, it was decided to set the variable wavelength detector at either 280 nm or 320 nm, while the fixed wavelength detector monitors 254 nm. Thus, ratios of A_{280}/A_{254} or A_{320}/A_{254} could be obtained for each of the acids.

B. Selection of Mobile Phase and Solvent Program:

The decision to make Reservoir A 98% 1-heptanesulfonic acid (.05M) was based on a paper by P. A. Realini, dealing with the separation of gallic and pyrogallic acids.[12] It was found that addition of the 1-heptanesulfonic acid at a concentration of .05M increases retention time sufficiently to allow separation of very similar carboxyl containing aromatic molecules. The way in which the 1-heptanesulfonic acid increases retention times can be seen in Figure 1. Essentially, the column packing is a long 18-carbon chain which is very non-polar. The 1-heptanesulfonic acid has a long non-polar end but also has a negatively charged SO_3^- on the other end. Were it not for the presence of the acid in Reservoir A, the moderately polar aromatic acids would pass through the non-polar column without adsorbing

TABLE 3

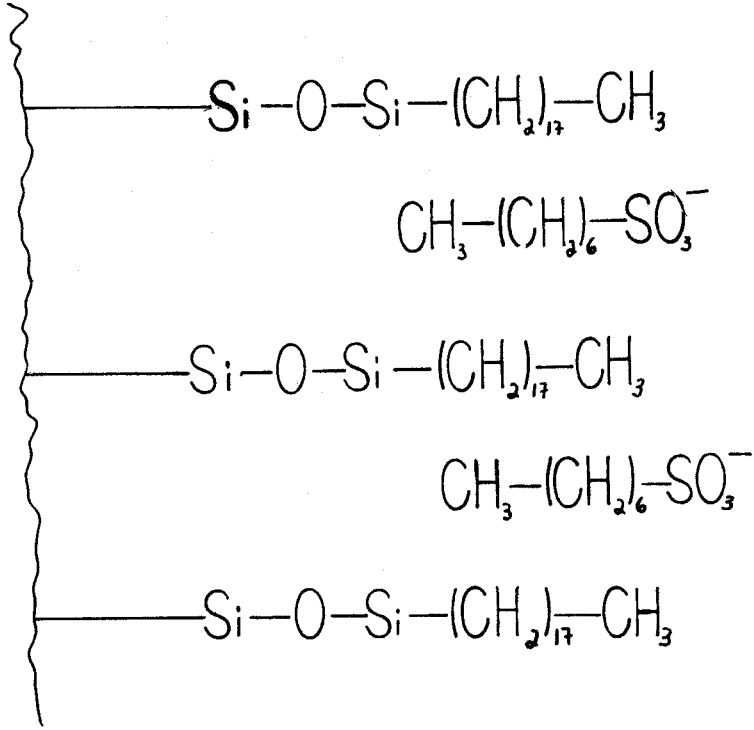
Experimental λ_{max} Values for the Individual Acids

Acid	λ_{max}(nm)
Caffeic	320
Cinnamic	270
p-Coumaric	320
Ferulic	320
Gallic	270
Gentisic	323
β-Resorcylic	293
Syringic	274

* * * * *

FIGURE 1

Interaction of Column Stationary Phase with 1-Heptanesulfonic Acid


Si–O–Si–(CH2)17–CH3
CH3–(CH2)6–SO3−
Si–O–Si–(CH2)17–CH3
CH3–(CH2)6–SO3−
Si–O–Si–(CH2)17–CH3

onto the packing surface for any significant length of time, and the acids would therefore be difficult to resolve. With the 1-heptanesulfonic acid to provide a polar site for adsorption, however, the acids are retained and are resolvable. Reservoir B was 100% acetonitrile.

1. Choice of Reservoir A pH:

In early work, it was noted that a broad group of peaks appeared consistently on every run, eluting at an acetonitrile concentration of 50%. Efforts were made to isolate the source of the "impurity". First, a blank (i.e. no injection) was run with water as Reservoir A, taking the acetonitrile concentration up to 50%. This showed a good baseline with no substantial peaks of any kind. However, when the reservoir containing 1-heptanesulfonic acid (1-HSA) and 1% acetic acid was used in its place, the group of peaks appeared again. Through running a series of injections of the individual components of Reservoir A, and seeing the same set of peaks for each, it was determined that no individual component was responsible for the peaks. The only remaining difference between the water reservoir and the one containing 1-HSA was pH. Two blanks were run: One blank used a reservoir at pH 2.9, the other used a reservoir at pH 3.5. The lower pH blank showed the peaks, while the high one did not. By bracketing the pH to find a low pH at which the peaks will not appear,

a cutoff value of pH 3.0 was found to yield a good baseline. Perhaps the lower pH somehow breaks the carbon-silane bonds and degrades the column.

2. Choice of Ethanol as Solvent for Acid Solutions:

Originally, all acid solutions were dissolved in 10 ml of water. Due to the relatively high polarity of water as compared to the acids, the solutions had to be made up 24 hours prior to use to insure that the acids were fully dissolved. This created a possible source of error, in that the stability of the acids in solution is questionable, so decomposition could occur during the 24 hour period of sitting in water. Ethanol, being less polar than water, dissolved the acids immediately, allowing the use of fresh solutions for all chromatograms.

Theoretically, a change in solvent will not alter a chromatogram, while a change in mobile phase will. However, the new chromatogram of all eight acids in ethanol showed broad, overlapping peaks for the first four components to elute. Attempts to change the solvent program to resolve the peaks proved fruitless, so further investigations were conducted in an attempt to rectify the problem. In checking the pH of Reservoir A, it was found that the pH was approximately 3.5. When the pH was lowered to its proper value of 3.0, three of the peaks returned to normal Gaussian peaks, while gentisic acid still showed a broad,

asymmetric peak. It was therefore decided to carry out further studies without gentisic acid present.

C. Evaluation of Separation Procedure:

1. Final Gradient Elution Program:

The final gradient program which was used is as follows:

Time(mins)	%Acetonitrile
0	0
5	28
6	39
8	50

flow rate = 1.5 ml/min

The seven component solution was run in triplicate with the above program while monitoring 280 nm and 254 nm. A run was also done while monitoring at 320 nm and 254 nm, but the background noise was so high that it was decided that where a A_{320}/A_{254} ratio is needed, the component(s) can be run individually. The average values of all relevant quantities from these runs are tabulated in Tables 4-6.

2. Evaluation of Data in Tables 4-6:

In examining the t'_r values (corrected retention time), it can be seen that cinnamic acid, which elutes last, has a corrected retention of 8.47 minutes. In an optimum chromatogram, the retention time of the last component to elute should be as small as possible, while at the same time allowing for maximum resolution

FIGURE 2

Final chromatogram on 7-component acid solution, monitoring at 280 nm and 254 nm.

The gradient elution program used is as follows:

Time(mins)	%Acetonitrile
0	0
5	28
6	39
8	50

flow rate = 1.5 ml/min

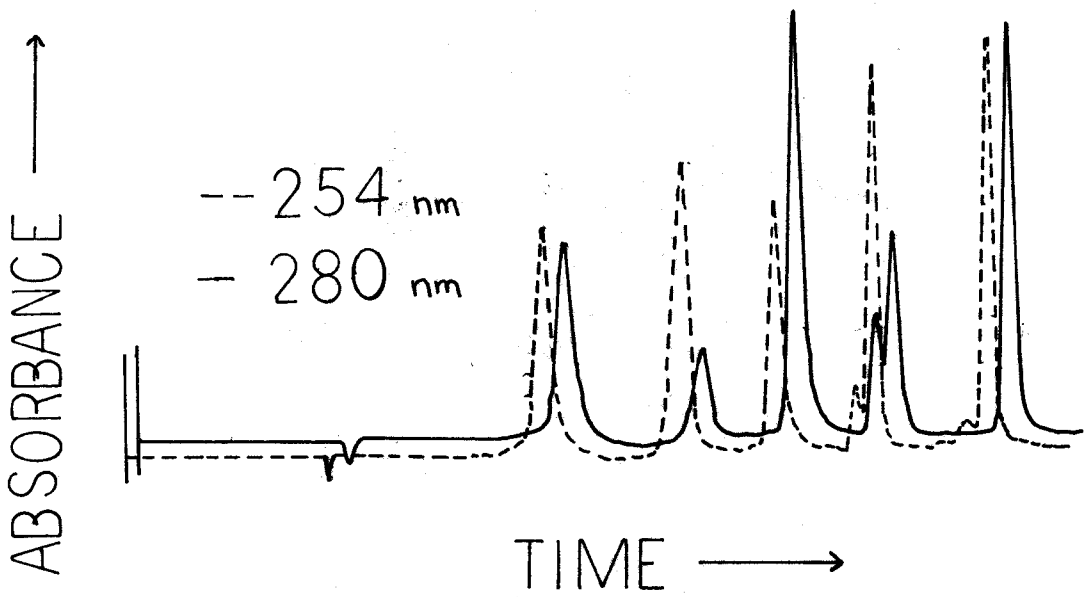

ABSORBANCE
-- 254 nm
— 280 nm
TIME

TABLE 4

Average Data for Triplicate Runs of 7-Component Acid Solution

Peak #	Acid	t_r'(min)[b]	A_{280}/A_{254}	R^c_{280}	R^c_{254}
1	Gallic	2.71 ± .04[a]	1.76 ± .01[a]	48.7 ± 1.7[a]	24.8 ± .9[a]
2	β-Resorcylic	4.51 ± .02	.311 ± .002	23.3 ± .5	66.4 ± 3.2
3	Caffeic & Syringic	5.71 ± .08	-[d]	-[d]	-[d]
4	p-Coumaric	6.80 ± .12	3.89 ± .22	40.9 ± 2.0	8.78 ± .53
5	Ferulic	7.02 ± .12	1.07 ± .01	31.4 ± 3.2	28.9 ± 3.5
6	Cinnamic	8.47 ± .04	2.01 ± .01	268.7 ± 1.4	118.7 ± 3.6

* * * * *

a. Average deviation.

b. Corrected retention time ($t_r - t_m$), where t_m = retention time of unretained solvent.

c. Response (area in cm^2)/ppm at indicated wavelength x 10^3.

d. These values can only be obtained from pure samples of the individual components due to their overlap when in solution together. (See TABLE 5)

TABLE 5

Data for Pure Samples of Caffeic and Syringic Acid

Acid	A_{280}/A_{254}[a]	A_{320}/A_{254}[a]	R_{320}[b]	R_{280}[b]	R_{254}[b]
Caffeic	.964 ± .005[c]	2.14 ± .00[c]	37.0 ± 1.5[c]	17.3 ± .4[c]	15.7 ± .7[c]
Syringic	2.40 ± .01	.192 ± .003	4.46 ± .10	64.1 ± 4.6	21.6 ± 2.1

* * * * *

a. Peak absorbance ratio at the indicated wavelengths.

b. Response (area in cm^2)/ppm at indicated wavelength x 10^3

c. Average Deviation.

TABLE 6

Retention Times, Column Capacity Factors, and Column Selectivity Values

Peak #	Acid	t_r'(min)[b]	k'[c]	α[d]
1	Gallic	2.71 ± .04[a]	1.05 ± .02[a]	
				1.67 ± .02[a]
2	β-Resorcylic	4.51 ± .02	1.75 ± .02	
				1.27 ± .00
3	Caffeic & Syringic	5.71 ± .08	2.21 ± .03	
				1.19 ± .03
4	p-Coumaric	6.80 ± .12	2.63 ± .02	
				1.03 ± .00
5	Ferulic	7.02 ± .12	2.72 ± .02	
				1.21 ± .00
6	Cinnamic	8.47 ± .04	3.28 ± .02	

* * * * *

a. Average Deviation.

b. Corrected Retention Time ($t_r - t_m$), where t_m = retention time of unretained solvent.

c. Column Capacity Factor (t_r'/t_m), where t_m = retention time of unretained solvent.

d. Column Selectivity (k_2'/k_1').

of the other components. With two exceptions, these requirements are met in the chromatogram in Figure 2. The first exception occurs in the fact that caffeic acid and syringic acid elute simultaneously. This can be shown by running syringic and caffeic acids separately and comparing their retention times. With the help of the variable wavelength detector, these overlapping peaks can be resolved mathematically. The procedure will be discussed later. The second exception to the "ideal" chromatogram occurs when p-coumaric acid elutes almost simultaneously with ferulic acid. Although p-coumaric and ferulic are not completely resolved, there is a sufficient gap between the two peaks to allow measurements with a fair amount of accuracy.

The next value in the tables is the A_{280}/A_{254} ratio. This is a ratio of the peak absorbances at 280 nm and 254 nm, and it often serves as a means of identification of a component in a chromatogram. As can be seen from the data in Tables 4 and 5, the ratios of the components differ from each other to such an extent that a simple comparison of t'_r and A_{280}/A_{254} from a solution of unknowns to those from a solution of knowns should provide fairly conclusive evidence for assignment of peak identifications.

The response values listed in Tables 4 and 5 do not aid in the identification of the peaks, but once

the peaks have been identified, the response values allow us to make quantitative calculations of acid concentrations.

Table 6 contains values for k' (column capacity factor) and α (column selectivity). Accepted values for k' are between 1 and 10, and all of those values in Table 6 fall within this range. The range for k' values is very important, because a small k' could possibly overlap the unretained solvent peak, while an exceedingly large value would indicate a very long retention time. The column selectivity provides a measure of the relative retentions of two adjacent peaks. Values greater than 1.05 are generally accepted to provide 98% separation of peaks. The only α value which falls below 1.05 is α_A the previously mentioned pair of p-coumaric and ferulic acids.

3. Mathematical Resolution of Overlapping Peaks:

As can be seen in Figure 2, there are only six peaks present for a seven component solution. It was mentioned previously that caffeic and syringic acids elute simultaneously, and that a set of simultaneous equations will allow mathematical resolution of the peaks. Following is the method for resolution:

$$\text{Area}_{320}^{mix} = \text{ppm}_{caffeic} \times R_{320}^{c} + \text{ppm}_{syringic} \times R_{320}^{s}$$

$$\text{Area}_{280}^{mix} = \text{ppm}_{caffeic} \times R_{280}^{c} + \text{ppm}_{syringic} \times R_{280}^{s}$$

where R_{320}^{c} is the area/ppm for caffeic acid at 320 nm,

and the other R values are the area/ppm for the acid and the wavelength indicated. This method points out the value of having a variable wavelength detector in addition to the fixed wavelength detector which is standard with the instrument.

Using a solution in which both components were in a concentration of 253 ppm, data was collected and inserted into the simultaneous equations. The results are 232 ppm syringic acid and 412 ppm caffeic acid. Based on these results, one might conclude that the equations do not provide us with accurate values for concentrations. However, all solutions consisted of such small quantities of solute and solvent that a small error in weighing would constitute a substantial percentage error. Hopefully, if larger quantities are used, we will see better agreement with the expected solutions to the equation.

FUTURE WORK

The next logical step in this project is to begin running chromatograms on soil extracts. Although an extract from red pine soil was carried out, time did not allow us to collect chromatographic data from this extract. Once data are collected, an extract from Scotch pine soil can be carried out and the data compared to that from the red pine extract. Once the component(s) causing the fungus growth inhibition has been isolated, further biological testing can be done to verify the findings.

There is no reason why techniques described in this paper cannot be applied to similar situations in which separations of similar phenolic acids might be desirable.

Other possibilities for further work might include experimentation with different soil extraction techniques in order to find a quantitative method for removal of polar aromatic acids from soil samples.

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