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HPLC SEPARATION AND WAVELENGTH AREA RATIOS OF MORE THAN 50 PHENOLIC ACIDS AND FLAVONOIDS

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Abstract—Relative retention times and wavelength area ratios for over 50 standard compounds were calculated using reverse-phase HPLC. The standard compounds analyzed included benzoic acids, cinnamic acids, benzene carboxylic acids, acetic acids, coumarins, benzaldehydes and a variety of flavonoid compounds including flavanones, flavones, isoflavones, and their glycosides. Each standard compound was chromatographed by three different gradient elutions. Compounds were detected by UV absorption at 254 nm and 280 nm. Relative retention times with respect to two different internal references and the 254nm:280nm wavelength area ratio was determined for each standard. Soybean root and seed extracts were analyzed for the presence of the standard compounds using the chromatographic conditions described.

Key Words—HPLC, retention time, area ratio, phenolic acids, flavonoids, soybean, *Glycine max*.

INTRODUCTION

HPLC has become a popular analytical tool for the separation of complex mixtures of metabolic intermediates. Phenolic acids have been implicated as active allelochemicals from living and decomposing plant tissues and are often precursors to flavonoids (Vickery and Vickery, 1981; Prikryl and Vancura, 1980; Bokari et al., 1979; Kuc, 1972; Rovira, 1969). Roston and Kissinger (1982) review recent work on the use of HPLC in the determination of phenolic acids, including those endogenous to plant material. They draw attention to the fact that many of the communications dealing with bonded-phase separation of mixtures of phenolic acids have also considerable flavonoid compounds because the

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two are often found in a common matrix. However, the greater hydrophobicity of the larger flavonoid molecules necessitates the use of gradients if they are to be determined simultaneously with phenolic acids. Several researchers have analyzed soybeans by HPLC for these types of compounds (Eldridge, 1982; Hardin and Stutte, 1980; Lookhart et al., 1978; Murphy and Stutte, 1978). In this study over 50 standard phenolic compounds, as well as soybean root and seed extracts, were analyzed by a modification of the HPLC procedure of Granato et al. (1983).

The procedures described in this paper attempt to circumvent the possibility that two or more compounds may have similar retention times (coelution) under a specific gradient analysis by measuring the absorbance of the sample simultaneously at two different wavelengths so that a wavelength area ratio could be determined for each component of the sample and by analyzing each sample by three different gradient conditions. To correct for any potential variations in column conditions, and to maximize the utility to other workers, retention times relative to internal references are reported rather than absolute retention times. Relative retention times are the most reliable index for compound identification (Johnson and Stevenson, 1978).

METHODS AND MATERIALS

Apparatus. The liquid chromatograph system employed a Beckman model 420 controller/programmer equipped with Beckman models 100A and 110A solvent metering systems, a Beckman 25-cm C_{18} Ultrasphere ODS column, and a Micromeritics 725 autoinjector with a 20-μl sample loop. Detection was by means of two Beckman variable wavelength detectors (models 100-40 and 100-10). Retention times and peak areas were obtained with two recording integrators (Beckman model C-R1A and Hewlett Packard 3390A). The two detectors were connected in series. Detection was achieved first at 254 nm by the model 100-40 detector and recorded by the Beckman C-R1A integrator. Approximately 0.1 min later detection was achieved at 280 nm by the model 100-10 detector and recorded by the HP 3390A integrator. The range for both detectors was 0.05, the absorbance mode was 0–2, and the time constant was 0. During the analyses, the pressure varied from approximately 13,700 kP_a (at 10% B and a flow rate of 1.00 ml/min) to approximately 26,110 kP_a (at 100% B and a flow rate of 1.20 ml/min).

Chemicals. Table 1 lists the source of the chemical standards and solvents. All chemicals were purchased as high-purity standards and were used without further purification. Solvents were HPLC spectral grade, and distilled water was deionized before use. All solvent ratios are given on a volume basis.

Plant Material. Wells II soybean [*Glycine max* (L.) Merrill] roots were taken from greenhouse-grown plants in the R-2 stage of growth (Fehr and Caviness, 1977) and analyzed. Root tissue was extracted by dimethyl sulfoxide

TABLE 1. STANDARD COMPOUNDS AND SOLVENTS

Common name	Chemical name	Abbreviation	Source[a]
STANDARD COMPOUNDS			
Benzoic acids			
Gallic acid	3,4,5-trihydroxybenzoic acid	3,4,5-THBA	S
α-Resorcylic acid	3,5-dihydroxybenzoic acid	3,5-DHBA	S
Protocatechuic acid	3,4-dihydroxybenzoic acid	3,4-DHBA	S
Gentisic acid	2,5-dihydroxybenzoic acid	2,5-DHBA	S
β-Resorcylic acid	2,4-dihydroxybenzoic acid	2,4-DHBA	S
	4-hydroxybenzoic acid	4-HBA	A
Salicylic acid	2-hydroxybenzoic acid	2-HBA	A
	4-ethoxybenzoic acid	4-EBA	E
	4-aminobenzoic acid	4-ABA	A
Vanillic acid	4-hydroxy-3-methoxybenzoic acid	4-H,3-MBA	A
Syringic acid	4-hydroxy-3,5-dimethoxybenzoic acid	4-H,3,5-DMBA	A
Benzoic acid		BA	A
Benzaldehydes			
	4-hydroxybenzaldehyde	4-HBald	S
	2,3-dihydroxybenzaldehyde	2,3-DHBald	S
Vanillin	4-hydroxy-3-methoxybenzaldehyde	4-H,3-MBald	S
Ethylvanillin	4-hydroxy-3-ethoxybenzaldehyde	4-H,3-EBald	E
Syringaldehyde	4-hydroxy-3,5-dimethoxybenzaldehyde	4-H,3,5-DMBald	M
Phenols			
Pyrogallol	1,2,3-benzenetriol	pyrogallol	A
Catechol	1,2-benzenediol	catechol	E
Benzene carboxylic acids			
Terephthalic acid	4-benzene carboxylic acid	terephthalic	E
Phthalic acid	1,2-benzene dicarboxylic acid	phthalic	E
Diphenic acid	1,1′-biphenol-2,2′-dicarboxylic acid	diphenic	E
Pyromellitic acid	1,2,4,5-benzene tetracarboxylic acid	pyromellitic	E
Acetic acid			
Homophthalic acid	2-carboxyphenyl acetic acid	homophthalic	K
	3-indole acetic acid	IAA	E
	1-naphthalene acetic acid	NAA	E
Cinnamic acids			
Caffeic acid	3,4-dihydroxycinnamic acid	3,4-DHCA	A
Ferulic acid	4-hydroxy-3-methoxycinnamic acid	4-H,3-MCA	A
p-Coumaric acid	4-hydroxycinnamic acid	4-HCA	A
m-Coumaric acid	3-hydroxycinnamic acid	3-HCA	S
o-Coumaric acid	2-hydroxycinnamic acid	2-HCA	M
Cinnamic acid	3-phenyl-2-propenoic acid	CA	A
Coumarins			
Scopoletin	7-hydroxy-6-methoxycoumarin	7-H,6-MC	S
Umbelliferone	7-hydroxycoumarin	7-HC	S
	4-hydroxycoumarin	4-HC	S
Coumarin	1,2-benzopyrone	coumarin	A

TABLE 1. Continued

Common name	Chemical name	Abbreviation	Source[a]
STANDARD COMPOUNDS Cont.			
Flavanones			
Naringenin	4′,5,7-trihydroxyflavanone	naringenin	S
Hesperetin	4′-methoxy-3′,5,7-trihydroxyflavanone	hesperetin	S
d-Catechin	3,3′,4′,5,7-pentahydroxyflavanone	d-catechin	S
Flavanone glycosides			
Naringin	naringenin-7-rhamoglucoside	naringin	A
Hesperedin	hesperetin-7-rutinoside	hesperedin	S
Flavones			
Kaempferol	3,4′,5,7-tetrahydroxyflavone	kaempferol	S
Morin	2′,3,4′,5,7-pentahydroxyflavone	morin	S
Quercetin	3,3′,4′5,7-pentahydroxyflavone	quercetin	S
Myricetin	3,3′,4′,5,5′,7-hexahydroxyflavone	myricetin	S
Flavone glycosides			
Rutin	quercetin-3-rutinoside	rutin	S
Isoflavones			
Daidzein	4′,7-dihydroxyisoflavone	daidzein	I
Genistein	4′,5,7-trihydroxyisoflavone	genistein	K
Coumestans			
Coumestrol	dihydrocoumarinocoumarone	coumestrol	P
Other standards			
	methyl-4-aminobenzoate	M-4-ABenz	E
Indole	1-benzo [b] pyrrole	indole	A
Phloretin	dihydronaringenin	phloretin	S
Uracil	2,4-pyrimidinedione	uracil	N
P-Anisidine	4-methoxyaniline	anisidine	E
Shikimic acid	trihydroxycyclohexenecarboxylic acid	shikimic	S
Chlorogenic	3-caffeoylquinic acid	chlorogenic	A
SOLVENTS			
Glacial acetic acid			F
Ammonium acetate			F
n-Butanol			B
Methanol			B
Dimethyl sulfoxide		DMSO	F
Water			W

[a] A = Aldrich Chemical Company; B = Burdick & Jackson Laboratories Inc.; E = Eastman Kodak Company; F = Fisher Scientific Company; I = from Dr. John Ingham, University of Reading; K = K & K Company; M = Mann Research Laboratories; N = Nutritional Biochemicals Corporation; P = Pfaltz & Bauer, Inc.; S = Sigma Chemical Company; W = purified by a Millipore Milli-Q Water Purification System.

(DMSO) according to methods previously described by Granato et al. (1983). The seed extract was from the soybean cultivar Pella. Fifty seeds were cracked by running them four times through a Wiley mill grinder with no retaining screen in place. The cracked seeds were mixed with DMSO in a 2 : 1 (w/w) ratio, and this mixture was shaken for 30 min (at low speed on an Eberbach shaker). The DMSO extract was decanted off, centrifuged (by a Damon IEC B-20A centri fuge) at 294,000 m/sec^2 for 10 min and filtered through a 5-μm Millipore filter. Prior to analysis two internal references, 2,4-DHBA and indole, were added to the soybean extracts.

Separation Procedure. The mobile phase consisted of solvents A and B. Solvent A contained 98% water and 2% glacial acetic acid in 0.018 M ammonium acetate. Solvent B was 70% solvent A and 30% organic solvent. The organic solvent contained 82% methanol, 16% *n*-butanol, and 2% glacial acetic acid in 0.018 M ammonium acetate. Solvent A was pumped by the model 100A pump and solvent B by the model 110A pump. All the standards were analyzed by the three gradient elutions. The initial flow rate for all gradients was 1.00 ml/min. The three gradient elutions consisted of the following steps:

For gradient 1: (a) 0.0–1.0 min isocratic at 10% B; (b) 1.0–21.0 min linear gradient from 10 to 25% B; (c) 21.0–36.0 min linear gradient from 25 to 45% B; (d) 36.0–56.0 min linear gradient from 45 to 100% B; (e) 50.0–50.15 min flow increased to 1.20 ml/min; (f) 82.0–82.15 min linear gradient from 100 to 10% B; (g) 92.0–92.15 min flow decreased to 1.00 ml/min; (h) at 99.0 min sample loop rinsed and gradient repeated.

For gradient 2: (a) 0.0–1.0 min isocratic at 20% B; (b) 1.0–21.0 min linear gradient from 20 to 35% B; (c) 21.0–36.0 min linear gradient from 35 to 45% B; (d) 36.0–56.0 min linear gradient from 45 to 90% B; (e) 50.0–50.15 min flow increased to 1.20 ml/min; (f) 60.0–61.0 min linear gradient from 90 to 100% B; (g) 85.0–85.15 min linear gradient from 100 to 20% B; (h) 92.0–92.15 min flow decreased to 1.00 ml/min; (i) at 99.0 min sample loop rinsed and gradient repeated.

For gradient 3: (a) 0.0–1.0 min isocratic at 20% B; (b) 1.0–21.0 min linear gradient from 20 to 45% B; (c) 21.0–61.0 min linear gradient from 45 to 90% B; (d) 50.0–50.15 min flow increased to 1.20 ml/min; (e) 61.0–66.0 min linear gradient from 90 to 100% B; (f) 85.0–85.15 min linear gradient from 100 to 20% B; (g) 92.0–92.15 min flow decreased to 1.00 ml/min; (h) at 99.0 min sample loop rinsed and gradient repeated.

Each standard compound was first subjected to analysis by gradient 1 to determine its absolute retention time. Standard mixes were made which grouped together up to 10 standard compounds whose retention times differed sufficiently to allow for good resolution. These standard mixes were then subjected to analysis by the three gradients. The absolute retention times of some of the standard compounds analyzed both individually and in their respective standard mix were compared to verify that their absolute retention times did not change upon mixing of the standard compounds.

TABLE 2. RELATIVE RETENTION TIMES AND WAVELENGTH AREA RATIOS FOR STANDARD COMPOUNDS

Standard Compound	Standard Mix[a]	RRT(2,4-DHBA) gradient			RRT(indole) gradient			Wavelength area ratio (254nm:280nm)	Peak area at 280nm[b]
		1	2	3	1	2	3		
1. Uracil	D	sf[c]	sf	sf	sf	sf	sf	sf	sf
2. Pyromellitic	E	sf	sf	sf	sf	sf	sf	sf	sf
3. Pyrogallol	F	0.38	0.47	0.49	0.10	0.10	0.11	4.2	1
4. 3,4,5-THBA	A	0.40	0.45 [d]	0.46	0.11	0.10 [d]	0.11	0.6	750
5. 2,5-DHBA	G	0.59	0.65	0.65	0.16	0.14	0.15	1	45
6. 3,5-DHBA	D	0.65	0.63	0.65	0.18	0.14	0.15	6.0	110
7. 3,4-DHBA	C	0.72	0.75	0.74 [d]	0.20	0.16	0.17 [d]	2.0	530
8. 4-ABA	B	0.74	0.79 [d]	0.79 [d]	0.20	0.17 [d]	0.19 [d]	0.4	1900
9. Phthalic	E	0.77 [d]	0.84 [d]	0.92 [d]	0.21 [d]	0.18 [d]	0.21 [d]	1.2	120
10. Catechol	G	0.82	0.98	0.94	0.22	0.21	0.22	0.1	420
11. Anisidine	A	1.00	1.00	0.95	0.27	0.21	0.22	0.1	190
12. 2,4-DHBA	B	1.00	1.00	1.00	0.27	0.21	0.23	3.5	410
13. 4-HBA	C	1.18	1.21	1.20	0.32	0.26	0.28	3.7	570
14. Terephthalic	F	1.36	1.32	1.31	0.37	0.28	0.31	6.7	220
15. *d*-Catechin	D	1.42	1.16	1.15	0.39	0.25	0.27	0.2	190
16. Shikimic	G	1.47	1.49	1.44	0.40	0.32	0.34	0.1	18
17. 4-HBald	D	1.47	1.49	1.44	0.40	0.32	0.34	0.3	2400
18. Chlorogenic	E	1.48	1.28	1.27	0.41	0.27	0.30	1	300
19. 4-H,3-MBA	A	1.55	1.52	1.46	0.43	0.32	0.34	2.0	590
20. 2,3-DHBald	B	1.58	1.60	1.52	0.42	0.34	0.36	1.3	960
21. 3,4-DHCA	C	1.76	1.67	1.63	0.48	0.34	0.37	0.6	890
22. 2-HBA	F	1.80	1.95	1.89	0.49	0.41	0.44	0.2	260
23. 4-H,3,5-DHBA	A	1.87	1.77	1.65	0.52	0.37	0.39	0.5	1000
24. Homophthalic	B	1.92	1.89	1.79	0.52	0.40	0.42	1.0	110
25. 4-H,3,4-DHBald	B	2.28	2.33	2.23	0.61	0.49	0.51	0.7	78
26. M-4-ABenz	A	2.44	2.59	2.35	0.67	0.55	0.55	0.3	504
27. 7-HC	C	2.49	2.58	2.35	0.67	0.55	0.55	0.5	500
28. 4-HCA	B	2.53	2.66	2.44	0.68	0.56	0.58	0.2	2900

29. 4-H,3-MCA	A	2.83	3.13	2.74	0.78	0.66	0.64	0.6	930
30. BA	F	2.83	3.33	3.06	0.78	0.71	0.72	0.9	130
31. 7-H,6-MC	C	2.86	3.08	2.68	0.78	0.65	0.63	1.2	380
32. 4-H,3MBald	G	2.88	3.24	2.86	0.79	0.69	0.67	1.7	580
33. 3-E,4-HBald	D	2.92	3.25	2.85	0.80	0.69	0.67	0.3	1300
34. 3-HCA	E	3.02	3.44	3.08	0.83	0.73	0.72	0.4	2100
35. IAA	B	3.06	3.44	3.03	0.82	0.73	0.72	0.5	600
36. Kaempferol	G	ur[e]	ur	ur	ur	ur	ur	ur	ur
37. Coumarin	C	3.43	4.14	3.60	0.93	0.88	0.84	0.4	1500
38. Myricetin	F	ur	ur	ur	ur	ur	ur	ur	ur
39. 2-HCA	A	3.57	4.57	3.93	0.98	0.97	0.92	0.4	1900
40. Indole	A	3.66	4.73	4.27	1.00	1.00	1.00	0.6	410
41. Naringin	B	3.80	4.99	4.03	1.02	1.06	0.95	0.2	450
42. Rutin	D	3.80	5.09	4.27	1.04	1.08	1.00	2.3	190
43. 4-HC	E	3.82	5.04	4.40	1.05	1.06	1.02	0.4	1300
44. Hesperidin	C	3.84	5.13	4.29	1.04	1.09	1.00	0.2	400
45. Diphenic	B	3.40	5.28	4.49	1.08	1.12	1.06	1.7	150
46. Daidzein	C	4.41	5.99	5.89	1.20	1.26	1.37	2.4	460
47. CA	H	4.46	6.00	5.85	1.21	1.27	1.36	0.4	2400
48. 4-EBA	A	4.62	6.30	6.12	1.27	1.33	1.44	2.8	490
49. Morin	G	ur	ur	ur	ur	ur	ur	ur	ur
50. Naringenin	B	4.79	6.57	6.47	1.29	1.39	1.53	0.1	620
51. Hesperetin	C	4.94	6.98[d]	7.09	1.34	1.74[d]	1.67	0.1	730
52. Quercetin	D	4.95	7.10	7.21	1.35	1.51	1.69	2	2000
53. Genistein	A	4.96	7.00	7.09	1.36	1.48	1.66	2.5	690
54. NAA	B	5.09	7.04	6.95	1.37	1.49	1.64	0.4	520
55. Phloretin	C	5.18	7.27	7.61	1.40	1.54	1.78	0.2	920
56. Coumestrol	E	5.80	8.21	8.55	1.59	1.73	1.99	4.1	250

[a] Each standard mix was comprised of up to 10 standard compounds designated by the same letter.
[b] Peak area is in integration units $\times 10^{-3}$ for standard mixes containing 10 μg/ml of individual compounds.
[c] sf= No data reported because of solvent front interference.
[d] More than one peak was observed for this standard compound. RRT of major peak observed is recorded.
[e] ur = No data reported because of broad, undefined response.

The standards were dissolved in dimethyl sulfoxide and the concentration of each standard compound upon analysis was 10.0 μg/ml. Each standard mix also contained two internal references: 2,4-DHBA and indole. Both internal references absorbed well at the wavelengths of interest. 2,4-DHBA eluted early in the HPLC analyses, whereas indole eluted much later. The concentration of the two internal references upon analysis was 10.0 μg/ml and 5.0 μg/ml for 2,4-DHBA and indole, respectively.

Calculations. Peak retention times and peak areas were monitored and computed by integrating recorders. The relative retention time (RRT) for each standard compound with respect to each of the two internal references was calculated by dividing the absolute retention time (RT) of the standard compound by the RT of the internal reference. Table 2 lists the relative retention times of all the standard compounds. They are listed according to increasing RRT with respect to 2,4-DHBA for gradient 1. In instances where the standard compounds eluted at the solvent front, and those where the recorder failed to detect the standard compound because the peak was too broad, no RRT was calculated.

Since each sample was analyzed simultaneously at 254 nm and 280 nm, a ratio of the integrated area at 254 nm compared to 280 nm was calculated and is called the wavelength area ratio. For two or more detectors operated under fixed conditions the ratio of response of a single compound to two different wavelengths is characteristic of that compound (Johnson and Stevenson, 1978). Additional evidence for compound identification is possible when wavelength area ratios of a standard compound are very similar to those for an unknown of similar retention time. These ratios can best be obtained by connecting two detectors in series or by information obtained from a rapid scan detector that records absorbance and peak areas for two or more wavelengths simultaneously.

Because our studies used two absorbance detectors connected in series, each with a separate integrating device, a correlation between the peak areas (integration units) obtained from the two recording integrators was determined. The conversion factor for normalizing areas from recorder 1 to recorder 2 was 7.69. Thus the integrated areas of the peaks analyzed at 254 nm by the first recording integrator were adjusted by a factor of 7.69 to give normalized areas that correspond to the integrated areas had the analysis been done at 254 nm by the second recording integrator.

The normalized integrated area of each standard compound at the two wavelengths analyzed for each of the three gradients was determined. Thus three wavelength area ratios could be calculated for each standard compound. Table 2 lists the mean wavelength area ratio and the mean normalized integrated area (integration units) at 280 nm for each of the standard compounds.

RESULTS AND DISCUSSION

Figure 1 shows a representative chromatogram of a standard mix detected at 280 nm. Table 2 reports retention times relative to two internal references

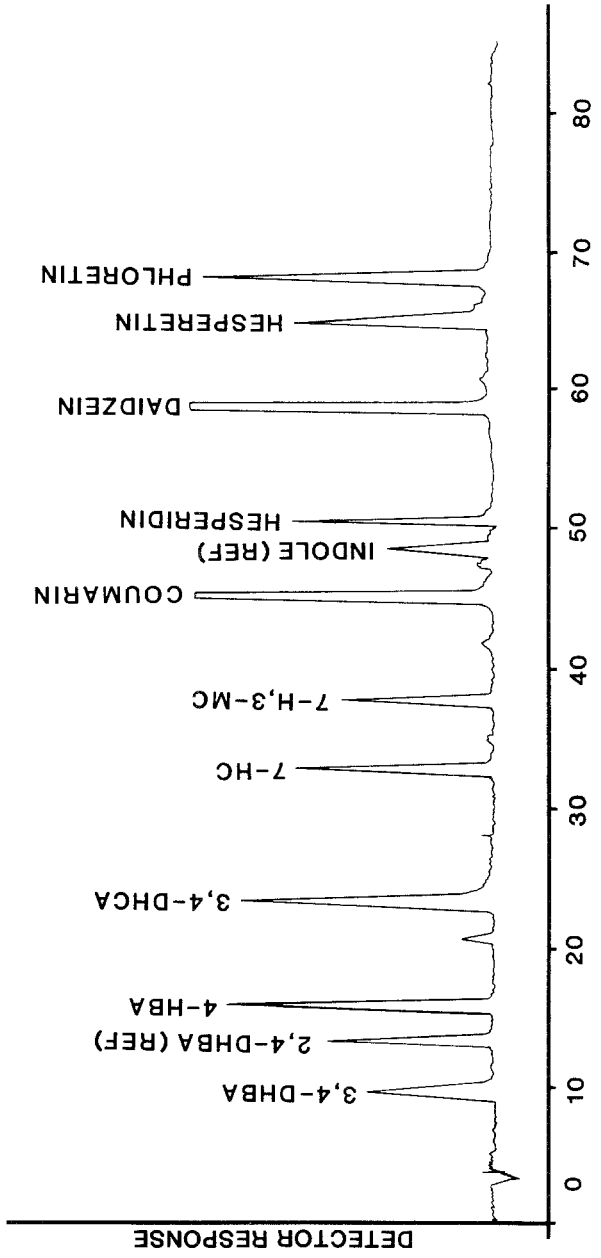

FIG 1. HPLC chromatogram of standard mix C (gradient 1, 280 nm).

and the 254nm:280nm wavelength area ratios for each of 56 standard compounds. The use of three gradients is intended to increase the probability that compounds will be properly identified. The data in Table 2 show that a single gradient of the type proposed would not allow separation of all compounds. Anisidine, for example, could not be distinguished from 2,4-DHBA on gradients 1 and 2 (RRT = 1.00), but it has a somewhat smaller RRT (0.95) on gradient 3. Because of the large number of compounds of interest in many characterization studies and in an effort to improve the probability of proper compound identification, the 254nm:280nm wavelength area ratios were also reported. Anisidine, for example, has a wavelength area ratio of 0.1 whereas 2,4-DHBA has a wavelength area ratio of 3.5. Comparison of wavelength area ratios in this case is a valuable tool in helping to establish compound identification.

There are at least two limitations to wavelength area ratios that should be noted. First, chromatograms from five of the 56 compounds tested resulted in two poorly resolved peaks from one or more of the gradients used. This may be the result of impure standards or such possibilities as isomers of the same standard that absorb differently at one wavelength than they do at another. In cases where two peaks were observed, the total peak area was used for the wavelength area ratioing (Table 2). When the two poorly resolved peaks occur, the wavelength area ratios are undoubtably less precise. Secondly, if the absorbance of an unknown is very small, the integrated peak area will be more subject to integration parameters and errors. The areas reported in Table 2 indicate the peak areas for some compounds are several orders of magnitude larger than the peak areas of compounds like pyrogallol, for example. Thus wavelength area ratios other than 254nm:280nm may be more appropriate for specific compounds. These techniques (multiple gradients and wavelength area ratios) are not intended to replace the value of additional compound identification methods such as mass spectrometry or infrared analysis. They can, however, serve to add evidence for positive identification and, in many cases, can be used to rule out tentative identifications made on a single chromatogram with a single detector.

Results shown in Table 2 indicate that the gradients used in this study will not detect 10 μg/ml uracil, pyromellitic acid, kaempferol, myricetin, and morin. If these compounds are of specific interest, modifications of the proposed gradients would be needed.

Figures 2 and 3 show examples of chromatograms obtained by applying the methods described to soybean root and seed extracts. Using the information in Table 2, we were able to determine that daidzein and genistein were present in soybean roots extracted with DMSO, and these same compounds were present in extracts from soybean seeds. Benzoic and cinnamic acids were not found in the free form in roots or in the seeds of soybeans.

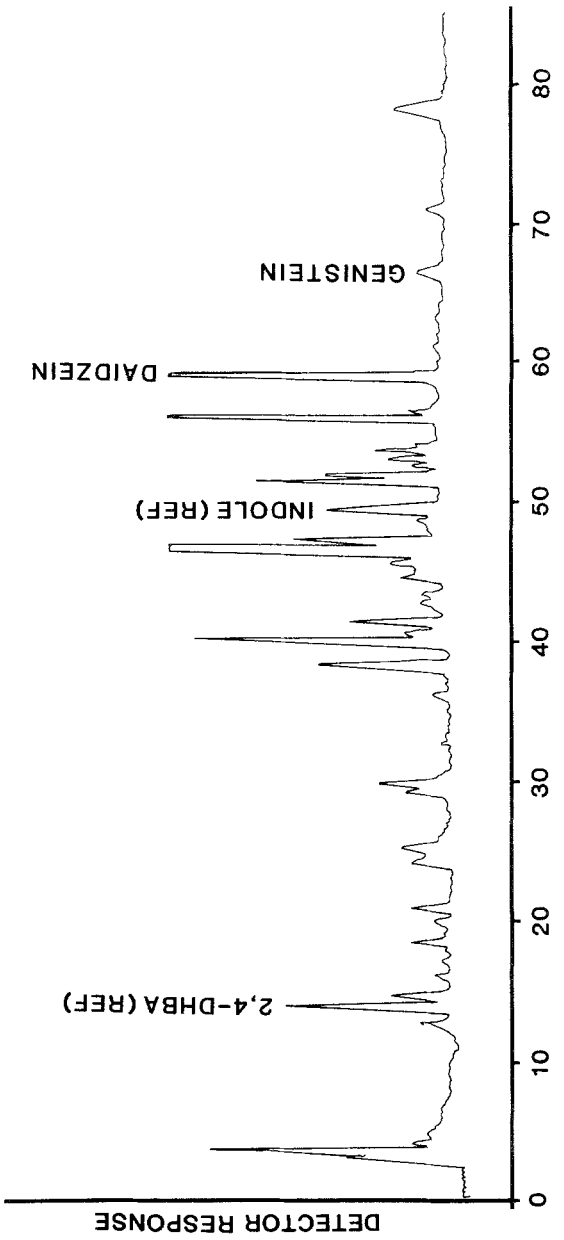

FIG. 2. HPLC chromatogram of a soybean root extract with 2,4-DHBA and indole added as internal references (gradient 1, 280 nm).

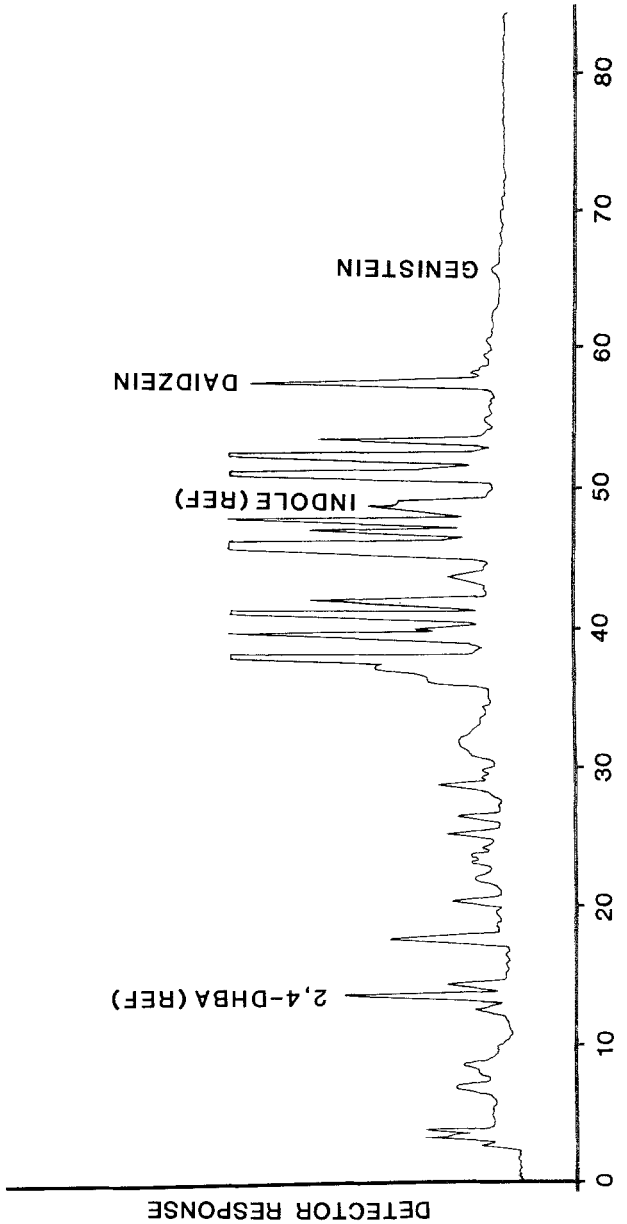

FIG. 3. HPLC chromatogram of a soybean seed extract with 2,4-DHBA and indole added as internal references (gradient 1, 280 nm).

The absence of benzoic, cinnamic, and related acids in the free form in soybean root and seed extracts is somewhat surprising because of the emphasis placed on these compounds as potential allelochemicals. Further isolation and evidence for positive identification of daidzein and genistein will be presented in a subsequent report. The methods reported in this paper should be applicable to extracts of a wide variety of biological materials. They allow detection of a large number of phenolic acids and flavonoids. Relative retention times with three different gradients and use of wavelength area ratios greatly improve the confidence of the analytical technique.

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