Journal of Chromatography, 248 (1982) 446–450
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,129

Note

Phenolic acid analyses comparing two high-performance liquid chromatographic techniques*

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(Received June 21st, 1982)

As researchers in the plant sciences recognize the influence of phenolics on plant growth, crop production, and pest control, methods to facilitate phenolic analyses become increasingly important. Prior to the introduction of gas–liquid (GLC) and high-performance liquid chromatography (HPLC), paper chromatography was the major analytical device for the study of phenolic compounds. The analysis of phenolics has been greatly aided by the advent of new chromatographic tools such as GLC and HPLC. HPLC has been used to successfully isolate phenolic acids[1–3], as well as many other more complex phenolic compounds such as anthocyanins[4,5] and flavonoids[3,6–8].

Procedures isolating phenolic compounds have been performed on stainless-steel columns[1–8]. A radially compressed system containing flexible cartridges has been successfully reported for use in isolating plant hormones[9] and proteins[10]. The purpose of this study was to explore the possible use in phenolic separation of the radial compression system (using reversed-phase packing) and to compare the results with the separations achieved on the μBondapak C_{18} column, a stainless-steel column used for many different phenolic analyses.

MATERIALS AND METHODS**

A HPLC system (Waters Assoc.) as previously described[1,2] was used in this study. The columns were either the μBondapak C_{18} or the Radial-Pak A (C_{18}) cartridge used in the Radial Compression Module-100. All compounds were detected by absorbance at 254 nm.

Mixtures of standard compounds included gallic, gentisic, protocatechuic, *p*-hydroxybenzoic, salicylic, vanillic, caffeic, syringic, benzoic, *p*-coumaric, ferulic, sinapic, and cinnamic acids at 10^{-4} *M* concentrations.

Extraction of phenolics from plant tissue and hydrolysis of the conjugates have been described[1,2]. Methanol and butanol were high-purity, spectrophotometric-grade solvents. The initial solvent used for separation of the phenolics with the Radial-Pak A was a mixture of 2.25 m*M* ammonium acetate in 1.5% acetic acid and methanol

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0021-9673/82/0000–0000/$02.75

(80:20). This mixture was pumped isocratically for 1400 sec and followed by a 10-min convex gradient (gradient 10) to 45% methanol. The flow-rate was 2 ml/min. Conditions for separating the phenolic compounds on the μBondapak C_{18} column were the same as previously described by Hardin and Stutte[2]. Separation at 2 ml/min flow-rate was achieved by halfing the time required for each stage of the solvent scheme.

The following chromatographic data were calculated on the various components of each analytical procedure:

$$k' = \frac{t_R - t_0}{t_0} \qquad \alpha = \frac{k'_2}{k'_1} \qquad R_s = \frac{1}{4}\frac{\alpha - 1}{\alpha}\sqrt{N}\frac{k'_2}{1 + k'_2}$$

where k' = capacity factor, t_0 = retention time of non-sorbed solvent, t_R = retention time of component, α = relative retention, R_s = resolution of two peaks, N = theoretical plate count (μBondapak C_{18} = 3000, Radial-Pak A = 5000).

TABLE I

RETENTION TIMES AND RESOLUTION DATA FOR SEPARATION OF PHENOLIC ACIDS ON RADIAL-PAK A

Flow-rate = 2 ml/min; t_0 = 108 sec.

Phenolic acid	*Retention time (sec)*	k'	α	R_s
Gallic	169	0.56		
			2.77	6.9
Protocatechuic	275	1.55		
			1.21	2.0
Gentisic	310	1.87		
			1.71	5.6
p-Hydroxybenzoic	453	3.19		
			1.45	4.5
Vanillic	607	4.62		
			1.14	1.8
Caffeic	678	5.28		
			1.26	3.2
Syringic	824	6.63		
			1.38	4.4
Salicylic	1098	9.17		
			1.21	2.8
p-Coumaric	1301	11.05		
			1.13	1.9
Benzoic	1453	12.45		
			1.30	3.8
Ferulic	1851	16.14		
			1.34	4.3
Sinapic	2445	21.64		
			1.13	2.0
Cinnamic	2747	24.44		

TABLE II

RETENTION TIMES AND RESOLUTION DATA FOR SEPARATION OF PHENOLIC ACIDS ON μBONDAPAK C_{18}

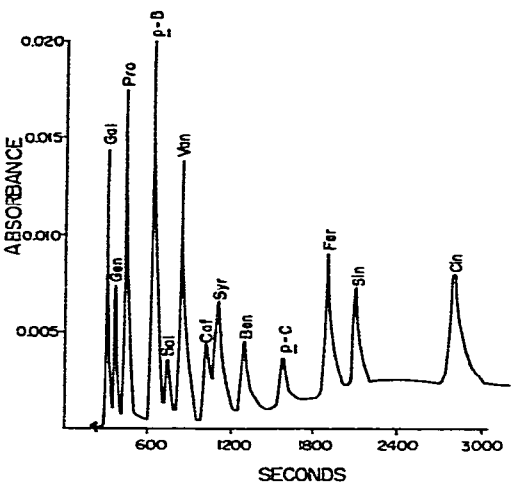
Flow-rate = 1 ml/min; t_0 = 231 sec.

Phenolic acid	*Retention time (sec)*	*k'*	*α*	R_s
Gallic	321	0.390		
			1.73	2.3
Gentisic	387	0.675		
			1.35	1.7
Protocatechuic	441	0.909		
			1.89	4.1
p-Hydroxybenzoic	627	1.714		
			1.27	2.0
Salicylic	735	2.182		
			1.14	1.2
Vanillic	807	2.494		
			1.34	2.7
Caffeic	1002	3.338		
			1.10	1.0
Syringic	1080	3.675		
			1.18	1.7
Benzoic	1236	4.351		
			1.33	2.9
p-Coumaric	1563	5.766		
			1.25	2.4
Ferulic	1899	7.221		
			1.15	1.6
Sinapic	2154	8.325		
			1.35	3.3
Cinnamic	2832	11.260		

RESULTS AND DISCUSSION

The retention times and resolution data for the separation of the phenolic acids on the μBondapak C_{18} and Radial-Pak A are presented in Tables I and II. Gallic, *p*-hydroxybenzoic, ferulic, sinapic, and cinnamic acids eluted in the same relative positions on both columns. The relative positions of the remaining eight phenolic acids were dependent on the column used in the analysis. Optimal *k'* values should range between 1 and 10 for the multicomponent separation[11]. Four values from the μBondapak C_{18} data were outside the desired range and six were not within the range on the Radial-Pak A separation. At an R_s value of 1.0, peak overlap is near 2%[12]. All R_s values were 1.0 or above for procedures on both columns. Without regard to the different elution orders of the phenolic acids on each column, many of the R_s values between the relative positions were greater for the separation on the Radial-Pak A.

The total time required for the analysis was comparable between the two procedures even though the flow-rates were different. Chromatograms representing the separation of the phenolic acid mixture on the two columns are presented in Figs. 1 and 2. Visual assessment of the chromatograms indicates that near baseline resolution of the components was achieved on the Radial-Pak A. Baseline resolution was observed for a few of the components of the phenolic mixture using the μBondapak C_{18}.



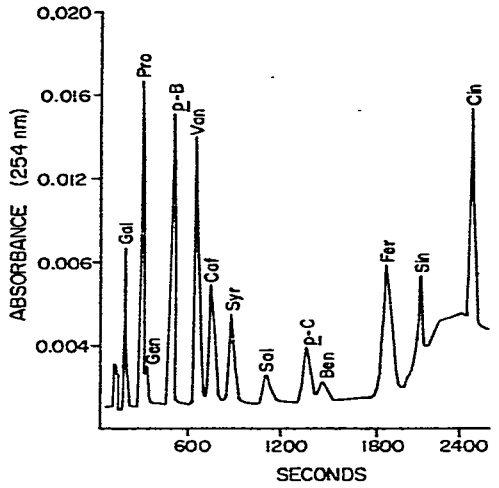


Fig. 1. Chromatogram of separation of phenolic acids on μBondapak C_{18} at 1 ml/min flow-rate. Gal = Gallic acid; Gen = gentisic acid; Pro = protocatechuic acid; p-B = *p*-hydroxybenzoic acid; Sal = salicylic acid; Van = vanillic acid; Caf = caffeic acid; Syr = syringic acid; Ben = benzoic acid; p-C = *p*-coumaric acid; Fer = ferulic acid; Sin = sinapic acid; Cin = cinnamic acid.

Fig. 2. Chromatogram of separation of phenolic acids of Radial-Pak A at 2 ml/min flow-rate. Peaks as in Fig. 1.

Several advantages may be afforded by using the Radial-Pak A as compared to the μBondapak C_{18} system. Even though the actual length of the analyses was comparable between the two systems, more samples were processed in the same amount of time using the Radial-Pak A. The minimal 5-min gradient required for return to initial conditions on the μBondapak C_{18} was not required with the Radial-Pak A column. Several samples could be analyzed prior to purging the Radial-Pak A with methanol as compared to purging after each sample processed on the μBondapak C_{18}. Pressure and clogging problems often plague the user of the stainless steel columns; however, no such problems were encountered with the Radial-Pak A. The end result was that more samples could be analyzed in a shorter time on the Radial-Pak A.

Both procedures were used in the analysis of plant phenolics isolated from soybeans, (*Glycine max* L. Merrill). Chromatograms representing the separation of phenolic acid aglycones from "Davis" cultivar are presented in Fig. 3. The isolation on the μBondapak C_{18} column represents a separation achieved using a flow-rate of 2 ml/min. At the 2 ml/min flow-rate peaks appear closer together; however, there is no difference in the number of peaks. A better separation of the initial eluting compounds was achieved on the Radial-Pak A. Identification of gallic acid and protocatechuic acids was facilitated by the enhanced separation of the initial substances on the Radial-Pak A.

Previous publications suggest that flavonoid compounds may be present in plant tissue along with the simple benzoic and cinnamic acid conjugates[2,3]. Flavonoids such as naringenin, hesperetin, quercetin, and kaempferol have been included in analytical procedures along with the phenolic acids[2,3]. The Radial-Pak A offers a selectivity not proffered by the μBondapak C_{18} column. Under the conditions defined, neither the coumarins nor flavonoids included in other studies elute from the

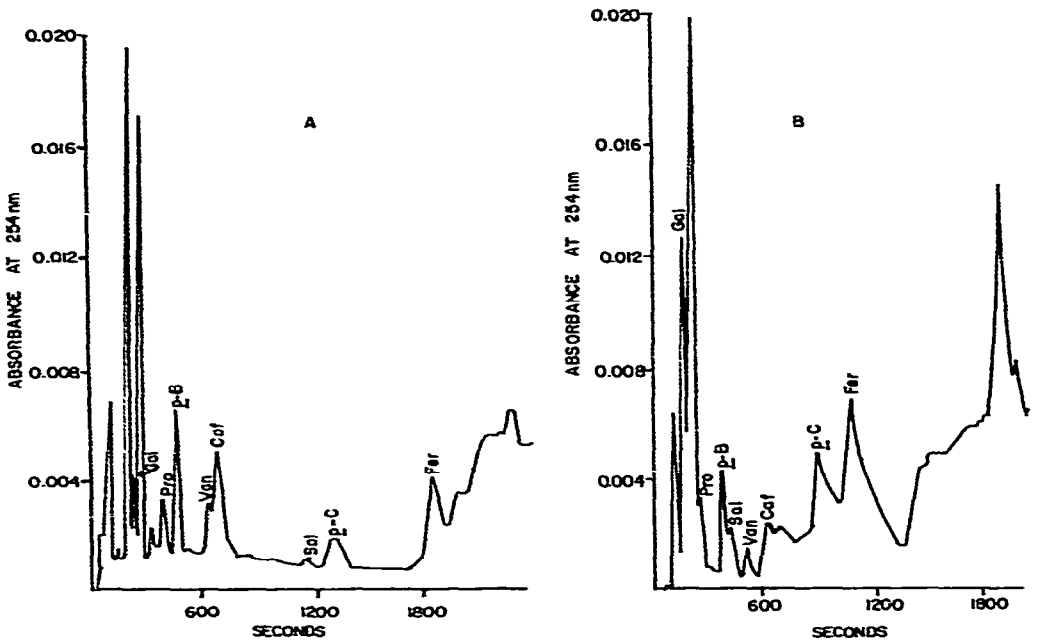


Fig. 3. Chromatogram of separation of phenolic acids extracted from soybean tissue on Radial-Pak A (A) and μBondapak C_{18} (B) (flow-rate 2 ml/min). Peaks as in Fig. 1.

Radial-Pak A column. Under previously described conditions, satisfactory separation of all the phenolic acids, coumarins, and flavonoids included in the study was not achieved[2]. Thus, the use of the solvent scheme on the Radial-Pak A selects for analysis of the benzoic and cinnamic acids alone.

Satisfactory separation of the benzoic and cinnamic acids has been achieved on both the μBondapak C_{18} and the Radial-Pak A columns. Enhanced resolution of the early eluting phenolics was observed on the Radial-Pak A. More efficient use of time and exclusion of the higher phenolic compounds from the separation may also be considered advantages of the procedure for isolating the benzoic and cinnamic acids on the Radial-Pak A. These advantages can facilitate the evaluation of phenolic content in plant tissue.

REFERENCES

1 J. B. Murphy and C. A. Stutte, *Anal. Biochem.*, 86 (1978) 220–228.
2 J. M. Hardin and C. A. Stutte, *Anal. Biochem.*, 102 (1980) 124–128.
3 L. W. Wulf and C. W. Nagel, *J. Chromatogr.*, 116 (1976) 271–279.
4 C. W. Nagel and L. W. Wulf, *Amer. J. Enol. Vitic.*, 30 (1979) 111–116.
5 M. Wilkinson, J. G. Sweeney and G. A. Iacobucci, *J. Chromatogr.*, 132 (1977) 349–351.
6 R. E. Carlson and D. Dolphin, *J. Chromatogr.*, 198 (1980) 193–197.
7 M. Vanhaelen and R. Vanhaelen-Fastré, *J. Chromatogr.*, 187 (1980) 255–260.
8 D. Strack and J. Krause, *J. Chromatogr.*, 156 (1978) 359–361.
9 J. M. Hardin and C. A. Stutte, *J. Chromatogr.*, 208 (1981) 124–128.
10 W. S. Hancock, J. D. Capra, W. A. Bradley and J. T. Sparrow, *J. Chromatogr.*, 206 (1981) 59–70.
11 B. L. Karger, in J. J. Kirkland (Editor), *Modern Practice of Liquid Chromatography*, Wiley-Interscience, New York, 1971, p. 3.
12 L. R. Snyder, in J. J. Kirkland (Editor), *Modern Practice of Liquid Chromatography*, Wiley-Interscience, New York, 1971, p. 125.