Analysis of Phenolic Acids by High Performance Liquid Chromatography Using a Step-Wise Gradient

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A high performance liquid chromatographic method for analyzing phenolic acids on reverse phase C-18 is described. Twelve substituted benzoic and cinnamic acid derivatives as well as the parent acids were resolved in less than 25 min. Separation was achieved by using a step-wise gradient of three different solvent systems and by adding ammonium acetate for preventing intramolecular hydrogen bonding of the acids.

The applicability of the described method to crude plant extracts is demonstrated with *Adenostoma fasciculatum, Adenostoma sparsifolium* and *Phyllospadix scouleri.* The percent composition of the phenolic acids within the three extracts is given.

Introduction

Interest in phenolic acids has increased with the discovery of their ability to act as protective agents against pathogens, as feeding deterrents against insect pests and grazing animals [1, 2] and as allelopathic agents [3]. The growing importance of phenolics as biologically active agents has produced several efforts to characterize them quantitatively. Paper chromatography (PC) or thin-layer chromatography (TLC) are the most common methods used for the detection and separation of phenolic acids from plant extracts [4, 5] but often these traditional chromatographic techniques are time consuming and lack exact quantitative measurements. Gas-liquid chromatography (GLC) provides both separation and quantification but requires purification and derivatization [5].

These disadvantages are easily avoided with the use of high performance liquid chromatography (HPLC) by which crude plant extracts can be analyzed qualitatively and quantitatively requiring only a short analysis time. The use of the HPLC in separating phenolic acids has been reviewed in recent articles [6–10]. In this present study, we report the separation of 14 common phenolic acids on reverse phase HPLC using a step-wise gradient with three different solvent-systems. The application of the described method to plant extracts is shown with two Californian Chaparral shrubs, *Adenostoma fasciculatum* and *A. sparsifolium,* and with a seagrass, *Phyllospadix scouleri. Adenostoma fasciculatum* (chamise) has previously been shown to contain phytotoxic phenolic acids that inhibit seed germination and seedling growth [11].

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Materials and Methods

Adenostoma fasciculatum and *A. sparsifolium* were collected in October 1980 at Oak Grove Campground and at Highway 79, North San Diego County, California. *Phyllospadix scouleri* was collected at Cape Arago, Oregon in July, 1980.

Extraction of phenolic acids from young leafy stems of *Adenostoma* was carried out in boiling methanol [4]. Phenolic acids from *Phyllospadix* were extracted with water on a shaker overnight after grinding the dry material [12]. The extracts were filtered and bound phenolic acids hydrolized with 2 N NaOH under nitrogen agitating on a shaker for four hours and then acidified with dilute HCL [4, 12]. The free phenolic acids were then extracted by partitioning against ethyl-acetate.

The ethyl-acetate fractions of *Adenostoma* were evaporated to dryness, dissolved in 80% aqueous methanol and partitioned against *n*-pentane. The methanol was evaporated, the remaining water-fractions slightly acidified to keep the acids protonized and partitioned against 1,2-dichloroethane and ethyl-acetate [13]. The obtained organic fractions were evaporated to dryness and dissolved in methanol for HPLC analysis.

Standard phenolic acids were purchased from Sigma Chemical Company, St. Louis, Mo. and from Aldrich Chemical Company, Milwaukee, Wisc. Cinnamic acid and its derivatives were of the *trans* configuration.

The liquid chromatograph used was Waters and included a solvent pump model 6000A and an universal injector model U6K. Detection of the acids was achieved with an ultraviolet absorbance detector model 440 at 254 nm. Solvent changes were recorded by the use of a refractive index model R401. The chromatographic column (250 × 4 mm) was prepacked with Lichrosorb RP 18 (10 µm, porous size), Alltech Associates, Los Altos, Ca.

Elution was achieved by switching between three different solvent systems. System 1 was H_2O/MeOH/HOAc (75:25:1 v/v), system 2 was H_2O/MeOH/HOAc (70:30:1 v/v) containing 9.0×10^{-2} M ammonium acetate and system 3 H_2O/MeOH/HOAc (50:50:1 v/v). Switching was done from 1 to system 2 eight minutes after injection and from system 2 to system 3 sixteen minutes after injection. Retention times were obtained by measuring the chromatograms, chart-speed was 1 cm/min. Peak areas were calculated by height times width at half height. For calculating the capacity factor k' and the relative retention α the equations

$$k' = \frac{t_R - t_0}{t_0} \quad \text{and} \quad \alpha = \frac{k'_2}{k'_1} \quad \text{were used [14].}$$

t_R = retention time of compound, t_0 = retention time of non-retained solvent, k'_2 = capacity factor of compound 2, k'_1 = capacity factor of compound 1. t_0 was 2.2 min.

Results and Discussion

In recent years, numerous articles have appeared on the use of HPLC to analyze phenolic acids from plant extracts [6–10]. Attempts to use isocratic separations required high flow rates (3–4 ml/min) when trying to avoid extreme peak broadening and

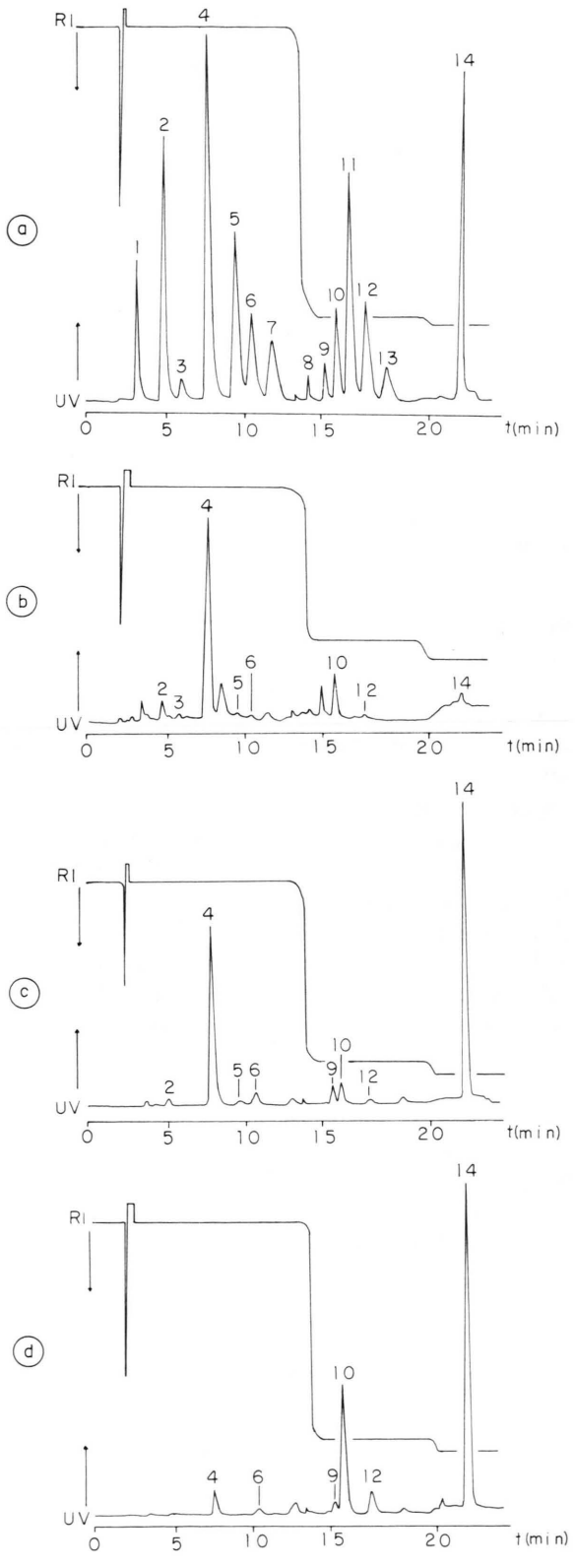

Fig. 1 a. HPLC resolution of a standard mixture of substituted benzoic and cinnamic acids on Lichrosorb RP-18. b–d. HPLC resolution of extracted phenolic acids from b) *Phyllospadix scouleri;* c) *Adenostoma fasciculatum;* d) *Adenostoma sparsifolium.* For peak identification see No. in Table I; UV = absorbance of UV detector at 254 nm; RI = response of refractive index detector caused by solvent changes.

Table I. Retention times (t_R), capacity factors (k'), and relative retentions (α) of analyzed substituted benzoic and cinnamic acids.

No.	Name	t_R [min:sec]	k'	α
1	gallic	3:18	0.5	
2	protocatechuic	5:00	1.27	2.54
3	gentisic	6:21	1.88	1.48
4	*p*-hydroxybenzoic	8:00	2.63	1.39
5	vanillic	10:15	3.70	1.40
6	caffeic	11:18	4.13	1.11
7	syringic	13:00	4.90	1.18
8	salicyclic	14:18	5.50	1.12
9	benzoic	15:36	6.09	1.10
10	*p*-coumaric	16:24	6.45	1.05
11	veratric	17:24	6.90	1.06
12	ferulic	18:24	7.36	1.06
13	sinapic	19:54	8.04	1.09
14	cinnamic	23:57	9.86	1.22

Numbers refer to Fig. 1 a.

long retention times [15]. The resulting high back-pressures can rapidly decrease the separation quality and shorten the life of analytical columns. One of the most extensive studies so far has been done by Murphy and Stutte [16], who demonstrated the separation of thirteen phenolic acids on reversed phase HPLC using a linear gradient program. Since not all liquid chromatographs are equipped for linear gradient programing it was our aim to develop a separation technique using only one solvent pump but resembling the quality and speed of linear gradient programing. Our results show that good separations can be obtained by using a step-wise gradient system. Fig. 1 a illustrates the separation of twelve common substituted benzoic and cinnamic acids as well as the two parent acids within a total time of less than 25 minutes by switching between three different solvent systems and using a reverse phase C-18 column. The applied flow rates were 1.5 ml/min resulting in back-pressures of less than 3500 psi. Table I lists the analyzed acids, and their retention times. Two chromatographic parameters, the capacity factor k' and the relative retention α were calculated from the retention times and are also given in Table I to represent the selectivity and resolution of the demonstrated method. In a multi-component sample the k' values should be in an optimum range of $1 \leqq k' \leqq 10$ [17]. Only one of our k' values falls slightly outside that range. As a rough guide α values $\geqq 1.05-1.10$ are required for a resolution of 1 (98% separation) between two components on the used analytical column representating a number of effective theoretical plates of approximately 4000 [18]. Table I shows that all of the calculated α values fit well into that range.

Salicyclic, benzoic, *p*-coumaric, veratric and ferulic acid were only partially resolved when solvent systems consisting of various amounts of water, methanol and acetic acid were applied. Only addition of ammonium acetate to the solvents, as suggested by Murphy and Stutte to prevent intramolecular hydrogen bonding [16], yielded clear separation within this group. Whereas these two authors recommended a concentration of 1.8×10^{-2} M ammonium acetate we could achieve our reported separations only with a content of 9.0×10^{-2} M ammonium acetate in solvent system 2. Lower concentrations caused increasing tendency within this group to cochromatograph. Addition of ammonium acetate also to solvent 1 resulted in decreasing peak symmetry and separation quality of gallic, protocatechuic and gentisic acid and the elution of caffeic and syringic acid as one peak.

Fig. 1 b, c, d demonstrates the applicability of the described method to crude plant extracts. Nine phenolic acids from the hydrolized extracts of *Adenostoma*

Table II. Percentage content of phenolic acids extracted from *Phyllospadix scouleri*, *Adenostoma fasciculatum* and *Adenostoma sparsifolium*.

No.	Compound	*Phyllospadix scouleri*	*Adenostoma fasciculatum*	*Adenostoma sparsifolium*
2	protocatechuic acid	+	+	+
3	gentisic acid	+		
4	*p*-hydroxybenzoic acid	52.9	26.8	+
5	vanillic acid	+	+	+
6	caffeic acid		4.0	+
9	benzoic acid		6.1	+
10	*p*-coumaric acid	47.1	10.6	63.0
12	ferulic acid	+	+	2.7
14	cinnamic acid	+	52.5	34.3

Values are given in (%). (+) means less than 0.5%.
Numbers refer to Fig. 1 b, c, d.

and *Phyllospadix* could be tentatively identified by comparison of retention times and co-chromatography with known standards. Purification of the plant extracts previous to HPLC analysis was not necessary. For more complex extracts we recommend the partitioning procedure as outlined for *Adenostoma* in this work. None of the reported phenolic acids were found in the pentane layer, whereas all cinnamic acids were contained in the 1,2-dichloroethane layer. Other phenolic acids were detected in the ethylacetate layer. Quantitative analyses of the phenolic acid concentrations was carried out using external standards to determine the UV response curve. Amounts of 0.6–5 µg of acid were within the Beers-Lambert linear range. Percent concentrations of the plant extracts are given in Table II. The extract of *Phyllospadix scouleri* consisted mainly of *p*-hydroxybenzoic and *p*-coumaric acid in nearly equal amounts. *Adenostoma fasciculatum* and *A. sparsifolium* showed hardly any qualitative but significant quantitative differences in the amounts of their phenolic acids. The main component of *Adenostoma fasciculatum* was cinnamic acid (52.5%), followed by *p*-hydroxybenzoic acid (26.8%) and *p*-coumaric acid (10.6%). *p*-Coumaric acid predominant (63%) within *Adenostoma sparsifolium*, cinnamic acid was here found at a lower content (34.3%), whereas *p*-hydroxybenzoic acid could only be detected in minor amounts (below 0.5%).

Acknowledgements

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