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Chromatographic Determination of Phenolic Acids in the Snowdrop by HPLC

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Free and esterified phenolic acids of *Galanthus elwesii* (snowdrop) were extracted with petroleum ether and determined by HPLC. Cinnamic, ferulic, vanillic, p-coumaric, p-hydroxy benzoic, and caffeic acids were detected by a linear gradient elution with acetic acid/water (2:98 v/v) and acetic acid/acetonitrile/water (2:30:68 v/v) as the mobile phases in 30 min with a flow rate of 1.5 mL/min. Among the acids from snowdrop extracts, p-hydroxy benzoic was the major constituent followed by vanillic and ferulic acids.

Key Words: Galanthus elwesii (snowdrop), phenolic acids, HPLC.

Introduction

The species of *Galanthus* are an important genus of the family Amaryllidaceae. Of the species, *Galanthus elwesii* Hooker (snowdrop) (Kardelen in Turkish) is a native of Asia Minor and is locally distributed in the North and South Anatolian mountainous districts. Studies have been carried out on the alkaloids[1,2] and mannose specific lectins[3,4] of snowdrop samples. There is no report regarding the phenolic acids of this plant.

Various plants have been analysed with respect to phenolic acids by HPLC[5−8]. Plant acids are known to have anticarcinogenic activity[9]. Phenolic compounds are believed to be an important part of the general defence mechanism of many plants to infections[10,11] and they may play an important role in the resistance of bulbous plant parts to soft rot bacteria[12−15]. Purification of phenolic acids is very difficult due to their isomeric similarities and various effects such as acid-base treatment, temperature and light on their labile structures[16].

The determination of phenolic acids is important both for their characterization and to facilitate more efficient uses of the important plant resources[17,18].

There are several HPLC methods for the determination of phenolic acids in fruit juices and plant bulbs[19−21]. In order to separate the phenolics chemically, potassium salts of phenolic acids, present in the

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saponification solution, are converted to phenols by bubbling carbon dioxide[22], and the formed free phenolic acids are extracted with petroleum ether[23].

This article describes a determination of nonvolatile phenolic acids obtained on TLC and column chromatography, which were confirmed by HPLC with acetic acid-water and acetonitrile-acetic acid-water solvent systems.

Experimental

Chemicals. Standard acids (p-coumaric acid, vanillic acid, p-hydroxy benzoic acid, caffeic acid, ferulic acid, and cinnamic acid) were purchased from E. Merck. Solvents, acetic acid, acetonitrile, chloroform, ethyl acetate, petroleum ether (b.p. 40° -60°), diethyl ether, and methanol were supplied by Fluka and E. Merck. *Galanthus elwesii* Hooker (snowdrop) samples were collected from the Sürmene district in Trabzon (Turkey).

Standard Solutions. Stock solutions of the standard acids were prepared in a concentration of l.0 g/100 mL in pure methanol. The working solutions of samples obtained from snowdrop stalk and leaves were prepared at a concentration of 1.0 g/100 mL in methanol.

Chromatographic Equipment. The acid mixtures were separated on a Shimadzu LC-9A model HPLC equipped with a manual injector, a programmable wavelength photodiode array UV detector (200-400 nm), and column packing with modified silicagel (C_{18} column). The column was thermostated to 25°C by a column temperature control module. Integrations and data storage were carried out by means of a Pentium-III computer.

Extraction of the acids. The acids are present as free and esterified forms in the plant. Ground snowdrop samples (65 g) were extracted with petroleum ether (b.p. 40-60°C) and the extract was acidified using concentrated H_2SO_4 to liberate free acids. The free acids were extracted with ethyl acetate from aqueous solution. In order to recover phenolic acids, the extract was washed with 2.0% $NaHCO_3$ aqueous solution using a separatory funnel. The aqueous $NaHCO_3$ layer was acidified and then hydrolysed separately by using 8.0% HCl and 2N NaOH solutions for 4 h under N_2 atmosphere. The mixture was filtered, acidified and then subsequently extracted with petroleum ether, chloroform, diethyl ether and ethyl acetate.

HPLC Analysis. HPLC separation was accomplished on a Shimadzu LC-9A model instrument. For analysis a linear gradient elution programme was applied, and elution was carried out with solvent A (acetic acid/water (2:98 v/v)) and solvent B (acetic acid/acetonitrile/water (2:30:68 v/v)) as mobile phase. During HPLC analysis the solvent gradient was programmed from 10 to 100% B in A in 30 min with a flow rate of 1.5 mL/min. The standards were analysed under the same conditions, before the mixture of acids obtained from snowdrop samples.

Results and Discussion

The principal tests for the determination of phenolic acids on chromatograms are given in Table 1. The effect of eluent polarity on the phenolic acids in column chromatography is given in Table 2. Elute fractions taken from the column were connected with respect to similarity on TLC with R_f values, 1-7, 8-14 and 15-85. The retention factor (R_f) of each standard acid obtained by different solvent systems is presented in Table 3. The polarity and choice of solvent system are important factors in the separation of phenolic acids which can be eluted on the silicagel column or the preparative TLC. The isolation of phenolic acids

was carried out by preparative TLC.

In this study, we report the results of a simple and rapid gradient HPLC method with photodiode array UV detection for six phenolic acids in snowdrop samples. The phenolic acids were detected at both 280 nm and 360 nm. All phenolic acids were identified by matching the retention time and their spectral characteristics against those of standards. Absorption spectra were recorded on a Jasco V-530 model UV-vis spectrophotometer. The retention times (min) and UV-vis maximum absorption wavelengths (nm) of phenolic acids were 16.663 and 274 for cinnamic acid, 13.754 and 321 for ferulic acid, 8.700 and 259, 290 for vanillic acid, 11.386 and 307 for p-coumaric acid, 6.269 and 254 for p-hydroxy benzoic acid and 9.779 and 217, 324 for caffeic acid, respectively. These values are in agreement with the standards. Figure 2 shows the HPLC chromatograms of the phenolic acids present in different elute fractions from column chromatography. The weight percent of eluents and total acids in the extracts obtained from snowdrop samples by HPLC are given in Table 4. The average yields acid from all of elute fractions are p-hydroxy benzoic 26.9%, vanillic 17.3%, ferulic 16.6%, p-coumaric 15.1%, caffeic 15.8%, and cinnamic 8.3%. Among the phenolic acids from snowdrop extracts, p-hydroxy benzoic was the major constituent followed by vanillic and ferulic acids. The retention times for each acid are given in Figure 2 (a,b,c).

Table 1. Principal Test for the Determination of Phenolic Acids on Chromatograms.

Acids	UV	UV + NH_3	$FeCl_3$	$FeCl_3$ + $K_3Fe(CN)_6$	Bromcresol green	Vanillin + H_2SO_4
Cinnamic	-	blue-green	dark yellow	yellow	yellow	-
Ferulic	blue	blue-green	red coffee	dark blue	yellow	light violet
Vanillic	-	-	-	blue	yellow	pink
p-coumaric	-	blue-violet	orange	blue	yellow	dark violet
p-hydroxy benzoic	-	-	yellow	light blue	yellow	-
Caffeic	blue	bright blue-green	green	dark blue	yellow	violet

Table 2. Effect of Eluent on the Phenolic Acid in Column Chromatography.

Elute Fractions	Solvent System (v/v)	Acid
1-7	chloroform-ether (95:5)	cinnamic, ferulic, and vanillic
8-14	chloroform-ether (90:10)	vanillic, p-hydroxy benzoic, and p-coumaric
15-62	chloroform-ether (75:25)	p-coumaric, ferulic
63-75	chloroform-ether (40-60)	caffeic
76-85	ether-methanol (95:5)	caffeic
86-89	ether-methanol (90:10)	polar impurity

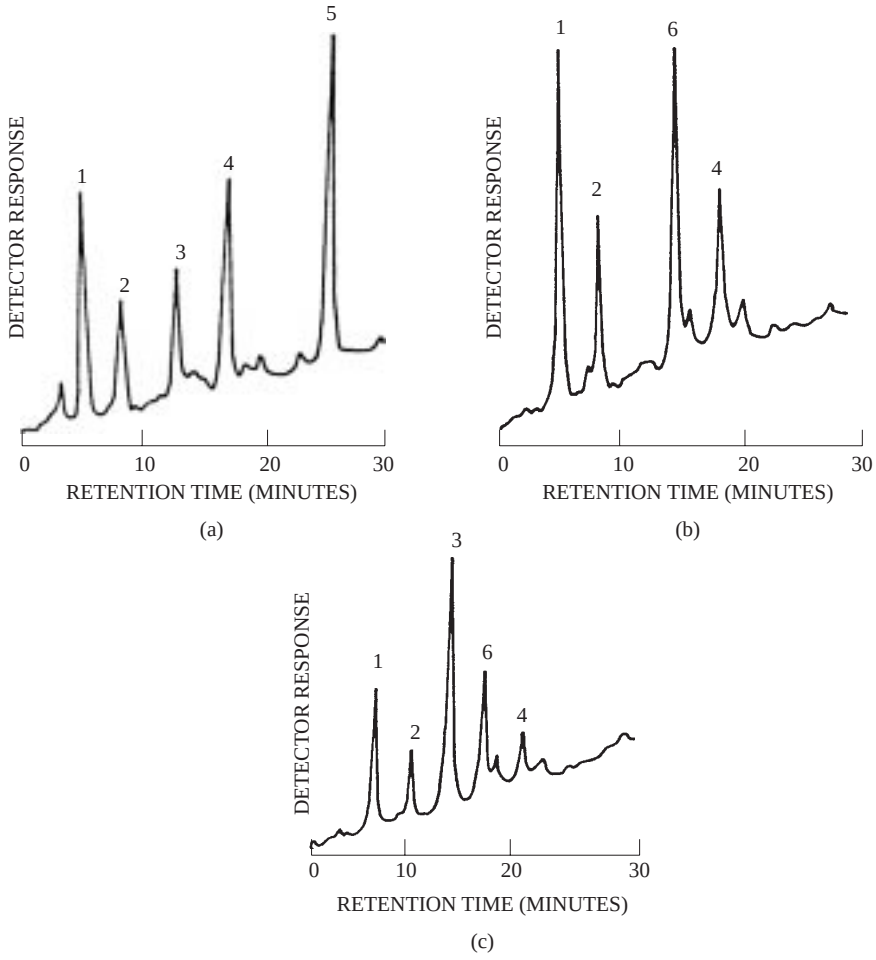

Figure 1. HPLC Chromatograms of the Phenolic Acids Present Different Elute Fractions from Column Chromatography. (a) 1-7, (b) 8-14, (c) 15-85, Elute Fractions: (1) p-hydroxy benzoic acid, (2) vanillic acid, (3) caffeic acid, (4) ferulic acid, (5) cinnamic acid, (6) p-coumaric acid.

Table 3. TLC R_f Values and HPLC Retention Times of Phenolic Acids.

Acids	R_f values				Retention Times (min)
	I	II	III	IV	
Cinnamic	0.96	0.90	0.68	0.93	16.663
Ferulic	0.92	0.83	0.51	0.87	13.754
Vanillic	0.92	0.83	0.56	0.87	8.700
p-coumaric	0.92	0.68	0.34	0.73	11.386
p-hydroxy benzoic	0.92	0.62	0.34	0.73	6.269
Caffeic	0.86	0.42	0.13	0.55	9.779

I: Et OAC - Toluene- CH_3COOH (50:40:20)
II: $CHCl_3$-CH_3COOH (90:10)
III: C_6H_6-CH_3COOH-H_2O (37:45:18)
IV: $CHCl_3$-CH_3 COOH (80:20)

Table 4. Weight Percent of Eluents and Total Acids in the Extracts Obtained from Snowdrop by HPLC.

Elute Fractions	Acids	% of Elute	% of Total Acids
1-7	p-hydroxy benzoic	9.6	24.0
	vanillic	4.7	11.9
	caffeic	6.0	15.0
	ferulic	9.7	24.1
	cinnamic	10.0	25.0
8-14	p-hydroxy benzoic	15.6	32.1
	vanillic	10.5	21.6
	p-coumaric	13.6	28.0
	ferulic	8.9	18.3
15-85	p-hydroxy benzoic	15.3	24.7
	vanillic	11.4	18.4
	caffeic	20.2	32.6
	p-coumaric	10.3	17.0
	ferulic	4.5	7.3

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