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Plant hormones, specialized chemical substances produced by plants, are the main internal factors controlling growth and development. In this study the pH and polarity of the mobile phase were taken into consideration to optimize the mobile phase for the chromatographic separation of 3 important plant hormones: abscisic acid (ABA), indole-3-acetic acid (IAA) and gibberellic acid (GA_3). pK_a values of ABA, IAA and GA_3 were determined using retention factors. These 3 hormones were extracted from 99 R (*Vitis berlandieri* x *Vitis rupestris*) and rose oil (*Rosa damascena* Mill.) and the chromatographic method developed was used for the separation of these hormones.

Key Words: Abscisic acid, gibberellic acid, indole-3-acetic acid, plant hormones, resolution, RP-HPLC.

Introduction

Plant hormones, specialized chemical substances produced by plants, are the main internal factors controlling growth and development[1−3]. They regulate most of the life cycle events in plants such as cell division and extension, seed and bud dormancy, seed germination, flowering, fruit set and ripening and cutting rooting[4,5].

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Hormones occur in plant material at a very low concentration (ng g^{-1}), together with many other compounds. Accurate analysis of trace quantities of hormones in complex mixtures such as plant extracts requires a sophisticated analytical technique. Recently reversed phase high performance liquid chromatography (HPLC) is the most preferable method for the separation and determination of plant hormones, but unfortunately there are few studies on optimizing the chromatographic separation of plant hormones[6−12].

This research was carried out to optimize mobile phase composition during the separation of indole-3-acetic acid (IAA), gibberellic acid (GA_3), and abscisic acid (ABA). The pH of the mobile phase has an important effect on optimizing the chromatographic separation for acidic compounds, since the degree of ionization of acids, stationary phases and mobile phase additives may be affected by the pH. In this study the influence of pH of the mobile phase on the chromatographic separation was investigated, and pK_a values of the plant hormones studied were determined from chromatographic data.

Moreover, in order to investigate the optimized condition, IAA, GA_3 and ABA were studied in 99 R (*Vitis berlandieri* x *Vitis rupestris*) and rose oil (*Rosa damascena* Mill.) by reversed phase HPLC.

Experimental

Chemicals and reagents

Analytical reagent grade chemicals were used, unless otherwise indicated. Water, with a conductivity lower than 0.05 μS/cm, and acetonitrile (Merck) were of HPLC grade. Sodium hydroxide (Merck) and phosphoric acid (Merck) were used for pH adjustment. Potassium hydrogen phthalate (dried at 110 °C before use, Fluka) was used as the referance value standard. Methanol, phosphate buffer components, ethyl acetate, hydrochloric acid, diethyl ether and sodium sulfate were all of analytical purity (Merck) and were used for extraction of the plant hormones.

ABA, IAA and GA_3 were all of standard purity (Sigma) and used without further purification. The stock standard solutions (1000 ppm) of these hormones for LC studies were prepared by dissolving in the HPLC mobile phase. Working solutions were prepared by diluting the stock solutions with the same HPLC mobile phase at appropriate concentrations. Working solutions were prepared fresh on the day of use. These solutions were filtered before injections.

Plant material

Shoots of 99 R (*Vitis berlandieri* x *Vitis rupestris*), a very important rootstock for grape varieties, and rose oil (*Rosa damascena* Mill.), a very important essential oil plant, were used as plant material. The samples were taken at the beginning of the flowering stage.

Extraction

Ten grams of fresh tissue per sample was homogenized with 70% (v/v) methanol and stirred overnight at 4 °C. The extract was filtered through a Whatman filter and the methanol evaporated under vacuum. The aqueous phase was adjusted to pH 8.5 with 0.1 M phosphate buffer and then partitioned with ethyl acetate 3 times. After removal of the ethyl acetate phase, the pH of the aqueous phase was adjusted to 2.5 with 1 N HCl. The solution was partitioned with diethyl ether 3 times, and then passed through andydrous sodium

sulfate. After that the diethyl ether phase was evaporated under vacuum and the dry residue containing hormones was dissolved in 2.0 mL of methanol and stored in vials at 4 °C[13,14].

Apparatus

The chromatographic analysis was performed on a Shimadzu Model LC. The chromatographic system consists of a Shimadzu Model LC 10 ADVP pump with an auto injector (SIL 10 AD VP) and diode array detector (SPDM 10 A DAD). This equipment has a column oven (CTO 10 AVP) and a degasser system (DGU 14 A). The column used was a Luna C_{18}, 250 mm x 4.6 I.D. stainless steel analytical column with 5 μm particle size (Phenomonex).

The e.m.f. values used to evalute the pH of the mobile phase were measured with a Mettler Toledo MA 235 pH/ion analysis apparatus using a Hanna HI 1332 Ag/AgCl combination pH electrode. All solutions were externally thermostated at 25 °C ± 0.1 °C. The electrode was stabilized in appropriate acetonitrile-water mixtures before the e.m.f. measurements. In this study, pH measurements in acetonitrile-water binary mixtures were performed by taking into account the operational definition of pH[15−18].

Chromatographic procedure

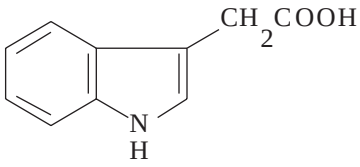
Throughout this study, the mobile phases used were acetonitrile-water (26:74; 30:70% ; v/v). In these media, 30 mM phosphoric acid was adjusted to different pH values with sodium hydroxide. The Luna C_{18} column was equilibrated for each mobile phase condition with a time limit of 30 min. The column temperature was maintained at constant 25 ± 0.1 °C. The separation was carried out by isocratic elution with a flow rate of 0.8 mL/min. An injection volume of 10 μL was used for each analysis.

The standard solution of the individual acid was prepared in the mobile phase and chromatographed separately to determine the retention time for each acid. The signal of the compounds was monitored at 208, 265 and 280 nm for GA_3, ABA and IAA, respectively. Capacity factors were calculated from $k = (t_R\text{-}t_o)/t_o$, where $t_o$was the hold-up time, and $t_R$was the retention time of each hormone for each mobile phase. In this equation the hold-up time, t_o, was established for every mobile phase composition using potassium bromide solution [Merck, 0.01% (w/v) in water, λ_{max}= 200 nm]. The retention times and capacity factors of the solutes were determined from 3 different injections. Peak identification was based on retention time and spiking of the sample.

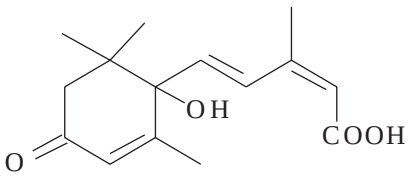
Results and discussion

Reversed phase HPLC has become popular for analyzing mixtures of plant hormones because of its simplicity, rapidity and reliability. In this study, we investigated the effect of 2 properties of the mobile phase on chromatographic separation. The pH of the mobile phase is a major factor influencing the chromatographic behavior of acidic compounds. GA_3, IAA and ABA contain carboxylic groups and their retention depends on the percentage of ionized and non-ionized species. The optimum pH of the mobile phase should be taken into account to study the influence of pH on retention in LC. In our study, taking this into account, we examined the dependence of the retention factor on the pH of the eluent. The mobile phase was adjusted to different pH values in order to select a suitable pH condition for chromatographic separation. Retention factor values, k, for the plant hormones studied (Figure 1) were determined in ACN-water mixtures at 26%

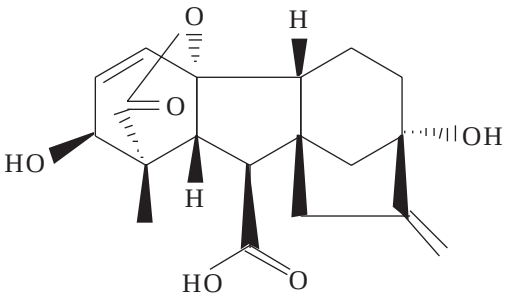
and 30% (v/v) of acetonitrile. Six pH values (4.0, 4.5, 5.0, 5.5, 6.0 and 7.0) were investigated for the mobile phase.



Indole-3-acetic acid



Abscisic acid



Gibberellic acid

Figure 1. Chemical formulae of the plant hormones studied.

The retention factor values obtained for the 2 mobile phases investigated are given in Table 1.

Table 1. Chromatographic capacity and selectivity factors of IAA, GA_3 and ABA at different pH values with 26% (v/v) acetonitrile and 30% (v/v) acetonitrile.

Compounds	pH											
	4.00		4.50		5.00		5.50		6.00		7.00	
	k	α	k	α	k	α	k	α	k	α	k	α
GA_3,26% (v/v)	1.03		1.05		0.91		0.62		0.41		0.35	
GA_3,30% (v/v)	0.82		0.77		0.64		0.45		0.19		0.11	
IAA,26% (v/v)	5.03	4.87	4.72	4.51	3.97	4.34	2.68	4.32	1.26	3.09	0.47	1.34
IAA,30% (v/v)	3.00	3.66	2.99	3.88	2.87	4.48	2.24	4.98	1.33	7.00	0.32	2.91
ABA,26% (v/v)	6.79	1.35	6.46	1.37	5.81	1.47	3.81	1.42	1.86	1.48	0.80	1.71
ABA,30% (v/v)	3.53	1.18	3.52	1.18	3.41	1.19	2.55	1.14	1.54	1.16	0.48	1.50

As seen in Figure 2 the relationships between k and pH are sigmoidal. Using these data and the program NLREG, the pKa values of the compounds were calculated (Table 2). All these results proved that the compounds are present in non-ionized form at approximately pH 4.0.

Table 2. pKa values of plant hormones from liquid chromatography measurements.

Compounds	Percentage of acetonitrile	
	26% (v/v)	30% (v/v)
Gibberellic acid	5.36 (0.15) *	5.42 (0.08)
Indole-3-acetic acid	5.46 (0.04)	5.88 (0.06)
Abscisic acid	5.51 (0.07)	5.82 (0.08)

*Standard deviation

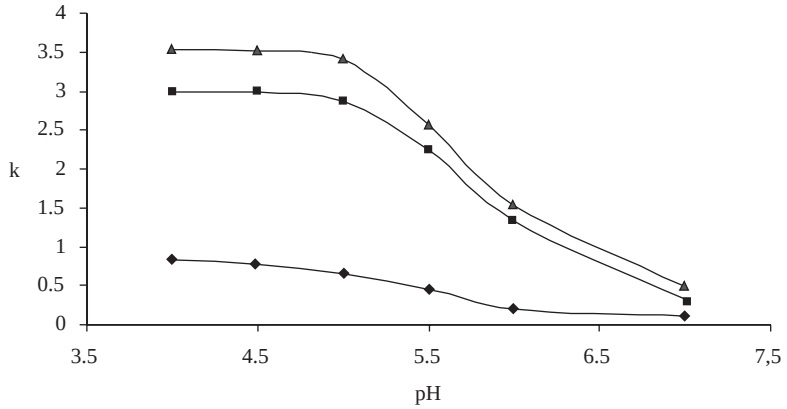


Figure 2. Plot of chromatographic capacity factor, k, of studied plant hormones vs. pH of mobile phase at 30% (v/v) ACN:, ♦, GA_3; ■, IAA; ▲, ABA.

We examined the effect of pH in the mobile phase on the resolution to select appropriate pH values for chromatographic separation. After examining pH 4.0 we concluded that this pH value was suitable for an original sample.

Based on the result of experiments we carried out the separation of the 3 compounds using this mobile phase and, as seen in Figure 3, the mobile phase is appropriate for the chromatographic separation.

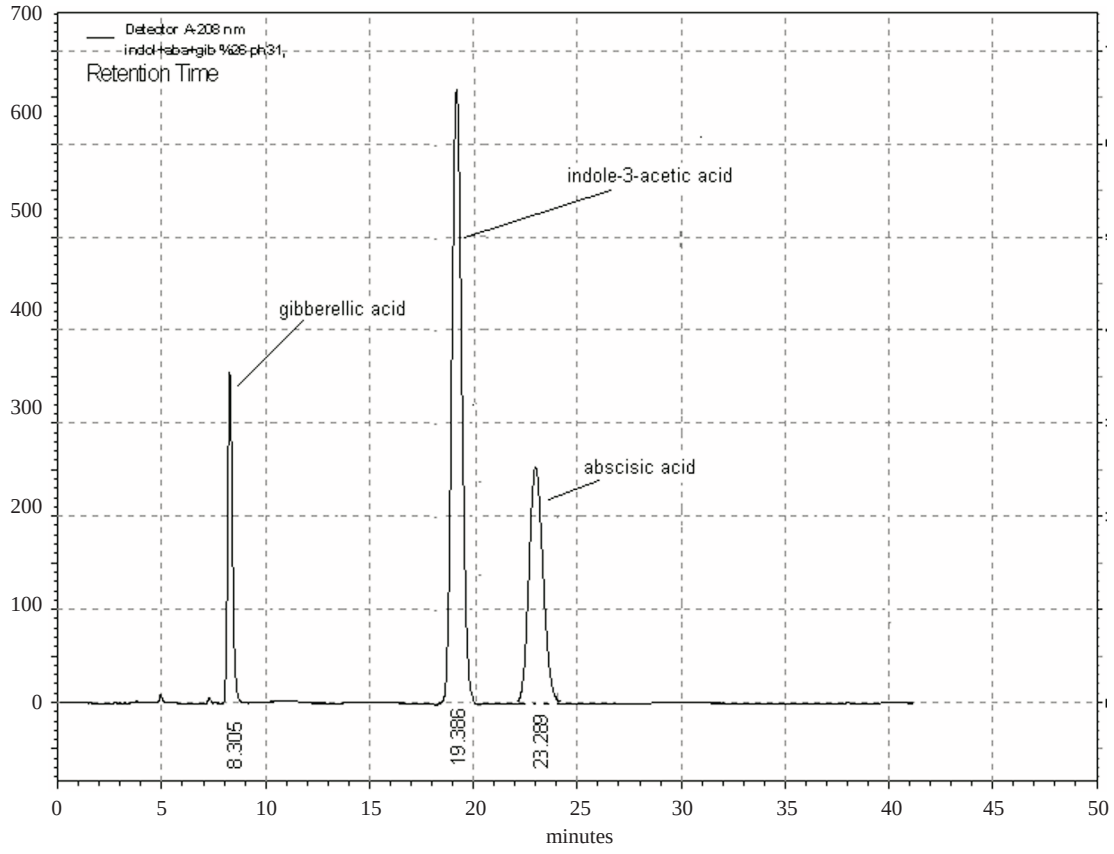


Figure 3. The chromatogram of the plant hormones studied in the acetonitrile-water, 26% (v/v); pH 4.00; Flow rate: 0.8 mL/min.

In this study, extracts of 99 R and rose oil were analyzed for these 3 plant hormones using these chromatographic conditions. Typical chromatograms for these acids in plant materials are shown in Figures 4 and 5.

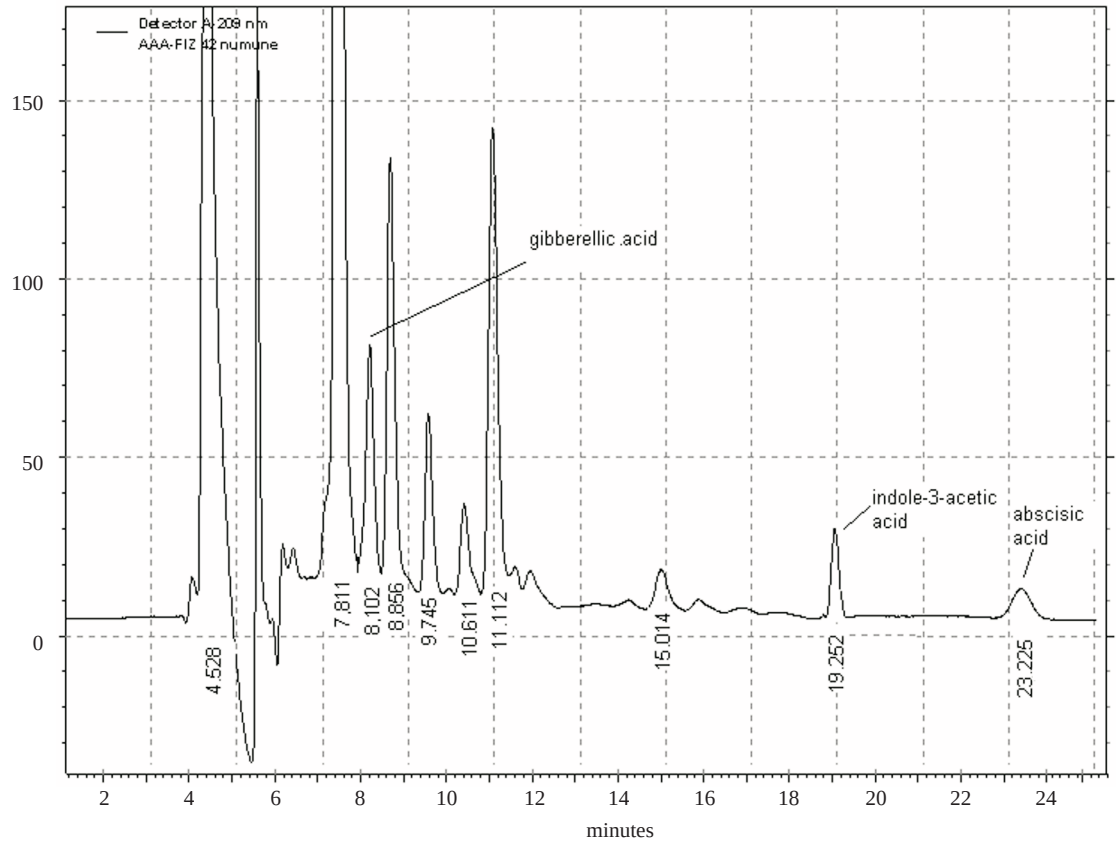


Figure 4. The chromatogram of 99 R (*Vitis berlandieri* x *Vitis rupestris*), 26% (v/v) acetonitrile containing 30 mM phosphoric acid at pH 4.00.

The GA_3, ABA and IAA content of 2 plant samples were determined in acetonitrile-water, 26% (v/v), containing 30 mM phosphoric acid at pH 4.00. Calibration graphs of these 3 substances were prepared using analytical reagent grade standards and the linear calibration range was evaluated statistically. Linear regression data for GA_3, ABA and IAA are listed in Table 3. The plant samples were spiked with the standards for calculation of their recovery percentages. The recovery percentages of GA_3, ABA and IAA were 98%, 108% and 97%, respectively. Concentrations of these 3 compounds were calculated from 1 sample of rose oil (*Rosa damascena* Mill.) and 99R (*Vitis berlandieri x Vitis rupestris*) using the area values and the results obtained were corrected for their recovery percentages. GA_3, ABA and IAA 95% confidence limits obtained by HPLC in rose oil were 15.54 ± 0.01, 4.68 ± 0.17 and 2.57 ± 0.01 ppm, respectively. Confidence limits of GA_3, ABA and IAA in 99R (*Vitis berlandieri* x *Vitis rupestris*) wer 9.42 ± 0.01, 2.96 ± 0.17 and 0.51 ± 0.02, respectively.

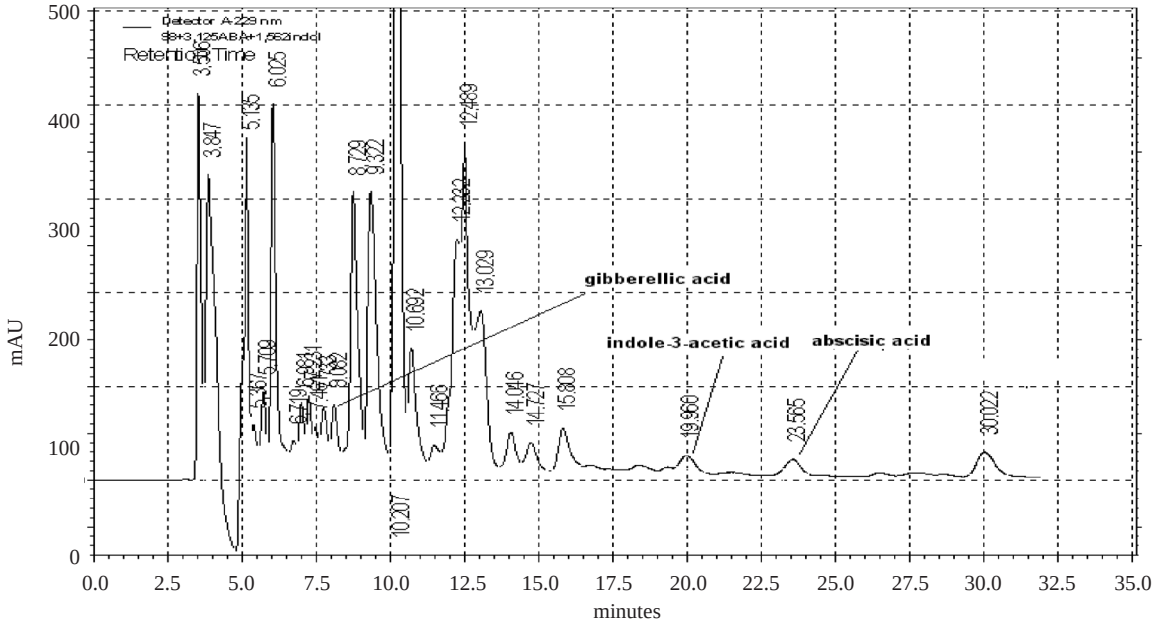


Figure 5. The chromatogram of rose oil (*Rosa damascena* Mill.), 26% (v/v) acetonitrile containing 30 mM phosphoric acid at pH 4.00.

Tablo 3. Linear regression data for GA_3, ABA and IAA.

Compounds	Calibration function	r	s^*_{xo}	CV**	Lineer range, ppm	N
GA_3	$(4478.4 \pm 12.7)C + (1692.3 \pm 655.7)$	0.9999	0.307	0.886	(5.23 – 125)	8
ABA	$(56856.7 \pm 664.8)C + (816.9 \pm 891.6)$	0.9998	0.020	1.803	(0.31 – 2.50)	5
IAA	$(29902.5 \pm 419.9)C + (2683.2 \pm 299.7)$	0.9996	0.012	1.911	(0.24 – 1.25)	6

*Standard deviation of method
**Coefficient variation of method

Conclusion

The pH and polarity of the mobile phase are 2 important parameters in the chromatographic separation of acidic compounds. In the present study, these 2 parameters were taken into consideration to optimize the mobile phase for the chromatographic separation of IAA, ABA and GA_3.

The percentage of ACN in the mobile phase is 26 by volume and the pH of the eluent is 4.00 under optimized conditions. The plant hormones studied can be separated using these conditions in 99 R (*Vitis berlandieri* x *Vitis rupestris)* and rose oil (*Rosa damascena* Mill.). We expect that these conditions can be used for the analysis of these hormones in other plant materials.

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