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Short communication

Reversed-phase C_{18} high-performance liquid chromatography of gibberellins GA_3 and GA_1 [1]

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Abstract

Gibberellins (GAs) are a group of plant growth hormones that are involved in the regulation of shoot elongation in many crop plants and the regulation of flowering in some biennials or photoperiodic plants. A reversed-phase HPLC procedure was applied for the separation of GA_3 and GA_1 with high resolution by means of ionic suppression, without the necessity for derivatization, using UV detection in fermentation broths. The reproducibility at these chromatographic conditions showed a 2% R.S.D. for GA_3, the recovery of GA_3 was higher than 95% and the detection limit for GA_3 standard solution was ca. 50 ng. GA_3 and GA_1 concentrations were determined for different fermentation cultures of the fungus *Gibberella fujikuroi*.

Keywords: Gibberellins; Plant hormones

1. Introduction

Gibberellins (GAs) belong to an important group of plant growth hormones involved in the control of different physiological processes such as: regulation of shoot elongation in many crop plants, regulation of flowering in some biennials or photoperiodic plants, fruit set, fruit external ripening and fruit development, and others [1–3].

At present, plant growth hormones are applied in agriculture to improve crop quality and yields. Industrial production of these plant growth hormones have been possible using fermentative processes from the action of some fungus on specific substrates and different methods have been established for their recovery and purification from fermentation medium [4].

High-performance liquid chromatography (HPLC) is now a routine procedure for the purification and separation of gibberellins (GAs). Reversed-phase C_{18} HPLC of gibberellins has been the most frequently used and reported HPLC system for separating free GAs [1–3,5–10]. The HPLC of some gibberellin derivatives have also been reported [6]. Different chromatographic methods have been developed for the determination of GAs such as: thin-layer chromatography (TLC) [11], gas chromatography (GC) [11], gas chromatography–mass spectrometry (GC–MS) [1,3,9] and gas chromatography–selected ion monitoring MS (GC–SIM-MS) [1,2].

According to the method developed by Barendse et al. in [7] we modified it for quantitative de-

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termination of GA_3 and GA_1 in a fermentation culture of the fungus *Gibberella fujikuroi*. An isocratic reversed-phase C_{18} HPLC of GA_3 and GA_1 is described in this paper. The ionic suppression technique, which involves the regulation of pH, was considered suitable for separating GAs, without the necessity for derivatization, using UV detection.

2. Experimental

2.1. High-performance liquid chromatography

The HPLC system consisted of the following components from Philips analytical (Cambridge, UK): a pump (PU 4100) and a variable-wavelength UV–Vis detector (PU 4110). The injector was a Rheodyne (Cotati, CA, USA) Model 7125. The column was a reversed-phase C_{18} column Spherisorb S5ODS1 (25 cm×4.6 mm I.D., 5 μm, Philips Scientific, Cambridge, UK). The mobile phase consisted of 30% methanol (HPLC; Panreac Montplet y Esteban, Barcelona, Spain), containing 0.01 *M* H_3PO_4, adjusted with KOH to pH 3. The detection took place at 206 nm. Routine sample calculations were based on the comparison of peak areas with external standard peak areas using BIOCROM software for chromatography (CIGB, Havana, Cuba). The chromatographic conditions are given in the figure legends.

2.2. Gibberellins

GA_3 was purchased from Sigma (St. Louis, MO, USA) and GA_1 was provided by the ICIDCA's Biochemical Laboratory. GA_3 and GA_1 standard solutions were prepared by dissolving them in the mobile phase. Calibration curves were developed for a GA_3 concentration range from 0.1 to 1 mg/ml and GA_1 concentration range from 0.01 to 0.5 mg/ml.

2.3. Sample preparation

Cultures were filtered through Whatman No 1 paper. The filtrates were adjusted to pH 2.5 and diluted when necessary in mobile phase so that the detector response was within the concentration range used to prepare the calibration curve. The filtrates were then filtered by ISO DISC Syringe Tip Filter Unit 0.45 μm. The sample was injected in 20 μl portions.

3. Results and discussion

A reversed-phase HPLC procedure was developed for the separation of Gibberellins GA_3 and GA_1. The linearity of the analysis was evaluated for GA_3 and GA_1 calibration curve concentration range. A slope of 248.78 with a standard error of 0.65 and the origin as intercept were obtained for GA_3 standard curve for $n=5$. A slope of 104.73 with an standard error of 0.97 and the origin as intercept were obtained for GA_1 standard curve for $n=5$. The correlation coefficients calculated for the compound concentration versus the peak area were 0.9999 ($P<0.05$) for GA_3 and 0.9996 ($P<0.05$) for GA_1. The detection limit of this HPLC technique was ca. 50 ng on column for GA_3 standard solution.

A 1.49 resolution was achieved with Spherisorb S5ODS1 column but it was not necessary working at the lower limit of pH (2.3) as in the method described by Barendse. This corroborates the statement by Barendse et al. in Ref. [7] that by choosing a particular methanol concentration, containing 0.01 *M* H_3PO_4 and an appropriate pH, a particular set of GAs can be analyzed with good resolution.

The more polar compounds elute sooner from reversed-phase C_{18} HPLC [5,6]. The gibberellin having a double bond (GA_3) is more polar than the saturated analogue. Fig. 1 shows the separation of standards of GA_3 and GA_1. The retention times were 11.00 min for GA_3 and 12.62 min for GA_1.

A good analytical within-day precision was obtained for GA_3 in a fermentation culture. The results obtained were $\bar{X}$ (mg GA_3/ml)=0.381, $\sigma_{n-1}=7\times 10^{-3}$ and R.S.D.=2.2% for $n=5$.

To evaluate the recovery of GA_3, a fermentation culture of known GA_3 concentration was taken and GA_3 standard was added at two different concentrations: 0.1 and 0.5 mg/ml. The sample was submitted to the described sample preparation procedure and recoveries of 96.4% and 98.7% were found, respectively.

Fig. 2 shows a typical chromatogram of a fermentation culture. At this wavelength no signifi-

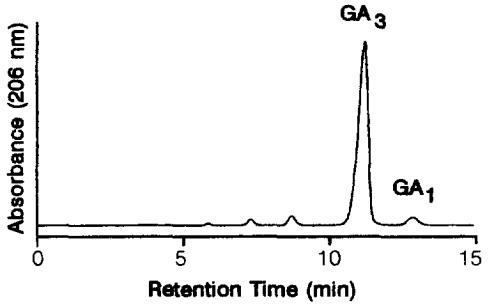

Fig. 1. Chromatogram of C_{18} HPLC of GA_1 and GA_3 (20 μl, 1 μg/μl). Column: Spherisorb S5ODS1, 25 cm×4.6 mm I.D. Mobile phase: 30% MeOH, containing 0.01 *M* H_3PO_4 adjusted with KOH to pH 3; flow-rate 1 ml/min. Detection at 206 nm, 1.0 a.u.f s.

cant interferences for GA_3 and GA_1 from contaminants in the acidic fermentation culture filtrate have been observed. This agrees with the results obtained by J.-T. Lin and E. Heftmann [5].

Results from different fermentation cultures achieved with the method described in this paper are presented in Table 1.

The results shown in this table are obtained under different fermentation conditions such as: pH, fermentation technique, scaling-up, and others, used in the optimization study of the fermentative process conditions.

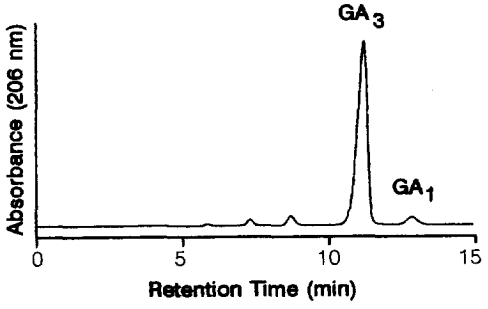

Fig. 2. Chromatogram of C_{18} HPLC of a fermentation culture (20 μl). Column: Spherisorb S5ODS1, 25 cm×4.6 mm I.D. Mobile phase: 30% MeOH, containing 0.01 *M* H_3PO_4 adjusted with KOH to pH 3; flow-rate 1 ml/min. Detection at 206 nm, 1.0 a.u.f.s.

Table 1
GA_3 and GA_1 concentrations from different fermentation cultures

Sample	Concentrations (mg/ml)	
	GA_3	GA_1
1	0.425	0.099
2	0.301	0.063
3	0.388	0.171
4	0.229	0.029
5	0.415	0.058

For HPLC conditions see Fig. 2.

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