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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF GIBBERELLINS

G. W. M. BARENDSE* and P. H. VAN DE WERKEN
Department of Botany, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen (The Netherlands)
and
N. TAKAHASHI
Department of Agricultural Chemistry, Bunkyo-ku, Tokyo (Japan)
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SUMMARY

The analysis of different gibberellins (GAs) by high-performance liquid chromatography (HPLC) was carried out with two types of columns: μBondapak C_{18} and the recently developed Radial-Pak-A. A reversed-phase HPLC procedure has been developed which enables the separation of different GAs with high resolution by means of ionic suppression, including the separation of double bond isomers such as GA_1 and GA_3, GA_4 and GA_7, GA_5 and GA_{20}, without the necessity for derivatization. A combined HPLC–bioassay procedure proved to be suitable for rapid routine assay of GA-activities in plant extracts.

INTRODUCTION

The most extensive and rigorous analysis of gibberellins (GAs) in plant extracts has been carried out by MacMillan and co-workers[1,2] who used combined gas chromatography–mass spectrometry (GC–MS). Their techniques required, however, a relatively high degree of purity of extracts and the availability of sophisticated GC–MS facilities for signal processing[2], and are thus not suitable for routine analyses of GAs in plant extracts.

Reeve and Crozier[3] have described the analysis of GAs by means of preparative and subsequent analytical high-performance liquid chromatography (HPLC). One of the problems in using HPLC for GA analyses is detection of these compounds. Reeve and Crozier[3] chose to derivatize the GAs by esterification of the carboxyl group at C-7; the resulting GA-benzyl esters have an absorption maximum at 256 nm and can thus be detected with a standard UV monitor at 254 nm. Morris and Zaerr[4] described the 4-bromophenacyl esters of gibberellins as useful derivatives for separations of GAs by HPLC. The separation of isomers of GAs containing double bonds, such as GA_1 and GA_3, GA_4 and GA_7, GA_5 and GA_{20}, by HPLC was achieved by Heftmann *et al.*[5] in the form of their *p*-nitrobenzyl esters. Yamaguchi *et al.*[6] have used HPLC to separate conjugated GAs. Finally, Jones *et al.*[7] recently reported the fractionation of GAs in plant extracts by reversed-phase HPLC.

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The studies in refs. 1–7 required derivatization of the GAs, particularly in order to separate the above pairs of double bond isomers. Since we have found the derivatization of GAs, particularly in extracts, to be rather troublesome and unreliable, we used the wavelength of 203 or 206 nm for detection of GAs.

The GAs are weak acids. Therefore, the ionic suppression technique, which involves the regulation of pH, was considered suitable for the separation of GAs. A reversed-phase HPLC procedure has been developed which enables the separation of the GA double bond isomers without derivatization, as well as a combined HPLC–bioassay procedure which enables rapid assay of GA activities in plant extracts without the necessity of extensive and time-consuming purification.

EXPERIMENTAL

High-performance liquid chromatography

The HPLC system consisted of the following components from Waters Assoc. (Milford, MA, U.S.A.): two sequentially connected solvent-delivery systems (Model 6000 A); a universal liquid chromatography injector (Model U6K); a solvent programmer (Model 660); a variable-wavelength detector (Model 450); an Omniscribe strip-chart recorder and a LKB 7000 Ultrorac fraction collector. Two types of columns (Waters Assoc.) were employed, *i.e.*, μBondapak C_{18} (30 cm × 3.9 mm I.D.) and Radial-Pak-A liquid chromatography cartridges. The latter were used in a Radial compression system (RCM-100). The mobile phase for the reversed phase HPLC consisted of double distilled water–methanol (LiChrosolv; E. Merck, Darmstadt, G.F.R.) mixtures or gradients, containing 0.01 *M* H_3PO_4 and adjusted to the appropriate pH with KOH. The detection took place at either 203 nm or 206 nm.

Gibberellins

GA_3 was purchased from Sigma (St. Louis, MO, U.S.A.) and a mixture of GA_4 and GA_7 from United States Biochemical Corp. (Cleveland, OH, U.S.A.); the other samples of relatively pure GAs, *e.g.*, GA_1, GA_4, GA_5, GA_7, GA_9 and GA_{20}, were obtained from the laboratory of Professor N. Takahashi. The GA samples were dissolved in methanol. Except for GA_3, the exact amounts of injected GAs were not known, but are estimated as 1–5 μg GA per injection.

Extraction and purification

Immature seeds of *Pharbitis nil* Choisy cv. Violet (5 g) were homogenized in methanol (50 ml) in a Sorvall omnimixer at maximum speed, filtered and the residue reextracted in methanol (50 ml) for another 24 h at 5°C. The combined filtrate was evaporated to a small volume (40 ml) under vacuum at 40°C (Rotavapor), and subsequently purified by forcing the solution through a Sep-Pak C_{18} cartridge (Waters Assoc.) with the aid of a 10-ml syringe, which removes the non-polar compounds from the solution. The filtrate is then further reduced in volume (3 ml) and once again forced through a Sep-Pak C_{18} cartridge. The filtrate is subsequently evaporated to dryness and taken up in 1 ml of the starting mobile phase for reversed-phase HPLC. It is essential that the pH of the sample to be injected is checked and if necessary adjusted to a value, *e.g.*, pH = 2.5, at which the GAs are retained on the column. The sample is injected in portions of 200 μl. The corresponding collected

fractions of 2.5 ml (1 min) were pooled, evaporated to dryness, taken up again in 0.5 ml of distilled water and used for bioassay.

Bioassay

The bioassay was carried out with dwarf corn d_1 seedlings. Seeds were soaked for 24 h in tap-water, sown in vermiculite and after 5–6 days selected for homogeneity and transplanted. The plants were treated with 0.1 ml of the above fractions, four plants per treatment. The elongation of the leaf sheaths of the first and second leaf were measured 7 days after treatment and compared with a standard concentration range of GA_3 solutions. The biological activity is expressed as the percentage increase over the control (water) treatment.

RESULTS AND DISCUSSION

There are two main problems associated with the analysis of the many naturally occurring GAs, *i.e.*, detection and separation, particularly the separation of double bond isomers. Because GAs do not absorb at the standard UV monitoring wavelength of 254 nm they were monitored at 203 or 206 nm.

The first efforts were aimed at the separation of the double bond isomers GA_1 and GA_3. This separation was finally achieved by reversed-phase chromatography on a μBondapak C_{18} column with 20% methanol, containing 0.01 *M* H_3PO_4 adjusted to pH = 2.3 as mobile phase (Fig. 1). It was established that the presence of 0.01 *M* H_3PO_4 and a low pH were essential for a clear separation of the double bond isomers. Several non-isomeric GAs, *e.g.*, GA_3, GA_5, GA_4 and GA_9, can be separated by an increasing methanol gradient without the presence of H_3PO_4. Subsequently, other

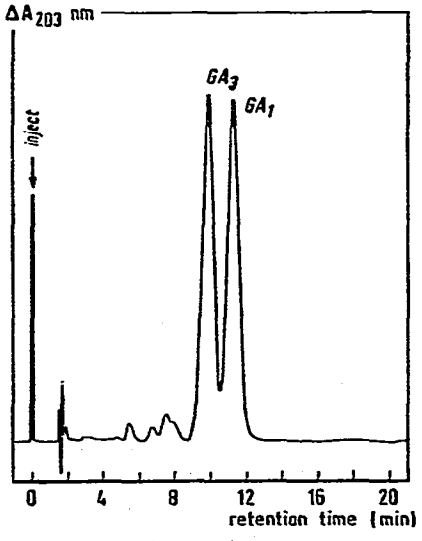

Fig. 1. Reversed-phase HPLC of GA_1 and GA_3 (2 μl, 1 μg/μl). Column: μBondapak C_{18}, 30 cm × 3.9 mm I.D. Mobile phase: 20% methanol, containing 0.01 *M* H_3PO_4 adjusted with KOH to pH = 2.3; flow-rate 2 ml/min. Detection: 0.1 absorption units full scale (a.u.f.s.) at 203 nm. Chart speed: 0.25 in./min.

double bond isomers, *i.e.*, GA_5 and GA_{20}, GA_4 and GA_7, were separated by increasing the methanol concentration at the low pH of 2.3. Finally, a methanol gradient was developed whereby the available three pairs of double bond isomers and GA_9 could be separated in one run (Fig. 2). The baseline shift caused by the increasing concentration of methanol was suppressed by adding a small amount of acetone to the lowest methanol concentration. The gradient is easily adapted for the separation of particular GAs.

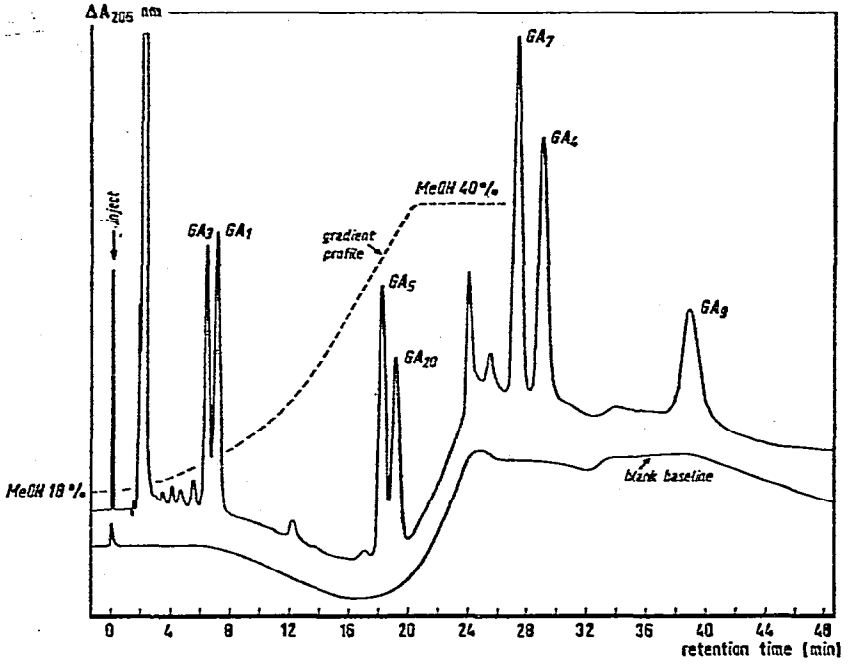

Fig. 2. Reversed phase HPLC of GA_1, GA_3, GA_4, GA_5, GA_7, GA_9 and GA_{20}. Mobile phase: gradient (curve 7) from 18% methanol, containing 0.01 *M* H_3PO_4 and acetone (10 ml/l) to 40% methanol, 0.01 *M* H_3PO_4 at pH = 2.2 in 20 min. Flow rate: 1.8 ml/min. Detection at 206 nm. Other details as in Fig. 1.

In the following experiments the new Waters Assoc. column involving a Radial-Pak-A column in a Radial compression module was used for the analysis of the different GAs. With this type of column the resolution of double bond isomers is even better than with the μBondapak C_{18} column, *cf.*, Figs. 2 and 3. With the Radial-Pak-A column one does not have to resort to a methanol gradient which causes large baseline shifts, but merely to apply a pH gradient (Fig. 3). A change in pH results in a baseline shift caused by differences in the potassium concentration which is smaller and acceptable at 206 nm. 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. An example is given in Fig. 4 which shows the separation of the the double bond isomers GA_4 and GA_7 and of GA_9 with a mobile phase consisting of 35% methanol at pH = 7.2.

The GAs separate according to their degree of hydroxylation. Those having

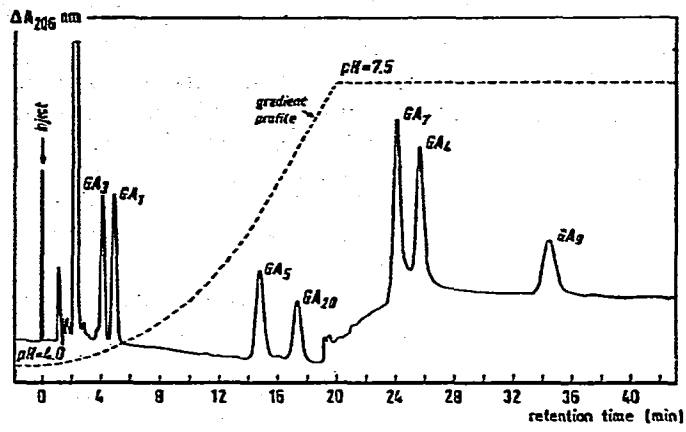

Fig. 3. Reversed phase HPLC of GA_1, GA_3, GA_4, GA_5, GA_7, GA_9 and GA_{20}. Column: Radial-Pak-A. Mobile phase: gradient (curve 9) from 35% methanol, 0.01 *M* H_3PO_4 at pH = 4.0 to 35% methanol, 0.01 *M* H_3PO_4 at pH = 7.5 in 20 min. Flow-rate: 2 ml/min. Detection: 0.1 a.u.f.s at 206 nm. Chart speed: 0.25 in./min.

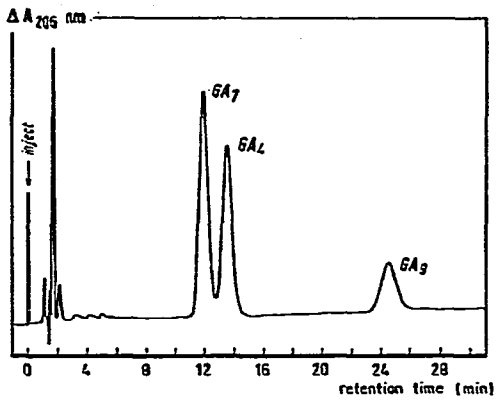

Fig. 4. Reversed-phase HPLC of GA_4, GA_7 and GA_9. Column: Radial-Pak-A. Mobile phase: 35% methanol, 0.01 *M* H_3PO_4 at pH = 7.2. Other details as in Fig. 3.

no hydroxyl groups such as GA_9 have the greatest retention, and they are preceded by the monohydroxylated GAs, *e.g.*, GA_4 and GA_7, GA_{20} and GA_5, which in turn are preceded by the dihydroxylated GA_1 and GA_3. Taking this into account, the Radial-Pak-A column will be more suitable than the μBondapak C_{18} column for reversed-phase HPLC for the separation of GAs with three, *e.g.*, GA_8, or even with four hydroxyl groups, *e.g.*, GA_{32}, because with μBondapak C_{18} columns GA_1 and GA_3 show retention at 18% methanol and below this methanol concentration both retention as well as resolution cannot be improved, and also the lower limit of pH (2.3) has been reached. In contrast, with the Radial-Pak-A column, GA_1 and GA_3 already show retention at 35% methanol and pH = 4.0. Thus with this column both the methanol concentration and the pH can be further manipulated in order to separate the GAs having more hydroxyl groups, such as GA_8 and GA_{32}. In addition

the resolution of GAs on Radial-Pak-A columns appears also to be somewhat better than with μBondapak C_{18} columns.

Finally, the crucial test for the practical usefulness of a particular method lies in its suitability for analyzing GAs in plant extracts. In spite of the great advances in analytical instrumentation, the bioassay remains an effective means of detecting minute quantities of GAs, *e.g.*, 1–10 ng, in plant extracts. In comparison, the detection limit of the above HPLC techniques is *ca.* 100 ng for relatively pure GA samples. However, the quantitative accuracy of a bioassay depends greatly upon the purity of the extract as the bioassay response reflects the interaction of GA-activity and inhibitory compounds present in the samples. Therefore, laborious purification procedures, including partitioning, thin-layer chromatography (TLC) and/or column chromatography, of extracts are employed before the sample is ready for bioassay. In this respect reversed-phase HPLC combined with a bioassay offers potential advantages with regard to speed as well as degree of purification compared with the conventional methods.

We have developed a procedure which involves extraction of the plant material with methanol, followed by a two-step purification with a Sep-Pak C_{18} cartridge as described under Experimental, whereafter the sample can be directly injected into the HPLC under the appropriate conditions, followed by bioassay of the collected fractions. An example of the results of such a procedure is presented in Fig. 5, which

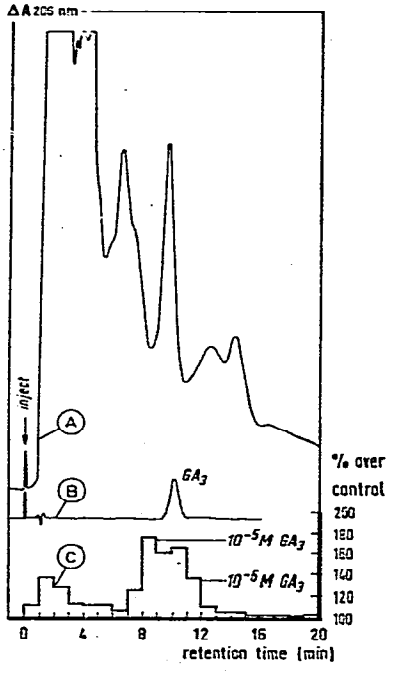

Fig. 5. Reversed-phase HPLC of a plant extract. Column: Radial-Pak-A. Mobile phase: 35% methanol, 0.01 *M* H_3PO_4 at pH = 2.5. Flow-rate: 2 ml/min. Detection: 1 a.u.f.s. at 206 nm. A, Profile of an injection with 200 μl of extract; B, reference chromatogram of GA_3 (15 μg); C. Biological activity of the collected 2-ml (1-min) fractions on the dwarf corn d_1 assay compared with standards of 10^{-6} *M* and 10^{-5} *M* GA_3, respectively.

gives the elution profile of the sample (A), the GA_3 reference chromatogram (B) and the biological activities of the collected fractions (C).

A disadvantage of monitoring at 206 nm is that at this wavelength many other compounds present in the plant extract have a relatively large absorption, as shown in the profile A of Fig. 5. At 254 nm this extract looked "clean" since, there were hardly any peaks beyond 4 min after injection (not shown). However, monitoring of derivatized extracts at 254 nm results also in new unknown absorption peaks. The reversed-phase HPLC results in considerable purification of the extract which compares favourably with conventional partitioning and TLC procedures (see also ref. 7). Consequently, the total biological activity obtained after HPLC is higher than with conventional purification procedures. The total calculated GA-activity, after extraction of equal amounts of plant material, was 1.5 μg GA_3-equivalent per 10 seeds for the Sep-Pak-HPLC procedure and 0.7 μg for the conventional method, *i.e.*, liquid–liquid partitioning followed by TLC. This Sep-Pak-HPLC purification and separation procedure is now routinely and successfully used in our laboratory either to investigate endogenous GA-activities or to analyse metabolites of exogenously applied radioactive GAs in plant extracts. Moreover, this reversed-phase HPLC procedure is promising for the relatively rapid purification of extracts by recycling, to a degree that the samples become suitable for further analyses by GC–MS techniques.

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