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Note

Separation of gibberellins by normal-phase high-performance liquid chromatography

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A major difficulty with the study of endogenous gibberellins (GAs) in plants is to remove substances interfering with the subsequent qualitative or quantitative analyses, for a general review see, *e.g.*, ref. 1. Since vegetative plant tissues usually contain small amounts of GAs together with large amounts of closely related impurities, the efficiences of the chromatographic procedures employed are of great importance. Therefore, high-performance liquid chromatography (HPLC) is now increasingly preferred to other chromatographic techniques[1,2].

Reversed-phase HPLC has proved to be very suitable for the separation of GAs[3–7]. If even higher purity is required, or as an alternative, normal-phase HPLC, can be used. The combination of a reversed-phase HPLC purification step with a normal-phase HPLC purification step increases the probability that the impurities are separated from the GAs and thereby facilitates the subsequent qualitative and quantitative analyses[2].

A frequently used normal-phase HPLC method for GAs was developed by Reeve *et al.*[8] from an open column system described by Powel and Tautvydas[9]. The stationary phase of this system was aqueous formic acid adsorbed to a microparticulate silica gel. The mobile phase used consisted of gradients or certain proportions of *n*-hexane saturated with aqueous formic acid, and ethyl acetate saturated with aqueous formic acid. This system is difficult to handle since the liquid stationary phase is easily stripped off the silica support, resulting in adsorption chromatography rather than partition chromatography. The development of HPLC has, however, resulted in covalently bonded polar stationary phases and such a system would be preferable. Bonded normal-phase HPLC of free GAs has been reported by Yamaguchi *et al.*[10] who used Nucleosil $N(CH_3)_2$ as a stationary phase, isocratically eluted with methanol plus 0.05% acetic acid.

The purpose of the present investigation was to develop a normal-phase HPLC method for semi-preparative and analytical separations of GAs. The method should be used for the purification of GAs in extracts of vegatative conifer tissues.

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MATERIALS AND METHODS

Preparation of radiolabelled GAs

Carbon-14 labelled GAs were prepared from mevalonic acid by enzymatic conversion in cell-free systems from pumpkin (*Cucurbita maxima*) and pea (*Pisum sativum*). Mevalonic acid lactone (1.96 GBq/mmol, Amersham) was hydrolysed with 1 *M* KOH at 30°C for 15 min and was dissolved in 2.35 ml of water. The solution was added to an incubation mixture consisting of 100 μl 0.05 *M* $MgCl_2$, 100 μl 0.1 *M* $MnCl_2$, 100 μl 0.5 *M* ATP, 150 μl 0.5 *M* phosphoenolpyruvate (PEP), 150 μl 0.05 *M* reduced nicotinamide–adenine dinucleotide phosphate (NADPH) and 7 ml of liquid pumpkin endosperm[11]. After 2 h of incubation at 30°C the conversion was stopped and the gibberellins were extracted at pH 3.0 with ethyl acetate. The products were purified by thin-layer chromatography and gradient elution reversed-phase HPLC and their identities were established by gas chromatography–mass spectrometry (GC–MS). In this manner kaurene, kaurenal, kaurenol, kaurenoic acid, GA_{12}-aldehyde, GA_{12} and GA_{15} were obtained.

Cell-free systems from pea seeds (for more details see ref. 12) were used for the preparation of ^{14}C-labelled GA_{19}, GA_{20}, GA_{29}, GA_{44} and GA_{53}. First the [^{14}C]GA_{12} was incubated with the low-speed supernatant (S-2, 2000 *g*) together with NADPH, ATP and PEP to yield [^{14}C]GA_{53}. The GA_{53} was then incubated with the high-speed supernatant (S-200, 200 000 *g*) and Fe^{2+} and ascorbate. This resulted in the production of ^{14}C-labelled GA_{19}, GA_{20}, GA_{29} and GA_{44}. All GAs were further purified by reversed-phase HPLC and their identities were established by GC–MS.

The other GAs were commercially available or obtained as gifts: [^{3}H]GA_1 (New England Nuclear, U.K.), [^{14}C]GA_3 (Amersham, U.K.) [^{3}H]GA_4 (R.P. Pharis, Calgary, Canada), [^{3}H]GA_8 (R.P. Pharis) and [^{3}H]GA_9 (Alan Crozier, Glasgow, U.K.).

Plant material

Seeds of Norway spruce [(*Picea abies* (L) Karst.)] were collected from a stand outside Umeå (lat. 63°50′ N). The seeds were germinated and grown in a greenhouse under artificial long day conditions during the period from July to December. Supplementary light was given 20 h a day with Osram metal halogen lamps, HQI-TS 400 W/DH (*ca.* 100 μmol m^{-2} s^{-1}, 400–700 nm). The seedlings were watered daily and given a complete nutrient solution twice a week. At the age of *ca.* 6 months the actively growing, non-lignified upper part of the seedlings was harvested and frozen in liquid nitrogen. Thereafter the plant material was stored at −80°C until analyzed.

Extraction and purification

Shoot samples of 200 g fresh weight were homogenized in 1000 ml cold (+4°C) methanol. After 2 h of extraction the tissue debris was filtered off and washed with another 500 ml of methanol. The methanol fractions were pooled, and 10 ml of 2% diethyldithiocarbamic acid were added. The organic phase was evaporated *in vacuo* at 35°C and the water phase was adjusted to a volume of 10 ml with 0.5 *M* phosphate buffer and, if necessary, adjusted to pH 8.0 with 6 *M* NaOH. The extract was applied to a 30 cm × 10 mm I.D. column packed with insoluble poly-(N-vinylpolypyrrolidone) (PVPP). The column was eluted with 0.1 *M* phosphate buffer pH 8.0 and the

fraction 0–200 ml was collected. This semipurified extract was then purified by semi-preparative normal-phase HPLC with a covalently bonded stationary phase.

High-performance liquid chromatography

The solvent delivery system consisted of one Waters M 510 pump, one Waters M 45 pump controlled by a Waters M 680 system controller. The samples were introduced to the columns via a Waters U6K injector. The GAs were detected by a Reeve Analytical radioactivity detector equipped with a 500-μl homogeneous flow cell. Instafluor (Packard) liquid scintillation fluid was delivered by a Reeve scintillation pump and mixed with the column effluent in the homogeneous flow cell.

The analytical separations were made with 150 mm × 4.6 mm I.D. steel columns packed with Nucleosil 5-μm particulate NO_2 phase. The phase is bonded to the silica support via a propyl group. The mobile phase consisted of gradients of *n*-heptane half saturated with 1 *M* formic acid to ethyl acetate with 1% water and 0.5% formic acid. Water and formic acid were added as polar modifiers and ion suppression agents in the mobile phase.

The semi-preparative separations were made with a 250 mm × 10 mm I.D. column packed with 5-μm Polygosil NO_2. This system was used for the first separation of GA-like substances in a Norway spruce extract. The GA-like substances were detected by the Tan-ginbozu dwarf rice bioassay according to Murakami[13].

RESULTS AND DISCUSSION

Since our aim was to use the system for preparative purifications of unknown GA-like substances we concentrated on gradient elution, which makes it possible to chromatograph GAs with different chemical characteristics in the same experiment. The mobile phase of choice was gradients of *n*-heptane to ethyl acetate. The use of formic acid and water in the mobile phase was necessary to suppress the ionization of the carboxyl groups and thereby avoid high capacity factors and poor peak symmetry. The water and acid content was very critical for good selectivities and 1% of water and 0.5% of formic acid in the ethyl acetate proved to be optimal. The *n*-heptane was half saturated with 1 *M* formic acid in water. A problem with formic acid in water in *n*-heptane and ethyl acetate is that the two solvents separately hold more water and acid than they do in mixtures. If the water and acid content is too high, water and formic acid will separate during gradient elution thus risking deterioration of the stationary phase.

The separation of the available GAs and GA precursors is shown in Fig. 1. A comparison of the elution order of the γ-lactonic C_{19}-GAs indicates that the number and also the position of the hydroxyl groups on the GAs are very important for the solubility in the stationary phase. GA_9 with no hydroxyl group is eluted first (*ca.* 40 ml) and is followed by GA_4 (*ca.* 56 ml) and GA_{20} (*ca.* 63 ml), each having one hydroxyl group. GAs with two hydroxyl groups, GA_1, GA_3 and GA_{29}, are eluted at *ca.* 85, 88 and 101 ml, respectively. The GA possessing three hydroxyl groups, GA_8, is eluted at *ca.* 117 ml, thus exhibiting the highest solubility in the polar stationary phase. The same effect of the number of hydroxyl groups is observed when comparing the δ-lactonic C_{20}-GAs, GA_{15} and GA_{44}, and the C_{20}-GAs with a C_{20}-methyl group. GA_{12} and GA_{53}. Ga_{15} and GA_{12} have no free hydroxyl group and are eluted earlier

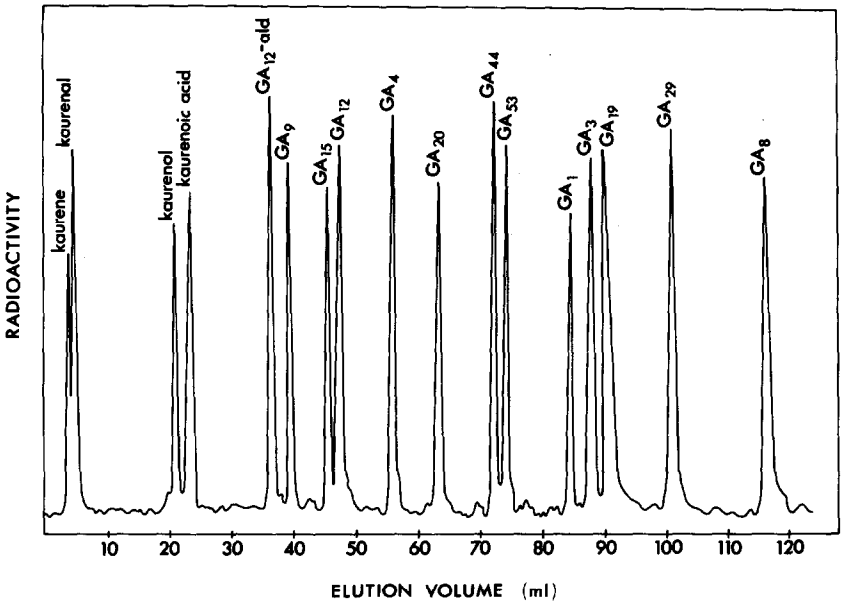

Fig. 1. Analytical normal-phase HPLC of radioactively labelled gibberellins and gibberellin precursors. Kaurene, kaurenol, kaurenal, kaurenoic acid, GA_{12}-aldehyde, GA_{15}, GA_{12}, GA_{20}, GA_{44}, GA_{53}, GA_3 and GA_{29} were ^{14}C-labelled and the other GAs were ^{3}H-labelled. Stationary phase: 5-μm Nucleosil NO_2 packed in a 125 mm × 4.6 mm I.D. Column. The mobile phase was a gradient of *n*-heptane half saturated with 1 *M* formic acid to ethyl acetate with 1% water and 0.5% formic acid. Gradient sweep time: 60 min. Flow-rate: 2 ml/min.

than the corresponding GAs with one hydroxyl group, GA_{44} and GA_{53}. It is also possible to refer the elution order to the position of the hydroxyl groups. By comparing the elution volume of GA_4, having the hydroxyl group in 3-position, with the elution volume of GA_{20}, having the OH group in 13-position, it is observed that GA_4 is eluted earlier, probably because the interaction of the 3-OH group with the stationary phase is reduced by hydrogen bonding to the C-7 carboxyl group. This type of hydrogen bonding is not possible if the OH group is in 13-position. This explains also the difference in elution volumes of GA_1, GA_3 and GA_{29}, all possessing two OH groups. GA_1 and GA_3, hydroxylated in the 3- and 13-positions are eluted earlier than GA_{29} which is hydroxylated in the 2- and 13-positions. The distance between the 2-OH group and the C-7 carboxyl group in GA_{29} is, however, too long for such hydrogen bonding. The slightly higher elution volume of GA_3 compared to GA_1 is probably caused by a higher interaction of the 1,2-double bond of GA_3 in the stationary phase.

By comparing the elution volumes of the γ-lactonic and non-OH C_{19}-GA, GA_9 with the δ-lactonic, non-OH C_{20}-GA, GA_{15}, it can be concluded that the δ-lactone form is more soluble in the stationary phase. The same is observed when comparing GA_4 and GA_{20}, both γ-lactones with one OH-group, with GA_{44} which is a δ-lactone also with one OH-group. The effect of the oxidation state of the C_{20}-carbon on the elution volume is demonstrated by the difference between GA_{53}, with a methyl in the C_{20} position, and GA_{19}, where this position is occupied by an aldehyde. The more

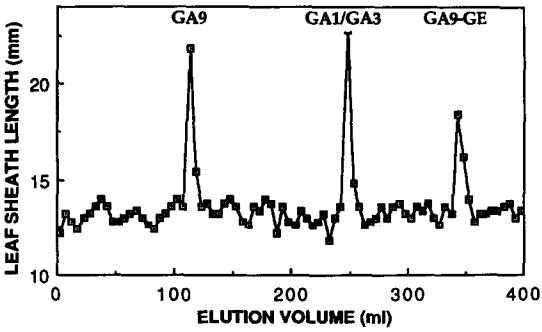

Fig. 2. Separation of gibberellins in a Norway spruce extract by semi-preparative normal-phase HPLC. Gibberellin-like substances detected by Tan-ginbozu dwarf bioassay. Stationary phase: 5 μm Polygosil NO_2 packed in a 250 mm × 10 mm I.D. column. Mobile phase: gradient from *n*-heptane half saturated with 1 *M* formic acid to ethyl acetate with 1% water and 0.5% formic acid. Gradient sweep time: 60 min. Flow-rate: 5 ml/min. Fraction size: 5 ml.

polar nature of the aldehyde group makes GA_{19} more soluble in the stationary phase and thereby increases the elution volume compared with GA_{53}. The difference in retention characteristic between GAs possessing a carboxyl group or an aldehyde group is demonstrated by the difference in elution volume between GA_{12}-aldehyde, with an aldehyde at C-6, and GA_{12}, which has a carboxyl group at C-6. The more polar nature of the carboxyl group increases the solubility of GA_{12} in the stationary phase compared to GA_{12}-aldehyde.

From these observations it may be possible to predict the approximate elution volume also of other GAs by consideration of: (1) the number of OH groups; (2) the position of the OH groups; (3) the type of GA, γ-lactonic, δ-lactonic, and the oxidation state of a retained C_{20}.

The semi-preparative separation of a semi-purified spruce extract corresponding to 400 g fresh weigth resulted in GA-like activity as shown in Fig. 2. The first region of activity (around 110 ml) was, after further purification by reversed phase HPLC, identified as GA_9 by (GC–MS)[14]. The second region (250 ml) was, after further purification by reversed phase HPLC, identified as GA_1 and GA_3 by GC–MS[15]. This means that GA_1 and GA_3 did not separate in the gradient elution preparative system. The third region of activity, 350 ml (not detected when formic acid coated on a silica support was used as the stationary phase), was further purified by reversed-phase HPLC. After cellulase hydrolysis, GA_9 was identified by GC–MS, thus indicating that this compound is GA_9-glucosyl ester[16]. This normal-phase HPLC system is easy to handle, reproducible, efficient, selective and the solvents are easily evaporated which makes it well suited for both semi-preparative and analytical separations of GAs. It has been used for the semi-preparative purification of large extracts (200–400 g fresh weight) of Norway spruce tissue. An additional purification of the GA-like substances by reversed-phase HPLC made it possible to identify them by GC–MS. The analytical system has been routinely used for the separation of GA_9, GA_1 and GA_3 in small extracts (5 g fresh weigth) of Norway spruce prior to quantitation by radioimmunoasay.

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