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Review

Chromatography of gibberellins

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ABSTRACT

The recent literature on the use of chromatography in the analysis of gibberellins in plants is reviewed. Particular emphasis is placed on the application of solid-phase purification techniques, immunoaffinity chromatography, HPLC, GC–MS and LC–MS.

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1. INTRODUCTION

Methods of gibberellin (GA) analysis have been recently summarized in reviews by Hedden [1,2], Beale and Willis [3], Barendse [4], Takahashi *et al.* [5] and Crozier and Durley [6]. Gaskin and MacMillan [7] have described in detail the procedures for GC–MS analysis of the GAs and related compounds. In this review the emphasis is on recent developments in the use of chromatography in GA analysis, particularly in the application of solid-phase purification techniques, immunoaffinity chromatography, HPLC, GC–MS and LC–MS. The availability of GC–MS in particular as a sensitive and specific means of detecting and quantifying GAs has led to the increasing adoption of small-scale chromatographic procedures for the purification of GAs from small amounts of plant material.

2. STRUCTURES

The gibberellins are a group of diterpenoids, currently with some 90 members recognized (Fig. 1), and a number of others awaiting the allocation of GA numbers. Their chemistry has been recently reviewed by Mander [8]. A number of structural variations on the *ent*-gibberellane skeleton (Fig. 2) influence their behaviour in chromatography. Two major divisions of structure exist; the C_{19} GAs have a 19-carbon pentacyclic skeleton which includes in most cases a C-19,10 lactone ring (*e.g.*, GA_9), and the C_{20} GAs have a 20-carbon skeleton. In the C_{20} GAs, C-20 is present as either a methyl- (*e.g.*, GA_{12}), carboxy- (*e.g.*, GA_{25}) or aldehyde- (*e.g.*, GA_{24}) function, or is included in a C-19,20 lactone ring (*e.g.*, GA_{15}). All of the GAs are carboxylic acids, at C-7, and may also be carboxylated at C-18 or -19. One, two, three or four hydroxy functions may be present at C-1, -2, -3, -11, -12, -13, -15, -16, -17 or -18. Two GAs have epoxy functions, and two have oxo functions. A number of the C_{19} GAs are dehydrogenated at C-1,2 or C-2,3, and two at C-9,11. Several C-15-ene GAs have been identified, and while such compounds may arise as artefacts of acidic extraction procedures it seems likely that they also occur naturally.

A number of endogenous GA–glucosyl conjugates have been identified. These include esters, which are neutral, and ethers (glucosides), which are acidic (Fig. 3). In addition a small number of other naturally occurring esters have been found [8]. The function of the glucosyl conjugates in plants is uncertain. Their formation may convert GAs to inactive, water-soluble forms suitable for transport or storage. It may also serve in regulation of the pool of active GAs, by removing biosynthetic precursors or inactivating those GAs directly active in growth. The glucosyl ester conjugates may be hydrolyzed to release the free GA [9,10] and thus may serve as a repository of potentially active GAs [9–11]. These recent findings should prompt increased interest in analysis of the GA conjugates.

The immediate precursors of the GAs are kauranoids. Because of the relevance of their physiology to the GAs and since they are sometimes isolated in conjunction with the GAs and GA conjugates, the use of chromatography in their analysis is discussed briefly. Those which are part of the biosynthetic pathway leading to the GAs include compounds with a range of properties, from *ent*-kaurene with no polar functional groups to the acidic and hydroxylated *ent*-7α-hydroxy-kaurenoic acid (Fig. 4).

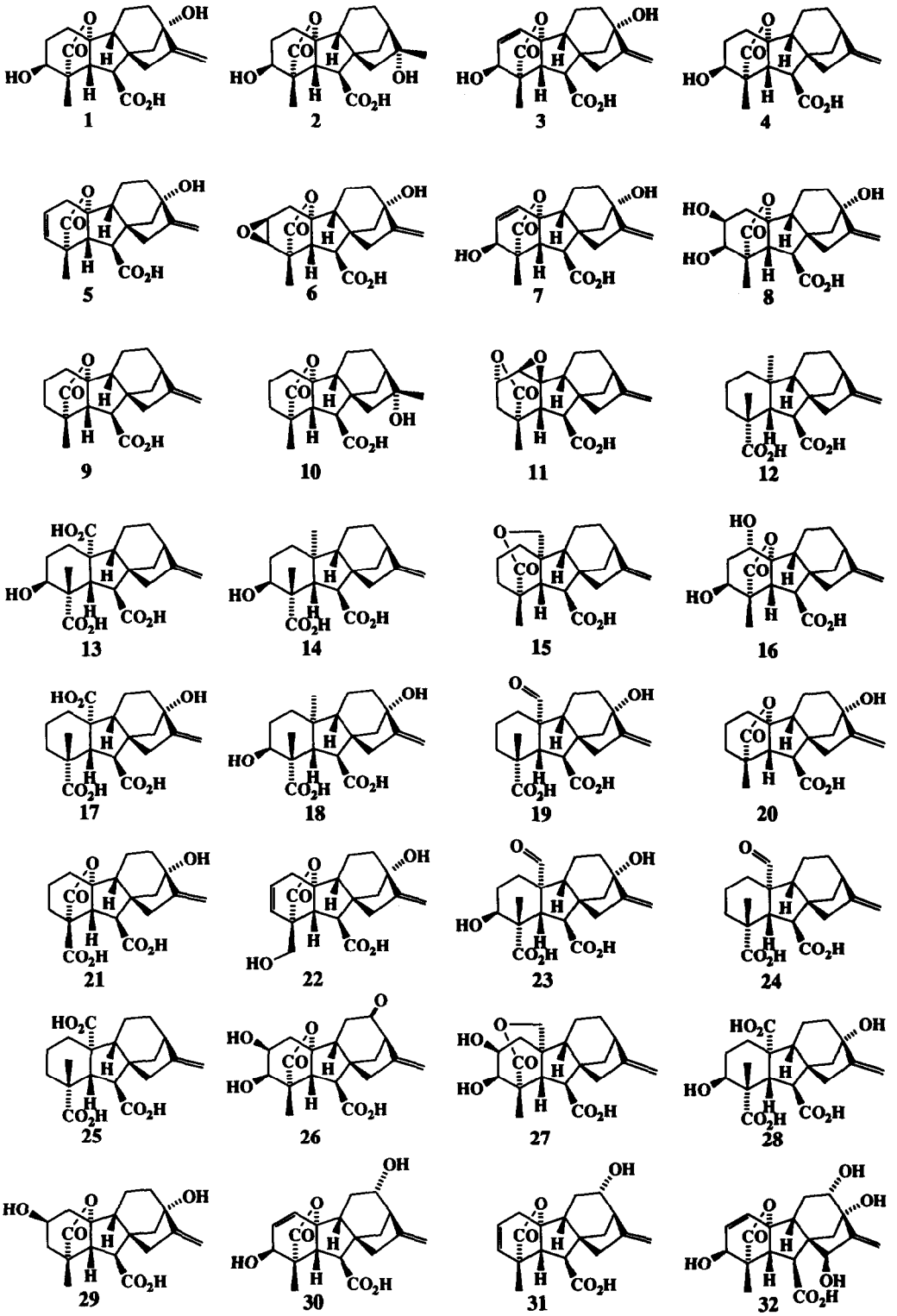

Fig. 1.

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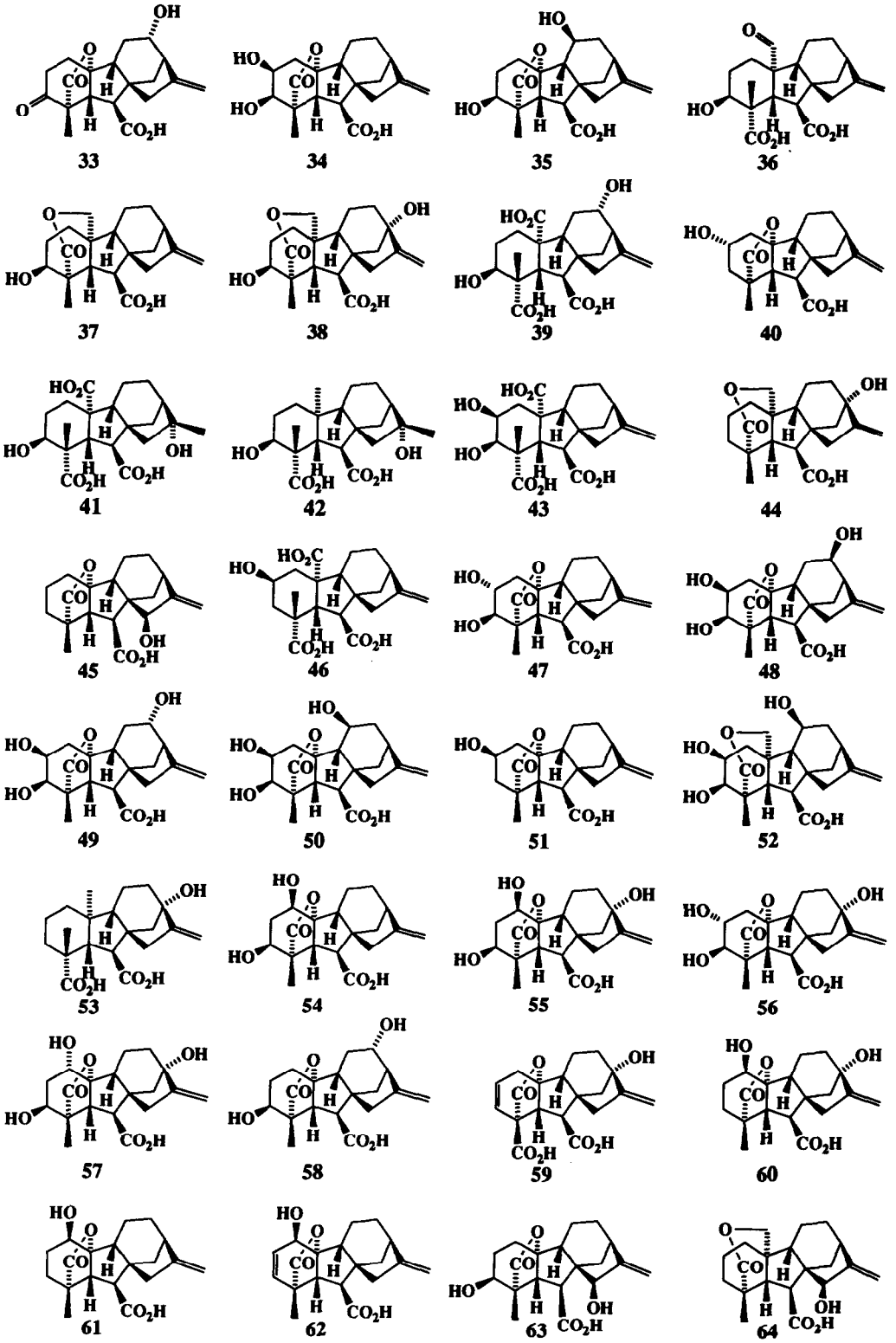

Fig. 1 (*continued*)

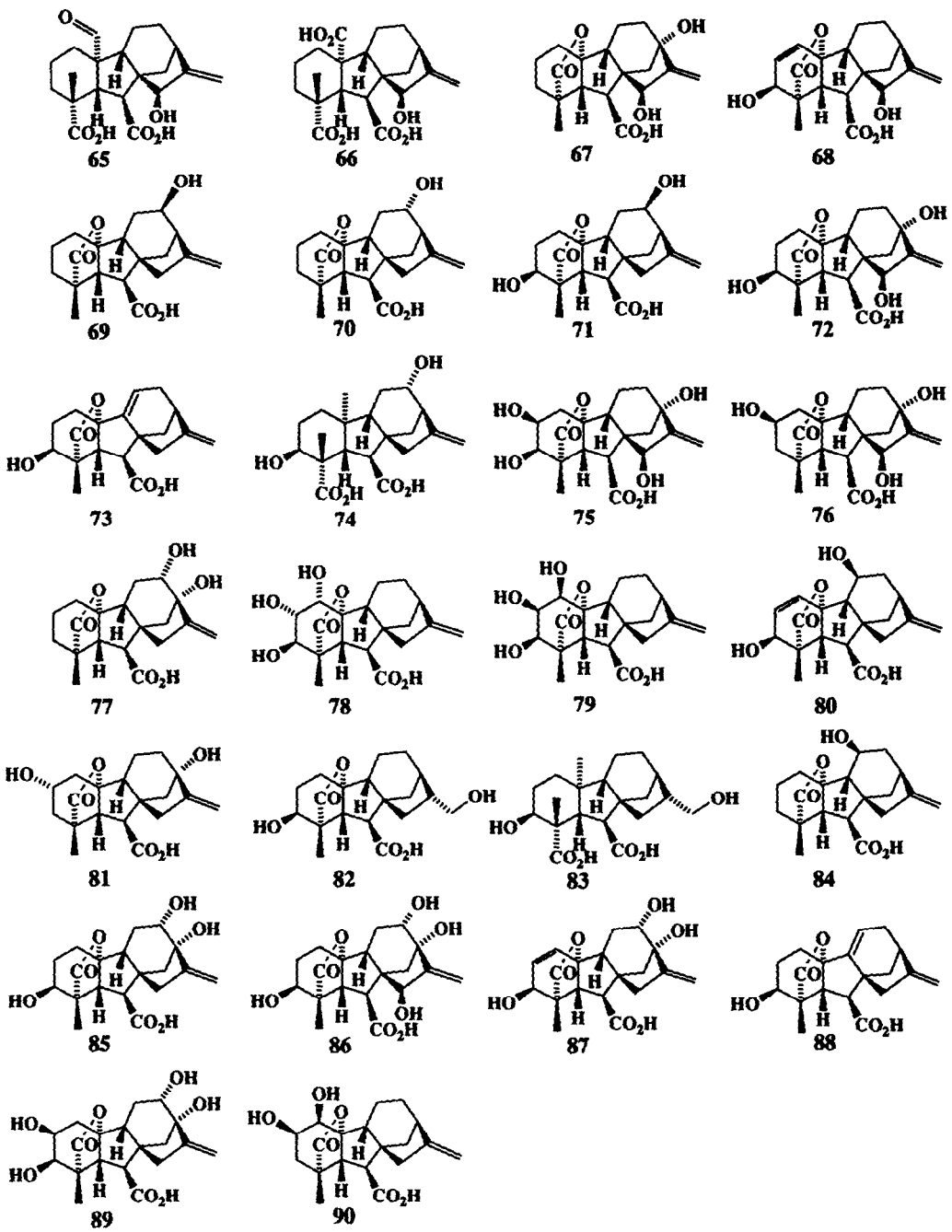

Fig. 1. Structures of the gibberellins. Numbers correspond to n in GA_n.

3. PREPARATIVE CHROMATOGRAPHY

Where samples are destined for GC–MS, the purpose of preparative purification is to concentrate the GAs so that an aliquot containing sufficient mass for identification or quantification (usually in the range ng to pg) can be introduced to GC. At the same time extraneous compounds are reduced so that they do not interfere with chromatography, contribute excessively to the

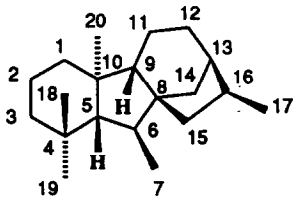

Fig. 2. C-numbering of the *ent*-gibberellane skeleton.

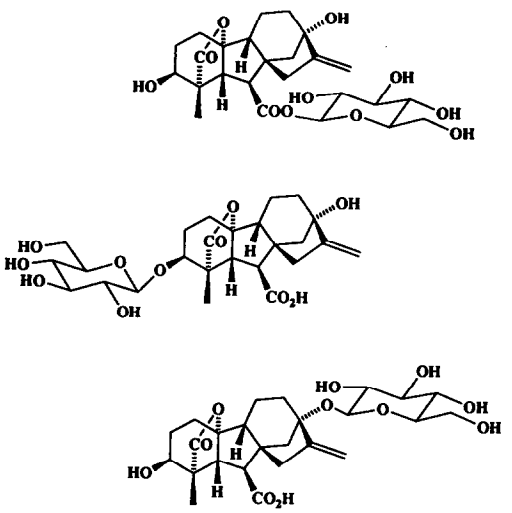

Fig. 3. Structures of GA_1–glucosyl conjugates. Top, GA_1–glucosyl ester; middle, GA_1–3-O-glucoside; lower, GA_1–13-O-glucoside.

background signal, or contaminate the mass spectra or ion chromatograms of the compounds of interest. For convenience in minimizing the number of samples which are submitted to GC–MS analysis, the GAs are fractionated as little as possible during purification. Where immunological methods are used to detect and quantify the GAs, purification is required to eliminate compounds which would otherwise interfere with binding, and often the GAs must be resolved by HPLC to provide additional evidence of identity. Clearly the purification procedures required will vary from sample to sample.

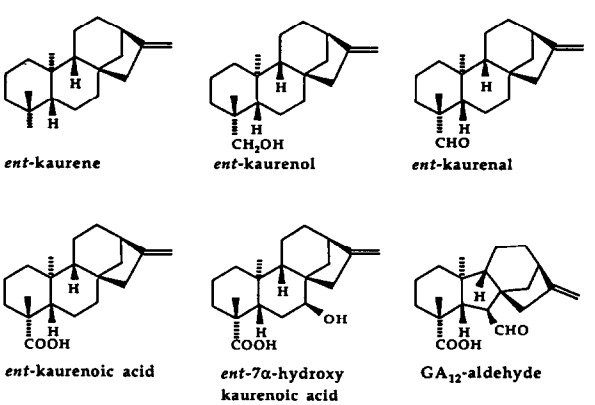

Fig. 4. Structures of GA_{12}-aldehyde and the *ent*-kauranoid precursors of the gibberellins.

The usual approach in preparative purification of GAs is to retain the free GAs as a group, insofar as possible, separate from the GA conjugates and from the less polar kauranoids. The methods employed have been described in the reviews cited above; that of Hedden [1] is a particularly instructive practical guide to GA analysis. A brief summary of the methods employed in group separation and purification is included here.

3.1. Solvent partitioning

This traditional method of preliminary purification of GAs is still in widespread use. Typical schemes have been summarized in the reviews [1,3,5]. After extraction, usually in 80% aqueous methanol, the methanol is evaporated *in vacuo* and the majority of the GAs are extracted from the aqueous residue by partitioning into ethyl acetate at pH 2.5–3.0. The most polar GAs, such as GA_{32} [12] and other tetrahydroxylated- and some trihydroxylated species (GA_{72}, GA_{75}, GA_{76} [13]) remain to a large extent in the aqueous phase, from which they can be extracted with *n*-butanol. Subsequent partitioning of the initial ethyl acetate phase, against 0.1 *M* phosphate buffer at pH 8.5, leaves neutral compounds in the ethyl acetate. The acidic GAs partition into the aqueous fraction and can be extracted by partitioning into ethyl acetate after the pH is adjusted to 2.5–3. Alternative procedures modify the order of the partition steps and may incorporate early removal of less polar impurities by extraction from the aqueous residue into ethyl acetate or other solvents such as light petroleum, diethyl ether or *n*-hexane at pH 8–9. A significant proportion of the less polar GAs, such as GA_4 and GA_9, can also be expected to partition into ethyl acetate at pH 8, but not into diethyl ether at pH 9 [14].

The GA conjugates are usually isolated from extracts together with free GAs, with solvent partitioning which results in them being divided amongst neutral ethyl acetate (GA–glucosyl esters) and neutral and acidic *n*-butanol (GA–glucosyl esters and glucosides) fractions [15] (see refs. 1 and 5). Schneider [16] ascribed such partitioning characteristics to the dominating effect on solubility of the hydroxy groups of the glucose moiety, rather than the degree of ionization of the –COOH function of the GA. An alternative approach which avoids this division is to extract the GA-conjugates as a group from aqueous solution into acidic *n*-butanol, to be subsequently divided into neutral and acidic fractions if required [17]. The acidic fraction will also contain the free GAs, and these can later be separated from the conjugates by other methods such as size-exclusion chromatography or on HPLC as the permethyl derivatives.

The kauranoids have also been obtained from the usual extraction in 80% aqueous methanol, or where these are the only class of compounds of interest have been extracted in less polar solvents such as ethyl acetate [18–20] or acetone [21], and then further purified by methods more applicable to their chemistry. *ent*-Kaurene, *ent*-kaurenol and *ent*-kaurenal are neutral, and *ent*-kaurenoic acid is a weak acid which may occur in both neutral and acidic fractions [7].

A number of chromatographic methods have been applied to purify the various fractions from solvent partitioning. Some of these have been successfully used in lieu of solvent partitioning, in keeping with the increasing use of solid-phase extraction techniques in the preparative purification of natural compounds. Other methods, used formerly as analytical procedures, have been supplanted in this rôle by methods which offer improved resolution and are used now mainly as preparative techniques.

3.2. Normal-phase chromatography

Several types of sorbents have been widely used. These include polyvinylpyrrolidone (PVP) or polyvinylpolypyrrolidone (PVPP), charcoal, silica and Sephadex in various forms. Small columns of bonded-phase material have become increasingly popular for preparative normal-phase chromatography.

In recent studies the most-used adsorbent for larger-scale purification has been PVPP or PVP, to bind and remove phenolic compounds from aqueous extracts of free GAs [22]. Samples are applied to columns in phosphate buffer at pH 8.0–8.5 and the GAs washed from the columns with buffer; alternatively PVP is simply filtered from slurries. Recent examples of PVP use include the purification of extracts of apple seeds [23], shoots of *Brassica* [24], *Zea* [25] and *Cucumis* [26], and shoots [27] and fruits [28] of *Citrus*. While acidic GAs and their glucosyl conjugates are recovered well from PVP, it seems possible that some might be retained if associated with phenolics [29] which bind to the PVP. GA–glucosyl conjugates could also be purified on PVP [22], but few examples are found. A PVP column was used in the normal manner in the purification of GA_9–glucosyl ester from shoots of *Picea* [30].

Charcoal or charcoal–Celite columns were used routinely in earlier studies to separate salts and polar impurities such as sugars from the GAs. The impurities are eluted with aqueous acetone or sometimes with aqueous methanol, and GAs are eluted subsequently in an increasing proportion of acetone, the most polar first [31]. While providing effective purification, the activity of charcoal can be variable, and acid-induced artefacts may be formed. These include isopropylidene derivatives (acetonides) of GAs with vicinal diols [32,33]. Recoveries may be poor because of irreversible adsorption especially of more polar compounds. These considerations have resulted in recent use being confined to only a few examples, such as the purification of GAs from leaves of *Silene* [34], and shoots of *Citrus* [27] and *Spinacia* [35]. Charcoal columns eluted with aqueous methanol or acetone have been used successfully as a means of purifying GA conjugates from large quantities of seeds in a number of earlier studies [15,36–39].

Normal-phase chromatography on silica also entails risk of poor recovery of strongly adsorbed polar compounds. Nonetheless, silica is often used in preparative purification, either in columns or in TLC. The latter largely replaced

paper chromatography and was originally popular as an analytical technique in GA analysis. It is used now only occasionally, mostly in preparative purification. The behaviour of free GAs and GA methyl esters in a number of acidic and basic solvent systems has been described in a number of studies [40,41]. Analytical use is now confined to situations where only a few compounds need to be resolved, as is often the case in analysis of the products of feeds of various substrates. Thus Ozga *et al.* [42] used silica TLC, with a solvent system of ethyl acetate–chloroform–acetic acid (90:30:1), to separate [^{14}C]GA_{20} and [^{14}C]GA_{19} from extracts of pericarp of *Pisum* fed with [^{14}C]GA_{12}. Such an acidic solvent system is typical of those used. There are a number of recent examples of schemes incorporating similar use of TLC in preparative chromatography of endogenous free GAs [43–45].

Takahashi *et al.* [5] have included tabulations of the R_F values of a range of GA conjugates in TLC obtained in early studies, and Schneider [16] has summarized the early widespread use of TLC in the analysis of GA conjugates formed from the metabolism of applied ^{3}H-, ^{14}C- or unlabelled GAs. Schneider *et al.* [46] have described a more recent use of silica TLC in the purification of permethylated GA–glucosyl conjugates before GC–MS analysis. A solvent system of toluene–ethyl acetate–acetic acid was used to purify the permethylated GA–glucosides as a group. A similar separation was also achieved on a column of silica eluted with a gradient of methanol in toluene [47].

Column chromatography, commonly on deactivated silica, has been more widely used in the purification of free GAs. The procedure pioneered for GAs by Powell and Tautvydas [48] and further developed by Durley *et al.* [49] (see also [6]), continues in routine use in several laboratories. Samples are applied to a column of silica with 0.5 *M* formic acid as the stationary phase and eluted with formate-saturated ethyl acetate in *n*-hexane, in a gradient or steps of increasing proportion of ethyl acetate if some separation of the GAs is required. The GAs elute in order of increasing hydroxylation or increasing carboxylation [49]. A 20×1.3 cm column of Woelm silica gel (deactivated with 20% water) had high capacity (100 mg of plant extract) and recoveries of the GAs exceeded 80%. Smaller columns (5 g, 10×1 cm) have been used in the preparative purification and separation of free GAs [9], or, eluted with 70 ml of ethyl acetate–*n*-hexane (95:5), to separate the GAs as a group from more polar impurities [50]. In these conditions [^{3}H]GA_8 elutes in 20–50 ml [51]. There are a number of other examples of the recent use of these procedures [23,26,45,52]. Less frequently, active silica has been used. Examples include Beale *et al.* [53], who used silica eluted in steps of increasing proportion of ethyl acetate in light petroleum (b.p. 40–60°C) in the purification of GA_{58} from endosperm of *Cucurbita*. Extracts of leaves of *Spinacia* [35] and *Silene* [34] were purified on columns of silica–Celite (1:2, w/w; 12×1.5 cm), with the GAs eluted in chloroform–ethyl acetate to leave adsorbed polar impurities.

Koshioka *et al.* [50] have used the "short-column" deactivated silica method to separate GA conjugates as a group (eluted from the column in methanol), from the free GAs, eluted previously with ethyl acetate–*n*-hexane. Evidence of the separation was based on the analysis of extracts of several [^{3}H]GA-fed plant tissues, with tentative identification of metabolites as being conjugated or otherwise. Subsequently, the procedure has been used routinely to separate conjugated GA-conjugates from free acids in studies of the metabolism of [^{3}H]GAs [9,54–57]. Although the GA conjugates can be expected to be much more polar than their respective aglycones, as seen for example in the separation of GA_9 and GA_9–glucosyl ester in normal-phase HPLC [58], it is now clear that GAs of a wide range of polarity cannot be cleanly separated as a group from their glucosyl esters and glucosides in this manner. Earlier examples of the use of silica in column chromatography for the purification of GA–glucosyl conjugates have been discussed in the reviews [5].

Silica columns have commonly been used to purify kauranoids, to remove impurities such as chloropyll and to provide preparative separation of the various kauranoids. Recent examples include that of Zeevaart and Gage [20], who separated *ent*-kaurene from the more strongly

retained chlorophyll in extracts of *Spinacia* and *Agrostemma* shoots on a 20-ml column of silica eluted with 40 ml of *n*-hexanes. Hazebroek and Metzger [21] purified a more polar kauranoid-containing fraction from shoots of *Thlapsi* on a 10 × 1.5 cm column of silica eluted with 100 ml of ethyl acetate–chloroform, and a less polar fraction on silica eluted in steps of increasing proportion of ethyl acetate in *n*-hexane. Suzuki *et al.* [19] separated kauranoids in the acidic ethyl acetate extract of Zea shoots on a 20-g column of silica eluted with 100 ml volumes of light petroleum (4×); light petroleum–ethyl acetate (95:5), 4×; light petroleum–ethyl acetate–acetic acid (90:10:0.2), 4×; light petroleum–ethyl acetate–acetic acid (85:15:0.2), 2×; and light petroleum–ethyl acetate–acetic acid (80:20:0.2), 2×. *ent*-Kaurene was contained in the first fraction, *ent*-kaurenal in fractions 2–4, *ent*-kaurenol in fractions 7–8, *ent*-kaurenoic acid in fractions 9–13 and *ent*-7α-hydroxy kaurenoic acid and GA_{12}-aldehyde in fractions 11–13. Silica Sep-Pak cartridges have been used to separate fatty acids from *ent*-kaurene in extracts of germinating caryopses of *Hordeum* [59]. The sample was applied in 5 ml of light petroleum (b.p. 60–80°C) and the cartridge washed with a further 5 ml of light petroleum to completely elute the *ent*-kaurene.

Columns of Sephadex G-25, G-50 and LH-20 have been used in normal-phase separations of GAs and kauranoids. MacMillan and Wels [60] described an analytical procedure with capacity for 100–200 mg of plant extract which yielded excellent separation of a range of GAs, but which required 30 h to complete. A 145 × 1.5 cm column was packed with LH-20 which had been equilibrated with the aqueous phase of a solvent mixture of light petroleum–ethyl acetate–acetic acid–methanol–water (100:80:5:40:7). The column was eluted with the organic phase of the mixture. Another column used with the solvents described above but in the proportions 50:15:10:10:2 provided better separation of the less polar GAs and kauranoids. A "non-polar" column used with light petroleum–acetic acid–methanol (100:1:40) separated *ent*-kaurene, *ent*-kaurenol and *ent*-kaurenoic acid. While partition chromatography clearly predominated in these cases, the mechanism of separation operating with this type of support may also include a component of size-exclusion chromatography. The use by Yamaguchi *et al.* [61] of LH-20 eluted with methanol–acetone, in which seven different GAs eluted in a narrow range of volumes, was described by those authors as "rough gel permeation". This procedure has been used subsequently in several studies as a means of preparative group purification of the free GAs [62–64]. Sephadex G-50 [15,36,39] and Sephadex LH-20 [17,38] have also been used for the normal-phase purification of GA conjugates.

Poling and Maier [27] described the use of bonded-NH_2 Sep-Pak cartridges for the purification of samples in preparation for HPLC on $N(CH_3)_2$ columns. Samples were loaded in methanol, the cartridge eluted with 15 ml of methanol, and the GAs eluted with 10-ml volumes of each of methanol–acetic acid (99:1) and methanol–acetic acid (98:2). From extracts of shoots of *Citrus* and of seeds of *Pisum*, GA_9, GA_{20}, GA_{29}, GA_{51} and GA_{29} catabolite eluted mainly in the methanol–acetic acid (99:1) fraction, with 15–20% of GA_{20} and GA_{29} eluting in the methanol, and 20–25% of the GA_{29} catabolite eluting in the methanol–acetic acid (98:2) fraction. With 20 mg loading, the sample mass was reduced by 90%. We have purified various samples using a similar procedure with NH_2-PrepSep cartridges and found that the retention of GAs on loading, and also when the cartridge was eluted with non-acidic solvent, was not always predictable [65]. As might be expected, the GAs were better retained initially if loaded in ethyl acetate–methanol (4:1) instead of methanol. After the cartridge was eluted with 1-ml volumes of ethyl acetate–methanol (4:1 and then 1:1), $[^3H]GA_3$, $[^3H]GA_8$, $[^2H_2]GA_{19}$, $[^3H]GA_{20}$ and $[^{14}C]GA_{53}$ were eluted in 1 ml of methanol–acetic acid (99:1) and 3 ml of methanol–acetic acid (98:2). The method could be further modified by adjusting the pH of the sample to ensure that the GAs were ionized and so better retained on loading.

A number of Japanese workers in particular have reported use of a similar procedure, entailing chromatography on cartridges such as Bond Elut DEA, as a routine preparative step in GA

analysis. Samples are applied in methanol, impurities eluted with methanol, and the GAs eluted with methanol containing a small percentage of acetic acid. While the behaviour of standard GAs in this system does not seem to have been described, GAs representing a range of structures have been identified in samples purified by this method [28,66–69].

3.3. Reversed-phase chromatography

Small cartridges of reversed-phase material, such as C_{18} Sep-Pak, are now often used in preparative purification schemes. An example of the use of the method has been described by Hedden [1]. After washing the cartridges with methanol and then with 5% acetic acid, samples are loaded 0.1 *M* phosphate buffer pH 2.5. Polar impurities are eluted with 5 ml of 5% aq. acetic acid, and then with 5 ml of water, and the GAs subsequently eluted with 5 ml of 80% aq. methanol, leaving less polar impurities on the cartridge. Very polar GAs may elute from the cartridge in the wash with 5% acetic acid and water, and this step can be omitted. There are many recent examples of the use of C_{18} cartridges, which include various minor variations on the procedure described above [23,24,26,70–72].

Commercial C_{18} cartridges have limited capacity and are thus suitable for use later in purification schemes or for small samples. For larger samples, small columns packed with C_{18} material can be used in the same fashion as described for cartridges, with the volumes of eluting solvents increased accordingly. Such columns have also been used in lieu of solvent partitioning of aqueous extracts (against light petroleum, diethyl ether or hexane) to effectively separate chlorophyll, carotenoids and other non-polar impurities from the GAs in aqueous methanolic extracts [50].

These procedures are also suitable for separating non-polar impurities from the GA conjugates, which elute from reversed-phase chromatography before their aglycones (see section 4.1.3). Similarly, some of the more polar kauranoids can be purified in this manner [19].

3.4. Size-exclusion chromatography

The use of size-exclusion chromatography (SEC) for GA purification was first described by Reeve and Crozier [73,74]. Bio-Beads SX-4 (porous polystyrene, exclusion limit M_r 1500) was packed into two 100×2.5 cm columns connected in series which were eluted with tetrahydrofuran at 2 ml min^{-1}. Solutes elute in order of decreasing size, and the free GAs were obtained in an elution volume of 450–570 ml. This method has high capacity and recoveries are high, but is time-consuming. Faster, small-scale HPLC-based methods were subsequently developed [75]. In chromatography on a 300×8 mm column of PL gel (cross-linked polystyrene–divinylbenzene copolymer) in tetrahydrofuran–acetic acid (99.5:0.05), free GAs (defined by [^{3}H]GA_{43}, M_r 394, and [^{3}H]GA_9, M_r 316) were eluted in 6.8–7.8 ml. Acetic acid was added to suppress ionization of the GAs and its use resulted in improved chromatography. The capacity of this column was 100 mg of plant extract. In similar fashion a variety of C_{19} and C_{20} GAs ranging in molecular mass from 316 (GA_9) to 364 (GA_8) were eluted from a 500×8 mm column of Shodex A-801 (polystyrene–divinylbenzene polymer, with an exclusion limit of about M_r 1000) in terahydrofuran at 1 ml min^{-1}, with retention times between 11.9 and 13.7 min [61]. The method has been used recently for preparative purification of GAs in a number of studies [63,71,76–78]. SEC may be of general practical value when used early in a purification procedure to reduce sample mass for subsequent chromatography [75]. Its usefulness later in purification will depend on the nature of the particular sample. Thus when used after preparative RP-HPLC in the purification of GA_9 from extracts of *Picea*, HPSEC yielded little useful additional purification of the GA_9-containing fractions [79].

The large difference in mass between free GAs and GA conjugates has been exploited to separate these two groups of compounds by SEC. Thus ^{3}H-labelled GA-glucosides and glucosyl esters, produced in shoots of *Phaseolus* seedlings fed with [^{3}H]GA_4, have been separated as a group from [^{3}H]GAs [80] with the

open column SEC procedure of Reeve and Crozier [73]. The ^{3}H-labelled high-molecular-mass compounds eluted in 340–365 ml, and the ^{3}H-labelled low-molecular-mass compounds eluted in 370–440 ml.

SEC has also proved useful in the purification of kauranoids. From an extract of shoots of *Thlapsi*, several of the kauranoids were separated as a group from impurities of higher-molecular-mass (notably chlorophyll), on a 500 × 6 mm column of Bio-Beads SX-8, exclusion limit M_r 1000 [18]. About 90% of the sample mass eluted in the first 90 ml of ethyl acetate, and the next 30 ml contained *ent*-kaurene, *ent*-kaurenoic acid and *ent*-kaurenol. This method is a valuable alternative to chromatography on silica in such purifications.

3.5. Anion-exchange chromatography

Anion-exchange chromatography on DEAE-Sephadex A-25 was used by Gräbner *et al.* [81] to separate GA_3, GA_7 and GA_3-3-O-glucoside. Adaptations of this procedure, including chromatography on other anion-exchange materials, have become increasingly popular as a means of separating neutral and weakly acidic impurities from the more acidic GAs, or for separating GA–glucosyl esters from the free GAs. The DEAE-Sephadex is prepared in the acetate form, neutral compounds are eluted with methanol, and increasingly acidic compounds eluted with increasing amounts of acetic acid in methanol, as described, for example, by Fujioka *et al.* [25]. In this study a 100-ml column was eluted with successive 100-ml volumes of methanol, 0.25 *M* acetic acid in methanol, 0.5 *M* acetic acid in methanol, 0.75 *M* acetic acid in methanol, 1 *M* acetic acid in methanol and 3 *M* acetic acid in methanol. Free GAs apparently eluted in all of the acidic fractions. This procedure yields some resolution of the acidic compounds, but if this is not required a simple two-step elution with methanol or weakly acidic methanol and then with more acidic methanol should suffice [6].

The stronger anion-exchanger QAE-Sephadex A-25 has also been used in a number of studies. Aqueous extracts at pH 7.5–8.0 were applied to a 5-ml column with had been equilibrated with sodium formate, neutral impurities were eluted with 15 ml of water, and the GAs eluted with 20 ml of 0.2 *M* formic acid [23,24]. QAE-Sephadex A-25 in the acetate form has been used in a similar fashion [34,35]. Other materials which have been used in recent studies include DEAE-cellulose (DE-52) in the hydroxylated form [45].

DEAE-Sephadex A-25 has been widely used in preparative chromatography of GA conjugates, to separate the neutral GA–glucosyl esters from the acidic GA–glucosides [10,46,80,81]. The method is the same as described above for the free GAs. QAE-Sephadex A-25 has been used to purify GA glucosyl esters from shoots of *Picea* [30]. The sample was applied to a 10 × 1 cm column in 5 m*M* sodium phosphate pH 8 and the GA–glucosyl esters eluted with 25 ml of the same buffer.

ent-Kaurenoic acid, *ent*-7α-hydroxy kaurenoic acid and GA_{12}-aldehyde from extracts of *Zea* shoots have also been purified on DEAE-Sephadex A-25 [19]. On a 25-ml column eluted with 10-ml volumes of methanol (4×), 0.25 *M* acetic acid in methanol (4×), and 0.5 *M* acetic acid in methanol (4×), these compounds eluted with the last of the methanol fractions and in the first three weakly acidic fractions.

3.6. Immunoaffinity chromatography

Immunoaffinity chromatography (IAC) is a valuable technique for selectively purifying small quantities of GAs before identification or quantification, with a minimum of other preparative steps. Antibodies for use in immunoassays for GAs have been developed in a number of laboratories, and used in studies which include those of Fuchs and Fuchs [82], Weiler and Wieczorek [83], Atzorn and Weiler [84,85], Eberle *et al.* [86], Knox *et al.* [87,88], Odén *et al.* [79], Yamaguchi and co-workers [78,89,90], Durley *et al.* [91] and Nakajima and co-workers [92,93]. Such antibodies were first used in IAC by Fuchs and Gertman [94]. However, not until recently has the application been revived, and reports of use are confined to only a few examples. The principles of IAC have been described in one of these [70]. Briefly, anti-GA antibodies (monoclonal or polyclonal) are raised against

GAs linked to a carrier protein [bovine serum albumin (BSA) or keyhole limpet haemocyanin (KLH)]. The position of attachment of the protein to the GA-hapten determines the portion of the GA molecule which is presented for immune recognition, and thus determines the specificity of the antibodies. The antibodies are subsequently bound to a support, usually Sepharose, which is used to prepare a column. Partially purified samples are added to the column, and antigenic GAs form ligands with the antibodies. After non-antigenic impurities are eluted from the column, the bound GAs can be eluted with water, aqueous methanol or methanol, in order of increasing affinity for the antibody [95]. Depending on the specificity of the antibody or antibodies which are used, IAC may be used to selectively bind a single GA, a certain class of GAs (*e.g.*, C-3- or C-13-hydroxy), or a wider range of GAs. In common with immunological techniques, the binding of the primary antigen(s) to the antibody in IAC is subject to interference by inhibitory components in plant extracts, and by cross-reactive compounds which bind with similar affinity as the GA(s) of interest. Various preparative purification steps may be required to remove such compounds, as illustrated in the examples which follow. Results of these studies emphasize the need for verification of identities and quantities by a less equivocal method such as GC–MS.

Polyclonal antibodies, which might be expected to offer a range of specificities and thus be most useful in binding a wide range of GAs in IAC, were used in IAC by Durley *et al.* [91]. Antibodies were prepared against a mixture of GAs (GA_1, GA_3, GA_4, GA_7 and GA_9) each conjugated to BSA via *p*-aminohippuric acid at the carboxy function. These five GAs include common structural features found in C_{19} GAs (C-3 and C-13 hydroxylation, and C-1,2 and C-2,3 double bonds). The anti-GA antibodies, immobilized on matrices of Affi-Gel 10 or Sepharose CL-4B, retained C_{19} GA methyl esters, but did not retain free GAs or any of the C_{20} GA methyl esters which were examined (GA_{13}, GA_{14}, GA_{18}, GA_{23} and GA_{27}). Spikes of the methyl esters of the GAs used for antibody preparation, and in addition the methyl esters of GA_{20}, GA_{29}, GA_{34}, GA_{54} and GA_{57}, were recovered from a soybean leaf extract with greater than 90% yield. In order to avoid non-specific binding of substances such as phenolics to the column, extracts were first purified by anion-exchange chromatography on DEAE-cellulose (DE-52), and then by chromatography on a small (1.5–2 ml) C_{18} column. The methylated samples were taken up in methanol, diluted with phosphate-buffered saline (PBS), and applied to the column. Impurities were washed from the column with PBS and water, and the bound GA methyl esters eluted with methanol, which was found to be the most effective of a variety of chaotropic agents and changes in pH. A 3-ml column of Affi-Gel 10 loaded with 35 mg ml^{-1} of immunoglobulin G (IgG) could retain 390 ng ml^{-1} of GA_1 methyl ester, 1090 ng ml^{-1} of GA_4 methyl ester and 1280 ng ml^{-1} of GA_9 methyl ester. Columns could be reused as many as 500 times. Durley *et al.* noted that high IgG loading is an advantage in immunoaffinity chromatography of small molecules such as GAs, since the column capacity is maximized, the amount of immunosorbent is minimized, the flow-rate is maximized, and the amount of methanol required to elute the GAs is minimized. A high column capacity (10- to 100-fold greater than the content of the sample) is necessary to ensure complete recovery of the GAs of interest in the face of competition from cross-reacting GAs and interference from other components in plant extracts.

Knox *et al.* [87,88] and Nester-Hudson *et al.* [96] have prepared a number of monoclonal antibodies raised against GAs coupled to KLH at either C-3 or C-17. These antibodies recognize free acids, and have been used in IAC in a number of studies for the purification of GAs from a variety of materials.

The antibody MAC 183 has a high affinity for GA_4 and discriminates between GA_4 and GA_9, while MAC 136 binds C-13-hydroxy GAs. These were tested for use in IAC by Smith and MacMillan [95]. An aqueous extract of *Pisum* cotyledons was effectively purified by MAC 136, and yielded GA_{29} by GC–MS in similar quantity to that found in an extract purified by other methods. In a test of the possible effect of

interference by components in extracts, the binding of labelled GA_{20} to the matrix was found to be constant in the presence of increasing amounts of an extract of internodes of *Pisum*. Results with MAC 183, however, illustrated some of the potential problems with IAC, particularly if immunoassay is used subsequently to identify and quantify GAs. The MAC 183 column bound a small proportion of the large amount of GA_{43} and GA_{46} present in *Marah* endosperm, and these GAs were subsequently also bound by MAC 183 in radioimmunoassay (RIA). The amount of GA_4, the primary antigen, was negligible. The method thus erroneously indicated the presence of GA_4. Given these problems, Smith and MacMillan concluded (of the successful results with MAC 136) that "... confidence in the reliability of these results was entirely dependent on the information provided by GC–MS."

Smith *et al.* [70] used MAC 136, and MAC 213 (which binds C-3-hydroxy GAs), in IAC to purify GAs from stems and leaves of *Cucumis*. The aqueous methanolic extracts were first partitioned against light petroleum (b.p. 60–80°C), and passed through a C_{18} Sep-Pak, to remove components which interfered with antigen–antibody binding. It was also necessary to pass the extract through foetal calf serum (FCS)–Sepharose to remove components which would bind non-specifically and irreversibly to FCS in the IAC matrix, and thus also interfere with binding. FCS was the major non-antibody protein present in the culture medium of the antibody-producing cells and as such was the major bound protein in the IAC matrix. The purified extracts were then passed through MAC 136–Sepharose and the GAs which were bound to the immunosorbent were eluted with water. The non-bound component was applied to MAC 213–Sepharose and the bound GAs were then eluted with 30% aq. methanol. Gibberellins $A_{1,3,4,8,20,29 \text{ and } 34}$ were subsequently identified and quantified by GC–MS.

The C-13-hydroxy GAs from shoots of *Pisum* [97] and *Lactuca* [98] have also been purified on MAC 136–Sepharose. Preparative purification entailed adsorption chromatography on PVPP, the usual sequence of solvent partitioning steps, and chromatography on DEAE-Sephadex A-25. A final partitioning step was necessary to remove lipids which remained after earlier extraction with hexanes and which prevented binding of GAs to the MAC 136 matrix. Immunoaffinity chromatography effectively purified the samples for GC–MS, whereas RP-HPLC did not provide sufficient purification.

Nakajima *et al.* [93] raised monoclonal antibodies against GA4 coupled to BSA at C-16. One antibody, 1-A8(1)/B8, with a relatively low affinity constant for GA_1 and broad cross-reactivity with a number of free acid C_{19} and C_{20} GAs, was selected for IAC. After preparative chromatography on a Bond Elut DEA cartridge, GA-containing fractions from extracts of small amounts of anthers of *Oryza* and immature seeds of *Phaseolus* (27 and 50 mg fresh mass, respectively) were purified on a column of 1-A8(1)/B8-Sepharose. The GAs which were bound to the matrix were eluted with 40% methanol in 0.1 *M* acetic acid buffer pH 4.0 containing 0.5 *M* potassium thiocyanate. After RP-HPLC, fractions were analyzed by RIA. The amount of GA_1 in the *Phaseolus* seeds as determined by RIA was in good agreement with that obtained by GC–MS.

4. ANALYTICAL CHROMATOGRAPHY

The methods used in the identification and quantification of GAs include HPLC, GC and GC–MS, and LC–MS. Each method has valuable applications. For analysis of endogenous GAs, GC–MS and LC–MS yield positive identification and are the methods of choice, given the large number of GAs which may be present in any sample. More than 20 of the 90 known GAs, and other as yet unnumbered GAs, have been identified in extracts from single source [23,35,99]. Given expected precursors for the several biosynthetic pathways, and logical metabolites, presumably a number of other GAs would also be present in small amounts. Gradient-elution HPLC is not adequate to resolve all of these compounds in admixture, and with the exception of mass spectrometers or certain bioassays, HPLC detectors lack either the specificity or the sensitivity to enable unequivocal identifi-

cation of the GAs. A smaller number of GAs, metabolites of feeds of various substrates for example, can be separated either by gradient HPLC or isocratic HPLC, and provided that the system is well characterized can be identified with reasonable certainty.

Detectors for HPLC which have been used for the GAs include UV, bioassay, immunoassay and radiocounters. The GAs absorb only weakly in the UV at 200–210 nm in common with other carboxy acids, with the limit of detection about 50 ng in optimal conditions [6]. Certain GA derivatives such as benzyl esters [74], *p*-nitrobenzyl esters [100] and *p*-bromophenylacyl esters [101] absorb at wavelengths more amenable to analysis (256, 265 and 256 nm, respectively). The limit of detection for GA-monobenzyl esters, however, is only about 300 ng. The limit for the latter two derivatives, as the mono esters, is about 10 ng [6] and 5 ng, respectively. Greater sensitivity can be obtained with methoxycoumaryl esters [102] which fluoresce strongly (excitation at 320 nm, emission at 400 nm) and can be detected at about 1 pg for the GA3 mono ester [102]. While these derivatives can be detected in smaller amounts than the free GAs, the method is not selective for the GAs; other carboxy acids in natural samples will form derivatives which absorb in a similar fashion. Crozier and Durley [6] have provided a detailed discussion of the use of such GA derivatives in HPLC.

Sensitive bioassays are available to detect those GAs active in promoting elongation. The dwarf rice microdrop assay [103] is a widely used method for detection of a range of active GAs. The sensitivity of this assay can be improved by treating the seeds during imbibition with the GA biosynthesis inhibitor uniconazole [104] or with both uniconazole and the inhibitor prohexadione calcium [105]. Normal rice can be substituted for the less-easily obtained dwarf varieties [105,106]. The minimum detectable dose of GA_1, GA_3, GA_4 and GA_7 is 1–3 fmol (*ca.* 0.3–1 pg) when applied to the dwarf cultivar Waito C, and 3–30 fmol when applied to the cultivar Tan-ginbozu or the normal variety Koshihikari [105]. This remarkable sensitivity compares favourably with that of immunoassay. Other bioassays in use include lettuce hypocotyl, cucumber hypocotyl, barley aleurone, dwarf pea and dwarf maize [6]. Bioassays can be used for quantitative analysis, but are limited to detection of active GAs and are subject to interference from other components in plant extracts. A comparison of results from bioassay, radioimmunoassay, and GC–MS in the analysis of GA_9 in shoots of *Picea* [79] illustrates this point.

RIA and enzyme-linked immunoassay (EIA) are sensitive methods for the detection of GAs with limits of detection ranging to the fmol level. For example, in the EIA of Atzorn and Weiler [85], the limits were 0.5 fmol for GA_3, 1.0 fmol for GA_4 and 1.5 fmol for GA_7. Antibodies to the GAs have been raised in a number of laboratories (see section 3.6). Their use in immunoassay has been outlined in Hedden [1] and Beale and Willis [3]. The specificity of the immunoassays varies, depending on the cross-reactivity of the antibodies. In common with bioassays, immunoassays are subject to interference from other components in plant extracts.

Radiocounters have been used in many studies to detect metabolities from feeds of radio-labelled substrates. Where the substrate was ^{14}C-labelled, or also labelled with a stable isotope, or accompanied by a stable isotope-labelled analogue, the fractions containing the heavy isotope-labelled metabolites can be identified for subsequent analysis by GC–MS [19,21,72,76,107–109]. If used on-line [110,111], radiocounters provide accurate measurements of retention time, but on this basis alone enable only tentative identification of potential metabolites. Where fractions are collected from HPLC and analysed for radioactivity [50,54] confidence in identifications is less certain, unless the system has been well characterized [112,113]. In other cases identification may be confounded not only by other GAs with properties similar to those of expected metabolites but also by the formation of labile associations of GAs with compounds such as phenolics [29] with unpredictable chromatographic behaviour. Confidence in the identification can be improved by chromatography on more than one type of column [114].

For the endogenous GAs the use of HPLC in analysis has been confined largely to the prepara-

tive separation and purification of GAs before definitive analysis by GC–MS. Compilations of retention times can be useful in aiding identification in such cases, and also in guiding the choice of fractions for pooling before GC–MS, often in conjunction with information from bioassay and the elution of radioactive internal standards. They also serve to indicate possible candidates for search by GC–(selected ion monitoring (SIM), if there is not a sufficient amount of the GA present for detection by GC–MS.

With the use of immunological methods as a means of identifying and quantifying GAs, HPLC retention times of immuno-reactive fractions provide additional evidence of identity. More recently, however, with the introduction of LC–MS has come the beginning of use of HPLC as an integral part of the analytical method by which GAs and GA conjugates may be identified.

4.1. Reversed-phase HPLC

4.1.1. Free GAs

Ion-suppressed reversed-phase HPLC has been the most favoured means of separating the GAs. The established practice is to separate GAs of a wide range of polarity on columns of bonded octadecylsilica (ODS, C_{18}) with a polar mobile phase consisting of a gradient of increasing methanol in acidic water (*e.g.*, refs. 115 and 116). Aqueous acetonitrile has also been used in a few studies [42,92,117]. Acetic acid is widely used as an ionic suppressor in concentrations ranging to 1 ml l^{-1}, although 50 μl l^{-1} is sufficient [1]. It can be readily evaporated from the fractions after HPLC with little danger to the GAs. It is less suitable for use where the elution of GA standards is being monitored by UV absorption since it yields a high background. Phosphoric acid has also been used [97,118] though it is not recommended in preparative purification for risk of degrading the GAs [1]. Formic acid was used by Lin and Heftmann [119].

In such conditions the GAs elute in order of decreasing polarity, first described in Jones *et al.* [120], who reported the separation of 20 GAs in gradient elution. Barendse and Van den Werken [121] reported the development of methods for the separation of isomers such as GA_1 and GA_3, GA_5 and GA_{20}, and GA_4 and GA_7 in isocratic and gradient-elution HPLC, and Lin and Heftmann [119] described the separation of ten GAs in isocratic HPLC.

Koshioka *et al.* [115] extended the earlier results [120,121] to include the retention times in gradient-elution HPLC of three additional GAs and a range of derivatives, isomers and related compounds. The most extensive original list, with precise retention times in isocratic HPLC, is that of Jensen *et al.* [118], which includes 41 of the first 53 numbered GAs plus GA_{12}-aldehyde. A list of the retention times in gradient-elution HPLC of 24 GAs, including three of those (GA_{32}, GA_{54} and GA_{55}) not described previously [122] was later extended by Lin *et al.* [116] to include GA_2, GA_{61}, GA_{62}, GA_{63} and GA_{68}. With interpolation of retention times from Jensen *et al.* [118] and Koshioka *et al.* [115] 66 compounds were represented, including 51 of the numbered GAs. This list is reproduced in Table 1, with retention times of several other compounds added, estimated from recent reports. Koshioka *et al.* [115] and Jensen *et al.* [123] provided analyses of the effects of the various structural features on elution of the GAs, and these were extended by Lin *et al.* [116]. The other of elution of the non-hydroxylated GAs reflects the relative polarities conferred on the molecules by the substituent at C-20 and the C-19 carboxy function, for the C_{20} GAs (GA_{24}, GA_{25}, GA_{12} and GA_{15}), or by the C-19,10-lactone, for GA_9. Hydroxylation of these GAs reduces their retention. The effect of hydroxylation at different positions on the polarity of the C_{19} GAs is $12\alpha > 13 > 11\beta > 16\beta > 1\alpha > 2\alpha > 15\beta > 1\beta > 2\beta > 3\alpha > 3\beta$ [116,123]. Local interactions, such as that of the C-13 hydroxyl with the exocyclic methylene at C-16, may modify the effect that individual hydroxyl functions might have otherwise [123]. With the exception of hydroxylation at C-15, hydroxylation in the C- and D-rings has greater effect in reducing retention time than does hydroxylation in the A-ring, possibly a consequence of the already hydrophilic nature of the A/B region of the molecule [123]. This generalization does not hold

TABLE 1

RETENTION TIMES OF GIBBERELLINS, GIBBERELLIN METHYL ESTERS AND RELATED COMPOUNDS IN ION-SUPPRESSED REVERSED-PHASE HPLC

Adapted from Lin *et al.* [116] and Lin and Stafford [122]. Unless otherwise indicated, analysis on Ultrasphere ODS (5 μm, 250 × 4.6 mm); gradient: A = linear from 35% aqueous methanol containing 0.05% acetic acid to 100% methanol with 0.05% acetic acid in 40 min, B = linear from 40% methanol to 100% methanol in 30 min; 1 ml min^{-1}.

Compound	Retention time (min)		Compound	Retention time (min)	
	A	B (methyl ester)		A	B (methyl ester)
GA_{89}	2–5[a]		GA_{10}	17.8–18.4[e]	
12α-Hydroxy GA_{43}	2–5[b]		GA_{16}	18.42	
GA_{55}	5.03	6.64	GA_{20}	18.97	19.41
GA_{8}	5.08	7.36	GA_{46}	19.0–19.5[b]	
GA_{85}	5.1–6[c]		GA_{27}	19.61	
GA_{81}	5.0–6.5[d]		GA_{47}	19.6–20.4[e]	
GA_{29}	6.0–6.5[e]		GA_{36}	20.44	20.44
GA_{39}	6.0–6.5[e]		GA_{13}	20.47	26.10
Iso-GA_3	6.36		GA_{68}	20.5[i]	
GA_{32}	6.50		GA_{40}	20.5–22.0[e]	
GA_{33}	6.98		Allogibberic acid	20.5–22.5[e]	
Gibberellenic acid	7.42		12α-Hydroxy GA_{25}	20.5–21.4[b]	
GA_{30}	7.46		GA_{44}	21.4	
GA_{23}	7.7–8.2[e]		GA_{63}	21.5[i]	
GA_{28}	8.0–8.4[e]		GA_{19}	22.41	23.54
GA_{38}	8.3–8.6[e]		GA_{54}	22.58	22.85
GA_{41}	9.1–9.3[e]		GA_{34}	22.83	22.80
GA_{26}	9.1–9.3[e]		GA_{62}	23.5[i]	
GA_3	9.38	10.32	GA_{17}	23.74	25.35
C-3-*epi* GA_1	9.41	11.51	*epi*-Allogibberic acid	23.5–24.0[e]	
GA_1	10.51	11.41	GA_{37}-15-ene	24–26[f]	
GA_{29}-catabolite	10.5–11.0[e]		C-3-*epi*-GA_{37}	24–26[f]	
$\Delta^{1,10}$ GA_1-counterpart	11.32		GA_{51}	24.5[i]	
GA_6	11.5–12.3[e]		GA_{37}	24.07	22.94
GA_{18}	12.0–12.5[e]		GA_{61}	24.5[i]	
GA_{77}	12–14[g]		GA_{88}	24.5[h,i]	
GA_{35}	12.89		C-3-*epi*-GA_4	24.42	
GA_1 methyl ester	13.40		GA_7	24.92	23.69
$\Delta^{1,10}$ GA_1-counterpart-7-methyl ester	13.2–13.6[e]		Iso-GA_7	25.0–26.0[e]	
			12α-Hydroxy GA_{12}	24–26[b]	
GA_{22}	13.61		GA_4	26.07	24.60
GA_{21}	14.25		GA_{53}	27.81	27.34
GA_2	16.07		GA_{14}	28.17	28.64
GA_{31}	16.07		GA_{24}	28.86	29.91
GA_{43}	14.5–17.5[e]		GA_9	29.36	27.79
GA_5	17.86	18.67	GA_{25}	29.54	31.21
			GA_{15}	29.76	27.87
			GA_4 methyl ester	29.0–31.0[e]	
			GA_{12}	32.0–38.0[e]	
			GA_{12}-aldehyde	38.0–44.0[e]	
			ent-Kaurenoic acid	44.82	
			ent-Kaurene	60.72	

[a] Estimated from ref. 126.
[b] Estimated from ref. 72.
[c] Estimated from ref. 127.
[d] Estimated from ref. 128.
[e] Estimated from refs. 115 and 118.
[f] Estimated from ref. 129.
[g] Estimated from ref. 68.
[h] From ref. 130.
[i] ±0.5 min.

for the C_{20} GAs, where GA_{17} and GA_{19} elute from RP-HPLC later than their C-3 hydroxy counterparts, GA_{13} and GA_{36}. Those GAs which are dehydrogenated in the A-ring at C-1,2 or C-2,3, or in the C-ring at C-9,11, elute before their saturated counterparts. Precise retention times for other GAs not included in Table 1 are not readily available but the approximate elution volumes of GAs of particular structure can be confidently predicted. Description of the behaviour of these other GAs in RP-HPLC have not been published or are limited to large elution volumes for the natural isolates.

Heavy isotope-labelled GAs are frequently included in extracts for quantification of their endogenous counterparts. Since ^2H-labelled GAs elute from RP-HPLC slightly before their endogenous counterparts, as observed for similarly labelled standards of other hormones [124,125], care must be taken not to separate the two.

4.1.2. GA derivatives

Lin and Stafford [122] listed the retention times of 23 GA methyl esters in gradient-eluted RP-HPLC (Table 1), and described the method as having the highest separation efficiency in comparison with RP-HPLC of the free GAs and silica HPLC of free GAs and GA methyl esters. The GA methyl esters elute from RP-HPLC in approximately the same order as the free acids, with exceptions expected of C_{20} GAs which have more than one carboxy function (which, of those included in Table 1, are GA_{13}, GA_{14}, GA_{17}, GA_{19}, GA_{24}, GA_{25} and GA_{36}). Gibberellins in methylated plant extracts may be better separated by RP-HPLC from certain impurities than they would otherwise, but biologically active free GAs are virtually inactive if methylated, and so the opportunity to detect these subsequently by bioassay is lost. The method is relevant in studies where the GAs have been methylated for recognition in certain immunoassays, and also where radio-labelled products of feeds of radiolabelled substrates are to be purified, since these compounds can be easily detected by HPLC–radiocounting. There are only a few reports of the purification of GAs from methylated plant extracts [42,131,132].

p-Bromophenylacyl esters have been analyzed on RP-HPLC by Morris and Zaerr [101]. Separations of the highly fluorescent methoxycoumaryl esters on RP-HPLC [102] are generally similar to those obtained for the free GAs (as shown, for example, in Table 1). Thus GA_1, GA_4 and GA_{20} are easily distinguished from their more polar A-ring dehydro analogues (GA_3, GA_7 and GA_5, respectively); increasing hydroxylation increases polarity; and an increasing number of methoxycoumaryl groups decreases polarity. The derivatives are not sufficiently volatile for analysis by GC–MS, and so their use has been confined to identification of GAs in only few studies [80,114].

RP-HPLC has also been used to introduce GA methyl esters and free GAs to MS. This is discussed in section 4.4.

4.1.3. GA–glucosyl conjugates

RP-HPLC has been popular for the purification and separation of the GA conjugates, polar compounds that are strongly adsorbed and often not well recovered from other types of chromatography. Comprehensive descriptions of the behaviour of GA conjugates in isocratic RP-HPLC are found in Yamaguchi *et al.* [39], Schneider [16], Jensen *et al.* [118] and Sembdner and Schneider [133]. Koshioka *et al.* [115] have described the behaviour of GA conjugates in gradient-elution RP-HPLC.

All of the GAs whose conjugates are represented in these studies can be separated from their glucosyl esters or glucosides, and all are less polar in RP-HPLC than their conjugates (Table 2). The glucosyl esters of GA_1, GA_3, GA_4 and GA_7 elute after their 3-O-glucoside counterparts. Sembdner and Schneider [133], however, have shown GA_4- and GA_7-glucosyl esters to elute before the 3-O-glucosides. For GA_1 and GA_3, the 3-O-glucosides are less polar than the 13-O-glucosides. Jensen *et al.* [123] suggested that the order of elution of the GA_1–glucosyl conjugates (GA_1–13-O-glucoside > GA_1–3-O-glucoside > GA_1–glucosyl ester) reflects (a) the polarity conferred by the highly polar carboxyl group, and (b) the orientation, primarily, and also the position, of the glucose moiety. In GA_1–3-O-glucoside the glucose is

TABLE 2

RETENTION TIMES OF GIBBERELLIN CONJUGATES AND THEIR AGLYCONES IN ISOCRATIC REVERSED-PHASE HPLC

Adapted from Jensen *et al.* [118]. Supelcosil LC C18 (5 μm, 250 × 4.6 mm), isocratic methanol in aqueous phosphoric acid pH 3.0, 1 ml min^{-1}.

Compound	Methanol (%)									
	10	15	20	25	30	35	40	45	50	55
GA_{29}-2-O-glucoside	19.9	11.2	7.4							
GA_8-2-O-glucoside	23.8	12.6	8.0							
GA_8			11.5	8.2						
Gibberellenic acid-2-O-glucoside		25.7	13.6	8.9						
GA_3-13-O-glucoside		26.1	13.9	8.9						
GA_{29}			14.8	10.3	8.2					
GA_1-13-O-glucoside		30.0	15.9	10.0						
GA_3-3-O-glucoside			22.1	12.8	8.4					
GA_1-3-O-glucoside			23.3	13.5	8.7					
GA_{38}-glucosyl ester			23.9	14.5	9.2					
GA_{26}-2-O-glucoside			25.1	15.6	9.6					
GA_3-glucosyl ester			26.4	16.0	9.8					
GA_1-glucosyl ester			31.1	17.8	11.2					
GA_{38}				19.1	12.4	8.2				
GA_{35}-11-O-glucoside				19.6	11.3	8.0				
GA_{26}				22.3	14.0	9.1				
GA_3				23.0	14.0	9.2				
GA_1				26.5	16.5	10.8				
GA_{35}					24.2	15.8	10.5			
GA_5-13-O-glucoside					24.4	14.5	9.2			
GA_{20}-13-O-glucoside					25.2	15.2	9.8			
GA_5-glucosyl ester					44.9	23.1	13.1			
GA_{20}-glucosyl ester						24.3	14.0	9.4		
GA_{37}-glucosyl ester						25.3	17.3	11.2		
GA_7-3-O-glucoside							20.1	12.6	8.4	
GA_5							24.3	14.6	9.6	
GA_4-glucosyl ester							24.5	15.3	9.8	
GA_{20}							28.0	17.2	11.2	8.4
GA_7-glucosyl ester								22.6	13.6	8.9
GA_{37}									24.2	16.8
GA_7									30.0	20.6
GA_4									36.8	25.0

axial, while in GA_1-13-O-glucoside it is equatorial.

Endogenous GA conjugates have been identified in recent studies from a variety of sources by MS after final isolation on RP-HPLC (see section 4.5). In a recent novel use of RP-HPLC which culminated in the identification of GA–glucosides from *Pisum*, *Hordeum* and *Zea*, the permethylated GA–glucosides were separated as a group on RP-HPLC from the GA permethyl derivatives [11,47]. The retention times are shown in Table 3.

Radiolabelled GA conjugates, produced from feeds of a labelled free GA substrate to various systems, have been tentatively identified by comparison of retention times on analytical RP-HPLC with the retention times of authentic standards of GA conjugates [9,50,54,55,80]. Further evidence of identity was provided by hydrolysis of the presumed [^{3}H]conjugates to yield products which eluted at the retention times of the expected ^{3}H-free GAs on HPLC and/or GC. The hydrolysis of fractions containing presumed GA conjugates, to yield free GAs which can be further purified on RP-HPLC and/or identified by GC–MS, has been a common technique in studies where definitive methods of identifying conjugates have not been available.

TABLE 3

RETENTION TIMES OF PERMETHYLATED (PME) GIBBERELLINS AND GIBBERELLIN-O-GLUCOSIDES IN REVERSED-PHASE HPLC

Adapted from Schneider *et al.* [11]. LiChrospher 100 RP-18 (5 μm, 125 × 4 mm); isocratic methanol–water (70:30), 1 ml min^{-1}.

Compound	Retention time (min)
GA_1-PME	2.87
GA_{29}-PME	3.32
GA_5-PME	3.40
GA_3-PME	3.45
GA_8-PME	3.60
GA_{20}-PME	3.78
GA_3–13-O-glucoside-PME	5.27
GA_{29}–13-O-glucoside-PME	5.27 [134]
GA_5–13-O-glucoside-PME	5.50 [134]
GA_{29}–2-O-glucoside-PME	5.67
GA_{20}–13-O-glucoside-PME	6.23
GA_8–2-O-glucoside-PME	6.35
GA_1–13-O-glucoside-PME	6.67
GA_3–3-O-glucoside-PME	6.67
GA_1–3-O-glucoside-PME	7.33

RP-HPLC has also been used to introduced GA conjugates to MS, as described in section 4.4.

4.1.4. Kauranoids

If included with fractions that also contain GAs, these compounds, most of which are less polar than the GAs, can be separated by RP-HPLC in 100% methanol at the end of the usual gradient of aqueous methanol (Table 1). Where the kauranoids have been examined as a separate group of compounds, solvent programs more appropriate to their polarity have been used. Examples include that of Turnbull *et al.* [135] (Table 4). The behaviour in RP-HPLC of a number of other kauranoids which are not intermediates in the pathway to the GAs and which are not included in Table 4, including kaurenolides, and mono-, di- and trihydroxylated kaurenoic acid, has been described by Metzger and Hazebroek [18]. Suzuki *et al.* [19] have described examples of solvent programs

TABLE 4

RETENTION TIMES OF KAURANOIDS IN REVERSED-PHASE HPLC

Retention times have been estimated from Turnbull *et al.* (ref. 135, Fig. 1). ODS Hypersil (5 μm, 250 × 5 mm); aqueous methanol in dilute acetic acid (pH 3): 0–15 min, 50–80% methanol; 15–19 min, 80–100% methanol; 19–44 min, 100% methanol; 1 ml min^{-1}.

Compound	Retention time (min)
ent-6α,7α-Hydroxy kaurenoic acid	19.1
ent-7α-Hydroxy kaurenoic acid	19.8
GA_{12}	20.8
GA_{12}-aldehyde	21.9
ent-Kaurenoic acid	24.7
ent-Kaurenol	24.7
ent-Kaurenal	25.9
ent-Kaurene	32.4

suited for the purification of *ent*-kaurene alone, and for groups of kauranoids of similar polarity (*ent*-kaurenal, *ent*-kaurenol, *ent*-kaurenoic acid; and *ent*-kaurenoic acid, GA_{12}-aldehyde and *ent*-7α-hydroxy kaurenoic acid).

4.2. Normal-phase HPLC

Normal-phase HPLC on unmodified silica has been little-used in GA separation. This is a reflection of the likelihood of poor recoveries especially of the more polar compounds because of irreversible adsorption. Also, resolution of such compounds is likely to be poor (*cf.* ref. 122). Lin and Heftmann [119] defined the behaviour of ten GAs in silica HPLC, eluted with *n*-hexane–ethanol–acetic acid (93:7:0.05). This work was extended by Lin and Stafford [122], who determined the retention times of 23 GAs and their methyl esters eluted from silica with *n*-hexane–ethanol (90:10) containing 0.05% acetic acid (free GAs) or *n*-hexane–ethanol (92:8; methyl esters) (Table 5). As expected, most efficient separation was achieved with the methyl esters. The C_{19} GAs elute more or less in the reverse of their order from RP-HPLC. Notably, C-3-*epi*-GA_1 is better retained than expected from its behaviour in RP-HPLC and thus

TABLE 5

RETENTION TIMES OF FREE GIBBERELLINS AND THEIR METHYL ESTERS IN SILICA HPLC

Adapted from ref. 122. Conditions: free GAs: Spherisorb S5W (5 μm, 250 × 4.6 mm), isocratic *n*-hexane–ethanol (90:10) containing 0.05% acetic acid, 2 ml min^{-1}. GA methyl esters: isocratic *n*-hexane–ethanol (92:8).

Gibberellin	Retention time (min)	
	Free GA	GA methyl ester
GA_9	2.77	2.08
GA_{24}	2.90	1.84
GA_{15}	3.20	2.38
GA_{25}	3.31	1.74
GA_4	4.00	3.36
GA_7	4.03	3.50
GA_{14}	4.36	2.74
GA_{36}	4.73	3.31
GA_{37}	4.84	4.38
GA_{34}	4.93	4.30
GA_{53}	4.96	3.13
GA_{54}	5.32	4.45
GA_{20}	5.73	4.70
GA_5	5.75	4.87
GA_{19}	5.81	3.93
GA_{17}	6.67	3.89
GA_{13}	6.74	3.05
GA_1	8.99	9.24
GA_3	9.34	9.73
GA_8	11.69	12.49
GA_{55}	12.21	12.57
GA_{32}	12.61	12.21
C-3-*epi*-GA_1	12.68	12.31

well separated from GA_1. The order of elution of the free C_{20} GAs is determined by the substituent at C-20 and the position of hydroxylation. The polar –COOH group at C-20 dominates behaviour. Thus GA_{25} is the best retained of the non-hydroxylated C_{20} GAs, eluting after GA_{15} (δ-lactone) and GA_{24} (CHO). This order is maintained for the representatives of the C-3- and C-13-hydroxylated C_{20} GAs. Amongst these hydroxylation at C-13 increases retention more than does hydroxylation at C-3. Examples of the use of silica HPLC in preparative purification schemes include Birnberg *et al.* [131] and Maki *et al.* [136]. Other uses include the separation of GA benzyl esters [74] and GA *p*-nitrobenzyl esters [100].

Normal-phase chromatography of GAs on open columns of deactivated silica, with a mobile phase of formate-saturated ethyl acetate in *n*-hexane, has been described in section 3.2. The early use of this system in HPLC for the separation of free GAs by Reeve *et al.* [110] has been superseded by chromatography on bonded phases. However, few applications are reported. Methoxycoumaryl esters of GAs were separated on bonded cyanopropyl (CPS Hypersil) eluted with 3% ethanol in *n*-hexane–dichloromethane (88:12 or 80:20) [102] and more recently on CN nitrile eluted isocratically with dichloromethane–*n*-hexane or ethyl acetate–*n*-hexane [114]. Gibberellin-4-bromophenacyl esters have also been separated on cyanopropyl silica [101]. Odén *et al.* [58] used a column of bonded NO_2, eluted with a gradient of *n*-heptane (half-saturated with 1 *M* formic acid) to ethyl acetate containing 1% water and 0.5% formic acid, to separate 12 GAs, GA_{12}-aldehyde, and several kauranoids (Table 6). The water and formic acid

TABLE 6

RETENTION TIMES OF GIBBERELLINS AND KAURANOIDS IN NORMAL PHASE HPLC

Retention times estimated from Odén *et al.* (ref. 58, Fig. 1). Nucleosil NO_2 (5 μm, 125 × 4.6 mm), gradient of *n*-heptane (half-saturated with 1 *M* formic acid) to ethyl acetate containing 1% water and 0.5% formic acid over 60 min; 2 ml min^{-1}.

Compound	Retention time (min)
ent-Kaurene	1.8
ent-Kaurenal	2.4
ent-Kaurenol	10.3
ent-Kaurenoic acid	11.5
GA_{12}-aldehyde	18.2
GA_9	19.4
GA_{15}	22.7
GA_{12}	23.6
GA_4	27.9
GA_{20}	31.8
GA_{44}	36.4
GA_{53}	37.3
GA_1	42.4
GA_3	43.9
GA_{19}	44.8
GA_{29}	50.3
GA_8	57.9

were added as polar modifiers and ion suppressors. The compounds elute in the order expected from the earlier work [60,110]. The C_{19} GAs elute in order of increasing hydroxylation, the reverse of that observed in RP-HPLC (section 4.1.1). The C-13 hydroxylated C_{20} GAs (GA_{19}, GA_{44} and GA_{53}) elute after the non-hydroxylated C_{20} GAs (GA_{12} and GA_{15}). The method has proved effective in the semi-preparative purification of GAs from *Picea*, in combination with subsequent analytical RP-HPLC [58]. From similar material Odén *et al.* [79] purified GA_9 on μBondapak NH_2 eluted with *n*-hexane–ethanol–acetic acid (96:2:2).

Normal-phase HPLC has not been routinely applied in the analysis of GA conjugates. Odén *et al.* [58] tentatively identified GA_9–glucosyl ester from extracts of *Picea* after chromatography on a column of Polygosil NO_2 with a mobile phase of *n*-heptane (half-saturated with 1 *M* formic acid) to ethyl acetate containing 1% water and 0.5% formic acid. The GA conjugate eluted at 350 ml, much later than GA_9 (110 ml) and GA_1 and GA_3 (250 ml), reflecting the influence of the glucose moiety on polarity. In similar fashion Moritz [30] purified GA_9–glucosyl ester on a column of Nucleosil NO_2.

4.3. Dimethylamino Nucleosil HPLC

Yamaguchi *et al.* [61] introduced the use of HPLC on columns of dimethylamino [$N(CH_3)_2$] Nucleosil to GA analysis. The application, in which the GAs are eluted in a mobile phase of isocratic methanol with 0.05% acetic acid, has gained considerable acceptance because of the unusual separations afforded. Retention of the C_{20} GAs is determined largely by the degree of oxidation at C-20 (Table 7); thus the GAs with $-CH_3$ are least retained, eluting ahead of -δ-lactone, –COOH and –CHO. Separation of the C_{19} GAs is determined by the position and orientation of hydroxyl functions, which can increase (C-3β-hydroxy) or decrease (C-2α, -2β or -13-hydroxy) retention. Hydrogenation in the A-ring increases retention. Thus $N(CH_3)_2$ HPLC is particularly useful for the separation of GA_1, GA_4 and GA_{20} from their A-ring dehydro counterparts (respectively GA_3, GA_7 and GA_5).

TABLE 7

RETENTION TIMES OF GIBBERELLINS IN DIMETHYLAMINO NUCLEOSIL HPLC

Adapted from Yamaguchi *et al.* [61]. Nucleosil $N(CH_3)_2$ (10 μm, 250 × 6 mm), isocratic methanol containing 0.05% acetic acid, 2 ml min^{-1}, 50°C.

Gibberellin	Retention time (min)
GA_{12}	10.7
GA_{14}	11.4
GA_{18}	11.7
GA_{37}	13.8
GA_{27}	15.6
GA_4	20.0
GA_{17}	21.4
GA_1	22.0
GA_{35}	22.5
GA_{16}	25.6
GA_8	26.0
GA_9	27.0
GA_7	27.8
GA_{13}	28.0
GA_{40}	28.0
GA_{30}	28.6
GA_3	28.9
GA_{20}	30.0
GA_{51}	30.2
GA_{36}	31.4
GA_{24}	32.4
GA_{31}	37.6
GA_5	38.2
GA_{19}	41.6
GA_{26}	48.2
GA_{22}	49.0

The isomers GA_1 and C-3-*epi*-GA_1 are also well separated [137]. These pairs of GAs are often not well resolved in gradient-eluted RP-HPLC. The method has been frequently used as an additional purification step after separation on RP-HPLC [28,64,68], or as a single HPLC step before GC–MS [25,76,138].

4.4. LC–MS

The recent introduction of LC–MS has permitted GAs and their conjugates to be introduced to MS for analysis usually at low temperature without converting them to a derivative. This is an advantage for analysis of the GA–glucosyl

ester conjugates in particular. Since LC–MS is not yet widely available, and improved methods for transferring the solutes from LC to MS are still being sought, techniques for analysis of GAs and GA conjugates by LC–MS are in their infancy.

Two of the available LC–MS interfaces have been used in GA-conjugate analysis. Murofushi *et al.* [139] employed an atmospheric pressure ionization interface to introduce GA–glucosides and GA–glucosyl esters from RP-HPLC to MS. The GA conjugates were separated in gradients of 10 m*M* ammonium acetate–methanol, at 1 ml min^{-1}. In the interface, the eluate from HPLC is nebulized (at 380°C) and then vaporized (at 380°C) and the solutes subsequently ionized by atmospheric pressure chemical ionization (APCI). A 1-ng amount of GA_3–3-O-glucoside could be detected by single ion monitoring. The method can accommodate various flow rates (0.1 to 2 ml min^{-1}) without the need for splitting.

In the second approach Moritz [30] and Moritz *et al.* [140] have used capillary RP-HPLC–frit fast atom bombardment (FAB)-MS for the analysis of GA conjugates. In this method the eluate from capillary HPLC, at a flow-rate of about 5 μl min^{-1}, is introduced through a frit into the ion source, and is then ionized by accelerated xenon atoms. Moritz *et al.* [140] first separated GA–glucosyl esters in HPLC in a solvent program of methanol–water–acetic acid–glycerol, and found negative ion mass spectra to be the more useful in identification. The GA–glucosides were introduced from the capillary column without any attempt to provide separation, and positive ion spectra were found to be the more useful. Full mass spectra were obtained from injections of 10 to 100 ng of compound; in the identification of GA_9–glucosyl ester from shoots of *Picea* by this method 5 ng of GA_9–glucosyl ester were considered sufficient to obtain a spectrum [30]. Capillary HPLC offers the advantage that there is no need to split the effluent before the detector; the disadvantage is that only a small volume can be injected. The FAB mass spectra of other GA conjugates have been described by Voigt and Dube [141].

Free GAs and their methyl esters have also been analyzed by LC–APCI-MS [139]. The compounds were introduced to MS from RP-HPLC with a mobile phase of 10 m*M* ammonium acetate–methanol (20:80). A 10-ng amount was required to obtain spectra, and several hundred pg was required for detection by single ion monitoring. A thermospray interface was used by Hansen *et al.* [142] in the LC–MS analysis of free GAs and GA methyl esters. The free GAs were separated with a mobile phase of increasing acetonitrile in aqueous 0.1 *M* ammonium acetate. The minimum amount needed to obtain the very simple positive ion thermospray mass spectra was about 100 ng of GA_3 methyl ester and about 250–500 ng of GA_3. Negative ion spectra were obtained at 10× less sensitivity. The isomers GA_3 and GA_{30} were not separated on HPLC and could not be distinguished by their positive ion spectra, but tandem mass spectra obtained from the collisionally activated dissociation of the $[M + NH_4]^+$ ions did show differences.

Provided that the limited resolution of HPLC is not an obstacle in analysis, likely developments in LC–MS interfaces which result in improved sensitivity should lead to increased application of LC–MS for the direct analysis of both free GAs and GA–glucosyl conjugates from plant extracts.

4.5. GC and GC–MS

The use of GC in conjunction with MS in GA analysis was introduced in 1967 [143] and developed since then by MacMillan and co-workers, in particular. A useful practical summary of methodology is found in Hedden [1], and Gaskin and MacMillan [7] have provided an authoritative reference to current methods. GC–MS is now the most widely used method for identifying known GAs. It is the method of choice for establishing the characteristics of new GAs isolated in small amounts from plant extracts, with identity confirmed through comparison of Kováts retention index (*I*) and mass spectrum with that of the synthetic equivalent of known structure. This is illustrated in the identification of most of the GAs recently assigned A-numbers (see refs. 64,126–128 and 144, and the refs. cited in ref. 7). GC–MS is also a sensitive and specific method

for quantifying GAs, as discussed briefly in section 4.5.4.

The usual approach in analysis of GAs from plant extracts is to prepare derivatives of purified samples (groups of fractions after HPLC, for example) which are more suited for GC–MS analysis than are the parent compounds. These include methyl- and trimethylsilyl esters and ethers. These are then introduced onto capillary columns for GC and separated in a temperature gradient, with the volatile compounds emerging directly into the ion source of a mass spectrometer. The mass spectra, and retention indices, provide sufficient evidence to distinguish all of the known GAs.

Detectors other than mass spectrometers have also been used in GA analysis. These include flame ionization and electron-capture (which can be used for GA derivatives which have electron-capturing properties) detectors, and radiocounters. None of these is specific for the GAs; the only evidence of identity obtained is the retention time. Packed-column GC–radioactivity counting has been used to provide tentative identifications of metabolites from the application of radiolabelled substrates in a number of studies [145]. In such circumstances where the identity of the metabolites was predictable and where chromatography was carried out on several different columns, the identifications have proved reliable [6]. However, for unequivocal evidence of identity GC–MS provides not only retention times but also mass spectra. Compounds entering into the ion source of the mass spectrometer are ionized, usually by electron impact (EI) at 70 eV, and characteristic fragmentation of the molecule results. The positive ions produced are detected by scanning of the mass range of interest to yield a mass spectrum. With a cycle time of about one second, a peak from GC can be sampled about 5–10 times. The total ion current (TIC) at each scan yields a chromatogram which can be integrated for estimates of the relative amounts of the various compounds. From the spectra, chromatograms of individual ion currents can be reconstructed. These are often useful in pointing out the location of GAs present in small amounts or hidden in co-eluting impurities [1,7]. The mass spectrometer can also be used to sample only certain ions of those produced in the fragmention process. In GC–SIM sensitivity is improved about 100-fold over GC–MS, since each ion can be sampled more frequently. The method is used to provide ion chromatograms which can be integrated for quantitative analysis [1], and to provide evidence of identity of GAs present in amounts too small to yield full mass spectra. In our experience the limit of detection of the GAs in analysis on commonly used benchtop gas chromatograph–mass spectrometers is of the order of 3 pmol (*ca.* 1 ng) in GC–MS and 30 fmol (*ca.* 10 pg) in GC–SIM. Greater sensitivity can be achieved on other instruments; Gaskin and MacMillan [7] have reported 50–100 pg in GC–MS and 0.1–1 pg in GC–SIM. The use of GC–SIM in particular often allows for GAs to be identified or quantified from samples which have not been highly purified. This is particularly the case for the more hydroxylated GAs, with derivatives which yield molecular ions of high molecular mass which are unlikely to be contaminated by ions from the fragmentation of co-eluting compounds.

4.5.1. Derivatives and spectra

Hedden [1] and Gaskin and MacMillan [7] have provided details of the methods of preparation of the various derivatives for GC–MS. The best suited for the hydroxylated free GAs and kauranoids is the methyl ester trimethylsilyl ether (MeTMSi). It is sufficiently volatile and stable for GC and yields MS spectra which provide useful diagnostic information on structure. Free GAs are usually methylated with ethereal diazomethane which can be synthesized using a convenient small-scale procedure described by Cohen [146]. Hydroxylated GAs are then converted to trimethylsilyl ethers with (N,O - bis - trimethylsilyltrifluoroacetamide (BSTFA), N-methyl-O-trimethylsilyltrifluoroacetamide (MSTFA) or hexamethyldisilazane–trimethylsilyl chloride–pyridine (3:1:9) (Sweeleys reagent). The compendium of Gaskin and MacMillan [7] includes the spectra of the methyl esters of non-hydroxylated GAs, and the MeTMSi derivatives of hydroxylated GAs, for GAs up to GA_{86}. In addition the spectra of a

host of other GAs, kauranoids and related compounds are shown. They have also provided a comprehensive analysis of the fragmentation of GA methyl and MeTMSi derivatives. Binks *et al.* [147] have provided the only other compilation of the full spectra of MeTMSi and methyl ester derivatives, of GAs up to GA_{24}. Takahashi *et al.* [148] have also described and discussed the fragmention of GA methyl esters. Tabulations of major ions in the spectra of GA methyl- or MeTMSi derivatives have been given by Beale and Willis [3], Hedden [1], Takahashi *et al.* [5] and Crozier and Durley [6]. Otherwise full spectra or tabulations of major ions can be gleaned from the literature, with references to the identification of all GAs up to GA_{86} found in Mander [8]. For the hydroxylated free GAs, other derivatives occasionally used include methyl esters, but these are not as amenable to chromatography as the MeTMSi derivatives [7]. Also occasionally used are TMSi esters, and TMSi ester TMSi ethers [76], and since the ester and ether bonds are readily hydrolyzed in water these offer the opportunity to recover the free GA. A limited number of spectra of these derivatives have been recorded by Gaskin and MacMillan [7] and their fragmentation discussed.

There has been very limited use of permethyl derivatives of the free GAs [149,150]. The usual permethylation procedure with sodium hydride and methyl iodide in dimethylformamide [149] is somewhat involved and much less suitable for routine use than methylation and silylation. However, the use of an alternative simple procedure for permethylation has recently been reported [150]. MethElute (0.2 *M* trimethylanilinium hydroxide in methanol) is added to the sample which is then injected onto GC, with the derivative being formed on-column. Fang and Rappaport [150] have described the spectra and fragmentation of 23 permethylated GAs; the spectra of a number of permethyl GAs have also been recorded by Gaskin and MacMillan [7].

Unequivocal identification of small amounts of GA conjugates requires their analysis either by GC–MS, as permethyl or silyl derivatives, or by LC–MS, where they may be introduced to MS without modification. Neither of the derivatives for GC–MS is ideal, since they yield weak or non-existent M^+ and the EI mass spectra are dominated by fragmentation of the glucosyl moiety [151,152]. However, permethyl derivatives are preferred because their molecular masses are within the range of commonly used gas chromatographs–mass spectrometers (often 10–800 u), and they yield fragmentation by EI which is of more value in indicating structure [149,152]. Schmidt *et al.* [152] have described the spectra of 14 permethylated GA–glucosides and provided a detailed discussion of their fragmentation. The spectra of the permethyl derivatives of 15 GA–glucosides have been recorded by Gaskin and MacMillan [7]. A number of GA–glucosides have been successfully identified as permethyl derivatives either as endogenous constituents or as products of metabolism of feeds [10,46,47,149,153]. Rivier *et al.* [149] have reported the identification of two glucosyl esters (of GA_1 and GA_4) as the permethyl ethers, but it seems that in general GA-glucosyl esters cannot be easily permethylated since transesterification occurs to produce the permethylated aglycone [154].

The spectra and fragmentation of the methyl ester TMSi ether derivatives of six GA–glucosides, and of the TMSi ethers of five GA–glucosyl esters, have been described by Yokota *et al.* [151]. However, these derivatives are less suitable for routine analysis by GC–MS since they have high molecular masses and may decompose at the high temperatures required for GC analysis [155]. Both GA–glucosides and glucosyl esters have been identified from natural samples by GC–MS as the TMSi derivatives [17,39], but their use has been very limited.

4.5.2. Columns, injection techniques and temperature programmes

Packed-column GC is little used now in GA analysis. While the capacity is high, resolution is low compared with that which can be obtained in capillary GC. Crozier and Durley [6] detail the conditions for chromatography, and list the retention times, of MeTMSi derivatives of 43 GAs and other related compounds on columns of 2% QF-1, 2% SE-30 and 1% XE-60.

In most laboratories analyses have been standardized on fused-silica capillary columns with

non-polar dimethylpolysiloxane silicone coatings such as OV-1 and equivalents (*e.g.*, DB-1, BP-1, CPSil-5, HP-1). Such practise permits ready comparison of I values obtained in different laboratories. Columns of 12 or 15 m are suitable for most routine analyses, but for complex separations or resolution of GAs with similar I a longer 25-m [7] or 30-m column is preferred. Columns used have included those with internal diameters which range from 0.18 to 0.32 mm. The capacity for individual GA components is about 50–100 ng, quite sufficient to obtain spectra of high quality.

GAs have also been successfully analyzed on columns with slightly more polar coatings such as DB-5 (5% diphenyl 95% dimethyl polysiloxane). Separations for most of the GAs examined are similar to those obtained in analyses on DB-1 [156].

Analyses of permethylated GA conjugates have been made on the same type of capillary columns used for analysis of the free GAs [11,149,152], and also on packed columns of SP-2100 or QF-1 [46]. The packed-column GC of TMSi and MeTMSi derivatives of a number of GA conjugates has been described by Hiraga *et al.* [157] and Schneider *et al.* [158]. Since the derivatives are volatile at relatively high temperatures, analysis time in capillary GC can be shortened by increasing the oven temperature rapidly after injection to the maximum required, as illustrated in Table 9 [152].

Samples are introduced onto the column usually by splitless injection into a heated injection port. Details of common methods have been given by Hedden [1] and Gaskin and MacMillan [7]. Briefly, the sample is vaporized on injection (at 280°C) and refocussed at the head of the column which is held at low temperature (30–35°C). The oven temperature is increased rapidly (to 150°C) and then slowly at 3°C min^{-1} to 300°C [7]. This type of programme has been used in most GA analyses in various laboratories. Alternatively, cool on-column injection can be used. Here, the sample is introduced directly onto the column at a temperature below the boiling point of the solvent, and the temperature is increased from there in similar fashion to that described above. In order to extend column life the injection should be made into a precolumn which is connected to the main column. The precolumn is inert (uncoated, deactivated fused silica), 0.32 or 0.53 mm I.D. to accommodate the syringe, and 0.5 to 1 m long. It can be readily and cheaply replaced when it becomes contaminated with involatile residues. If required, a longer precolumn will also serve as a retention gap to permit greater volumes than the usual (*ca.* 1 μl) to be injected. This might be of particular advantage in analyses where the GA content of the sample was approaching the lower limit for quantification or identification, but requires that the sample be well purified. A clear discussion of these sample introduction techniques is found in Poole and Poole [159]. The cool on-column method is useful for the quantitative analysis of compounds of varying volatility, since there is little of the discrimination in transfer of solutes from syringe to the column which can occur in splitless injection. Also, there is less likelihood that thermally labile compounds will decompose. We have successfully used this method in all of our GC–MS analyses [126,160].

4.5.3. Retention indices

Mass spectra alone are sometimes not sufficient evidence of identity, since some isomers, such as GA_1 and C-3-*epi*-GA_1, have spectra which are very similar. Identity can be confirmed by retention time, or preferably, by I [161]. The I value is calculated from the retention times of n-alkanes co-injected with the sample. A suitable range of n-alkanes, from $C_{21}H_{44}$ to $C_{36}H_{74}$, can be conveniently extracted from Parafilm [162]. The retention index is calculated from a calibration curve of carbon number of the n-alkanes plotted against retention times, or from the formula below as $I = 100\ [(t_{R(x)} - t_{R(C_n)})/(t_{R(C_{n+1})} - t_{R(C_n)})] + 100n$, where $t_{R(x)}$ is the retention time of the compound of interest, $t_{R(C_n)}$ is the retention time of the n-alkane of carbon number n which elutes immediately before the unknown, and $t_{R(C_{n+1})}$ is the retention time of the n-alkane of carbon number $n+1$ which elutes immediately after the compound of interest.

Absolute I values will differ from system to system, dependent on temperature program, gas

TABLE 8

KOVÁTS RETENTION INDICES OF GIBBERELLINS AND KAURANOIDS

Calculated from analyses of methyl esters or MeTMSi derivatives in capillary GC-MS on DB1-15N (15 m × 0.25 mm I.D., 0.25 μm dimethylpolysiloxane film); 60°C (0.1 min) to 200°C at 20°C min^{-1}, to 300°C at 5°C min^{-1}. Values are also included for a number of common isomers, and also for some GAs (synthetized by Professor L.N. Mander) which have not been identified in natural samples.

Type	–OH groups	Gibberellin	*I*	M^+
C_{20} Gibberellins				
C-20 CH_3				
C-7 CHO	–	GA_{12}-aldehyde	2356	330
	–	GA_{12}	2345	360
	3β	GA_{14}	2496	448
	13	GA_{53}	2508	448
	3β,13	GA_{18}	2646	536
	3β,12α	GA_{74}	2682[a]	536
C-16,17-dihydro	3β,16α	GA_{42}	2718[a]	538
	3β,17	GA_{83}	2778	538
δ-Lactone	–	GA_{15}	2628	344
	15β	GA_{64}	2754[a]	432
	3β	GA_{37}	2773	432
	13	GA_{44}	2800	432
	2β,3β	GA_{27}	2897	520
	3β,13	GA_{38}	2940	520
	2β,3β,11β	GA_{52}	3076[a]	608
C-20 CHO	–	GA_{24}	2469	374
	15β	GA_{65}	2580[a]	462
	3β	GA_{36}	2606	462
	13	GA_{19}	2608	462
	3β,13	GA_{23}	2747	550
C-20 COOH	–	GA_{25}	2460	404
	15β	GA_{66}	2563[a]	492
	13	GA_{17}	2585	492
	3β	GA_{13}	2597	492
	2β	GA_{46}	2616[a]	492
	3β,13	GA_{28}	2720	580
	2β,3β	GA_{43}	2725[a]	580
	3β,12α	GA_{39}	2776[a]	580
C-16,17-dihydro	3β,16α	GA_{41}	2817	582
C_{19} Gibberellins				
Non-hydroxy				
C-2,3-dehydro	–	$\Delta^{2,3}GA_9$	2333	328
C-9,11-dehydro	–	GA_{73}	2344	328
C-15,16-dehydro	–	Iso-GA_9	2292	328
	–	GA_9	2332	330
C-1,10-epoxy	–	GA_{11}	2402[a]	344
Monohydroxy				
C-1,2-dehydro	1β	GA_{62}	2439	416
	13	$\Delta^{1,2}$-GA_{20}	2503	416

TABLE 8 (*continued*)

Type	–OH groups	Gibberellin	*I*	M^+
	3β	GA_7	2541	416
	12α	GA_{31}	2564	416
C-2,3-dehydro	13	GA_5	2495	416
C-9,11-dehydro	3β	GA_{88}	2512	416
C-1,10-dehydro, 19,2-lactone	3β	Iso-GA_7	2509	416
	1β	GA_{61}	2405	418
	11β	GA_{84}	2488	418
	13	GA_{20}	2499	418
	15β	GA_{45}	2499	418
	12β	GA_{69}	2512	418
	3β	GA_4	2519	418
	2β	GA_{51}	2534	418
	2α	GA_{40}	2543	418
	12α	GA_{70}	2561	418
	3α	*epi*-GA_4	2639	418
C-16,17-dihydro	16α	GA_{10}	2591	420
C-2,3-epoxy	13	GA_6	2590	432
C-2,3-dehydro, C-18 COOH	13	GA_{59}	2727	460
C-18 COOH	13	GA_{21}	2729	462
Dihydroxy				
C-1,2-dehydro	3β,11β	GA_{80}	2672	504
	3β,15β	GA_{68}	2695	504
	3β,13	GA_3	2700	504
	3β,12β	12β-OH-GA_7	2719	504
	3β,12α	GA_{30}	2771	504
C-1,10-dehydro, 19,2-lactone	3β,13	Iso-GA_3	2642	504
C-2,3-dehydro	1β,13	1β-OH-GA_5	2592	504
	12β,13	12β-OH-GA_5	2635	504
	12α,13	12α-OH-GA_5	2641	504
	1α,13	1α-OH-GA_5	2675	504
	13,18	GA_{22}	2700	504
	1β,13	GA_{60}	2583	506
	1β,3β	GA_{54}	2609	506
	13,15β	GA_{67}	2625	506
	12β,13	12β-OH-GA_{20}	2637	506
	2α,3β	GA_{47}	2638	506
	1α,3β	GA_{16}	2641	506
	12α,13	GA_{77}	2643	506
	3β,11β	GA_{35}	2644	506
	2β,3β	GA_{34}	2669	506
	3β,13	GA_1	2675	506
	2α,13	GA_{81}	2681	506
	2β,13	GA_{29}	2690	506
	1α,13	1α-OH-GA_{20}	2694	506
	3β,12β	GA_{71}	2695[a]	506

(Continued on p. 118)

TABLE 8 (*continued*)

Type	-OH groups	Gibberellin	*I*	M^+
	3β,15β	GA_{63}	2712	506
	3β,12α	GA_{58}	2742[a]	506
	3α,13	*epi*-GA_1	2794	506
C-16,17-dihydro	3β,16α	GA_2	2761	508
	3β,17	GA_{82}	2818[a]	508
C-3-oxo	1β,12α	GA_{33}	2695	520
C-12-oxo	2β,3β	GA_{26}	2844	520
Trihydroxy				
C-1,2-dehydro	3β,13,15β	15β-OH-GA_3	2814	592
	3β,12β,13	12β-OH-GA_3	2843	592
	3β,12α,13	GA_{87}	2854	592
	1α,2α,3β	GA_{78}	2740[a]	594
	1β,2β,3β	GA_{79}	2753[a]	594
	1β,3β,13	GA_{55}	2764	594
	2α,3β,13	GA_{56}	2769	594
	2β,13,15β	GA_{76}	2776[a]	594
	1α,3β,13	GA_{57}	2791	594
	2β,3β,12β	GA_{48}	2796[a]	594
	3β,12β,13	12β-OH-GA_1	2803	594
	3β,12α,13	GA_{85}	2817	594
	2β,3β,13	GA_8	2821	594
	3β,13,15β	GA_{72}	2830	594
	2β,3β,12α	GA_{49}	2837[a]	594
	2β,3β,11β	GA_{50}	2847	594
	1α,3α,13	*epi*-GA_{57}	2898	594
Tetrahydroxy				
C-1,2-dehydro	3β,12α,13,15β	GA_{32}	2973	680
	2β,3β,12α,13	GA_{89}	2910	682
	2β,3β,13,15β	GA_{75}	2947[a]	682
	3β,12α,13,15β	GA_{86}	2975	682
Kauranoids				
ent-kaurene			2044	272
ent-kaurenol			2302	260
ent-kaurenal			2249	286
ent-kaurenoic acid			2270	316
ent-7α-hydroxy-kaurenoic acid			2430[a]	404

[a] Adapted from Gaskin and MacMillan [7] based on a comparision of *I* values of GAs of similar structure.

flow, column coating and condition, and other components in the sample [7], but relative retention times can usually be predicted with reasonable accuracy.

The reader is referred to Gaskin and MacMillan [7] for the most comprehensive published list of *I* values which includes those for all GAs up to GA_{86} as well as a host of isomers and related compounds. Beale and Willis [3] and Hedden [1] have provided less extensive compilations. Table 8 is a list of *I* values from our analyses of GA methyl ester or MeTMSi derivatives on DB1-15N columns, complemented with additional values estimated from the data of Gaskin and MacMillan [7].

The most extensive list of *I* values of a number

TABLE 9

RETENTION TIMES (t_R) AND KOVÁTS RETENTION INDICES (I) OF PERMETHYLATED GA–GLUCOSIDES IN GC

Adapted from Schmidt *et al.* [152]. HP1, 25 m × 0.31 mm i.d., 0.17 μm methylsilicone film; injection at 275°C, GC temperature programme 60°C (1 min) to 260°C (25°C min^{-1}).

Compound	t_R	KRI
GA_1–3-O-glucoside PME	24.369	3602
GA_1–13-O-glucoside PME	22.042	3532
C-3-*epi*-GA_1–3-O-glucoside PME	25.361	3625
C-3-*epi*-GA_1–13-O-glucoside PME	24.208	3597
GA_3–3-O-glucoside PME	21.460	3514
GA_3–13-O-glucoside PME	21.480	3515
GA_4–3-O-glucoside PME	19.419	3440
GA_5–13-O-glucoside PME	18.196	3392
GA_7–3-O-glucoside PME	18.168	3391
GA_8–2-O-glucoside PME	26.014	3640
GA_8–13-O-glucoside PME	24.484	3604
GA_{20}–13-O-glucoside PME	18.320	3398
GA_{29}–2-O-glucoside PME	23.287	3569
GA_{29}–13-O-glucoside PME		3502[a]
GA_{35}–11-O-glucoside PME	18.755	3415

[a] Estimated from Schneider *et al.* [10].

of permethyl GA–glucosides in such conditions has been provided by Schmidt *et al.* [152] (Table 9). These values together with the mass spectra were considered sufficiently distinctive for positive identification of the 14 GA-glucosides examined in that study.

4.5.4. *Quantitative analysis*

GC–MS is the method of choice for quantitative analysis of endogenous GAs, and results obtained by other methods (immunoassay, for example) should be verified by GC–MS. The principle of the method is well known. A known amount of an internal standard (IS), ideally the GA of interest labelled with a heavy isotope, is added to the sample after extraction. GAs labelled with atoms of ^2H, ^{13}C and ^{14}C have been used [2,19,25,76]. [17,17-$^2H_2$]-Labelled standards of a variety of GAs have been synthesized by Professor L. Mander (Research School of Chemistry, Australian National University, Canberra, Australia) and made available for purchase. These have been used in numerous studies [24,34,160,163]. If such standards are not available, another GA of similar structure to the endogenous GAs of interest can be used. Care must be taken to ensure that such an internal standard is carried through the various chromatographic steps with the endogenous GAs. The relative quantities of the IS and the endogenous GA are calculated finally after analysis by GC–MS or GC–SIM. The ion chromatograms are integrated and the ratio of areas of the signals for the IS and endogenous GA entered into a calibration curve to determine the ratio of the amounts of the two, as described, for example, by Hedden [1,2]. Alternatively, the distribution of masses in the molecular ion clusters can be used to determine isotope dilution [7,25]. Hence the absolute amount of endogenous GA in the sample can be found.

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REFERENCES

1 P. Hedden, in L. Rivier and A. Crozier (Editors), *Principles and Practice of Plant Hormone Analysis*, Academic Press, London, 1987, Vol. 1, p. 9.
2 P. Hedden, *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 44 (1993) 107.
3 M.H. Beale and C.L. Willis, in D. Banthorpe and B.V. Charlwood (Editors), *Methods in Plant Biochemistry*, Academic Press, London, 1991, Vol. 7, p. 289.
4 G.W.M. Barendse, in H.F. Linskens and J.F. Jackson (Editors), *High Performance Liquid Chromatography in Plant Sciences*, Springer, Berlin, Heidelberg, 1987, p. 1.
5 N. Takahashi, I. Yamaguchi and H. Yamane, in N. Takahashi (Editor), *Chemistry of the Plant Hormones*, CRC Press, Boca Raton, FL, 1986, p. 57.
6 A. Crozier and R.C. Durley, in A. Crozier (Editor), *The Biochemistry and Physiology of Gibberellins*, Praeger, New York, 1983, Vol. 1, p. 485.
7 P. Gaskin and J. MacMillan, *GC–MS of the Gibberellins and Related Compounds: Methodology and a Library of Spectra*, University of Bristol (Cantock's Enterprises), Bristol, 1991.
8 L.N. Mander, *Chem. Rev.*, 92 (1992) 573.
9 S.B. Rood, R.P. Pharis and M. Koshioka, *Plant Physiol.*, 73 (1983) 340.

10 G. Schneider, E. Jensen, C.R. Spray and B.O. Phinney, *Proc. Natl. Acad. Sci. U.S.A.*, 89 (1992) 8045.
11 G. Schneider, W. Schliemann, B. Schaller and E. Jensen, in C.M. Karssen, L.C. van Loon and D. Vreugenhil (Editors), *Progress in Plant Growth Regulation —Proceedings of the 14th International Conference on Plant Growth Substances, Amsterdam, 1991*, Kluwer, Dordrecht, 1992, p. 566.
12 I. Yamaguchi, T. Yokota, N. Murofushi, Y. Ogawa and N. Takahashi, *Agric. Biol. Chem.*, 34 (1970) 1439.
13 S.J. Castellaro, J. MacMillan, A.K. Singh and C.L. Willis, *J. Chem. Soc., Perkin Trans. 1*, (1990) 145.
14 R.C. Durley and R.P. Pharis, *Phytochemistry*, 11 (1972) 317.
15 K. Hiraga, T. Yokota, N. Murofushi and N. Takahashi, *Agric. Biol. Chem.*, 38 (1974) 2511.
16 G. Schneider, in A. Crozier (Editor), *The Biochemistry and Physiology of Gibberellins*, Praeger, New York, 1983, Vol. 1, p. 389.
17 I. Yamaguchi, M. Kobayashi and N. Takahashi, *Agric. Biol. Chem.*, 44 (1980) 1975.
18 J.D. Metzger and J.P. Hazebroek, *Plant Physiol.*, 91 (1989) 1488.
19 Y. Suzuki, H. Yamane, C.R. Spray, P. Gaskin, J. MacMillan and B.O. Phinney, *Plant Physiol.*, 98 (1992) 602.
20 J.A.D. Zeevaart and D.A. Gage, *Plant Physiol.*, 101 (1993) 25.
21 J.P. Hazebroek and J.D. Metzger, *Plant Physiol.*, 94 (1990) 157.
22 J.L. Glenn, C.C. Kuo, R.C. Durley and R.P. Pharis, *Phytochemistry*, 11 (1972) 345.
23 P. Hedden, G.V. Hoad, P. Gaskin, M.J. Lewis, J.R. Green, M. Furber and L.N. Mander, *Phytochemistry*, 32 (1993) 231.
24 P. Hedden, S.J. Croker, W. Rademacher and J. Jung, *Physiol. Plant.*, 75 (1989) 445.
25 S. Fujioka, H. Yamane, C.R. Spray, P. Gaskin, J. MacMillan, B.O. Phinney and N. Takahashi, *Plant Physiol.*, 88 (1988) 1367.
26 M. Nakayama, H. Yamane, I. Yamaguchi, N. Murofushi, N. Takahashi and M. Katsumi, *J. Plant Growth Regul.*, 8 (1989) 237.
27 S.M. Poling and V.P. Maier, *Plant Physiol.*, 88 (1988) 639.
28 A. Goto, H. Yamane, N. Takahashi and K. Hirose, *Agric. Biol. Chem.*, 53 (1989) 2817.
29 A.R. Nutbeam and D.E. Briggs, *Phytochemistry*, 21 (1982) 2217.
30 T. Moritz, *Phytochem. Anal.*, 3 (1992) 32.
31 R.C. Durley, J. MacMillan and R.J. Pryce, *Phytochemistry*, 10 (1971) 1891.
32 P. Gaskin and J. MacMillan, *Phytochemistry*, 14 (1975) 1575.
33 I. Yamaguchi, T. Yokota, N. Murofushi, N. Takahashi and Y. Ogawa, *Agric. Biol. Chem.*, 39 (1975) 2399.
34 M. Talon and J.D. Zeevaart, *Plant Physiol.*, 92 (1990) 1094.
35 M. Talon, J.A.D. Zeevaart and D.A. Gage, *Plant Physiol.*, 97 (1991) 1521.
36 T. Yokota, N. Murofushi, N. Takahashi and S. Tamura, *Agric. Biol. Chem.*, 35 (1971) 583.
37 H. Yamane, I. Yamaguchi, N. Murofushi and N. Takahashi, *Agric. Biol. Chem.*, 38 (1974) 649.
38 T. Yokota, S. Kobayashi, H. Yamane and N. Takahashi, *Agric. Biol. Chem.*, 42 (1978) 1811.
39 I. Yamaguchi, T. Yokota, S. Yoshida and N. Takahashi, *Phytochemistry*, 18 (1979) 1699.
40 T. Kagawa, T. Fukinbara and Y. Sumiki, *Agric. Biol. Chem.*, 27 (1963) 598.
41 B.D. Cavell, J. MacMillan, R.J. Pryce and A.C. Sheppard, *Phytochemistry*, 6 (1967) 867.
42 J.A. Ozga, M.L. Brenner and D.M. Reinecke, *Plant Physiol.*, 100 (1992) 88.
43 H. Saimoto, S. Nakagawa, M. Kobayashi, S. Fujioka, M.C.C. Barreto, A. Sakurai and K. Shono, *Plant Cell Physiol.*, 31 (1990) 365.
44 W. Dathe, H. Oliva, O. Miersch, J. Schmidt, I. Yamaguchi and N. Takahashi, *Agric. Biol. Chem.*, 55 (1991) 2491.
45 P. Gaskin, G.V. Hoad, J. MacMillan, I.K. Makinson and J.E. Readman, *Phytochemistry*, 31 (1992) 1869.
46 G. Schneider, J. Schmidt and B.O. Phinney, *J. Plant Growth Regul.*, 5 (1987) 217.
47 G. Schneider, G. Sembdner, E. Jensen, U. Bernard and D. Wagenbreth, *J. Plant Growth Regul.*, 11 (1992) 15.
48 L.E. Powell and K.J. Tautvydas, *Nature*, 213 (1967) 292.
49 R.C. Durley, A. Crozier, R.P. Pharis and G.E. McLaughlin, *Phytochemistry*, 11 (1972) 3029.
50 M. Koshioka, K. Takeno, F.D. Beall and R.P. Pharis, *Plant Physiol.*, 73 (1983) 398.
51 L.M. Janzen and R.P. Pharis, unpublished results.
52 K. Endo, H. Yamane, M. Nakayama, I. Yamaguchi, N. Murofushi, N. Takahashi and M. Katsumi, *Plant Cell Physiol.*, 30 (1989) 137.
53 M.H. Beale, J.R. Bearder, P. Hedden, J.E. Graebe and J. MacMillan, *Phytochemistry*, 23 (1984) 565.
54 M. Koshioka, T.J. Douglas, D. Ernst, J. Huber and R.P. Pharis, *Phytochemistry*, 22 (1983) 1577.
55 M. Koshioka, A. Jones, M.N. Koshioka and R.P. Pharis, *Phytochemistry*, 22 (1983) 1585.
56 M. Koshioka, R.P. Pharis, R.W. King, N. Murofushi and R.C. Durley, *Phytochemistry*, 24 (1985) 663.
57 M. Koshioka, R.P. Pharis, N. Matsuta and L.N. Mander, *Phytochemistry*, 27 (1988) 3799.
58 P.C. Odén, L. Schwenen and J.E. Graebe, *J. Chromatogr.*, 464 (1989) 195.
59 E. Grosselindemann, J.E. Graebe, D. Stöckl and P. Hedden, *Plant Physiol.*, 96 (1991) 1099.
60 J. MacMillan and C.M. Wels, *J. Chromatogr.*, 87 (1973) 271.
61 I. Yamaguchi, S. Fujisawa and N. Takahashi, *Phytochemistry*, 21 (1982) 2049.
62 M. Kobayashi, I. Yamaguchi, N. Murofushi, Y. Ota and N. Takahashi, *Agric. Biol. Chem.*, 52 (1988) 1189.

63 R. Mettrie, J. De Greef, S. Nakagawa and A. Sakurai, *Plant Cell Physiol.*, 29 (1988) 777.
64 E. Yuda, S. Nakagawa, N. Murofushi, T. Yokota, N. Takahashi, M. Koshioka, Y. Murakami, D. Pearce, R.P. Pharis, G.L. Patrick, L.N. Mander and P. Kraft-Klaunzer, *Biosci. Biotech. Biochem*, 56 (1992) 17.
65 D.W. Pearce, F.D. Beall and R.P. Pharis, unpublished results.
66 M. Kobayashi, Y. Kamiya, A. Sakurai, H. Saka and N. Takahashi, *Plant Cell Physiol.*, 31 (1990) 289.
67 I. Nakayama, Y. Kamiya, M. Kobayashi, H. Abe and A. Sakurai, *Plant Cell Physiol.*, 31 (1990) 1183.
68 M. Nakayama, H. Yamane, T. Yokota, I. Yamaguchi, N. Murofushi, N. Takahashi, T. Nishijima, N. Katsura, M. Nonaka, P. Gaskin, J. MacMillan, L.N. Mander and A. Chu, *Agric. Biol. Chem.*, 54 (1990) 837.
69 M. Koshioka, S. Yamaguchi, T. Nishijima, H. Yamazaki, D.O. Ferraren and L.N. Mander, *Biosci. Biotech. Biochem.*, 57 (1993) 1586.
70 V.A. Smith, V.M. Sponsel, C. Knatt, P. Gaskin and J. MacMillan, *Planta*, 185 (1991) 583.
71 I. Nakayama, M. Kobayashi, Y. Kamiya, H. Abe and A. Sakurai, *Plant Cell Physiol.*, 33 (1992) 59.
72 T. Lange, P. Hedden and J.E. Graebe, *Planta*, 189 (1993) 340–349.
73 D.R. Reeve and A. Crozier, *Phytochemistry*, 15 (1976) 791.
74 D.R. Reeve and A. Crozier, in J.R. Hillman, *Isolation of Plant Growth Substances*, Cambridge University Press, 1978, p. 41.
75 A. Crozier, J.B. Zaerr and R.O. Morris, *J. Chromatogr.*, 198 (1980) 57.
76 M. Takahashi, Y. Kamiya, N. Takahashi and J.E. Graebe, *Planta*, 168 (1986) 190.
77 M. Kobayashi, A. Sakurai, H. Saka and N. Takahashi, *Plant Cell Physiol.*, 30 (1989) 963.
78 I. Yamaguchi, H. Nakazawa, R. Nakagawa, Y. Suzuki, S. Kurogochi, N. Murofushi, N. Takahashi and E.W. Weiler, *Plant Cell Physiol.*, 31 (1990) 1063.
79 P.C. Odén, E.W. Weiler, L. Schwenen and J.E. Graebe, *Planta*, 171 (1987) 212.
80 C.G.N. Turnbull, A. Crozier and G. Schneider, *Phytochemistry*, 25 (1986) 1823.
81 R. Gräbner, G. Schneider and G. Sembdner, *J. Chromatogr.*, 121 (1976) 110.
82 S. Fuchs and Y. Fuchs, *Biochim. Biophys. Acta*, 192 (1969) 528.
83 E.W. Weiler and U. Wieczorek, *Planta*, 152 (1981) 159.
84 R. Atzorn and E. Weiler, *Planta*, 159 (1983) 1.
85 R. Atzorn and E. Weiler, *Planta*, 159 (1983) 7.
86 J. Eberle, I. Yamaguchi, R. Nakagawa, N. Takahashi and E.W. Weiler, *FEBS Lett.*, 202 (1986) 27.
87 J.P. Knox, M.H. Beale, G.W. Butcher and J. MacMillan, *Planta*, 170 (1987) 86.
88 J.P. Knox, M.H. Beale, G.W. Butcher and J. MacMillan, *Plant Physiol.*, 88 (1988) 959.
89 I. Yamaguchi, R. Nakagawa, S. Kurogochi, N. Murofushi, N. Takahashi and E.W. Weiler, *Plant Cell. Physiol.*, 28 (1987) 815.
90 I. Yamaguchi, M. Nakajima, K. Kanazawa, L.N. Mander, N. Murofushi and N. Takahashi, in C.M. Karssen, L.C. van Loon and D. Vreugenhil (Editors), *Progress in Plant Growth Regulation —Proceedings of the 14th International Conference on Plant Growth Substances, Amsterdam, 1991*, Kluwer, Dordrecht, 1992, p. 875.
91 R.C. Durley, C.R. Sharp, S.L. Maki, M.L. Brenner and M.G. Carnes, *Plant Physiol.*, 90 (1989) 445.
92 M. Nakajima, I. Yamaguchi, S. Kizawa, N. Murofushi and N. Takahashi, *Plant Cell Physiol.*, 32 (1991) 505.
93 M. Nakajima, I. Yamaguchi, A. Nagatani, S. Kizawa, N. Murofushi, M. Furuya and N. Takahashi, *Plant Cell Physiol.*, 32 (1991) 515.
94 Y. Fuchs and E. Gertman, *Plant Cell. Physiol.*, 15 (1974) 629.
95 V.A. Smith and J. MacMillan, *Plant Physiol.*, 90 (1989) 1148.
96 J.E. Nester-Hudson, F.M. Semenenko, M.H. Beale and J. MacMillan, *Phytochemistry*, 29 (1990) 1041.
97 V.A. Smith, C.J. Knatt, P. Gaskin and J.B. Reid, *Plant Physiol.*, 99 (1992) 368.
98 W. Waycott, V.A. Smith, P. Gaskin, J. MacMillan and L. Taiz, *Plant Physiol.*, 95 (1991) 1169.
99 M. Talon, M. Koornneef and J.A.D. Zeevaart, *Planta*, 182 (1990) 501.
100 E. Heftmann, G.A. Saunders and W.F. Haddon, *J. Chromatogr.*, 156 (1978) 71.
101 R.O. Morris and J.B. Zaerr, *Anal. Lett.*, AII(i) (1978) 73.
102 A. Crozier, J.B. Zaerr and R.O. Morris, *J. Chromatogr.*, 238 (1982) 157.
103 Y. Murakami, *Bot. Mag.*, 79 (1968) 33.
104 T. Nishijima and N. Katsura, *Plant Cell. Physiol.*, 30 (1989) 623.
105 T. Nishijima, M. Koshioka and H. Yamazaki, *Plant Growth Regul.*, 12 (1993) in press.
106 T. Nishijima, M. Koshioka and H. Yamaji, *Plant Physiol.*, 98 (1992) 962.
107 C.G.N. Turnbull, A. Crozier, L. Schwenen and J.E. Graebe, *Planta*, 165 (1985) 108.
108 S. Fujioka, H. Yamane, C.R. Spray, B.O. Phinney, P. Gaskin, J. MacMillan and N. Takahashi, *Plant Physiol.*, 94 (1990) 127.
109 E. Grosselindemann, M.J. Lewis, P. Hedden and J.E. Graebe, *Planta*, 188 (1992) 252.
110 D.R. Reeve, T. Yokota, L.J. Nash and A. Crozier, *J. Exp. Bot.*, 21 (1976) 1241.
111 D.R. Reeve and A. Crozier, *J. Chromatogr.*, 137 (1977) 271.
112 J.J. Ross and J.B. Reid, *Physiol. Plant.*, 76 (1989) 164.
113 V.A. Smith, *Plant Physiol.*, 99 (1992) 372.
114 J.M. Malcolm, A. Crozier, C.G.N. Turnbull and E. Jensen, *Physiol. Plant.*, 82 (1991) 57.
115 M. Koshioka, J. Harada, K. Takeno, M. Noma, T. Sassa, K. Ogiyama, J.S. Taylor, S.B. Rood, R.L. Legge and R.P. Pharis, *J. Chromatogr.*, 256 (1983) 101.
116 J.T. Lin, A.E. Stafford, G.L. Steffens and N. Murofushi, *J. Chromatogr.*, 543 (1991) 471.

117 N. Murofushi, M. Nakayama, N. Takahashi, P. Gaskin and J. MacMillan, *Agric. Biol. Chem.*, 52 (1988) 1825.
118 E. Jensen, A. Crozier and A.M. Monteiro, *J. Chromatogr.*, 367 (1986) 377.
119 J.T. Lin and E. Heftmann, *J. Chromatogr.*, 213 (1981) 507.
120 M.G. Jones, J.D. Metzger and J.A.D. Zeevaart, *Plant Physiol.*, 65 (1980) 218.
121 G.W.M. Barendse and P.H. van den Werken, *J. Chromatogr.*, 198 (1980) 449.
122 J.T. Lin and A.E. Stafford, *J. Chromatogr.*, 452 (1988) 519.
123 E. Jensen, G. Schneider, A.M. Monteiro and A. Crozier, in K. Schreiber, H.R. Schütte and G. Sembdner (Editors), *Conjugated Plant Hormones —Structure, Metabolism and Function; Proceedings of the International Symposium, Gera, 1986*, Institute für Biochemie der Pflanzen Halle der Akademie der Wissenschaften der DDR, Halle, 1987, p. 216.
124 B.J. Brown, S.J. Neill and R. Horgan, *Planta*, 167 (1986) 421.
125 T.J. Wodzicki, H. Abe, A.B. Wodzicki, R.P. Pharis and J.D. Cohen, *Plant Physiol.*, 84 (1987) 135.
126 C. Sheng, K.V. Bhaskar, L.N. Mander, D.W. Pearce, R.P. Pharis and S. Young, *Phytochemistry*, 31 (1992) 4055.
127 C. Sheng, K.V. Bhaskar, W.L.A. Chu, L.N. Mander, D.W. Pearce, R.P. Pharis and S. Young, *Biosci. Biotech. Biochem.*, 56 (1992) 564.
128 V.M. Sponsel, *Planta*, 168 (1986) 119.
129 T. Lange, P. Hedden and J.E. Graebe, *Planta*, 189 (1993) 350.
130 J.T. Lin, G.L. Steffens and J.D. Metzger, unpublished results.
131 P.R. Birnberg, M.L. Brenner, M.C. Mardaus, H. Abe and R.P. Pharis, *Plant Physiol.*, 82 (1986) 241.
132 Y.X. Zhu and P.J. Davies, *Plant Growth Regul.*, 10 (1991) 13.
133 G. Sembdner and G. Schneider, in M. Kutácek, M.C. Elliott and I. Machácková (Editors), *Molecular Aspects of Hormonal Regulation of Plant Development —Proceedings of Symposium 39 and Colloquia 30 and 31 of the 14th Biochemical Congress, Prague, 1988*, SPB Academic Publishing, The Hague, 1990, p. 151.
134 G. Schneider, unpublished results.
135 C.G.N. Turnbull, A. Crozier, L. Schwenen and J.E. Graebe, *Phytochemistry*, 25 (1986) 97.
136 S.L. Maki, M.L. Brenner, P.R. Birnberg, P.J. Davies and T.P. Krick, *Plant Physiol.*, 81 (1986) 984.
137 D.W. Pearce and R.P. Pharis, unpublished results.
138 H. Yamane, I. Yamaguchi, M. Kobayashi, M. Takahashi, Y. Sato, N. Takahashi, K. Iwatsuki, B.O. Phinney, C.R. Spray, P. Gaskin and J. MacMillan, *Plant Physiol.*, 78 (1985) 899.
139 N. Murofushi, Y.Y. Yang, I. Yamaguchi, G. Schneider and Y. Kato, in C.M. Karssen, L.C. van Loon and D. Vreugenhil (Editors), *Progress in Plant Growth Regulation —Proceedings of the 14th International Conference on Plant Growth Substances, Amsterdam, 1991*, Kluwer, Dordrecht, 1992, p. 900.
140 T. Moritz, G. Schneider and E. Jensen, *Biol. Mass Spectrom.*, 21 (1992) 554.
141 D. Voigt and G. Dube, *J. Prakt. Chem.*, 327 (1985) 682.
142 E.B.J. Hansen, Jr., J. Abian, T.A. Getek, J.S.J. Choinski and W.A. Korfmacher, *J. Chromatogr.*, 603 (1992) 157.
143 J. MacMillan, R.J. Pryce, G. Eglington and A. McCormick, *Tetrahedron Lett.*, (1967) 2241.
144 K.V. Bhaskar, W.L.A. Chu, P.A. Gaskin, L.N. Mander, N. Murofushi, D.W. Pearce, R.P. Pharis, N. Takahashi and I. Yamaguchi, *Tetrahedron Lett.*, 32 (1991) 6203.
145 R.C. Durley, T. Sassa and R.P. Pharis, *Plant Physiol.*, 64 (1979) 214.
146 J.D. Cohen, *J. Chromatogr.*, 303 (1984) 193.
147 R. Binks, J. MacMillan and R.J. Pryce, *Phytochemistry*, 8 (1969) 271.
148 N. Takahashi, N. Murofushi, S. Tamura, N. Wasada, H. Hoshino, T. Tsuchiya, S.I. Sasaki, T. Aoyama and E. Watanabe, *Org. Mass Spectrom.*, 2 (1969) 711.
149 L. Rivier, P. Gaskin, K.Y.S. Albone and J. MacMillan, *Phytochemistry*, 20 (1981) 687.
150 N. Fang and L. Rappaport, *Biomed. Environ. Mass Spectrom.*, 19 (1990) 123.
151 T. Yokota, K. Hiraga, H. Yamane and N. Takahashi, *Phytochemistry*, 14 (1975) 1569.
152 J. Schmidt, G. Schneider and E. Jensen, *Biomed. Environ. Mass Spectrom.*, 16 (1988) 7.
153 G. Schneider and J. Schmidt, in R.P. Pharis and S.B. Rood (Editors), *Plant Growth Substances 1988 —Proceedings of the 13th International Conference on Plant Growth Substances, Calgary, 1988*, Springer, Berlin, Heidelberg, 1990, p. 300.
154 J. MacMillan, personal communication.
155 R. Lorenzi, R. Horgan and J.K. Heald, *Phytochemistry*, 15 (1976) 789.
156 K.P. Zanewich and S.B. Rood, *J. Plant Growth Regul.*, 12 (1993) 41.
157 K. Hiraga, H. Yamane and N. Takahashi, *Phytochemistry*, 13 (1974) 2371.
158 G. Schneider, S. Jänicke and G. Sembdner, *J. Chromatogr.*, 109 (1975) 409.
159 C.F. Poole and S.K. Poole, in E. Heftmann (Editor), *Chromatography —Fundamentals and Applications of Chromatography and Related Differential Migration Methods, Part A: Fundamentals and Techniques*, Elsevier, Amsterdam, 5th ed., 1992, p. A393.
160 S.B. Rood, R.I. Buzzell, L.N. Mander, D. Pearce and R.P. Pharis, *Science*, 241 (1988) 1216.
161 E. Kováts, *Helv. Chim. Acta*, 41 (1958) 1915.
162 P. Gaskin, J. MacMillan, R.D. Firn and R.J. Pryce, *Phytochemistry*, 10 (1971) 1155.
163 J.J. Ross, J.B. Reid and H.S. Dungey, *Planta*, 186 (1992) 166.