Journal of Chromatography, 238 (1982) 157–166
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,559

REVERSED- AND NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF GIBBERELLIN METHOXYCOUMARYL ESTERS

ALAN CROZIER*
Department of Botany, The University, Glasgow G12 8QQ (Great Britain)
JOE B. ZAERR
Department of Forest Science, Oregon State University, Corvallis, OR 97331 (U.S.A.)
and
ROY O. MORRIS
Department of Agricultural Chemistry, Oregon State University, Corvallis, OR 97331 (U.S.A.)
(Received November 19th, 1981)

SUMMARY

The methoxycoumaryl esters of thirteen gibberellins were synthesized by crown ether catalysis. These derivatives are highly fluorescent (λ_{max}^{ex} 320 nm, λ_{max}^{em} 400 nm) and can be detected at the low picogram level with a spectrophotofluorimeter after reversed-phase high-performance liquid chromatography. The various gibberellin esters were readily resolved by high-performance liquid chromatography on both ODS-Hypersil and CPS-Hypersil supports. There were marked differences in the selectivity of the reversed- and normal-phase systems especially in relation to the behaviour of mono, bis and tris esters. Chemical ionization negative-ion mass spectra of the gibberellin methoxycoumaryl esters were obtained by direct-probe mass spectrometry.

INTRODUCTION

The application of high-performance liquid chromatography (HPLC) to the analysis of gibberellins (GAs) is still in its infancy. Although GAs are readily chromatographed they exhibit only low UV absorbance, and the main dilemma confronting potential users of the technique is the choice of a detector system. Although an absorbance monitor operating at *ca.* 210 nm can detect *ca.* 50 ng of GA, the UV cut-off point of most solvents restricts this level of sensitivity to a very limited range of mobile-phase conditions. The problem is compounded when analysing trace amounts of GAs in multicomponent plant extracts as many of the impurities induce a strong detector response. One course of action is to use selective GA bioassays to detect active components in HPLC eluates[1,2]. However, if chromatographic peak capacity is to be maintained many fractions have to be collected and assayed. This is time-consuming and much of the practicality of HPLC is lost. More often only small numbers of fractions are assayed, and in these circumstances resolution is clearly

0021-9673/82/0000–0000/$02.75

being traded for enhanced speed of analysis. This can be an acceptable compromise when HPLC is being used to purify unknown endogenous GA-like compounds prior to analysis by mass spectrometric techniques.

An alternative approach, that involves sacrificing detector selectivity in favour of the resolution and general flexibility of HPLC, is to convert GAs into derivatives that absorb in an accessible region of the UV spectrum. This option is feasible when either radioactive GAs are being analysed or the identity of the individual GAs in a sample are known or suspected. Reeve and Crozier[3] made use of GA benzyl esters which were synthesized by esterification of N,N′-dimethylformamide dibenzylacetal and have a λ_{max} of 256 nm. The GA benzyl esters were chromatographed on a silica gel adsorption column which readily separated several isomers because of its ability to distinguish subtle differences in the spatial relationships of the polar groupings of structurally similar molecules. Marked changes in the selectivity of the silica gel column were achieved by using different reagents to modify the mobile phase. The procedures have been used in conjunction with a radioactivity monitor to analyse [^{3}H]GA metabolites from *Phaseolus coccineus* seedlings[3–5] and lettuce hypocotyl sections[6]. A comprehensive discussion of silica gel adsorption HPLC of GA benzyl esters has also been published[3].

Although GA benzyl esters have proved useful in metabolism studies it should be noted that the ε_{max} of mono derivatives is 205 l mol^{-1} cm^{-1} and that the limit of detection at 254 nm is only 300 ng. This lack of sensitivity is a serious constraint when it comes to utilizing fully the high resolving power of HPLC to analyse sub-microgram amounts of endogenous GAs. Other derivatives do, however, offer much greater potential in this regard. Heftmann *et al.*[7] prepared *p*-nitrobenzyl GA esters (λ_{max} 265 nm, $\varepsilon_{max} > 6000$) using O-*p*-nitrobenzyl-N,N′-diisopropylurea[8]. Unfortunately when the esters were chromatographed on a preparative silver nitrate impregnated silica gel column the speed of analysis was very slow and the performance was poor (number of theoretical plates, $N = 1500$; height equivalent to a theoretical plate, $H = 3.25$ mm). As 50–200-ml peak volumes were obtained the limit of detection at A_{265} was 100 ng rather than the <10 ng that might have been anticipated if conventional HPLC techniques had been used. Morris and Zaerr[9] used 18-crown-6 according to the procedures of Durst *et al.*[10] to catalyse the conversion of GAs into GA *p*-bromophenacyl esters (λ_{max} 256 nm, ε_{max} 19,100). The limit of detection at A_{254} for mono esters eluting from reversed- and normal-phase HPLC columns was <5 ng.

This paper reports on the synthesis and HPLC of GA methoxycoumaryl esters (GACE) which are strongly fluorescent derivatives (λ_{max}^{excit} 320 nm, λ_{max}^{emiss} 400 nm) that can be detected at the low picogram level.

MATERIALS AND METHODS

Solvents were delivered at a flow-rate of 1 ml min^{-1} by an Altex Model 332 gradient liquid chromatograph. Samples were introduced off-column via an Altex Model 210 valve fitted with a 20-μl loop. A 250 × 5 mm I.D. ODS-Hypersil (5 μm) column eluted with varying ratios of either methanol or ethanol in 20 m*M* ammonium acetate buffer (pH 3.5) was used for reversed-phase HPLC, while normal-phase separations were carried out on a 250 × 5 mm I.D. CPS-Hypersil (5 μm) column eluted isocratically with various ratios of hexane and dichloromethane con-

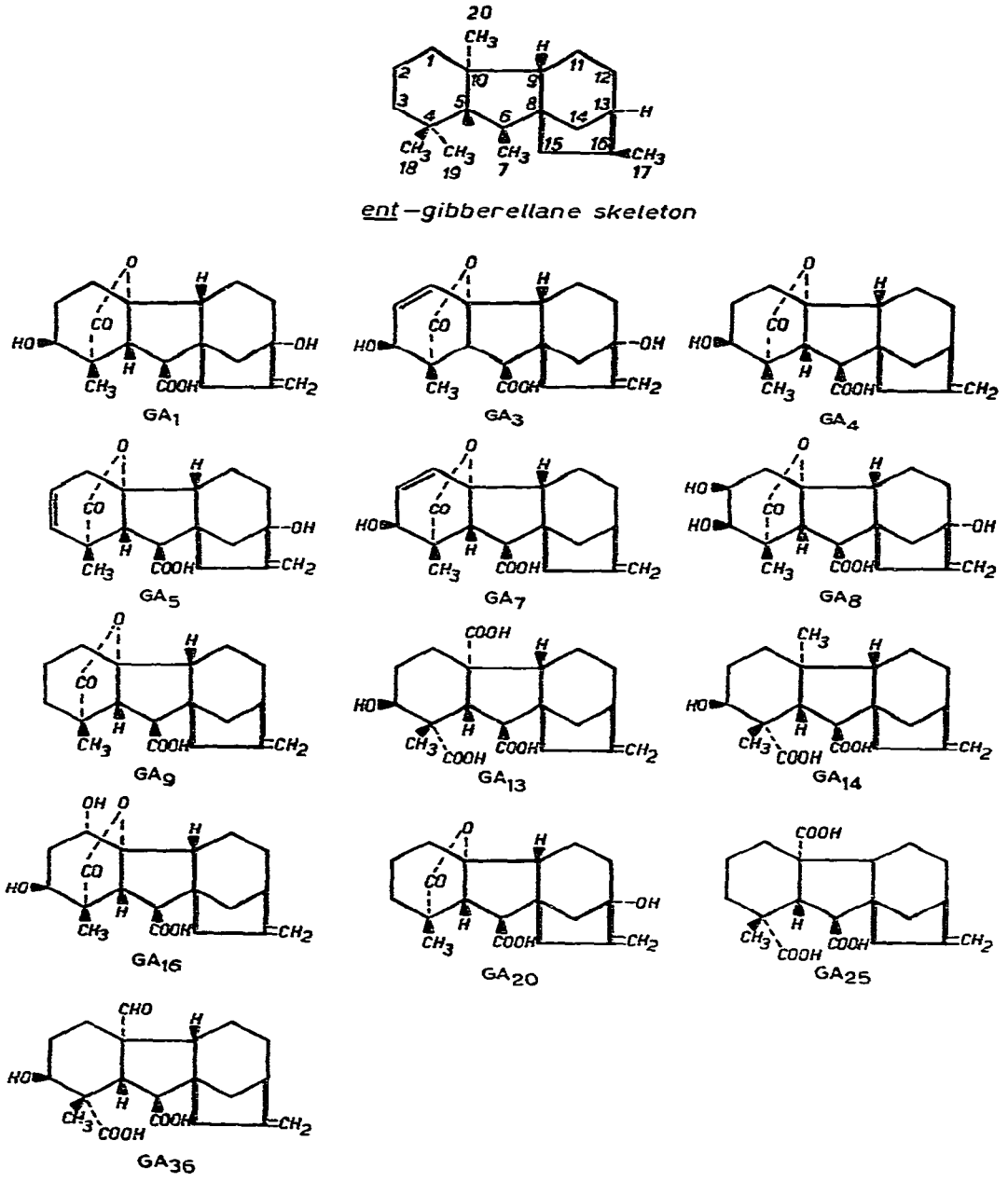

Fig. 1. Gibberellin structures.

taining 3% ethanol. Column effluent was monitored with either a Perkin-Elmer 650-10S spectrophotofluorimeter (excitation 320 nm, emission 400 nm) fitted with an 18 μl flow cell or an ISCO Model UA-5 absorbance monitor at 254 nm with an 8-μl flow cell. When radioactive samples were analysed, effluent leaving the fluorimeter was

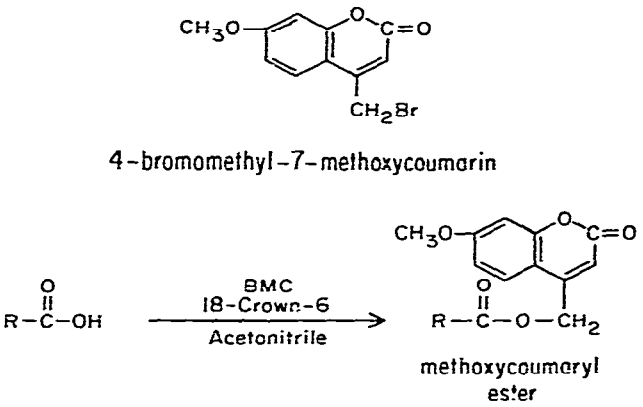

Fig. 2. Synthesis of a methoxycoumaryl ester.

directed to a Reeve Analytical (Glasgow, Great Britain) Splitter–Mixer, mixed with scintillant and passed to an on-stream radioactivity monitor[11]. A scintillant composed of 10 g of PPO, 330 ml of Triton X-100, 670 ml of distilled xylene and 150 ml of methanol was used for reversed-phase HPLC analyses. A 3:1 scintillant–eluent ratio was compatible with all methanol–ethanol concentrations from 0–100% giving 12–15% counting efficiencies for ^3H when using reagent grade chemicals. The mobile phase from the cyanopropyl column was mixed with scintillant containing 12 g of PPO, 150 g of naphthalene, 50 ml of Triton X-100 and 1 l of distilled toluene. An efficiency of 25% for ^3H was obtained with a 2:1 scintillant–eluent ratio.

Methoxycoumaryl esters of GA_1, GA_3, GA_4, GA_5, GA_7, GA_8, GA_9, [17^3H]GA_9 (11.2 mCi mmol^{-1}), GA_{13}, GA_{14}, GA_{16}, GA_{20}, GA_{25} and GA_{36} (see Fig. 1) were prepared according to the procedures of Dünges[12] by reaction of each of the free acids with an equimolar amount of 4-bromomethyl-7-methoxycoumarin (BMMC), a one-tenth molar equivalent of 18-crown-6 and a crystal of K_2CO_3 in 100 μl of dry acetonitrile at 60°C for 2 h. The reaction is shown in Fig. 2. The reaction mixture was taken to dryness, water was added and the GACEs were extracted into chloroform and dried prior to purification by steric exclusion[13] and reversed-phase HPLC. Electron impact and chemical ionization mass spectra of GACEs were obtained by direct insertion probe using a Finnigan 4023 mass spectrometer.

RESULTS AND DISCUSSION

The efficacy of the 18-crown-6-catalysed conversion of GAs into GACEs was determined by analysing a [^{3}H]GA_9 reaction mixture by reversed-phase HPLC using fluorescence and radioactivity monitors. The data obtained are illustrated in Fig. 3. The traces show that the GA_9 underwent complete conversion into GA_9CE, which was the predominant fluorescent component in the sample.

Although fluorimetry offers inherently high sensitivity, practical performance is very much instrument-dependent and limits of detection can vary by as much as three orders of magnitude. We assessed several commercial HPLC fluorescence monitors and obtained the best results with a Perkin-Elmer 650-10S spectrofluorimeter,

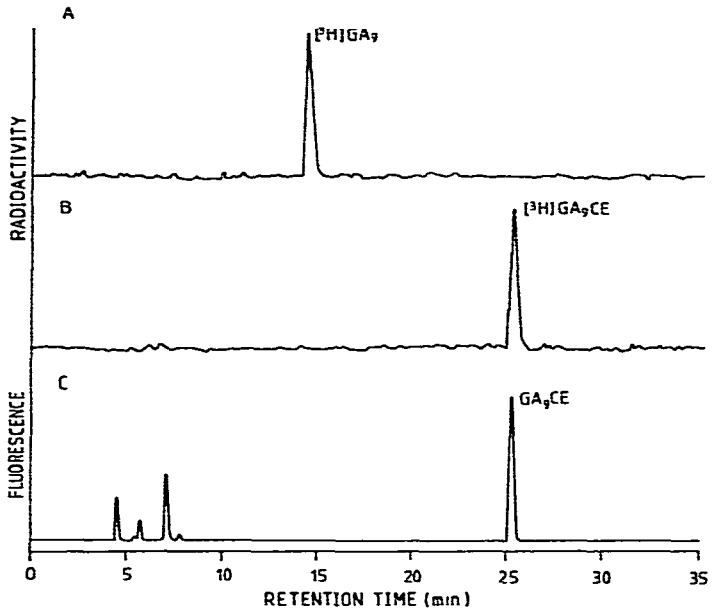

Fig. 3. Conversion of [^{3}H]GA_9 into [^{3}H]GA_9 methoxycoumaryl ester. Column, 250 × 5 mm I.D. ODS-Hypersil; mobile-phase, 30 min gradient, 60–100% methanol in 20 m*M* ammonium acetate buffer (pH 3.5); flow-rate, 1 ml min^{-1}. Samples: (A) *ca.* 15 × 10^3 dpm aliquot of [^{3}H]GA_9-BMC-18-crown-6 reaction mixture at 0 h; (B, C) *ca.* 15 × 10^3 dpm aliquot of [^{3}H]GA_9-BMC-18-crown-6-reaction mixture after 2 h at 60°C. Detectors, homogeneous radioactivity monitor and spectrofluorimeter (excitation 320 nm, emission 400 nm).

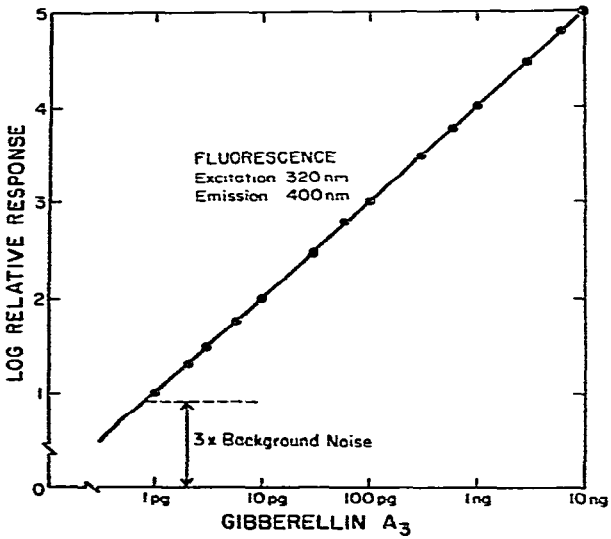

Fig. 4. Fluorescence detection limit of the methoxycoumaryl ester of GA_3 after reversed-phase HPLC. Column, 250 × 5 mm I.D. ODS-Hypersil; mobile phase, 45% ethanol in 20 m*M* ammonium acetate buffer (pH 3.5); flow-rate, 1 ml min^{-1}; sample, GA_3CE (k' = 2.3), load as indicated; detector, spectrofluorimeter (excitation 320 nm, emission 400 nm).

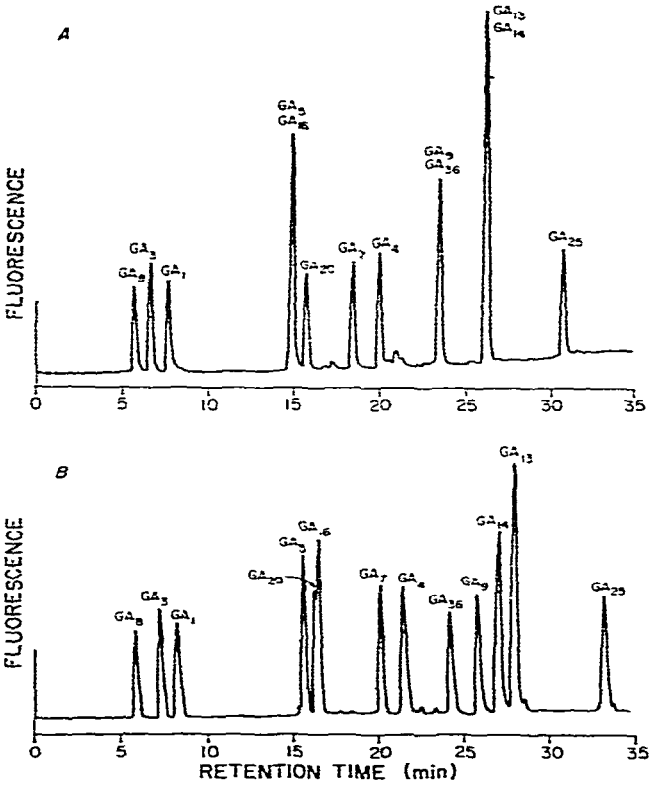

Fig. 5. Reversed-phase HPLC of gibberellin methoxycoumaryl esters. Column, 250 × 5 mm I.D. ODS-Hypersil; mobile phase. 30 min gradient (A) 60–100% methanol in 20 m*M* ammonium acetate buffer (pH 3.5). (B) 40–80% ethanol in 20 m*M* ammonium acetate buffer (pH 3.5); flow-rate, 1 ml min^{-1}; sample, methoxycoumaryl esters of GA_1, GA_3, GA_4, GA_5, GA_7, GA_8, GA_9, GA_{13}, GA_{14}, GA_{16}, GA_{20}, GA_{25} and GA_{36}; *ca.* 9.0 ng mono, 4.5 ng bis and 3.0 ng tris esters; detector, spectrofluorimeter (excitation 320 nm, emission 400 nm).

which was capable of detecting GA_3CE at the low picogram level after reversed-phase HPLC. This is shown in Fig. 4, where a log–log plot of relative response against sample size gives a line with a slope of 1.0, linear over almost four orders of magnitude. The limit of detection for GA_3, which forms a mono ester, is *ca.* 1 pg (2.8 fmol) as determined by the point at which the curve intersects the ordinate equivalent to three times the level of background noise. With bis and tris derivatives the figure is correspondingly lower.

Reversed-phase separations of a range of GACEs obtained by gradient elution from an ODS-Hypersil column are illustrated in Fig. 5. The recovery of $[^3H]GA_9CE$ was greater than 90%. The system was able to distinguish between closely related GAs. The double-bond isomers GA_1CE/GA_3CE, GA_4CE/GA_7CE and $GA_5CE/GA_{20}CE$ all separated with baseline resolution with the $\Delta^{1,2}$ and $\Delta^{2,3}$ derivatives eluting before their saturated analogs. It is of interest to note the effect of solvents on column selectivity. When a methanol–buffer mobile phase was employed $GA_{13}CE$ and $GA_{14}CE$ co-chromatographed as did GA_9CE and $GA_{36}CE$ (Fig. 5A).

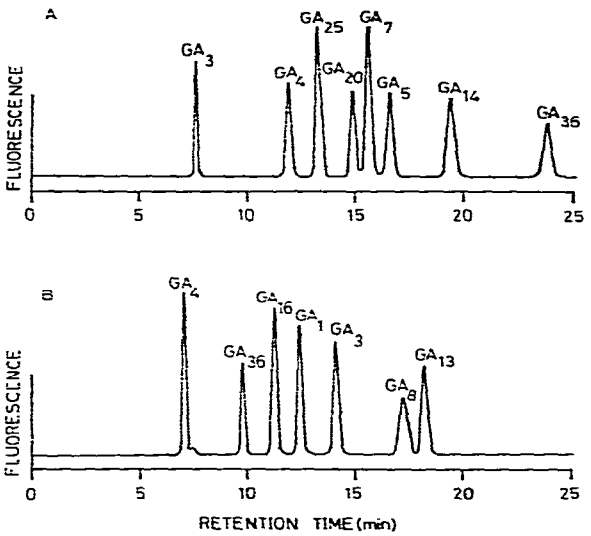

Fig. 6. Normal-phase HPLC of gibberellin methoxycoumaryl esters. Column, 250 × 5 m I.D. CPS-Hypersil; mobile phase, (A) 3% ethanol in dichloromethane–hexane (12:88) and (B) 3% ethanol in dichloromethane–hexane (20:80); flow-rate, 1 ml min^{-1}; sample, methoxycoumaryl esters of GA_1, GA_3, GA_4, GA_5, GA_7, GA_8, GA_9, GA_{13}, GA_{14}, GA_{16}, GA_{20}, GA_{25} and GA_{36} as indicated; *ca.* 3.0 ng mono, 1.5 ng bis and 1.0 ng tris esters; detector, spectrofluorimeter (excitation 320 nm, emission 400 nm).

However, when ethanol was substituted for methanol the compounds were well resolved (Fig. 5B). In general, increasing the number of free hydroxyl groups decreases retention, 13α-hydroxylation to a much greater extent than 3β-hydroxylation which, in turn, is more effective than hydroxylation at either the 1α or 2β positions. A comparison of the retention characteristics of GA_4CE, GA_{36}CE, GA_{14}CE and GA_{13}CE as well as GA_9CE and GA_{25}CE shows an elution order of mono > bis > tris esters indicating that increasing the number of methoxycoumaryl functions decreases polarity. The elution of GA_{14}CE and GA_{38}CE implies that C-20 methyl GACEs are less strongly retained than the C-20 aldehydic counterparts.

The ODS-Hypersil column generated 9300 theoretical plates when GACEs were analysed. This compares unfavourably with a figure of 16,500 obtained when a test mixture containing biphenyl was chromatographed and monitored at A_{254}. In both instances retention volumes were more than 20 ml. This minimized extra-column effects and ensured that the differences in column performance were not a consequence of the larger flow-cell volume of the fluorimeter used to detect the GACEs. The reduced efficiency with GACEs was much more marked on Ultrasphere ODS and MicroPak MCH-5 columns and was not counteracted by increasing the molarity of the ammonium acetate buffer in the mobile phase. The symptoms may therefore reflect the effectiveness with which the various supports were end capped.

Normal-phase separations of GACEs on a CPS-Hypersil column eluted isocratically with 3% ethanol in either 12% or 20% dichloromethane in hexane are illustrated in Fig. 6. Recoveries of the [^{3}H]GA_9CE from the cyanopropyl column were *ca.* 85%. Although the effects of 3β- and 13α-hydroxylation and 1,2 and 2,3 double bonds are, as anticipated, the opposite of those observed in reversed-phase

TABLE I

METHANE CHEMICAL IONIZATION NEGATIVE-ION MASS SPECTRA OF GIBBERELLIN METHOXYCOUMARYL ESTERS

Compound	*Mol.wt.*	
GA_1CE	536	347—100% (M − 189)
GA_3CE	534	534—100% (M^-), 345—43% (M − 189), 301—7% (M − 233), 283—7% (M − 251)
GA_4CE	520	331—100% (M − 189)
GA_5CE	518	329—100% (M − 189)
GA-CE	518	518—100% (M^-), 329—34% (M − 189), 285—5% (M − 233), 267—15% (M − 251), 265—5% (M − 253)
GA_8CE	552	363—100% (M − 189)
GA_9CE	504	315—100% (M − 189)
$GA_{13}CE$	942	565—8% (M − 377 [− 189 − 188]), 547—100% (M − 395 [− 189 − 206]), 359—54% (M − 583 [− 189 − 188 − 206 and/or − 189 − 206 − 188]), 315—5% (M − 627), 314—6% (M − 628)
$GA_{14}CE$	724	535—100% (M − 189), 347—18% (M − 377 [− 189 − 188])
$GA_{16}CE$	536	347—100% (M − 189), 329—6% (M − 207), 303—9% (M − 233)
$GA_{20}CE$	520	331—100% (M − 189)
GA_{36}	738	549—10% (M − 189), 343—100% (M − 395 [− 189 − 206])

analyses, the overal elution pattern is not a mirror image of the ODS-Hypersil profiles in Fig. 5. This is primarily due to the behaviour of bis and tris GACEs which exhibit increased capacity factors (k') with respect to their increased number of methoxycoumaryl groups. The polarity of GACEs, in this regard is, thus, the opposite of that observed on the reversed-phase support. The other noticeable difference, as indicated by the elution of $GA_{16}CE$ close to GA_1CE rather than $GA_{20}CE$, is that 1β-hydroxylation has a more marked effect on retention in the normal than the reversed-phase system. There was no reduction in the performance of the CPS-Hypersil column when GACEs were analysed as *ca.* 14,000 theoretical plates were generated with both GACEs and a test mixture containing 2,6-dinitrotoluene. The only exception was the 2β-hydroxy derivative GA_8CE which chromatographed with an efficiency of only 6100 theoretical plates. Because of increased levels of background fluorescence and/or quenching in the mobile phase the limit of detection of mono GACEs eluting from the CPS column was no better than 30 pg.

GACEs are not readily volatile and as a consequence mass spectra have to be obtained by direct-probe mass spectrometry rather than combined gas chromatography–mass spectrometry. Direct-probe electron impact and chemical ionization positive-ion spectra were of no practical value as the dominant fragment in all instances is *m/e* 191 with no other ions of significant intensity being present. However, chemical ionization negative-ion spectra proved to be of more diagnostic value (Table I). A strong molecular ion was obtained with the $\Delta^{1,2}$ GACEs, GA_3CE and GA_7CE. M − 189 arising from the loss of the methoxycoumaryl moiety was the main fragment in the spectra the other C_{19}-GA derivatives that were tested. The C_{19}-GA isomers GA_4CE and $GA_{20}CE$ yielded identical spectra. These compounds can however be readily distinguished on the basis of their HPLC retention characteristics. Spectra were obtained from the methoxycoumaryl esters of three C_{20}-GAs. M − 189 was the strongest ion produced by $GA_{14}CE$ while M − 395 was the base peak in the spectra of both $GA_{13}CE$ and $GA_{36}CE$.

The picogram limits of detection of the fluorescence monitor to GACEs enhance the overall flexibility of HPLC as an analytical tool, especially in the investigation of trace amounts of endogenous GAs in vegetative tissues. It should be noted, however, that the high sensitivity is not accompanied by any degree of detector selectivity. GAs do not exhibit native fluorescence. Thus, when plant extracts are derivatized not only endogenous GAs but also any other carboxylic acids in the sample will form methoxycoumaryl esters and so acquire fluorescent properties. As a consequence considerable purification will be required before homogeneity of detector response can be obtained and an accurate analysis achieved. Such an approach appears feasible with HPLC because of the wide array of diverse separatory mechanisms that can be employed and the ease and efficiency of sample recovery. Procedures for use in the verification of accuracy of such analyses have been proposed by Reeve and Crozier[14] and further discussed by Crozier[5,15,16].

The HPLC fluorescence procedures that have been described have the potential to quantitatively analyse endogenous GAs as their methoxycoumaryl esters only when the identity of individual GA(s) likely to be present in a sample is known or suspected and when reference compounds are available to determine HPLC retention characteristics and quantify the response of the fluorimetric detector. If estimates are to be truely quantitative a radioactive internal standard must also be available to account for sample handling losses and variability in derivatizing efficiency. With the exception of [^{3}H]GA$_1$ and [^{3}H]GA$_4$, labelled GAs suitable for use as internal markers are not currently available from commercial sources so investigators are faced with the choice of either accepting such errors or investing time in the synthesis of either ^{14}C-labelled or, more preferably, high specific activity ^{3}H-labelled GAs.

ACKNOWLEDGEMENTS

This investigation was initiated at Oregon State University, when Alan Crozier was on leave of absence, and completed at the University of Glasgow. Studies at Oregon State University were supported by grants from the Weyerhauser Company, Crown Zellerbach Corporation, Sun Studs Company, International Paper Company and St. Regis Paper Company. Research at the University of Glasgow made use of an HPLC purchased with a Royal Society Grant-in-Aid and a spectrofluorimeter acquired with funds from the Science Research Council. We wish to thank Alison Sutcliffe (University of Glasgow) for expert technical assistance and Don Griffin (Oregon State University) for obtaining the mass spectra.

REFERENCES

1 G. W. M. Barendse, P. H. van de Werken and H. Takahashi, *J. Chromatogr.*, 198 (1980) 449.
2 M. G. Jones, J. D. Metzger and J. A. D. Zeevaart, *Plant Physiology*, 65 (1980) 218.
3 D. R. Reeve and A. Crozier, in J. Hillman (Editor), *Isolation of Plant Growth Substances*, Cambridge Univ. Press, London, New York, 1978, p. 41.
4 A. Crozier and D. R. Reeve, in P. E. Pilet (Editor), *Plant Growth Regulation*, Springer, Berlin, 1977, p. 67.
5 A. Crozier, in H. W. Woolhouse (Editor), *Advan. Bot. Res.*, 9 (1981) 33.
6 L. J. Nash, R. L. Jones and J. L. Stoddart, *Planta*, 140 (1978) 143.
7 E. Heftmann, G. A. Saunders and W. F. Haddon, *J. Chromatogr.*, 156 (1978) 71.
8 D. R. Knapp and S. Kruger, *Anal. Lett.*, 8 (1975) 603.

9 R. O. Morris and J. B. Zaerr, *Anal. Lett.*, AII(i) (1978) 73.
10 D. Durst, M. Milano, E. J. Kitka, Jr., S. A. Connelly and E. Grushka, *Anal. Chem.*, 47 (1975) 1797.
11 D. R. Reeve and A. Crozier, *J. Chromatogr.*, 137 (1977) 271.
12 W. Dünges, *Anal. Chem.*, 49 (1977) 442.
13 A. Crozier, J. B. Zaerr and R. O. Morris, *J. Chromatogr.*, 198 (1980) 57.
14 D. R. Reeve and A. Crozier, in J. MacMillan (Editor), *Hormonal Regulation of Development 1. Molecular Aspects of Plant Hormones, Encyclopeadia of Plant Physiology New Series*, Vol. 9, Springer, Berlin, 1980, p. 203.
15 A. Crozier, in C. H. A. Little (Editor), *Control of Shoot Growth in Trees*, Canadian Forest Service, Fredericton, 1980, p. 325.
16 A. Crozier, in J. R. Lenton (Editor), *Gibberellins-Chemistry, Physiology and Use*, Monograph No. 5, British Plant Growth Regulator Group, Wantage, 1980, p. 17.