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PLANT HORMONES—V.

THIN-LAYER AND GAS–LIQUID CHROMATOGRAPHY OF THE GIBBERELLINS; DIRECT IDENTIFICATION OF THE GIBBERELLINS IN A CRUDE PLANT EXTRACT BY GAS–LIQUID CHROMATOGRAPHY

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Abstract—Thin-layer chromatographic data in five solvent systems are presented which enable the separation of the seventeen known gibberellins. The R_f values are not reproducible and cannot be standardized. They can only be used for positive identification in conjunction with authentic samples. All seventeen gibberellins can be separated and identified by gas–liquid chromatography of the methyl esters and trimethyl silyl ethers of the methyl esters on 2% QF-1 and 2% SE-33 columns. The retention times can be standardized. These methods have been applied to the crude acidic extract from immature seed of *Phaseolus multiflorus* and indicate the presence of gibberellins A_1, A_4, A_5, A_6, A_8, and A_{13}. In subsequent column chromatography of the crude extract, gas–liquid chromatography showed the presence of gibberellins A_1, A_5, A_6 and A_8 in the appropriate fractions, eluted with the expected percentage of acetone in water.

THIN-LAYER chromatography (TLC) of the gibberellins[1–3] has proved a useful method for the identification of gibberellins in higher plants[4–6] and in culture filtrate of *Gibberella fujikuroi*.[7] We have extended our studies on the TLC of the gibberellins to include the seventeen gibberellins which have so far been isolated and characterized. The structures of these gibberellins are shown in Fig. 1. The R_f values presented in Table 1 were obtained for all gibberellins simultaneously on one plate for each solvent system. All gibberellins were detected by their fluorescence in u.v. light after spraying with 5% sulphuric acid–ethanol then heating at 120°. The Bamboo and Lupinus-I gibberellins showed weak fluorescence after long heating and were easier to detect using a ceric sulphate spray. Using the solvent systems described in Table 1 on silica or Kieselguhr, the seventeen gibberellins can be distinguished from each other. However, for a decisive identification of gibberellins by this method, the appropriate authentic specimens are required. The R_f values are not reproducible and cannot be standardized in terms of reference compounds. For example, the R_f values in Table 1 for gibberellins A_1–A_9 in solvent systems 1, 2, and 3 differ from those previously reported[2] as do the R_f values relative to gibberellins A_3 and A_7, which are reasonably available and therefore attractive reference compounds. The need for reference specimens of the scarce gibberellins is a serious disadvantage. We are seeking alternative methods and wish to report the use of gas–liquid chromatography in the direct identification of gibberellins in the crude acid extract from immature seeds of *Phaseolus multiflorus*.

[1] G. SEMBDNER, R. GOSS and K. SCHREIBER, *Experientia* **18**, 584 (1962).
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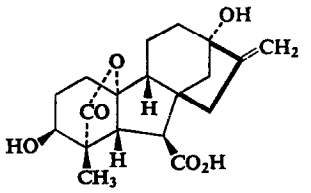
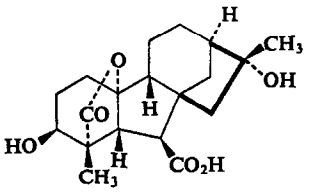
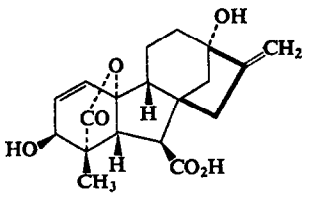


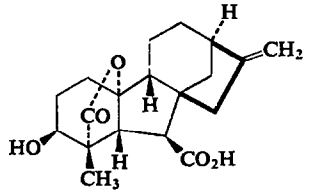
(1) Gibberellin A_1[a]



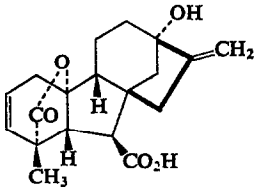
(2) Gibberellin A_2[b, c]



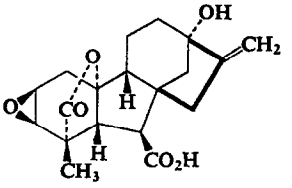
(3) Gibberellin A_3=Gibberellic acid[d, e]



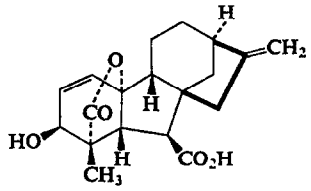
(4) Gibberellin A_4[f, g, h]



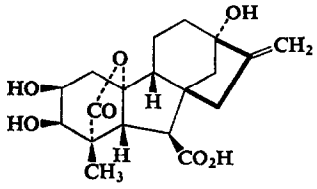
(5) Gibberellin A_5[i]



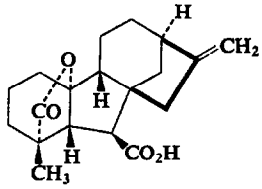
(6) Gibberellin A_6[j]



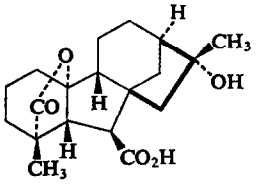
(7) Gibberellin A_7[h]



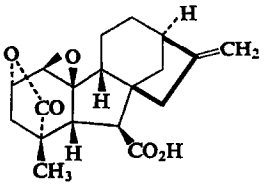
(8) Gibberellin A_8[j]



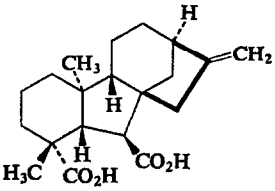
(9) Gibberellin A_9[h]



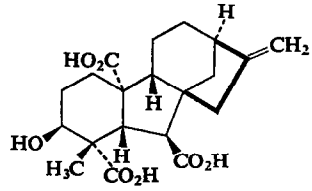
(10) Gibberellin A_{10}[c]



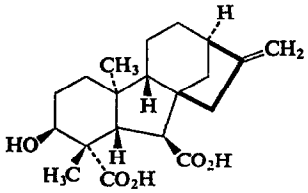
(11) Gibberellin A_{11}[k]



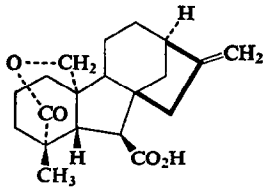
(12) Gibberellin A_{12}[l]



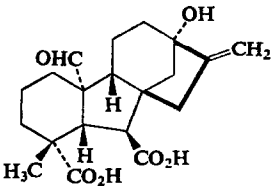
(13) Gibberellin A_{13}[m]



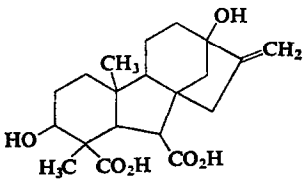
(14) Gibberellin A_{14}[n]



(15) Gibberellin A_{15}[o]



(16) Bamboo gibberellin[p]



(17) Lupinus gibberellin—I[q]

FIG. 1. STRUCTURES OF THE GIBBERELLINS.

TABLE 1. TLC R_f VALUES OF THE GIBBERELLINS

Gibberellin	Structure (see Fig. 1)	Kieselgel Solvent system* 1	2	4	5	Kieselguhr 2	3
A_1	1	0·06	0·00	0·37	0·95	0·20	0·55
A_2	2	0·01	0·00	0·19	0·85	0·24	0·67
A_3	3	0·06	0·00	0·37	0·95	0·13	0·45
A_4	4	0·20	0·69	0·61	0·80	1·00	1·00
A_5	5	0·16	0·29	0·59	0·80	0·87	1·00
A_6	6	0·13	0·16	0·57	0·90	0·82	1·00
A_7	7	0·19	0·60	0·62	0·80	1·00	1·00
A_8	8	0·01	0·00	0·24	1·00	0·04	0·20
A_9	9	0·59	1·00	0·78	0·65	1·00	1·00
A_{10}	10	0·06	0·36	0·33	0·60	0·91	1·00
A_{11}	11	0·49	1·00	0·74	0·80	1·00	0·88
A_{12}	12	0·67	1·00	0·78	0·70	1·00	1·00
A_{13}	13	0·14	0·11	0·46	0·90	0·39	1·00
A_{14}	14	0·26	0·75	0·63	0·80	0·86	0·72
A_{15}	15	0·44	1·00	0·78	0 57	1·00	1·00
Bamboo	16	0·80	0·00	0·42	0·95	0·12	0·82
Lupinus-I	17	0·04	0·02	0·35	0·95	0·34	1·00

* Solvent systems: (1) Di-isopropyl ether/acetic acid (95:5); (2) benzene/acetic acid/water (8:3:5); (3) benzene/propionic acid/water (8:3:5); (4) ethyl acetate/chloroform/acetic acid (15:5:1); (5) Water.

Gas–liquid chromatography (GLC) of the gibberellin methyl esters A_1–A_9 has previously been reported.[8,9] We now report GLC of the seventeen characterized gibberellins (Fig. 1) as their methyl esters and methyl ester trimethyl silyl ethers (where applicable). The derivatives were chromatographed on a 2% QF-1 and 2% SE-33 column and the results are shown in Table 2. It was found that the gibberellin methyl ester trimethyl silyl ethers were an essential addition to GLC analysis of gibberellins.

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Footnotes to Fig. 1.

[a] J. F. GROVE, P. W. JEFFS and T. P. C. MULHOLLAND, *J. Chem. Soc.* 1236 (1958).
[b] J. F. GROVE, *J. Chem. Soc.* 3545 (1961).
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[k] B. E. CROSS and J. R. HANSON, Personal communication.
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[m] R. H. B. GALT, *J. Chem. Soc.* 3143 (1965).
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[o] J. R. HANSON, *Tetrahedron* **23**, 733 (1967).
[p] S. TAMURA, N. TAKAHASHI, N. MUROFUSHI, S. IRIUCHIJIMA, J. KATO, Y. WADA, E. WATANABE and T. AOYAMA, *Tetrahedron Letters* 2465 (1966).
[q] K. KOSHIMIYU, H. FUKUI, T. KUSAKI, T. MITSUI and Y. OGAWA, *Tetrahedron Letters* 2459 (1966).

TABLE 2. GLC RETENTION TIMES OF THE GIBBERELLINS

Gibberellin	Structure (see Fig. 1)	Retention time (mm)			
		Methyl esters		Trimethyl silyl ethers of methyl esters	
		2% QF-1*	2% SE-33†	2% QF-1‡	2% SE-33§
A_1	1	19·7	14·7	16·3	16·8
A_2	2	20·7	15·1	23·1	8·2
A_3	3	20·6	17·6	19·1	18·3
A_4	4	8·4	7·4	11·2	8·9
A_5	5	10·4	6·7	11·3	8·1
A_6	6	17·1	9·4	19·1	11·4
A_7	7	9·1	7·9	12·8	9·5
A_8	8	38·6	30·7	20·7	29·3
A_9	9	4·3	3·9	(7·4)**	(4·5)**
A_{10}	10	10·4	6·9	16·0	10·8
A_{11}	11	6·9	5·1	(12·3)**	(6·0)**
A_{12}	12	2·0	4·0	(3·5)**	(4·9)**
A_{13}	13	6·2	11·9	6·2	12·2
A_{14}	14	4·8	8·4	4·5	8·7
A_{15}	15	14·6	9·9	(24·9)**	(12·4)**
Bamboo	16	8·7	9·9	9·8	12·9
Lupinus-I	17	10·7	16·5	6·9	13·3
5α-Cholestane	—	3·1	20·4	5·1	23·3

* Column temp., 201°; carrier gas, N_2, 60 ml min^{-1}.
† Column temp., 190°; carrier gas, N_2, 80 ml min^{-1}.
‡ Column temp., 179°; carrier gas, N_2, 84 ml min^{-1}.
§ Column temp., 187°; carrier gas, N_2, 75 ml min^{-1}.
** Methyl ester only.

The methyl esters on the QF-1 column show broadening due to decomposition at longer retention times, the degree of broadening depending on the quality of the column used. Sumiki *et al.*[8,9] reported a reasonable separation of gibberellins A_1, A_2, and A_3 methyl esters on a 2% QF-1 column. We were unable to achieve this separation under our conditions. On the SE-33 column all peaks are correspondingly broader. Gibberellins A_1, A_2, and A_3 are again unresolved due to the broadness of their peaks. Gibberellin A_8 methyl ester shows a very broad peak on the QF-1 column and on the SE-33 column it is very difficult to discern. Gibberellins A_5 and A_{10} could not be separated as their methyl esters on either column. The gibberellin methyl ester trimethyl silyl ethers (of those gibberellins with hydroxyls—see Fig. 1), however, all show sharp peaks on both columns. Using this derivative, the gibberellins A_1, A_2, and A_3, and the gibberellins A_5 and A_{10} can now be distinguished from each other. Gibberellin A_8 is easily detected as its methyl ester trimethyl silyl ether, and most conveniently on the SE-33 column.

The gibberellin methyl esters can be arranged in groups of increasing retention time on QF-1 (Table 2) according to the number of hydroxyl and/or epoxide functions. Thus gibberellins A_{12} and A_9 with none of these oxygen functions have lowest retention times followed by the group of gibberellins, A_{14}, A_{13}, A_{11}, A_4, Bamboo, A_7, A_5, and A_{10} with one such function, and finally the group, Lupinus-I, A_6, A_1, A_3, and A_2, with two such functions. Gibberellin A_8 with three hydroxyls has the longest retention time. Only gibberellin A_{15}, with no hydroxyl or epoxide rings does not fit this classification.

The retention times shown in Table 2 are reproducible. Columns could be standardized for either derivative of all gibberellins using only the more readily available gibberellins, A_3, A_7, and A_9 and the appropriate conversion factors derived from the retention times in Table 2. Using the data given in Table 2 we have attempted to analyse the gibberellin content of the crude acid extract from immature seeds of *P. multiflorus* by GLC.

Extraction of the immature seeds was carried out as previously described[10,11] with an additional ethyl acetate extraction of the concentrated aqueous ethanolic extract at pH 7·2–7·5. Extraction at this pH has been shown to remove gibberellin inhibitors in related plant material.[12] The GLC's of a sample of the crude acid extract methylated and trimethyl silylated, are shown in Figs. 2–5. These chromatograms were obtained from a fraction of the total crude acid extract corresponding to 56 g fresh weight of seed. However, this figure by no means represents a minimum.

On each of the chromatograms (Figs. 2–5), six of the more definite assignments are indicated. The four chromatograms clearly indicate the presence of gibberellins A_1, A_5, A_6, A_8, and possibly A_4 and A_{13} in the crude acid extract. It is possible that many other gibberellins are present, but only those which can be detected from all four chromatograms have been considered. The presence of gibberellins A_1, A_5, A_6, and A_8 in the acid extract of immature seeds of *P. multiflorus* has been shown by isolation.[10,11] Subsequent charcoal–celite chromatography of the crude acid extract and GLC analysis of representative fractions supported the presence of gibberellins A_1, A_5, A_6, and A_8 which were detected in fractions eluted with acetone–water ratios similar to those previously reported. So far gibberellin A_1 has been successfully isolated from the appropriate fraction, by celite–silicic acid chromatography.[10] GLC analysis, and bioassay, of fractions from the charcoal–celite column indicated the presence of additional gibberellins and other plant-growth substances which are under investigation.

TABLE 3. GLC RETENTION TIMES (2% QF-1) OF METHYLATED ACIDS COMMONLY OCCURRING IN EXTRACTS OF PLANT MATERIAL

Acid	Retention times (min)	Column temperature (carrier gas, N_2, 60 ml min^{-1})
Succinic	Not seen even at 50°	—
Benzoic	2·0	82°
p-Hydroxybenzoic	2·0	117°
Pimelic	2·1	117°
p-Hydroxyphenylacetic	2·4	117°
p-Coumaric	4·4	117°
Phthalic	4·7	117°
Azelaic	5·3	117°
Protocatechuic, Vanillic	6·1	117°
Gallic	11·4	117°
Fumaric	12·8	117°
	0·4	200°
Indolyl-3-acetic	25·8	117°
	0·6	200°

[10] J. MACMILLAN, J. C. SEATON and P. J. SUTER, *Tetrahedron* **11**, 60 (1960).
[11] J. MACMILLAN, J. C. SEATON and P. J. SUTER, *Tetrahedron* **18**, 349 (1962).
[12] D. KOHLER and A. LANG, *Plant Physiol.* **38**, 555 (1963).

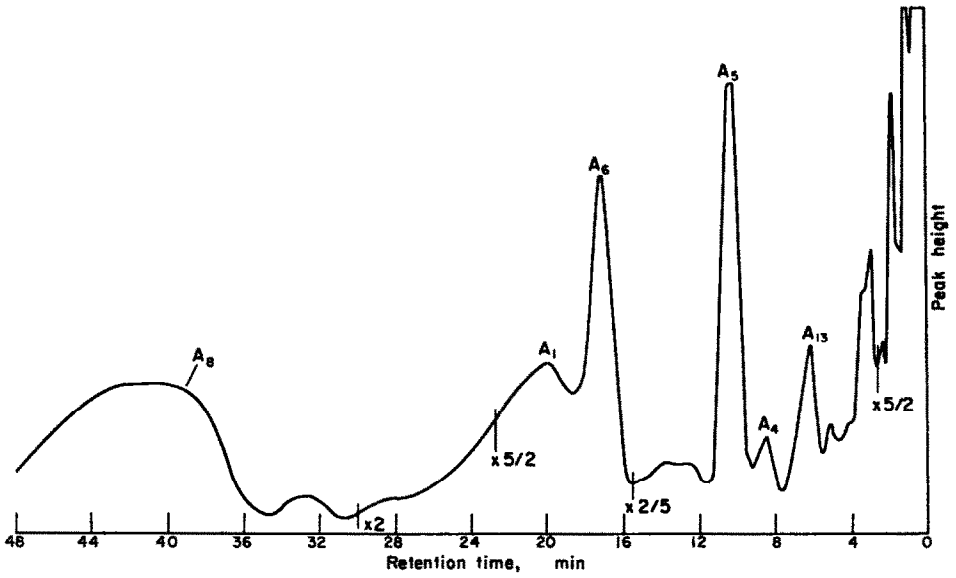

FIG. 2. GAS–LIQUID CHROMATOGRAPHY OF THE METHYLATED CRUDE ACID EXTRACT OF IMMATURE SEED OF *Phaseolus multiflorus* ON 2% QF-1.

(The breaks represent changes in sensitivity by the factors indicated.)

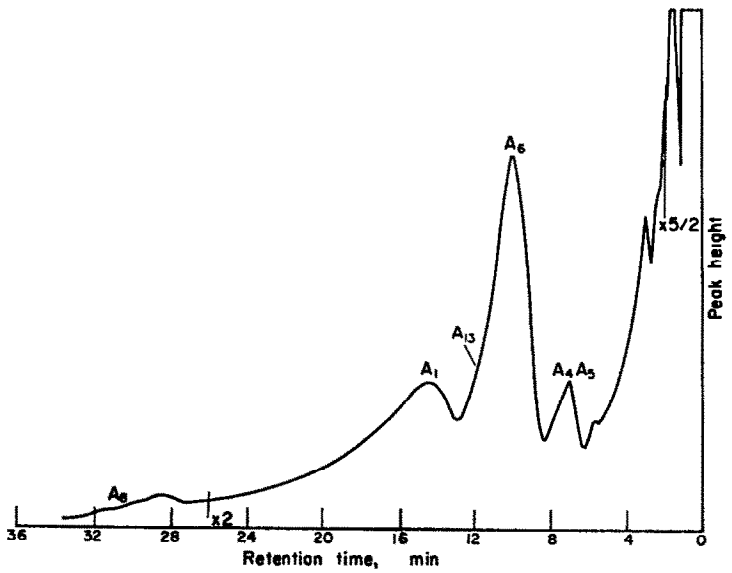

FIG. 3. GAS–LIQUID CHROMATOGRAPHY OF THE METHYLATED CRUDE ACID EXTRACT OF IMMATURE SEED OF *Phaseolus multiflorus* ON 2% SE-33.

(The breaks represent changes in sensitivity by the factors indicated.)

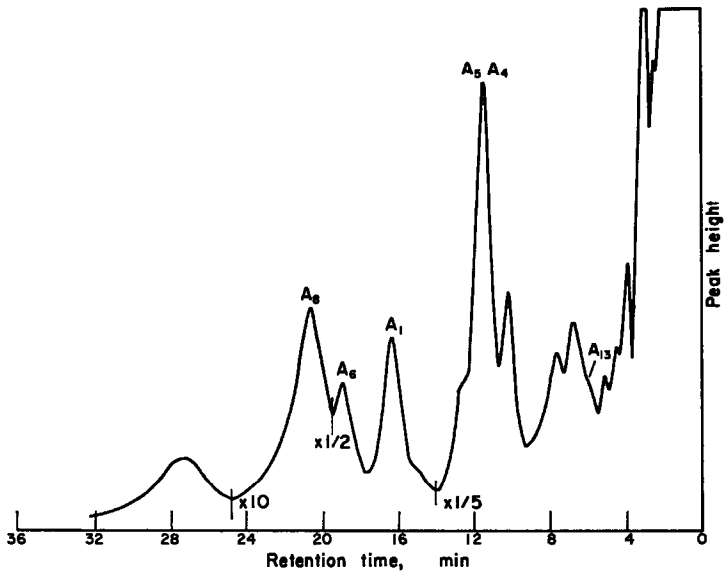

FIG. 4. GAS–LIQUID CHROMATOGRAPHY OF THE METHYLATED AND TRIMETHYL SILYLATED CRUDE ACID EXTRACT OF IMMATURE SEED OF *Phaseolus multiflorus* ON 2% QF-1.

(The breaks represent changes in sensitivity by the factors indicated.)

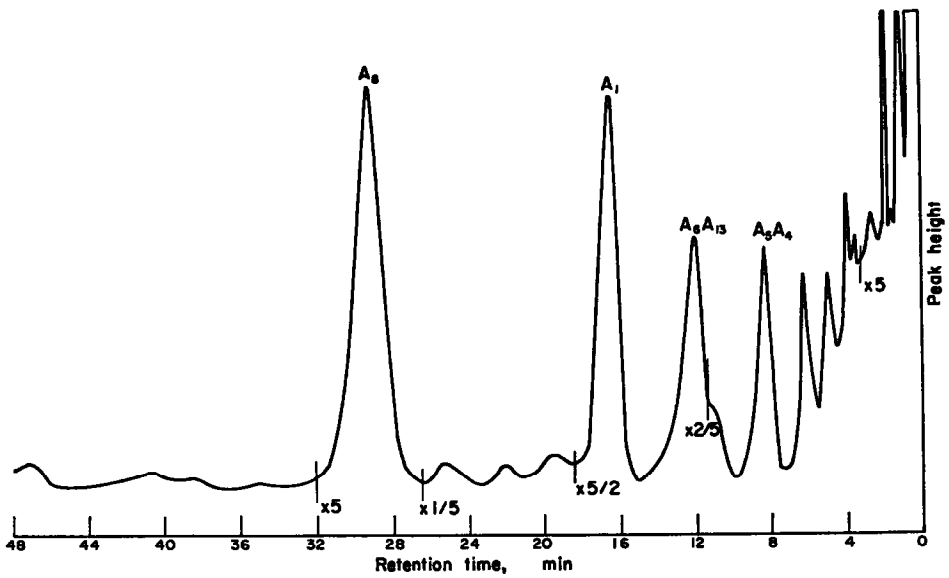

FIG. 5. GAS–LIQUID CHROMATOGRAPHY OF THE METHYLATED AND TRIMETHYL SILYLATED CRUDE ACID EXTRACT OF IMMATURE SEED OF *Phaseolus multiflorus* ON 2% SE-33.

(The breaks represent changes in sensitivity by the factors indicated.)

Many simple acids are known to occur in extracts of *P. multiflorus* seeds and similar plant material. These acids can interfere badly with gibberellin analysis by TLC but are found not to interfere with GLC analysis. They all have very short retention times compared with the gibberellins and appear mainly in the solvent front under the conditions shown in Table 2. The acids tested are shown in Table 3; they were chromatographed as their methylated derivatives obtained with diazomethane.

Further evidence for the presence of gibberellins and other unidentified compounds in the acid extract of immature seeds of *P. multiflorus* is being sought by combined GLC–mass spectrometry.

EXPERIMENTAL

Thin-layer chromatography was carried out as previously described.[2]

Gas–liquid chromatography

Instrument and columns. Retention times were determined with a Pye 104 dual column gas chromatograph fitted with flame-ionization detectors. Silanized glass columns, 5 ft × ¼ in. i.d., were packed with 2% QF-1 or 2% SE-33 on silanized Gaschrom A. Columns were pretreated by baking overnight at 300° with carrier gas (nitrogen) flowing at 60 ml min^{-1}. The 2% QF-1 and 2% SE-33 columns had efficiencies of 450 and 1060 theoretical plates respectively, as calculated with 5α-cholestane.

Preparation of samples: methyl esters. When pure methyl esters were not available they were prepared by treating a methanolic solution of the acid with diazomethane—no purification was attempted after evaporation of the reaction mixtures. Usually injections (1 μg) were made in methanol (1 μl).

Methyl ester trimethyl silyl ethers. Dry samples of the methyl esters, obtained as above, were dissolved in dry pyridine and treated with a solution of pyridine, hexamethyldisilizane and trimethyl silyl chloride (10:2:2). Normal quantities were methyl ester (*ca.* 0·1 mg) dissolved in pyridine (*ca.* 0·05 ml) and silylating reagent (*ca.* 0·05 ml). After standing for about 10 min silylation was complete and ammonium chloride had been precipitated. The gibberellin methyl ester trimethyl silyl ethers were chromatographed by injecting a portion (1 μl) of the total reaction mixture. It was found that the trimethyl silyl ethers could be stored for about 1 month in a desiccator over potassium hydroxide in the reaction mixture described; if it had gone dry in this time, addition of more pyridine made it usable again.

The Acid Extract of Immature Seeds of Phaseolus multiflorus

Immature seeds of *Phaseolus multiflorus* (25·8 kg) were extracted, as described in the discussion, to yield a crude acid extract (16·1 g). A portion of this (35 mg) was methylated and dissolved in methanol (0·5 ml). The chromatograms shown in Figs. 2 and 3 were obtained by injecting 1 μl (containing *ca.* 70 μg) onto the GLC columns. Half of this methylated acid extract was trimethyl silylated as above and chromatographed similarly to give the chromatograms shown in Figs. 4 and 5.

The crude acid extract (15·6 g) was adsorbed on silica (35 g) and placed on top of a column (70 × 5·5 cm) of celite (350 g): charcoal (175 g). The column was eluted in 200-ml fractions by gradient elution from an aspirator containing water (7·9 l.) connected to a similar aspirator containing acetone (10 l.).[11] Representative fractions were analysed by GLC in their methylated and methylated, trimethyl silylated forms. The results indicated that fraction 35, eluted with water containing 39–40% acetone, contained gibberellin A_8; fraction 40, 47% acetone, gibberellin A_1; fraction 45, 53% acetone, gibberellin A_6; fraction 51, 60% acetone, gibberellins A_4 and A_5; fraction 53, 62% acetone, gibberellins A_4, A_5, and A_{13}; and fraction 57, 65–66% acetone, gibberellin A_{13}.

Celite–silicic acid chromatography[10] of fraction 40 (368 mg) and crystallization from ethyl acetate–light petroleum (b.p. 60–80°) gave gibberellin A_1 (160 mg), m.p. 243–249·5° (decomposition)—i.r. spectrum identical with an authentic specimen.

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