Journal of Chromatography, 236 (1982) 181–188
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,397

LEAF ANALYSIS FOR ABSCISIC, PHASEIC AND 3-INDOLYLACETIC ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

RICHARD C. DURLEY*, TISSA KANNANGARA and GRAHAM M. SIMPSON
University of Saskatchewan, Crop Science Dept., Saskatoon, Sask. S7N 0W0 (Canada)
(Received September 17th, 1981)

SUMMARY

High-performance liquid chromatography (HPLC) is described for the purification and analysis of abscisic acid (AbA), phaseic acid (PA) and 3-indolylacetic acid (IAA) extracted from *Sorghum bicolor* leaves. The method is rapid, suitable for automation and capable of accommodating large numbers of samples. Detection limits are less than 1 ng for each hormone. Recovery efficiency is 75% for AbA and PA and 64% for IAA.

After initial extraction and partition, AbA, PA and IAA were purified together as ammonium salts (ion pairs) on polyvinylpyrrolidone. This was followed by further purification and separation of the three hormones on preparative C_{18} reversed-phase HPLC (5-μm spherical particles). Analysis of AbA and PA was by absorption phase silica HPLC (5-μm spherical particles) with detection by UV absorption. Analysis of IAA was by C_{18} reversed-phase HPLC (5-μm spherical particles), with fluorescence detection.

INTRODUCTION

Since hormones are present in low concentrations in most plant tissues, special techniques are needed to measure them accurately. The most sensitive chromatographic techniques for hormone analysis are capillary gas chromatography (GC), gas chromatography–mass spectrometry with selected ion current monitoring (GC–MS–SICM) and high-performance liquid chromatography (HPLC).

Capillary GC is effective for trace analysis due to high column resolution and high sample sensitivity. However the sensitivity is limited by the sample size which can be injected onto the column and hence the samples must be rigorously purified prior to injection. For plant hormones the sample must also be derivatized. GC–MS–SICM is an extremely accurate and sensitive method[1], but has similar disadvantages as capillary GC. As well, the high cost of GC–MS instrumentation, makes this method out of range for routine analysis.

On the other hand HPLC is effective for both purification and analysis of endogenous hormones[2]. The method has several advantages. Preparative and analytical high-resolution columns are available. Run times are short and the samples usu-

0021-9673/82/0000–0000/$02.75

ally do not require derivatization. Variations in selectivity may be obtained by changes in mobile or stationary phases. Thus the method is applicable to the separation of a wide range of chemically diverse compounds. An added advantage over GC methods is that the sample is not destroyed.

Reversed-phase preparative HPLC is well suited for the purification of plant extracts prior to hormone analysis. Efficiency, resolution and recoveries from the columns are high[2]. Polar compounds are not retained, so that the columns do not deteriorate over a period of time. Reversed-phase HPLC has been used for the purification of extracts for analysis of abscisic acid (AbA)[3–6], 3-indolylacetic acid (IAA)[4,6,7], phaseic acid (PA)[4] and dihydrophaseic acid (DPA)[4] and other hormones. As well, high-performance steric exclusion chromatography was effective in purification of plant extracts for the analysis of IAA[8].

Various methods of analysis of plant hormones by HPLC have been reported. The methods for AbA and IAA include ion exchange[9–12], reversed phase[4,6,7,13,14,24], ion-pair reversed phase[15], partition[3], and normal absorption phase[3,5]. Abscisins and IAA can be detected by UV absorption. More selective and sensitive detection of IAA may be achieved by fluorescence[12,13] or by electrochemical means[12].

We have been studying the effects of drought stress on the levels of endogenous hormones in field grown *Sorghum bicolor*[16,17]. HPLC was found to be effective for the purification and analysis of AbA, PA, DPA, IAA, zeatin and zeatin riboside extracted from leaf tissue[4,18]. As HPLC technology has advanced, we have improved our methods. Presented herein is a rapid and sensitive method for the analysis of AbA, PA and IAA in *Sorghum* leaf tissue.

EXPERIMENTAL

High-performance liquid chromatography

The instrument was a Beckman Model 322 microprocessor controlled HPLC system, including two Model 100A pumps, Model 210 sample injector and Model 420 microprocessor controller. A Model 440 absorbance monitor (Waters Assoc.) detected UV absorption at 254 nm. This detector was coupled in series with a Model 420 fluorescence detector (Waters Assoc.).

All solvents were either HPLC grade or fractionally distilled and filtered through Fluoropore (Millipore), pore size 0.5 μm. In addition, all aqueous solvents were boiled to remove dissolved air.

Extraction and initial purification

The top three leaves from 60-day old plants of *Sorghum bicolor* Moench cv. NK 300 were cut and plunged into liquid nitrogen. After measurement of fresh weight, the tissue was freeze dried and stored at −70°C. The equivalent of 5 g fresh weight (about 1.25 g dry weight) of tissue was extracted for analysis of free and conjugated hormones. The tissue was homogenized in pre-cooled (5°C) methanol–water (80:20; 50 ml), which contained 200 mg/l sodium diethyldithiocarbamate as antioxidant. The homogenized tissue was stirred for 4 h at 5°C, the mixture filtered, and the residue resuspended in methanol–water (80:20) and stirred overnight at 5°C. The mixture was again filtered and the two filtrates combined. The methanol was evaporated *in vacuo* and the aqueous solution at pH 8.0 was centrifuged for 1 h (14,000 g). The supernatant was carefully removed.

To release conjugated hormones, half of the aqueous solution was treated with an equal volume of 0.1 M sodium hydroxide solution at 50°C for 30 min. Each of the treated (total hormone) and untreated (free hormone) samples were adjusted to pH 3.0 and extracted (3 ×) with diethyl ether. The residue from the evaporated ether phase was dissolved in 1 ml methanol–diethyl ether (1:1) and treated dropwise with concentrated ammonium hydroxide solution, with shaking, until the ammonia was in excess (6–8 drops). The solution was evaporated *in vacuo*. Polyvinylpyrrolidone (PVP) powder was sieved (100 mesh size) to remove fines and washed with tap, then distilled water. The PVP was mixed thoroughly with distilled water and the fines decanted after settling for 5 min. When five decantations were complete, columns (8 × 1.5 cm) were prepared in distilled water. The ammonium salts prepared above were dissolved in 1 ml water and chromatographed on the PVP columns. The columns were eluted with distilled water containing sodium diethyldithiocarbamate (200 mg/l). The first 25 ml were collected, adjusted to pH 3.0, extracted (3 ×) with diethyl ether, which was evaporated.

High-performance liquid chromatography of extracts

The partially purified extracts from the PVP treatment were dissolved in water–methanol (1:1; 2 ml) and filtered through Fluoropore, pore size 0.5 μm. The solvent was evaporated. The residue was injected onto a 25 × 1 cm I.D. Beckman Ultrasphere ODS column. The mobile phase was a linear gradient of water–methanol–acetic acid starting with composition (60:40:0.5) and ending with composition (30:70:0.5) over a period of 37 min. The flow-rate was 1.6 ml/min. The sample injection solvent (150 μl) was the same as the starting solvent. Three fractions were collected corresponding to PA, IAA and AbA (Fig. 1). These were evaporated *in vacuo*. After completion of each run, the solvent composition was changed to water–meth-

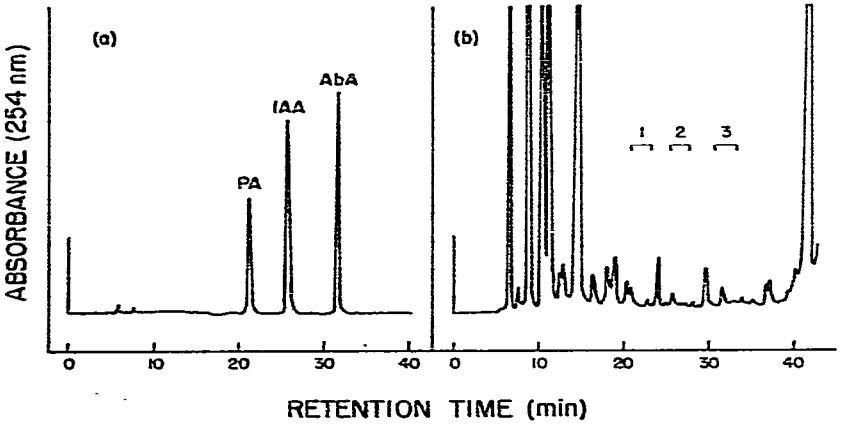


Fig. 1. (a) Separation of AbA, IAA and PA on a reversed-phase preparative HPLC column. Column, 25 × 1 cm I.D. Beckman Ultrasphere ODS; mobile phase, linear gradient over 37 min of water–methanol–acetic acid, starting with composition (60:40:0.5) and ending with composition (30:70:0.5); flow-rate, 1.6 ml/min; detection, UV absorption. *trans,trans*-AbA (not shown) elutes at 29.1 min. (b) Purification of AbA–IAA–PA extract from *Sorghum* leaves on same column and with same conditions as in (a). At the end of gradient, the solvent composition was changed to water–methanol–acetic acid (20:80:0.5) in order to remove impurities still remaining on the column. Three fractions were collected corresponding to the elution of 1 = PA, 2 = IAA and 3 = AbA.

anol–acetic acid (20:80:0.5) and kept for 10 min to remove impurities still remaining on the column. The column required equilibration for 15 min on initial conditions before the next sample was injected.

AbA and PA samples were analysed on a 25 × 0.46 cm I.D. Beckman Ultrasphere Si column. The mobile phase was chloroform–acetonitrile–acetic acid (94:5:1) and flow-rates were 1.0 ml/min for AbA and 1.5 ml/min for PA. Samples were dissolved in 50 μl of mobile phase and either 10 or 20 μl of this solution were injected. Peaks were detected by UV absorption at 254 nm.

IAA samples were analysed on a 25 × 0.46 cm I.D. Beckman Ultrasphere ODS column. The mobile phase was water–acetonitrile–acetic acid (71:28:1) and flow-rate was 1.0 ml/min. Samples were dissolved in 40 μl of the mobile phase and 10 or 20 μl of this solution were injected. Peaks were detected by fluorescence, with excitation at 254 nm and emission at 360 nm using a band pass filter.

The recorder response, in terms of peak height, for both UV absorption and fluorescence (IAA only) was found to be linearly proportional to the amount of hormone injected. Thus hormone levels were estimated by measuring peak heights.

Gas–liquid chromatography

Methyl esters of hormones were prepared as previously described[4]. Glass columns 1.83 m × 2 mm I.D. were packed with 2% OV-1 or 2% QF-1. Helium carrier gas flowed at 50 ml/min and detection was by flame ionization. The retention times at the temperatures indicated were: 2% OV-1: *cis,trans*-AbA methyl ester, 4.8 min (165°C); PA methyl ester, 5.9 min (165°C); and IAA methyl ester, 4.8 min (148°C). 2% QF-1: *cis,trans*-AbA methyl ester, 6.0 min (182°C); PA methyl ester, 6.8 min (182°C); and IAA methyl ester, 5.3 min (150°C).

RESULTS

Batches of 5 g fresh weight of leaves were sufficient for measurement of both free and conjugated AbA, PA and IAA. The antioxidant sodium diethyldithiocarbamate was added to the initial extraction medium and the PVP eluent to reduce oxidation of IAA in aqueous solutions[19]. Efficiency of recovery was improved from 27%, without the antioxidant, to 64%, with the antioxidant.

The extracts were initially purified by treatment with ammonia and chromatography on short PVP columns. The conversion to ammonium salts took about 5 min and the elution of each column with water (25 ml) took about 10 min. The procedure is therefore very rapid, but nevertheless highly effective in reducing extract dry weight (Table I). Acids in the extract form ammonium salts, which rapidly pass through the column as ion pairs. Phenols do not form salts with ammonia and hence are retained on the column. This method, first described for AbA analysis[20] was bound to be more effective in reducing extract dry weight than chromatography of untreated extracts on PVP[21], on Sephadex[4] or on PVP using methanol as eluent[22] (Table I). The PVP method with methanol as eluent was moderately effective in reducing extract dry weight, but did not remove interfering pigments co-chromatographing with AbA and IAA on the HPLC purification column.

After treatment with PVP, the extract was sufficiently pure for reversed-phase preparative HPLC. A high-resolution 25 × 1.0 cm I.D. Beckman Ultrasphere ODS

TABLE I

COMPARISON OF EFFECTIVENESS OF FOUR COLUMN TREATMENTS FOR REDUCING THE DRY WEIGHT OF THE RESIDUE FROM AN ETHER EXTRACT OF *SORGHUM* LEAVES

The ether extract (340 mg) was obtained from 5 g fresh weight (1.25 g dry weight) of leaf tissue (see Experimental). The extract was not hydrolysed. Indicated volume of mobile phase was the retention volume required to elute AbA, PA and IAA in an extract, plus 20% extra mobile phase. Method includes recovery from mobile phase. If the solvent was aqueous, it was extracted at pH 3.0 into diethyl ether (3 ×), and the ether evaporated.

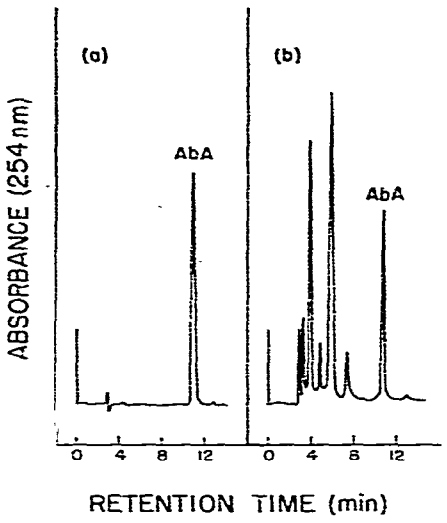
Treatment	*Weight (mg) of extract following treatment (average of duplicates)*
Convert to ammonium salts, 8 × 1.5 cm PVP column, mobile phase: water (25 ml)	61
12 × 1.5 cm PVP column, mobile phase: methanol (50 ml)	89
15 × 1.5 cm PVP column, mobile phase: 0.1 *M* phosphate buffer, pH 7.0 (80 ml)	119
20 × 1.5 cm Sephadex G-10 column mobile phase: 0.25 *M* phosphate buffer, pH 7.0 (150 ml)	129

(C_{18}, 5 μm spherical particles) column was used with a mobile phase of a gradient of acidified water–methanol (Fig. 1). Acetic acid was added to the mobile phase to suppress ionization of the hormones and to minimize the effect of absorption on exposed silica[2]. In this way peak tailing was eliminated. Three fractions were collected, corresponding to the retention volumes of AbA, PA and IAA (Fig. 1). Judging by the UV absorbing peaks, most impurities eluted either before or after the elution of the hormones.

Analysis of AbA and PA was by normal-phase chromatography with a Beckman Ultrasphere Si column (5-μm spherical particles) with a mobile phase of acidified chloroform–acetonitrile. Peaks corresponding to the elution of AbA and PA (Figs. 2 and 3) were well resolved from impurity peaks. Detection was by UV absorption at 254 nm. The detection limit for each hormone was about 1 ng.

Analysis of IAA was by reversed-phase on a Beckman Ultrasphere ODS analytical column)5-μm spherical particles), with mobile phase of acidified water–acetonitrile (Fig. 4). Detection was by fluorescence. The detection limit was about 100 pg.

In order to ensure that peaks were correctly identified and that they did not represent more than one compound, samples corresponding to each hormone peak were collected and examined by gas–liquid chromatography (GLC). The samples were first methylated, and the methyl esters chromatographed on 2% OV-1 and 2% QF-1 GLC columns as previously described[4]. In each case chromatography gave a single peak corresponding to the appropriate standards. Furthermore, by careful comparison of peak areas to those of the standards, the amounts observed on the GLC columns were the same as those observed on the HPLC columns. The overall recovery of the hormones, estimated by spiking five extracts with known amounts of



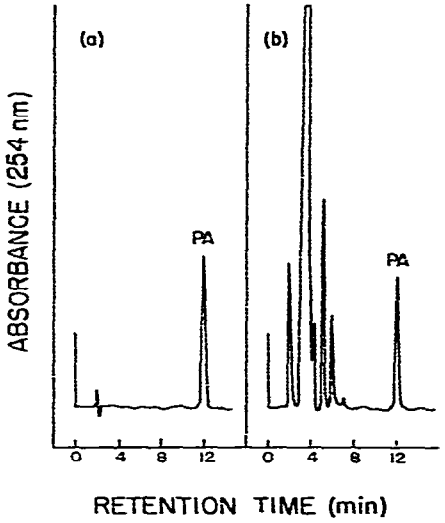


Fig. 2. (a) HPLC of AbA. Column, 25 × 0.46 cm I.D. Beckman Ultrasphere Si; mobile phase, chloroform–acetonitrile–acetic acid (94:5:1); flow-rate 1.0 ml/min; detection, UV absorption. (b) HPLC of residue recovered from fraction 3 (Fig. 1) on same column and with same conditions as in (a).

Fig. 3. (a) HPLC of PA. Column, 25 × 0.46 cm I.D. Beckman Ultrasphere Si; mobile phase, chloroform–acetonitrile–acetic acid (94:5:1); flow-rate 1.5 ml/min; detection, UV absorption. (b) HPLC of residue from fraction 1 (Fig. 1) on same column and with same conditions as in (a).

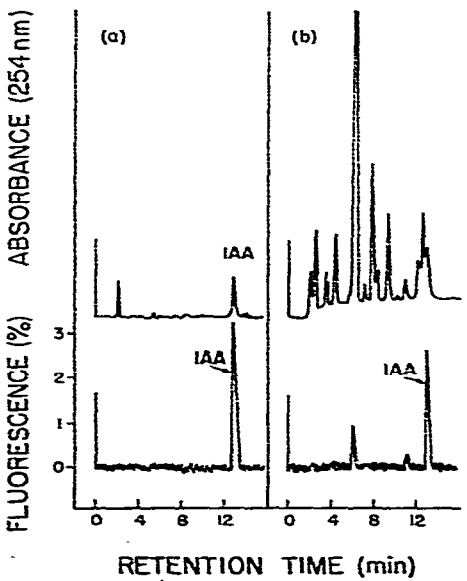


Fig. 4. (a) HPLC of IAA. Column, 25 × 0.46 cm I.D. Beckman Ultrasphere ODS; mobile phase, water–acetonitrile–acetic acid (71:28:1); flow-rate, 1 ml/min; detection, fluorescence, with excitation at 254 nm and emission at 360 nm using a band pass filter. (b); HPLC of residue from fraction 2 (Fig. 1) on same column and with same conditions as in (a).

hormones (and in similar amounts to those found in the tissue) were 75 ± 3% for both abscisins and 64 ± 3% for IAA.

Quantities of hormones (adjusted for extraction losses) in the top three leaves of 60-day old plants are given in Table II. Conjugated hormones, defined as those hormones which were released by 0.1 *M* sodium hydroxide solution at 50°C, were also estimated. These hydrolysed extracts had large dry weights, presumably due to the release of many other compounds by the treatment. In these extracts, UV-absorbing peaks were 2–5 times larger than those of unhydrolysed extracts. The increased dry weight did not affect the performance of the columns or the separation of the hormones from impurities. IAA conjugates were present in much larger amounts than free IAA (Table II and ref. 23).

TABLE II

LEVELS OF HORMONES IN THE TOP THREE LEAVES OF 60-DAY OLD PLANTS OF *SORGHUM BICOLOR* CV. NK300

Values averaged from five extracts. Values for conjugated hormones were calculated by subtracting free hormone from total hormone.

Hormone	*Free (ng/g fresh weight)*	*Conjugated (ng/g fresh weight)*
AbA	65.1 ± 3.0	58.8 ± 4.1
IAA	26.5 ± 2.4	164 ± 21
PA	16.5 ± 1.3	9.9 ± 1.6

DISCUSSION

The HPLC analysis of AbA, PA and IAA reported herein is rapid, efficient and sensitive. Although two purification steps are required, these are relatively simple and could be automated. Thus large numbers of samples could be processed.

The combination of chromatographing the extracted hormones first as ammonium salts on a short PVP column and then as free acids on a preparative HPLC reversed-phase column proved effective for purification. The initial PVP treatment was required before the extract could be chromatographed on HPLC. We have had no success in chromatographing crude extracts directly on high performance columns due to contamination effects and loss of resolution. Preparative HPLC is most effective when peak resolution is high and narrow band collections are made. In a recent report[6] a high degree of purification of hormone extracts from *Solanum tuberosum* leaf and root tissue was claimed by repeated use of PVP and μBondapak C_{18}. In our hands this method tended to concentrate impurities in the hormone zones, rather than separate impurities from hormones.

Preparative HPLC was by reversed-phase, whereas analytical HPLC for AbA and PA was by adsorption phase. These contrasting systems enabled AbA and PA to be effectively resolved from UV-absorbing impurities. For IAA, both purification and analysis were by reversed-phase C_{18} columns. However the mobile phase for the preparative column was acidified methanol–water, whereas for the analytical column it was acidified acetonitrile–water. Thus the two systems had different selectivities,

enabling IAA to be separated from interfering impurities. As well, fluorescence detection was found to be more selective than UV absorption at 254 nm (Fig. 4). Since IAA has a low extinction at 254 nm, fluorescence detection was found to be more sensitive.

An important criterion of trace analysis is that the analytical chromatograms should contain few peaks, and that the hormones should be well resolved from impurities. A large impurity peak close to a hormone peak can distort the latter making quantitation inaccurate. In the present work, excellent separation of AbA and PA peaks from impurity peaks was observed with UV detection at 254 nm. For IAA, however, it was necessary to use a selective fluorescence detector.

ACKNOWLEDGEMENTS

The authors thank Mr. Peter Tse, Mr. Gordon Sookocheff, Mrs. Janet Wanner and Mrs. Jane Montgomery for skilled technical assistance. Professor J. A. D. Zeevaart (Michigan State University) is thanked for generously supplying us with samples of phaseic acid and dihydrophaseic acid. This research was funded by a grant from the International Development Research Centre, Ottawa.

REFERENCES

1 J. McDougall and J. R. Hillman, in J. R. Hillman (Editor), *Isolation of Plant Growth Substances*, Cambridge Univ. Press, Cambridge, 1978, p. 1.
2 M. L. Brenner, in N. B. Mandava (Editor), *Advances in Analitycal Methods for Plant Growth Substances (ACS Symposium Series 111, Plant Growth Substances)*, American Chemical Society, Washington, DC, 1979, p. 215.
3 A. J. Ciha, M. L. Brenner and W. A. Brun, *Plant Physiol.*, 59 (1977) 821.
4 R. C. Durley, T. Kannangara and G. M. Simpson, *Can. J. Bot.*, 56 (1978) 157.
5 N. L. Cargile, R. Borchert and J. D. McChesney, *Anal. Biochem.*, 97 (1979) 331.
6 R. N. Arteca, B. W. Poovaiah and O. E. Smith, *Plant Physiol.*, 65 (1980) 1216.
7 M. Wurst, Z. Přikryl and V. Vančura, *J. Chromatogr.*, 191 (1980) 129.
8 A. Crozier, J. B. Zaerr and R. O. Morris, *J. Chromatogr.*, 198 (1980) 57.
9 H. Düring, *Experimentia*, 33 (1977) 1666.
10 B. Quebedeaux, P. B. Sweetser and J. C. Rowell, *Plant Physiol.*, 58 (1976) 363.
11 P. B. Sweetser and A. Vatvars, *Anal. Biochem.*, 71 (1976) 68.
12 P. B. Sweetser and D. G. Swartzfager, *Plant Physiol.*, 61 (1978) 254.
13 A. Crozier, K. Loferski, J. B. Zaerr and R. O. Morris, *Planta*, 150 (1980) 366.
14 V. Sjut, *J. Chromatogr.*, 209 (1981) 107.
15 D. M. A. Mousdale, *J. Chromatogr.*, 209 (1981) 489.
16 G. M. Simpson, R. C. Durley and T. Kannangara, in T. K. Scott (Editor), *The Problem of Plant Breeders, Plant Regulation and World Agriculture*, Plenum, New York, 1979, p. 111.
17 T. Kannangara, R. C. Durley and G. M. Simpson, *Can. J. Plant Sci.*, 62 (1982) in press.
18 T. Kannangara, R. C. Durley and G. M. Simpson, *Physiol. Plant.*, 44 (1978) 295.
19 J. D. Mann and E. G. Jaworski, *Planta*, 92 (1970) 285.
20 J. R. Lenton, V. M. Perry and P. F. Saunders, *Planta*, 96 (1971) 271.
21 J. L. Glenn, C. C. Kuo, R. C. Durley and R. P. Pharis, *Phytochemistry*, 11 (1972) 345.
22 D. M. A. Mousdale and M. Knee, *J. Chromatogr.*, 177 (1979) 398.
23 T. Kannangara, R. C. Durley and G. M. Simpson, unpublished results.
24 J. R. Dunlap, A. H. Hanks and P. W. Morgan, *Crop Sci.*, 21 (1981) 82.