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A study of the analysis of abscisic acid from broad bean (Vicia faba L.) plants

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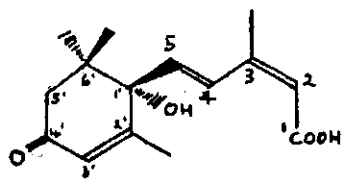
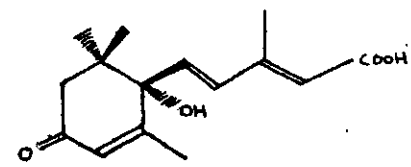
CONTENTS

INTRODUCTION

Abscisic acid (ABA) is considered to be an important endogenous regulator of plant growth and development. First isolated and characterised nearly twenty years ago by three separate research groups (Ohkuma *et.al.*,1963; Rothwell and Wain, 1964; Cornforth *et.al.*, 1965), it has been detected in almost all higher plant tissues but is often found in highest concentration in reproductive structures, such as buds, fruits and seeds. Abscisic acid has two isomeric forms, the 2-cis (cis-ABA) and the 2-trans (trans-ABA) configurations, as follows:



2-cis-ABA



2-trans ABA

Cis-ABA usually occurs in greatest abundance in plant tissues and has been found to be the only active isomer in many of the characteristic effects of ABA on plants. In addition to the free acid, ABA often exists in plants as a relatively minor 'bound', or glucosidic, form which may be a storage product of the acid (Milborrow, 1974).

In common with other classes of plant growth regulators, ABA has several possible functions in plant growth, development and physiology, including seed development and germination, dormancy of buds and seeds, ion movement, the abscission and senescence of various plant organs, and in the response to various environmental stresses such as salinity, mineral deficiency, water-logging and drought (for reviews see Milborrow, 1974; Zeevaart, 1979; Walton, 1980). These different modes of action of ABA are perhaps reflected by the way in which the concentration of ABA varies in plants, namely (1) the concentration of ABA often changes with the developmental stage of plant organs indicating that it may control normal plant growth and development, and (2) ABA may act as an intermediary in the response of plant function to changes in environmental conditions.

An example of (1) has been found with many seeds, such as wheat (King, 1976), broad bean (Grabner *et. al.*, 1980), kidney bean (Van Onckelen *et. al.*, 1980), soybean (Quebedeaux *et. al.*, 1976) and several others in which the concentration of ABA increases prior to, or during, the most active growth phase but then

declines during subsequent stages of seed maturation. In this case, ABA probably prevents premature germination and enzymic activity of the developing seeds (King, 1976; Van Onckelen *et. al.*, 1980) by inhibiting giberellin-induced mRNA synthesis (Chrispeels and Varner, 1966; Varner and Ho, 1976).

A clear and well-documented example where the ABA level in plants responds to environmental stimulation is seen in plants subjected to drought stress (for reviews see Milborrow, 1974; Zeevaart, 1979; Walton, 1980). Application of ABA to turgid leaves leads to a rapid and reversible closure of the stomata and reduced transpiration. In addition, when plants begin to wilt, there is a very rapid accumulation of ABA (up to 40 fold compared to turgid tissue) in the leaves and other organs within a matter of hours. This accumulation is followed by a decline in the levels of ABA after the drought stress is relieved. It appears that turgor is the critical component of water potential that determines the change in ABA levels in response to drought (Davies and Lakso, 1978; Pierce and Raschke, 1980), which may indicate that a change in pressure on cell membranes triggers the rise in ABA levels in drought-stressed plants.

One of the pre-requisites for confirming the regulatory role of natural plant growth substances, including ABA, in plant growth and development is the determination of their endogenous concentration or activity. Abscisic acid is probably more amenable to measurement than most other plant growth regulators because it is relatively stable, has good electron-capturing properties and has only a single known active species. However, ABA is generally found at low concentrations in plant tissues and has to be exhaustively purified before analysis; this fact has probably slowed down the rate of progress in our knowledge of the functions of ABA in plants.

Current methods for the analysis of ABA from plant tissues involve three main stages, namely extraction, purification and estimation, and each of these stages will be briefly outlined.

Extraction:

Frozen or freeze-dried material is exhaustively extracted, usually with 80% or 100% methanol followed by filtration or centrifugation. An internal standard of either radio-labelled or 'cold' ABA is added during extraction for estimating final recoveries, the assumption being that the internal standard equilibrates with the extracted endogenous ABA. If a 'cold' internal standard is used, either a pure trans-ABA standard may be added (since endogenous trans-ABA is usually absent or very low in relation to cis-ABA)(Lenton *et. al.*, 1971) or a mixed isomer standard

is added to a duplicate sample (Knegt *et. al.*, personal communication).

Purification:

Conventional acid-base ether fractionation is usually used in conjunction with other purification techniques, such as Sep-Pak cartridges to remove much of the solid matter and pigments, PVP (polyvinylpyrrolidone) columns for removing phenolics, and finally electrophoresis (Coombe and Hale, 1973), gas-liquid (Davies *et. al.*, 1968), thin-layer (Milborrow, 1972), silica gel column (Asmundson *et. al.*, 1969), Sephadex (Steen and Eliasson, 1969) or high pressure liquid (eg. Ciha *et. al.*, 1977) chromatography as final clean-up procedures. When small amounts of tissue are used, it is often possible to omit the final clean-up methods (Hubick and Reid, 1980; Knegt *et. al.*, personal communication), particularly when the tissue contains a high ABA concentration or when a highly-resolving GC capillary column is used in the final analysis.

Estimation:

Either GC or bioassays are usually used for ABA estimation but the former is now more widely employed because of its greater specificity. The purified plant samples are first methylated before injection. Conventional GC columns are often used but capillary columns are nowadays becoming more popular because of their high resolving capabilities. In addition, dry injection systems are considered superior to conventional solvent injections because the column life is extended and no interfering solvent peak is produced. When an internal standard of radio-labelled ABA is used, the radioactivity in part of the methylated plant sample is determined for the estimation of recovery efficiency. With 'cold' ABA internal standards, endogenous levels are calculated by comparison of the areas of the cis and trans peaks (for internal trans-ABA standards) or between unfortified and fortified duplicate plant samples (Lenton *et. al.*, 1971; Knegt *et. al.*, personal communication).

This report presents the results of a study which had the following aims and rationale:

(a) *Development of a suitable method for the analysis of ABA from different tissues of Vicia faba L. plants.*

Although various methods have been devised for the analysis of ABA from different plant tissues, it is often found necessary to modify them if they are used for tissues other than those for which they were developed. In addition, the method should be applicable to different tissues or physiological states of the same tissue if true comparisons are to be made. Finally, the method used should be as rapid as possible so that many plant samples can be routinely analysed with minimal

chemical conversion of the extracted ABA.

(b) *Comparison of the effect, if any, of irrigated and non-irrigated conditions on the ABA levels in various tissues of field-grown* Vicia faba *L. plants.*

Previous reports (eg. Sivakumaran and Hall, 1978; Davies and Lakso, 1978; Xiloyannis *et. al.*, 1980; Düring and Broquedis, 1980) show that the ABA levels of various crop plants change in response to drought stress, both diurnally and seasonally, and depend on the developmental stage of the plant or plant part. Drought may thus produce wide-ranging effects on the growth and development of crops, via an effect on their ABA levels, including seed set (Morgan, 1980) and photosynthesis (Loveys and Kriedemann, 1974), according to the developmental or seasonal stage at which it occurs. Consequently, information regarding the response of the ABA content of different tissues of crop plants to different water regimes under field conditions may help to rationalize the optimum levels of irrigation or drainage for producing maximum growth rates and yields.

Because of the limited time that was available for this project and due to the technical problems encountered, most of this report deals with aim (a), but a few general conclusions could be drawn from the limited results of aim (b).

GENERAL METHODS

(a) *Chemicals*

All the chemicals used were of Analar or Nanograde purity.

(b) *Growth, collection and storage of plant samples*

Broad bean (*Vicia faba* L. cv. Minica) seeds were sown in a set of three field plots (A,B and C) at the Centre for Agrobiological Research on March 6, 1980. The planting density was 18 plants m^{-2} with overhead roof protection. The plots were fertilized with 15 Kg ha^{-1} of K and 25 Kg ha^{-1} polyphosphate and irrigation was given from below ground level. In plot A, irrigation was terminated on June 2 (during flowering) and plant specimens collected on June 25. In plot B, irrigation was terminated on July 7 (pod-filling stage) and plant specimens collected at intervals up to July 29. The experiment was finalized on August 26 when abscission of almost all the leaves had occurred. For the collection dates and times see Table 5.

The harvested plants were quickly divided into various parts (excluding the roots), wrapped in cheesecloth and immediately frozen in liquid nitrogen. The samples were then stored in polyethylene bags and kept at -20°C prior to analysis. In order to obtain a representative sample of each tissue type from single plants, for most experiments the tissues were freeze-dried for 4 days and then ground to a powder in a IKA analysis mill A 10 (Janke and Kunkel KG, IKA Werk Staufen i Breisgau FRG). A fraction of the powder was then used for each analysis.

(c) *Gas chromatographic analysis of ABA*

Methylation of the samples:

Diazomethane in diethylether was prepared by the method of Schlenk and Gellerman (1960). To 0.132 or 0.25 g of N-nitrosomethylurea were added 0.5 ml distilled water, followed by 0.6 ml 5N NaOH and the resulting diazomethane gas was collected in 3 ml ice-cold diethylether. The diazomethane solution (0.2 ml) was added to 100 µl of sample in methanol contained in 300 µl capacity Reacti-vials fitted with valved caps. The mixture was left at room temperature for 1 h; persistence of the yellow colour of the added reagent was used as an indication of the presence of excess diazomethane required for methylation. After the reaction period, the sample was dried with N_2 gas and re-dissolved in either 100% cyclohexane or 500:1 cyclohexane:methanol prior to gas chromatography.

Gas chromatography:

A Becker 420 Research Gas Chromatograph was used in conjunction with a Kipp and Zonen BD9 twin-channel potentiometric recorder. Except where indicated in the text, the following conditions were used: *Column packing*, 4% OV-1 on 80/100 Gas Chrom Q; *dimensions*, 1.8 m x 1.9 mm; *carrier gas*, high purity N_2 at 32 cm^3 min^{-1};

column temp., 210°C; *ECD (^{63}Ni-foil) temp.*, 300°C; *injection port temp.*, 220°C; *pulse period*, 200 μsec; *pulse width*, 1 μsec., *attenuation*, 1; *recorder conditions*, 1 mv, 10 mm min^{-1}.

Samples of up to 1 μl were injected and ABA quantities in the peaks were calculated by comparison of the peak areas (height x width at half height) with a standard curve relating peak areas with different dilutions of 1 ng μl^{-1} authentic standard ABA (mixed isomers in ratio of 45% cis and 55% trans ABA) in cyclohexane. Aliquots of the methylated samples in cyclohexane were counted by liquid scintillation for determination of the recovery efficiences of the authentic ^{14}C-ABA internal standard added during extraction of the plant samples.

TRIALS OF METHODS FOR THE ANALYSIS OF ABA FROM *VICIA FABA* PLANTS

The following is an account of trials which were carried out in an attempt to develop a suitable method for the analysis of ABA from various tissues of *Vicia faba* L. plants. Except where indicated, the efficacy of each method for samples of leaves, stems, pod walls and seeds was tested. However, in most cases, only the results using leaves are presented as this was found to be the most difficult tissue to purify and hence gave the best indication of the suitability of the method for general use. The source of the plant material in each experiment is given by the plant number (see Table 5).

METHOD 1 (Ribôt, personal communication)

Plant material:

Plant 1 - leaves, 8.5 gFW (1.3 gDW)
- pod walls, 14.2 gFW (1.7 gDW)
- seeds, 1.9 gFW (0.3 gDW)

Plant 2 - pod walls, 22.5 gFW (2.2 gDW)
- seeds, 3.7 gFW (0.6 gDW)

Tissue extraction:

Tissue samples frozen at -20°C were extruded through a French press into 100% (v/v) aqueous methanol to give a final ratio of 10:1 (v/w) solvent:tissue gFW. After centrifugation at 3000 $g_{av.}$ for 10 min in a BHG Optima bench centrifuge at room temperature, the residue was extracted twice further with 100 ml of 100% methanol in a Virtis '23' omnimixer for 1 min. During this further extraction, a ^{14}C-ABA internal standard was added to give 6600 dpm (specific activity, 13.8 dpm per ng of pure ABA, containing 62.12% trans-ABA, 11.37% cis-ABA and 26.50% impurity). The combined methanolic supernatants were evaporated to dryness in a rotary evaporator at 40°C and then taken up in 13 ml of 0.1 M phosphate buffer, pH 8.0.

Purification of the samples:

(a) Solvent fractionation:

The buffer fraction was extracted with 3 x 10 ml portions of petroleum ether to remove pigments and the pet ether fractions were discarded. The aqueous residue was adjusted to pH 2.5 and extracted with 3 x 10 ml aliquots of diethylether; the aqueous residue was discarded. The ether fraction was evaporated to 10 ml and extracted with 3 x 10 ml portions of 5% (w/v) $NaHCO_3$; the ether fractions were discarded. The $NaHCO_3$ fraction was carefully adjusted to pH 2.5 with 6N HCl and fractionated against 3 x 10 ml portions of diethylether; the aqueous residue was discarded and the final ether fractions evaporated to dryness with high purity N_2 gas.

(b) Preliminary thin-layer chromatography (TLC):
The dried samples from the last ether extractions were reconstituted in 85% aqueous ethanol and layered as 3 cm wide strips on Merck TLC plates (20 x 20 cm, 0.5 mm thick Kieselgel 60 F_{254}), which had been pre-washed with 96% aqueous ethanol and activated at 110°C for 30 min. The strips were concentrated three times in methanol up to a second origin line, 1.5 cm from the first one. After the plates had been developed in chloroform:methanol:water (75:22:3), they were dried, visualized under UV light and scanned with a Dünnschicht-Scanner II (Berthold s.a. Benelux Analytical Instruments n.v., model LB 2723) radiochromatogram scanner to locate the regions containing ^{14}C-ABA.

(c) Second TLC:
The areas containing the ^{14}C-ABA were scraped from the plates and eluted with 3 x 5 ml aliquots of nanograde methanol. After concentrating the eluates with N_2 gas, a second TLC was carried out with benzene:acetic acid (50:20) as the developing solvent.

Following the second TLC purification, the regions containing the ^{14}C-ABA were eluted, the eluates concentrated and the sample methylated prior to GC analysis.

Results and discussion

The GC profile for the leaf sample of Plant 1 after purification by Method 1 is shown in Fig. 1. The results show a reasonably well-resolved peak with a retention time identical to that of cis-ABA in the authentic standard. Similarly clear peaks were found for cis-ABA in the samples purified from the pod walls and seeds of Plants 1 and 2. However, the elevated baseline of the GC trace indicated the presence of considerable quantities of impurities which increase the possibility of interfering peaks; this was evident with the trans-ABA peaks.

This method proved suitable for the extraction and analysis of ABA from *Vicia faba* tissues but was too time-consuming for routine use with large numbers of plant samples. In addition, a problem of low recovery efficiency was found with this method (Table 1). These results clearly show an apparent loss of ^{14}C-ABA either during or following methylation but the recoveries were high prior to methylation. This problem recurred in later analyses when TLC was used to purify the samples and was found to be caused by silica gel contaminants remaining in the samples after TLC. Further discussion of this problem is given in the section entitled 'A Study of the Methylation of ABA for GC analysis'.

The following experiments were conducted in order to improve the basic routine method for the analysis of ABA from *Vicia faba*. The main objective was to eliminate the need for the TLC purification steps which were time-consuming and also introduced silica contaminants which lowered the recovery efficiencies of the ^{14}C-ABA standard.

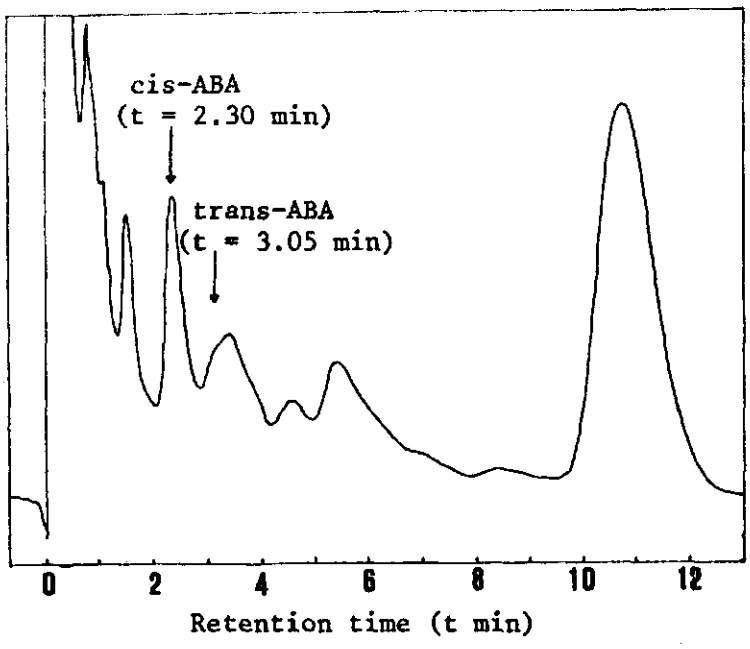


Fig.1. GC trace of an extract from *Vicia faba* leaves (Plant 1) after purification by Method 1.
Injection occurred at time 0 min and the cis- and trans-ABA peaks were identified by comparison with a methylated standard ABA sample.

Table 1. Recovery efficiencies with extracts from different tissues of *Vicia faba* (Plant 1) after purification by Method 1.

Plant no.	Tissue	Percentage recovery of added ^{14}C-ABA	
		Prior to methylation	Immediately after methylation
1	leaves	57	6
1	pod walls	69	9
1	seeds	65	7
2	pod walls	57	15
2	seeds	48	6

METHOD 2

An attempt was made in this method to replace the TLC steps with a purification method involving Sep-Pak cartridges (Waters Associates Inc.). Two types of Sep-Pak were used:

(a) C_{18} Sep-Paks: the sample, dissolved in 80% methanol is loaded onto the cartridge; polar compounds (eg. ABA) are eluted immediately with the first elution and relatively non-polar substances are absorbed by the cartridge.

(b) Silica Sep-Paks: the sample is loaded onto the cartridge in a relatively non-polar solvent (eg. methylene chloride) and the cartridges are then eluted with a series of solvents of increasing polarity. The more apolar contaminants are removed in the first elutions, followed by the desired polar compounds which are eluted with the more polar solvent elutions.

In these trials, the silica Sep-Paks were used with leaf samples while both types of cartridge were tested with seed tissue.

Plant material:

Plant 3 - leaves, 11.6 gFW (1.7 gDW)
- seeds, 1.6 gFW (0.2 gDW)

Procedures

Tissue extraction and solvent fractionations:

As described for Method 1.

Purification with Sep-Pak cartridges:

(a) Leaf extract:

The dried sample after ether fractionation was suspended in 7 ml of methylene chloride and loaded onto a silica Sep-Pak cartridge. The cartridge was then eluted with 5 ml aliquots of a series of solvents of increasing polarity according to the method of Hubick and Reid (1980) (see Fig. 2). The eluates containing 4% and 10% methanol in methylene chloride contained most of the radioactivity and so these were collected, evaporated to dryness, methylated and analysed by GC.

Results

The elution profile of the recovery of the internal standard radioactivity from the silica Sep-Pak is shown in Fig. 2. The total recovery of radioactivity after this method was 72% but the GC traces showed that the samples were insufficiently purified for analysis (Fig. 3). A second purification of the methylated sample was carried out with another silica Sep-Pak and the 100% methylene chloride fraction collected. However, no additional purification was obtained.

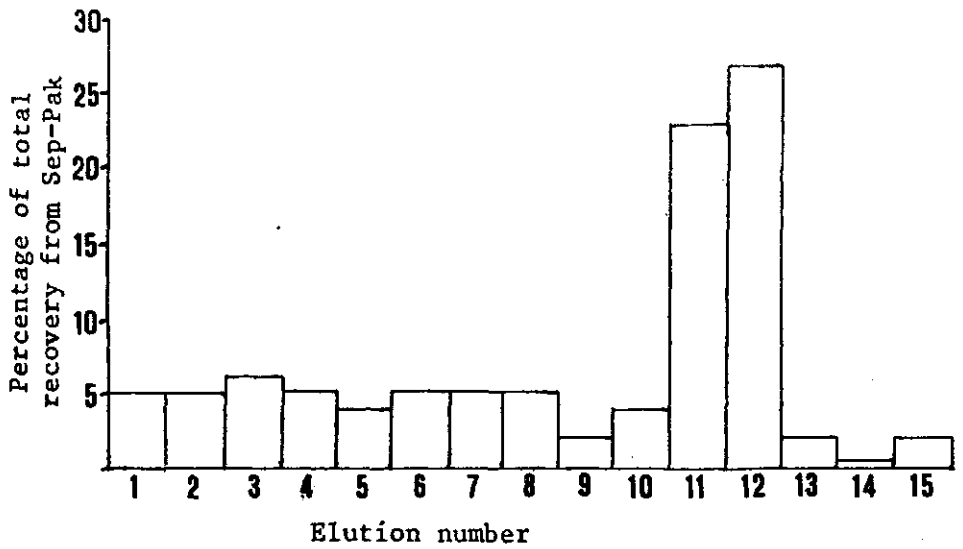


Fig. 2. Elution profile of the internal ^{14}C-ABA standard radioactivity from a silica Sep-Pak during purification of the leaf extract from Plant 3 by Method 2.
The following solvents dissolved in methylene chloride were used for the elutions: 1-7, methylene chloride; 8, 5% diethylether; 9, 5% ethyl acetate; 10, 5% acetone; 11, 4% methanol; 12, 10% methanol; 13, 20% methanol; 14, 60% methanol; 15, 100% methanol.

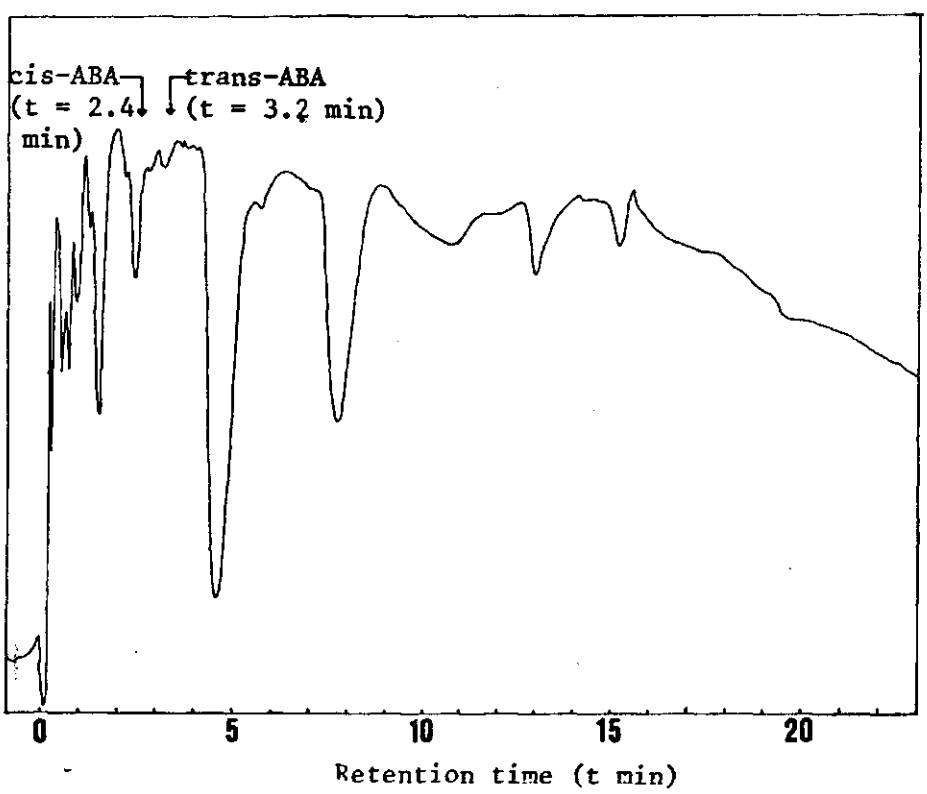


Fig. 3. GC trace of a leaf extract from Plant 3 after purification by Method 2(a).
Injection occurred at time 0 and the arrows indicate the retention times of cis- and trans-ABA obtained from an injection of standard methylated ABA.

(b) Seed extract:

The sample was first purified with a silica Sep-Pak cartridge, as described for the leaf material, and the eluates containing most of the radioactivity were collected and evaporated to dryness with N_2 gas. The dried samples were reconstituted in 80% methanol and injected through a C_{18} Sep-Pak cartridge which had been pre-wetted with 80% methanol. Most of the radioactivity from the internal standard passed through the cartridge during the sample injection with very little remaining in the cartridge. The radioactive fractions were collected for subsequent methylation and GC analysis.

Results

The GC profile of the seed sample using this method is shown in Fig. 4.

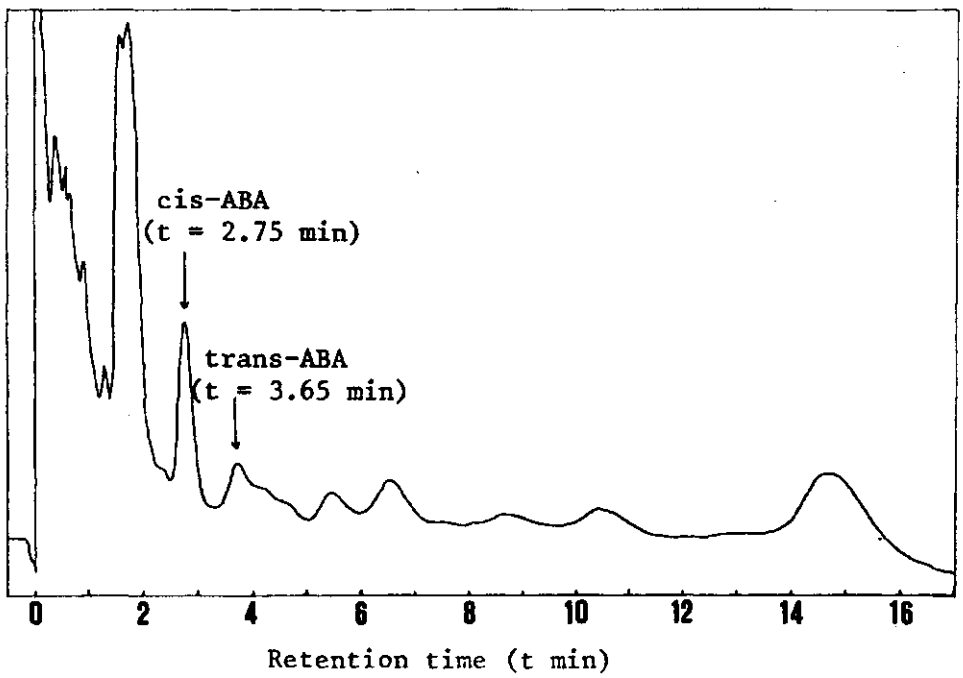


Fig. 4. GC trace of a seed extract from Plant 3 after purification by Method 2(b).
Injection occurred at time 0 and the arrows indicate the retention times of cis- and trans-ABA obtained from an injection of standard methylated ABA.

The GC profile shows that this method has potential for the analysis of ABA from seed samples of *Vicia faba*. A peak corresponding to the retention time of cis-ABA was sufficiently well-resolved for the calculation of the quantity of this isomer, although the trans-ABA peak was contaminated with impurities. This method, however, is unlikely to be suitable for use with tissues containing high levels of impurities, coupled with relatively low ABA concentrations, such as leaves, stems and pod walls.

METHOD 3

The method of Knegt *et. al.* (personal comm.) shows promise as being a simple and rapid routine method for small quantities of tissue (0.5 - 5.0 gFW). However, the method involves the use of a capillary GC column which gives very fine resolution of the ABA peaks during analysis. Consequently, leaf tissue was used in the following experiments in order to determine if the method is suitable for tissues with a high level of impurities, such as phenolics and pigments, in conjunction with a conventional GC column.

Plant material:

Plant 19 - freeze-dried, powdered leaf material, 0.26 gDW (1.7 gFW equivalent).

Procedure

To the leaf powder were added 20 ml 80% methanol and the suspension left to digest for 15 h at 4°C. The suspension was then filtered through a 75 ml capacity G4 sintered glass filter and the residue re-extracted twice, each time with 15 ml 80% methanol. Each of the last two extractions involved a 2 min homogenization with a Virtis '23' omnimixer and a 3 min sonication in a Megason ultrasonic bath (Ultrasonic Instruments International).

The following procedure was carried out by Dr. E. Knegt of the Agricultural University, Wageningen, as a demonstration of his method. The combined filtrates were divided into equal halves and to one of the duplicate samples was added an internal standard consisting of a 1 ml solution of 1.025 µg cm^{-3} of the mixed isomers of ABA (31.2% cis and 68.8% trans). Each of the duplicate samples was treated separately as follows. The samples were pressed through two pre-wetted C_{18} Sep-Pak cartridges in series with a 60 ml Plasti-Pak plastic syringe and the filtrates evaporated *in vacuo* at 30°C to approx. 5 ml of aqueous residue. The residues were taken up in 10 ml of 0.1 M K_2HPO_4 and pressed through a small column of 0.5 g PVP (Polyclar AT sieved to 35-100 mesh) in a 2 ml disposable plastic syringe, using a 60 ml plastic syringe as the reservoir. The resulting aqueous fraction was extracted once with 10 ml of diethylether and then the pH adjusted to 3.0 with 0.57 M H_3PO_4 and extracted once again with 10 ml diethylether. The ether fraction was further extracted with 10 ml 0.1 M K_2HPO_4 which, after adjusting to pH 3.0, was extracted again with 10 ml diethylether. Finally, the diethylether fraction was extracted once again with 0.1 M K_2HPO_4 which, after adjusting to pH 3.0, was extracted with ether. The resultant ether fraction was evaporated to dryness *in vacuo* at room temperature and the residue methylated prior to GC analysis. GC analysis was compared using two systems, namely (a) the capillary and dry injection system used by Knegt *et. al.*, and (b) the conventional column and wet injection system as described in the section entitled 'General Methods'.

The conditions for the capillary system using a Tracor 560 gas chromatograph were as follows: *column*, 25 m Scot, coated with SE-30; *carrier gas*, 5% CH_4 in Ar; *column temp.*, 200°C; *detector*, ^{63}Ni foil ECD; *injector*, all glass-coated system from Chrompack.

Results and Discussion

The GC profiles for the leaf extract of Plant 19 after purification by the method of Knegt *et. al.* and analysed with a capillary GC column and dry injection system are shown in Fig. 5. The standard solution of mixed isomers used for fortifying one of the duplicate plant samples showed a peak area ratio of 94.9:43.0 trans:cis ABA (Fig. 5A), whereas the comparable peak area ratio for the unfortified duplicate sample was 33.8:135.3 (Fig. 5B). The peak area ratio for the fortified duplicate was 146:32 (Fig. 5C). This latter ratio for the fortified sample was unexpectedly high and the reason for this is unclear but it means that the concentrations of endogenous trans-and cis-ABA cannot be calculated from these results using the formulae of Knegt *et. al.* and the experiment needs to be repeated. The total ABA concentration in this tissue was found by another method (Method 4) to be 704 ng per gDW (see Table 5). However, the results do show that this method has good potential for the analysis of ABA from the leaves, and probably other tissues, of *Vicia faba* using the conditions stipulated by Knegt *et. al.* The method was also found to be adequate for leaf samples from glasshouse-grown *Vicia faba* plants where the ABA concentration was very low (approx. 2 ng per g FW).

Using the conventional GC system, the standard (Fig. 6A) and fortified plant (Fig. 6C) samples showed well-resolved cis- and trans-ABA peaks but, in the unfortified plant sample (Fig. 6B), the cis-ABA peak was not resolved from impurities. Consequently, this method was not suitable for leaf samples of *Vicia faba* in conjunction with the conventional GC system. Several other attempts to use this method were made with leaf material from other plant samples of *Vicia faba* but in every case insufficiently clean samples were obtained for use with the conventional GC system described here. However, the method was found to work well with seed samples where the ABA concentration is relatively high and the level of impurities relatively low.

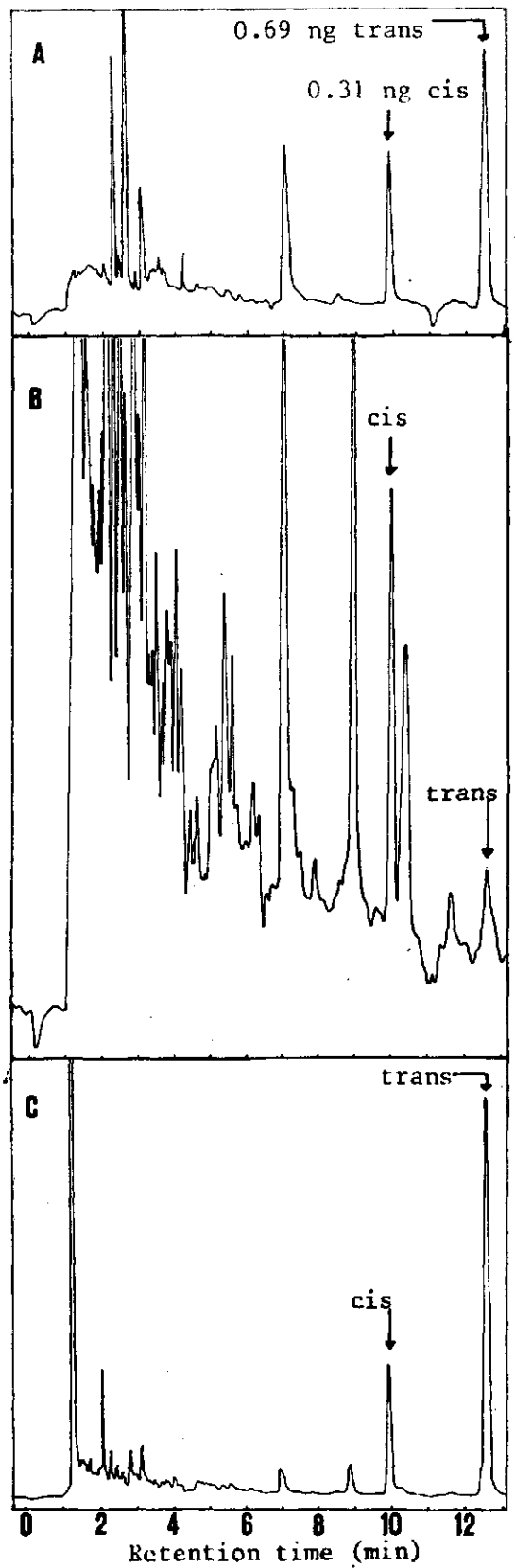


Fig. 5. GC profiles for the leaf sample of Plant 19 after purification by Method 3 and analysis with a capillary GC column and dry injection system.

A, standard ABA (attenuation 10); B, unfortified leaf sample (attenuation 5; C, leaf sample fortified with the standard ABA sample during extraction (attenuation 100). Injection occurred at time 0 and the cis- and trans-ABA peaks are indicated with arrows.

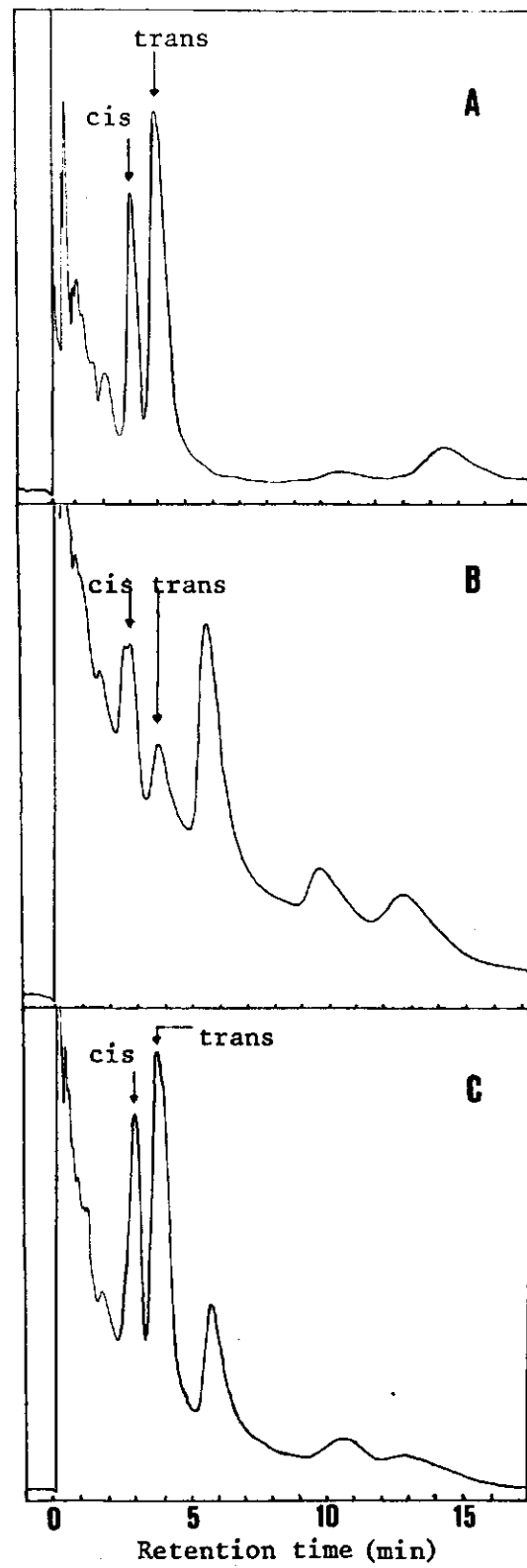


Fig. 6. GC profile of a leaf sample from Plant 19 after purification by Method 3 and analysed with a conventional (non-capillary) GC system.

The GC conditions are described in the section entitled 'General Methods'. A, standard ABA sample; B, unfortified leaf sample; C, leaf sample fortified with the standard ABA solution during extraction. Injection occurred at time 0 and the cis- and trans-ABA peaks are indicated by arrows.

METHOD 4

Method 3 produced insufficiently clean samples for the analysis of ABA from leaf tissue of *Vicia faba* when a conventional GC system was used. In the absence of a capillary GC system, therefore, an alternative method was investigated which involves the method of Knegt *et. al.* but which has an additional TLC purification step. The ratio of the ABA peak to baseline heights were increased with this method in two ways:

(a) The ABA peak heights were increased by using a larger quantity of tissue than that used in Method 3. In addition, these larger amounts of tissue compensated to some extent for the losses which were found to occur after TLC purification.

(b) The high baseline:peak ratio caused by the large quantity of impurities left after purification by Method 3 was reduced by the TLC step.

Plant material:

Freeze-dried tissue powder from Plant 19:

leaves - 23.2 gFW (3.5 gDW)

stem - 26.3 gFW (5.0 gDW)

pod walls - 35.5 gFW (5.0 gDW)

seeds - 12.0 gFW (3.5 gDW)

Procedure

The plant material was first extracted and partially purified according to Method 3. In the extraction, a final 80% methanol to tissue DW ratio of 100:1 was maintained. The first extraction involved half the solvent volume and at this point the internal standard of ^{14}C-ABA (5,500 dpm of cis-ABA, with a specific activity of 100 dpm per ng of ABA) was added. The other two extractions were with one quarter each of the solvent volume. Further purification of each sample with Sep-Pak cartridges, PVP columns and ether fractionation was carried out as described in Method 3, except that the aqueous fractions were each extracted with 2 x 10 ml portions of ether. The final ether extracts were combined, reduced to dryness with N_2 gas and taken up in a small volume of diethylether:methanol 1:1 (v/v), prior to application to TLC plates. Thin-layer chromatography was carried out according to Method 1 using chloroform:methanol:water (75:22:3) as the developing solvent. The methods used for elution of the spots, methylation and GC were described in the sections entitled 'General Methods' and Method 1.

Results and Discussion

The GC traces obtained with extracts from various tissues of Plant 19 after purification by Method 4 are shown in Fig. 7. The results show very well-resolved and 'clean' peaks corresponding to the retention times of cis- and trans-ABA. There were also very large peaks of impurities with retention times between 10-15 min.

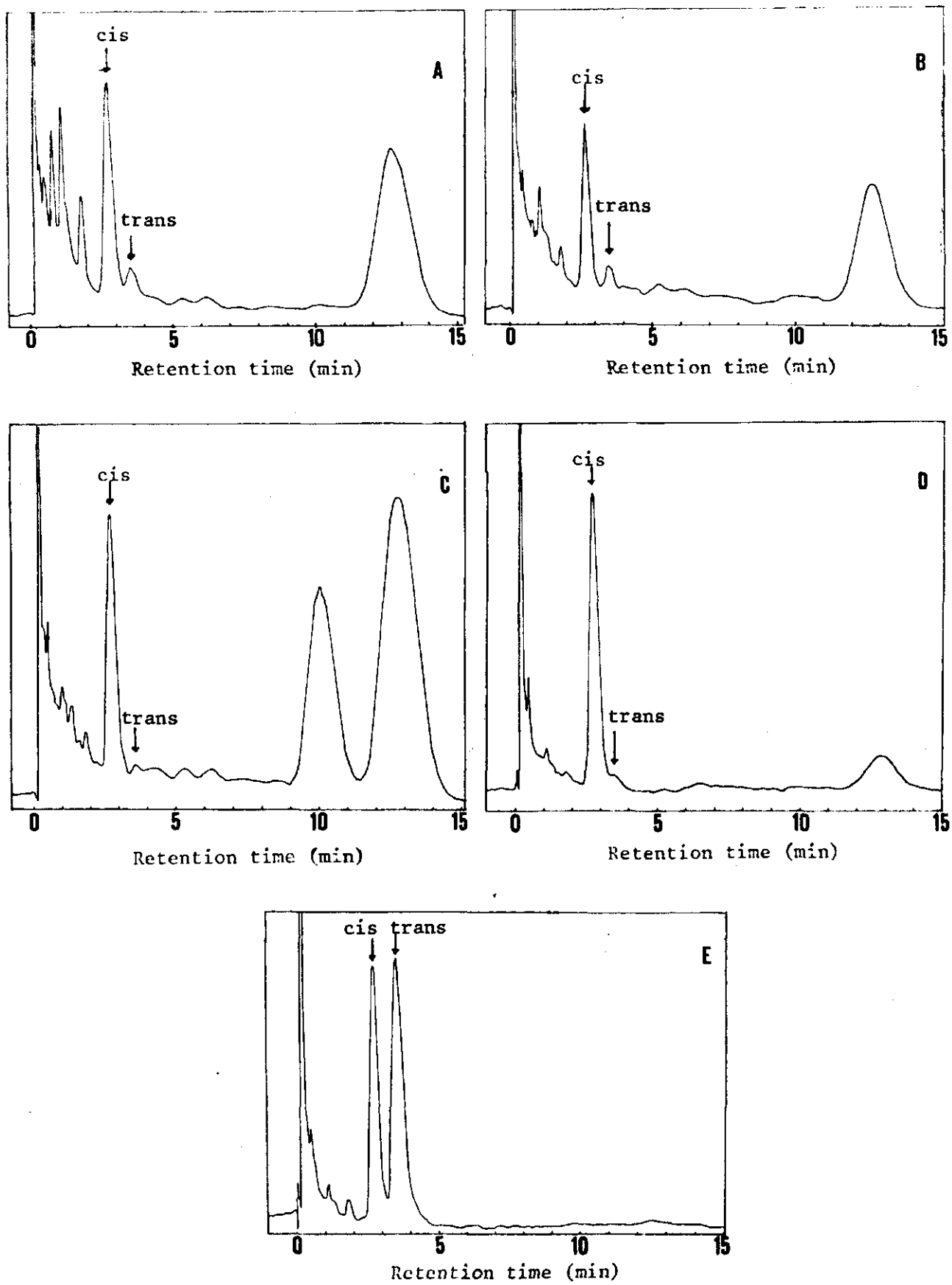


Fig. 7. GC profiles of samples from different tissues of Plant 19 after purification by Method 4.
The extracts were obtained from: A, leaf; B, stem; C, pod walls; D, seeds; E, standard methylated ABA containing 0.45 ng cis-ABA and 0.55 ng trans-ABA. Injection occurred at time 0, and the arrows indicate the cis- and trans-ABA peaks.

Further confirmation of the identities of the cis- and trans-ABA peaks was carried out as follows on a seed sample from another plant after purification by Method 4:

(a) *Co-chromatography of the sample with authentic ABA*

The co-injection of an aliquot of standard methylated ABA, containing significant amounts of both cis- and trans- ABA (Fig. 8C), and a sample of methylated seed extract with a large cis/trans ratio (Fig. 8B) produced a trace with two ABA peaks containing no shoulders and with additive peak areas (Fig. 8A).

(b) *UV-induced isomerization of the seed extract alongside an authentic ABA standard*

The seed extract and an authentic ^{14}C-cis-ABA standard, both methylated and dissolved in pure cyclohexane, were irradiated in 1 x 10 x 42 mm quartz cuvettes with a Camag TL 900 UV lamp at 254 nm. Aliquots were withdrawn at intervals, after adjusting the solutions to their original volumes to compensate for evaporation, and the quantities of cis- and trans-ABA determined by GC.

The results (Table 2) show that, up to 315 min, the quantity of cis-ABA declined and that of trans-ABA increased in each 1 ul aliquot, resulting in a comparable drop in the cis/trans ratio for both samples. This indicates that the presumptive cis-ABA peaks in both samples contained cis-ABA. However, in both samples, the total amount of ABA dropped sharply, which may have resulted from UV-induced breakdown of ABA. A non-irradiated methyl-ABA standard did not show a decline in ABA concentration so the sensitivity of the ECD detector on the GC had not changed during the experiment. Consequently, although the presumptive cis-ABA peaks probably contained cis-ABA, this experiment did not confirm that they contained *only* cis-ABA.

In conclusion, Method 4 shows promise for estimating ABA levels in different tissues of *Vicia faba*. However, in several trials of the method on different plant samples, final recovery efficiencies after methylation ranged from as low as 3% to as high as 32%. This contrasts with recoveries in the same preparations before the methylation step of from 48% to 73%. When the recoveries were extremely low, the ABA peak area to baseline height ratios were also low and this introduced inaccuracies into the calculations of ABA levels. These variable and unsatisfactory decreases in recoveries during methylation were probably caused by the introduction of silica gel into the samples during the elution of the TLC plates.

Consequently, although this method produced sufficiently purified samples for ABA analysis from quite large quantities of different tissues of *Vicia faba*, its use is limited until a method is devised to purify the TLC eluants from silica gel contaminants so that high and reproducible recovery efficiencies may be obtained (attempts to overcome this problem are discussed in the section entitled 'A Study of the Methylation of ABA for GC Analysis').

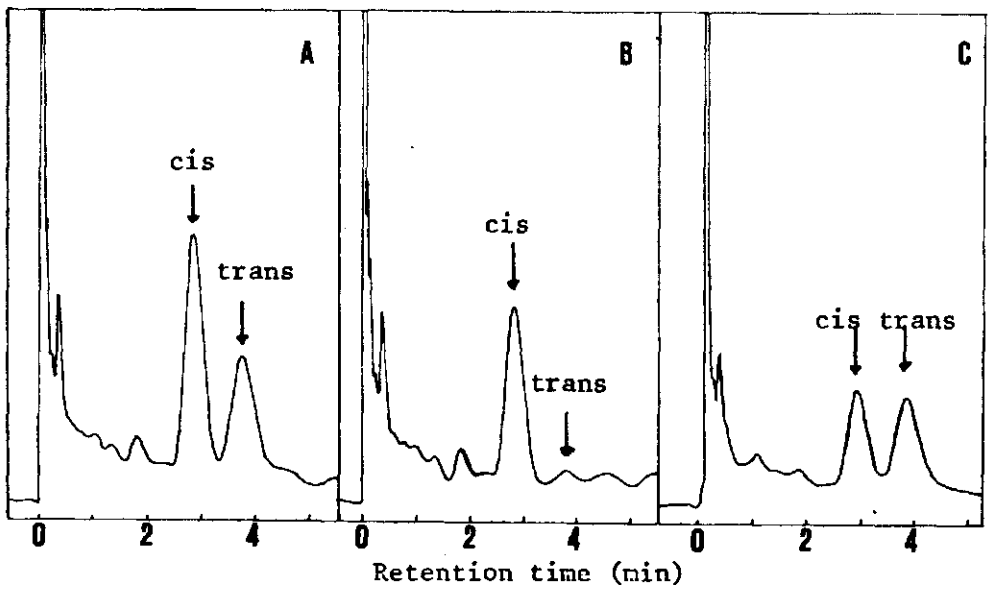


Fig. 8. Co-chromatography of authentic ABA with a seed sample of *Vicia faba* purified by Method 4.

Injection occurred at time 0 with: A, seed sample and authentic ABA; B, seed sample alone; C, authentic ABA alone.

Table 2. Comparison of the changes in the cis/trans ABA ratio during UV-induced isomerization of a seed sample purified by Method 4 and an authentic ^{14}C-ABA standard.

Time of UV-irradiation (min)	Seed sample (ng ABA)				^{14}C-ABA standard (ng ABA)			
	cis	trans	cis/trans	cis + trans	cis	trans	cis/trans	cis + trans
0	0.153	0.005	30.6	0.158	0.300	0.015	20.0	0.315
70	0.060	0.013	4.6	0.073	0.077	0.017	4.5	0.094
130	0.038	0.018	2.1	0.056	0.083	0.030	2.8	0.113
190	0.027	0.013	2.1	0.040	0.053	0.023	2.3	0.076
315	0.027	0.013	2.1	0.040	0.048	0.031	1.5	0.079

METHOD 5

Because of the problems associated with the use of TLC in reducing the recovery efficiencies of ABA with Method 4, an alternative method whereby the TLC step was replaced by an Extrelute purification column (Merck Diagnostica) was investigated.

Plant material:

Freeze-dried powder from:

Plant 12 - leaves, 0.67 gDW (5.0 gFW)(Method a)

Plant 17 - leaves, 0.51 gDW (4.0 gFW)(Method b)

General use of the Extrelute columns:

A plant extract with added ^{14}C-ABA internal standard in exactly 20 ml of 0.1 M K_2HPO_4, pH 3.0, was added to a pre-prepared Extrelute column and left for 20 min to allow the extract to penetrate the solid phase. The column was then eluted with 20 ml aliquots of diethylether and the eluates containing most of the radioactivity were collected.

Procedures

Two variations involving the basic method of Knegt *et. al.* (Method 3) and an Extrelute column were tested so that the column was employed either (a) after, or (b) before, the last ether fractionation.

Results and Discussion

Both methods produced visually clean preparations coupled with high recovery efficiencies (40% for method a and 78% for method b, although the reason for this difference is unclear). However, neither method produced sufficiently clean preparations for the GC analysis of ABA from the leaf samples (Fig. 9). In addition, there was evidence that the columns introduced additional impurities into the samples since an attempt to use the Extrelute columns to remove the silica gel contaminants from a leaf extract following TLC reduced the quality of the GC traces.

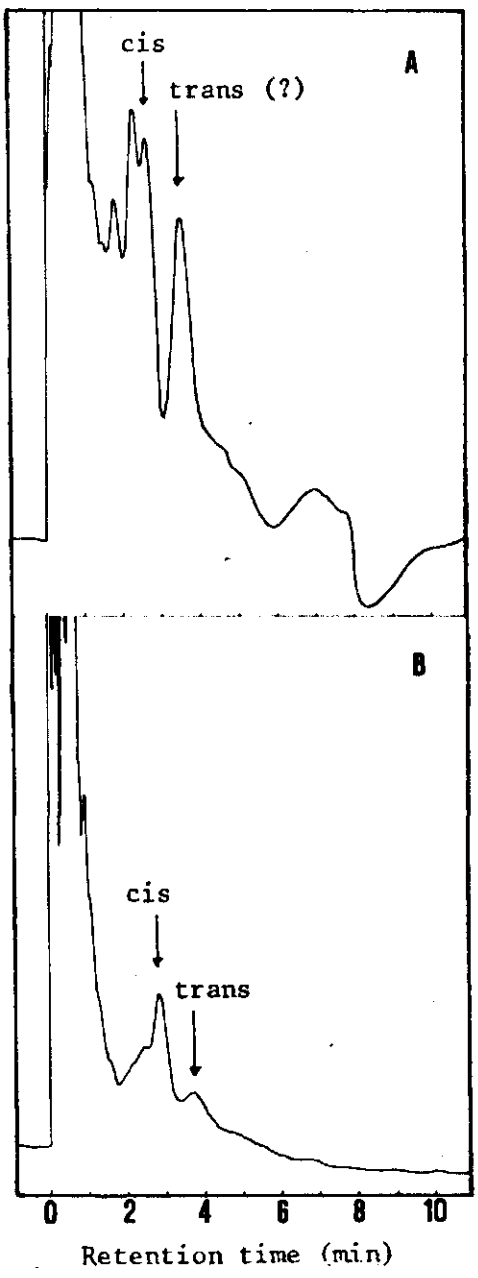


Fig. 9. GC traces of the leaf extracts of Vicia faba after purification using methods involving Extrelute columns.

See text for experimental details. A, Plant 12 (Method (a)); B, Plant 17 (Method (b)). Injection occurred at time 0 and the arrows indicate the retention times of cis- and trans-ABA as compared with an authentic standard methyl-ABA sample.

A STUDY OF THE METHYLATION OF ABA FOR GC ANALYSIS

Throughout this project, very low recovery efficiencies for the internal ^{14}C-ABA standard were obtained after methylation if TLC was incorporated in the method (eg., see Table 1). These low recoveries must have occurred either during or immediately after methylation because relatively high recoveries were routinely obtained before methylation. In addition, when TLC was used and the spots eluted with methanol, large residues of silica gel were introduced into the samples which could have interfered with the recoveries of ABA after methylation in several possible ways, including:

(a) By reducing the uptake of methyl-ABA into the cyclohexane solvent after the methylated samples were dried.

(b) By reducing the rate of methylation of ABA so that after the usual reaction period of 1 h only a small proportion of the ABA was methylated.

(c) A combination of these two possibilities.

A series of experiments were carried out to determine if any of these explanations were valid so that higher recovery efficiencies might be obtained after TLC purification of the plant samples.

1. Uptake of methyl-ABA and ABA by cyclohexane and methanol

Two experiments were carried out here: (a) one using ^{14}C-ABA to determine the solubility of dried ABA in cyclohexane and methanol in the absence of silica gel, and (b) the other using 'cold' methyl-ABA to determine the influence of silica gel on the uptake of dried methyl-ABA by cyclohexane and methanol.

(a) To each of 3 x 300 ul capacity glass Reacti-vials were added 50 μl of ^{14}C-cis-ABA (5479 dpm, sp. act. 100 dpm per ng cis-ABA) and the solutions evaporated to dryness with N_2 gas. To one vial were added 100 μl of 100% cyclohexane, to another 100 μl of 500:1 (v/v) cyclohexane:methanol, and to the third 100 μl of 100% methanol. The vials were mixed thoroughly with a whirlimixer and 20 μl of each solution taken for radioactivity determination.

Results

Table 3 shows the solubility of ^{14}C-ABA in three different solvents.

Table 3. The solubility of ^{14}C-ABA in three different solvents

Solvent	Percentage recovery of radioactivity from dried sample in solvent supernatant
100% cyclohexane	0.2
500:1 cyclohexane:methanol	2.1
100% methanol	101.2

The results show that ^{14}C-ABA is only slightly soluble in 100% cyclohexane but completely soluble in 100% methanol at the concentration tested. Addition of 1 part of methanol to 500 parts of cyclohexane slightly increased the uptake of dried ^{14}C-ABA compared to pure cyclohexane.

Consequently, when 100% cyclohexane or 500:1 cyclohexane:methanol is added to a dried, methylated sample of ABA, most of the unmethylated ABA should not be taken up.

(b) To a series of 300 µl capacity glass Reacti-vials were added 100 µl of standard methyl-ABA (1 µg/ml), The solutions were evaporated to dryness with N_2 gas and to half the number of vials were added 100 µl aliquots of a suspension of 120 mg silica gel (from a pre-prepared Merck 60 F_{254} TLC plate) in 100% methanol (this produced approximately similar residues as were often obtained with plant samples after TLC purification). 100 µl of 100% methanol were added to the remaining vials. The methanol was evaporated from each vial and different mixtures of methanol and cyclohexane added to separate vials. After thorough mixing with a whirlimixer, the recovery of methyl-ABA from the walls of the Reacti-vials by the solvent supernatants was determined with a GC calibrated with the original standard methyl-ABA solution in 100% cyclohexane,

Results

Pure cyclohexane was an excellent solvent for methyl-ABA in the absence of silica gel (Fig. 10B) but took up only a small quantity of methyl-ABA when silica gel was dried with the sample (Fig. 10A). Sonication of the solutions for up to 20 min did not improve the uptake of methyl-ABA by cyclohexane in the presence of silica gel.

Consequently, any silica gel (and possibly other) contaminants in the plant samples used for methylation may considerably reduce the apparent recovery efficiency of ^{14}C-ABA after methylation. A possible solution to this problem may be the use of cyclohexane:methanol mixtures which may be sufficiently hydrophilic to solvate the methyl-ABA from the surface or matrix of the contaminants, but which would not solvate significant amounts of any unmethylated ABA in the samples. Figure 11 shows the results of an experiment to determine the influence of cyclohexane: methanol mixtures on the recovery of methyl-ABA in the presence of silica gel. Increasing the percentage of methanol in cyclohexane increased the recovery of methyl-ABA from the silica contaminants with full recovery occurring at approximately 5.3% methanol in cyclohexane. However, methanol also produced highly-depressed baselines on the GC traces (Figs 11 and 12) which may indicate stripping of the stationary phase from the OV-1 column packing. This is obviously highly undesirable and also caused inaccurate calculation of the peak heights and ABA

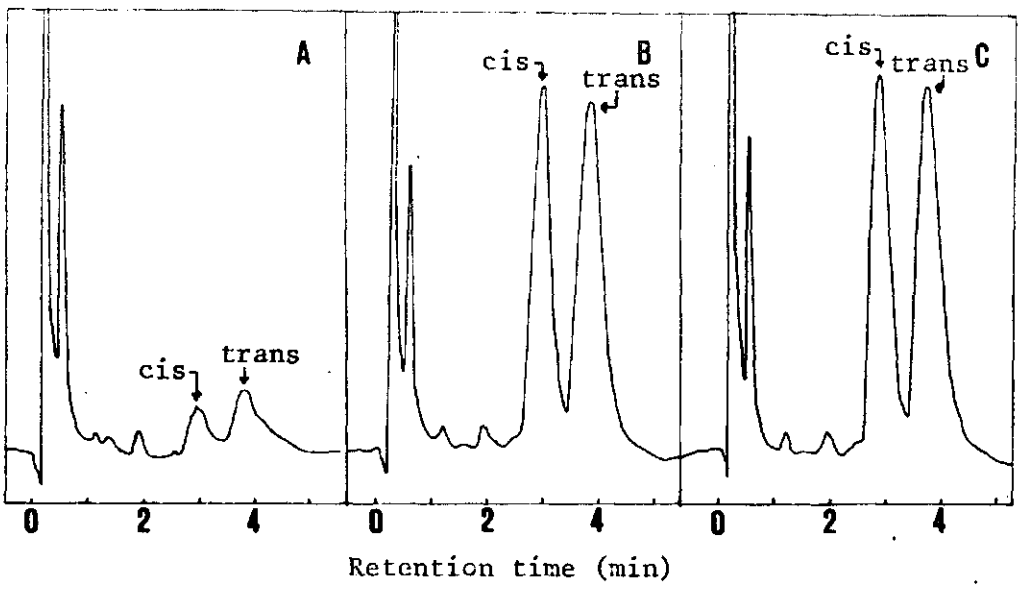


Fig. 10. GC traces showing the uptake of dried methyl-ABA by 100% cyclohexane from the glass walls of Reacti-vials in the presence and absence of silica gel.

Injection of 1 µl of standard methyl-ABA occurred at time 0 after:
A, drying with silica gel and resuspending in 100% cyclohexane;
B, drying without silica gel and resuspending in 100% cyclohexane
C, untreated methyl-ABA standard.

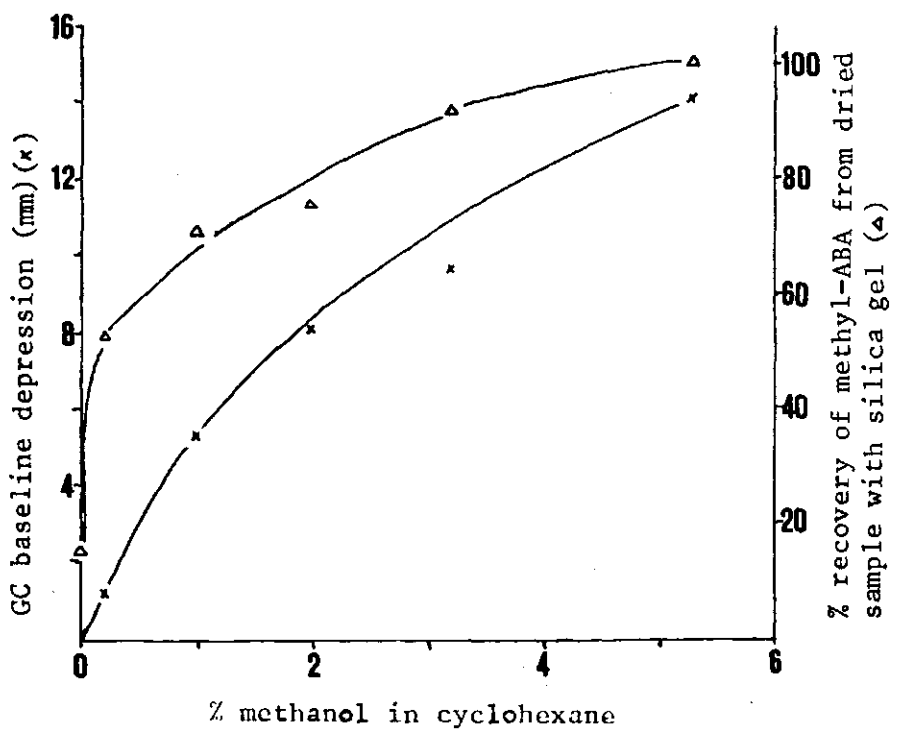


Fig. 11. The use of cyclohexane:methanol mixtures for solvating methyl-ABA in the presence of silica gel.

contents of experimental samples. However, a concentration of 0.2% methanol in cyclohexane (500:1 cyclohexane:methanol) was found to give very little baseline depression but recovered approximately 50% of the methyl-ABA from the silica contaminants. This 500:1 mixture was often used as the solvent for methyl-ABA in procedures where TLC was used because it tended to improve the recovery efficiencies, probably without producing highly inaccurate values due to the uptake of unmethylated ABA.

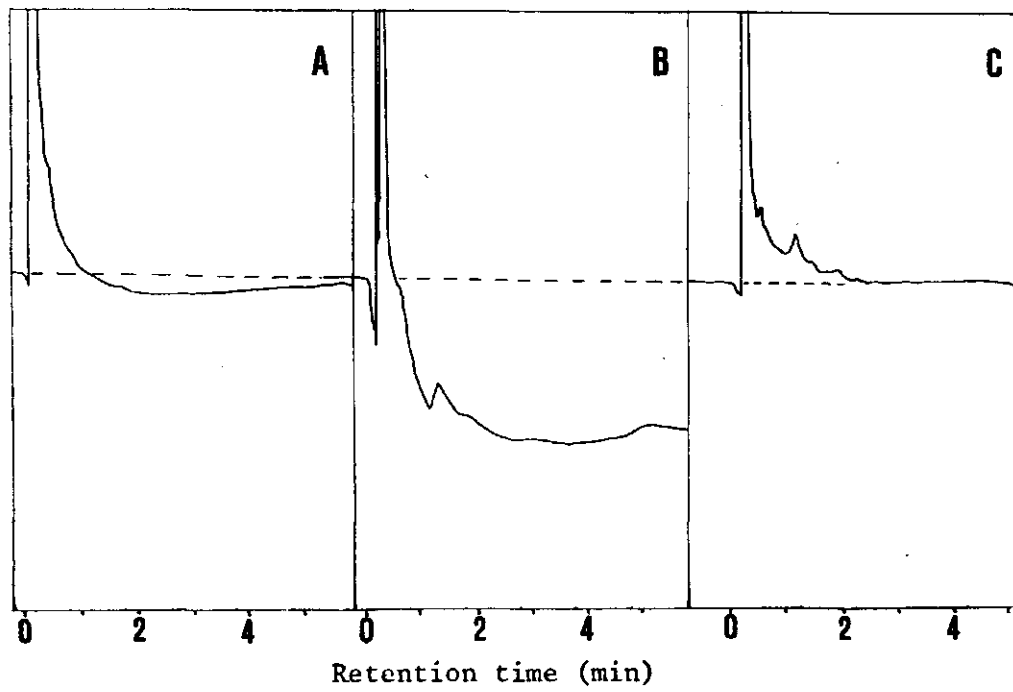


Fig. 12. Baseline depression in GC traces produced when methanol is used as the injection solvent with an OV-1 column.

Injection of 1 µl aliquots of blank solvents occurred at time 0 with: A, 500:1 (v/v) cyclohexane:methanol; B, 100% methanol; C, 100% cyclohexane. The dashed line represents the position of the baseline.

2. A third experiment was conducted to determine the optimum methylation time for ABA samples. In addition, the use of 100% cyclohexane as a specific solvent for methyl-ABA, but not unmethylated ABA, was also investigated.

Procedure

To each of 8 x 300 µl capacity glass Reacti-vials were added 100 µl of ^{14}C-cis-ABA (11,000 dpm with a sp. act. of 100 dpm per ng ABA). To one of the vials were added 10 µl of a suspension of 120 mg silica gel in methanol and to another 50 µl of the same suspension. Diazomethane in ether (0.2 ml) was added to each cooled vial and methylation carried out for different times at room temperature. The reaction was stopped by immediate evaporation of excess gas with a stream of air,

followed by evaporation to dryness with N_2 gas. 200 µl of 100% cyclohexane were then added to each vial to dissolve the ^{14}C-methyl-ABA and the vials thoroughly mixed with a whirlimixer. The cyclohexane fractions were carefully removed and 200 µl of 500:1 cyclohexane:methanol added to the two vials containing silica gel to collect any remaining ^{14}C-methyl-ABA. After thorough mixing of the vials, the cyclohexane: methanol fractions were carefully removed. Finally, the procedure was repeated with all the vials using 100% methanol as the solvent to remove any remaining radioactive compounds.

Half of each solvent fraction was counted for radioactivity and the other half was subjected to TLC on silica gel 60 F_{254} plates in benzene:acetic acid (50:20). An aliquot of ^{14}C-cis-ABA was used as the reference. After radiochromatogram scanning of the plates, each radioactive spot was scraped from the plates and the silica gel added directly to liquid scintillation vials containing 2 ml 100% methanol and 10 ml scintillation fluid added prior to counting.

Results

The R_f values for the radioactive spots resolved on the plates after TLC are shown in Table 4. It was not possible in this experiment to fully confirm the chemical identity of each spot because methyl-ABA and ABA were insufficiently well resolved in the solvent system used. However, the major proportion of the radioactivity in the 100% cyclohexane and 500:1 cyclohexane:methanol fractions should be methyl-ABA and not ABA (see Table 3), whereas the 100% methanol fraction should contain any radioactivity remaining after the dried samples had been eluted with one or both of the other solvents. Consequently, depending on the efficiency of solvation by the first two solvents, the 100% methanol fraction may contain both methyl-ABA and ABA. In addition, it should be recalled that the solvation by 100% cyclohexane of methyl-ABA dried on Reacti-vial glass walls is complete when no contaminants are present but highly inefficient in the presence of large amounts of silica gel residues (see previous experiment 1(b)).

Using the assumptions mentioned above, the results in Table 4 indicate that in the absence of silica gel, most of the ^{14}C-ABA is methylated within 20 min reaction time. Further methylation for up to 180 min produces only presumptive methyl-ABA spots from the cyclohexane fractions, with no spots from the 100% methanol fractions. These results were confirmed when both the unchromatographed solvent fractions were counted directly and when the radioactive spots were scraped off the TLC plates and counted (Fig. 13).

Whereas 100% cyclohexane solvated all the methyl-ABA from a small quantity (50 µl) of previously-added silica gel (Table 4), only 56% (as determined by direct counting) of the methyl-ABA was recovered by this solvent in the presence of a

Table 4. TLC of different solvent fractionations of methylated ABA samples

Methylation time (min)	No. of spots and R_f values*		
	Cyclohexane	500:1 CYCLOHEXANE : METHANOL	Methanol
5	1(0.67)	-	1(0.67)
10	1(0.65)	-	1(0.60)+
20	1(0.61)	-	None
30	1(0.61)	-	None
60	1(0.58)	-	None
180	1(0.60)	-	None
180 + 10 µl silica gel	1(0.66)	None	None
180 + 50 µl silica gel	1(0.58)	11(0.60)	None

* R_f values are given in parenthesis; - denotes not tested; the R_f value for the ^{14}C-ABA standard was 0.56.

\+ minor spot.

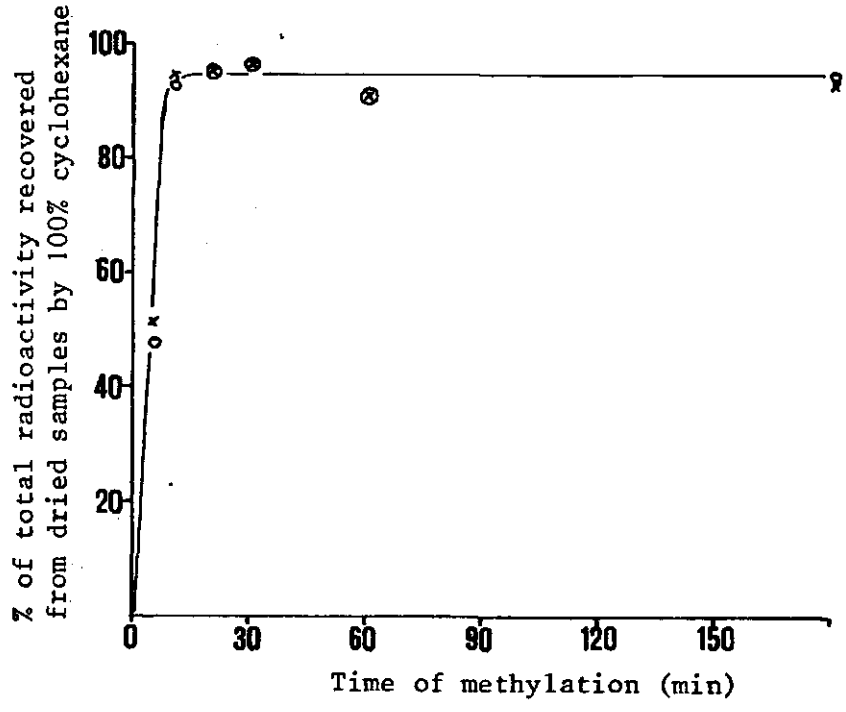


Fig. 13. Time-dependent methylation of standard ^{14}C-ABA samples.

The progress of methylation was determined by (×) direct counting of 100% cyclohexane fractions, and (○) by counting the radioactive spots produced after TLC of the cyclohexane fractions.

relatively large quantity (50 µl) of silica gel. In the latter case, the remaining methyl-ABA (41%) was solvated by the 500:1 cyclohexane:methanol solvent. No TLC spots and very few counts were found in the methanol fractions after 180 min methylation in the presence of either small or large quantities of silica gel.

Discussion

From the results presented above, it appears that silica gel contaminants from the TLC plates are responsible, at least in part, for the very low recovery efficiencies for the ABA in plant samples. Pure cyclohexane is probably too hydrophobic to penetrate the matrix of the hydrophilic silica particles to solvate the ABA. The slightly more hydrophilic solvent mixture consisting of 500:1 cyclohexane:methanol appears to solvate methyl-ABA more effectively than 100% cyclohexane in the presence of silica gel without introducing large counting errors by also solvating too much unmethylated ABA. However, further trials using plant samples after TLC and methylation for up to 3 h with 500:1 cyclohexane: methanol as the solvent system for taking up the methyl-ABA did not produce very large increases in recovery efficiencies. It may be that hydrophilic contaminants from the plant tissues, in addition to silica gel, produces a too hydrophilic matrix for the solvent system to penetrate. In addition, these experiments did not investigate the effect, if any, of silica gel on the *rate* of ABA methylation which may also be a factor contributing to reduced recoveries.

In the absence of a suitable method for the analysis of ABA from *Vicia faba* which does not involve TLC, one possible solution to this problem is to remove the silica gel from the plant extracts after TLC. Several trial experiments were conducted on this possible solution, as follows:

(a) *Cleaning of the chromatographed samples with ether fractionation or Extrelute columns*

Procedure

Leaf samples were purified by Method 4 and eluted from the TLC plates with 100% methanol. The dried samples were taken up in 0.1 M K_2HPO_4 which, after acidification to pH 3.0, was either extracted once with 10 ml diethylether or further purified with an Extrelute column (see Method 5). The Extrelute column packing was first washed thoroughly in the buffer, dried, re-washed in ether and finally dried before use. After purification, the ether fractions were evaporated to dryness and methylation of the sample carried out in methanol. Cyclohexane:methanol 500:1 was used as the injection solvent.

Results

Both methods purified the samples of silica gel but introduced other contaminants into the samples which reduced the quality of the GC traces.

(b) *Use of solvents other than methanol for eluting the TLC plates*

Procedure

A series of solvents and solvent mixtures which were found not to dissolve silica gel were tested for their ability to take up ^{14}C-ABA from dried samples containing silica gel. Diethylether and cyclohexane:methanol (50:1 and 22:1) eluted only 13, 17 and 25% respectively of the added ABA from the silica gel matrix. Consequentl these solvents are not useful for eluting ABA from TLC plates. It is probably impossible to prevent the solvation of silica gel into plant extracts during elutior because ABA requires a hydrophilic solvent and this type of solvent invariably dissolves silica gel.

In conclusion, the problem associated with the introduction of silica gel residues into plant samples after TLC purification must be solved if Method 4 is to be used for the routine analysis of ABA from *Vicia faba* tissues. The efficacy of this method, when used with most *Vicia faba* tissues containing low ABA concentrations, depends on high recovery efficiencies if high peak height:baseline ratios are to be obtained.

A scan of available recent literature gives clues as to why this problem associated with TLC has not been previously reported:

(a) TLC may not be used in the method.
(b) The plant sample is methylated just prior to TLC and the methyl-ABA spots eluted with a hydrophilic solvent, a portion of which is used directly for GC analysis (eg., Heilmann *et. al.*, 1980).
(c) Low recoveries may not be routinely observed when an internal non-radioactive ABA standard is used. Recovery efficiencies are not so important here because the quantity of endogenous ABA is calculated by direct comparison of peak areas (eg., Loveys, 1977).
(d) Hydrophilic solvents, eg. methanol or ethanol (Loveys and Kriedeman, 1974; Phillips and Hofmann, 1979) are used to take up methyl-ABA after methylation and drying. These solvents dissolve both methyl-ABA and any remaining unmethylated ABA, even in the presence of large silica gel deposits, and this may lead to an overestimation of the recovery efficiencies if a ^{14}C-ABA internal standard is used and methylation is incomplete. The extent of methylation may vary in different samples, particularly if the methylation time is short (eg., 10 min is often used) and if the samples contain different levels of silica gel and other impurities. Several papers do not provide sufficient details as to which solvents were used for the elution of TLC plates or for re-dissolving dried methyl-ABA prior to GC analysis Consequently, results obtained with these methods must be treated with caution.

The use of 100% cyclohexane is highly recommended for taking up methyl-ABA after methylation as it does not dissolve significant quantities of unmethylated ABA (Tab 3). Consequently, this is a simple and convenient method of estimating true recovery efficiencies for each sample when GC and ^{14}C-ABA internal standards are used togethe

COMPARISON OF THE ABA LEVELS IN VARIOUS TISSUES OF *Vicia faba* PLANTS GROWN UNDER IRRIGATED AND NON-IRRIGATED CONDITIONS

The ABA levels in various tissues of field-grown *Vicia faba* plants are shown in Table 5, along with values for the total fresh weights and percentage dry weights of the tissues.

These values are obviously incomplete and very few conclusions can be drawn from this experiment because of the following points:

(a) The values were obtained during trials of different methods for ABA analysis and usually relied on low recovery efficiencies which probably affected the relative accuracy of the results.
(b) The results were not replicated either between different plant specimens within a given treatment or between different samples of the same tissue for a given plant.
(c) There were several variables in the experiment, including developmental stage of each tissue, seasonal and diurnal changes in the environmental conditions, and possibly a varying influence of drought stress on different parts of the same tissue according to its position on the plant.

However, bearing in mind these points, it is possible to make a few observations on the results which may be useful for planning similar experiments in the future:

ABA levels

(1) The relative concentrations of ABA in the different tissues followed a fairly consistent pattern in the order: seeds $>>$ leaves $\approx$ pod walls $>$ stems.
(2) The ABA concentration in the seeds appeared to increase with the developmental stage but was not influenced by the irrigation regime of the parent plant.

Fresh weights and percentage dry weights of the tissues

(1) There was no consistent difference between the fresh weights of the plant tissues when grown under irrigated or non-irrigated conditions.
(2) Between June 7 and July 29 (Plots B and C), there were no consistent increases in any of the plots for the fresh weights and percentage dry weights of the leaves, stems and pod walls but these values markedly increased for the seeds.
(3) The percentage dry weights of the stems and pod walls were generally slightly higher in the non-irrigated plots compared to the irrigated one; there was not such a consistent pattern for the leaves and seeds.

Table 5. Total fresh weights, percentage dry weights and ABA concentrations of different tissues of *Vicia faba* plants grown in irrigated or non-irrigated field plots*

Plant no.	Harvest date	Harvest time	Plot type	Total fresh wt. (g)				Percent dry wt.				ABA concentration (ng/g DW)			
				L	St	W	Se	L	St	W	Se	L	St	W	Se
1+	June 25	09.30	A	-	-	-	-	15.3	-	12.3	15.8	547	-	358	2442
2+	June 25	11.00	C	-	-	-	-	-	-	11.1	15.7	-	-	191	2732
3	June 25	14.45	C	46.4	35.9	11.2	1.9	14.4	14.1	9.0	15.2	345	190	359	7458
5	July 7	09.30	B	39.7	38.5	54.4	24.0	16.7	19.2	12.7	17.5	-	-	-	-
6	July 7	11.15	C	50.3	75.9	35.2	11.4	14.7	15.8	11.7	15.7	-	-	-	-
7	July 7	14.15	C	54.9	66.0	45.4	15.2	12.9	13.7	9.4	16.0	-	-	-	-
8	July 7	15.45	B	17.7	40.7	34.5	18.6	23.4	20.7	14.0	19.0	-	-	-	-
12	July 14	10.45	B	45.6	44.5	57.0	25.1	13.4	15.6	10.5	18.7	-	-	-	-
13	July 14	14.00	C	38.8	47.6	45.5	23.1	14.4	14.7	10.7	19.2	75	0	30	12,665
16	July 21	10.40	B	46.5	35.7	50.7	31.9	12.3	17.1	12.6	22.5	-	-	-	11,067
17	July 21	13.50	C	70.9	84.3	84.8	47.4	12.7	15.8	11.5	20.1	-	-	-	-
19	July 28	10.00	B	34.5	49.5	61.8	54.0	15.1	19.0	14.1	29.1	704	309	526	11,323
21	July 28	-	C	41.9	55.7	51.5	36.6	15.3	17.2	13.8	22.8	160	49	194	12,087
24	July 29	-	B	38.4	57.6	61.6	44.6	18.5	21.3	14.7	28.7	-	-	-	-
25	July 29	-	C	47.2	51.2	75.3	64.1	16.3	18.6	11.5	29.1	-	-	-	-

* A, irrigation terminated on June 2; B, irrigation terminated on July 7; C, plot irrigated throughout experimental period. L, leaves; St., stem; W, pod walls; Se, seeds.

+ only part of each tissue for these plants was analysed.

SUGGESTIONS FOR FUTURE WORK ON THE ANALYSIS OF ABA FROM *Vicia faba* TISSUES

It is probably worth making a few suggestions at this point which may be useful to other workers involved in the ABA analysis of tissues from *Vicia faba* or other tissues.

(a) *Extraction of tissues*

It is probably wise to extract frozen material rather than freeze-dried powder. Freeze-drying takes several days, during which time the tissue reaches room temperature and the extent of ABA conversion is unknown. If a representative sample of a large quantity of tissue is required it is probably best to extract all the tissue and then take aliquots of the extract for smaller-scale analysis. Digestion of the tissue with 80% methanol overnight at +4°C is not recommended. Severe browning of the leaf extracts occurs during this time which may not only interfere with subsequent purification but possibly indicates chemical or enzymic oxidation which may lead to the conversion of ABA.

Previous reports (Hemberg and Tillberg, 1980; Horemans *et. al.*, 1980) indicate that the amount of IAA and ABA extracted from plant tissues is greatly influenced by the extraction method. Consequently, extraction of tissues should be rapid and exhaustive. Initial trials should be conducted on different tissues to determine whether more ABA is removed on further extraction of the residues, preferably using different solvents and extraction conditions.

(b) *Method of analysis*

In this authors opinion, the method of Knegt *et. al.* (Method 3) is the most convenient method available for the analysis of ABA from different tissues of *Vicia faba*, providing a capillary GC column is available. In the absence of a capillary GC column, this method was still found to be highly suitable, in conjunction with the conventional system described here, for tissues with high ABA concentrations such as *Vicia faba* seeds. If tissues of *Vicia faba* other than the seeds are to be analysed with the conventional system then additional purification must be performed. If TLC is used then the problem of silica gel residues must be resolved. One possible solution, suggested by Ribôt (personal comm.), is to methylate the plant extracts just prior to TLC and then the chromatographed methyl-ABA spots may be eluted with a suitable cyclohexane:methanol mixture that does not take up silica gel. After drying, the methyl-ABA should be taken up in 100% cyclohexane.

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