INDOLE - 3 - ACETIC ACID IN *PHASEOLUS VULGARIS* L.

by

JOHN McDOUGALL

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Department of Botany,
University of Glasgow,
Glasgow.

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
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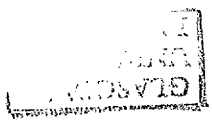

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ABSTRACT

An analytical method has been developed in order that the amount and distribution of IAA in shoot tissue of Phaseolus vulgaris could be determined.

A variety of extraction and purification procedures were investigated to devise a reliable method for the purification of shoot tissue extracts. By combining those methods that were shown to lead to an overall reduction in sample contamination, a procedure was developed that was capable of yielding a sample of suitable purity for subsequent analysis by gas chromatography and mass spectrometry. This procedure involved an initial methanol extraction period followed by solvent partitioning prior to the purification of the extract on columns of DEAE-cellulose and PVP, as well as thin layer chromatography.

For quantitative analysis of the total IAA in these purified samples an electron-capture GC system and a GC-MS procedure were tested on the basis of sensitivity and specificity. The GC-MS system using previously trimethylsilylated plant samples was found to be more suitable. In addition to this analytical procedure, a method involving radio-GLC was developed in order to gauge the recovery of radioactive IAA at the completion of the purification procedure.

The most efficient methods were then combined into one experimental procedure and applied to the assay of IAA in shoot tissue extracts of P. vulgaris. The results of this analysis indicated that the level of IAA was in the range of 1.7-3.8 $\mu g\ kg^{-1}$ fresh weight.

The distribution of IAA in the foliar and stem tissue was subsequently gauged. In the foliar tissue samples, the apex was found to contain the greatest level of IAA (7-62 $\mu g\ kg^{-1}$) with older trifoliate leaves having the second largest level (2-20 $\mu g\ kg^{-1}$). The lowest level of IAA was found in the primary leaf tissue (0.4

-3.5 μg kg^{-1}). For the stem tissue experiments no distinct gradient was apparent, but the internodal region between the cotyledons and the primary leaves was found to have the least amount of IAA.

Abbreviations

(1) General

ABS	absorbance
BF_3	boron trifluoride
BSA	N,O - bis - (trimethylsilyl) - acetamide
cpm	counts per minute
c.v.	cultivated variety
DEAE	diethylaminoethyl
dpm	disintegrations per minute
dry wt.	dry weight
ECD	electron - capture detector
et al.	et alia
eV	electron volt
FID	flame ionisation detector
fresh wt.	fresh weight
FSD	full scale deflection
GC	gas chromatograph(y)
GC-MS	combined gas chromatography - mass spectrometry
GLC	gas - liquid chromatograph (y)
HCl	hydrochloric acid
HFB	heptafluorobutyryl
HPLC	high performance liquid chromatograph
H_2SO_4	sulphuric acid
IAA	indole-3-acetic acid
log	logarithm
M	molar
m/e	mass-to-charge ratio
MS	mass spectrometer
PA	peak area

PH	peak height
PW	peak width
%	per cent
Rf	retention factor
RFE	rotary film evaporation
RT	room temperature
SID	selective ion detection
SOS	sums of squares
TIC	total ion current
TIM	total ion monitor
TLC	thin - layer chromatography
TMSi	trimethylsilyl
TP	temperature programming
T_R	retention time
T_R^1	retention time corrected to solvent front
UV	ultraviolet
viz.	videlicet
v/v	volume to volume

(2) Units

^oC	degrees Celsius
Ci	curie
d	day(s)
g	gram(s)
h	hours(s)
l	litre(s)
m	metre(s)
min	minute(s)
sec	second(s)

(3) Prefixes

k	kilo
c	centi
m	milli
μ	micro
n	nano
p	pico

CONTENTS

INTRODUCTION

PLANT HORMONES and AUXINS

The form and structure of any higher plant is dependent on the growth and development of its cells, tissues and organs. Many factors are known to influence these growth processes in the plant: these include various environmental parameters such as gravity, radiant energy, nutrient supply and competitors. It is therefore beneficial to the plant that various regulatory mechanisms exist to enable it to react to such factors. One basic method whereby a plant can alter its growth pattern may be by virtue of the action of endogenous plant hormones.

The concept of hormonal control of growth is not unique to plant physiology, since similar processes are thought to occur in mammals. The basic philosophy behind such control mechanisms is that chemical substances are produced by discrete areas of the organism, transported to a specific group of cells or target area where the action of the hormone is such that the metabolism of the target area is altered often resulting in a different growth pattern (Phillips, 1971).

In plants there are thought to be at least five types of hormones *viz.*: the auxins, cytokinins, gibberellins, ethylene and various inhibitory compounds such as abscisic acid (Phillips, 1971).

The discovery of auxins in plants has been authoritatively reviewed by Thimann (1972) and Audus (1972). Briefly, the experimental evidence and conclusions were as follows: (i) Darwin (1880) described the importance of the role of the apex in the phototropic response of coleoptiles; (ii) Boysen-Jensen (1910) introduced the idea of a chemical substance produced by the apex controlling the phototropic response; (iii) Paàl (1919) provided further evidence of a chemical substance that is produced and secreted by the apex effecting the growth of the sub-apical region of coleoptiles.

This type of growth substance that controls the growth or elongation of plant stems came to be called auxin. Auxin has subsequently been shown to exert a significant effect on other plant growth processes, e.g. root elongation (Street, 1969) and root initiation (Thimann, 1972).

It is surprising that the exact nature of auxin relied for several decades on a series of studies that provided circumstantial evidence that the compound in question was indole - 3 - acetic acid (IAA) (see Thimann, 1972). The actual identity of auxin was not unequivocally shown until Greenwood et al. (1972) identified IAA by mass spectral means in Zea coleoptiles.

In addition to IAA a variety of other compounds of a similiar nature have been isolated from various plant sources, these are summarised in Table 1. The structure of these indole auxins, including IAA, are shown in Figure 1.

Table 1: Indole compounds isolated from plants

Compound	Plant source	Reference
IAA	Zea mays	Greenwood et al. 1972
4-chloroindolyl-3-acetic acid	Pisum sativum	Marumo et al. 1968a
Methyl-4-chloro-IAA	"	Marumo et al. 1968b
Methyl-4-chloroindolyl -3-acetyl-L-aspartate	"	Hattori and Marumo, 1972
Indole-3-carboxylic acid	Undaria pinnatifida	Abe et al. 1972
Methyl-IAA	Citrus unshiu	Takahaski et al., 1975
Indole-acetamide (IAM)	"	"
α-malonyl-D-tryptophan	Triticum vulgare	Elliott, 1971
Indole-3-lactic acid	Endomycopsis vernalis	Glombitza and Hartmann, 1966
Indole-3-ethanol	Cucumber	Rayle and Purves, 1967

Figure 1: Structure of indole auxins present in plants

A : Indole-3-acetic acid

B : 4-chloro-indole acetic acid

C : Methyl-4-chloro-indole acetic acid

D : Indole-3-carboxylic acid

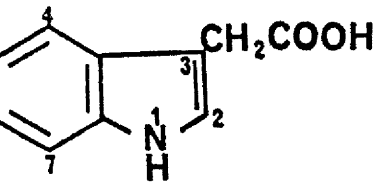
E : Indole ~~acetamide~~ ACETONITRILE

F : α-malonyl-D-tryptophan

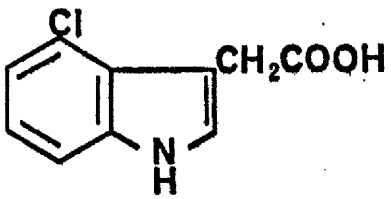
G : Indole-3-lactic acid

H : Indole-3-ethanol (Tryptophol)

I : Methyl-4-chloro-indoleacetyl asparate


4
3
2
1
7
N
H
CH2COOH

A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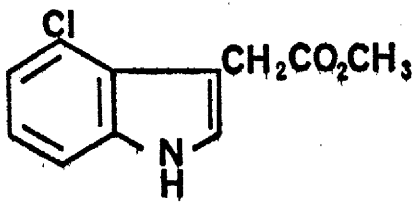
CH2COOH
N
H

B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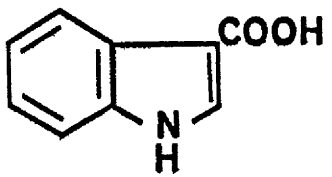
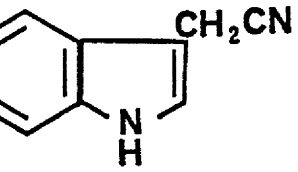
N
H

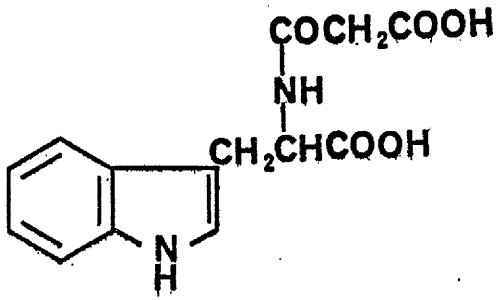
C


COOH
N
H

D


CH2CN
N
H

E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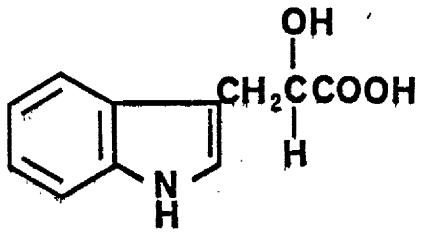
NH
CH2CHCOOH
N
H

F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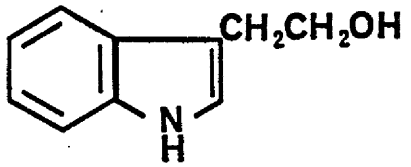
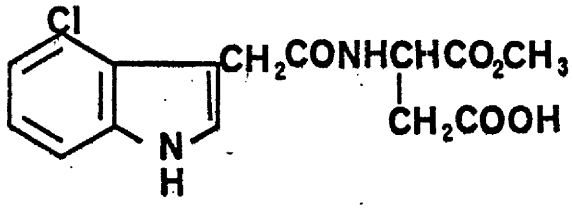
H
N
H

G


CH2CH2OH
N
H

H


Cl
CH2CONHCHCO2CH3
CH2COOH
N
H

I

Although most auxin compounds in plants have been shown to have a structure based on the indole nucleus; Khalifah et al. (1963, 1965) have claimed to extract an auxin from Citrus fruits that does not possess the characteristic fluorimetric properties of indoles. In contrast, Igoshi et al. (1971) identified IAA and indole acetamide (IAM) as two auxins in Citrus unshiu, with no evidence for the existence of any novel non-indolic auxin. Similarly, Takahashi et al. (1975) demonstrated the existence of three auxins in this species, which they identified as IAA, the methyl ester of IAA, and IAM. Thus the question of the existence of a non-indolic auxin in Citrus remains unanswered.

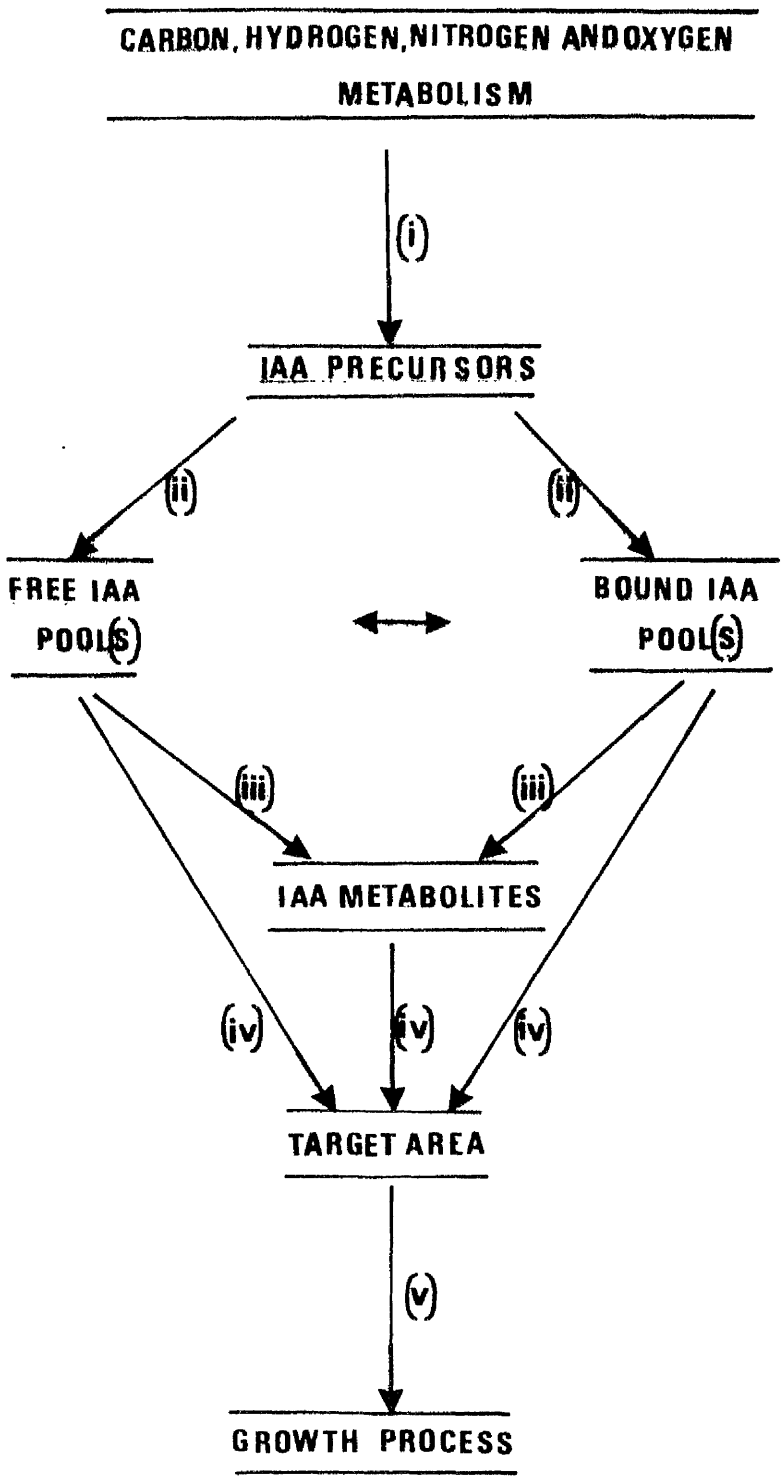
Another type of non-indole auxin is phenylacetic acid (PAA). Wightman and Rauthan (1973) described a biosynthetic route to PAA in plants, whilst Wightman (1977) has demonstrated the presence of PAA in developing leaves of tobacco plants by gas-chromatography. The unequivocal identification of this compound in plants has not so far been reported, thus the possible significance of PAA as an auxin in plants is obscure.

CONTROL OF PLANT GROWTH BY IAA

The concept of the ~~hormonal~~ HORMONAL control of plant growth is such that if IAA is a hormone, then it will be synthesised in specific parts of the plant from where it will be transported to the target areas to alter the pattern of cell metabolism. This basic theory in the light of subsequent evidence can, however, be expanded into a more elaborate scheme to explain the action of IAA in plants. An outline of this scheme is shown in Figure 2.

The theory behind such a scheme is that (i) precursors of IAA are formed from the general metabolism of carbon, hydrogen, nitrogen and oxygen compounds; (ii) these precursors are converted to IAA itself or are held as "bound" compounds in the cells where they are then converted to a "bound" form of IAA; (iii) conversion of IAA to

Figure 2: Regulation of plant growth by IAA


CARBON, HYDROGEN, NITROGEN AND OXYGEN METABOLISM
(i)
IAA PRECURSORS
(ii)
(ii)
FREE IAA POOL(S)
BOUND IAA POOL(S)
(iii)
(iii)
IAA METABOLITES
(iv)
(v)
(iv)
TARGET AREA
(v)
GROWTH PROCESS

metabolites occurs directly from the free pool of IAA, indirectly from the "bound"form of IAA via the free pool, or alternatively by direct conversion of IAA in the "bound" form; (iv) movement of IAA or its metabolites to the target area; or conversely the conversion of free IAA to a "bound" form may in itself be the transport mechanism; (v) action of the IAA or metabolites occurs at target areas resulting in an alteration of metabolism of the cells.

The evidence for the various stages may be briefly summarised as follows:-

(A) Schneider and Wightman (1974) recently reviewed the evidence for steps (i) and (ii). They have considered the main precursor of IAA to be tryptophan (TRY TPP) which arises from the shikimic acid pathway. Although a large body of evidence points to TRY TPP being the main precursor of IAA, some contradictory observations have been reported: for example, Davies et al. (1975) noted that in crown gall tissue the main precursor of labelled IAA was ^{14}C-indole or tryptamine; on the other hand only crude identification procedures were used.

Evidence for TRY TPP conversion via a number of intermediate compounds to IAA was provided by Gibson et al. (1972) who used identification procedures based on TLC analysis.

The main biosynthetic routes to IAA have been summarised by Gibson et al. (1972) and Schneider and Wightman (1974): (a) The indole pyruvic pathway - TRY TPP is converted by transamination to indole pyruvic acid (IPyA) which subsequently is decarboxylated to indoleacetaldehyde (IAAld). IAAld is then oxidised to IAA. (b) The tryptamine pathway - TRY TPP is decarboxylated to tryptamine which then undergoes oxidation and deamination to IAAld. Conversion of IAAld to IAA then occurs as above. (c) The indoleacetaldoxime pathway - This pathway is thought to be characteristic of Brassicacae and involves conversion of TRY TPP to IAA, via indoleacetaldoxime and indole acetonitrile. (d) The tryptophol

pathway - TRy TPP is transaminated to IPyA and converted to IAAld as for (a). IAAld is then altered to tryptophol which can then be reconverted to IAAld and finally IAA. Tryptophol in this case presumably acts as a storage form of IAA.

The nature of the particular route for conversion of TRy TPP to IAA is not known. All the routes may be important, but since these pathways have arisen from experimental evidence in which the identification procedures of IAA and intermediates are often unspecific, care in their interpretation is required.

(B) The exact nature of the "bound" forms of IAA in plants is varied, with the actual amounts of "bound" or esterified IAA thought in many cases to be in excess of free IAA levels. For example, Bandurski and Schulze (1974) found that the levels of esterified IAA in Zea mays seedlings were approximately 330μg per kg, whilst the amount of free IAA was only 24μg per kg. With Avena seedlings, however, they detected little esterified IAA. Similarly, Bandurski and Schulze (1975) quantified IAA and its esters in several plant species, with the esterified IAA concentrations in most cases being greater than those of free IAA.

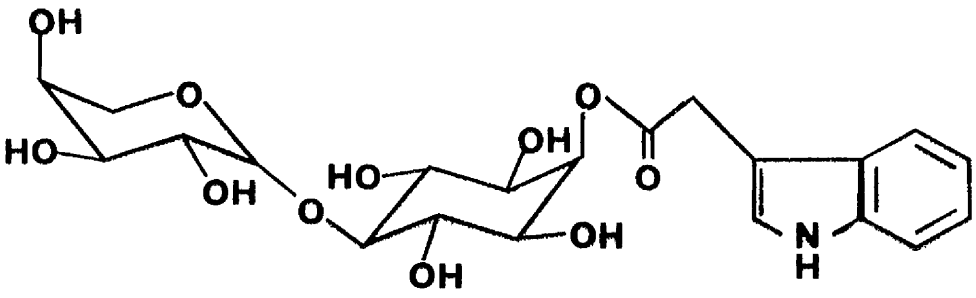
The nature of these esters has in part been elucidated by Kopcewicz et al. (1974) and Ueda and Bandurski (1974). They identified a series of inositol esters which are formed from IAA by the catalytic action of coenzyme A and ATP. Figure 3 shows the structure of these esters. Bandurski (1977) has suggested that the physiological role of these esters is three fold: "First, esterification of IAA to myo-inositol increases by 1000 fold the rate at which the hormone is transported from seed to shoot. Secondly, formation and hydrolysis of IAA esters plays a role in hormone homeostasis, thus governing the rate at which the seedling grows. Thirdly, esterification of IAA to myo-inositol protects the IAA from oxidative destruction."

Figure 3: Inositol esters of IAA

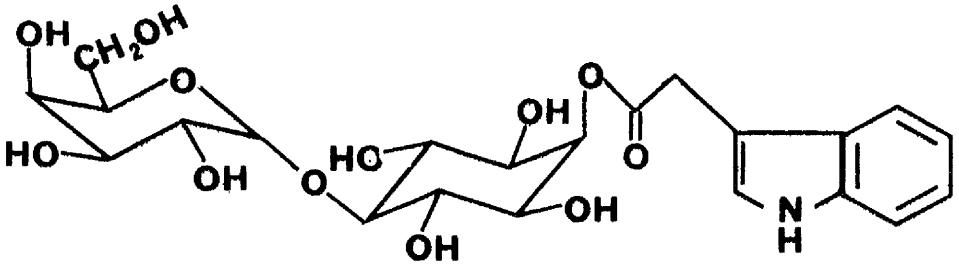
A : 5 - O - L - arabinopyranosyl - 2 - O - indole - 3 - acetyl - myo - inositol

B : 5 - O - D - galactopyranosyl - 2 - O - indole - 3 - acetyl - myo - inositol

C : 2 - O - indole - 3 - acetyl - myo - inositol


OH
O
HO
OH
O
HO
OH
O
O
OH
N
H

A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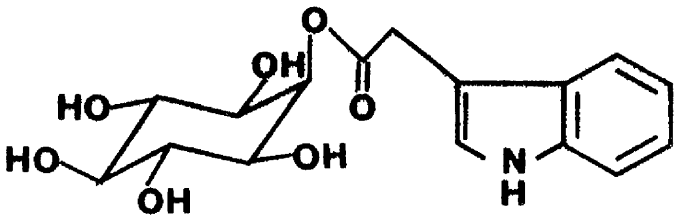
CH2OH
O
HO
OH
O
HO
OH
O
O
OH
N
H

B


O
OH
HO
HO
OH
O
O
N
H

C

A further series of esters of IAA has been identified by Feung et al. (1976) as metabolites of IAA in crown gall callus tissue. These esters were found to be IAA conjugates of aspartic acid, glutamic acid, alanine, glycine and valine; conjugation occurred as peptide linkages between the carboxyl group of IAA and the amino group of the amino acid.

Further information on the nature of "bound" IAA has come from hormone-binding experiments. For example, Batt et al. (1976) found that the binding of [^{14}C]-IAA to membrane preparations of Zea mays coleoptiles occurred at two sites, the first site being unspecific, whilst the second site was only active with auxins. Subsequently, Batt and Venis (1976) demonstrated that the first site was the plasma membrane and the second site was associated with golgi membranes and/or endoplasmic reticulum. Latterly, Ray (1977) has shown that one main binding site for auxins is located on the endoplasmic reticulum, the presence of this site not being dependent on ribosome attachment to the endoplasmic reticulum.

(C) The nature of the metabolites of IAA and their effect on growth is more complex. Schneider and Wightman (1974) examined the vast amount of evidence present on the metabolism of IAA and concluded that, "the principle product of oxidation of IAA in plants is 3-methyleneoxindole (3-MO)" and this "occurs via a series of intermediate compounds". The role of 3-MO may then be to act as the primary compound regulating the growth process. It is of interest to note that oxindoles can be formed from IAA in the absence of enzymes and presence of bisulphite and oxygen (Horng and Yang, 1975) by a free radical reaction.

(D) Auxin transport in plants is considered to take place by two types of mechanism (Goldsmith, 1977). The first system is thought to involve rapid transport of IAA in sieve tubes; evidence for this has come mainly from supplying radioactive auxin to mature leaves of the plant and observing the rate and direction of the transport. (Goldsmith

1977). Further evidence to support this view has been provided by Hall and Medlow (1974) who were able to identify IAA in phloem exudate of Ricinus communis.

The other mode of transport is a specific system for auxins, requires living cells, and can be stopped by 2,3,5-triidobenzoic acid (this compound is thought to be a membrane competitor of auxin). The route by which the IAA transport occurs is considered to be through the cells and is a function of pH at the membrane (Goldsmith, 1977).

On the basis of many experiments using the exogenous compound, IAA is observed to move throughout the plant, but transport in itself may not be necessary for growth. It is feasible that the transport of IAA or its metabolites may only occur from one intracellular location to another. In addition, care must be taken in the interpretation of many transport experiments since the methods involved often do not take into account the labile feature of the molecule (Yang and Saleh, 1973; Fujimori, 1972; Horngand Yang, 1975).

(E) Various theories are postulated for the mode of action of IAA; see reviews by Audus (1972), Thimann (1972), Davies (1973) and Schneider and Wightman (1974) ~~and Davies (1973)~~.

One proposal is that IAA acts on cell growth by increasing H^+ extrusion from cell walls, thus changing the pH of the wall which undergoes a resultant enlargement Davies (1973). However, Vanderhoef et al. (1977) has provided evidence that the extrusion of H^+ from Glycine max hypocotyls is unaffected by auxin.

Alternatively it has been suggested that IAA is metabolised to 3-MO which in turn causes the cell elongation (Tuli and Moyed, 1969). This theory has been disputed by Evans and Ray (1973) who synthesised 3-MO and found it to have no effect on the growth of pea stems or Avena coleoptiles.

Some plant hormones, among them IAA, have also been considered to

act primarily at the gene level (Key, 1969) with a resultant alteration in protein synthesis. The timing of plant responses in many cases, however, appears to refute this mode of action as being exclusive (e.g. Philipson et al., 1973).

In summary, it is thus conceivable that the mode of action may be in several ways, neither of which is mutually exclusive, the validity of these theories must await further experimental evidence.

DETERMINATION OF IAA IN PLANTS

Although much information on the metabolic routes to and from IAA in plants is available, there are surprisingly few accounts of the accurate measurement of free IAA. This crucial fact must be borne in mind when reviewing the physiology of IAA in the higher plant, since many of the conclusions that have been made are based on the application of exogenous IAA in amounts that are often well in excess of endogenous free IAA present, and therefore the observed effects may not be due to a normal physiological response but rather may reflect a pharmacological effect. In addition, several plant growth processes are thought to be the result of changes in IAA concentration or distribution, (e.g. geotropism in coleoptiles, apical dominance, abscission Thimann (1972)). The remainder of this thesis therefore will be confined to the determination of free IAA in the plant.

The methods by which IAA have been identified and quantified in plants are similar in approach. Firstly, the removal or extraction of the IAA from plant tissue; secondly, the application of various purification procedures to enable accurate identification; and lastly, the adoption of an analytical procedure by which IAA can be measured (Audus, 1972).

For the extraction of IAA fresh tissues are often used although other techniques of sample preparation such as freeze-drying and diffusion from the cut surfaces of plant parts have been used (Audus,

1972; Greenwood et al. 1972). In order to select a suitable extraction procedure, several criteria are considered to be important. Obviously all the free IAA should be extracted from the tissue; in addition, no synthesis of IAA should occur during the extraction procedure. Of the wide range of methods of extraction used for extracting IAA from plant tissue (see Weaver, 1972), the most widely quoted are those of Nitsch (1956) who employed various organic solvents, viz.: ethyl acetate, diethyl-ether, acetone and methanol; and found the latter gave the greatest extraction yields. Similarly, Hillman (pers. comm.) found highest yields of IAA from Zea coleoptiles were obtained when methanol was used for extraction as compared ~~to~~ WITH several other organic solvents. The conventional method is to immerse the tissue in the solvent for a suitable time period following which, with photosynthetic tissue, degreening of the material results, presumably indicating plastid breakdown.

Some synthesis of IAA from TPP during ether extraction of Pinus phloem and cambial tissue was observed by Whitmore and Zahner (1964). Similarly, Atsumi et al. (1976) claimed that transamination of TPP to IPyA and its subsequent decarboxylation to IAA could occur during methanolic extraction of plant tissue, thus leading to enhanced yields of IAA after extraction. It is therefore possible that IAA synthesis might occur during extraction but the situation obviously requires further investigation.

The possibility of contamination of plant extracts cannot be discounted since bacteria have been shown to be capable of synthesising a compound with similar chromatographic properties to IAA (Libbert et al., 1966); it therefore appears to be desirable to take precautions to exclude contamination from foreign material.

Following the extraction of IAA, various purification procedures have been employed (Audus, 1972), the purpose of these procedures being

to reduce (i) the total sample volume from several litres to a convenient size for analysis of several microlitres (Bandurski and Schulze, 1974); (ii) the degree of contamination of the IAA-containing sample and hence prevent other compounds interfering with the analysis (Eliasson et al., 1976). The procedures by which these have been achieved are commonly the application of an initial solvent partitioning stage with the subsequent use of chromatographic methods (Weaver, 1972).

Solvent partitioning usually involves the acidification of the IAA -containing fraction and mixing this fraction with an immiscible solvent often diethyl-ether, whereupon the ~~acidified~~ non-dissociated IAA dissolves in the ether layer. The total volume of the ether layer can then be conveniently reduced to a minimum, with the recovery of IAA from such a process being in excess of 90% (e.g. Mann and Jaworski, 1970).

Following the use of this preliminary purification procedure the IAA fraction - ideally now in a small volume - can be subjected to a variety of chromatographic procedures to purify the fraction further. These methods are common to most micro-analytical techniques (Vanden Heuvel and Zacchei, 1976).

Various column chromatographic methods have been employed in purifying IAA extracts. Glenn et al. (1972) used polyvinylpyrrolidone, whilst De Yoe and Zaerr (1976b) used silica gel in purifying plant hormone samples. Ion-exchange resins, notably DEAE-cellulose, have also proved useful in purifying IAA extracts (Elliott and Greenwood, 1974; Bridges et al., 1973; White et al., 1975; Robertson et al., 1976). Similarly, Sephadex gels were utilised by Steen and Eliasson (1969) and Bandurski and Schulze (1974). A high-performance liquid chromatography system (HPLC) has recently been described by Crozier and Reeve (1977) which employs the dual capability of both high sample capacity and high resolution.

Subsequent to or instead of column chromatography, a TLC stage has been adopted as a purification method for plant extracts. In the TLC of

IAA plant samples, a large variety of solvent systems have been tested (e.g. Gibson et al., 1972; and Stahl, 1965), nevertheless, little information is available on their relative efficiencies in purifying IAA from extracts of green tissue.

In recent times, a final purification step using GLC is often used, but as this chromatographic system is often run in conjunction with, or as an identification procedure, mention is made of this method later on p 16.

During these purification procedures it is inevitable that some IAA will be lost, thus making the accuracy of measurement impossible, unless some method is used to take account of these losses. One such method is to add an aliquot of a radioactive isotope of IAA at the onset of these procedures so that an assessment of the losses involved in the purification of the sample can be made at the completion of the procedure (Mann and Jaworski, 1970).

Once a suitably pure IAA fraction is obtained, an analytical method is applied in order to measure the "levels" of IAA present. In choosing any analytical system, several general considerations must be borne in mind:-

(i) sensitivity and detection limit of the method
(ii) precision or accuracy of the method
(iii) resolution or specificity achieved
(iv) effect of impurities on the system

(Ewing, 1975)

During the 1950's and 1960's the greatest proportion of measurements of IAA in plants relied on the use of a bioassay procedure (Thimann, 1972). These systems rely on the use of various plant parts that react in a particular growth mode to IAA. Several main objections can be listed against the use of bioassay systems to measure IAA, particularly (a) auxin bioassay systems react to other growth hormones as well as IAA

(Audus, 1972); (b) even if IAA is present in the sample, the resultant growth effect may be due to conversion of IAA to another compound that has growth promoting effects; (c) conversely, IAA may not be present but a precursor, e.g. TPP that is present may be converted by the tissue to IAA (Thimann, 1972); (d) substances, e.g. 4-chloro-indole -3-acetic acid may mimic the biological effects of IAA (Marumo et al., 1968a); (e) inhibitors or promotors may be present altering the effect of IAA on the bioassay.

Following the demise of the bioassay, there have been an increasing number of techniques employed to measure IAA; these are listed in Table 2 with their detection limits.

Table 2: Analytical methods for IAA

METHOD	DETECTION LIMIT (g)	REFERENCE
Specific dyes	12.5×10^{-9}	Tirimanna and Geevaratne 1972
High resolution chromatography	1×10^{-12}	Ewing, 1975
Spectrophotofluorometry	1×10^{-9}	Eliasson et al., 1976
Infra-red spectrometry	1×10^{-7}	Ewing, 1975
Mass spectrometry	1×10^{-11}	Ewing, 1975

Various dyes specific for indoles can be used to locate IAA on chromatograms; three types of these reagents in particular have been described in the literature: Salkowski's reagent, which is a solution of Ferric salts in hydrochloric acid, and reacts with IAA to give a purplish colour (Thimann, 1972); Ehrlich's reagent consisting of a solution p - dimethylaminobenzaldehyde in HCl and gives a pink colouration with IAA (Thimann, 1972); and Ehmann's reagent which is a modification of Salkowski's and Ehrlich's reagent and reacts with IAA to produce a violet colouration (Ehmann, 1977). The obvious disadvantage in using these reagents is that similar reactions are obtained with most

3-substituted indoles, thus identification must also rely on the ability of the chromatogram to resolve IAA from other indoles.

Two common methods of high resolution chromatography have been used in analysing IAA samples, these are GLC and HPLC.

Prior to GLC analysis, IAA is normally converted to a suitably stable derivative and various types of derivatives are available (Drozd, 1975). The methyl ester of IAA has been used by Bayer (1969) in its analysis in Nicotiana. Similarly, Wightman (1977) has described the use of GLC, using methyl esters, in analysing indole acids, among them IAA in various plant tissues. A disadvantage in using this type of ester is that its use is governed by the fact that it is sensitive to a flame-ionisation detector (FID) but does not show electron-capture properties. Thus the level of methylated IAA that can be detected is commonly only 10 to 100 ng (Powell, 1972). Powell (1972) has pointed out however, the possibility of using alkali-FID which has a higher sensitivity level than that of FID.

Increased detection of IAA by GLC has been attempted using an electron-capture detector (ECD) instead of FID. The ECD detector has an increased sensitivity when compared with FID (Powell, 1972). Brook et al. (1967) demonstrated that methylation of IAA followed by N-trifluoroacetylation resulted in a product that could be measured in the range of circa 100 pg by the ECD. Similarly, Seeley and Powell (1974) prepared the heptafluorobutyryl (HFB) derivative of IAA after initial methylation, and observed an ECD limit of sensitivity of 100 pg. Further attempts have been made by Bittner and Even - Chen (1975) to increase the sensitivity of IAA to ECD by preparing the tri-chloroethyl ester of IAA; however, on application of this derivative to plant samples, the degree of contamination was such that the accurate identification of IAA became impossible.

Analytical HPLC systems have also been used in the analysis of

IAA samples. Bausher and Cooper (1974) used a HPLC system employing a U.V. monitor to detect IAA in xylem sap of Citrus rootstocks. Similarly, Durley and Kannangara (1976) detected IAA in extracts of Sorghum leaves using HPLC.

The disadvantage of the above type of system is the lack of specificity of the chromatographic detector to IAA, thus making the unequivocal identification of IAA impossible (Ewing, 1975).

Another technique employed in the measurement of IAA is that of U.V. spectrophotofluorimetry. Studies by Burnett and Audus (1964) demonstrated that IAA could be identified by its activation and fluorescence properties in U.V. light. By employing this method, they were able to demonstrate that indole-3-acetonitrile and possibly IAA are constituents of white cabbage. Stowe and Schilke (1964) described a method in which GLC was used to purify plant extracts prior to fluorometric analysis. They were able to detect IAA in the 10 ng to 1µg range. Using a similar method Powell (1964) was able to identify IAA and IAN in maize and cabbage respectively.

The fluorescence spectrum of IAA is 285nm (activation maxima) and 365 nm (fluorescence maxima); unfortunately most indolic compounds have similar spectra (Burnett and Audus, 1964). They recommended that the fluorescence spectra of IAA must also be taken with several other properties of IAA, such as the pH effect on the fluorescence spectra and the chromatographic properties, if accurate identification of IAA is required.

In an attempt to overcome some of these difficulties, Stoessl and Venis (1970) demonstrated that if IAA were converted to indolo-α-pyrone (specific reaction) and then measured by fluorimetric analysis, then many of the above effects were negated. Nevertheless, as they point out, 5-hydroxy IAA reacted in a manner similar to IAA, and they did not test the reaction of any other 4 or 5 substituted IAA compounds.

A further disadvantage of this method is that samples for analysis must be relatively pure, this in itself presents considerable problems (Eliasson et al., 1976). Also, fluorescence spectrometry measurements of IAA must be extremely precise with several factors affecting them, viz.: (i) temperature (Stowe and Schilke, 1964), (ii) solvent concentrations (Kawski and Czako, 1974) and (iii) pH values (Burnett and Audus, 1964).

Infra-red (IR) spectroscopy is a technique that has been used to identify IAA in plants. Powell (1967) demonstrated that IR spectra could be obtained from microgram quantities of IAA. Marumo et al. (1968a) identified 4-chloro-IAA in seeds of Pisum sativum on the basis of IR studies. The disadvantage of the use of IR spectra is, however, that usually a few micrograms of the substance must be available for identification (Powell, 1972). These amounts of IAA required are usually too high for routine analysis of plant tissue; nevertheless, the technique of IR has proved useful in identifying novel auxins in plants (e.g. Hattori and Marumo, 1972; and Abe et al., 1972).

Mass spectrometry (MS) has been increasingly used in the last few years as a means of identifying and quantifying compounds in biological extracts (Games, 1975; Palmér and Holmstedt, 1975). By use of a MS it is possible to characterise a compound on the basis of its molecular weight and fragmentation pattern (Bonelli et al., 1975). Moreover, direct measurement or quantification of the compound is possible using techniques such as selective ion detection (SID). SID studies are carried out using a combined gas chromatograph-mass spectrometer (GC-MS), where the MS acts as a highly specific detector for the GC. Briefly, the method is to tune the MS to particular fragment ions that are highly characteristic of the compound, and then to monitor the GC effluent by the MS. The presence of the compound in question is indicated by a MS response at the GC retention time of the compound.

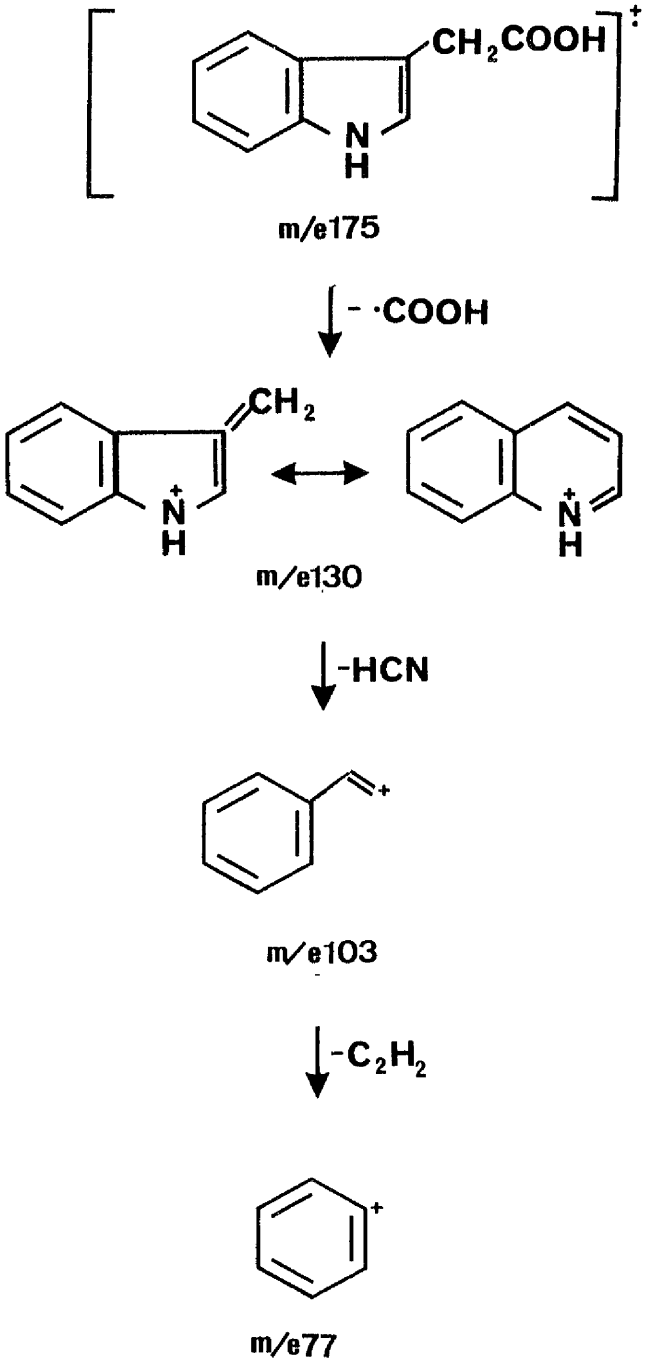
It is obvious that a SID trace for each ion will have an appearance similar to that of a conventional GC trace (Palmér and Holmstedt, 1975).

The electron-impact mass spectrum of indole consists mainly of the molecular ion as the basepeak (most abundant fragment ion) with very little fragmentation occurring. The introduction of alkyl substituents into the indole system at the 3-position results in α,β side chain cleavage giving 3-methylene indole as the base peak. It is thought that this ion (m/e 130) undergoes ring expansion to the quinolinium cation (Jamieson and Hutzinger, 1970; and Porter and Baldas, 1971). Thus the mass spectrum of IAA reveals an ion of m/e 175 as the molecular ion which fragments to give the m/e 130 ion. Fragmentation of this ion then occurs by loss of HCN resulting in an ion of m/e 103 with further loss of C_2H_2 yielding an ion of m/e 77 (Abe and Marumo, 1974; and Spiteller, 1971). Figure 4 shows the structure of these ions.

The identification and quantification of IAA in plants has been undertaken in two main ways. Firstly, by the direct insertion or direct probe of the compound into the MS and then the comparing the resultant spectrum with that of authentic IAA. This technique was applied by Greenwood *et al* (1972) to identify IAA in *Zea mays* coleoptiles. Similarly, Abe *et al.* (1972) identified IAA in the alga *Undaria* and Takahashi *et al.* (1975) identified IAA in young fruits of *Citrus*. Secondly, the plant extract is purified by GC prior to MS analysis by a GC-MS system. Analysis of the components resolved by the GC can be made by either or both of the following: (i) a full spectrum giving the detailed fragmentation pattern of each compound in the GC effluent (Middleditch and Desiderio, 1973); (ii) SID techniques; this method also enables direct quantification of the compound as the peak area of the compound in the SID trace is proportional to the amount of compound in question (Vanden Heuvel and Zacchei, 1976).

Elliott and Greenwood (1974) claim to be the first to use GC-MS to

Figure 4 : Mass spectral fragmentation of IAA



identify IAA as the auxin in plant material, in this case, in roots of Zea mays. At the same time, however, Bridges et al. (1973) identified and quantified IAA in the ste les, cortices and tips of primary roots of Zea mays by GC-MS.

The derivative used for the GC-MS studies by Bridges et al. (1973) was the trimethylsilyl derivative of IAA (TMSi-IAA). This derivative has also been used by White et al. (1975) to identify IAA in lateral buds of Phaseolus vulgaris; Hall and Medlow (1974) to measure IAA in phloem and root pressure sap; Bandurski and Schulze (1975) to identify IAA in Avena, Pisum and Zea; Shindy and Smith (1975) to identify IAA in cotton ovules; and Hillman et al. (1977) to quantify levels of IAA in lateral buds of Phaseolus vulgaris; all these analyses utilised GC-MS facilities.

Another derivative that has been used in GC-MS studies is the HFB derivative of IAA-Me. This was initially used by Bertilsson et al. (1972) to identify IAA in human cerebrospinal fluid. Later the same derivative was used by Rivier and Pilet (1974) to identify and measure IAA in the cap and apex of Zea mays roots.

The trifluoracetyl derivative of IAA-Me has been employed by Hopping and Bukovac (1975) to identify IAA in seeds of Prunus cerasus by GC-MS.

Recently, use has been made of pentafluoropropionic acid (PFPA) to acylate various indole acids and amines (Gelpi et al., 1974). This type of derivative was also used by Miyazaki et al. (1974) in GC-MS studies of various indole acids including IAA. The MS in this case was a chemical ionisation - mass spectrometer. It should be noted that the level of sensitivity of detection of norepinephrine using this method was 1 pg.

Thus in summarising the various analytical techniques at present used in IAA analysis, GC-MS appears to be the only method that is capable of high sensitivity, high resolution and high specificity; however, little information is present on the effect of impurities on

GC-MS analysis.

IAA MEASUREMENT IN SHOOT TISSUE

Since IAA is thought to control the growth of the shoots and roots of higher plants (Wareing and Phillips, 1970; Thimann, 1972; Phillips, 1975; Torrey, 1976) it is obviously necessary to ~~accurately~~ accurately measure the distribution and content of IAA in plant tissue.

The majority of GC-MS analyses of IAA has been carried out on plant tissue where the content of IAA would appear to be relatively high and the degree of pigmentation low, viz.: coleoptiles (Greenwood et al., 1972); seedling roots (Bridges et al., 1973; Rivier and Pilet, 1974); shoot apices (White et al., 1975); lettuce fruits (Robertson et al., 1976); and lateral buds (Hillman et al., 1977). The application of GC-MS techniques to the analyses of IAA in tissues such as green stems and senescing leaves has been difficult as a result of the impurity or the contamination of the extracts. Indeed, attempts using spectroscopic methods (Tillberg, 1974) and GC-MS methods (White, 1973) to analyse levels of IAA in Phaseolus vulgaris shoot tissue have proved unsuccessful.

The studies outlined in this thesis describe firstly, the development of a purification procedure and an analytical method to facilitate the routine measurement of IAA in extracts of P. vulgaris shoot tissue; secondly, the application of such procedures to determine the distribution of IAA in foliar and stem tissue of P. vulgaris.

GENERAL EXPERIMENTAL MATERIALS AND METHODS

In view of the fact that much of the experimental part of this thesis is devoted towards the development of analytical procedures, the substance of this section is confined to general methods common to a majority of the experiments.

(1) Plant Material

Seeds of Phaseolus vulgaris L. c.v. Canadian Wonder were purchased from Charles Sharpe and Co. Ltd., Sleaford, England, and stored at 4°C prior to sowing. When required, seeds were sown in John Innes No. 1 compost mixture in batches of 50 per box and left to germinate in the glasshouse. The plants were grown until the second trifoliate leaf had just attained the grand period of growth (White, 1973). Plate 1 shows the average plant type, whilst Plate 2 shows the comparative sizes of the leaves at the harvesting stage. At this stage the plants were harvested with care being taken to ensure that only vegetative plants were employed.

(2) Harvesting

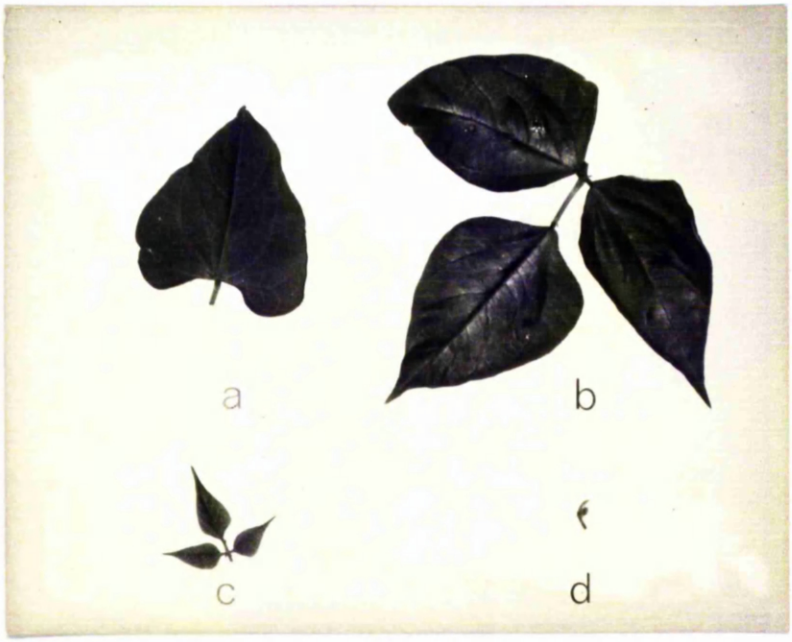
In "whole shoot" experiments the stems were excised through the base of the hypocotyl 5 cm above soil level and then divided into approximately 2 cm portions for extraction. In "foliar distribution" experiments, the laminae and petioles were detached at the pulvinar junction with the stem, with care being taken to ensure that no lateral buds were removed. For "stem distribution" experiments a 1 cm section of stem in the middle of a specific internode was removed with a clean razor blade on a plate-glass bed. In both types of distribution experiment the apex was also detached.

(3) Chemicals

Technical grade methanol and anhydrous diethyl ether were used as solvents for the major extraction experiments. They were supplied

Plate 1 : Typical Plant Material used for Extraction

Plate 2 : Typical Leaf Size of Plants at Harvest SCALE 1:2·5



a: Primary Leaf
c: 2nd Trifoliate Leaf
b: 1st Trifoliate Leaf
d: Apex

in bulk from several suppliers and redistilled prior to use, from and into acid-washed glassware.

Indole-3-acetic acid (Sigma London Chemical Company Ltd., Surrey) and L-tryptophan (B.D.H. Chemicals Ltd., Poole) were stored in darkness at 4°C in vacuum desiccators.

N,O-bis-(trimethylsilyl)-acetamide (BSA), N,O-bis-(trimethylsilyl) -trifluoroacetamide (BSTFA) and heptafluorobutyrylimidazole (HFBI), (Peirce and Warriner Ltd., Chester) were stored at 4°C, 4°C and -15°C respectively.

Other solvents and special grade reagents are discussed under the relevant section.

(4) General Extraction Procedures

During harvesting each fraction was weighed and then placed in methanol at intervals; at the completion of harvesting the volume of methanol was adjusted such that 1 g fresh weight of tissue was extracted in greater than 5 ml of methanol. The extracts were then placed in a deep freeze for 24 h at -15°C in darkness. Whenever necessary the details of tissue weights and solvent volumes are tabulated in each experimental section.

Each extraction experiment had a control or solvent blank carried out in tandem. This consisted of 25 ml of distilled water in 3 l of methanol, and was subjected to precisely the same procedures as those described for the plant materials.

(5) Chromatographic Methods

(a) Column Chromatography - The column support materials used were Sephadex LH20 (Pharmacia (Great Britain Ltd.), London), DE1 and DE23 (Whatman Biochemicals Ltd., Kent) and Polyclar AT (Gaf Great Britain Ltd., Manchester).

The method used in determining the relative effectiveness of each column system was as follows: a plant extract was prepared by

extracting 10 g of trifoliate leaf tissue in excess absolute methanol for 16 h in the dark at -15°C. The methanol extract was subsequently filtered and reduced to an aqueous fraction at 36°C on a rotary evaporator. The pH of the aqueous fraction was then adjusted to 3.0 by the addition of 2 N HCl and subsequently partitioned against equal volumes of diethyl ether three times, the ether layers were then collected and combined. This ether fraction was then left for 4 h at -15°C following which excess water was removed as ice by Büchner filtration. The ether fraction was then reduced to a dry residue by rotary film evaporation (RFE) and redissolved in methanol. It was in this form that the plant extract, together with an aliquot of [^{14}C]-IAA, was applied to the column. Thereafter the residual volume of the column was filled with eluent and a reservoir was attached to the column such that a constant volume of eluent was present above the column support material. The column eluate was collected on a L.K.B. fraction collector via a drop-counter attachment.

The absorbance between 450 and 850 nm of each fraction was subsequently determined on a Unicam SP.8000 Spectrophotometer. Similarly, the radioactivity of each fraction was assessed by liquid scintillation counting; the nature of the scintillant employed was dependent on the type of column eluent. Using these measurements the elution profile of the [^{14}C]-IAA and pigments contained in the plant extract were constructed for each column system.

The percentage recovery of [^{14}C]-IAA from each system was gauged by determining the mean dpm of three identical aliquots of the [^{14}C]-IAA applied to the column, assessing the total dpm present in the eluate (only those fractions whose dpm $>$100 were taken into account) and then calculating the % [^{14}C]-IAA present in the eluate as compared to the original mean dpm of [^{14}C]-IAA applied to the system.

(b) Thin-layer Chromatography - All chromatograms were developed at a constant temperature of 15°C in darkness. IAA was applied to

TLC plates as spots of 400 ng in 2 µl of methanol. Location of the IAA on TLC plates after development was achieved by spraying the plates with Ehrlich's reagent (0.1 g para-dimethylaminobenzaldehyde in 10 ml 2N HCl) and heating the plate, whereupon IAA reacted to give a blue colouration; or, alternatively, by examining the TLC plate under a UV_{254} light, with the position of IAA being indicated by a blue spot.

The procedure for testing the relative effectiveness of each thin -layer system was the same throughout. Initially a pigment extract was prepared as described for column chromatography methods. This was applied in 3 separate spots each with an aliquot of IAA in order that the plant extract and IAA were co-chromatographed. The TLC plate was then developed for 10 cm in the appropriate solvent system, following which the Rf values of the components of the plant extracts were noted after examining the plate under visible, $UV_{254\ nm}$ and $UV_{360\ nm}$ light. After this the position of IAA was located as above. Where complete resolution occurred between the detectable components of the plant extract and IAA the system was deemed successful.

The cellulose plates used were 20 x 20 cm pre-prepared Polygram Cel 200 with a 0.1 mm layer of cellulose (MN 300), and the silica gel plates were pre-prepared 20 x 20 cm Sil G-UV^{254} with a 0.25 mm layer of silica gel (Camlab, Cambridge).

(c) Gas-liquid Chromatography - A variety of GLC systems have been used, two were in conjunction with mass spectrometers and a third with a radioactivity monitor; these are discussed under their relevant sections.

For routine analysis of derivatives of IAA, a Pye 104 GLC equipped with a flame ionisation detector (FID) was used; and for electron capture studies a Pye ECD-GC with an electron capture detector (Ni^{63}) was employed.

A range of column systems were used; in the main however the gas

chromatographically equivalent non-polar phases OV-1, OV-101 and SE-30 coated on Gas Chrom Q (GCQ) or Chromosorb W in varying concentrations have been employed. The exact nature of the column and GLC operating conditions are summarised at each experiment.

(d) Resolution - Resolution is a measure of the degree of separation of adjacent compounds that have been eluted from a given chromatographic process. Where the response of two adjacent compounds from such a process gave rise to peaks that had Gaussian distributions, then the resolution between these compounds has been calculated, after triangulation, according to the following formula:

$$R = \frac{2\triangle x}{YA + YB}$$

where $\triangle x$ is the distance between the peak maxima; YA and YB are the relative peak widths at their bases; and R is the degree of resolution

(6) Mass Spectrometry

Two MS instruments have been used, both are double-focusing single-beam electron-impact instruments with directly coupled gas chromatographs.

In the main, an AEI MS-30 mass spectrometer with an on-line Pye 104 GC was used. The separations of sample mixtures on the GC were made on silanized glass columns usually packed with OV-101 or SE-30 on GCQ (100-120 mesh size). The GC-MS interphase consisted of a silicone -rubber membrane usually maintained at 220°C with a source temperature of 230°C and an ionizing voltage of 70 eV. Where these conditions differ they are described under the appropriate experimental section.

For quantitative studies using the AEI MS-30, an AEI Multipeak Selectro WF-055 (multipeak monitor) acted as an accelerating voltage alternator. This facility enabled the MS to act as a specific detector for the GC such that selective ion detection (mass fragmentography, selective ion current monitoring, multiple ion monitoring,

multiple peak monitoring) studies could be carried out.

In some studies a Jeol JMS-D100 mass spectrometer was used. The GC separations were performed on a Jeol JGC-20 K gas chromatograph linked to the MS via a metal double jet separator maintained usually at 193°C. The GC column consisted of a 1 m glass column packed with 3% OV-1 on Chromosorb W with separations being obtained by oven temperature programming from 100 - 200°C at 15° min^{-1}, 60 sec after injection.

An on-line Instem Data Mass Maxi Computer was available for data acquisition; and calibration of the overall system was achieved during sample runs by allowing the MS to scan samples of perfluorokerosene (PFK) and comparing the scan produced by the computer with a known spectrum of PFK.

(7) Radioassay

(a) Radiochemicals - The [^{14}C]-IAA used in all the experiments other than the "rotary evaporation" experiment was 3-indolyl [1-^{14}C] acetic acid (Radiochemical Centre, Amersham) with a specific activity of 294 μCi mg^{-1}. The sample total activity was 50 μCi and this was received in a solution of 400 μl of benzene (containing 10% of acetone) in a sealed glass ampoule. On receipt the ampoule was opened, the solution transferred to a 10 ml glass vial and the ampoule washed with benzene. The solvent was then removed under a light stream of nitrogen and the residue taken up in 5 ml of methanol such that 10 μl of this mixture contained approximately 0.1 μCi. The [^{14}C]-IAA was used in this concentration for all the following experiments except the TLC efficiency experiments. For these experiments an aliquot (100 μl) of the above solution was diluted with methanol such that 5 μl of the solution contained approximately 1,000 dpm of [^{14}C]-IAA. In order to assess the amount of [^{14}C]-IAA added to various extracts, three identical aliquots were subjected to liquid scintillation counting,

and the mean of these three values was taken as the true amount of [^{14}C] -IAA added initially.

From time to time a purity analysis of the above solutions was carried out by radio-TLC in two solvent systems ([methyl acetate: propan-2-ol: 25% ammonia; 45 : 35 : 20 : v/v] and [propan-2-ol: 0.88 ammonia: water; 10 : 1 : 1 : v/v]) to ensure that the sample was suitable for use. At no time was the radiochemical purity found to be less than 92%.

For the "rotary evaporation" experiments the [^{14}C]-IAA was a second stock of 3-indolyl [1-^{14}C] acetic acid which had been diluted to give a total activity of 50 μCi in 1 ml of methanol.

All of the above [^{14}C]-IAA solutions were stored at 4°C in darkness.

For the "extraction-metabolism" experiment, L-[5(n)-^{3}H]-tryptophan (Radiochemical Centre, Amersham) was used. This had a specific activity of 25 Ci mmol^{-1} and was supplied in ethanol : water (1 : 1; v/v) solution in a sealed rubber-capped multidose vial. This solution was stored in darkness at -15°C until ready for use. The radioactivity concentration in which this sample was received and used, was 1 μCi μl^{-1}.

(b) Liquid Scintillation Counting - Total radioactivity in samples was measured by Packard Tricarb scintillation spectrometers which determined the radioactivity present in each sample as cpm, with an AES ratio which indicated the quench level present in each sample. The efficiency of counting is governed by the quench of each sample. This efficiency was obtained by constructing quench correction curves for [^{14}C] and [^{3}H] compounds. These curves were constructed from the cpm found in vials containing n-[1,2(n-^{3}H)] hexadecane (specific activity 2.27 μCi g^{-1}) or n-[1-^{14}C] hexadecane (specific activity 0.509 μCi g^{-1}), (Radiochemical Centre, Amersham) in the appropriate scintillation fluid at various quench levels as indicated by the AES ratio. The degree of quenching in these standards was varied

by adding different amounts of plant extracts. Thus, by knowing the cpm and true dpm of these standards, the % counting efficiency of the particular isotope at a known quench level was calculated. Quench correction curves were constructed for each scintillation fluid and isotope.

For the actual estimation of radioactivity present in samples, aliquots of these samples were placed either in glass or plastic acintillation vials and a volume of the appropriate scintillation fluid was added. Where the sample was present in an organic solvent, the solvent was first removed in vacuo and the scintillation fluid added. Table 3 shows the scintillation fluid used with the sample type.

Table 3: Scintillation fluid used with a particular sample type

SAMPLE	SCINTILLATION FLUID
Dry residue	10 ml of 4 g of PPO (2,5 diphenyloxazole) l^{-1} of toluene OR 10 ml of 2 : 1 mixture of 4 g of PPO l^{-1} toluene : Triton x-100 (v/v)
Aqueous (1 or 5 ml volume)	10 ml of 2 : 1 mixture of 4 g of PPO l^{-1} toluene : Triton x-100 (v/v)
Aqueous (1 - 5 ml volume)	10 ml of Unisolve 1 (Koch Light Laboratories)
TLC Plate scraping	1 ml of 50 : 50 mixture of methanol : diethyl ether (v/v); plus 10 ml of 2 : 1 mixture of 4 g of PPO l^{-1} toluene : Triton x-100 (v/v)

(c) Radio-Thin-layer Chromatography (radio-TLC) - The estimation of the distribution of radioactivity present on TLC plates after development in the appropriate solvent was by two main methods: (i) the plate support was divided into equal zones, which were then scraped off and placed in scintillation vials to which was added the appropriate scintillation fluid (see Table 3) and the dpm of each zone was then determined by liquid scintillation counting. A complete radio-TLC profile was then constructed according to the dpm and Rf of each zone.

(ii) Radio-TLC was also determined by scanning the TLC plates with a Panax TLC radiochromatogram scanner. This scanner utilised a fixed Geiger counter with a 2% propane/98% argon carrier-gas mixture as the radioactivity detector. The detector was in a fixed position and the TLC plate to be scanned was placed on a moving stage such that a minimum distance between the TLC plate and detector was attained. As the TLC plate moved forward the radioactivity present was detected and portrayed on a flat bed recorder. Thus a trace of the cpm present throughout the TLC plate was constructed automatically.

Radio-TLC was used to gauge the % purity of radioactive isotopes by chromatographing adjacent spots of non-radioactive IAA with the radioactive sample. The cpm (as obtained by triangulation from the radio-TLC scan) equivalent to the Rf zone of the IAA reference spot was then taken as the value of radioactivity present as [^{14}C]-IAA, and by calculating the cpm of other compounds on the radio-TLC trace, the % purity of [^{14}C]-IAA in terms of total radioactivity present could be gauged.

(d) Radio-Gas-Liquid Chromatography (radio-GLC) - The radio-GLC apparatus consisted of a Perkin Elmer F11 gas chromatograph equipped with a stream splitter such that 90% of the column effluent was directed to a Packard gas proportional counter (Model 894) via a heated glass line, and 10% to the FID detector. Separations on the GC were achieved using a 5 ft silanized glass column packed with 5% OV-101 stationary phase coated on GCQ (80-100 mesh size) with nitrogen as the carrier gas.

Once eluted from the gas chromatograph the [^{14}C] samples in the gas proportional counter were converted to carbon dioxide in a quartz combustion tube containing copper oxide. Following this combustion, hydrogen gas was added to the flow (this reduced the hot iron in a second furnace where [^{3}H] samples that had previously been converted

to $[^3H]_2O$ in the copper oxide furnace were reduced to tritium gas). After passing through a water trap, the effluent of these furnaces was mixed with a quench gas (propane) prior to radioactivity estimation in the proportional tube. The record of radioactivity present resembled that of a conventional FID-GC detector. The radioactivity and FID traces were recorded simultaneously on a dual pen recorder.

The counting efficiency of this radioactivity monitor was gauged by chromatographing aliquots of bis-TMSi-[^{14}C]IAA and [^{14}C]-methyl palmitate. The net cpm of the resultant radio peak (which had an identical T_R value to a non-radioactive standard) was equivalent to the area of the peak as calculated by triangulation (peak height after background cpm correction x width at half height). From this net cpm value, and knowing the true dpm of the sample, the counting efficiency of the monitor was calculated according to the following formula:

$$\text{Percentage counting efficiency} = \frac{\text{net cpm x 100}}{\text{sample dpm x 20/CFR}}$$

where CFR refers to the combined flow rate which was the summed total of the carrier gas, hydrogen and make-up gas flow rates; 20 is the volume of the proportional flow tube (ml).

During analyses, an internal standard of 10 µg of non-radioactive bis-TMSi-IAA was co-injected to obviate column adsorption and to ascertain injection efficiency.

(8) Statistical Analysis

Linear and curvlinear regressions were calculated routinely for standard-response relationships on a Hewlett-Packard HP67 programmable calculator using pre-constructed programmes. Two types of regressions were commonly used, namely:

(i) Linear regression - This type of regression was calculated according to the formula $y = a + bx$ where y and x were two variables

and

$$a = \left[\frac{\sum yi}{n} - b\frac{\sum xi}{n}\right]$$

$$b = \frac{\sum xi\ yi - \frac{\sum xi \sum yi}{n}}{\sum xi^2 - \frac{(\sum xi)^2}{n}}$$

The coefficient of determination (r^2) was calculated from the following formulae:

$$r^2 = \frac{\left[\sum xi\ yi - \frac{\sum xi\ \sum yi}{n}\right]^2}{\left[\sum xi^2 - \frac{(\sum xi)^2}{n}\right]\left[\sum yi^2 - \frac{(\sum yi)^2}{n}\right]}$$

(ii) Power curve - The relationship of $y = ax^b$ (a power curve) for two variables y and x was calculated using the following formulae:

$$a = \exp\left[\frac{\sum \ln yi}{n} - b\frac{\sum \ln xi}{n}\right]$$

$$b = \frac{\sum (\ln xi)(\ln yi) - \frac{(\sum \ln xi)(\sum \ln yi)}{n}}{\sum (\ln xi)^2 - \frac{(\sum \ln xi)^2}{n}}$$

In addition, the coefficient of determination (r^2) for the power curve was simultaneously calculated from the following relationship:

$$r^2 = \frac{\left[\sum (\ln xi)(\ln yi) - \frac{(\sum \ln xi)(\sum \ln yi)}{n}\right]^2}{\left[\sum (\ln xi)^2 - \frac{(\sum \ln xi)^2}{n}\right]\left[\sum (\ln yi)^2 - \frac{(\sum \ln yi)^2}{n}\right]}$$

RESULTS

SECTION A : The Purification of P. vulgaris Shoot Extracts

This Section describes those experiments concerned with the development of a reliable purification procedure to enable the quantitative analysis of IAA in extracts of P. vulgaris shoot tissue.

(1) The Extraction and Preliminary Purification of Shoot Extracts

(a) Extraction Procedure

Since Atsumi et al. (1976) and Kuraishi (1976) claimed that IAA could arise as an artifact during the methanolic extraction of plant tissue, and because methanol would appear to be the most suitable solvent for extraction (Nitsch, 1956 and Hillman, unpublished results), an initial experiment was designed therefore to determine if, in fact, conversion of tryptophan to IAA could occur during methanolic extraction of P. vulgaris shoot tissue.

Three batches of 50 g of shoot tissue were harvested and placed in 500 ml of methanol. A control consisting of 20 ml of distilled water and 500 ml of methanol was also prepared. Both the plant extracts and the control were then subjected to the following procedure: an aliquot of 10 µl of [^{3}H]-tryptophan solution was added to each sample, following which the samples were left at -15^{o}C in darkness for 24 h. Each sample was then filtered through Whatman No. 1 paper with the shoot residues being re-washed with a further 500 ml of methanol and filtered as above. The combined methanolic fractions were then reduced to an aqueous residue in vacuo. The aqueous residue was then taken up in 250 ml of water and the pH altered to 3.0 by the addition of 2 N.HCl. The acidified fractions were then partitioned against equal volumes of diethyl ether three times with the ether layers being collected and reduced to dryness by RFE. The dry residues were then taken up in methanol to a volume of 10 ml.

Aliquots (100 µl) of each sample were then subjected to analysis by chromatographing each sample independently with two adjacent marker spots (2.5 µg) of tryptophan and IAA (applied in solutions of water and methanol respectively) on Sil G thin layer plates. Each sample was chromatographed in solvent system (a) propan-2-ol : ammonia : water (10 : 1 : 1; v/v) and (b) methyl acetate : propan-2-ol : ammonia (45 : 35 : 20; v/v). Following development, the position of the IAA and tryptophan reference spots were noted and the distribution of radioactivity in the chromatogram of the sample was determined by dividing the plate into 10 equal zones for subsequent liquid scintillation counting, as described under "Materials and Methods".

The radio-TLC profiles obtained are shown in Figures 5 and 6, with the positions of the reference spots of IAA and tryptophan (TRy) being indicated by bars.

In two samples (B and C) a small peak of radioactivity was observed using solvent system (a) that had a Rf value equivalent to that of IAA. This pattern, however, could not be confirmed when samples B and C were chromatographed in solvent system (b). In fact, only two significant areas of radioactivity were present on the chromatograms of each plant sample (in addition, the control (D) contained only one main peak which was always proportionately greater than the equivalent peak in the sample extracts), neither of which had a similar Rf to IAA. It was concluded that no evidence was found to confirm the proposition that conversion of tryptophan to IAA can occur during the methanolic extraction of P. vulgaris shoot tissue.

(b) Preliminary Purification

When methanol and other organic solvents are used to extract IAA from plant tissue, it is inevitable that large volumes of solvents are utilised to achieve effective extraction. Thus following the period of extraction various preliminary purification procedures are

Figure 5: Radio-TLC Profile of Samples after Development in Solvent System (a)

Key: A = Plant Extract No. 1

B = Plant Extract No. 2

C = Plant Extract No. 3

D = Control

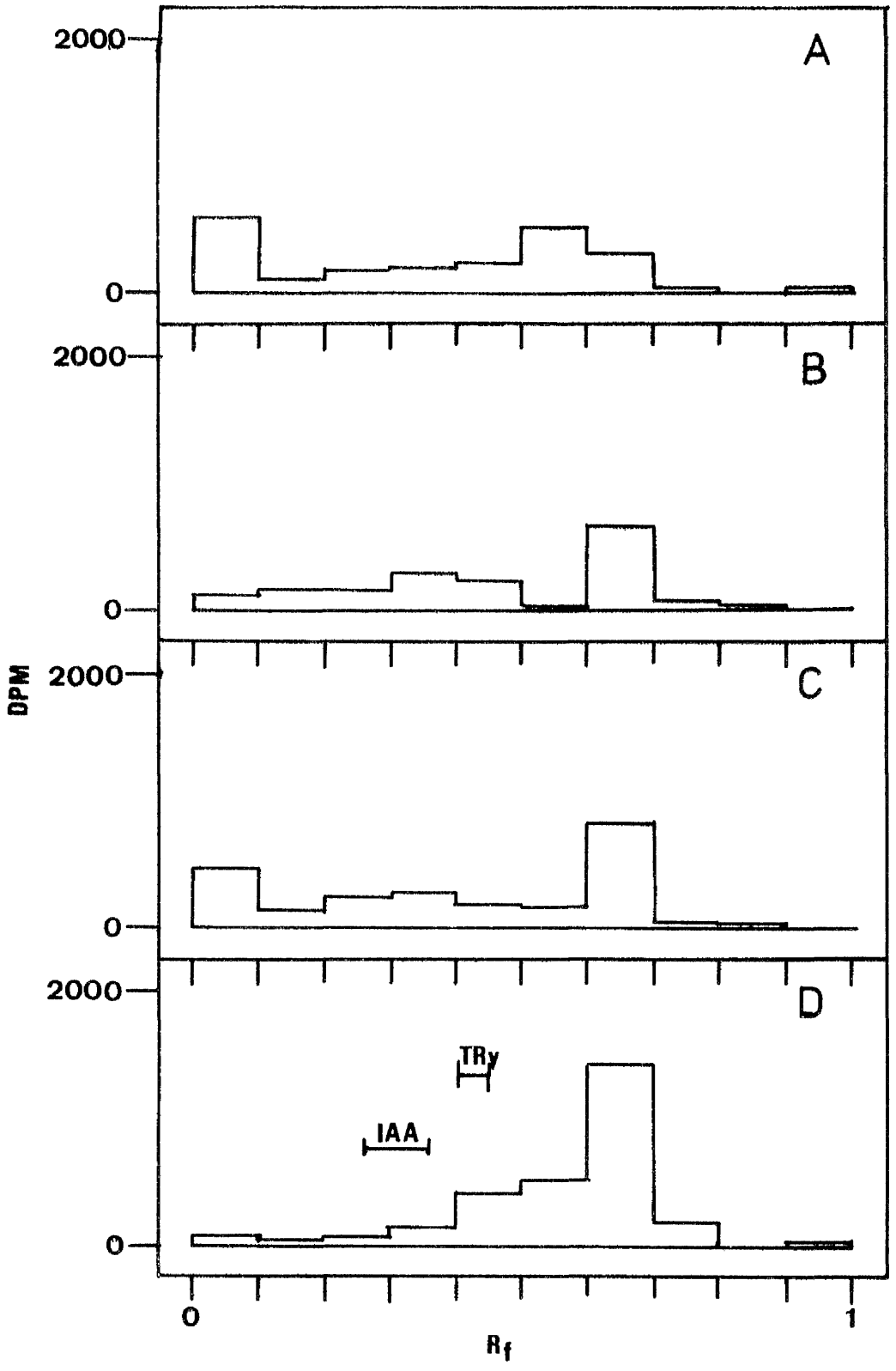

2000
A
0
2000
B
0
DPM
2000
C
0
2000
D
TRy
IAA
0
0
1
Rf

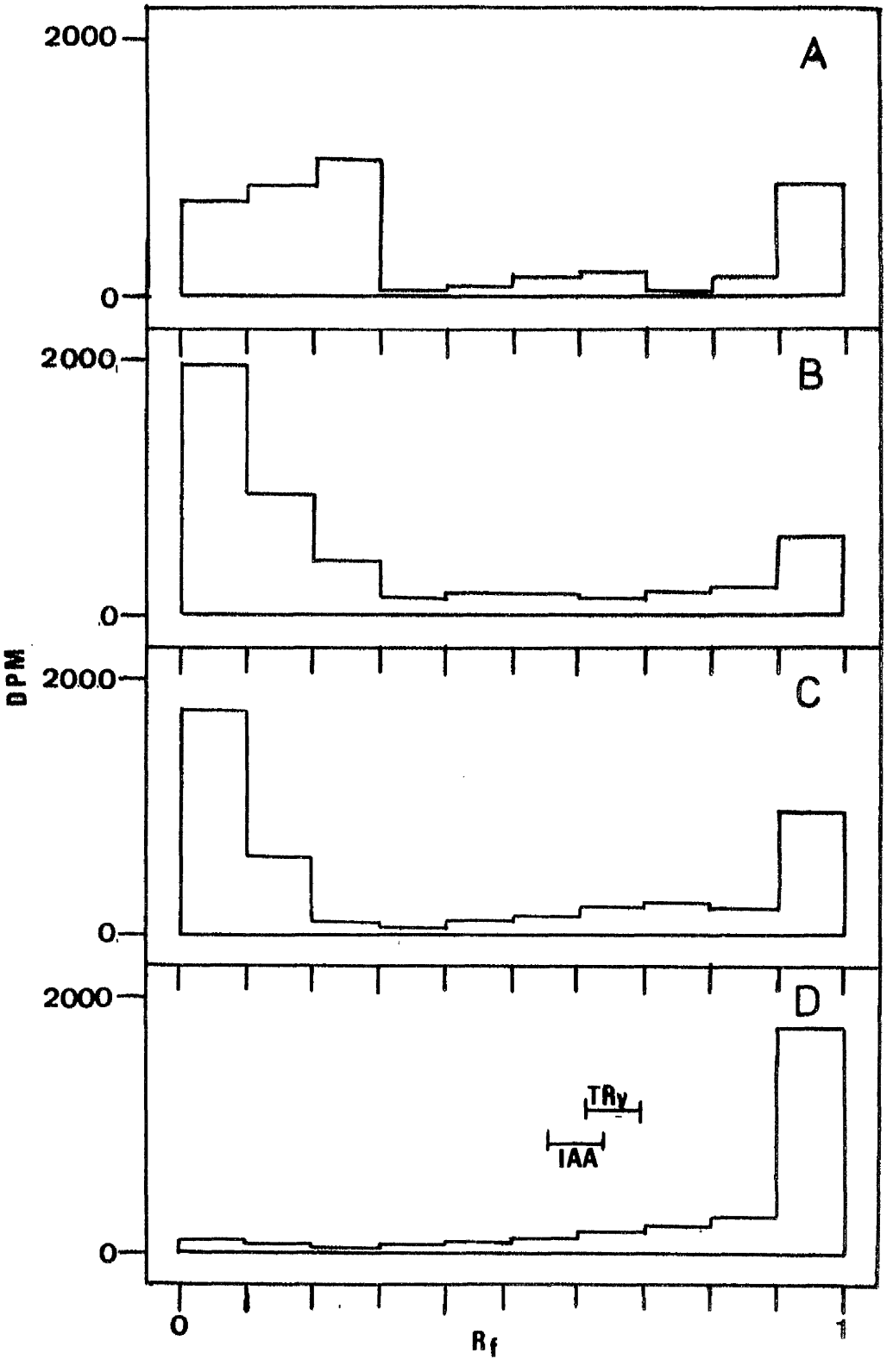
Figure 6: Radio-TLC Profile of Samples after Development in Solvent System (b)

Key: A = Plant Extract No. 1

B = Plant Extract No. 2

C = Plant Extract No. 3

D = Control

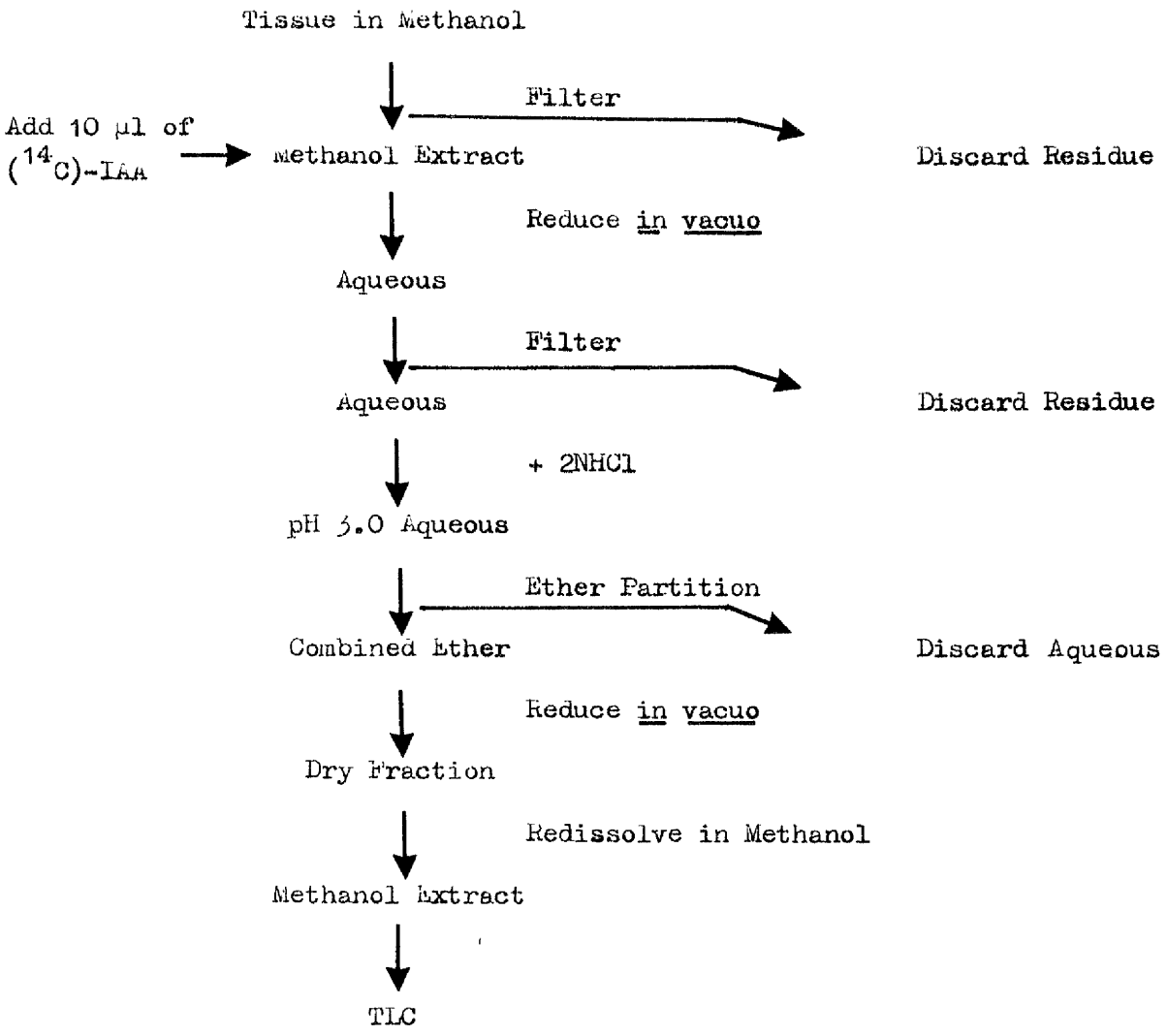

2000
A
0
2000
B
0
DPM
2000
C
0
2000
D
TRy
IAA
0
0
Rf
1

applied such that the total volume and dry weight of the sample is reduced to a convenient size. This is normally accomplished by employing a procedure similar to that of the previous experiment (e.g. White et al., 1975). In order to gauge the effect of such procedures on the dry weight of plant extracts, an initial experiment was designed where three batches of 500 g (fresh weight) of shoot tissue were harvested and extracted in 3 l of methanol; following which the extracts were purified as above. At the completion of the acidic -ether partitioning stage, each sample was reduced to a dry residue and the weight of this residue was then taken and found to be 1.501 g, 1.967 g and 1.740 g, respectively. In addition, it was noted that following the removal of methanol from the extract, a considerable amount of solid debris was present in the aqueous residue, and on acidic-ether partitioning, this residue was taken up in the ether layers. A method was then sought to overcome this effect and thereby reduce the dry weight of the sample, whilst not unduly affecting the levels of IAA present.

Two main methods were investigated: (i) introduction of a filtration stage to remove the solid debris from the aqueous fraction; (ii) introduction of an alkali-ether partition.

In the first series of three separate experiments, three batches of 500 g of shoot tissue were harvested and extracted as described previously. Following this, the extracts were filtered and the methanolic fraction collected. To this fraction was added 10 µl of the [^{14}C]-IAA solution. The methanolic extract was then subjected to Preliminary Purification method I (Figure 7). The aqueous filtration stage consisted of reducing the extract to an aqueous residue in vacuo and then filtering this through Whatman No. 3 paper by Büchner filtration. The flask with the extract residue was then washed with 50 ml of distilled water which was then filtered. This washing

Figure 7: Schematic Representation of Method I



procedure was repeated two more times following which the volume of the combined aqueous fraction was adjusted to 500 ml with distilled water. This aqueous fraction was then acidic-ether partitioned as described previously, and after reducing the ether layers to dryness, the residue dry weight was taken. Aliquots were also taken and the radioactivity assessed in order that the amount of [^{14}C]-IAA present after each stage and hence the % efficiency of recovery of IAA was determined. Table 4 shows the % efficiency of [^{14}C]-IAA recovery, as gauged by liquid scintillation counting, at each stage after correcting for sampling losses, with the final dry weight of the residue obtained from each extract.

The effect of this method on the dry weight of the final sample was obviously considerable. When compared with the results of the previous initial experiment, the dry weight was reduced by a mean factor of 85.4% whilst achieving a recovery of [^{14}C]-IAA in excess of 80%.

A radio-TLC analysis of the recovered [^{14}C]-IAA was also carried out by first dissolving the dry residue, after weighing, in 25 ml of methanol and then chromatographing a sample of this (200 µl) with an adjacent spot of 1 µl of [^{14}C]-IAA solution on Sil G TLC plates in methyl acetate : propan-2-ol : ammonia (45 : 35 : 20; v/v). Following development, the distribution of radioactivity on the plates was gauged using the radiochromatogram scanner, and thus the % purity of the [^{14}C]-IAA present in both the standard and sample was calculated as described in the "Materials and Methods".

Table 5 shows the % purity of the [^{14}C]-IAA present in each sample after this preliminary purification procedure. It is apparent that little change occurred in the overall composition of the [^{14}C]-IAA during this procedure.

In a second series of experiments, three batches of 500 g of shoot tissue were subjected to the procedure outlined in Method II (Figure 8)

Table 4: Efficiency of preliminary purification

	Replicate		
	A	B	C
METHOD I			
% efficiency of filtration stage	91.4	92.9	92.9
% efficiency of acid partition	92.3	92.2	90.9
Overall % efficiency	83.9	85.2	83.1
Dry wt. of final extract (g)	0.298	0.176	0.285
METHOD II			
% efficiency of filtration stage	91.4	99.8	96.1
% efficiency of alkali partition	94.4	91.3	96.2
% efficiency of acid partition	96.9	97.7	89.9
Overall % efficiency	83.2	88.6	82.7
Dry wt. of final extract (g)	0.156	0.246	0.342

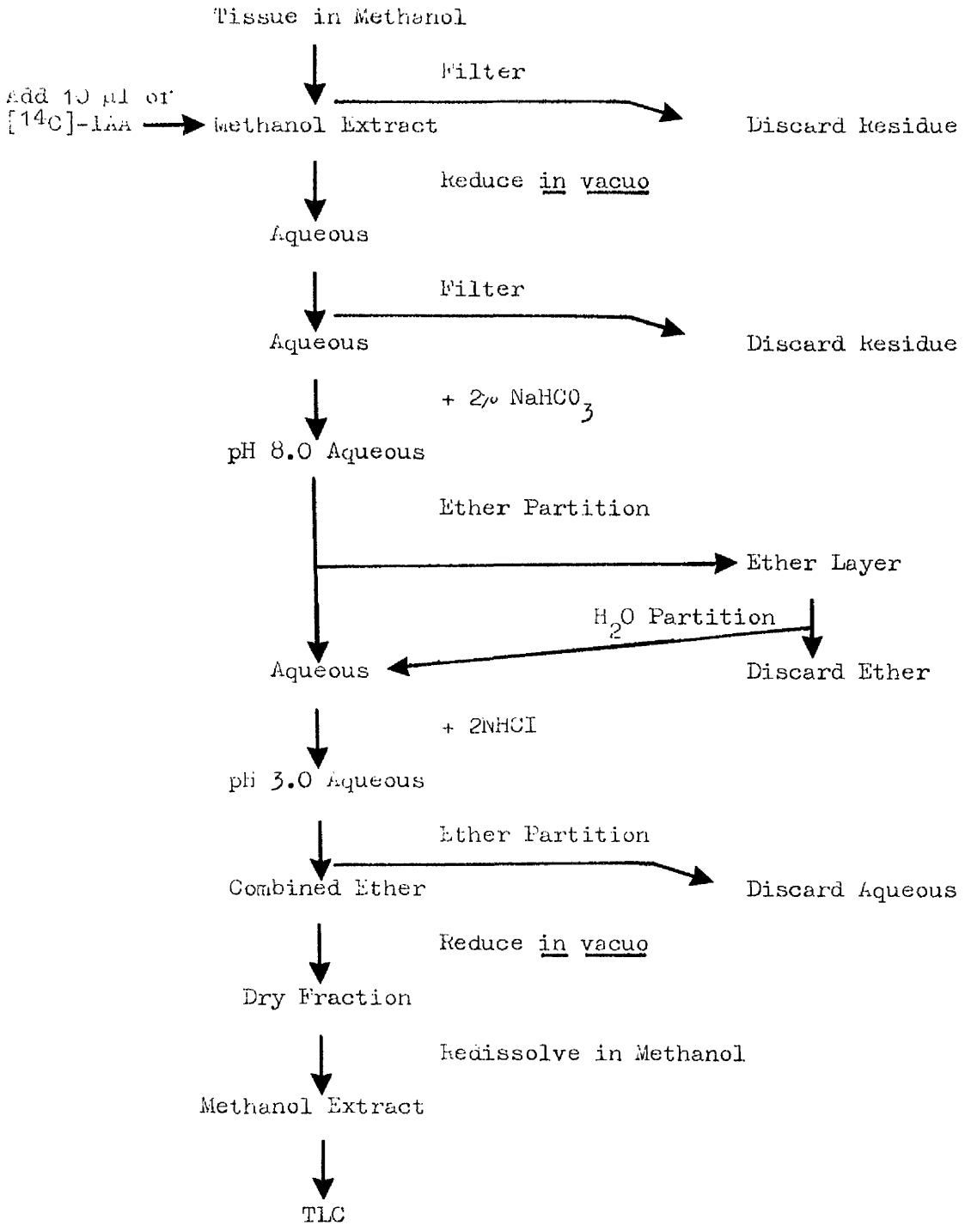
Table 5: % Purity and Rf zones of [^{14}C]-IAA in standard and Extract (Method I)

	Expt. 1	Expt. 2	Expt. 3
Rf zone of main peak in [^{14}C] -IAA standard	0.23 - 0.35	0.22 - 0.39	0.20 - 0.30
Rf zone of main peak in Extract	0.19 - 0.34	0.29 - 0.39	0.18 - 0.32
Rf zones of minor peaks in [^{14}C]-IAA standard	0.42 - 0.51 0.80 - 0.82	0.71 - 0.84 0.41 - 0.51	0.37 - 0.43
Rf zones of minor peaks in Extract	0.04 - 0.08	-	0.07 - 0.12
% Purity of [^{14}C]-IAA standard	97.96	96.6	98.5
% Purity of [^{14}C]-IAA in Extract	97.66	99	98.3

Table 6: % Purity and Rf zones of [^{14}C]-IAA in standard and extract (Method 2)

	Expt. 1	Expt. 2	Expt. 3
Rf zone of main peak in [^{14}C] -IAA standard	0.70 - 0.85	0.42 - 0.55	0.71 - 0.82
Rf zone of main peak in Extract	0.66 - 0.82	0.40 - 0.54	0.73 - 0.82
Rf zones of minor peaks in [^{14}C]-IAA standard	0.85 - 0.94	0.80 - 0.71	0.44 - 0.51
Rf zones of minor peaks in Extract	0.88 - 0.99	0.18 - 0.30	0.42 - 0.48
% Purity of [^{14}C]-IAA standard	97.8	96.3	95.6
% Purity of [^{14}C]-IAA in Extract	93.8	93.9	88.9

Figure 8: Schematic Representation of Method II



after extraction in 3 l of methanol. This procedure differed from Method I in that an alkali-ether partition stage had been introduced after the filtration stage and prior to the acidic-ether partitioning.

The alkali-ether partition was performed by altering the pH of the aqueous solution to 8.0. This alkaline aqueous fraction was then partitioned three times against an equal volume of diethyl-ether. The combined ether layers were then re-washed with 100 ml of distilled water, following which the ether layers were discarded. The combined aqueous layers were then subjected to acidic-ether partitioning as previously described, except that a $\frac{1}{2}$ volume of ether was used for each partition. The dry weight, % recovery of each stage, and the % purity of the [^{14}C]-IAA remaining was calculated as described for Method I.

Table 4 shows the % recovery of IAA at each stage of Method II after correction for sampling losses, and the final dry weight of each residue. The % recovery of [^{14}C]-IAA involving the acidic-ether partitioning and filtration stages were found to be similar to those obtained for Method I; in addition, the introduction of the alkali-ether partition did not have a significant effect on the overall efficiency of the method. Both methods had recovery rates of [^{14}C]-IAA in excess of 80%. The % recovery of the alkali-ether partition was found to be greater than 90%.

The dry weight of the residue recovered ranged from 0.15 to 0.34 g, and when compared to the dry weights obtained with Method I, was not deemed to be significantly different.

The radio-TLC analysis results are shown in Table 6, and demonstrated that by using Method II, the % purity of [^{14}C]-IAA in the sample was lowered from an initial value of 95-98% to a final value of 89-94%.

In summary, the introduction of an aqueous filtration step into the preliminary purification procedure of P. vulgaris shoot extracts

resulted in the lowering of the residue dry weight by a mean factor of 85%; on the other hand, the introduction of an alkali-ether partition had little effect on this weight. It was decided, therefore, that when extracts are to be purified for analysis, Method I would be used as a routine preliminary purification procedure prior to chromatography.

(2) Column Chromatography

Using the purification procedure of Method I, it was demonstrated that a dry weight residue of 0.2 to 0.3 g can be obtained from 500 g of P. vulgaris shoot tissue. On the basis of TLC analysis, the main components of this residue were the plant pigments with little other material being present. With this in mind, several column chromatographic systems were tested to assess their relative effectiveness in eliminating the pigments from the IAA-containing fractions. The procedure by which this was determined is described in Materials and Methods, part 5(a).

The column systems utilised were as follows:

(A) Sephadex LH20

Two eluents were employed, namely methanol and 80% methanol in water.

(Ai) Sephadex LH20 with methanol.

This column was prepared by adding 35 g of Sephadex LH20 to 300 ml of methanol and allowing the Sephadex to swell for 3 h. Excess methanol was then poured off until a fairly thick slurry was obtained. This was then poured carefully into a Pharmacia K25/45 column (internal diameter 25 mm, length 45 cm). After pouring, an eluent reservoir was connected to the column and the flow of the eluent started immediately, the height of the eluent reservoir with respect to the column being first adjusted to ensure a minimal pressure on the Sephadex gel, whilst not inhibiting the flow of the system too adversely. By this method an even "bed" surface of the gel was achieved.

The eluent was allowed to flow for at least one hour before the plant extract and [^{14}C]-IAA were applied to the column. After application, the column eluate, at a flow rate of 0.37 ml min^{-1}, was then collected in 2 ml fractions from the column whose "bed" volume was 140 ml. The elution profile of the [^{14}C]-IAA and the plant pigments was then determined (Figure 9).

The elution of [^{14}C]-IAA from the column was found to coincide with a pigment peak; since these eluted peaks had approximate Gaussian profiles, the resolution between the [^{14}C]-IAA and the second pigment zone was calculated and found to be 0.15. The recovery of the [^{14}C] -IAA from this column system was found to be 83.5%.

(Aii) Sephadex LH20 with 80% Methanol

The methods employed in the preparation and use of this column were exactly the same as that described for system (Ai) with the obvious change in solvent constitution. Specifications of the column were slightly different, however, in that 2 ml eluate fractions were collected from the column whose "bed" volume was 156 ml, at a flow rate of 0.38 ml min^{-1}.

From the elution profile obtained of the [^{14}C]-IAA and plant pigments (Figure 10) it was apparent that by having altered the composition of the mobile phase from system (Ai), an enhanced resolution between the IAA and pigment zones occurred. The resolution in this case was found to be 1.21.

(B) Diethylaminoethyl-Cellulose.

The DEAE cellulose (DE1 or DE23) was supplied as a dry material. It was prepared for use by sequentially washing 15 g batches in 2 l of 0.5 N HCl for 10 mins, removing the acid and re-washing the cellulose in 2 l aliquots of distilled water until an intermediate pH of 4 or greater was obtained. This procedure was repeated with substitution of the acid wash by a wash in 2 l of 0.5 N NaOH solution; the final

Figure 9: Elution Profiles from column of Sephadex LH20 and Methanol

Key: ▲ = DPM

△ = OD 450 nm

▽ = OD 660 nm

▼ = OD 670 nm

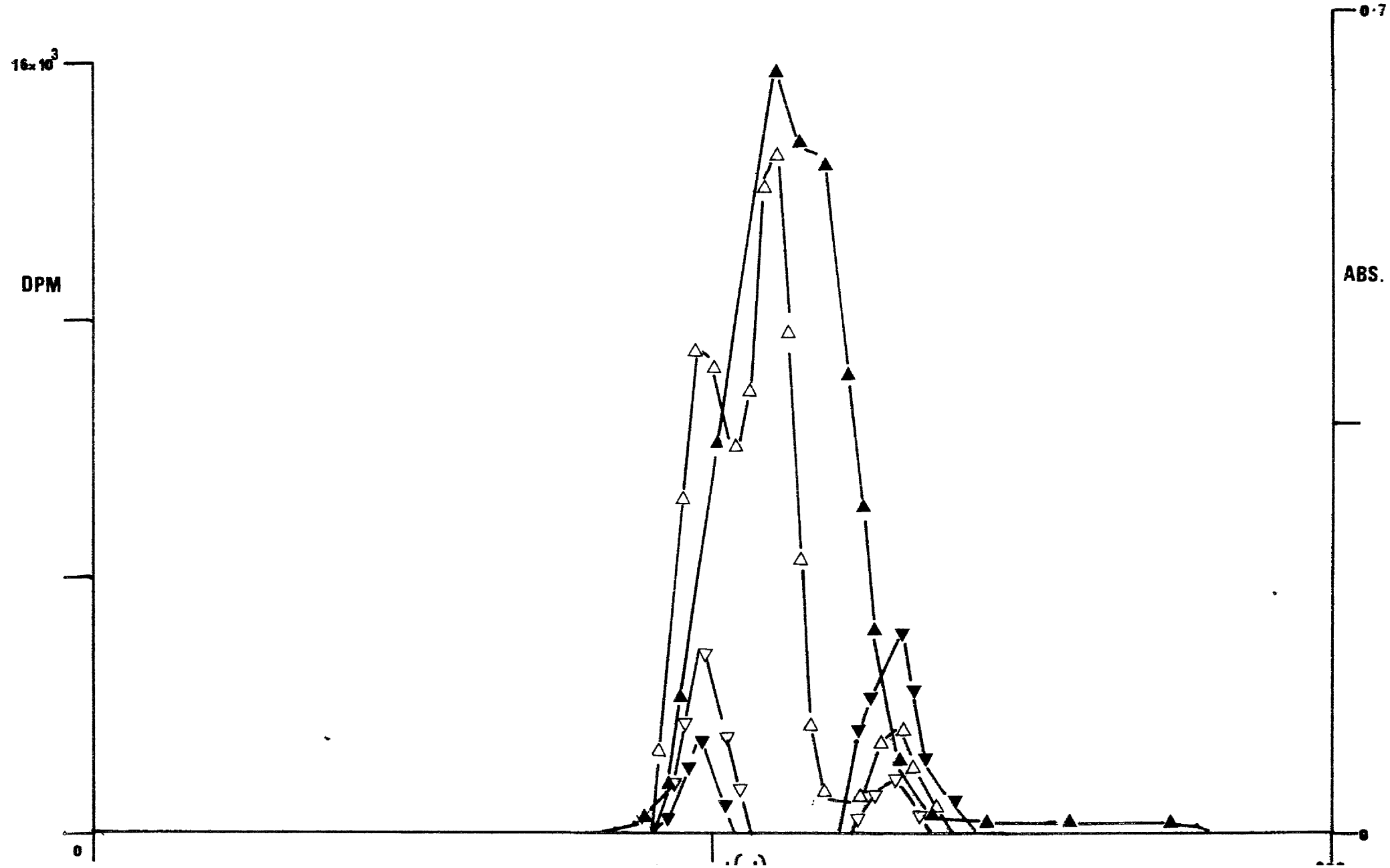

16x10^3
DPM
0
0·7
ABS.
0

Figure 10: Elution Profiles from column of Sephadex IH20 and 80% Methanol

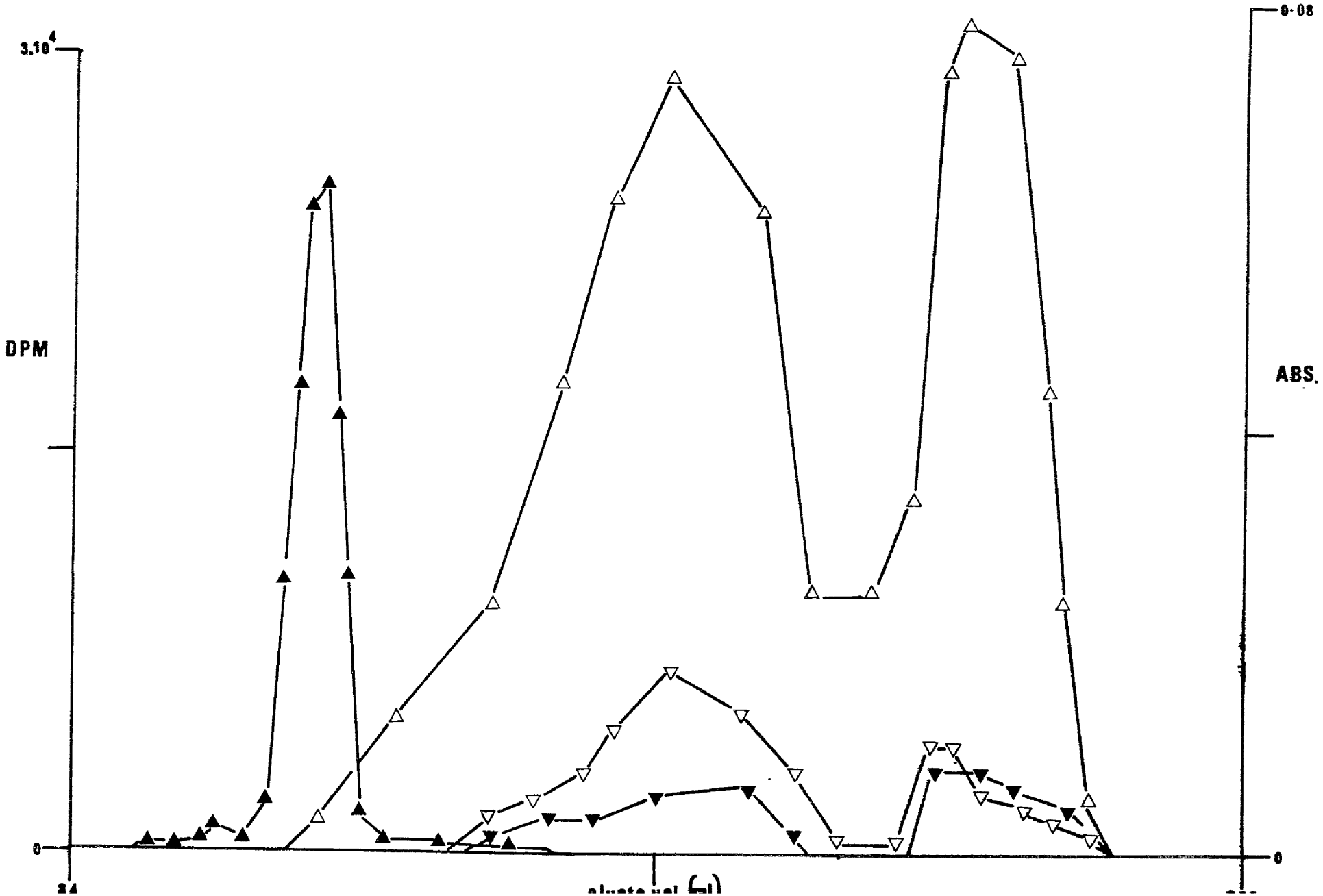
Key:

▲ = DPM

△ = OD 450 nm

▽ = OD 660 nm

▼ = OD 670 nm


3.10⁴
DPM
0
0·08
ABS.
0

pH of the solution after distilled water rinsing was 6.5 to 7.5

Following this pre-treatment, the cellulose in water suspension was poured into a column (internal diameter 1.8 cm, height 40 cm; with integral sinter overlaid by a 2 cm layer of glass beads of mesh size 40) and allowed to compact until the bed height was 30 cm.

After the application of the [^{14}C]-IAA and the plant extract to the column, three eluents were applied in succession. Firstly, 300 ml of distilled water, secondly, 350 ml of 0.05 M Na_2SO_4 solution, and finally, 200 ml of distilled water. After the flow of the column was commenced, the eluate was collected in 5 ml fractions and the elution profile of the [^{14}C]-IAA and the plant pigments was determined.

Using this column system, no plant pigments were detected in any of the elution fractions. Moreover, the [^{14}C]-IAA was found to be eluted as a single peak between 325 and 500 ml.

This experiment was repeated on two further occasions with similar elution profiles of [^{14}C]-IAA being obtained. The % recovery of the [^{14}C]-IAA from this system was found to be 98.3%, 89.7% and 95.7% .

(C) Polyvinylpyrrolidone.

Polyclar AT is an insoluble form of poly-N-vinyl pyrrolidone (PVP). The PVP was prepared by mixing 20 g of Polyclar AT in five times the volume of distilled water, leaving the suspension to settle for 15 min, after which the excess water was then decanted. This procedure was repeated two more times with distilled water and then with 0.1 M phosphate buffer of pH 8.0, the purpose of this washing procedure being the removal of fine material from the PVP. Following the final decantation, the slurry was poured into a column (internal diameter 2.0 cm with sinter overlaid by a 1 cm layer of glass beads) until the column height was 20 cm.

After the application of the plant extract and [^{14}C]-IAA, the

column was eluted with the 0.1 M phosphate buffer at a flow rate of 1.89 ml min^{-1}, and the elution profiles and % recovery of [^{14}C]-IAA were calculated. This experiment was repeated on two other occasions, with similar elution profiles in each experiment being obtained. The % recovery of [^{14}C]-IAA in these experiment experiment being obtained. 6%, respectively. Figure 11 shows the elution profile of the first experiment.

The elution of the [^{14}C]-IAA from this system was seen to occur as a single peak between 96-165 ml, with the pigment zone, similarly being eluted as a single peak, between 15 - 70 ml; the resolution in this case being 2.63.

In summary, four column systems were tested in order to gauge their relative effectiveness in separating IAA - present as [^{14}C]-IAA - from the pigments contained in plant extracts. Of the systems tested, DEAE cellulose completely absorbed all the plant pigments whilst exhibiting high recovery rates of IAA. PVP gave highly effective resolution between the pigment zones and the IAA, whilst Sephadex LH20 and 80% methanol achieved some degree of resolution. Sephadex LH20 used only with methanol was found to be ineffective with almost total overlap between the pigments and IAA.

(3) Thin-Layer Chromatography

TLC is often used to purify plant extracts for IAA (see Introduction). However, it suffers from the disadvantage of having a low sample capacity when compared to column chromatography (Snyder and Kirkland, 1974); thus TLC normally follows preliminary purification and column chromatography procedures. For the analysis of IAA in plant extracts, many solvent systems have been described (e.g. Gibson, Schneider and Wightman, 1972; Stahl, 1965). Hence an attempt was made to determine the relative effectiveness of certain solvent systems to separate IAA from the pigments present in the plant extracts by developing all the TLC solvent

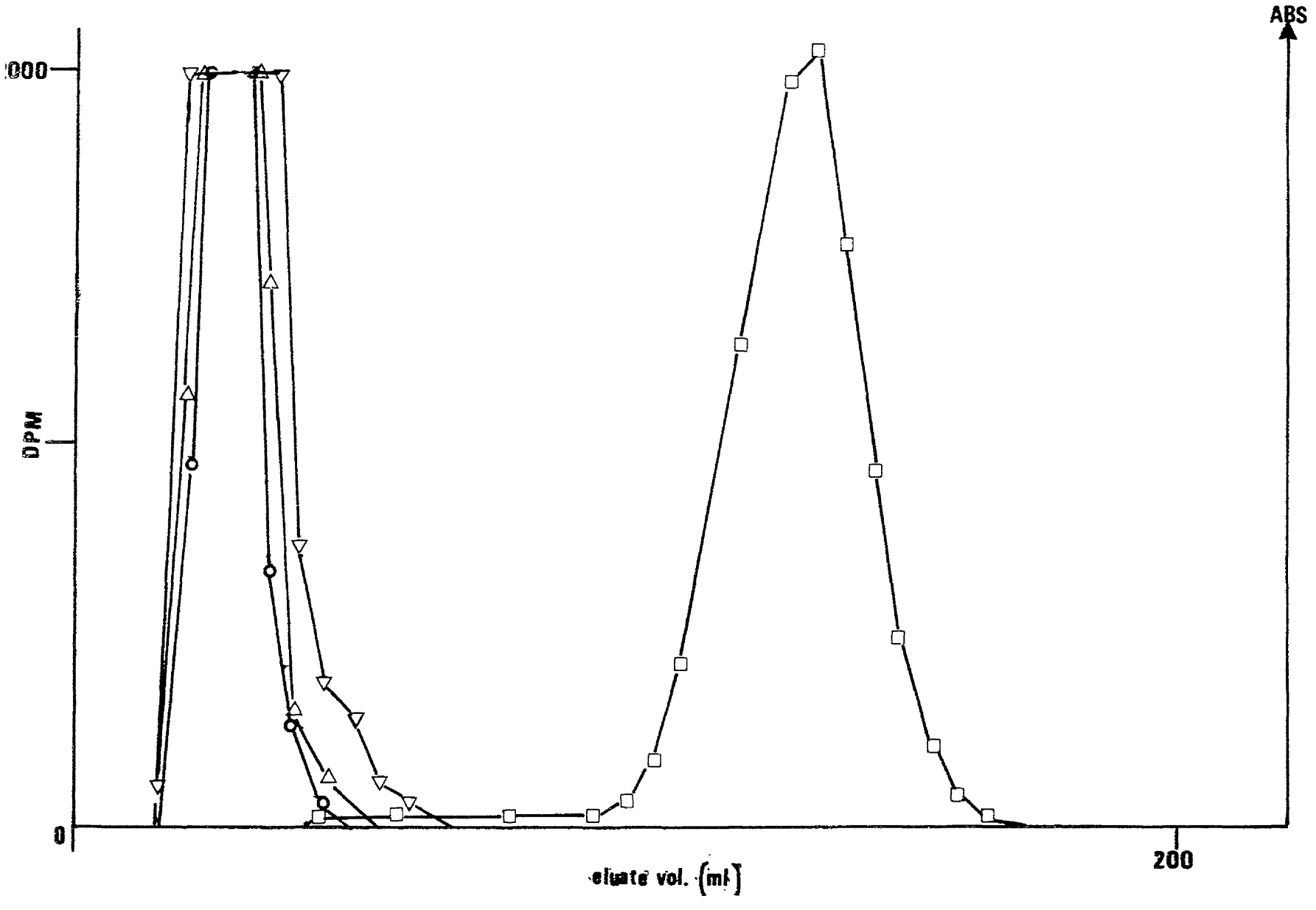
Figure 11: Elution Profiles from column of PVP

Key: □ = DPM

▽ = OD 450 nm

○ = OD 660 nm

△ = OD 670 nm


ABS
000
DPM
0
200
eluate vol. (ml)

systems under standard conditions (Materials and Methods, part 5 (b)).

The solvent systems employed are shown in Table 7, with solvent systems Nos. 1 - 7 being used on both cellulose and silica gel TLC plates, whilst solvent systems 8 - 17 were used only on silica gel plates.

Table 7: TLC solvent systems

1. Propan-2-ol:ammonia:water (8:1:1) (v/v)
2. n-Butanol:acetic acid:water (12:3:5) (v/v)
3. n-Butanol:acetic acid:water (5:1:2,2) (v/v)
4. Propan-2-ol:ammonia:water (10:1:1) (v/v)
5. n-Butanol:pyridine:water (1:1:1) (v/v)
6. Propan-2-ol:ammonia:water (100:5:10) (v/v)
7. 8% Sodium chloride solution
8. Benzene:acetone:pyridine (60:39:1) (v/v)
9. Methyl acetate:propan-2-ol:25% ammonia (aq.) (45:35:20) (v/v)
10. Chloroform:methanol:water (45:35:20) (v/v)
11. Chloroform:methanol:ammonia (80:25:0.1) (v/v)
12. Chloroform:acetic acid (95:5) (v/v)
13. Benzene:methanol:acetic acid (90:16:8) (v/v)
14. Propan-2-ol:ethyl acetate:water (24:65:11) (v/v)
15. Chloroform:ethyl acetate:formic acid (5:4:1) (v/v)
16. Chloroform:methanol:acetic acid (90:20:5) (v/v)
17. Ethyl acetate:methyl ethyl ketone:formic acid:water (5:3:3:1) (v/v)

Of the systems tested, only five were found to be of significant use in separating IAA from the plant extract components; these are shown in Table 8, with their respective Rf values; all used silica gel as the support medium.

Table 8: Rf Values and % Recovery of IAA from Sil G-TLC Plates in 5 Solvent Systems

Solvent System	Mean Rf Value	Mean % Recovery of [^{14}C]-IAA
6	0.24-0.33	
7	0.44-0.63	Mean % Recovery of [^{14}C]-IAA
9	0.32-0.42	74.5
11	0.01-0.08	67.7
4	0.25-0.34	71.2

The recovery of the IAA from the TLC plates after development, in these five systems, was determined by chromatographing three aliquots of [^{14}C]-IAA (circa 10^3 dpm) with adjacent reference spots of 400 ng of IAA. After development in the appropriate solvent systems, the position of the reference spots of IAA was noted by examining the plates under UV_{254} light. The Rf zone equivalent to this in the sample containing the [^{14}C]-IAA was then removed, eluted with 25 ml of methanol and the methanol was removed *in vacuo*, following which the dpm of the residue was taken. The mean % recovery of IAA from the TLC plates using these solvent systems was then calculated (Table 8).

Thus, of the 17 solvent systems tested, only five were found to be effective in separating IAA from the main components of plant extracts, and of these systems, four exhibited a percentage recovery value of IAA in excess of 60%. The highest degree of recovery of IAA from silica gel plates was those plates that had been developed in solvent system 9.

(4) Gas-Liquid Chromatography and Mass Spectrometry

In comparison with TLC, resolution using GLC is greater, but sample capacity is smaller. Moreover, many substances cannot be routinely subjected to GLC without conversion to a suitably stable derivative (Drozd, 1975). The following series of experiments describe the methods that were used to derivatise IAA for GLC studies, with the

intention of employing these methods to purify IAA in plant extract samples prior to analysis. In addition, mass spectrometry has been used to characterise the various derivatives of IAA that were prepared.

(a) GLC of Free IAA - Although the GC of free or underivatised polar compounds is usually unsatisfactory (Drozd, 1975), the successful GLC of free IAA has been claimed by Champault (1975) on columns of 3% DC-11 at an oven temperature of 160°C. Under these conditions, on injection of free IAA, he obtained a GC peak with a T_R of 6 min 15 sec. Since DC-11 is a methyl silicone stationary phase with a similar selectivity to the 100% methyl silicone stationary phases OV-101, OV-1 and SE-30 (Analabs Catalog 18, 1976), it would appear that the GC of free IAA can be successful on a non-polar stationary phase. Thus an initial experiment was undertaken to determine whether underivatised IAA can be successfully chromatographed on stationary phase OV-101.

The samples subjected to GLC were (i) IAA (10 mg) in methanol (800 µl); (ii) IAA (10 mg) in BSA (800 µl), mixed prior to injection. Solvent blanks of methanol and BSA were also injected. The GC conditions were:-

column: 3% OV-101, GCQ (100-120); 9 ft glass

oven temp: (A) TP from 135° to 247°C at 6° min^{-1} after 2 min

(B) 200°C

carrier gas: N_2 at 70 ml min^{-1}

detector: FID

The peaks observed on the injection of the two samples are expressed in terms of T_R and peak area (PA) in Table 9.

Following both the initial injectionsof sample (i), two peaks were noted; whilst an injection of methanol failed to give rise to similar peaks. However, when BSA was subsequently injected, a large peak with a T_R of 7.7 min at 200°C was detected. At first this compound gave an

Table 9:, GC of IAA on OV-101

Sample	Oven temp.	Peak No 1 T_R (min)	Peak No 1 PA (cm^2)	T_R (min)	PA (cm^2)
3 µl of sample (i)	A	2.1	0.2	4.3	0.12
3 µl of sample (i)	A	2.1	0.2	4.3	0.10
3 µl of methanol	A	-	-	-	-
3 µl of methanol	B	-	-	-	-
3 µl of BSA	B	7.7	OS	-	-
3 µl of BSA	B	7.6	OS	-	-
3 µl of BSA	B	7.7	4.59	-	-
3 µl of BSA	B	7.7	1.48	-	-
3 µl of BSA	B	7.7	0.96	-	-
3 µl of BSA	B	7.7	0.78	-	-
8 µl of BSA	B	7.7	1.75	-	-
1 µl of sample (ii)	B	7.7	3.9	-	-
3 µl of BSA	B	7.7	1.54	-	-
3 µl of sample (i)	B	-	-	-	-
3 µl of BSA	B	7.7	OS	-	-

off-scale response (OS) but on repeated injections of BSA the response fell until only a small amount could be detected. It was surmised that this compound arose as a result of on-column derivatisation of IAA, previously deposited on the column during the GLC of sample (i), by the BSA. Thus a freshly prepared aliquot of BSA and IAA (sample (ii)) was injected, and this yielded a peak with equivalent T_R to that previously found with BSA alone, but with a larger response. This observation supports the view that the compound detected on the injection of BSA after that of free IAA, was due to on-column silylation of the free IAA.

In order to confirm this, fresh injections of sample (i) and then BSA were carried out resulting in a compound with T_R equivalent to that of a freshly prepared sample of IAA and BSA. It is concluded therefore, that the GC of free IAA on OV-101 columns is unsuitable. Nevertheless, other non-polar methyl silicone stationary phases may be successful. Thus in the following experiment the equivalent stationary phase of SE-30 was used to attempt the GC of free IAA.

Two samples for this experiment were prepared: (i) IAA (10 mg) in methanol (800 µl); (ii) IAA (10 mg) in BSA (800 µl) after reacting for 2 h at 60°C. The GC conditions were as follows:-

column: 3% SE-30, GCQ (100-120); 5 ft glass

oven temp.: 225°C

carrier gas: N_2 at 60 ml min^{-1}

detector: FID

The peaks obtained are described in terms of T_R, peak width (PW) and peak height (PA) in Table 10.

Table 10: GC of IAA on SE-30

	Peak Description		
Sample	T_R(min)	PW (cm)	PH (cm)
0.5 µl of sample (ii)	2.4 3.0	0.25 0.4	1.8 8.9
3 µl of BSA	-	-	-
5 µl of sample (i)	0.95 2.8	0.4 1.9	7.0 23.2
5 µl of sample (i)	0.9 2.8	0.4 1.9	6.0 23.0
5 µl of BSA	-	-	-
5 µl of methanol	-	-	-
5 µl of sample (i)	0.9 2.8	0.35 1.8	5.0 18.0
1 µl of sample (ii)	2.4 3.0	0.3 0.4	3.0 17.8
1.5 µl of sample (ii)	2.4 3.0	0.3 0.4	7.0 OS

The GLC of the sample of the IAA in BSA after reaction for 2 h at 60°C provided two peaks with respective T_R values of 2.4 and 3.0 min, the second peak having the larger response. When a sample of free IAA was injected, two peaks were also noted, although their T_R values in this case were 0.4 and 1.9 min. In addition, the response of the second compound in terms of peak width was three to four times that of the first. Subsequent injections of BSA or methanol did not reveal any compounds other than the solvent front. Thus the GLC of free IAA, in this case, proved successful. However, the peak width of the second compound present in the free IAA sample was several times larger than that of sample (ii), even when similar mass responses in terms of peak height were observed. In view of the fact that the presence of polar groups is known to lead to the marked tailing of peaks in GLC, it is possible that the second compound is free IAA, with the previously eluted compound being a breakdown product.

This hypothesis was tested by subjecting the sample of IAA in methanol to GC-MS analysis (AEI MS-30) using the SE-30 column. The conditions were:-

oven temp.: 200°C

separator temp.: 220 + RT°C

source temp.: 210°C

ionising voltage: 70 eV

When 1 µl of sample (i) was injected, a response similar to that obtained above was noted on the TIM trace (Figure 12). Mass spectra of the two components A (T_R^1 = 0.7 min) and B (T_R^1 = 3.6 min) were taken (Tables 11 and 12 and Figure 13).

Both spectra contained m/e 130 as the base ion which is indicative of a 3-substituted indole (Porter and Baldas, 1971). The molecular ion of compound A was m/e 131, and compound B was m/e 175. The remaining ions in both spectra are typical of any 3-substituted indole, hence

Figure 12: TIM response of free IAA on SE-30

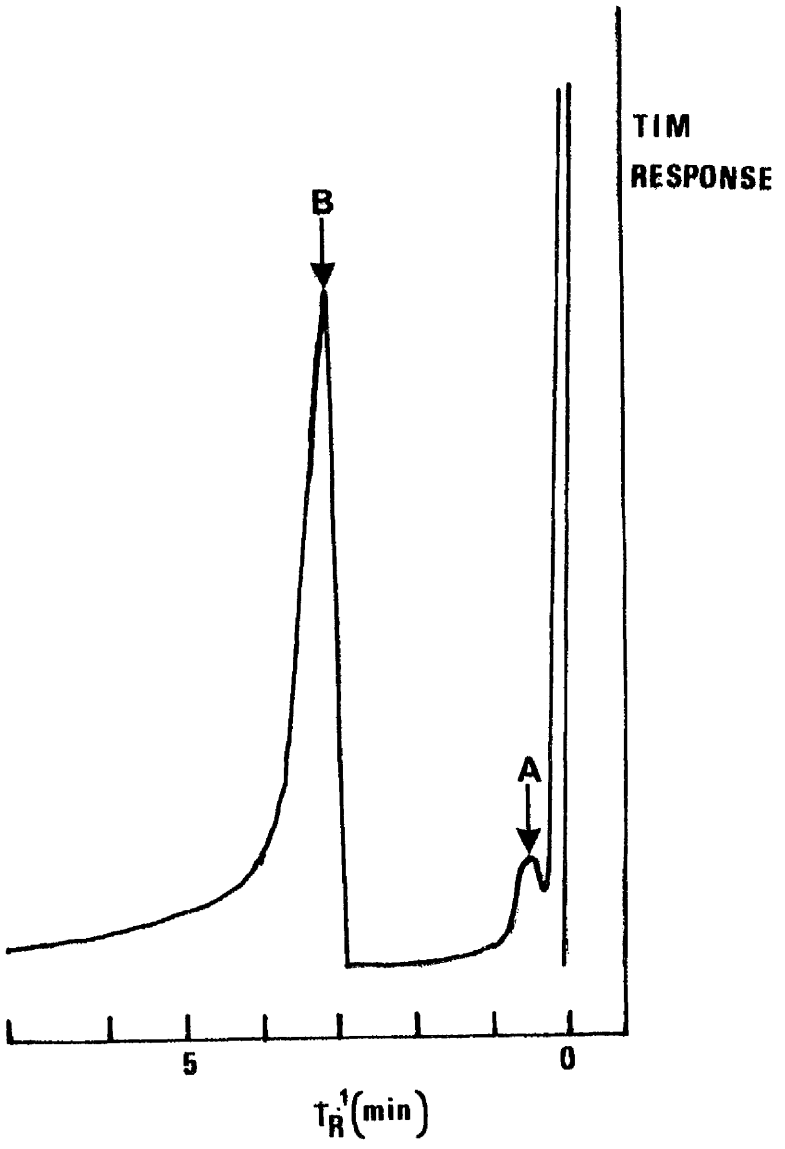

TIM
RESPONSE
B
A
5
0
t_R^1(min)

Figure 13: Mass Spectra of Compounds A and B

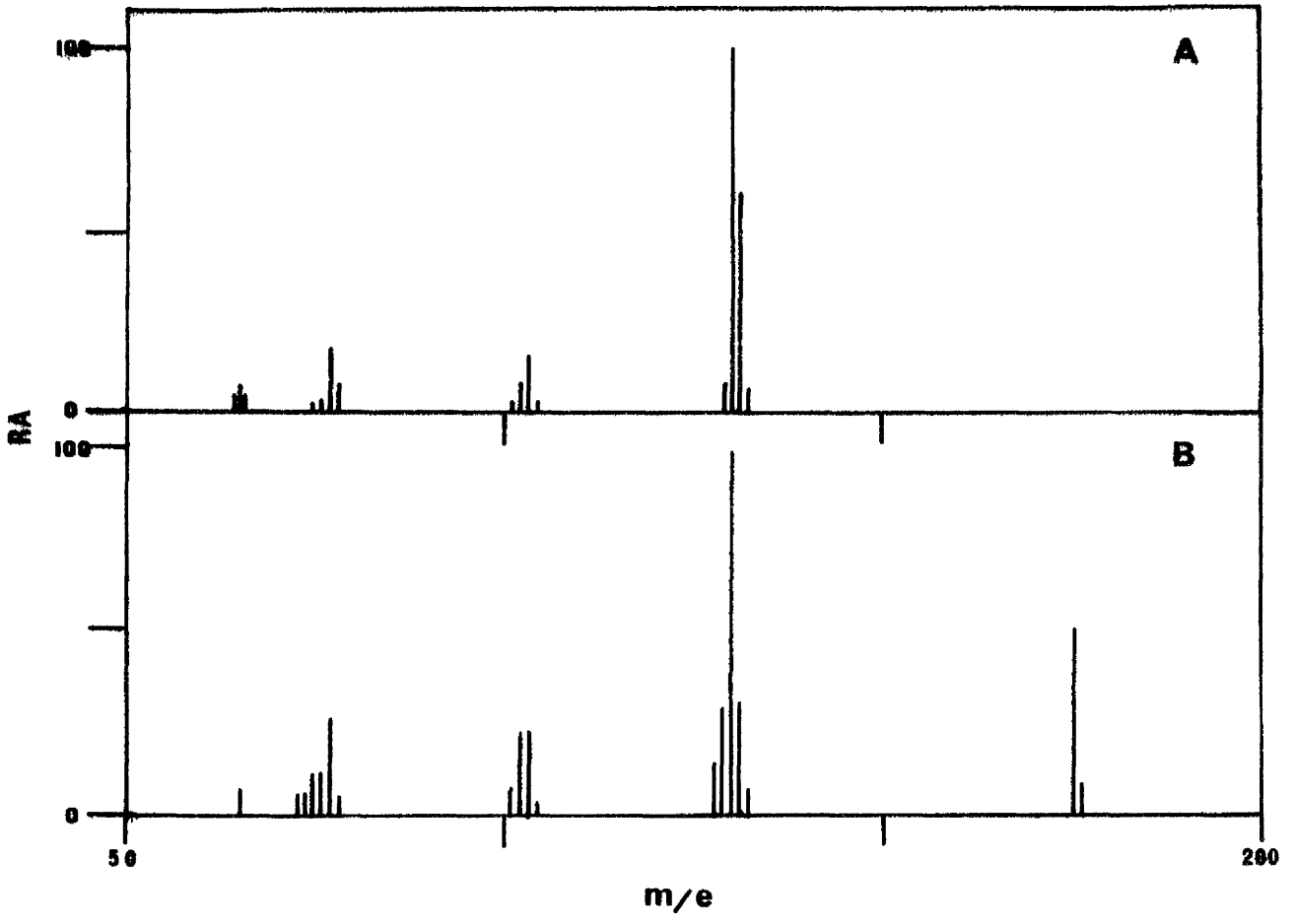

100
A
0
RA
100
B
0
50
200
m/e

Table 11: Mass Spectrum of Compound A

m/e	64.5	65	65.5	75	76	77	78	101
RA	6.4	6.7	4.8	1.7	1.7	18.3	3.3	1.6
m/e	102	103	104	129	130	131	132	
RA	6.7	15	1.7	5	100	60	6.7	

Table 12: Mass Spectrum of Compound B

m/e	65	73	74	75	76	77	78	101	102
RA	6.6	5.9	5.9	9.2	10.5	25.7	3.9	5.3	21.1
m/e	103	104	128	129	130	131	132	175	176
RA	22.3	2.6	13.2	27.7	100	28.9	5.3	51.2	6.6

identification of both compounds relied on the m/e value of the molecular ions. Compound B was, in fact, IAA, and compound A was a methyl indole, probably skatole (3-methyl indole) although other isomers exist with similar spectra (Porter and Baldas, 1971). The origin of the methyl indole in sample (i) could be either due to the decomposition of IAA during its GLC, or as an original impurity in the sample.

The second hypothesis was tested by subjecting sample (i) to a preparative HPLC system capable of separating and detecting acidic and neutral indoles (Reeve and Crozier, 1977). The HPLC conditions were:-

Column: Partasil 10 (10 x 450 mm); stationary phase 43% 0.5 M formic acid

mobile phase: 45% ethyl acetate in n-hexane at 4.6 ml min^{-1}

detector: $UV_{254\ nm}$ at 0.2 A FSD

On two successive injections of sample (i) (2 µl) only one peak with a T_R of 12.9 min was evident (Figure 14). An injection of 48 µl

Figure 14: HPLC trace of sample (i)

Figure 15: Mass spectrum of compound detected by HPLC in sample (i)

Fig.14

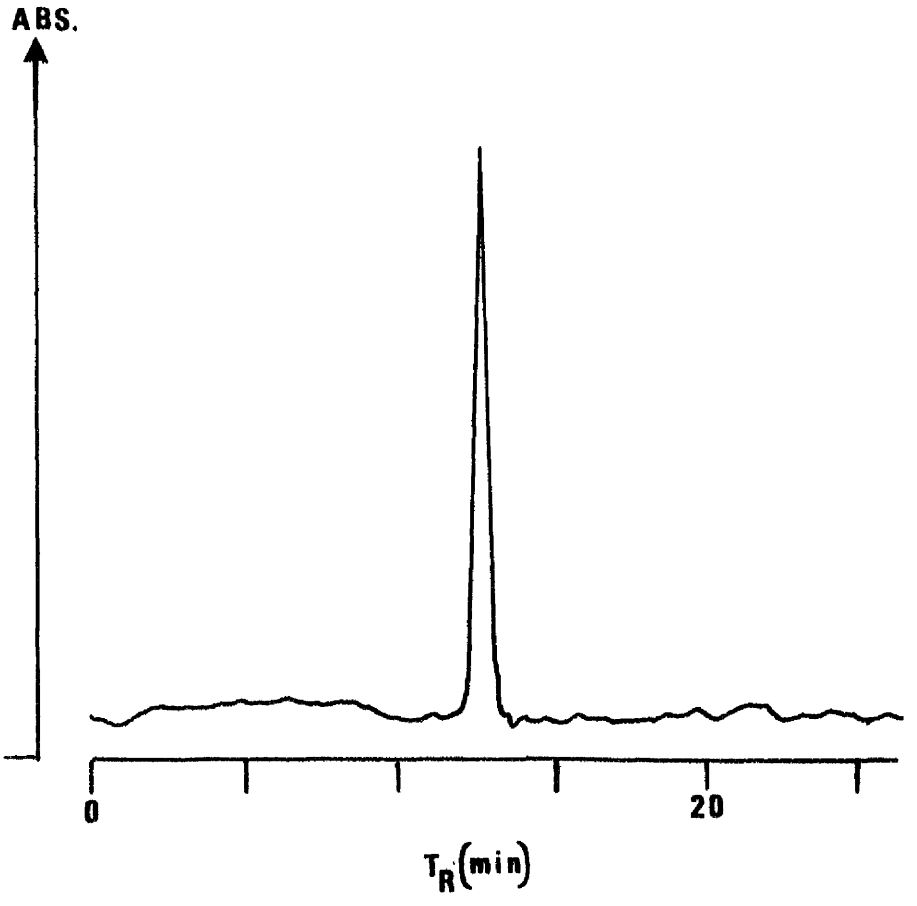
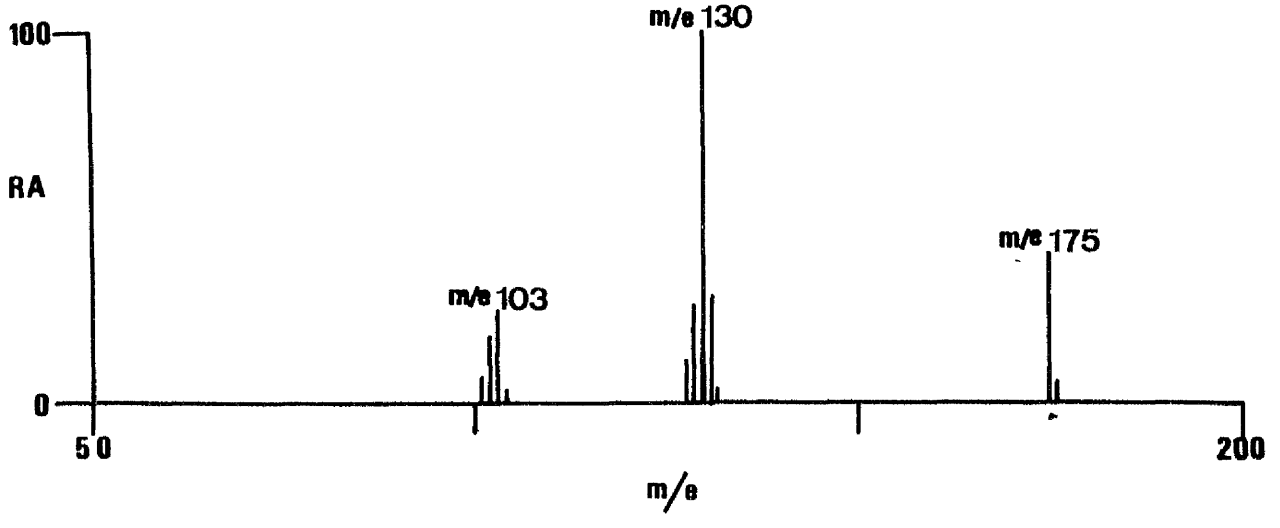


Fig.15



of sample (i), equivalent to 68 μg of IAA was then made, and at the same T_R a large response was observed. By means of an effluent splitter, whereby 10% of the column flow was diverted prior to the detector, an aliquot of this peak was collected. The solvent was then removed from the sample and the residue was subjected to MS analysis by direct probe insertion. The resulting mass spectrum (Figure 15) was identical to that of IAA.

In summary, IAA was found to yield two peaks on GLC, one being IAA, the other a methyl indole. Preparative HPLC and MS revealed that the sample subjected to GLC was pure IAA, hence the origin of methyl indole was due to breakdown of IAA during GLC. The Gaussian nature of the methyl indole peak indicates that this breakdown probably occurred at the injection port of the GLC.

(b) Derivatisation of IAA - The foregoing series of experiments has shown the unsatisfactory nature of using underivatised IAA for GLC studies on two stationary phases. On the other hand, stable derivatives of the compound can be prepared for GLC studies, and several types are available (Drozd, 1975). In the case of IAA, derivatisation can be achieved by (i) esterification of the carboxyl group, (ii) trimethylsilylation of the carboxyl and amine groups, and (iii) haloacylation of the amine group. Obviously, if such derivatives are intended for quantitative GLC studies, the yield of formation of such derivatives should be known. The following series of experiments describe the method of preparation and the yields obtained after preparation of the three types of derivatives.

(i) Methyl esterification of IAA

The esterification of carboxylic acids can be accomplished by diazomethane, or by methanol catalysed by HCl, H_2SO_4 or BF_3. Using these methods, Vorbeck et al. (1961) esterified several fatty acids and obtained high yields with diazomethane. Accordingly,

diazomethane was chosen as the derivatisation agent for the preparation of IAA-Me.

An etherial solution of diazomethane was initially prepared using a method similar to that of Schlenk and Gellerman (1960). Equal parts of "Nitrosan" (bis-n-methyl-n-nitrosoterephthalamide), ethylene glycol and diethyl ether were mixed in a 250 ml conical flask with a side-arm attachment. A small amount of strong sodium hydroxide solution was then added, and the flask was stoppered and heated on a hot-water bath. The open end of the side arm was placed under a few ml of diethyl ether in a 250 ml conical flask surrounded by ice. On heating the reaction mixture, diazomethane gas and ether distilled over and dissolved in the cold ether. Only when an intense yellow colour was observed in the ether was the solution considered ready for use. Fresh solutions were always used for derivatisation.

One mg of IAA was added to two Reacti-vials (~~Pelrce~~ PIERCE and Warriner, Ltd., Cheshire) and dissolved in 200 µl of methanol. To this solution, 1 ml of diazomethane solution was added and the vials sealed. These samples were then left for 20 min at 4°C, following which, each sample was reduced to dryness under a gentle stream of N_2. The resultant residues were dissolved in 200 µl of methanol and subjected to analysis by GLC and GC-MS.

For GLC analysis the conditions were:-

column: 3% OV-17, GCQ (100-120); 9 ft glass

oven temp.: 220°C

carrier gas: N_2 at 40 ml min^{-1}

detector: FID

Each sample on injection (1 µl), gave rise to a single peak with a T_R of 9.1 min. The mean peak area of the injections was 3.263 ± 0.08 cm^2.

To ensure that this peak was indeed IAA-Me, the samples were analysed on the AEI MS-30 under similar GC conditions. When injected,

each sample produced a single peak on the TIM trace, with a similar T_R value to that obtained above. A mass spectrum of this peak was taken from both samples, with the spectra in each case being similar. A representative spectrum is shown in Table 13 and Figure 16

Table 13: Mass spectrum of IAA-Me

m/e	50	51	52	65	75	76	77	78	79	89	90	91
RA	2	7	2	5	3	5	16	3	1	1	1	1
m/e	101	102	103	104	105	128	129	130	131	132	189	190
RA	3	10	15.5	1	0.5	7	11	100	24	2.5	51	5.5

The mass spectra obtained in all cases were consistent with that of IAA-Me (Abe and Marumo, 1974).

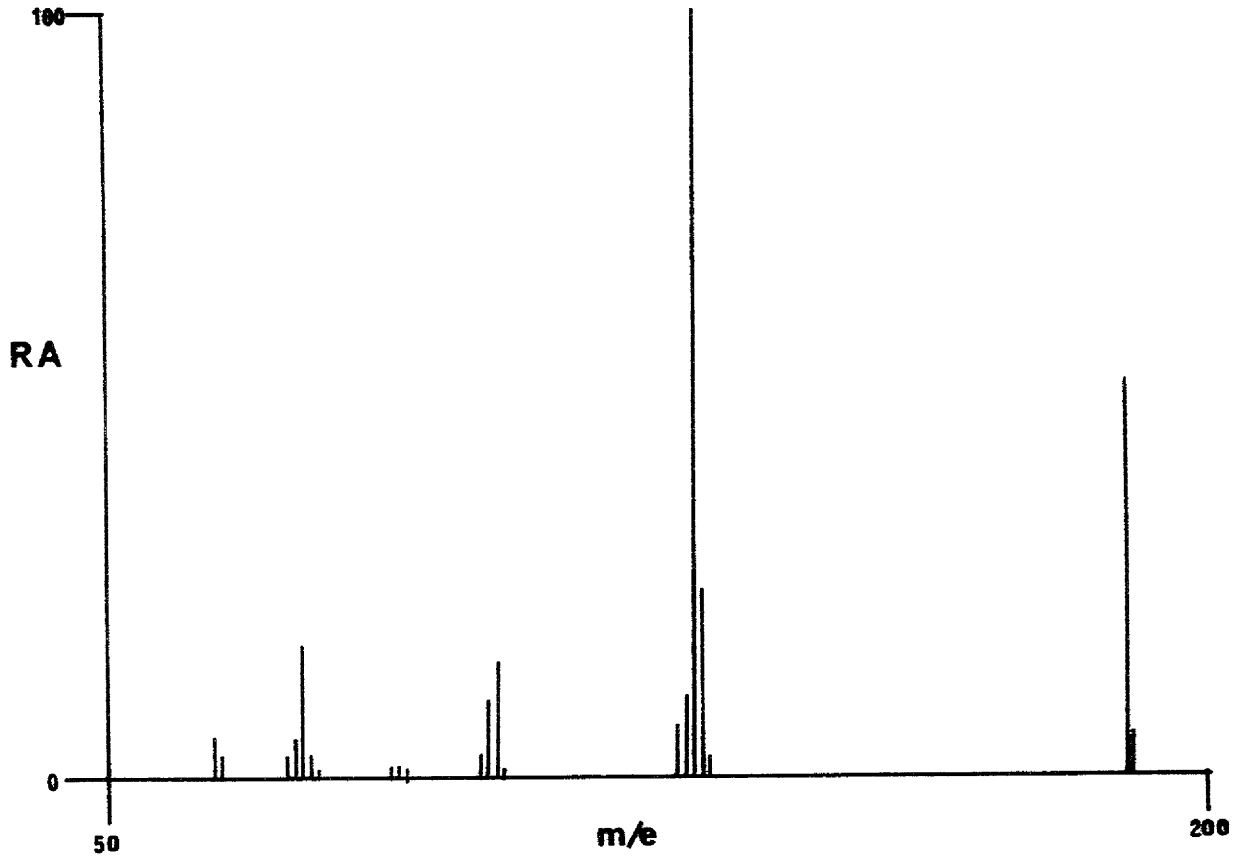
The yield obtained from the preparation of this derivative was obtained by methylating samples of [^{14}C]-IAA equivalent to approximately 300,000 dpm, 200,000 dpm and 100,000 dpm, using 100 µl of diazomethane solution. Following esterification, each sample was analysed by radio -TLC on silica gel plates, with adjacent reference spots of underivatised IAA using benzene:acetone (60:40; v/v) as the solvent system. The plates were developed for 15 cm following which the distribution of [^{14}C] activity was determined by use of the radiochromatogram scanner, with the position of the underivatised IAA prelocated by examination under $UV_{254\ nm}$ light.

The radio-TLC profiles of the three methylated [^{14}C]-IAA samples revealed the presence of only one peak with a Rf value of 0.53-0.61 in each case, whilst the Rf of the underivatised IAA was 0.12-0.20. Since no other peak of radioactivity was detected in the methylated samples, it was concluded that total conversion of IAA to IAA-Me had occurred.

(ii) Trimethylsilylation of IAA

The one-step derivatisation of hydroxyl, carboxyl and amine groups

Figure 16: Mass spectrum of IAA - Me


100
RA
0
50
m/e
200

can be carried out by trimethylsilylation (Drozd, 1975). BSA was originally demonstrated by Klebe et al. (1966) to silylate the functional groups of organic compounds under mild conditions to yield volatile, stable derivatives. It was demonstrated previously (Results Section A, Part 4 (a)) that mixing IAA with BSA at room temperature gave a single compound when the mixture was subjected to GLC. However, when such a mixture was left for 2 h at 60°C, then a larger second derivative also appeared in the GLC trace. The appearance of these two compounds is compatible with the silylation reaction of IAA and BSA occurring in two steps; firstly, the initial silylation of the carboxyl group; and secondly, the slower silylation of the amine group. The presence of multiple derivatives of compounds as a result of silylation has been previously reported (Coward and Smith, 1969).

In order to determine the reaction time required to form the bis -TMSi derivative of IAA, BSA and IAA were reacted over a specific time period, with the products of the reaction being examined by GLC. The GLC conditions were:

column: 5% OV-101 (GCQ100-120); 5 ft glass
oven temp.: 196°C
carrier gas: N_2 at 40 ml min^{-1}
detector: FID

The reaction mixture was prepared by dissolving IAA (2 mg) in BSA (200 µl). Upon mixing, 1 µl of this sample was injected into the GLC and the vial was placed in an oven at 60°C. At various time intervals, 1 µl samples of this mixture were subjected to GLC analysis. In addition, between each sample injection, aliquots of BSA (3 µl) were injected to ensure that no column effects such as adsorption had occurred.

The resultant GLC trace showed that two derivatives were formed. Initially, only one derivative appeared in large amounts, but with time the second derivative increased in amounts with a corresponding decrease

in the first derivative. The T_R of the first or mono-derivative was 11.0 min, whilst the T_R of the second or bis-derivative was 14.0 min. The amounts of bis-derivative detected was then calculated as a % of total IAA present and plotted against time (Figure 17). After 180 min of reaction, 99% of bis-TMSi derivative had been found.

This experiment was repeated on two other occasions, with the time of reaction to reach 99% of bis-TMSi derivative being 175 min and 180 min. Thus the overall reaction time was 178.3 min.

The precise identity of the two derivatives was investigated by GC-MS analysis. A fresh sample of IAA (10 mg) in BSA (800 µl) was prepared and reacted for 2 h at 60°C, and then subjected to GC-MS analysis (AEI MS-30) on a 5 ft 3% SE-30 (GCQ 100-120) column at 200°C. Injections of 0.1 µl of the mixture again revealed the characteristic two peaks, with T_R values of 4.2 and 5.2 min, respectively. Mass spectra of each peak were then taken (Tables 14 and 15).

Table 14: Mass spectrum of mono-derivative

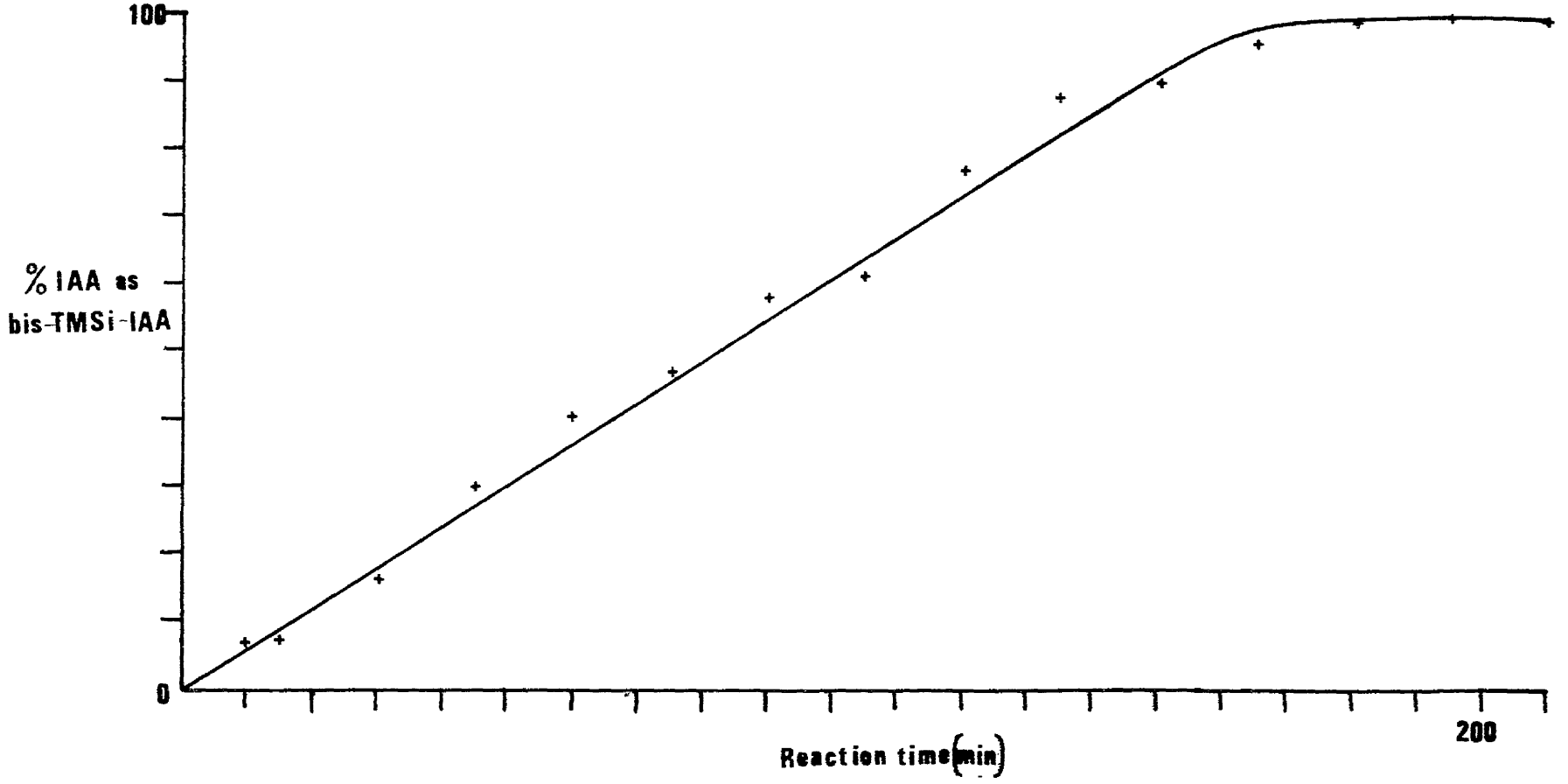
m/e	73	74	75	76	77	78	101	102	103	104
RA	39.2	13.7	37.2	7.8	33.3	2.9	2.9	11.7	17.6	1.9
m/e	128	129	130	131	232	233	247	248	249	
RA	7.8	17.6	100	31.3	17.6	3.9	49.0	9.8	3.9	

Table 15: Mass spectrum of bis-derivative

m/e	73	75	129	130	200	202
RA	38.2	11.7	5.9	8.8	3.9	100
m/e	203	204	304	319	320	321
RA	17.6	5.8	8.8	47.1	8.8	3.0

The spectrum of the mono-derivative was consistent with that of a 3-substituted indole and the molecular ion of m/e 247 indicated that the

Figure 17: % of bis - TMSi -, IAA formed with time


100
0
% IAA as
bis-TMSi-IAA
200
Reaction time(min)

compound was IAA silylated at the carboxyl group. The spectrum of the bis-derivative is also shown as a line diagram (Figure 18B) together with the structures of the ions present. Ions m/e 319, 304 and 202 were compatible with that of IAA silylated at the carboxyl and amine positions. When compared with spectra of 3-substituted indoles (e.g. Figure 13), little fragmentation was observed to occur after the formation of the base ion.

It is apparent, therefore, that the silylation of IAA is a two -stage process (Figure 18A) where silylation is initially confined to the carboxyl group, followed by a slower silylation at the amine group.

The yield of formation of the bis-TMSi-derivative was gauged by reacting two 10 µl aliquots of the [^{14}C]-IAA solution plus 2 mg of IAA in 200 µl of BSA for 3 h at 60^{o}C. At the start of the reaction, three 5 µl samples were taken from each reaction mixture for liquid scintillation counting, and the mean of these three values was taken as the dpm present originally in the sample. On this basis, the original amount of [^{14}C]-IAA present at the onset of the reaction was found to be 13,593:5 dpm/5 µl and 13,211:4 dpm/5 µl, respectively.

At the completion of the reaction, each sample was subjected to radio-GLC analysis as described in Materials and Methods, Part 7 (d). The % counting efficiency of the radio-GLC was found to be 36.9% for [^{14}C] samples, based on the response of [^{14}C]-methyl palmitate. Aliquots of the reaction mixtures were then injected and the dpm present in each sample was calculated from the net cpm results. The actual dpm attributable to bis-TMSi-IAA for each sample was found to be 14,724.3 dpm/5 µl and 14,182.2 dpm/5 µl, respectively. Thus, the % yield was calculated and found to be 108.3% and 107.3%, indicating that total conversion of IAA to bis-TMSi-IAA had occurred.

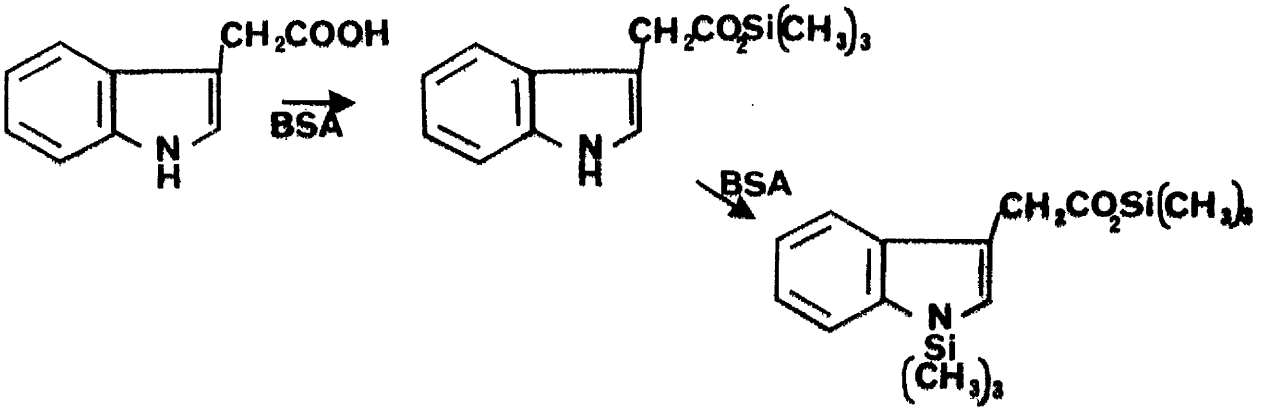
(iii) Haloacylation of IAA

Haloacyl derivatives of compounds can be prepared by the use of

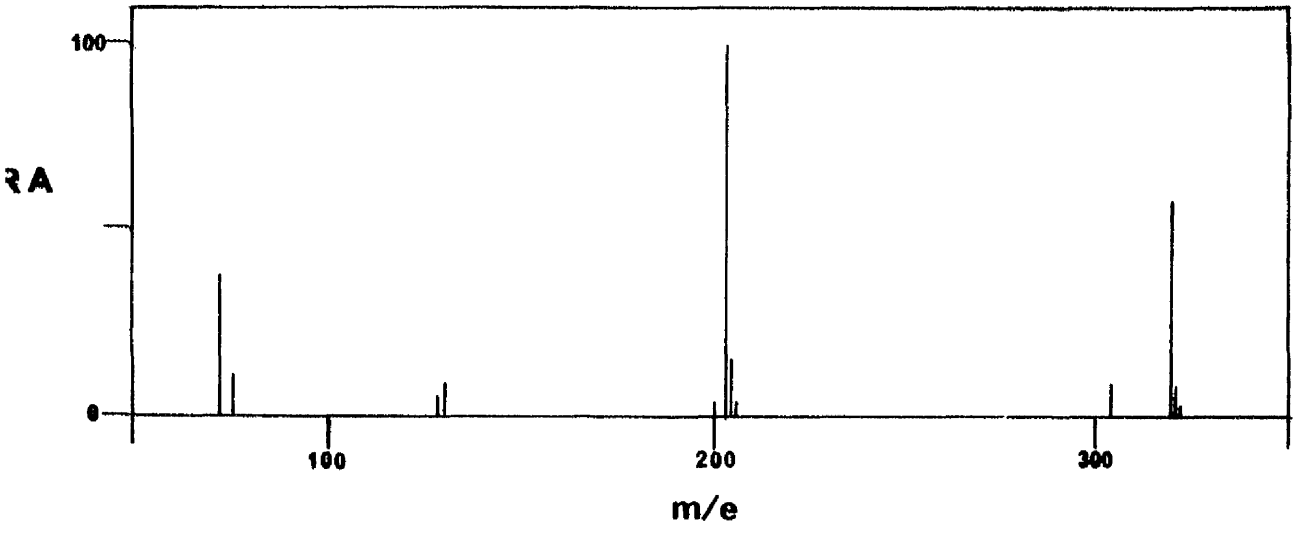
Figure 18A: Silylation of IAA by BSA

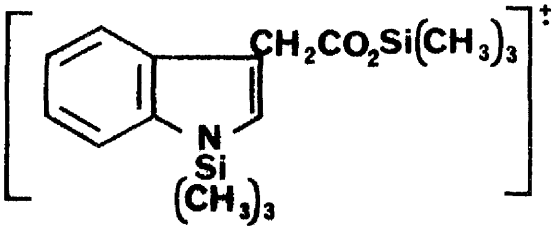
Figure 18B: Mass spectrum of bis-TMSi-derivative of IAA

A

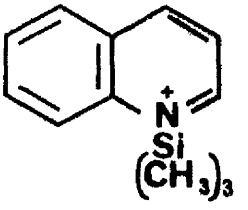


B





m/e319
(M^+)



m/e202

m/e304 $= [M^+ - CH_3]$

m/e73 $= \overset{+}{Si}(CH_3)_3$

m/e75 $= (CH_3)_2 Si{=}\overset{+}{O}H$

an appropriate anhydride or imidazole, although the anhydride can lead to undesirable side reactions. Use of the imidazole, however, does not cause such effects. These types of esters possess strong electron -capture properties, with the heptafluorobutyryl derivatives having particularly strong effects (Poole, 1976). Thus, HFB-esters of compounds have found use in quantitative ECD-GC studies (Seeley and Powell, 1974) as well as in GC-MS investigations (Rivier and Pilet, 1974).

The following series of experiments describes the preparation and mass spectral properties of the HFB-derivative of IAA-Me.

As a first step, the methyl ester of IAA was prepared by reacting two batches of IAA (1 mg) in methanol (200 µl), with etherial diazomethane solution (1 ml) for 20 min at 4°C in Reacti-vials. The solvent was then removed from each sample under a gentle stream of N_2 and HFBI (400 µl) was added to each sample. The vials were then sealed and placed in an oven at 90°C for 2 h. Following this treatment, the vials were cooled and n-hexane (2 ml) (HPLC grade, supplied by Rathburn Chemical Co., Peebleshire) and 1 N.H_2SO_4 (0.5 ml) were added to each sample. The lower acid layer was then removed and the hexane layer was rewashed twice with distilled water (0.5 ml). After the removal of the final water layer, the hexane was removed from the sample under a stream of N_2 and the residue redissolved in n-hexane (200 µl). The vials were then sealed and aliquots of each sample were subjected to GC-MS analysis. The synthetic route for this experiment is outlined in Figure 19.

GLC of the samples was carried out at the following conditions:

column: 3% OV-17, GCQ (100-120); 9 ft glass

oven temp.: 200°C

carrier gas: N_2 at 40 ml min^{-1}

detector: FID

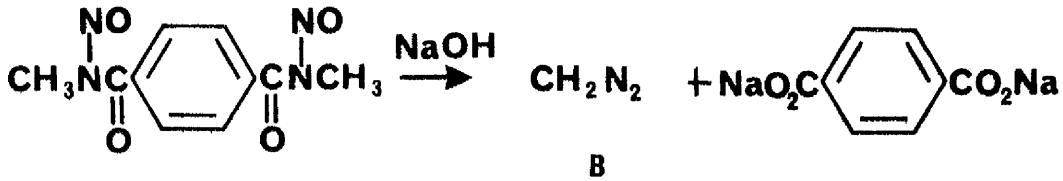
Figure 19: Synthetic route to HFB-IAA-Me

(a) Reaction of nitrosan (A) with sodium hydroxide to produce diazomethane (B)

(b) Reaction of diazomethane with IAA yielding IAA-Me (C)

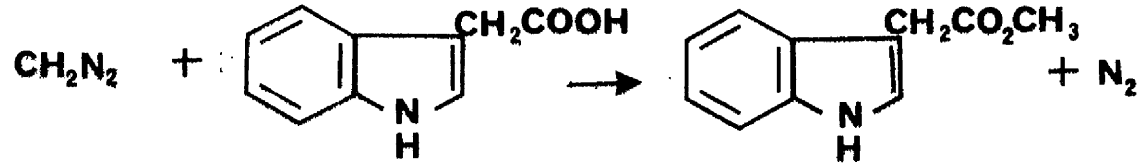
(c) Reaction of IAA-Me and HFBI (D) to give HFB-IAA-Me (E)

(a)


NO
NO
CH3NC
CNCH3
O
O
NaOH
CH2N2
+NaO2C
CO2Na

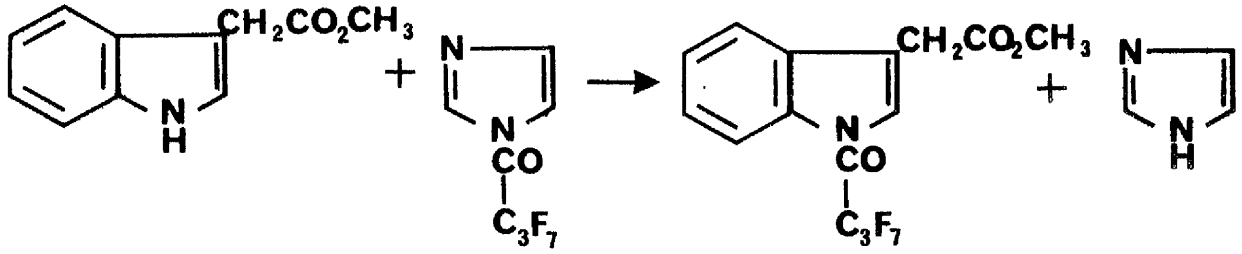
A B

(b)


CH2N2
+
CH2COOH
N
H
CH2CO2CH3
+ N2

C

(c)


CH2CO2CH3
N
H
+
N
N
CO
C3F7
CH2CO2CH3
+

D E

The T_R (min) and PA (cm^2) of each compound detected on the GLC of each reaction product are shown in Table 16. In addition, a fresh sample of IAA-Me was prepared according to the method outlined previously, and this was co-injected with the above two samples.

Table 16: GLC of reaction products

	Peak No. 1		Peak No. 2	
Sample	T_R	PA	TR	PA
2 µl sample 1	2.3	2.85	8.7	0.28
2 µl sample 1	2.3	2.15	8.7	0.18
2 µl sample 1	2.3	2.65	8.7	0.28
2 µl sample 2	2.3	2.34	8.7	0.30
2 µl sample 2	2.3	2.37	8.7	0.30
2 µl sample 2	2.3	2.25	8.7	0.28
1 µl sample 1 + IAA-Me	2.3	1.12	8.7	5.676
1 µl sample 1 + IAA-ME	2.3	1.46	8.7	OS

In both samples, a large volatile component was present that comprised 91.24 and 88.78% of the total derivatised IAA. A second component, that co-chromatographed with IAA-Me, was also present in each sample, suggesting incomplete conversion of IAA-Me to HFB-IAA-Me by HFBI.

Both samples were then analysed by GC-MS (AEI MS-30 and Jeol JMS -D100). GC-MS analysis on the AEI MS-30 was carried out under identical conditions to those outlined above. On injection of sample 1, two peaks were observed with respective T_R values of 2.8 and 9.2 min, and peak areas of 1.4 and 0.5 cm^2. A similar result was obtained with sample 2. Thus the TIM traces of the two samples revealed a lower ratio of the first compound to the second compound when compared to that obtained by GC-FID detection.

Mass spectra of the two peaks were then taken. The spectrum of

the second peak was identical to that previously shown for IAA-Me and the spectrum of the first peak is shown in Table 17.

Table 17: Mass spectrum of first derivative

m/e	69	100	101	102	103	128	129	130	131	169
RA	13.6	2.3	4.6	22.7	4.6	4.6	63.6	9.1	2.3	18.2
m/e	179	197	278	297	305	326	327	385	386	76 75
RA	4.6	4.6	4.6	4.6	4.6	100	15.9	57.3	8.3	6 4

In addition to the ions shown in Table 17, a metastable ion of m/e 276 at approximately 1-2% RA was observed.

Confirmation of this spectrum was made by subjecting the two samples to GC-MS analysis on the Jeol MS. The GC separations were carried out as described in Materials and Methods, Part 6. On injection, both samples contained a compound with T_R of 9.6 min. The spectrum of this compound (Figure 20) was found to be similar to that of Table 17. This spectrum was consistent with that of HFB-IAA-Me (Bertilsson and Palmér, 1972).

The fragmentation of this compound (Figure 21) is more elaborate than that of bis-TMSi-IAA. The metastable ion at m/e 276 can be correlated with the fragmentation of the molecular ion m/e 385 to the base ion m/e 326. Considerable fragmentation from the base ion then occurred to give ions of m/e 129, 102 and 76. The ions m/e 69 and 169 were due to CF_3 and C_3F_7, respectively.

The % conversion of IAA to HFB-IAA-Me by the above reaction was gauged in a manner similar to that described for bis-TMSi-IAA. Briefly, two samples (375, 634.2 dpm and 187, 817.1 dpm) of [^{14}C]-IAA were reacted with (i) etherial diazomethane solution (1 ml); and (ii) HFBI (40 µl). After reaction and washing, the residues of each sample were redissolved in n-hexane (200 µl) and subjected to radio-GLC analysis

Figure 20: Mass spectrum of HFB-IAA-Me

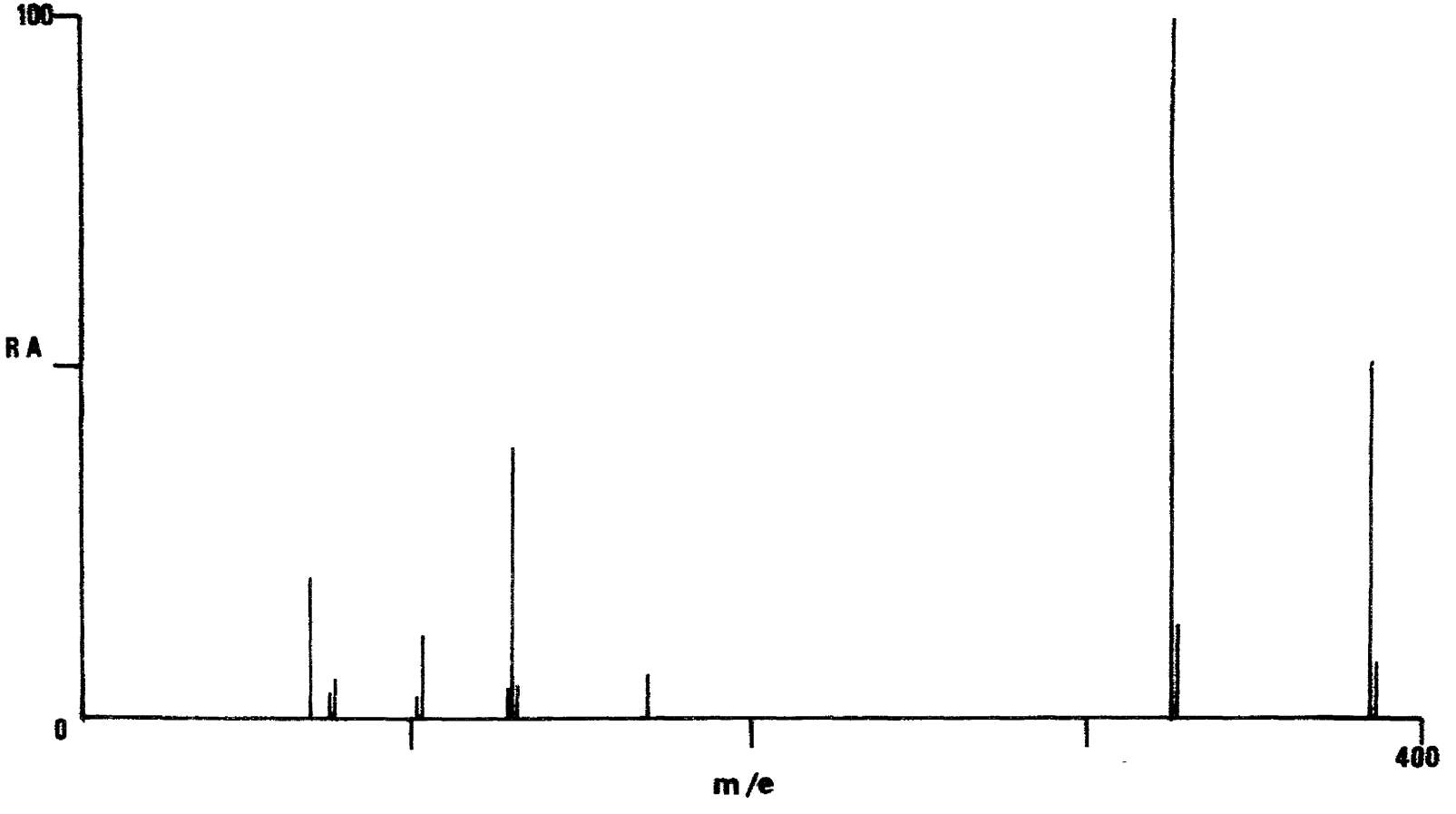
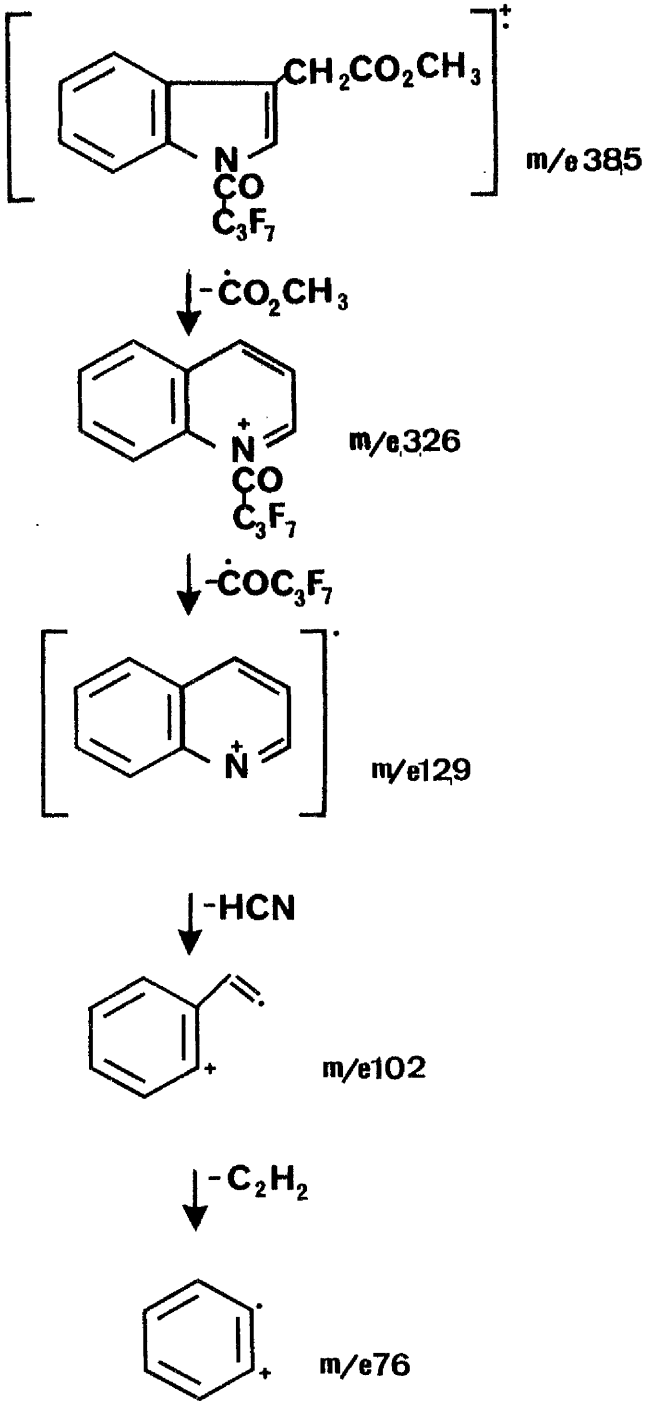

100
R A
0
400
m /e

Figure 21: Mass spectral fragmentation of HFB-IAA-Me



on a 5% OV-101 column at 170°C. From the net cpm values obtained for each sample the true dpm values attributable to HFB-IAA-Me were calculated to be 201,064.0 and 96,665.0, respectively; i.e. a 52.5% mean yield.

In summary, the GLC of underivatised IAA proved unsuccessful, although various derivatives of IAA were prepared with varying yields that were suitable for GLC. The highest reaction yields were obtained with the preparation of the IAA-Me and bis-TMSI-IAA derivatives. Moreover, the most convenient derivative to prepare was the bis-TMSI -IAA derivative, since preparation simply involved dissolving IAA in BSA (no other solvent necessary) and heating for 3 h at 60°C.

SECTION B : The Analysis of IAA

In the Introduction, several criteria were listed that should be applied to any chosen analytical method. These were (i) sensitivity, (ii) accuracy, (iii) specificity, and (iv) effect of impurities (Ewing, 1975). GLC analytical methods can satisfy many of these requirements, depending on the detector used, whilst acting as a purification system. The following series of experiments describes the use of three GLC techniques for the analysis of IAA.

(1) GC-MS Analysis

There are two common methods of GC-MS analysis: (a) repetitively scanned mass spectra (mass chromatography) or (b) selective ion detection (SID). Although both methods can yield information on the amount of a compound present, the sensitivity of SID is considerably greater than that of mass chromatography (Palmér and Holmstedt, 1975).

In order to gauge the response characteristics and relative sensitivity of SID technique to IAA, two standards of bis-TMSi-IAA were prepared at concentrations of 10 ng μl^{-1} and 1 ng μl^{-1} respectively. This derivative was chosen in preference to IAA-Me for SID studies by virtue of the ease of preparation.

The GC-MS system was the AEI MS-30 and the conditions were as follows:

column: 1% SE-30 (GCQ 100-120); 5 ft glass

oven temp.: 180°C

carrier gas: helium at 40 ml min^{-1}

separator temp.: 230 + RT °C

source temp.: 210°C

ionising voltage: 70 eV

The MS was adjusted so that only ions of m/e 202 (base ion of bis -TMSi-IAA) were detected. On the initial injection of bis-TMSi-IAA (50 ng), a peak with a T_R of 5.5 min and peak area of 12.54 cm^2 was

detected. A series of injections of varying concentrations were then made, and where a peak was observed at a T_R = 5.5 min, the PA and PH were calculated (Table 18 and Figure 22). Responses to a limit of 100 pg were achieved.

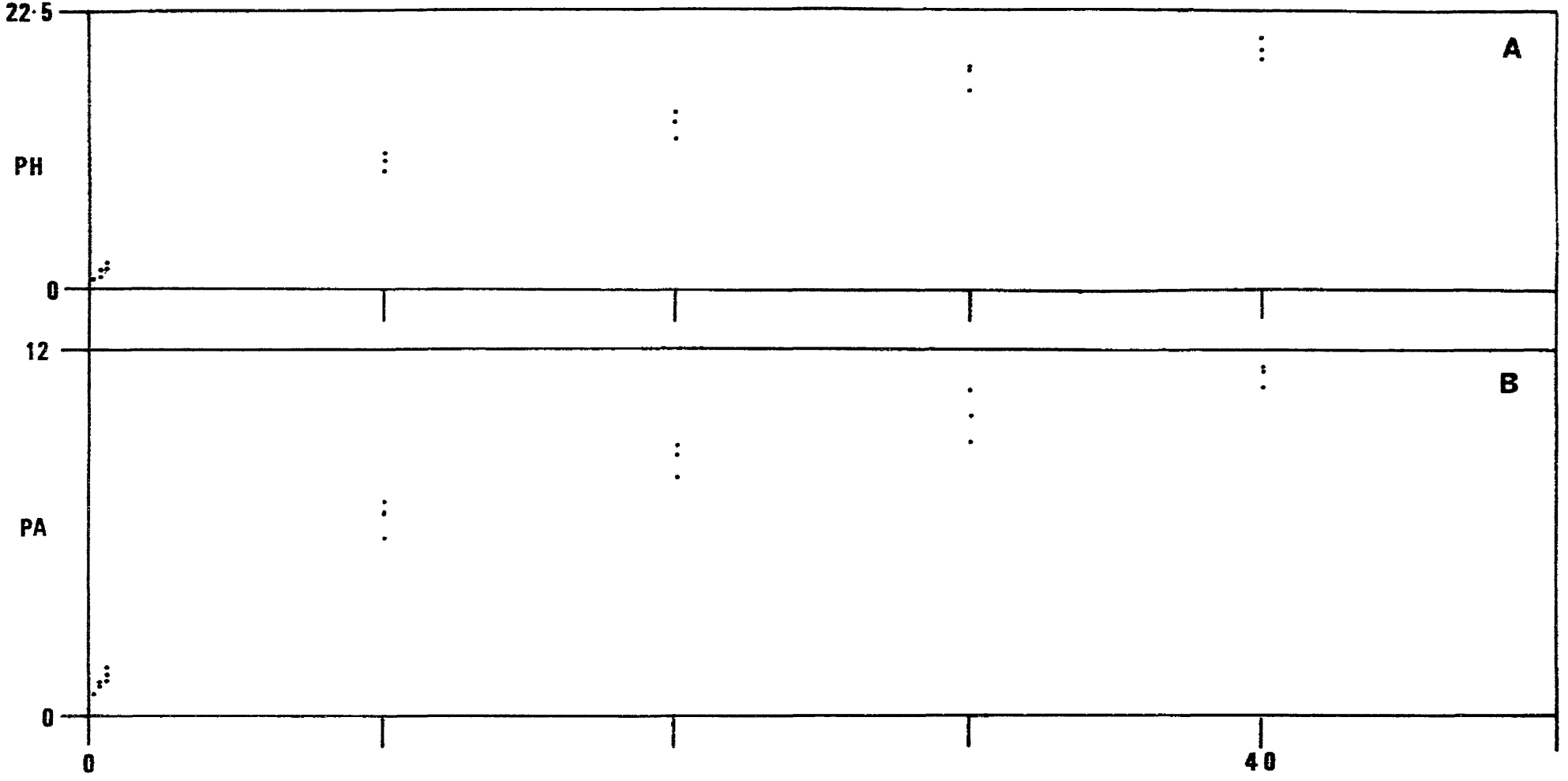
Table 18: MS response at m/e 202 to bis-TMSi-IAA

	m/e 202 Response	
Amount (ng)	PA (cm^2)	PH (cm)
0.5	1.62	2.3
0.5	1.38	2.1
0.5	1.20	1.8
0.3	1.05	1.6
0.3	1.14	1.8
0.1	0.66	0.96
10	5.88	9.85
10	6.72	10.8
10	7.02	11.2
20	8.88	14.8
20	8.4	13.4
20	7.95	12.4
30	9.9	17.8
30	10.7	18.0
30	9.02	16.2
40	11.5	20.4
40	10.73	19.3
40	11.4	18.8

The relationship between response (PA or PH) and concentration of a compound in SID studies is commonly linear (e.g. Bertilsson et al., 1972), but some non-linearity has been claimed for bis-TMSi-IAA (Robertson et al., 1977). Thus, linear correlations between PA and amount of derivatised IAA, and PH and amount were calculated for the data in Table 18:

Figure 22: A - Peak height (cm) values obtained at m/e 202 for bis-TMSi-IAA by GC-MS

B - Peak area (cm^2) values obtained at m/e 202 for bis-TMSi-IAA by GC-MS


22·5
A
PH
0
12
B
PA
0
0
40
ng of bis-TMSi-IAA

(i) $y = a + bx$; y = PA at m/e 202, x = amount of bis-TMSi-IAA (ng)

$r^2 = 0.9009$

$a = 2.1045$

$b = 0.2557$

(ii) $y = a + bx$; y = PH at m/e 202, x = amount of bis-TMSi-IAA (ng)

$r^2 = 0.9269$

$a = 3.1161$

$b = 0.4547$

The coefficient r^2 represents a "goodness of fit" value for linear correlations, where the greater the fit, the closer the value of r^2 is to 1 (Steel and Torrie, 1960). In addition, the y intercept value for a zero value of bis-TMSi-IAA was not zero, whereas injections of BSA alone gave no response. In order to determine if any other type of function could be found to express the relationship between response and amount better, the values in Table 18 were transformed to their equivalent $\log_e$ values (Table 19). Linear correlations for this transformed data were then calculated:

(i) $y = a + bx$; $y = \log_e$ PA, $x = \log_e$ amount (ng)

$r^2 = 0.9931$

$a = 0.6796$

$b = 0.4808$

(ii) $y = a + bx$; $y = \log_e$ PH, $x = \log_e$ amount (ng)

$r^2 = 0.9949$

$a = 1.1155$

$b = 0.5089$

It was thus found that a more linear relationship existed between the $\log_e$ of response and $\log_e$ of amount when compared to response and amount. This experiment was repeated on six other occasions and similar

Table 19: Log - log transformation of values in Table 18

$\log_e$ Amount (ng)	$\log_e$ PA	$\log_e$ PH
-0.69	0.48	0.83
-0.69	0.32	0.74
-0.69	0.18	0.59
-1.20	0.05	0.47
-1.20	0.13	0.59
-2.30	-0.42	-0.04
2.30	1.77	2.29
2.30	1.91	2.38
2.30	1.95	2.42
3.0	2.18	2.69
3.0	2.13	2.60
3.0	2.07	2.52
3.40	2.29	2.88
3.40	2.37	2.89
3.40	2.20	2.79
3.69	2.44	3.02
3.69	2.37	2.96
3.69	2.43	2.93

results were obtained. In one other experiment using the Jeol GC-MS system, however, a contradictory result was obtained.

The relationship $\log_e y = a + b \log_e x$ represents a transformation of $y = ax^b$ (Steel and Torrie, 1960) which can be conveniently calculated without adjustment of the raw data (see Materials and Methods, part 8). Thus, in later experiments on the AEI MS-30, where a series of bis-TMSI -IAA standard responses were made, the relationship $y = ax^b$ has been calculated.

Although SID has proved a sensitive technique for IAA analysis, little information is available on the effect of impurities on SID measurement. In order to gauge such possible effects, several standards of bis-TMSi-IAA containing varying amounts of dry plant extract were subjected to SID.

The plant extract was prepared by extracting 500 g of P. vulgaris shoot tissue in methanol, following which the extract was purified according to procedure No. 1. At the completion of the acid-ether partitioning, the extract was reduced to a dry residue by RFE. Aliquots of this were added to a series of Reacti-vials and the weight of the residue in each determined, such that a dry weight range of approximately 5 to 15 mg, for four samples, was attained. Table 20 shows the weight of extract in each sample.

A standard of bis-TMSi-IAA in BSA was then prepared at a concentration of 1 ng μl^{-1}, and 100 µl of this standard was added to each residue, after which the vials were sealed.

Table 20: Dry weight of plant extract in each sample

Sample No.	Wt. of extract (mg)
1	10.5
2	14.5
3	7.5
4	4.7

The samples were then subjected to SID analysis (AEI MS-30), by pre-tuning the MS to detect the base and molecular ions of bis-TMSI-IAA, namely m/e 202 and m/e 319. The GC-MS conditions were:

column: 3% SP 2250 on Supelcoat (100-120); 5 ft glass

oven temp.: 210°C

carrier gas: helium at 40 ml min^{-1}

separator temp.: 235 + RT°C

source temp.: 225°C

ionising voltage: 70 eV

Initially, an injection of 1 µl of the original standard, equivalent

to 1 ng of bis-TMSi- IAA was made, and peaks at both m/e 202 and m/e 319 were observed at a T_R of 8.9 min. Three separate injections of each sample were then carried out, and the response at a T_R of 8.9 min in terms of m/e 202 (Table 21) was gauged.

Table 21: Sample responses at m/e 202 (PA)

Sample	1	2	3
1 µl of No. 1	5.25	5.60	5.60
1 µl of No. 2	5.60	5.78	5.81
1 µl of No. 3	4.73	4.70	5.13
1 µl of No. 4	4.93	5.0	5.86
1 µl of Original Standard	5.0	5.0	6.0

In order to assess the variation between samples, one way analysis of variance was carried out using the values of the samples in Table 21. The results are shown in Table 22.

Table 22: Analysis of variance for Table 21

Source of Variation	SOS	DOF	Mean Square	Variance Ratio
Samples	1.52	4	0.38	2.95
Residuals	1.42	10	0.129	
Totals	2.94	14		

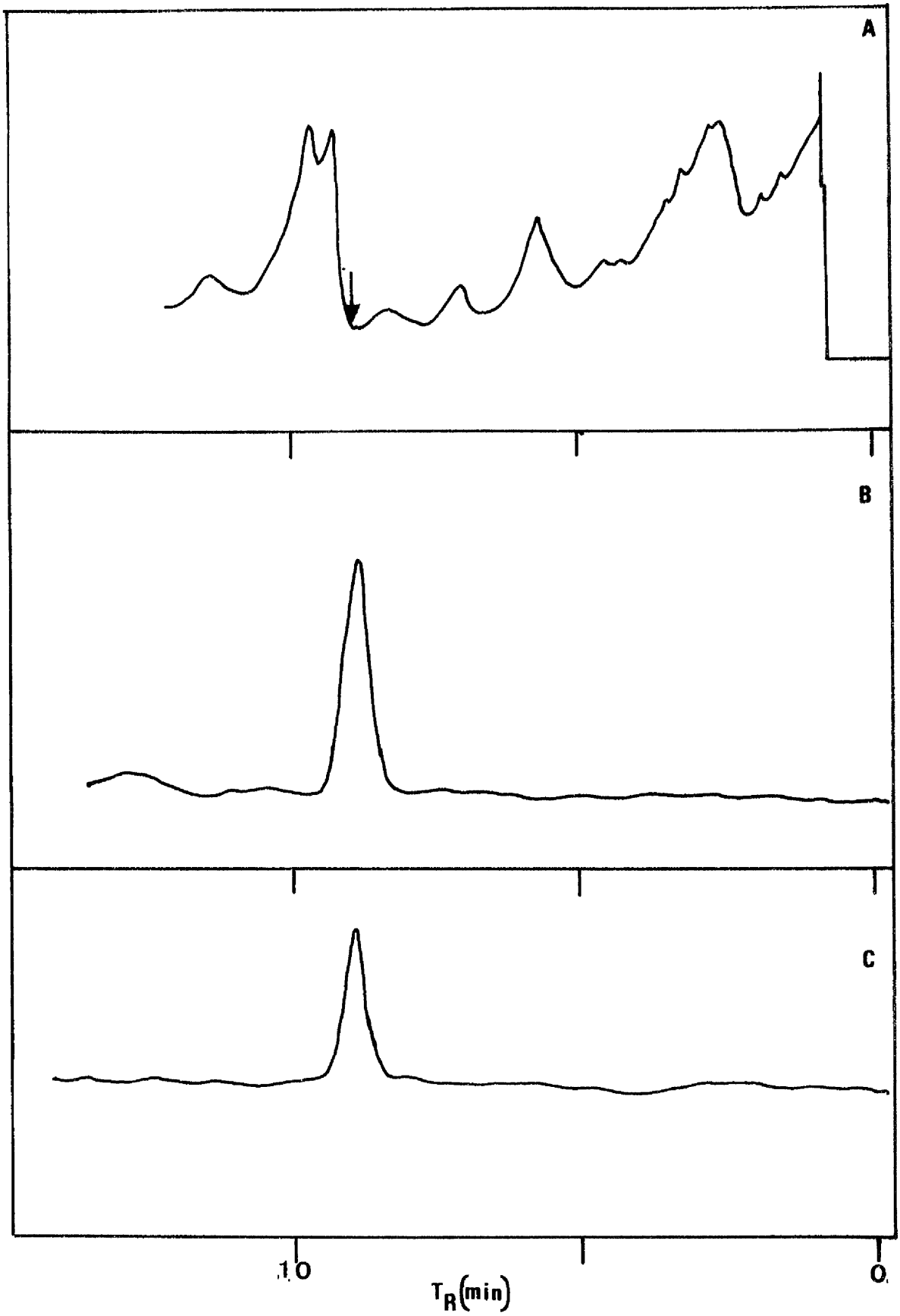
Thus, no significant variation in the sample responses was observed. It was therefore concluded that the effect of contaminants on the measurement of IAA by SID is negligible when the concentration of IAA was 100 ng in 5 - 15 mg of plant extract material. In addition, although many compounds appeared on the TIM trace, only IAA was detected at m/e 202 and m/e 319 with each sample (an example is shown in Figure 23); thereby demonstrating the high specificity of this technique. Thus,

Figure 23: GC-MS response of sample No. 2

A: TIC detected at TIM with arrow indicating the retention time of bis-TMSi-IAA

B: TIC detected at m/e 202

C: TIC detected at m/e 319


A
B
C
TIC
10
0
T_R(min)

each of the criteria laid down for an analytical system have been met by GC-MS.

(2) Electron-capture gas-chromatography

Considerable use has been made of ECD-GC in analysing other plant hormones, such as abscisic acid (Powell, 1972), but few measurements of IAA in plant tissue have employed ECD-GC, usually because of interfering impurities masking the IAA response (Seeley and Powell, 1974). Nevertheless, this technique may be useful in measuring IAA when more efficient purification procedures are used to "clean up" the plant extract.

In order to gauge the sensitivity and response of ECD-GC to IAA, the HFB-IAA-Me derivative was prepared (see Results section A, part 4); in addition, to minimise the risk of accidental contamination, the glassware was washed in 5 N HCl and rinsed in distilled water prior to the experiment. Two 1 mg samples of IAA were derivatised. After reaction, the residue containing the derivatised IAA was dissolved in "HPLC grade" n-hexane (200 µl). A solvent blank consisting of the reagent minus IAA was similarly prepared.

Both IAA samples were then diluted to give two standards whose concentrations were 250 pg of HFB-IAA-Me in 0.1 µl of n-hexane, and 50 pg of HFB-IAA-Me in 0.1 µl of n-hexane, respectively. The volume of the solvent blank was adjusted in a manner similar to that of the second standard.

Each sample was then analysed by ECD-GC at the following conditions:

column: 5% OV-17

oven temp.: 170°C

carrier gas: N_2 at 40 ml min^{-1}

detector: ECD

The IAA samples yielded only one peak at a T_R of 5.6 min. A series of injections of each standard were then made to establish a relationship

between PA (cm^2) and amount of HFB-IAA-Me; the results of both samples were found to be similar. Figure 24 represents the relationship between amount of derivatised IAA (pg) and response for the first sample of HFB-IAA-Me standard dilutions. No allowance for reaction yield has been made in the construction of Figure 24, thus the actual response of ECD-GC to HFB-IAA-Me must have been considerably lower, since the amount of IAA present as HFB-IAA-Me after derivatisation was previously shown to be 52.5%.

Although a linear response to a limit of 25 pg was noted with the derivatised samples, a response at a similar retention time was also detected with the solvent blank. For example, two injections of 1 µl of the solvent blank gave rise to peaks with a T_R of 5.6 min and PA of 0.4 and 0.25 cm^2, respectively. When compared to the IAA samples, an injection of 1 µl of the second diluted standard would have resulted in an off-scale response. It is apparent that the presence of a positive result in the solvent blank, albeit small in value, presents problems for quantitative estimations of IAA. The response of the ECD-GC to the solvent blank was substantiated in a repeat experiment.

Therefore, although the HFB derivative of IAA-Me proved useful for ECD-GC studies, the positive response of the solvent blank has made doubtful the suitability of such a technique for quantitative studies.

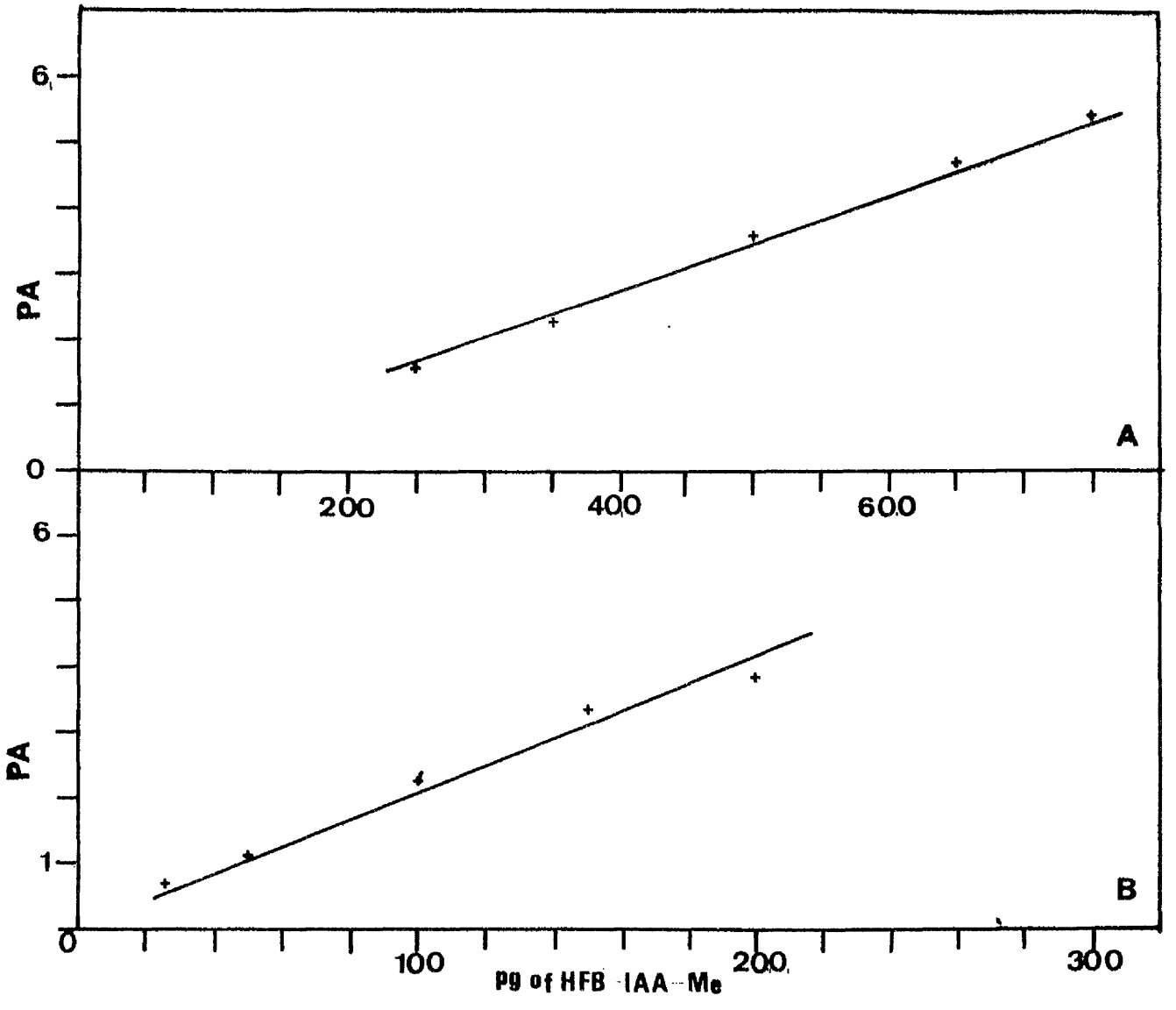
(3) Radio-GLC

The two previous series of experiments described two methods of analysing samples for IAA. If such techniques are used at the completion of a purification procedure, then an estimation of the losses of IAA incurred should be undertaken (Mann and Jaworski, 1970). The most common method of assessing these losses is by the inclusion of a radioactive isotope at the onset of the purification procedures; the percentage recovery of IAA is then calculated at the identification stage by monitoring the total radioactivity remaining in the sample,

Figure 24: Response of ECD-GC to HFB-IAA-Me

Key: A - Response of standard whose conc. was 250 pg 0.1 μl^{-1}

B - Response of standard whose conc. was 50 pg 0.1 μl^{-1}


6
PA
0
200
400
600
A
6
PA
1
0
100
200
300
B
pg of HFB -IAA--Me

usually by liquid scintillation spectrometry (e.g. Robertson et al., 1976). This method of assay has the obvious disadvantage of being unable to identify the components that contribute to the radioactivity present. In contrast, if a radioactivity monitor coupled to a chromatographic system is used, then any radioactive contaminants can be separated from the IAA prior to radioactivity analysis. There are two common systems, namely radio-TLC and radio-GLC. At present, radio -GLC has the advantage of greater resolution, in addition to the fact that it can analyse the radioactivity in a sample intended for either GC-MS or ECD-GC analysis.

In the following experiment, a sample of [^{14}C]-IAA was esterified with BSA and subjected to radio-GLC analysis to determine the response characteristics of the radio monitor. The sample was prepared by removing the solvent from 10 µl of [^{14}C]-IAA solution in Reacti-vials, adding BSA (200 µl), sealing the vials, and heating the mixture for 3 h at 60°C. After cooling, three aliquots of 5 µl were removed from the sample and the radioactivity assessed by liquid scintillation counting; the mean of these three values was found to be 14,711.6 dpm. Previously the original [^{14}C]-IAA sample had been shown by radio-TLC to have a radiochemical purity of 92.4%, and since the % conversion of IAA to bis-TMSi-IAA had previously been shown to be 100%, the radioactivity attributable to bis-TMSi-[^{14}C]-IAA was calculated as 13,593.5 dpm per 5 µl. The sample was then subjected to radio-GLC analysis (as described in Materials and Methods, part 7(d)) as a range of aliquots of varying volumes. From the resultant trace, the T_R^1 of bis-TMSi-[^{14}C]-IAA was found to be 5.0 min. After calculating the cpm detected in each sample (Table 23), the true dpm and net cpm were plotted as a graph (Figure 25).

It was observed that a response to at least 1,300 dpm could be obtained from the radio-GLC and that the relationship between net cpm

Table 23: Net cpm detected in bis-TMSi- [^{14}C]-IAA by radio-GLC

Volume of sample (µl)	Net cpm	True dpm
0.5	151.7	1359.4
1	220	2718.7
2	307.3	5437.4
3	391.6	8156.1
4	533.3	10874.8
5	660	13593.5
1	220	2718.7

and true dpm remained linear over a wide range.

In later experiments, the range of linearity was extended in some cases to include 200 dpm; this in itself may not represent a true indication of the lower sensitivity limit. Moreover, the % counting efficiency of the radioactivity monitor was found to vary by as much as 10% from experiment to experiment. In no case, however, did any significant variation occur during any sample analysis.

In conclusion, three different GLC detectors have been used in analysing IAA in samples. The GC-MS system proved more useful than ECD-GC, and it also met the criteria required for an analytical system. Radio-GLC was found to be suitable for detecting the true dpm attributable to bis-TMSi-[^{14}C]-IAA in samples intended for further GLC analysis.

Figure 25: Relationship between net cpm detected by radio-GLC and true dpm

NET
CPM
700
0
5000
10000
15000
TRUE DPM

SECTION C : The analysis of IAA in Phaseolus vulgaris

(1) Quantification of free IAA in shoot tissue

Normally, the measurement of any compound in material of a biological nature follows a basic method: (i) the isolation of the compound from the biological specimen, and (ii) the analysis of the compound by a suitably specific method (Vanden Heuvel and Zacchei, 1976).

For compounds present in low amounts in a tissue, the isolation stage will obviously require several independent steps. Commonly, these will involve extraction, solvent partitioning and chromatographic procedures. In order to develop a reliable purification procedure for free IAA in shoot tissue extracts of P. vulgaris, various systems were assessed on their relative ability to purify such extracts in Results section A. By the combination of those methods that were found to significantly reduce the degree of contamination of IAA extracts, it should be possible to obtain a reliable overall procedure suitable for the purification of shoot tissue extracts.

Those methods that were deemed suitable are:

A. Methanol for the extraction of the tissue

B. Preliminary purification of extracts by method I

C. Column-chromatography using either or both DEAE-cellulose or PVP as solid supports

D. TLC on silica gel layers in methyl acetate:propan-2-ol:25% ammonia (45:35:20; v/v)

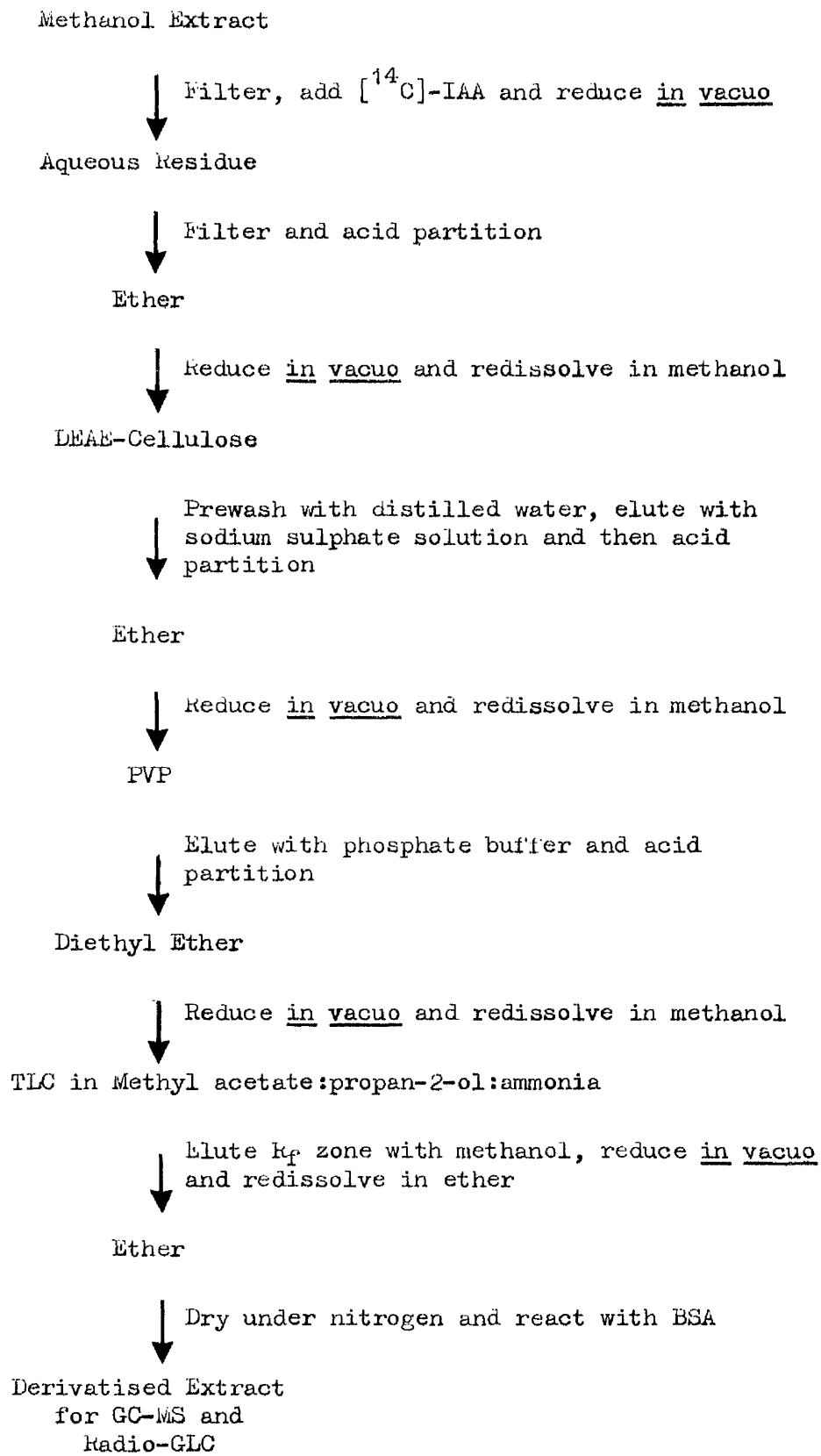
If, however, an extract of P. vulgaris is purified according to the above type of procedure, then it is apparent that the analysis stage should not only include an assessment of the IAA remaining, but should also indicate the amount of IAA that is inevitably lost during purification. The experiments conducted in Results section B indicated that these two requirements could be met by subjecting a purified extract, after reaction with BSA, to GC-MS and radio-GLC analysis, respectively.

The levels of IAA in shoot tissue of P. vulgaris were thus assessed by combining the above methods into one overall scheme (Figure 26).

Three batches of 500 g (fresh weight) of shoot tissue were extracted in 3 l of methanol for 24 h at -15°C in darkness; a solvent blank or control was also prepared. Thereafter the methanolic extracts were filtered through Whatman No. 1 paper, with the plant residue being rinsed in two further batches of 500 ml of methanol and re-filtered. An aliquot (10 µl) of [^{14}C]-IAA methanolic solution was then added to each filtrate, and the dry weight of the residues remaining were also taken. The methanolic fractions were subsequently reduced to an aqueous phase at 36°C by RFE, following which the aqueous fractions were filtered by Büchner filtration, with the residue remaining in the flask being re-washed with three lots of 50 ml of distilled water and filtered. The pH of these aqueous phases was subsequently adjusted to 3.0 by the addition of 2 N HCl, after which the acidified aqueous fractions were partitioned three times against equal volumes of diethyl ether. The combined ether layers of each sample were then taken to dryness by RFE and the residues were redissolved in approximately 20 ml of methanol (see Results section A, part 1 (B)).

For column chromatography, an initial column of DEAE-cellulose was used prior to a second column of PVP, by virtue of the higher loading capacity of the cellulose. Thus the methanolic extracts were applied to individual columns of DEAE-cellulose (see Results section A, part 2 (b)), and eluted, firstly with distilled water (300 ml), and then with 0.05 M Na_2SO_4 solution (350 ml). Following elution the IAA fractions were recovered by acidic-ether partitioning of the sulphate eluates. The resultant ether layers were reduced in vacuo and the residues redissolved in 1 - 2 ml of methanol. These methanolic extracts were applied to columns of PVP (see Results section A, part 2 (c)), and eluted with phosphate buffer (pH 8.0). The IAA-containing zone of the eluates,

Figure 26: Overall scheme for quantification of IAA in P. vulgaris shoot tissue



previously shown to be in the region of 90 - 170 ml, were collected. After acidification, these fractions were partitioned three times against equal volumes of ether, the ether removed *in vacuo*, and the residues remaining were taken up in a minimal volume of methanol.

After the above period of column chromatography, the methanol fractions were subjected to TLC by applying each extract to Sil G-UV 254 TLC plates as a streak, with adjacent lateral spots of authentic IAA. Following development of the TLC plates in methyl acetate:propan-2-ol: 25% ammonia (45:35:20, v/v), the position of the IAA reference spots was noted by examining the plates under UV light (Table 24). The zone on each TLC plate corresponding to these R_f values containing the samples was then rapidly removed and eluted with methanol (25 ml) in a 10 ml sintered glass funnel. These methanol eluates were subsequently taken to dryness *in vacuo*, and the dry weights of each ascertained. For the plant extracts the weights were 14.2, 3.98 and 3.14 mg, respectively, and for the solvent blank 1.98 mg; in addition each extract was seen to contain a significant amount of silica gel. The presence of silica gel in extracts after recovery from TLC plates using large solvent volumes has been previously described and noted by Hezel (1977). In order to remove this contamination, the samples were redissolved in ether and clarified by low speed centrifugation. After removal of the extracts from the centrifuge tubes, the samples were dried under a light stream of nitrogen, and the dry weights recorded (Table 24). Thus, the effect of redissolving the samples in ether was essentially to reduce considerably the overall contamination of the IAA zone. The dry residues of each sample were then reacted with BSA (20 µl) in sealed Reacti-vials for 3 h at 60°C.

By using the final extract dry weights, it was possible to calculate the amount of IAA that must be present for GC-MS analysis. In the experiment on the SID detection of IAA (see Results section B, part 1)

Table 24: Dry weights and R_f values of shoot tissue extracts

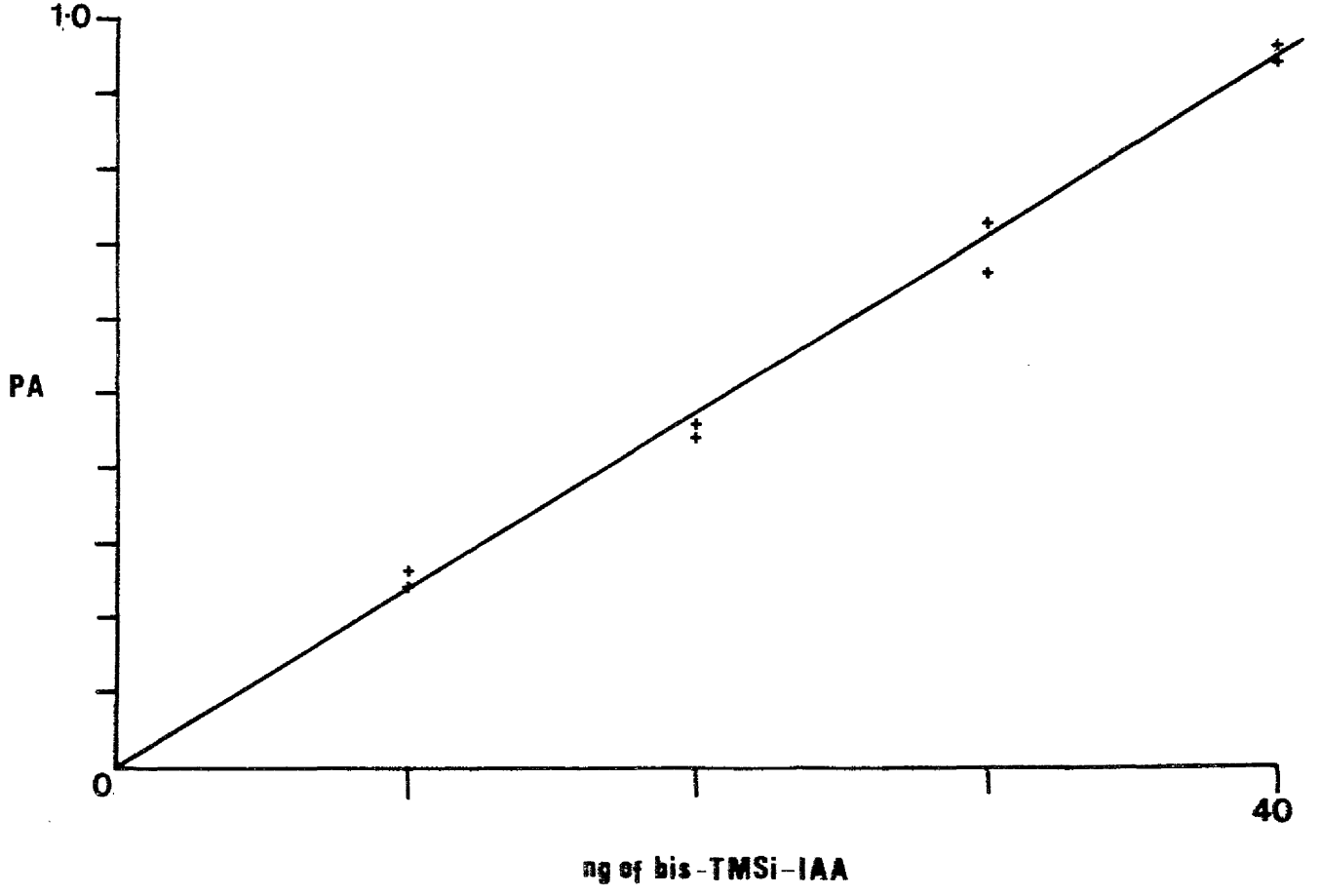
	Extract 1	Extract 2	Extract 3	Solvent Blank
Tissue dry wt (g)	28.9	47.3	52.6	-
R_f value of IAA standards	0.23-0.31	0.35-0.44	0.38-0.49	0.37-0.53
Tissue extract dry wt (mg)	0.79	0.82	1.70	0.22

it was shown that GC-MS techniques are capable of measuring IAA (100 ng) in extracts whose dry weight was 15 mg. By comparing this value to the dry weight obtained above, it was calculated that the amount of IAA in the samples could be as low as $\sim$10 ng to enable accurate quantification. In addition, if 10 ng is present in each sample, then a 2 µl injection should yield a response of 1 ng, which is a value larger than that of the least detectable amount of IAA.

The analysis of IAA in each sample was undertaken with the Jeol JMS D-100 MS, and GC separations were performed as described in Materials and Methods, part 6. Initially, only the sample from plant extract 1 was analysed, with the other samples being analysed on a different occasion.

The MS was carefully tuned to detect ions of m/e 202 only. A series of injections using standard bis-TMSi-IAA (10 ng μl^{-1}) were made in order to construct a standard-response curve. For the standard range of 10 to 40 ng, the peak response at T_R = 8.2 min was found to be linear (Figure 27), with the y intercept value being -0.0049 and r = 0.9936. Two injections of 1 µl of the sample were then made; both resulted in peaks whose T_R was equivalent to that of authentic bis-TMSi-IAA, and areas were 0.40 and 0.33 cm^2 (Figure 28). Calculation of the mean amount of bis-TMSi-IAA equivalent to these responses involved taking the y-intercept value of the response curve as 0. Thus, the sample was

Figure 27: Relationship between response at m/e 202 (PA) and amount of bis-TMSi-IAA injected


1·0
PA
0
40
ng of bis-TMSi-IAA

found to contain a mean value of 15.7155 ng of bis-TMSi-IAA per µl. Since the % yield of bis-TMSi-IAA formation was previously found to be 100%, the original sample at the derivatisation stage was calculated to contain 314.31 ng of IAA.

Although previous results indicated that sample responses in GC-MS analysis at this level of purity are unaffected, it is still desirable that some check is made on any possible effects on the sample responses that may arise by virtue of the sample and standards having a different total composition. Two methods were chosen to determine if such effects occurred. Firstly, an injection of the sample was made into the GC-MS and a mass spectrum was taken at the T_R value of authentic bis-TMSi-IAA. The resultant mass spectrum was consistent with that of bis-TMSi-IAA. Secondly, a co-injection of 1 µl of the plant extract sample and 10 ng of the standard was made, and the GC-MS response calculated. This was found to be 0.63 cm^2, thus indicating that no suppression of the bis-TMSi -IAA response by sample contaminants occurred.

In order to calculate the original amount of IAA in the plant extract, the value of IAA determined in the sample had to be corrected to take account of two factors, viz. the losses incurred of IAA during the purification procedure, and the contribution that the radioactive IAA, originally added at the onset of the purification procedure, would make to the GC-MS response. These two factors were determined by subjecting the plant sample to radio-GLC analysis as described in Materials and Methods, part 7 (d). The oven temperature of the GC was 200°C and the combined flow rate 52.6 ml min^{-1}. An initial injection of 6,922.0 dpm of bis-TMSi-[^{14}C]-IAA and 10 µg of bis-TMSi-IAA revealed the T_R of IAA on the radio-monitor to be 7.2 min. A series of injections of the bis-TMSi-[^{14}C]-IAA were then made, each yielding only one peak at the radio-monitor. From these injections, the mean counting efficiency of the radio-GLC was found to be 43.4% for [^{14}C] samples. Three injections

of the plant sample were then made with two radioactive compounds being detected on each injection (Figure 29). The peaks whose T_R were equivalent to bis-TMSi-[^{14}C]-IAA were then used to calculate the cpm attributable to [^{14}C]-IAA in the sample, which was found to be 540 cpm μl^{-1}. From this value, the true dpm and hence the % recovery of [^{14}C]-IAA was readily calculated (Table 26). In addition, since the specific activity of the [^{14}C]-IAA standard was known and the true dpm in the sample had been calculated, then the contribution of the radioactive IAA to the MS response of the sample was readily determined (Table 26).

After correction for these two factors, the content of IAA in the plant tissue in terms of both dry and fresh weight was finally calculated (Table 26).

The two other plant samples and the solvent blank were subjected to similar analytical procedures, and the m/e 202 traces, radio-GLC responses and IAA contents are shown in Figures 28 and 29, and Table 26. For these plant samples, however, no mass spectrum was taken, the method involving co-injection of sample and standard was used to ascertain if any sample effects on the GC-MS response were noted (Table 25). In fact, as noted previously, no such effects were observed.

Table 25: Results of sample standard additions

	m/e 202 response (PA, cm^2)
Std of 10 ng of bis-TMSi-IAA	0.29
1 µl of sample 2	0.69
1 µl of sample 3	0.19
1 µl of sample 2 + 10 ng std	1.10
1 µl of sample 3 + 10 ng std	0.49

Thus, the content of IAA was found to be in the range of 1.7 to 3.8 $\mu g\ kg^{-1}$ fresh weight of tissue. With regard to the solvent blank, the

Figure 28: SID responses of plant extract samples

A: Plant extract No. 1

B: " " " 2

C: " " " 3

D: Solvent blank

Arrow indicates T_R of authentic bis - TMSi - IAA

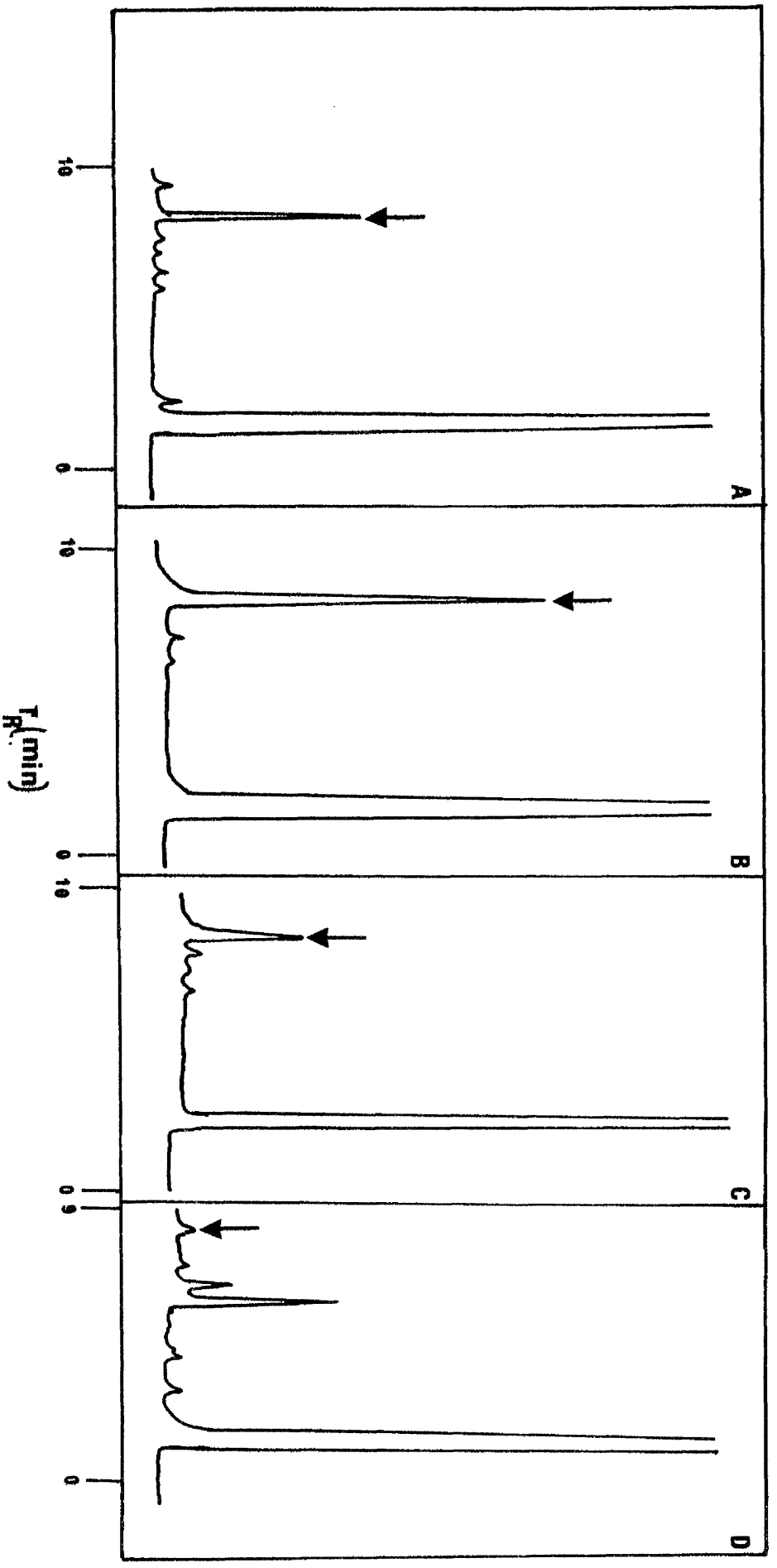

RECORDER RESPONSE
10
0
A
10
0
B
0 10
C
0 9
0
D
T_R (min)

Figure 29: Radio-GLC responses of plant extract samples

A: Plant extract No. 1

B: " " " 2

C: " " " 3

D: Solvent blank

T_R of authentic bis - TMSi - IAA for A = 7.2 min; for remainder 6.4 min.

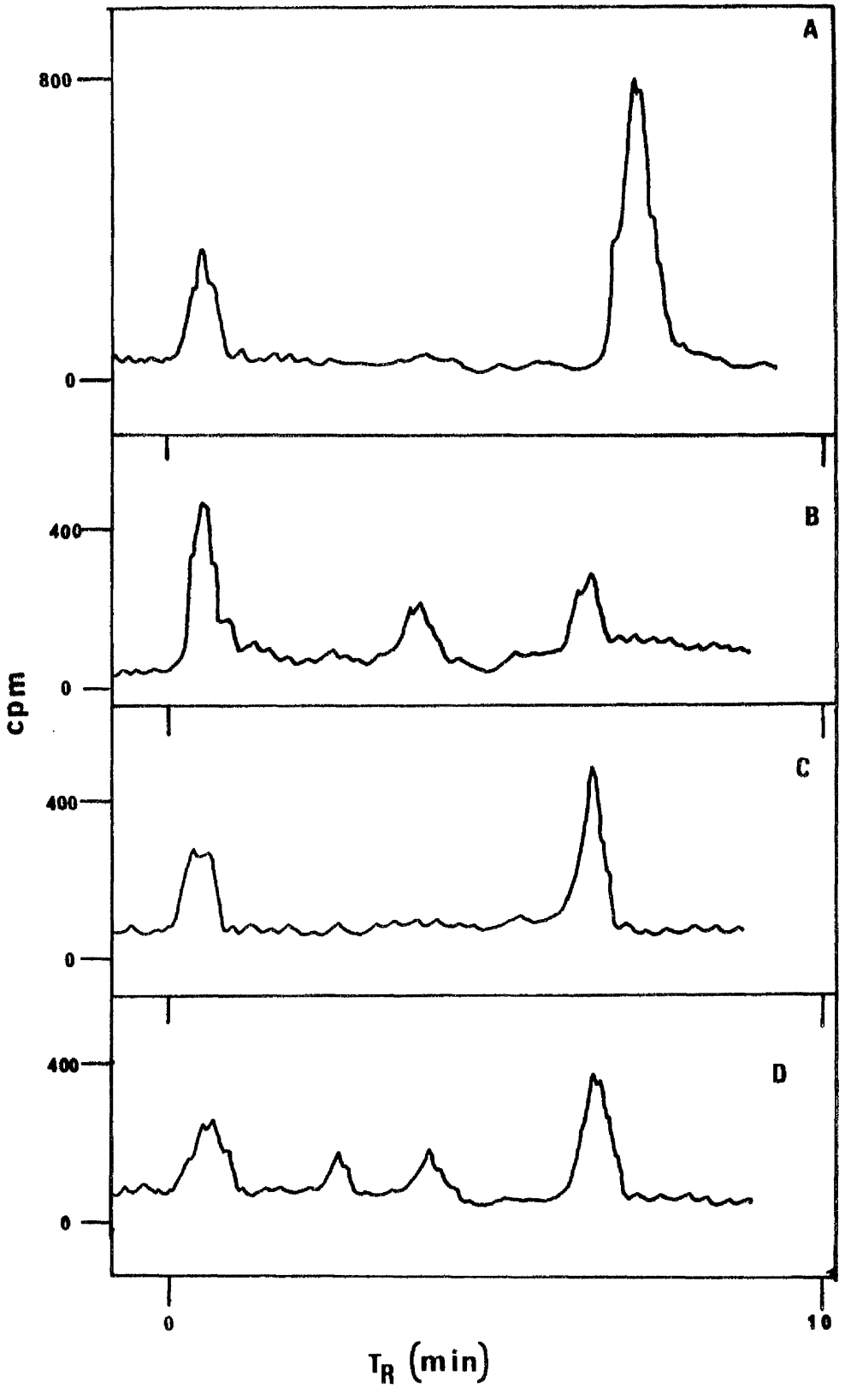

A
800
0
B
400
0
C
400
0
D
400
0
cpm
0
10
T_R (min)

Table 26: Levels of IAA in shoot tissue of Phaseolus vulgaris

	Plant Sample 1	2	3	Solvent Blank
% purification efficiency by radio-GLC	24.8	19.1	5.2	4.5
IAA detected by SID in total sample (ng)	314.3	440	110	20
Contribution to SID result by [^{14}C]-IAA (ng)	100.27	77.23	21.0	18.28
Content of IAA ($\mu g\ kg^{-1}$ fresh wt)	1.726	3.799	3.423	-
Content of IAA ($\mu g\ kg^{-1}$ dry wt)	29.864	40.15	32.54	-

value of IAA detected by SID and that contributed by [^{14}C]-IAA was close enough to assume that no IAA was accidentally incorporated into the samples from either the purification procedure or the materials used.

If, however, the radio-GLC profile of the four samples are compared (Figure 29 and Table 27) then the % of the total recovered radioactivity present as IAA can be seen to vary dramatically.

Table 27: cpm and retention time (T_R) of radioactive compounds detected in each sample by radio-GLC

Sample	T_R (min)	cpm	% total cpm as [^{14}C]-IAA
Plant extract No 1 (1 µl)	0.5	160	
	7.2	480	75
Plant extract No 2 (1 µl)	0.5	324	18.6
	3.9	48	
	6.4	85	
Plant extract No 3 (1 µl)	0.3	108	72.2
	6.4	280	
Solvent blank (3 µl)	0.45	216	
	2.5	60	38.2
	3.9	80	
	6.4	220	

T_R of authentic bis-TMSi-[^{14}C]-IAA in sample 1 was 7.2 min, in remainder 6.4 min

It is apparent from these radio-GLC profiles that breakdown of IAA occurred during the purification of the extracts, and that liquid scintillation spectrometry alone would be incapable of revealing the true percentage recovery of IAA. In addition, this breakdown, on the basis of % recovered IAA, appeared erratic. The mechanism for this variable breakdown might lie in the rotary evaporation stages, since Mann and Jaworski (1970) have shown that rotary evaporation of IAA extracts can lead to erratic recoveries of IAA.

As a next step, it was decided to examine whether such an effect occurred. Three samples of IAA in methanol were subjected to rotary film evaporation and then analysed for impurities by HPLC. Initially, three samples of IAA (6 mg) and [^{14}C]-IAA (10 µl) were dissolved in methanol (35 ml) in 50 ml round-bottom flasks. The first sample was reduced to dryness for 25 min at 36°C, the second for 20 min, and the third for 15 min. All the samples were dry after approximately 13 min; each sample was then taken up in methanol (200 µl). Two control samples, consisting of IAA (6 mg) plus [^{14}C]-IAA (10 µl) in methanol (200 µl), were also prepared; these were not subjected to rotary evaporation.

On a purely visual basis, after the period of evaporation, sample A appeared green in colour. All of the samples were then separated by HPLC, with detection by a UV and a radio-monitor. The radioactive monitor consisted of a Coru-flow manual scintillation counter with a PTFE flow cell, through which the column eluate, after the addition of scintillation fluid, passed. The HPLC conditions were as follows:

column : Partasil 10 (10 x 450 mm) at 30°C

stationary phase : 43% 0.5 M formic acid

mobile phase : 45% ethyl acetate in n-hexane

flow rate : 4.6 ml min^{-1}

radio-monitor : range 30 cps FSD; TC 10 S

UV monitor : 0.2 A FSD

Both the samples and controls yielded one peak at the radio and UV monitors, with identical T_R values and similar mass responses. It was concluded therefore that no appreciable breakdown of the IAA had occurred as a result of being subjected to rotary evaporation in methanolic solution. Some breakdown may have occurred to give rise to the green colouration, but only to a minimal extent.

Although no discernable change in the levels of IAA were found after rotary evaporation from methanolic solution, some degradation may occur following similar evaporation from etherial solutions, perhaps as a result of the presence of peroxides, which are known to affect the structure of indoles (Sundberg, 1970). This possibility was tested by carrying out an experiment using procedures similar to those above but with the substitution of diethyl-ether as the rotary evaporation solvent. No significant breakdown of IAA, however, was observed.

In summary, no obvious stage in the extraction and purification procedure was found to be the cause of the erratic breakdown of IAA. Most of the methods employed are common to many other purification methods, despite the fact that hitherto not all of the stages have been used together. Radio-GLC enabled measurement of the true content of [^{14}C]-IAA recovered, allowing compensation for the erratic breakdown during the purification procedure. GC-MS proved suitable for the quantification of IAA in these purified shoot tissue extracts.

(2) Quantification of free IAA in foliar tissue

The following series of experiments describes the application of the combined extraction, purification and analytical methods to the measurement of IAA in the foliar tissue of *P. vulgaris*.

For harvesting of tissue, a standard method was used throughout. In essence, the leaves (laminae plus petioles) and shoot apices were gathered from 5000 bean plants (approximately 33 d old). Four separate fractions were collected, namely, (1) apices, (2) the first or uppermost

trifoliate leaves, (3) the second or older trifoliate leaves, and (4) the primary leaves. Each fraction was weighed after harvesting, prior to extraction in the appropriate volume of methanol. A solvent blank was also processed (fraction 5).

Each methanolic extract was treated as described previously and the final extracts were then reacted with BSA prior to radio-GLC and GC-MS analysis.

Three separate experiments were carried out using this procedure, the results of which are summarised as follows:

Experiment 1

The fresh weight, residue dry weight and volume of methanol used for extraction of each fraction are shown in Table 28.

Table 28: Fresh wt., dry wt., and volume of methanol for extraction of each fraction

Fraction No.	Fresh wt. (g)	Dry wt. (g)	Volume of methanol (l)
1	78.2	10.9	1.5
2	500	61.8	3
3	500	49.0	3
4	500	34.3	3

Following purification, each extract was reacted with 20 µl of BSA, except for the solvent blank which was reacted with 30 µl of BSA. The GC-MS analysis (AEI MS-30) was performed on two occasions.

In the first analysis, fractions 1, 2 and 3 were analysed at the following conditions:

column : 3% OV-101 (GCQ 100-120); 9 ft glass

oven temp. : 200°C

carrier gas : helium at 40 ml min^{-1}

separator temp. : 220 + RT°C

source temp. : 230°C

ionising voltage : 70 eV

The MS was tuned to detect ions of m/e 202 and m/e 319. In a previous section (Results section B, part 1), it was shown that bis -TMSi-IAA would elicit a response at both m/e 202 and m/e 319 at an identical T_R. Thus, initially, a standard of bis-TMSi-IAA (5 ng μl^{-1}) was injected in varying amounts, and at the T_R value where such a dual response occurred, the PA of the resultant peaks were calculated. The samples were subsequently injected and where a response was observed equivalent to bis-TMSi-IAA, that response was noted. The overall results of this GC-MS analysis are shown in Table 29.

Table 29: GC-MS analysis of fractions 1, 2 and 3

Sample	PA m/e 202 (cm^2)	PA m/e 319 (cm^2)
10 ng bis-TMSi-IAA	6.93	0.675
20 ng "	9.945	0.9
30 ng "	15.3	1.35
BSA	-	-
1 µl fraction 1	3.68	
1 µl fraction 1	2.635	
10 ng bis-TMSi-IAA	5.20	0.795
1 µl fraction 2	1.33	-
1 µl fraction 2	0.96	-
1 ng bis-TMSi-IAA	0.28	-
1 ng bis-TMSi-IAA	0.35	-
1 µl fraction 3	2.61	0.765
1 µl fraction 3	1.785	0.27

T_R of above responses = 7.9 min

In this GC-MS analysis, no response could be obtained at the m/e 319 channel when the amount of bis-TMSi-IAA approached 1 ng, preventing determination of the ratio of m/e 202:m/e 319.

For quantification, a standard-response relationship was calculated and found to be $y = 0.331\ x^{1.1744}$ with $r^2 = 0.9878$, where y was the

response at m/e 202 in terms of peak area, and x was the amount of bis -TMSi-IAA in ng. By comparing the mean sample responses to this curve, the amount of bis-TMSi-IAA present in each sample was calculated (Table 33).

For the GC-MS analysis of the samples corresponding to fractions 4 and 5, a similar analytical procedure was adopted, with the conditions and injection procedures as beforehand. The sample and standard responses are shown in Table 30.

Table 30: GC-MS analysis of fractions 4 and 5

Sample	PA m/e 202 (cm^2)	PA m/e 319 (cm^2)
10 ng bis-TMSi-IAA	13.86	2.875
5 ng "	8.67	2.21
1 µl fraction 4	2.84	0.825
1 µl fraction 4	2.88	0.825
5 ng bis-TMSi-IAA	8.59	1.9
2 µl fraction 5	0.24	-
2 µl fraction 5	0.34	-
10 ng bis-TMSi-IAA	13.69	2.86
1 ng "	1.62	-
1 ng "	1.68	-

The standard-response curve was calculated to be $y = 1.7045\ x^{0.9404}$ where $r^2 = 0.9924$; y = response at m/e 202 and x = ng of bis-TMSi-IAA. The mean amount of bis-TMSi-IAA in the fractions was then calculated (Table 33).

Radio-GLC was then used to estimate the amount of [^{14}C]-IAA present in each fraction. A series of initial injections of bis-TMSi-[^{14}C]-IAA revealed that the counting efficiency of the radio-monitor was 21.2% for [^{14}C] samples. Each fraction was then subjected to radio-GLC analysis, and where a radio response at a T_R equivalent to that of authentic bis -TMSi-[^{14}C]-IAA was observed, the net cpm was calculated (Table 31).

Table 31: Net cpm of bis-TMSi-[^{14}C]-IAA in each fraction

Sample	net cpm
1.5 µl fraction 3	62
1.5 µl fraction 3	62
1.5 µl fraction 2	92
1.5 µl fraction 2	108
1.5 µl fraction 1	44
1.5 µl fraction 1	38
1.5 µl fraction 4	78
1.5 µl fraction 4	72
1.5 µl fraction 5	no significant peak
1.5 µl fraction 5	"

These cpm values detected for each sample were then converted to the actual dpm present in each sample, and hence the % recovery of [^{14}C]-IAA and the contribution of the [^{14}C]-IAA to the amount of IAA detected by SID were calculated (Table 32).

Table 32: True dpm and % recovery of [^{14}C]-IAA, and contribution to SID result by [^{14}C]-IAA

	Fraction No.			
	1	2	3	4
DPM present	7,885.3	20,217.3	12,534.5	15,162.7
% recovery of [^{14}C]-IAA	2.56	6.57	4.08	4.93
contribution to SID result by [^{14}C]-IAA (ng)	12.08	30.97	19.2	23.23

Thus, by calculating the amount of IAA detected by SID, and by taking into account the losses of [^{14}C]-IAA and the contribution of [^{14}C]-IAA to the SID result, the original amount of IAA present in each sample was calculated (Table 33).

Table 33: Amount of IAA present in foliar tissue of P. vulgaris

	Fraction No. 1	2	3	4	Solvent Blank
Amount of IAA detected by SID (ng)	136.49	57.54	82.18	34.68	1.5208
Content of IAA ($\mu g\ kg^{-1}$ fresh wt)	62.15	0.81	3.09	0.46	-
Content of IAA ($\mu g\ kg^{-1}$ dry wt)	445.9	6.54	31.5	6.77	-

The amount of IAA present in the apical tissue was found to be in excess of that of the foliar tissues. Of the foliar tissues, the largest amounts of IAA were detected in the older trifoliate leaves, with the levels in the younger trifoliates and the primary leaves being similar.

A small amount of IAA was detected in the solvent blank. On the basis of the previous experiment, it is probable that this IAA was the [^{14}C]-IAA originally added to the extract. This could not, however, be substantiated by radio-GLC since the level of IAA detected by GC-MS, were it [^{14}C]-IAA, would be too low for detection by radio-GLC.

Experiment 2

This experiment was essentially a repeat of experiment 1. Table 34 lists the fresh weight and volume of methanol used in extraction of each fraction and the residue dry weights.

Table 34: Fresh wt., dry wt., and volume of methanol for extraction of each fraction

Fraction No.	Fresh wt. (g)	Dry wt. (g)	Volume of methanol (l)
1	92.5	13.7	1.5
2	500	59.0	3
3	500	56.6	3
4	500	47.6	3

After extraction and purification, each fraction was reacted with BSA (20 µl) prior to analysis. For GC-MS analysis (AEI MS-30), the following conditions were different from those employed in experiment 1:

column: 1% SE-30 (GCQ 100-120); 5 ft glass

oven temp.: 180°C

Following a series of standard injections, the standard curve was calculated to be $y = 1.4369\ x^{0.7291}$ with $r^2 = 0.9944$. The T_R of the bis-TMSi-IAA standard was 9.2 min. Injections of each sample were then made, and where a response was obtained that was equivalent to the T_R of bis-TMSi-IAA, the PA at m/e 202 and m/e 319 were calculated (Table 35).

Table 35: GC-MS response of fractions 1 to 5

Sample	PA m/e 202 (cm^2)	PA m/e 319 (cm^2)	PA m/e 202 : PA m/e 319
1 µl fraction 1	2.85	0.65	4.3846
1 µl fraction 1	2.66	0.55	4.8364
1 µl fraction 2	2.44	0.54	4.5185
1 µl fraction 2	2.52	0.80	3.1500
1 µl fraction 3	5.525	1.275	4.3333
1 µl fraction 3	6.95	1.47	4.7279
1 µl fraction 4	3.30	0.60	5.5000
1 µl fraction 4	2.64	0.55	4.8000
1 µl fraction 5	1.20	0.25	4.8000
1 µl fraction 5	0.88	-	-

The mean sample response was compared to the standard-response curve, and the equivalent amount of IAA, present as bis-TMSi-IAA, was calculated (Table 38).

In this experiment, the mean ratio of the response at m/e 202:m/e 319 for the series of standards was calculated to be 4.5934. Only two sample injection results were found to vary significantly from this value, but this was not verified on repeat injections. In addition, no sample

effects on the SID responses were detected on co-injection of each sample with an aliquot of the standard bis-TMSi-IAA.

For radio-GLC analysis of the fractions, the % counting efficiency of the radio-monitor for [^{14}C] samples was found to be 22.37% at a combined flow rate of 54.55 ml min^{-1}. The net cpm attributable to bis-TMSi-[^{14}C] -IAA in each sample was then determined as previously (Table 36), and hence the true dpm and % recovery of [^{14}C]-IAA and the contribution that this [^{14}C]-IAA made to the GC-MS result were calculated (Table 37).

Table 36: Net cpm of bis-TMSi-[^{14}C]-IAA in each fraction

Sample	net cpm
1 µl of fraction 5	96
1 µl of fraction 5	63
1 µl of fraction 4	98
1 µl of fraction 4	105
1.5 µl of fraction 3	210
1.5 µl of fraction 3	225
1.5 µl of fraction 2	70
1.5 µl of fraction 2	84
1 µl of fraction 1	55
1 µl of fraction 1	63

Table 37: True dpm and % recovery of [^{14}C]-IAA, and contribution of [^{14}C]-IAA to GC-MS result

	Fraction No.				
	1	2	3	4	Solvent Blank
DPM present	14,452	12,574.7	35,517.3	24,862	19,496
% recovery of [^{14}C]-IAA	3,65	3.17	8.97	6.28	4.94
contribution to GC-MS result by [^{14}C]-IAA (ng)	22.04	19.18	54.17	37.92	29.86

After taking into account the values shown in Table 37, the original amount of IAA present in each sample was then calculated (Table 38). The

solvent blank (fraction 5) has not been included as the value of IAA revealed by GC-MS was less than the amount of IAA present as [^{14}C]-IAA (amount detected by GC-MS = 12.84 ng).

Table 38: Amount of IAA present in foliar tissue of P. vulgaris

	Fraction			
	1	2	3	4
Amount of IAA detected by GC-MS (ng)	48.84	42.28	149.80	54.14
Content of IAA ($\mu g\ kg^{-1}$ fresh wt.)	7.94	1.46	2.13	0.52
Content of IAA ($\mu g\ kg^{-1}$ dry wt.)	53.59	12.35	18.84	5.42

As in experiment 1, the apex contained considerably more IAA than the other fractions, and of the the remainder, the older trifoliate leaf tissue was found to have the highest content of IAA. In contrast to experiment 1, the younger trifoliate sample had a level of IAA in excess of the primary leaves.

Experiment 3

This experiment was conducted in a manner similar to the previous two experiments. Table 39 lists the details recorded at the extraction stage.

Table 39: Fresh wt., dry wt., and volume of methanol for extraction of each fraction

Fraction No.	Fresh wt. (g)	Dry wt. (g)	Volume of methanol (l)
1	60.8	9.1	1
2	500	67.9	3
3	500	77.7	3
4	500	44.6	3

The residues remaining after the extraction and purification of each sample were reacted with BSA (40 µl) prior to analysis.

For GC-MS analysis (AEI MS-30), the following conditions used, differed from those of experiment 1:

column : 1% SE-30 (GCQ 100-120); 5 ft glass

oven temp. : 180°C

The T_R of the derivatised IAA was found to be 10.2 min, and by calculating the response of each standard aliquot at this T_R, the standard -response relationship was found to be $y = 0.6067\ x^{1.3163}$, with $r^2 = 0.9937$. The response of each sample at this particular T_R value was then calculated (Table 40).

Table 40: GC-MS response of fractions 1 to 5

Sample	PA m/e 202 (cm^2)
0.5 µl fraction 4	0.8125
0.5 µl fraction 4	0.66
2 µl fraction 5	0.825
2 µl fraction 5	0.84
1 µl fraction 1	0.87
1 µl fraction 1	0.83
0.5 µl fraction 3	3.72
0.5 µl fraction 3	3.83
0.5 µl fraction 2	4.82
0.5 µl fraction 2	4.60

No sample effects were observed when co-injection of samples and standards were made. The amount of IAA detected in each fraction was then calculated (Table 43)

For radio-GLC analysis, the T_R of bis-TMSi-[^{14}C]-IAA was found to be 3.1 min, with a 17.6% counting efficiency for the monitor in respect of [^{14}C] samples. The net cpm of [^{14}C]-IAA present in each fraction was

then determined (Table 41) and thus the true dpm, % recovery of [^{14}C]-IAA and the contribution that this [^{14}C]-IAA made to the GC-MS values were calculated (Table 42).

Table 41: Net cpm of bis-TMSi-[^{14}C]-IAA in each fraction

Sample	net cpm
3 µl fraction 5	96
3 µl fraction 5	84
1.5 µl fraction 2	78
1.5 µl fraction 2	68
6 µl fraction 1	72
6 µl fraction 1	72
3 µl fraction 3	72
3 µl fraction 3	68
4 µl fraction 4	132
4 µl fraction 4	108

Table 42: True dpm and % recovery of [^{14}C]-IAA, and contribution of [^{14}C]-IAA to GC-MS result

	Fraction				Solvent
	1	2	3	4	Blank
DPM present	6,920	25,524	12,236	15,732	15,732
% recovery of [^{14}C]-IAA	1.53	6.16	2.98	3.84	3.84
contribution of [^{14}C]-IAA to GC-MS result (ng)	9.64	39.105	18.747	24.103	24.103

The amount of IAA originally present in each fraction was then calculated (Table 43).

It was noted that the amount of IAA detected by GC-MS in the solvent blank was essentially similar to that shown to be present as [^{14}C]-IAA.

The pattern of distribution of IAA in this experiment substantiated that of experiment 2 except that the actual values obtained were at least six fold higher.

Table 43: Amount of IAA present in foliar tissue of P. vulgaris

	Fraction				Solvent
	1	2	3	4	Blank
Amount of IAA detected by GC-MS (ng)	51.68	379.5	320.8	92.67	25.434
Content of IAA ($\mu g\ kg^{-1}$ fresh wt)	45.19	11.05	20.27	3.57	-
Content of IAA ($\mu g\ kg^{-1}$ dry wt)	301.95	81.4	130.5	40.0	-

(3) Quantification of free IAA in stem tissue

Attempts were also made to measure free IAA in stem tissue. Stem segments were harvested from batches of 5000 bean plants.

There were four fractions:

(1) apices;

(2) 1 cm portion of stem midway between nodes of younger and older trifoliate leaves;

(3) 1 cm portion of stem midway between nodes of older trifoliates and primary leaves;

(4) 1 cm portion of stem midway between nodes of primary leaves and cotyledons.

As in previous experiments, a control or solvent blank (fraction 5) was also prepared. Each fraction was extracted, purified and analysed as before. Three individual experiments were carried out.

Experiment 1

Table 44 lists the fresh and dry weights of the fractions with the volume of methanol used for extraction.

After processing, each fraction was reacted with BSA (20 µl) prior to analysis. GC-MS analysis (AEI MS-30) was conducted under the conditions used in the first leaf distribution experiment. The GC-MS analysis of the samples was conducted in two separate runs.

Table 44: Fresh wt. and dry wt., and volume of methanol used in extraction of each fraction

Fraction	Fresh wt. (g)	Dry wt. (g)	Volume of methanol (l)
1	31.0	5.5	1
2	57.9	4.0	1
3	133.3	9.6	1
4	255.4	20.7	2

In the first run, an initial series of injections of the standard bis-TMSi-IAA were made to establish the standard response relationship. The T_R of the derivatised IAA was found to be 6.0 min and standard -response curve was calculated to be $y = 2.5574\ x^{0.6768}$ with $r^2 = 0.9557$. The GC-MS responses of the samples are listed in Table 45.

Table 45: GC-MS results of fractions 1, 2, 3 and 5

Sample	PA m/e 202 (cm^2)	PA m/e 319 (cm^2)	PA m/e 202 : PA m/e 319
1 µl fraction 2	4.715	2.38	1.9811
1 µl fraction 2	5.32	2.86	1.8601
1 µl fraction 5	1.92	1.08	1.7778
1 µl fraction 5	2.17	1.35	1.6074
0.5 µl fraction 3	2.97	1.25	2.376
0.5 µl fraction 3	2.80	1.02	2.7451
1 µl fraction 1	6.56	2.2	2.9818
1 µl fraction 1	6.848	2.88	2.3778

For the second run, the GC-MS conditions were identical. The standard response relationship was calculated to be $y = 4.3614\ x^{0.9205}$ with $r^2 = 0.9926$ and the response at m/e 202 of fraction 4 (0.5 µl) at the T_R value of the standard was found to be 18.233 and 17.866 cm^2 respectively. The amount of IAA present in each sample equivalent to

their mean responses was then calculated (Table 48).

Although some variation was observed in the ratio of the m/e 202 response to that of the m/e 319, co-injection of samples with aliquots of standard revealed that no sample effects were present.

Subsequent radio-GLC analysis at a counting efficiency of 15.55% for [^{14}C] samples yielded the net cpm of bis-TMSi-[^{14}C]-IAA in each fraction (Table 46). From these values the actual dpm and % recovery of [^{14}C]-IAA, and the contribution that the radioactive IAA made to GC-MS results, was readily calculated (Table 47).

Table 46: Net cpm of bis-TMSi-[^{14}C]-IAA in each fraction

Sample	net cpm
1 µl fraction 5	24
1 µl fraction 5	32
0.5 µl fraction 4	62
0.5 µl fraction 4	56
0.5 µl fraction 2	20
0.5 µl fraction 2	20
0.5 µl fraction 1	22
0.5 µl fraction 1	36
1 µl fraction 3	22
1 µl fraction 3	16

Table 47: True dpm and % recovery of [^{14}C]-IAA, and contribution of [^{14}C]-IAA to GC-MS result

	Fraction				Solvent
	1	2	3	4	Blank
DPM present	22,380	15,436	7,332	45,532	10,804
% recovery of [^{14}C]-IAA	6.61	4.56	2.16	13.44	3.19
Contribution to GC-MS result by [^{14}C]-IAA (ng)	34.29	23.65	11.23	69.76	16.55

After correction for the [^{14}C]-IAA results, the content of IAA in each fraction was then calculated (Table 48).

Table 48: Amount of IAA present in stem tissue of P. vulgaris

	Fraction				Solvent
	1	2	3	4	Blank
Amount of IAA detected by GC-MS (ng)	83.07	54.14	47.80	187.14	14.37
Content of IAA ($\mu g\ kg^{-1}$ fresh wt.)	23.81	11.55	12.70	3.42	-
Content of IAA ($\mu g\ kg^{-1}$ dry wt.)	134.18	167.16	176.34	42.19	-

In terms of fresh weight content, the largest IAA concentration was found to occur in the apices, with the smallest levels detected in the internode between the primary leaves and the cotyledons. In contrast, on the basis of dry weight content, the internode between the older trifoliate and the primary leaves contained the largest amount of IAA, although this value was only marginally greater than that of the uppermost internode.

Experiment 2

This experiment was basically a repetition of the first experiment, and the various details of the extraction are listed in Table 49.

Table 49: Fresh wt. and dry wt., and volume of methanol used in extraction of each fraction

Fraction	Fresh wt. (g)	Dry wt. (g)	Volume of methanol (l)
1	73.2	12.2	1
2	71.5	8.0	1
3	122.6	19.0	1
4	216.95	49.3	2

After purification, each extract was reacted with BSA (40 µl) and subjected to GC-MS analysis (AEI MS-30) at the following conditions:

column : 1% SE-30 (GCQ 100-120); 5 ft glass

oven temp. : 180°C

carrier gas : helium at 40 ml min^{-1}

separator temp. : 230 + RT°C

source temp. : 210°C

ionising voltage : 70 eV

The standard-response curve, after a series of injections of bis-TMSi-IAA, was calculated to be $y = 1.9782x^{0.4804}$ with $r^2 = 0.9933$. Injections of the samples were then made and the response equivalent to that of bis-TMSi-IAA for each sample (Table 50) and hence the amount of IAA present was determined (Table 53).

Table 50: GC-MS results of fractions 1 to 5

Sample	PA m/e 202 (cm^2)	PA m/e 319 (cm^2)	PA m/e 202 : PA m/e 319
1 µl fraction 2	2.47	0.84	2.9405
1 µl fraction 2	2.31	0.44	5.25
0.5 µl fraction 3	1.52	0.63	2.4127
0.5 µl fraction 3	1.43	0.62	2.3065
0.5 µl fraction 1	1.33	0.66	2.0152
0.5 µl fraction 1	1.12	0.49	2.2857
1 µl fraction 4	0.84	0.40	2.1
1 µl fraction 4	0.90	0.40	2.25
2 µl fraction 5	2.16	1.0	2.16
2 µl fraction 5	1.96	0.7	2.8

Radio-GLC analysis of the fractions was carried out at a counting efficiency of 17.6% for [^{14}C] samples. The net cpm of bis-TMSi-[^{14}C]-IAA in each fraction was determined (Table 51), and from these values

the actual dpm, % recovery and the amount of [^{14}C]-IAA (ng) were calculated (Table 52).

Table 51: Net cpm of bis-TMSi-[^{14}C]-IAA in each fraction

Sample	Net cpm
3 µl fraction 5	100
3 µl fraction 5	90
3 µl fraction 1	55
3 µl fraction 1	65
2 µl fraction 3	60
2 µl fraction 3	45
4 µl fraction 4	60
4 µl fraction 4	50
2 µl fraction 2	45
2 µl fraction 2	50

Table 52: True dpm and % recovery of [^{14}C]-IAA, and contribution of [^{14}C]-IAA to GC-MS result

	Fraction				Solvent
	1	2	3	4	Blank
DPM present	10,488	12,456	13,768	7,212	16,608
% recovery of [^{14}C]-IAA	2.57	3.05	3.37	1.77	4.1
Contribution of [^{14}C]-IAA to GC-MS result (ng)	16.07	19.08	21.09	11.05	25.45

The original content of IAA present in each fraction was calculated after taking into account the values in Table 52 (Table 53).

No detectable level of IAA was found to be present in fraction 4 after correcting the GC-MS result. In addition, the largest concentration of IAA was detected in the internodal region between the younger and older trifoliate leaves.

Table 53: Amount of IAA present in shoot tissue of P. vulgaris

	Fraction				Solvent
	1	2	3	4	Blank
Amount of IAA detected by GC-MS (ng)	29.52	59.28	43.44	7.24	21.76
Content of IAA ($\mu g\ kg^{-1}$ fresh wt.)	7.15	18.43	5.41	-	-
Content of IAA ($\mu g\ kg^{-1}$ dry wt.)	42.89	164.75	34.91	-	-

Experiment 3

The fresh and dry weights of the tissue fractions and the volume of methanol used for each extraction are listed in Table 54.

Table 54: Fresh wt., dry wt., and volume of methanol for extraction of each fraction

Fraction No.	Fresh wt. (g)	Dry wt. (g)	Volume of Methanol (l)
1	52.2	8.4	1
2	80.9	7.7	1
3	163.0	19.3	1
4	235.8	36.8	2

After the application of the combined purification method, each extract was reacted with BSA (40 µl) prior to analysis. GC-MS analysis (AEI MS-30) was applied at the conditions of the previous experiment, except for the following:

column : 3% OV-17 (Chromosorb W 80-100); 5 ft glass

oven temp. : 185°C

The T_R of bis-TMSi-IAA was found to be 6.7 min, and the standard-response relationship was calculated to be $y = 1.1411\,x^{0.7669}$ with $r^2 = 0.9601$. In this analysis, only ions of m/e 202 were monitored. The

response of the extracts at this T_R value was then gauged (Table 55).

Table 55: GC-MS results of fractions 1 to 5

Sample	PA m/e 202 (cm^2)
1 µl fraction 3	0.9
1 µl fraction 3	0.99
1 µl fraction 2	1.22
1 µl fraction 2	0.99
1 µl fraction 5	0.65
1 µl fraction 5	0.55
1 µl fraction 1	1.50
1 µl fraction 1	1.96
1 µl fraction 4	1.10
1 µl fraction 4	1.70

After calculating the equivalent amount of IAA present in each sample (Table 58), the samples were subjected to radio-GLC analysis. Initially, a series of injections of authentic bis-TMSi-[^{14}C]-IAA established that the counting efficiency of the radio-monitor for [^{14}C] sample was 12.69%. Subsequently, the samples were injected and the net cpm of bis-TMSi-[^{14}C] -IAA determined (Table 56). From these values the dpm and % recovery of [^{14}C]-IAA, and the contribution that this made to the GC-MS results were calculated (Table 57).

Table 56: Net cpm of bis-TMSi-[^{14}C]-IAA in each fraction

Sample	Net cpm
2 µl fraction 5	33
2 µl fraction 5	36
2 µl fraction 4	36
2 µl fraction 4	35
2 µl fraction 3	30
2 µl fraction 3	27
2 µl fraction 1	38
2 µl fraction 1	42
2 µl fraction 2	33
2 µl fraction 2	30

Table 57: True dpm and % recovery of [^{14}C]-IAA, and contribution of [^{14}C]-IAA to GC-MS result

	Fraction				Solvent
	1	2	3	4	Blank
DPM present	15,760	12,412	11,232	13,988	13,596
% recovery of [^{14}C]-IAA	3.91	3.08	2.79	3.47	3.37
Contribution of [^{14}C]-IAA to GC-MS result (ng)	21.15	19.02	17.21	21.43	20.83

After correcting the GC-MS results by the values shown in Table 57, the original amount of IAA present in each fraction was calculated (Table 58).

Table 58: Amount of IAA in shoot tissue of P. vulgaris

	Fraction				Solvent
	1	2	3	4	Blank
Amount of IAA detected by GC-MS (ng)	68.82	38.36	31.28	52.24	17.30
Content of IAA ($\mu g\ kg^{-1}$ fresh wt.)	23.36	7.76	3.09	3.77	-
Content of IAA ($\mu g\ kg^{-1}$ dry wt.)	145.14	81.53	26.13	24.13	-

On the basis of both dry and fresh weight contents, the apical fraction was shown to have the largest concentration of IAA. Of the internodal fractions, the amount of IAA in fraction 2 was considerably greater than the lower two internodes.

(4) Summary of distribution experiments

(a) Foliar tissue

In order to compare the results of the three distribution experiments involving foliar tissue, the content of IAA in each fraction was converted to a value relative to the apical tissue concentration. For this purpose, a value of 1 was taken to indicate the content of IAA in apical tissue

and the amount of IAA in the remaining tissue fractions were calculated relative to this (Table 59).

Table 59: Relative amounts of IAA in each fraction based on their fresh and dry weight values

		Fraction number			
	Experiment	1	2	3	4
Fresh wt.	1	1	0.013	0.0497	0.0075
	2	1	0.1836	0.2675	0.0651
	3	1	0.2446	0.4486	0.0790
	mean value	1	0.1471	0.2553	0.0505
Dry wt.	1	1	0.0147	0.0706	0.0152
	2	1	0.2305	0.3516	0.1012
	3	1	0.2696	0.4323	0.1325
	mean value	1	0.1716	0.2848	0.083

In the three experiments, a definite pattern in the distribution of IAA was evident. Consistently more IAA was detected in the apical fractions, with the older trifoliate tissue having the second largest concentration of IAA. Of the remaining tissue fractions, the younger trifoliate leaves were found to have more IAA than the primary leaf tissue, although the converse was true with the dry weight results of the first experiment.

(b) Stem tissue

The results of the three stem distribution experiments were compared in a similar manner to that of the foliar tissue. The relative contents of each fraction are shown in Table 60.

In two experiments on the basis of dry weight content, the apical region contained the largest concentration of IAA. In contrast, in the other experiment, the uppermost internode was shown to have the largest amount of IAA. In the first two experiments, the region of the lowest

Table 60: Relative amounts of IAA in each fraction based on their fresh and dry weight values

		Fraction number			
	Experiment	1	2	3	4
Fresh wt.	1	1	0.4851	0.5335	0.1437
	2	1	2.5785	0.7566	0
	3	1	0.3322	0.1325	0.1612
	mean value	1	1.1319	0.4742	0.1016
Dry wt.	1	1	1.2458	1.3144	0.3145
	2	1	3.8412	0.8139	0
	3	1	0.5617	0.1800	0.1662
	mean value	1	1.8829	0.7694	0.1602

internode was found to contain the least amount of IAA; indeed, in one case, no IAA was detected.

DISCUSSION

It is accepted that many aspects of the growth and differentiation of higher plants are under the control of specific plant growth substances or hormones (Audus, 1972). One of the earliest discovered type of plant hormone was auxin. The main auxin in higher plants is generally considered to be indole-3-acetic acid and its status as the most widespread auxin is based on a large body of evidence from a variety of plant sources; this evidence includes many identifications using various chromatographic, colorimetric and biological assays (Thimann, 1972). In view of the equivocal nature of these techniques, several physico-chemical methods have recently been applied in identifying IAA in several plant species (e.g. Greenwood et al., 1972). The investigation of IAA in Phaseolus vulgaris began with an examination of the techniques involved when such a system is used.

In general, the analysis of components of complex biological mixtures is conducted by a three stage process involving extraction, purification and identification (Vanden Heuvel and Zacchei, 1976). This classical approach is common to most types of hormone, metabolite or drug analyses, where the chemical in question is often present in extremely low concentrations. The degree of elaboration of each particular stage of the analysis will not only be dependent on the concentration of the compound, but will be also influenced by the nature of the contamination present. Although many analyses have been made in non-photosynthetic tissue, little success has been gained when similar analytical methods are applied to photosynthetic tissue (see Introduction). In this thesis a combined extraction, purification and analytical method has enabled precise measurements of the levels of IAA in shoots of P. vulgaris. This method was also applied to both stem and leaf tissues in order to determine the distribution of IAA throughout the shoot.

Extraction of IAA from plant tissue involves certain methodological as well as site of origin problems (Dennis, 1977). The main criterion applied in the monitoring of extraction efficiency in this thesis has been the degreening of tissue, which presumably indicates plastid breakdown. Nevertheless, the intracellular location of the IAA remains a matter of conjecture. It is often assumed that a pool or pools of "free" IAA, that is IAA not chemically or physically bound to a substrate, exists within cells (Dennis, 1977). However, the extraction method used in this thesis may well be incapable of fully removing this IAA, and in addition, the possibility that "bound" forms of IAA are converted to "free" IAA during methanolic extraction cannot be discounted. It is apparent that the prime difficulty in determining extraction efficiency occurs in identifying the intracellular location of IAA.

Exhaustive extraction studies have indicated that methanol would appear to be the most suitable organic solvent for many tissues (Nitsch, 1956; Hillman, pers. comm.), but little information is available on biosynthesis or metabolism of IAA during extraction. Whitmore and Zahner (1964) have shown that IAA synthesis can occur during diethyl -ether extraction of Pinus phloem and cambial tissue. Similarly, studies by Atsumi et al. (1976) and Kuraishi (1976) have indicated that IAA can arise as an artefact from indole pyruvic acid during methanolic extraction, and that synthesis of indole pyruvic acid from tryptophan can also occur. These latter conclusions are open to the criticism that their method of assay was unspecific, in that it involved the chromatography of a crude extract followed by a mung bean hypocotyl bioassay. They did not present any evidence that their chromatographic system was capable of discriminating between IAA and other indole auxins. In addition, De Yoe and Zaerr (1976b) have pointed out that in examining extraction techniques, methods other than paper chromatography and bioassay must be applied, particularly since phenols, which may be

present in extracts, are known to affect the bioassay responses.

In the experiment involving the incorporation of [^{3}H]-tryptophan in a leaf extract (Results section 1, part 1 (A)), no radioactive IAA could be detected after 24 h incubation, suggesting that once extraction of tryptophan has occurred from tissue, no conversion to IAA will occur. This result does not exclude the possibility that endogenous indole pyruvic acid may well breakdown to IAA during extraction. In fact, indole pyruvic acid is particularly unstable and will readily degrade to IAA (Thimann, 1972); thus such a process as well as occurring during solvent extraction may also take place during the purification procedure. Therefore, the role of indole pyruvic acid in these types of studies requires further elucidation.

In addition to solvent extraction, the technique of diffusion has also been used for the collection of IAA from plant tissue. This involves the placing of the excised plant part cut ends in blocks of agar. After a suitable period to allow diffusion of the IAA into the agar, the blocks are extracted with an organic solvent (White et al., 1975). Dennis (1977) has noted that according to its proponents, diffusion techniques result in a better measure of IAA levels since true hormones must move out of the organ or tissue. This Dennis pointed out, assumes that the process occurring in vitro is identical with that in vivo. What is more, Larsen (1955) claimed that the yield of active material in a diffusion study depends on the duration of secretion, rate of production and rate of destruction at the cut surface. Nevertheless, in one comparative study, White et al. (1975) collected IAA from the shoot tips of Phaseolus vulgaris by both methanol extraction and by diffusion into agar blocks. After purification, both types of extracts were analysed for IAA content by bioassay and mass spectrometry. The results of the bioassay indicated that more IAA was obtained from the shoot tips by diffusion into agar. This pattern was not substantiated

in the mass spectrometry results, which in fact suggested that both methods of extraction will yield similar amounts of IAA from apical tissue. If, however, the technique of diffusion into agar for IAA collection is to be of use for a variety of plant species and organs, then the contribution of the cut surface effects to IAA values should be gauged.

Collections of IAA from xylem plants has also been made by centrifugation (De Yoe and Zaerr, 1976b) and by exudation into glass pipettes (Hall and Medlow, 1974). The centrifugation method utilised by De Yoe and Zaerr was found to effectively remove more IAA from shoots of Douglas fir than diethyl ether solvent extraction. Undoubtedly for IAA quantification studies in xylem and phloem sap, such techniques will yield much purer extracts than solvent extraction.

Water has also been recommended as an extractant of "free" auxin (Larsen, 1955). This method, however, allows enzymatic processes, which may alter IAA levels, to continue, although such effects can be overcome by lowering the extraction temperature and the inclusion of Na -diethyldithiocarbamate (see Larsen, 1955 and Bentley, 1961).

For the other acidic plant growth hormones, namely the gibberellins and abscisic acid, the method of extraction that has been suggested is the use of methanol. Reeve and Crozier (1978) have stated that maceration of plant tissue in cold methanol will remove all the precursors and metabolites of [^{3}H] gibberellins present. Similarly, Saunders (1978) has recommended extraction of abscisic acid from plant tissue by 80% methanol, thus the use of solvent extraction of plant tissue is not confined to IAA studies.

Following the period of solvent extraction, various purification procedures are employed in order to alleviate the difficulties that will certainly arise in measuring IAA in samples that are highly contaminated. In the initial purification methods involving solvent reduction and

partitioning, a simple method that consisted of filtration of the aqueous phase, was found to dramatically lower the dry weight of the extracts without unduly altering the IAA levels. Moreover, it was noted that the introduction of an alkali-ether partition, which should eliminate much phenolic and neutral material, did not lead to a significant reduction in the sample dry weight. Nevertheless, the possibility exists that several compounds, which might otherwise be present in the final sample, may be eliminated. This may arise from the fact that monitoring of extracts on a dry weight basis will not reveal any subtle changes in their composition. Dry weight assessments are, however, a convenient method for the examination of purification efficiencies since they do reveal the overall quantitative degree of contamination of the extract.

In contrast to filtration as a method of removing particulate matter from the aqueous phase of IAA extracts, Robertson et al. (1976) employed a centrifugation stage. Both these systems have also been recommended as a method for purifying abscisic acid extracts (Saunders, 1978).

The recovery of IAA during the two types of partitioning in these preliminary purification experiments was found to be closely similar to that of Mann and Jaworski (1970), who reported recoveries of IAA of 92-93% for the acidic-ether partitions at a pH of 3.0. In addition, it was noted that little change was found to occur in the radiochemical purity of the [^{14}C]-IAA during these preliminary purification experiments.

The main parameters by which the performance of preparative chromatographic systems are judged are (1) resolution (2) sample capacity (3) speed of operation (Reeve et al., 1976); and in the context of this thesis, high recovery rates for [^{14}C]-IAA. In the experiments on column chromatography, the main criterion applied in testing their relative efficiency was essentially their respective ability to resolve the plant pigments from the IAA containing zone.

Since these systems were gravity-fed, this obviously necessitated a loss in speed of operation. Based on their resolving ability, however, the DEAE-cellulose and PVP systems were found to be particularly effective, whilst exhibiting high recovery values for [^{14}C]-IAA. These values compare favourably with those of Bandurski and Schulze (1974) and Glenn et al. (1972), who reported recoveries of 78% from DEAE-cellulose when eluted with acetic acid, and 93% from PVP, respectively. In terms of sample capacity, the DEAE-cellulose system will obviously be preferable to the PVP system.

In using these systems it was apparent that the main disadvantage lay in the time involved in their preparation and use. Such disadvantages could be overcome in the future by the development of rapid flow column systems with solvent flow being controlled by a doseage pump. There are in use many high-performance liquid chromatographic systems that are capable of generating excellent resolution (Durley and Kannangara, 1976; Sweetser and Swartzfager, 1978), but their use is often negated by virtue of their low sample capacity and high cost. In general, it may be preferable to utilise a low pressure liquid chromatographic system which can satisfy the general requirements of speed and capacity with an acceptable lowering in resolution, prior to the utilisation of a high performance chromatograph.

In evaluating the relative merits of the various solvent systems for the TLC of IAA-containing extracts, the main consideration was to provide a reliable preparative chromatographic method although widespread use has been made of TLC as a means of analysing plant extracts for IAA (e.g. Powell, 1972). Of the systems tested, five were found to resolve completely IAA from the contaminants in the co-chromatographed plant extract. On the other hand, when methanol was used to recover IAA from the TLC support media, those plates that had been developed in methyl acetate : propan-2-ol : ammonia (45 : 35 : 20; v/v) exhibited the

highest recoveries. Indications are that the time involved in recovery is critical, since Sagi (1969) demonstrated that breakdown of IAA will occur on TLC plates if recovery is not attempted within two hours. Thus, in the experiments described in this thesis, the TLC zone was always eluted immediately following development.

When recovery from TLC plates involved elution with methanol, a high degree of contamination was noted, especially in the shoot tissue quantification experiments. In particular, when the chromatographic systems were combined into one purification procedure and applied to shoot tissue extracts, a large sample dry weight was noted after eluting the IAA from TLC plates using methanol. This effect could nevertheless be reduced by redissolving the sample in ether, presumably indicating the polar nature of the contamination. It was surmised that the bulk of this contamination was due to the elution of the UV indicator and silica gel from the TLC plate.

In contrast to most types of TLC, GLC is a more powerful separatory technique although this is tempered by a lower sample capacity (Keeve et al., 1976). Therefore, GLC was considered only as a separatory process for final sample purification.

Derivatisation of polar compounds to a suitably stable derivate is usual in order to avoid undesirable effects during GLC. These effects are commonly adsorption and thermal instability which are manifest often by peak asymmetry (Drozd, 1975). A report by Champault (1975) involving the GLC of underivatised or free IAA, indicated that such processes may not occur with IAA. In an attempt to repeat this report, two stationary phases with similar GC selectivity to that of Champault were chosen; the results of these experiments were, however, contradictory. In fact, two effects were noted, viz. the breakdown of IAA and either complete or partial adsorption of the IAA on to the stationary phase. Indeed, in one experiment a large volatile component,

methyl-indole, was noted presumably as a breakdown product of IAA. It is thus possible that the GC peak claimed by Champault (1975) to be IAA was in fact a decomposition product. This type of result illustrates the necessity of accurately identifying components in GC traces, particularly when a non-specific detector such as the FID is used.

Three methods of derivatisation were tested in order to prepare a suitably stable derivative of IAA for GLC. These were: (i) methyl esterification, (ii) trimethylsilylation, and (iii) haloacylation. Each was successful in producing stable derivatives, but as the ultimate object was the production of a quantitative analytical method, then the degree of conversion or yield of the derivatives from the IAA had of necessity to be known. Without this information, the conditions of reaction would have to be identical in all respects for each sample. It is patently obvious that in the case of plant extracts involving unknown amounts of IAA this would not have been the case. Accordingly, radio-GLC and radio-TLC techniques were used to gauge yields. For the haloacyl derivative, the yield was found to be 52.5%, even when the concentration of derivatisation agent was increased substantially above the values shown in the literature (e.g. Rivier and Pilet, 1974). Such a result demonstrates the necessity of appreciating the yield of derivatisation for GC quantitative methods, because it cannot be assumed that the reaction yield for unknown samples is similiar (Drozd, 1975).

The prepatory yields of the other two types of derivatives were found to be 100%. In choosing the TMSi derivative it had to be borne in mind its labile nature in the presence of water (Pierce catalog, 1977). Although trimethylsilylation was carried out using BSA, Grunwald and Lockard (1970) and Bandurski and Schulze (1974) have demonstrated that BTSFA can also act as a silyl donor, and that the reaction rate with this derivatisation agent and IAA is much faster.

The mass spectra of all three types of derivative were seen to

share a similar pattern. Initial fragmentation was at the α - β linkage in the side chain resulting in the formation of the quinolinium ion as the base ion. For the TMSi derivative little fragmentation was found to occur beyond this point; this was in marked contrast to the case of HFB-IAA-Me and IAA-Me. The low intensity of fragment ions beyond the base ion for bis-TMSi-IAA has previously been noted by Bridges et al. (1973) and Bandurski and Schulze (1974). The m/e losses for IAA and IAA-Me were similar after the formation of the base ion, presumably indicating a similar expulsion pattern, which is reported to be the initial loss of HCN followed by acetylene (Spiteller, 1971). Spiteller also noted that the cleavage pattern of 3-substituted indoles is similar to that of 2-substituted indoles, thus the use of mass spectrum analysis alone for unequivocal identification of indole-3-acetic acid may not be possible. No reports, however, appear to substantiate this proposition. A comparative mass spectral study of indole substituted with acetic acid at various ring positions could undoubtedly overcome this problem.

In addition to establishing the identity of each derivative, the MS results indicated that the TMSi reaction was a two-stage process. This has been previously reported by Grunwald and Lockard (1970), who used BSA in the presence of acetonitrile as the silyl donor.

Much of the difficulty that is experienced in analysing plant extracts for hormones occurs as a result of their low concentrations (Powell, 1972). Of the more generally applied identification procedures (see Introduction), only two methods can be considered to be highly specific, these are spectrofluorometry and mass spectrometry. Although spectrofluorometric methods that involve conversion of IAA to indolo-α -pyrone are precise, some difficulties have been experienced with pigmented and etiolated extracts (Eliasson et al., 1976; Mousedale et al., 1978). Mass spectrometry, when run in conjunction with gas chromatography, can provide information of a qualitative and quantitative

nature on many types of compounds. Despite this, little information has been presented on the effect of impurities on the precision of the quantitative technique. It was therefore necessary to evaluate the effect that shoot tissue extract contaminants would have on the GC-MS measurements of IAA. The most widely used type of mass spectrometer is one in which ions of sample molecules are produced by electron impact. For any of these ions to be in focus at the collector system, the following relationship must apply:-

$$m/e = \frac{H^2R^2}{2V}$$

where H is the magnetic field, R is the construction radius, and V is the accelerating voltage (Palmér and Holmstedt, 1975). For the quantitative MS studies outlined in this thesis, SID was used. This was achieved by having one or more ions in focus at the MS collectors. In order to focus one ion, the parameters H and V were kept constant, whilst for more than one ion, the values of V corresponding to the ions m/e 202 and m/e 319 were rapidly alternated by use of the multipeak monitor. Additionally, it was apparent that in these studies a careful check had to be made on the operating parameters of the MS. However, Eyem (1978) has shown that by careful focusing, the precision of the above type of analysis can be 0.2% for a given set of standards in the nanogram range. In theory, careful focusing should take determinations down to the pg range.

In Results section B, it was shown that a level of IAA as low as 100 pg could be detected. However, the relationship of peak response to the amount of IAA injected on the AEI MS-30 was found to depart from linearity. Robertson et al. (1976) have previously noted a non-linearity of response for bis-TMSi-IAA using this system. With the Jeol MS, the converse was found to be the case and no departure from linearity was detected. These two types of GC-MS systems differ in the design of

their interphase, with the Jeol MS having a metal double-jet separator. It is therefore conceivable that the non-linearity observed on the AEI MS-30 may in part be due to adsorption at the GC-MS interphase, a proposal already made by De Ridder et al. (1978) in explaining the non-linearity of testosterone measurement.

When the precision of the SID techniques was assessed by measuring the response of several samples of bis-TMSi-IAA containing varying amounts of plant extracts, no significant variation was found to occur. In addition, no peaks other than IAA were detected, thus demonstrating the specificity of the technique.

Although the SID method proved a precise and specific technique, its use is still somewhat restricted by its limits of detection. With this in mind, an ECD-GC quantitative method was then assessed, in order to develop a system for IAA quantification in a single plant. A survey of the literature revealed that the heptafluorobutyryl derivative of compounds is particularly powerful in its electron-capturing properties (Poole, 1976). This derivative of IAA-Me was therefore prepared and investigated by ECD-GC. The results demonstrated that the derivatised IAA could be detected to at least 25 pg, but as the yield was known to be approximately 50%, this value could be corrected to 12-15 pg. These results were, however, confounded by the fact that when a solvent blank was carried through the same procedure, a response equivalent in retention time but considerably lower in proportions was obtained. The origin of this impurity is not known, but possibly it arises from the original solvents or reagents. Thus, although this type of derivative would be suitable for detection of IAA in single plants, a considerable degree of purification of all experimental materials would be necessary, plus extreme care in the interpretation of results.

Radioactive IAA was used as an internal standard, and thus techniques were essential to measure it accurately bearing in mind the three

essential points of sensitivity, the effect of the radioactive IAA on total IAA measurements and the lability of IAA. If a specific analytical technique such as GC-MS is used to measure the level of total IAA, then two possibilities are available with regard to the choice of isotope: either all of the IAA in the radioactive sample should contribute to the total IAA measured or none. There are no types of radioactive IAA that would be suitable for the second alternative. Therefore [^{14}C]-IAA labelled at the carboxyl position was selected, since although the molecular ion of this type when silylated is m/e 321, the base ion is identical in m/e value to that of non radioactive IAA.

With regard to the lability of IAA, Mann and Jaworski (1970) have shown that erratic breakdown of IAA can occur during purification procedures. Hence it is feasible that if scintillation counting is used alone to measure the radioactive IAA of a sample, then breakdown products of IAA that are not excluded by the purification procedure will also be included in this measurement. In order to overcome this difficulty, radio-GLC was chosen as the means of assessing the radioactive IAA present in the samples intended for IAA measurement. This method conveniently combines a system operating at a high degree of resolution with a sensitive radioactive detector (Matucha and Smolkova, 1976). In a preliminary experiment this system was in fact shown to be a sensitive technique for the measurement of [^{14}C]-IAA present as the trimethylsilyl derivative.

To arrive at a suitable technique for IAA quantification in shoot tissue, the methods that were shown to be effective in Results sections A and B were combined into one experimental sequence. Prior to this, the problem of sample contamination was considered. Martin et al. (1975) have shown that the materials used in extracting and purifying plant extracts can be contaminated. Therefore, all the

glassware used in the procedure was thoroughly cleansed with dilute acid and the solvents were redistilled prior to use. After applying the combined method of extraction and purification to shoot tissue of P. vulgaris, a final sample dry weight of 1 mg was attained. This value was found to be well within the requirements for accurate SID quantification as described in Results section B. Nevertheless, it was noted that the solvent blank, when carried through this procedure, had a dry weight of 0.22 mg. The presence of this material could not solely be attritubed to the [^{14}C]-IAA originally added. Thus although precautions were taken to lessen sample contamination, some impurities were still present. It is probable that the bulk of this contamination originated in the materials used for purification. The dry weight thus obtained still appeared within the range acceptable for SID measurement.

After each extract had been derivatised with BSA, radio-GLC and SID analyses were carried out. Some consideration had, however, to be given to the question of GC-MS controls and standards. The solvent blank or control carried out in tandem with plant extracts is essential for the monitoring of possible cross-contamination from other sources of IAA. Also, the losses incurred during purification were monitored by the inclusion of a radioactive isotope. Although radioactive internal standards are suitable for this purpose, the commercially available samples were not deemed appropriate for estimating operator and instrument variation during GC-MS analysis. For the radio-GLC, the radioactive samples were co-injected with "cold" IAA as a means of estimating such effects. In devising a suitable system for the MS, several alternatives were considered. Closely similar molecules, e.g. 5-methyl-IAA have been used (Bertilsson and Palmér, 1972), but since these compounds are not identical in physical properties to IAA, it can be argued that they are thus not wholly suitable. In theory, IAA labelled with stable isotopes should provide the most suitable type of standard for GC-MS measurements, since

these compounds should possess identical chromatographic properties to non-labelled IAA. Such types of derivatives have been prepared for GC-MS quantification studies of steroids by Maume et al. (1973). They synthesised a perdeutero-trimethylsilyl derivative of estradiol as an internal standard, but noted that problems could occur with proton exchange between the deuterated TMSi group and the silyl derivatisation agent used on the biological material. This effect was found not to be critical for estradiol if analysis was conducted within ten hours of derivatisation. For IAA GC-MS studies, when IAA deuterated at the 2^1 position of the side chain was mixed with biological samples, exchange of protons was found to occur (Hillman, pers. comm.), thereby negating the use of this compound as an internal standard. It would appear, therefore, that the application of ^{13}C or ^{15}N for stable-isotope labelling of IAA is required.

In order to overcome this difficulty, the use of a standard sample addition method was made. This system for monitoring sample responses has been described by Ewing (1975). It could be argued that such a method is not necessary in view of the results of the experiment on the effect of sample dry weight contamination on GC-MS measurements of IAA. Despite this, the possibility cannot be excluded that the nature of these contaminants may be variable. However, the standard-sample addition method will detect any alteration between the slope of the observed sample response relationship and the calibration curve, since the added standard will also be affected by sample contaminants.

From the radio-GLC profiles of the shoot tissue extracts, several radiochemicals in addition to [^{14}C]-IAA were present, notwithstanding the fact that the nature and amount of these varied from extract to extract. Obviously if liquid scintillation spectrometry had been solely used to gauge the amount of recovered [^{14}C]-IAA, it would have been incapable of discriminating between these compounds, leading to enhanced

recovery values of IAA and consequently incorrect measurements of the [^{14}C]-IAA present in extracts. It was also noted that the recovery of [^{14}C]-IAA varied from experiment to experiment. Little et al. (1978) have recently reported erratic recoveries of [^{14}C]-IAA from Picea extracts even when liquid scintillation counting was used. In contrast to this, recovery of [^{14}C]ABA from similar extracts was not found to vary dramatically. Their purification procedure involved PVP adsorption, and paper and thin-layer chromatography with several precautions being taken to minimise IAA losses. These precautions involved (1) the rotary evaporation of extracts to partial dryness, (2) rapid loading and development of chromatograms, and (3) storage of extracts during purification at -15^{o}C. Thus in spite of these procedures, low recovery of values of [^{14}C]-IAA were still obtained. The suggestion that rotary evaporation might be the origin of such effects has been proposed by Mann and Jaworski (1970). However, when solutions of IAA were first subjected to this procedure and then analysed by HPLC no breakdown or conversion of IAA was noted. It would appear therefore that the cause responsible for the above results has not yet been detected. The implication of the appearance of several radiocompounds as an artefact is most disturbing. A large proportion of the relevant literature on IAA metabolism has come from studies where radioactive IAA is applied to a plant tissue, incubated, extracted, purified and then analysed by chromatography for radiocompounds other than IAA (Morris et al, 1969; Lepp and Peel, 1971; Davies, 1972; Minchin and Harmey, 1974). Because IAA undergoes decomposition during purification and as this decomposition is erratic, then the common control of simply using [^{14}C] -IAA incubated without plant tissue for such metabolic studies is manifestly inadequate. It is therefore important that the cause of this decomposition is identified. In addition to this, other methods used in analysing plant extracts for IAA metabolites are critical.

For example, Davies (1972) and Minchin and Harmey (1974) used the TLC solvent system chloroform : glacial acetic acid (95 : 5) as a method of analysing extracts for metabolites of exogenously applied radioactive IAA, but Bandurski and Schulze (1974) stated later that this solvent system will lead to destruction of IAA during TLC and hence result in pseudo-metabolic profiles. It can be concluded that it is necessary to investigate the effects of such experimental methods on IAA prior to undertaking IAA metabolic studies.

The GC-MS analysis results after correction for [^{14}C]-IAA values were found to be variable. The content of IAA ($\mu g\ kg^{-1}$ fresh wt.) for the shoot tissue varied from 1.7 to 3.8. These results are lower than those of Schulze and Bandurski (1976) who detected 18 $\mu g\ kg^{-1}$ of IAA in seeds of Phaseolus vulgaris. However, by use of a fluorescence method, Eliasson et al. (1976) found the IAA content in Phaseolus seeds to be 2 - 36 $\mu g\ kg^{-1}$, depending on the time of seed incubation. In addition, Hillman et al. (1977) used GC-MS procedures to detect a level of 20 - 80 $\mu g\ kg^{-1}$ of IAA in lateral buds of P. vulgaris. It is obvious that in view of the different natures of the tissue, no direct comparison is possible, whilst in addition, the distribution of this IAA may vary dramatically. It was appropriate therefore to determine how this IAA was distributed throughout the shoot tissues.

In the distribution experiments involving foliar tissue, a gradient was noted in IAA distribution from the apex. Of the tissues extracted the apex was found to contain the most IAA. In addition, considerably more IAA was detected in the older trifoliate leaves when compared with the younger, with the lowest levels of IAA being found in the primary leaves. Sweetser and Swartzfager (1978) have summarised the results of previous investigations on IAA distribution in Phaseolus vulgaris as gauged by bioassay. These results indicated that the shoot tips contain 7 - 12 $\mu g\ kg^{-1}$ fresh weight of tissue, the mature trifoliates

approximately 3 μg kg^{-1} and the primary leaves 9 - 15 μg kg^{-1}. They did, however, emphasise that these data were summarised from several literature sources and hence these values cannot be readily compared. Nevertheless, they repeated these types of measurements using a HPLC method and their results are summarised in Table 61.

Table 61: Levels of IAA in Phaseolus vulgaris (data from Sweetser and Swartzfager, 1978)

Plant part	IAA μg kg^{-1} (fresh wt.)
seeds - immature	200 - 336
trifoliates (older)	4 - 5
trifoliates (younger)	14 - 16
stem (lower)	22 - 36
stem (upper)	50 - 54

Thus, although they did detect more IAA in the apical region of Phaseolus vulgaris than in the remaining tissue, the results of the older and younger trifoliate leaf contents are in direct contrast to the leaf distribution results in this thesis. In fact, these results of Sweetser and Swartzfager suggest that the level of IAA in leaf tissue decreases with age (cf. the leaf distribution experiments). In contrast, Wheeler (1968) has shown by paper chromatography and wheat coleoptile bioassay techniques that the amount of IAA (auxin) increases with age in primary leaves of P. vulgaris to a maximum of 332 μg kg^{-1}. Wheeler (1966), using an identical technique was also able to demonstrate that there was a correlation between the levels of "free" and "bound" "IAA" in primary leaf tissue of P. vulgaris. Nevertheless, it is difficult to draw any direct comparison between such types of data, particularly in view of variation in the physiological ages between the plants used in these investigations, and the lack of specificity of the analytical technique used by these investigators.

The amounts of IAA detected in the apical tissue in both the leaf and stem distribution experiments were found to range from 7 to 62 μg kg^{-1} fresh weight. White et al. (1975) using a similar technique, found the amount of IAA in the same tissue to be 2.4 to 29.0 μg kg^{-1} fresh weight. Thus it would appear that the level of IAA in the apical tissue of P. vulgaris can vary dramatically.

In contrast to the leaf distribution experiments, no distinct pattern of IAA distribution was revealed in the complete shoot. In two experiments the largest concentration of IAA was detected in the apex; in the other experiment, however, the uppermost internode region was found to have the greatest amount of IAA. On the basis of dry weight content, the lowermost internode was consistently seen to have the largest amount of IAA. This observation is in agreement with that of Pegg and Selman (1959) who assayed the amount of IAA in shoots of tomato plants by bioassay, although no indication was given of IAA recovery rates. Sweetser and Swartzfager (1978) have indicated that more IAA is present in the upper stem portions of P. vulgaris but no precise measurements of IAA were made in specific internodal regions. Scott and Briggs (1960) have measured the amount of auxin in specific internodal regions of Pisum sativum, by extracting stem portions into ether. These ether extracts were then taken up in agar, and the amount of auxin present determined by Avena coleoptile curvature. In addition, they also collected auxin from these internodes by diffusion into agar. The auxin yield in diffusate extracts was observed to decline rapidly in the internode regions below the apex. Although the yields by extraction also fell, the level in the first two internodes remained similar to that of the apex. Indeed, after the third internode, the level of auxin fell until at the lowermost internode only one third of the auxin at the apex was present. The results of the stem distribution experiment did not reveal any similar pattern.

These results of Scott and Briggs are not accurate measurements of IAA since their method of analysis involved the application of relatively crude extracts to a relatively non-specific method of detection. In particular, no extensive purification of the extract was undertaken, thus the resultant curvature of the Avena coleoptiles cannot be attributed solely to IAA since many other compounds present in plant extracts are known to exert similar bioassay responses (Thimann, 1972; Marumo et al., 1968a; De Yoe and Zaerr, 1976b). Nevertheless, Scott and Briggs did relate these auxin levels to the relative growth of each internode. They found that the topmost internode accounted for 85% of the total stem growth, with remaining stem growth occurring in the second internode. Thus no direct correlation was found between the auxin level and the amount of stem growth. White (1973) investigated the pattern of stem growth in P. vulgaris and demonstrated that when the second trifoliate is starting to expand (the stage at which the plants in this thesis were harvested) the maximum amount of stem growth occurred in the internodal regions between the first trifoliate leaf and the primary leaf, and the primary leaf and the cotyledon. It is apparent that the levels of IAA gauged in the stem tissue do not correlate directly with this growth.

Failure to correlate IAA levels with growth of the tissue concerned has led to the hypothesis that the turnover rate of the IAA pool in tissue may be the determining factor in growth repationships. To gauge the metabolic rate of a compound, radioactive precursors or the compound itself are often applied to plant tissue and the changes in their concentration and nature determined with time (Dennis, 1977). In respect of this, Dennis has listed the precautions and assumptions involved in these types of studies, namely (1) that the compound applied should occur naturally in the tissue, (2) the amount of exogenous compound should not exceed the endogenous level if overloading effects

are not to occur, and (3) the compound fed should reach the normal site of metabolism. An alternative method may lie in the use of specific inhibitors of IAA metabolism, whereby one stage of the biosynthetic or metabolic routes of IAA is blocked thereby leading to alterations in the rate of IAA metabolism, and hence reveal any developmental phenomena controlled by such a process.

The role of IAA in apical dominance had been reviewed by Phillips (1969). It has been demonstrated that the young, growing leaves rather than the apex exerts the main inhibition on lateral bud growth in Phaseolus (White, 1973). In addition to this, IAA is known to inhibit the growth of lateral buds after release from apical dominance (Phillips, 1969). If IAA concentration in the foliar tissue of the apical region is a controlling factor in this process, then the level of IAA in this region might be expected to be high with respect to the rest of the stem and leaf tissue. The results of the leaf distribution experiments reveal considerably more IAA in the apex than the young trifoliate leaves which had just commenced expansion. Also, the level of IAA was higher in older trifoliates when compared to young trifoliates. These results again fail to reveal any correlation between IAA levels and growth control. In fact, the only correlation between IAA and growth was that of the foliar tissue, where the IAA was found to be highest in older leaves when compared to young leaves, except in the primary leaves which were starting to undergo senescence, where the IAA level was lowest.

Although the shoot tips contain the highest level of IAA when compared to leaf tissue, they did not consistently have more IAA than the remainder of the stem tissue. This variation could in part be due to active transport of the IAA throughout the shoot, since xylem and phloem exudates are known to contain IAA (Hall and Medlow, 1974), and the shoot tips are capable of synthesising IAA from precursors (Erdmann

and Schiewer, 1971; Black and Hamilton, 1971). Bacterial contamination of the plants might, however, contribute to the amounts of IAA detected. Libbert et al. (1966) have demonstrated the importance of bacteria in the normal production of auxin in Phaseolus vulgaris plants, although some auxin was produced by sterile plants. Thus the actual amount of IAA in the shoot may well vary from one group of plants to another. In addition to differences in IAA levels between plants, daily changes in IAA levels in root exudates of Citrus has been demonstrated by Bausher (1977). On the basis of this experiment, Bausher suggested that in analysing plant hormone levels, the time of harvesting may be important. Although every effort was made to harvest the plants used in this thesis at the same daily time, such effects observed by Bausher may obviously have occurred.

Although many studies involving the measurement of endogenous hormone levels have been undertaken, a large part of the available evidence is often open to modification in the light of new analytical techniques. The use of these techniques may provide precise measurements of IAA which are themselves related primarily to the extraction of the compound, but little evidence is available on the precision of the extraction method which might in the end fail to justify the use of sophisticated and expensive analytical instruments. It is therefore important that close attention is made to the intracellular location of IAA, and in so doing, determine a means of gauging extraction efficiency.

The experiments conducted in this thesis have by necessity relied on a large sample number for each extraction; this is both labour and time consuming. If, however, an analytical technique can be devised for the assaying of IAA in single plants, then much of these difficulties will be removed. A similar argument can be proposed for the development of more efficient "clean-up" systems for the extracts. Ideally, the extraction and purification should be simplified to one stage prior to

the actual identification and measurement. Moreover, IAA determinations in tissue will eventually have to be coupled to specific cells and cellular components if a profile of IAA and related compounds in a plant can be established.

In conclusion, although determinations of IAA levels in plants can be achieved with varying degrees of precision and accuracy, depending on the system used, further studies are necessary before a complete correlation between IAA and growth phenomena can be established. These investigations will by necessity include identification of the complete pattern of the biosynthesis and metabolism of IAA, the regulation and rate of these processes, the relationship of IAA metabolism to the general pool of nutrient supply, and the localisation of IAA within cells.

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