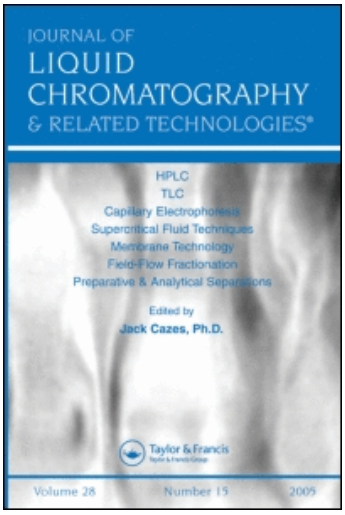


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# A RAPID METHOD FOR EXTRACTION AND ESTIMATION OF ABSCISIC ACID FROM PLANT TISSUE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple, rapid and economical method for the determination of the abscisic acid content in different plant organs was described. Silica Sep-Pak prepacked cartridges were used for prepurification of plant extracts. The abscisic acid content in the extract was determined by HPLC.

## INTRODUCTION

The phytohormone abscisic acid (ABA) is involved in a large range of physiological processes of plants (1). A well documented role for ABA is as a stress hormone. ABA is known to mediate the hydroactive stomatal

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closure, and water stress avoidance at least in some species seems to be directly related to the capacity of plants to increase their ABA levels in response to a decrease of leaf water potential or turgor (8, 6). Other stress factors like salinity (12, 9), nutrient deficiency (5), or waterlogging (11) are also known to increase ABA levels in plants.

The endogenous ABA level may be useful as an indicator of stress in plants. Nevertheless the low concentration of this hormone present in plant tissues makes its determination difficult. The purification process normally involves the partition of plant extracts against organic solvents, followed by chromatography (paper, thin layer, HPLC or GLCEC). The extraction procedures are time-consuming and each extraction step generally results in some ABA loss. These problems are increasing if it is necessary to determine the ABA content in a large number of samples, as occurs in stress physiology or crop improvement research.



A simple method using a Sep-Pak cartridge for prepurification of plant extracts and GLC for ABA analysis has been described (7). In this paper we describe a modification of this prepurification method, which is more rapid, relatively inexpensive, and allows quantification of ABA by HPLC. The method was used to determine the ABA content of different organs of bush bean plants at different developmental growth stages.

## MATERIALS AND METHODS

Bush bean plants (*Phaseolus vulgaris* L. cv. Contender) were grown on perlite with modified Long Ashton's nutrient solution (2) at pH 5 under normal glasshouse conditions. At different growth stages (primary leaf

stage, first trifoliate leaf stage, flowering and fruit development) plants were harvested, divided into roots, stems, primary and trifoliate leaves. After weighing, these plant organs were immediately frozen in liquid nitrogen and stored at -20°C until ABA extraction.

ABA extraction and prepurification

Ten g fresh weight (f.w.) of frozen leaf tissue, or 20 g f.w. of frozen stem or root tissue were homogenized for 2 min in 100 ml methanol 90% (v/v) at pH 8 (adjusted with $NaHCO_3$) (5), with 20 mg $l^{-1}$ butylated hydroxitoluene added as an antioxidant (7), using a Sorvall omnimixer (Du Pont Instruments) at position 7.5. Fifteen µl of (2-$^{14}$C)-ABA, S=25.6 µCi µmol$^{-1}$ (Amersham Corp.) were added to the methanol extract for monitoring ABA recovery. The homogenate was shaken for 12 h at 4°C and filtered through Whatman n. 1 filter paper. The filtrate was evaporated in a rotary evaporator at 35°C to the aqueous phase. The pH of the aqueous phase was adjusted to 8 with 6N NaOH, and partitioned three times with equal volumes of ethyl acetate, the fraction of which was discarded. The pH of the aqueous phase was adjusted to 2.5 with 6N HCl and extracted three times with equal volumes of ethyl acetate. The aqueous phase was discarded and the combined ethyl acetate phases dried under vacuum at 35°C. The residue was dissolved in 2 ml methylene chloride and loaded with a glass syringe onto a silica Sep-Pak (Waters) prewashed with 5 ml methylene chloride (7). The cartridge was washed with aliquots of organic solvents with increasing polarities: 1) 2 ml 5% acetone in methylene chloride. 2) 5 ml 4% methanol in methylene chloride. 3) 3 ml 10% methanol in methylene chloride. Fractions 2 and 3 were bulked and taken to dryness in a rotary evaporator at 35°C. The dried residue was dissolved in 0.5 ml of 60% (v/v) hexane in ethyl acetate

with 1% acetic acid (10), and filtered through a 0.45 μm pore size filter. Ten μl of naftalenacetic acid were added as internal standard. Fifty μl of this prepurified extract were injected into an HPLC system for ABA quantification. Figure 1 represents the different prepurification steps.

ABA quantification

For ABA quantification a high performance liquid chromatograph (Du Pont Instruments 850) equipped with an UV detector (254 nm) was used. The column employed was a Zorbax-$NH_2$ (Du Pont). The operation pressure was 11 MPa, the flux was 2 ml $min^{-1}$, and the operation temperature 35°C. As solvents, a mixture of 15% acetonitrile in chloroform, acidified with acetic acid (0.17N), was used (3). The retention time for ABA under these conditions was approximately 16 min.

For confirmation of the authenticity of the ABA peak cis-trans ABA (Sigma) was co-chromatographed with the extract. The exact quantity of the ABA content in the plant extract was calculated from a standard curve constructed from peak height measurements obtained from known amounts of ABA. For the calibration curve 5 a 500 ng ABA were used (r=0.99). Figure 2 shows a chromatography obtained using cis-trans ABA as a standard and naphtaleneacetic acid as internal standard.

The efficiency of the extraction and purification procedures was determined by scintillation counting of $^{14}C$-ABA, removing aliquots of a known quantity in each operation step. The $^{14}C$ activity was determined using a liquid scintillation spectrometer (Beckmann LS 1800). The scintillation solution was 30% triton-X-100 in xylene containing 0.6 PPO and 0.06 POPOP. The efficiency for $^{14}C$ was 78.8%. No interference with the colour of the samples could be detected.

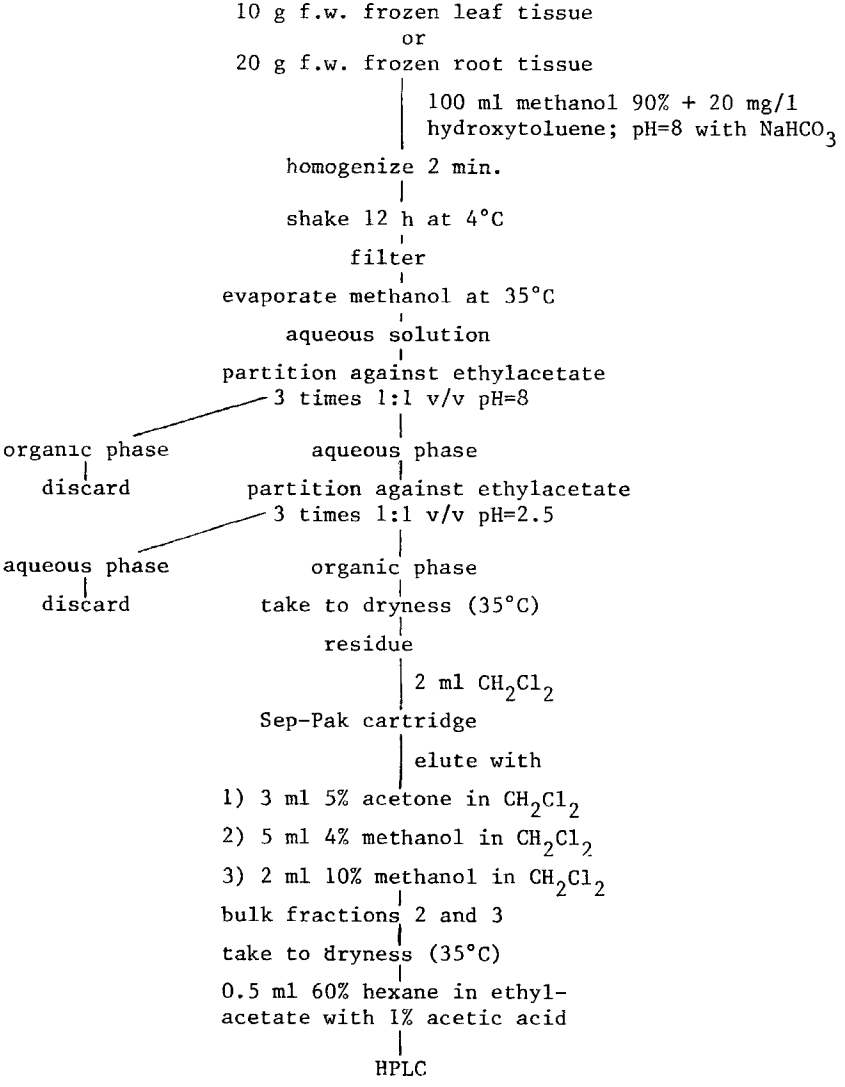


FIGURE 1: Flow Diagram for the Extraction and Purification of Abscisic Acid (ABA) from Plant Tissue.

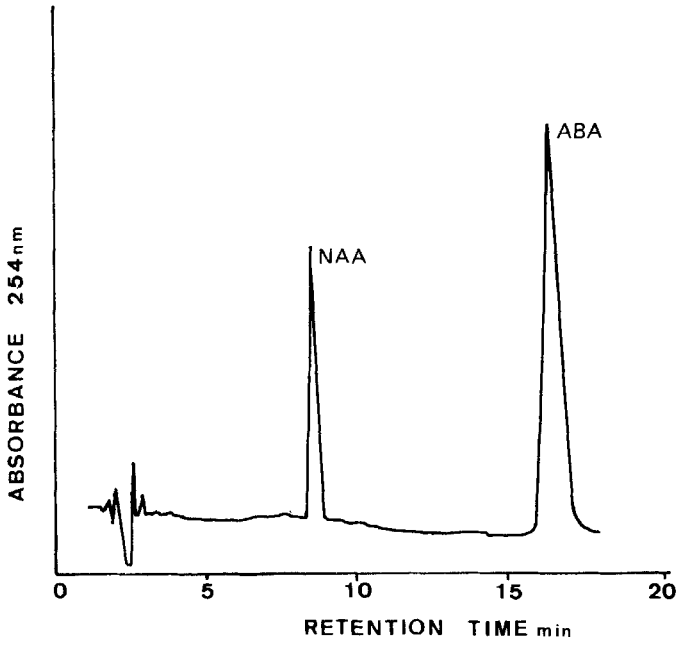


FIGURE 2: Chromatogram from Standard Solution Containing Abscisic Acid (ABA) and Naphtaleneacetic Acid (NAA).



## RESULTS AND DISCUSSION

The purification method used rendered a high and very constant recovery rate for ABA of 78±1.64% as measured with $^{14}$C-ABA. To achieve this recovery rate it was necessary to assure the complete homogenization of the plant material, indicated by no retention of colour in the filter after filtration of the extract. The filtration of the extract represents a small loss of ABA of 1% or less. Maintenance of pH 8 in the extract retained ABA in the aqueous phase and no ABA loss occurred during methanol evaporation. Partitioning against ethyl acetate was used because of the high partition coefficient of this solvent for ABA at both pH values 2.5 and 8 (3),

and so allows the elimination of most of the coloured components of the extract with a relatively low loss of ABA (less than 10%).

The first fraction eluted from the Sep-Pak with 4% acetone in methylene chloride eliminated most of the remaining coloured compounds and caused only small ABA loss (0.05% or less). The elution with 4% and 10% methanol in methylene chloride recovered 50 to 65% and 7 to 22% of ABA respectively. An increase in volume of the solvent did not allow any further increase in the ABA recovery and, on the contrary substancially increased the number of impurities. The use of solvents with higher polarity did not render better results either.

Silica Sep-Pak and not Sep-Pak $C_{18}$ cartridges were used for purification of the extracts because silica Sep-Pak allows the extraction with organic solvents which are much easier and faster to evaporate at low temperatures (35°C) than aqueous solvents. All evaporation steps of our extraction method were performed on the organic phase, thus avoiding time-consuming evaporation of aqueous solvents and making liophilization or drying under nitrogen unnecessary.

Figure 3 gives examples of chromatographies obtained with samples from different plant organs. Our extraction method rendered a clearly separated peak for ABA in all kinds of plant organs investigated. Table 1 shows the results on ABA contents in different plant organs at different stages of development.

The major limitation of the HPLC technique in ABA analysis is the lower selectivity and sensibility of the UV detector in comparison to the electron capture detector often used in the determination of ABA by GLC. The lower sensibility can be partially compensated by injecting larger amounts (50 µl) (3). Our prepurification and quantification method not only gives good results in

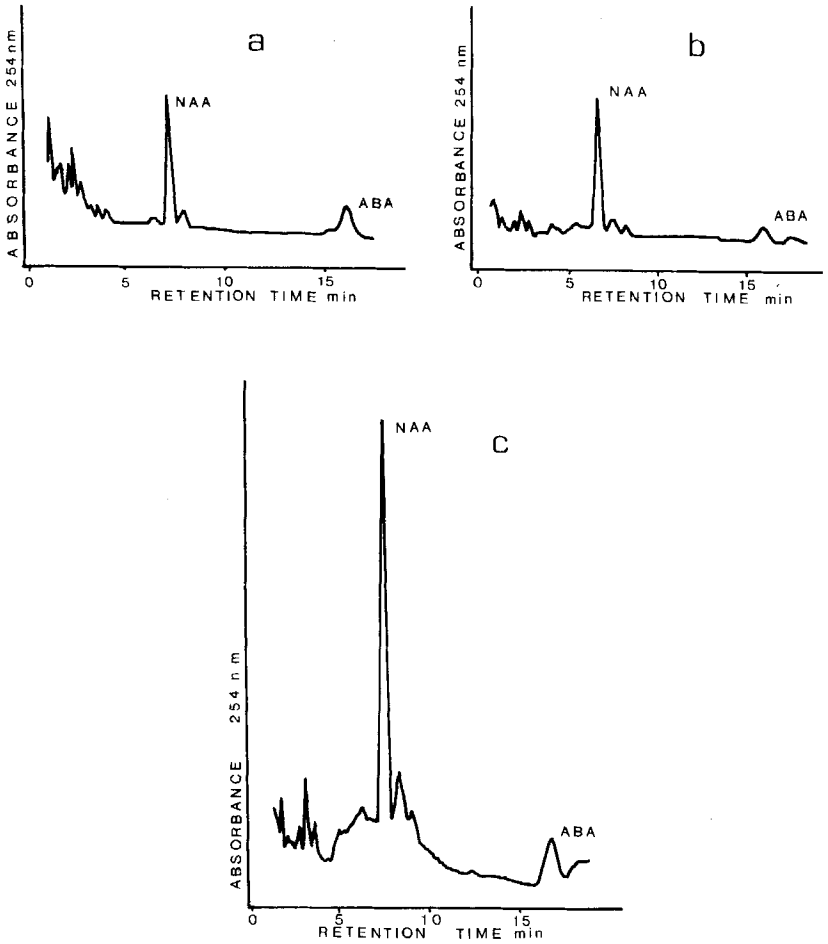


FIGURE 3: Chromatograms from Extracts of Different Plant Organs. a) Leaves, b) Stems, c) Roots.

TABLE 1

ABA Content (ng $g^{-1}$ fresh weight) of Different Organs of Bush Bean Plants at Different Developmental Stages. 1) primary leaf stage, 2) first trifoliate leaf stage, 3) flowering, 4) fruit development.

| Sample number | Primary leaf | Trifoliate leaf |
|---|---|---|
| 1 | 29.23 ± 9.93 | ------ |
| 2 | 98.61 ± 12.89 | 110.54 ± 3.56 |
| 3 | 173.01 ± 15.59 | 84.92 ± 5.03 |
| 4 | ------ | 166.83 ± 14.22 |

| Sample number | Stem | Root |
|---|---|---|
| 1 | 28.09 ± 7.84 | 30.45 ± 12.24 |
| 2 | 65.42 ± 4.77 | 32.54 ± 10.24 |
| 3 | 65.13 ± 8.94 | 42.29 ± 10.27 |
| 4 | 101.69 ± 8.68 | 47.38 ± 10.86 |



plant organs with relatively high ABA content like leaves, but also allows estimation of the lower ABA content in stems and roots, if greater amounts of plant material were used (20 g fresh weight). HPLC offers the advantage that substances need not be derivatized and can be easily collected after detection (4).

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