Plant Physiol. (1987) 85, 46–50
0032-0889/87/85/0046/05/$01.00/0

# The Development of an Indirect Enzyme Linked Immunoassay for Abscisic Acid[1]

Received for publication January 13, 1987 and in revised form May 4, 1987

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## ABSTRACT

**An indirect method of enzyme-linked-immunosorbent-assay (ELISA) is reported for abscisic acid (ABA), utilising a thyroglobulin-ABA conjugate for coating wells. The assay can use commercially available monoclonal antibodies, is sensitive to as little as 20 picograms ABA per well, and is much more conservative of antibody than direct methods. The most dilute ABA standards did not retain their antigenicity during storage, so ABA standard sets were diluted immediately prior to use. The indirect ELISA was used successfully to estimate ABA concentrations in developing cotyledons of *Pisum sativum* L., after only little preliminary purification. It was validated for this tissue through the use of gas chromatography-electron capture detection (GC-EC), and capillary GC-selected ion monitoring (GC-MS-SIM) using [$^2H_6$]ABA as an internal standard. Full spectrum GC-mass spectrometry was also used to verify that ABA was present in a sample assayed quantitatively by both ELISA and GC-MS-SIM.**

Immunoassays are gaining in popularity as a method of quantitating growth regulators in plants. A number of methods based on RIA[2] or ELISA have been developed (7, 8, 10, 12, 14). Many of these assays involve polyclonal antibodies, where the antisera contain multiple populations of antibodies with varying specificity. More recently, monoclonal antibodies have been used in assays for plant hormones (7, 8, 10, 12). Use of monoclonal antibodies should eliminate much of the variability due to cross-reactivity that is observed with polyclonal antisera.

ELISA can be more sensitive than RIA (1, 13), and often is less expensive. Additionally, ELISA is well suited for assay of large numbers of samples.

A number of different methods of ELISA are available and variations have been illustrated (11). Most ELISAs reported to date for the various PGRs have used the direct method of ELISA (4, 13). There are also several reports of an indirect method for cytokinins (10) and ABA (12), utilizing BSA conjugates of PGRs to coat the wells, although neither have used definitive methods for validation of the technique with extracts from plant tissue.

Herein we report a validated (GC-EC and GC-MS-SIM) indirect ELISA for ABA in developing cotyledons of pea seeds. The method utilizes a thyroglobulin-ABA conjugate for coating wells, commercially available antibody, and is highly sensitive and conservative of antibody.

## MATERIALS AND METHODS

**ABA-Thyroglobulin Conjugation.** Twenty mg of *cis,trans* (±) ABA (Sigma), spiked with $2 \times 10^{-4}$ Bq [G-$^3$H]ABA (Amersham; 688 GBq mmol$^{-1}$) was methylated with ethereal diazomethane according to the methods of Schlenk and Gellerman (9).

Abscisic acid-(*o*-carboxymethyl)oxime derivatives were prepared by a method modified from Erlanger *et al.* (6): 20 mg ABA-Me, 20 mg carboxymethoxylamine hemihydrochloride (Aldrich Chem. Co.), 1 ml MeOH (dried over molecular sieves), 50% saturated with $NaHCO_3$. The mixture was stirred overnight at room temperature. Progress of the reaction was checked by TLC on silica gel, using toluene:acetone:MeOH (1:1:1 v/v) as the solvent. A minimum of 3 h was required for quantitative conversion of the ABA-Me. The mixture was adjusted to pH 8.4 with NaOH, diluted with water and partitioned with diethyl ether. The alkaline aqueous phase was acidified to pH 3.0 with concentrated HCl and partitioned into ether. The ether extract, containing the ABA-oxime fraction was used with water and taken to dryness *in vacuo*.

Coupling of the oxime to thyroglobulin was via a mixed anhydride reaction (*Erlanger et al.* [6]). The ABA oxime was dissolved in 200 μl dioxane. Forty μl aliquots of 20% v/v *tert*-butyl amine in dioxane, and 5% v/v isobutylchloroformate in dioxane, were added to the ABA-oxime. The reaction mixture was stirred at 10°C for 10 min, added to 30 mg thyroglobulin dissolved in 1 ml 0.05 M $Na_2HPO_4$ (pH 9), and stirred at 4°C for 12 h.

The methyl ester on the ABA side chain was hydrolyzed in 2 N NaOH at 60°C for 20 min. The conjugate was dialyzed against 1 L water for 3 h and 3 L PBS (pH 7.4) for 3 d, with constant stirring.

The conjugate was stored at 4°C, after addition of 100 μl 0.1% $NaN_3$ and Thiomersal. From the radioactivity of ABA present in the conjugate, the binding ratio was estimated to be 40.

**Antibody and Conjugate Dilutions.** Monoclonal antibodies to free *cis,trans*(+)ABA were purchased from IDETEK Inc. (1057 Sneath Lane, San Bruno, CA 94066). IDETEK Inc. have claimed there is no cross-reactivity of antibody with *cis,trans*(−)ABA, *trans,trans*(+)ABA, *cis,trans*(+)ABA-D-glucopyranosyl ester, *cis, trans*(+)ABA-*cis*-diol, xanthoxin, and all-*trans*-farnesol, and very

[1] Supported in part by Natural Sciences and Engineering Research Council of Canada Research Grants IC-0211 (Intl. Collaborative) a Canadian Forestry Service PRUF contract, and A-2585 (operating) to R.P.P.

[2] Abbreviations: RIA, radioimmunoassay; GC-EC, gas chromatography-electron capture; SIM, selected ion monitoring; ABA-Me, ABA methyl ester; MeOH, methanol; Rt, retention time; HAc, acetic acid; ELISA, enzyme-linked-immunosorbent assay.

light (<0.1%) cross-reactivity with *cis,trans*(+)ABA-Me, phaseic acid, dihydrophaseic acid.

The 2 mg of purchased material containing the antibody was reconstituted in 20 ml PBS containing 0.1% w/v sodium azide. The solution was divided into 20 × 1 ml stock vials, and stored at −20°C. For each assay a stock vial was thawed, and diluted in ASSAY buffer (see "Buffers") to a total volume of 20 ml. Each stock vial thus contained sufficient antibody to assay 200 wells.

The thyroglobulin conjugate was diluted in 6 M guanidine-HCl (Sigma), to give a concentration of 2.5 $\mu g\ ml^{-1}$.

**Standards.** *cis,trans*(+)ABA standards were prepared by dilutions of *cis,trans*(+)ABA (Sigma) in assay buffer. The standards ranged from 12.4 pg 50 $\mu l^{-1}$ to 1000 pg 50 $\mu l^{-1}$.

**Buffers.** Buffers were: (a) PBS-Tween: 7.8 g $NaH_2PO_4$, 8.8 g NaCl, and 1 ml Tween 20 were dissolved in 1 L distilled water and the pH adjusted to 7.4. (b) Assay buffer: PBS-Tween with addition of 1 g $L^{-1}$ gelatin. (c) Substrate buffer: 7.1 g $Na_2HPO_4$ and 5.25 g citric acid dissolved in 1 L distilled water. (d) Enzyme substrate: 40 mg OPD (*o*-phenylenediamine dihydrochloride) and 60 $\mu l$ $H_2O_2$ (30% w/v) were added to 100 ml substrate buffer.

**Procedure.** The ELISA procedure is outlined in Figure 1. Falcon 3912 Microtest III flexible assay plates (Becton Dickinson Labware), containing 96 wells were used. The outer rows and columns were shown to produce inconsistent results, so only the inner 60 wells on each plate were used, the outer wells being filled with assay buffer. Triplicate standard curves were included on every plate.

*Coating*
Coat each well with 200 $\mu l$ diluted ABA-Thyroglobulin
Incubate at 4°C overnight
▼
*Wash*
Wash 4 times with PBS-Tween
▼
*Blocking*
Add 150 $\mu l$ assay buffer
Incubate at 4°C for 20–30 min
Empty wells
▼
*Sample and conjugate competition*
Add 50 $\mu l$ of sample or standard ABA solution
Add 50 $\mu l$ diluted antibody
Incubate for 4 h at room temperature
▼
*Wash*
Wash 4 times with PBS-Tween
▼
*Second antibody*
Add 100 $\mu l$ of 0.1% horse-radish peroxidase linked-
Anti-mouse antibody (from sheep) (Amersham)
(Diluted in assay buffer)
Incubate for 2 h at room temperature
▼
*Wash*
Wash 4 times with PBS-Tween
▼
*Substrate reaction*
Add 100 $\mu l$ enzyme substrate
Incubate in darkness at room temperature for 15–20 min
▼
*Stop*
Add 100 $\mu l$ 1.25 M $H_2SO_4$ stop solution
▼
*Read at 492 nm*

FIG. 1. Flowchart of procedures for indirect ELISA of ABA.

**Equipment.** All washing steps and dispensing of antibodies, substrate, and stopping reagent were performed using a Behring ELISA Processor M, which also contains the spectrophotometer used in detecting absorbance at 492 nm.

**Tissue Extraction.** Fresh cotyledons of *Pisum sativum* L. cv Tere were homogenized in 15 ml 80% (v/v) MeOH/$H_2O$ (0.5% v/v HAc) containing 20 mg $L^{-1}$ butylated hydroxytoluene. 2 × $10^4$ Bq [$^3$H](±)ABA was added to check losses during purification and locate discrete peaks of ABA during preparative chromatography. The macerated tissue was extracted with continual shaking for 24 h, with filtering and fresh MeOH/$H_2O$ added to the tissue macerate after 2 and 6 h. Supernatants at each solvent change were pooled.

**Purification.** The extract was then diluted to 50% MeOH/$H_2O$ (v/v) (0.5% v/v HAc) with 0.5 % v/v HAc/$H_2O$ and passed through a column containing 1 g reverse phase C18 material. This removed pigments and many nonpolar compounds from the extract, which were retained on the column. At this methanol concentration and extract pH (4.2–4.6), ABA washed through the column. After removal of MeOH from the 50% solution under reduced pressure, and appropriate dilution with assay buffer the semipurified extract was found by appropriate validation procedures (see below) to be sufficiently pure for use in the ELISA. Further purification involving HPLC of the seed extract did not improve performance of the assay, and was therefore deemed unnecessary.

**Validation.** Three samples of cotyledon tissue were extracted and after addition of [$^3$H]ABA to monitor losses, passed through the preparatory C-18 column as outlined above. Most of the methanol was removed under reduced pressure and an aliquot taken from one sample for ABA quantitation by ELISA (semi-purified sample C″). All extracts were then further purified on a Varian Vista 5500 HPLC using a Brownlee 22 cm reversed-phase OD-224 column. The solvent program used was: 0 to 15 min 28% v/v MeOH/$H_2O$ (0.8% HAc), 15 to 17 min linear gradient to 38% v/v MeOH/$H_2O$ (0.8% v/v HAc), and 17 to 28 min 38% v/v MeOH/$H_2O$ (0.8% v/v HAc). The flow rate was 2.00 ml $min^{-1}$. For each of the three samples the fraction containing [$^3$H] ABA (Rt 27–28 min) was collected and divided into two equal samples; one extract for ABA quantitation by ELISA and the other by GC-EC. All six extracts were taken to dryness with a stream of $N_2$.

The three samples destined for ELISA were redissolved in a small quantity of methanol, diluted in ASSAY buffer and the ABA levels quantitated by ELISA. For each extract a dilution series was prepared so that at least three dilutions fell on to the standard curve. Each dilution was assayed in triplicate.

The remaining samples were methylated with ethereal diazomethane and ABA levels quantified by GC-EC, using ABA-Me standards for comparison. A 2 m × 2 mm glass column of 3% OV-210 on Chromosorb was used with a Tracor 550 GC equipped with a Nickel-53 EC Detector and electron capture linearizer. Column temperature was 210°C; detector temperature was 235°C. The carrier was $N_2$ at a flow rate of 40 ml $min^{-1}$; $N_2$ at a flow of 30 ml $min^{-1}$ was also used as a detector purge. A Varian 4270 Integrator operating in external standard mode quantified peak areas.

Prior to all assays, by ELISA or GC-EC, an aliquot was taken for radioactivity counting. Estimates were corrected for losses during purification and methylation, which were up to 30%.

In a second validation comparison, two fresh samples were passed through the preparatory C18 column and methanol removed as above. Half was dedicated to analysis by ELISA (D and E). The remainder was dried carefully with $N_2$, prior to subsequent analysis by GC-MS-SIM (D′ and E′) with [$^2H_6$]ABA as an internal standard. To each residue portion of D′ and E′ plant extract samples, 100 ng of [$^2H_6$]ABA was added. The

sample residue was dissolved in 100% MeOH, then diluted to 32.5% MeOH with additional 10% MeOH/1% HAc. The samples were then filtered (Millipore type HATF, 0.45 μm) before injection on HPLC.

The HPLC was performed on a Waters C18 μBondapak column, 30 cm × 3.9 mm. The solvent program used was: 0 to 25 min 32.5% v/v MeOH/$H_2O$ (1% HAc), 25 to 30 min linear gradient to 100% MeOH (1% HAc), and 30 to 60 min 100% MeOH (1% HAc). The flow rate was 2 ml $min^{-1}$. This program separates *cis,trans*-ABA (Rt 18–19 min) from *trans,trans*-ABA (Rt 15–16 min). One min (2 ml) fractions were collected and 100 μl aliquots were taken for determination of radioactivity by liquid scintillation spectrometry. Fractions containing [$^3$H]*cis, trans*-ABA were collected, dried at 35°C under partial vacuum, transferred to reactivials with MeOH, and aliquots again taken for determination of radioactivity. The samples were then dried with $N_2$, residues dissolved in 10 μl of MeOH, and methylated with ethereal diazomethane for 30 min. The methylation procedure was repeated once. One of these samples (E′) required additional purification prior to GC-MS-SIM, and this was accomplished as ABA-Me, solvent program: 0 to 20 mm 32.5% v/v MeOH/$H_2O$, 20 to 25 min linear gradient to 100% MeOH, and 25 to 55 min 100% MeOH. The flow rate was 2.0 ml $min^{-1}$. The fraction containing [$^3$H]*cis,trans*-ABA-Me (Rt 27 min) was collected and dried as noted above.

The analysis by GC-MS-SIM was performed on a Hewlett-Packard 5970A GC with capillary direct interface to a Hewlett-Packard 5790A mass selective detector (MSD). The GC program

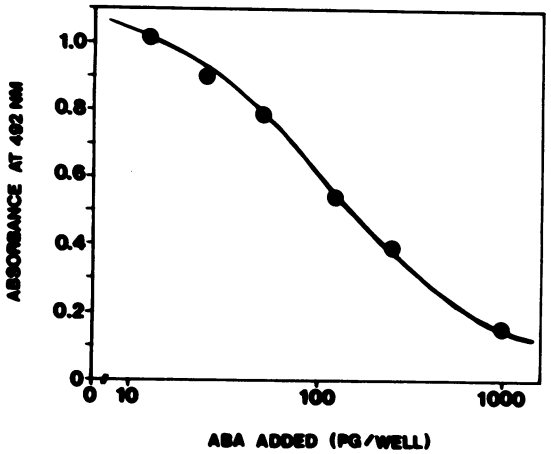


FIG. 2. A typical standard curve for the ELISA. All standards were assayed in triplicate.

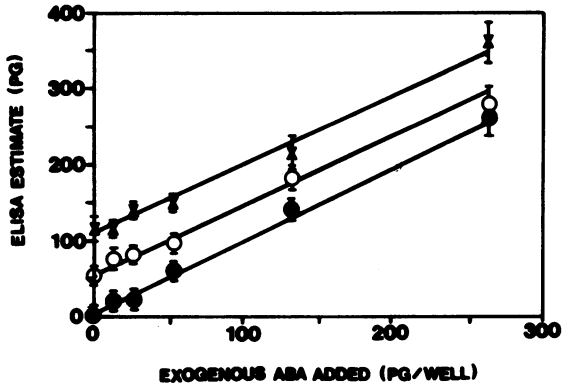


FIG. 3. Effect of sample dilution of ELISA estimate of cotyledon samples, after addition of exogenous ABA; (●) no sample added; (○) sample diluted 1/500 added; (×) sample diluted 1/250 added; 95% confidence intervals are plotted.

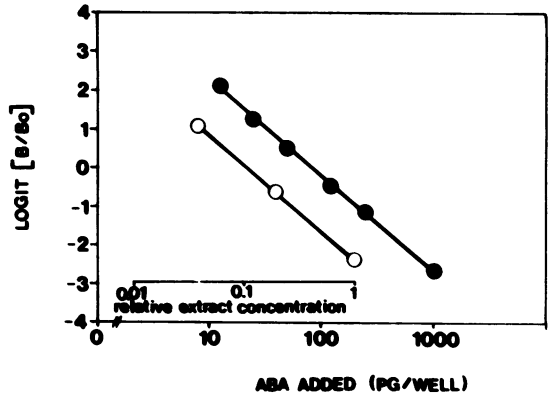


FIG. 4. Parallelism of extract dilution and standard curves; (●) standard curve; (○) semipurified *P. sativum* cotyledon extract added.

was 60°C isothermal for 1 min, then 25°C $min^{-1}$ to 250°C. The He gas flow rate was 1 ml $min^{-1}$. The capillary column was a DB1-15N (J&W Scientific Ltd). The interface temperature was 280°C. Data was collected using the SIM program, monitoring for m/e 190 (endogenous ABA) and m/e 194 ([$^2H_6$]ABA). The Rt of [$^2H_6$]ABA was 8.12 min, and ABA was 8.14 min under these conditions; a minor separation of [$^2H_6$]ABA from ABA would be expected (3). The calibration curve for [$^2H_6$]ABA was prepared by monitoring the same m/e ratios (190/194) for 100 ng [$^2H_6$]ABA in standard samples of ABA (Sigma grade IV, >95% ABA) of 50, 100, 200, and 500 ng. The ABA amounts in the natural samples were estimated by entering the 190/194 peak area value into a regression equation calculated from the calibration curve (*e.g.* [$^2H_6$]ABA calibrated with increasing amounts of Sigma ABA). A full mass spectrum of endogenous ABA was also obtained from sample D′.

## RESULTS AND DISCUSSION

An example of a standard curve attained is shown in Figure 2. The working range of the assay is between about 20 and 500 pg ABA per well, although this will vary with different dilutions of antibody and conjugate.

Estimates of ABA content by ELISA made following the addition of increasing amounts of exogenous (±)ABA, were not affected by addition of semipurified cotyledon extracts. This is shown by the parallel lines in Figure 3, which are offset only by the hormone content in the plant extracts. If regression lines were not parallel, it could be assumed that one or more substances in the extract was interfering with the binding of antibody to ABA. Additionally, when extracts were diluted, the ELISA estimate was consistent with the appropriate dilution. The extract dilution curve shown is parallel to the logit standard curve (Fig. 4), also indicating the absence of potentially interfering compounds in the semipurified extract.

Results of the validation by GC-EC are shown in Table I. There were no significant differences between ELISA estimates and the GC estimates of ABA content. The estimate of about 1 μg ABA $g^{-1}$ fresh weight is consistent with the results of Browning (2) who reported peaks in ABA content of between 0.80 μg $g^{-1}$ and 14 μg $g^{-1}$ fresh weight, using GC-MS-SIM. Eeuwens and Schwabe (5), using bioassay found that ABA levels peaked at 1 μg $g^{-1}$.

Results of the validation by GC-MS-SIM are shown in Table II. These results are somewhat less satisfactory than those shown in Table I, primarily because of problems with ABA standards at that time. It was found that standards with very low ABA concentrations could not be stored for any length of time, while more concentrated standards were 'safe.' If an 'old' standard set was used, ELISA estimates varied with position on the standard

Table I. *Comparison of Estimates of ABA Content by ELISA (A, B, C, and C″) and GC-EC (A′, B′, and C′)*

Sample C″ was 'semi-pure' (see text), while samples A, B, and C had been purified by HPLC prior to ELISA. Estimates are given with 95% confidence intervals.

| Sample | Method | Estimate |
|---|---|---|
| | | *μg ABA $g^{-1}$ fresh weight* |
| A | ELISA | 1.26 ± 0.10 |
| A′ | GC-EC | 1.34 ± 0.16 |
| B | ELISA | 1.26 ± 0.23 |
| B′ | GC-EC | 1.20 ± 0.10 |
| C | ELISA | 0.97 ± 0.19 |
| C″ | ELISA | 0.73 ± 0.17 |
| C′ | GC-EC | 0.91 ± 0.14 |

Table II. *Comparison of Estimates of ABA Concentration in Developing Pea Cotyledons by GC-MS-SIM (using [$^2H_6$]ABA as an Internal Standard) and by ELISA*

Estimates are given with 95% confidence intervals.

| Sample | Method | Estimate |
|---|---|---|
| | | *μg ABA $g^{-1}$ fresh weight* |
| D | ELISA | 0.51 ± 0.09 |
| D′ | GC-MS-SIM | 0.68 ± 0.04 |
| E | ELISA | 0.57 ± 0.08 |
| E′ | GC-MS-SIM | 0.76 ± 0.12 |

Table III. *Mass Spectra from Capillary Column GC-MS of the Methyl Esters of ABA, [$^2H_6$] ABA, and a Purified Fraction (D′; Table II) from Developing Pea seed Cotyledons*

Sample D′ co-chromatographed with [$^3$H]ABA as the free acid.

| ABA Methyl Ester | | Sample D′ (from Pea Cotyledons) | | [$^2H_6$]ABA Methyl Ester | |
|---|---|---|---|---|---|
| *m/e* | *intensity* | *m/e* | *intensity* | *m/e* | *intensity* |
| 123 | 5 | 123 | 8 | 123 | 9 |
| 124 | | 124 | 5 | 124 | 7 |
| 125 | 34 | 125 | 55 | 125 | 51 |
| 126 | 5 | 126 | 9 | 126 | 9 |
| 133 | 14 | 133 | 15 | | |
| 134 | 64 | 134 | 68 | | |
| 135 | 22 | 135 | 26 | | |
| | | | | 137 | 22 |
| 138 | | 138 | 11 | 138 | 57 |
| | | | | 139 | 17 |
| 161 | 22 | 161 | 23 | | |
| 162 | 57 | 162 | 70 | | |
| 163 | 11 | 163 | 10 | | |
| 165 | 5 | 165 | 10 | 165 | 23 |
| 166 | | 166 | 8 | 166 | 50 |
| 167 | 3 | 167 | | 167 | 13 |
| 190 | 100 | 190 | 100 | | |
| 191 | 16 | 191 | 16 | | |
| | | | | 193 | 24 |
| 194 | 3 | 194 | 14 | 194 | 100 |
| | | | | 195 | 17 |
| 222 | 3 | 222 | | | |
| | | | | 225 | 9 |
| 278 ($M^+$) | ND[a] | 278 ($M^+$) | ND | 284 ($M^+$) | ND |

[a] Not detectable, *i.e.* intensity <1% that of the base peak.

curve. This problem was overcome in all other plates by diluting the most concentrated standard on the day of processing the ELISA plate.

Nonetheless, estimates by ELISA in Table II were only 25% lower than estimates by GC-MS-SIM. The estimate of ABA concentration in the cotyledons was lower in Table II than Table I, but this was not surprising as different cotyledon samples were used. Again, the estimates were similar to those in the published literature (2, 5). The age of seeds strongly influences ABA levels (5) and this was not monitored carefully in the seeds used in the present study. However, the seeds were nearing the end of a phase of maximum growth, so ABA levels can be expected to be close to the maximum level (2).

In addition to the quantitative validation by two more definitive methods (GC-EC and GC-MS-SIM), the use of capillary GC-MS-SIM in the presence of [$^2H_6$]ABA gives a qualitative validation (*i.e.* Rt of m/e 190 peak is appropriate relative to Rt of m/e 194 peak), especially since the GC-MS-SIM procedure followed a relatively definitive analytical reversed phase C18 HPLC procedure. Finally, sample D also yielded a full mass spectrum at 8.12 min Rt which was consistent with that of authentic ABA-Me (Table III).

This indirect ELISA uses only small amounts of anti-ABA antibody. Raising quantities of specific antibody can be difficult and time-consuming. Although monoclonal antibodies to ABA can be purchased commercially, large quantities are required for use in direct ELISAs similar to those reported, *e.g.* Daie and Wyse (4), Weiler (13). This is because in these assays it is the antibody, rather than the ABA-conjugate, which is first coated to the plate. A large percentage of the antibody is thus washed off in a direct ELISA as only small amounts adhere to the plate. In the indirect ELISA, antibody is conserved by adding it later to a plate already coated with the ABA-conjugate to which the antibody has a high affinity. The indirect ELISA is thus particularly advantageous when there is limited supply of antibody since large quantities of the ABA-conjugate can be made easily.

The indirect ELISA presented here is quick and relatively inexpensive. For pea seed material very little purification is required when used with monoclonal antibody. However, some types of tissue may require more purification if they contain other interfering (or large amounts of cross-reactive substances. The amount of purification required for each type of tissue, and at each stage of development for that tissue, should be determined before large scale use of the assay. Definitive validation trials should be conducted before any immunoassay is used to quantitate endogenous growth substances. Finally, use of a radioactive [$^3$H] internal standard of high specific activity is very useful, indeed almost essential in developing an appropriate purification and/or chromatography protocol. Such an internal standard may also be useful as part of the routine procedure for individual sample extracts in case of unexplained losses, or unusual variation in the ELISA. Once an appropriate protocol has been established, and validated, then an automated ELISA processor can be used to assay very large numbers of samples.

*Acknowledgments*—The reversed phase C18 material used herein was a gift from the Chemistry Department, University of Canterbury, Christchurch N.Z. The [$^2H_6$] ABA was kindly provided by Dr. L. Rivier.

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