An Indirect Enzyme-Linked Immunosorbent Assay for (+)-Abscisic Acid in *Citrus*, *Ricinus*, and *Xanthium* Leaves

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An indirect enzyme-linked immunosorbent assay (ELISA) specific for free (+)-abscisic acid (ABA) (useful quantitation 20–400 pg/100 μL) was developed with 25 μg of commercial monoclonal antibody (MAb)/96-well plate. Cross-reactivity with several ABA-related compounds was negligible. (+)-ABA in culture media of the fungus *Cercospora rosicola* was quantified without cleanup. The most suitable purification procedure for leaves of *Ricinus*, *Xanthium*, and *Citrus* was filtration through a 0.2-μm nylon 66 filter. Quantification of ABA was validated by high-pressure liquid chromatography and UV detection (HPLC–UV) and gas chromatography flame ionization (GC-FID). The presence of ABA was confirmed by GC–mass spectrometry (GC–MS). The ABA contents of mature leaves of Valencia orange and grapefruit trees and *Ricinus*, new shoots of navel orange trees, and nonstressed and water-stressed Valencia orange and *Xanthium* leaves were compared.

ABA appears to be involved in the regulation of many physiological processes in plants (Addicott and Carns, 1983). ABA appears to be a central factor in the adaptation of plants to water stress in part by causing stomatal closure (Davies and Carns, 1983; Wright, 1978). We have some insight into the biosynthetic pathway of ABA in the fungus *Cercospora rosicola* (Bennett et al., 1984; Horgan et al., 1983), but little information about the biosynthetic pathway of ABA in higher plants is known beyond mevalonate (Milborrow, 1983). Past research into the mechanism of action and physiological roles of ABA in plants has been slow because of the difficulty in measuring small amounts of endogenous ABA in various plant organs, or parts thereof.

The most common methods of quantification of ABA are by HPLC–UV followed by GC-FID or GC-ECD and require rigorous cleanup procedures to ensure elimination of contaminants that may cochromatograph with ABA (Dorffling and Dietman, 1983). Immunoassays provide a technique to use the selectivity of Ab to identify and quantify the low physiological concentrations of hormones in plant material. Several RIA methods for ABA quantitation have been reported (Kannangara et al., 1984; Le Page-Degivry et al., 1984; Le Page-Degivry and Bulard, 1984; Leroux et al., 1985; Walton et al., 1979; Weiler, 1979, 1980; Rosher et al., 1985). Direct ELISA assays for free and conjugated ABA using PAb have been reported (Daie and Wyse, 1982; Weiler, 1982; Harris and Dugger, 1986). Rcently an indirect ELISA assay for ABA using a PAb and an avidin biotin system was reported (Leroux et al., 1985; Maldiney et al., 1986). These methods required sample purification by partition and/or HPLC for crude extracts of wheat, tomato, Douglas fir, tobacco, and citrus fruit followed by methylation of the ABA fraction prior to their immunoassays.

Mertens et al. (1983) developed a specific MAb directed at only free ABA. This MAb is now available commercially, and a direct ELISA procedure (Weiler, 1982) is recommended by the producer of the antibody (Idetek, 1986). Raikhel et al. (1987) evaluated this method for quantitation of ABA and successfully used it for wheat embryos and seedlings. Unfortunately, this method uses large amounts (2 mg/plate) of MAb and is thus very expensive. Concurrent with our research, Walker-Simmons and Sesing (1986) and Walker-Simmons (1987) used an indirect ELISA for crude extracts of wheat grain. After the preparation of this report, Ross et al. (1987) reported an indirect assay for cotyledons of *Pisum sativum* L.

Herein, we report an indirect ELISA procedure for leaf tissues of *Citrus*, *Xanthium*, and *Ricinus* and cultures of the fungus *C. rosicola* that requires only 25 μg of the commercial MAb for each microtiter plate. This rapid, sensitive, and inexpensive technique can be applied to filtered, but still crude, plant extracts. The identity and quantity of ABA were validated by HPLC–UV and GC–MS.

MATERIALS AND METHODS

Chemicals and Materials. MAb for (+)-ABA was purchased from Idetek, Inc., San Bruno, CA 94066. Rabbit antimouse IgG (whole molecule) antisera alkaline phosphatase conjugate (A-1902), *p*-nitrophenyl phosphate (5-mg tablets, 104–105), tyrosine hydrazide (T-3135), *p*-aminohippuric acid (A-7129), bovine serum albumin (BSA, A-7030), Tween 20 (P-1379), poly(ethylene glycol) 8000 (PEG, P-2139), polyamide (P-6755), and 2-*cis*-(±)-ABA methyl ester (A7391) were from Sigma Chemical Co., St. Louis, MO. Cellulose powder (CF11) was from Whatman. (±)-ABA was purchased from Burdick and Jackson Laboratories, Inc., Muskegon, MI. 4-Aminobenzoyl hydrazide (A4, 190-9) and *trans,trans*-farnesol (27,754-1) were from Aldrich Chemical Co., Milwaukee, WI. (+)-*cis*-4′-Keto-α-ionylideneactic acid was isolated from cultures of *C. rosicola* and separated and purified by HPLC–UV. (±)-*cis*-α-Ionylideneacetic acid, 4′-keto-α-ionone, 1′-hydroxy-α-ionone, and dehydrovomifoliol were previously synthesized (Hasagawa et al., 1984; Norman et al., 1985a,b). A MeOH solution of 2-*cis*-(+)-ABA under short-wave UV light for 4 h resulted in isomerization to a mixture of 59.4% *cis*-(+)-ABA and 40.6% *trans*-(+)-ABA, as measured by HPLC–UV. PA was isolated from the culture media of a *Corynebacterium* that was cultured with ABA as the sole carbon source (Hasegawa et al., 1984; Hasegawa and Poling, 1984). Insoluble polyvinylpolypyrrolidone (PVPP) (502538) was from Calbiochem, La Jolla, CA. Silica and C_{18} Sep Paks and Millex-GS 0.22-μm filters were from Millipore Corp., Milford, MA. Disposable filtration columns (7020-3) were from J. T. Baker, Phillipsburg, NJ. Nylon 66 (0.2- and 0.45-μm) filters were Lid/x AQOR from Xydex Corp., Bedford, MA, and Centrex DF101/1 from Schleicher & Schuell, Inc. Centrifree tubes were from Amicon, Danvers, MA.

Plant Material. Grapefruit (*Citrus paradisi* Macfad.) and Valencia (*Citrus sinensis* L. Osb.) orange trees were 3 years old, grown from seed in pots in a greenhouse.

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Washington navel (*Citrus sinensis* L. Osb.) orange tissue was from a mature tree growing outdoors in Pasadena, CA. *Xanthium strumarium* L. (cocklebur) seed was collected at an elevation of 600 m on the Wizernow Wildlife Preserve, Banning, CA. Plants from seed were grown in a greenhouse under a 20-h photoperiod. *Ricinus cummunis* L. (castorbean) leaves were collected in Carbon Canyon, CA. All leaves were harvested, weighed, immediately frozen on dry ice, lyophilized, and weighed again to determine percent dry weight. *Citrus* leaves were difficult to lyophilize unless crushed first. For wilting experiments leaves were held in the laboratory until 12% of the fresh weight was lost (20 min, *X. strumarium*; 2 h, *Citrus* leaves). Detached leaves of *Citrus* wilt very slowly because of a thick cuticle, thick leaves, and high dry-matter content. The average percent dry weight of the Valencia orange leaves was 32.0 compared with 18.0 for *Xanthium* leaves. The leaves were then incubated in plastic bags in the dark for 24 h at room temperature to allow ABA to accumulate, then frozen, and lyophilized.

Extraction of Plant Material. Dry plant material was ground to a fine powder in a blender. The weighed sample (5–10 g) was ground in 200 mL of 80% acetone with a Tissumizer (Tekmar, Cincinnati, OH) and the solution placed in a sonic bath 15 min and then filtered. The residue was extracted twice more with 200 mL of 80% acetone. The pH of the combined acetone extract was usually 2.5–2.6. The acetone extract was then divided in half for ELISA and HPLC–UV.

Treatment for ELISA. After evaporation in vacuo of half of the acetone extract at 40 °C, the resultant aqueous phase was passed through filter paper and diluted to 0.01 g dry weight tissue equiv/mL with TBST. Various cleanup procedures were then compared. Aliquots of the extract were passed through different syringe filters and various absorbents or centrifuged in a microcentrifuge 2 min at 10000 rpm. C_{18} Sep Paks were prepared by passing 5 mL of MeOH followed by 5 mL of H_2O through or prewashed with 10 mL of $CHCl_3$ before the MeOH and H_2O. Aliquots of the extract (2 mL) were then passed through C_{18} Sep Paks, and the sample containing ABA was eluted with 5 mL of MeOH. The MeOH was then evaporated to dryness and the residue dissolved in 2 mL of TBST. Other aliquots were loaded (1 mL) onto silica Sep Paks, and the sample containing ABA eluted with 15 mL of EtOAc. The EtOAc was evaporated to dryness and the residue dissolved in 1 mL of TBST. For charcoal cleanup, a 5-mL aliquot was passed through a 1-cm column of charcoal–cellulose (2:6) in a 3-mL disposable extraction tube. The sample was then eluted with 10 mL of 60% acetone, and the acetone was evaporated to the aqueous phase, which was diluted to 5 mL with TBST. For polyamide or PVPP cleanup, a 10-mL aliquot was stirred with 0.5 g of either material for 1 h. The sample was filtered through disposable extraction columns, the material washed with 5 mL of TBST, and the aqueous sample tested by ELISA.

After all the experimental parameters of the ELISA assay were optimized, the procedure for routine analyses of *Citrus* tissue was changed. Leaf tissue (20 mg dry weight) was extracted in 1.5 mL of 80% acetone containing 100 mg/L of BHT by storing 2–3 weeks at 4 °C. A 0.75-mL aliquot of the extract was diluted to 5 mL with TBST in a Centrex tube and filtered through a 0.2-μm nylon 66 filter by centrifugation 9 min at 1200 rpm.

Treatment prior to HPLC–UV. The other half of the 80% acetone extract was stirred 30 min with 10 g of HCl-washed charcoal and 5 g of Celite 545 and filtered. After evaporation in vacuo, the resultant aqueous residue was adjusted to pH 2.5 and extracted three times with 60 mL of EtOAc. After the combined EtOAc extracts were taken to dryness in vacuo, the residue was dissolved in 10 mL of H_2O, the pH adjusted to 2.5, and this solution passed through a C_{18} Sep Pak. The sample was eluted with 5 mL of MeOH and the entire 5 mL loaded on the preparative HPLC (see Chromatography).

***Cercospora rosicola* Culture.** Strain No. 138.35 was purchased from C.B.S., The Netherlands. Cultures were grown in a defined medium and resuspended as previously described (Norman et al., 1981). A 2-mL portion of the media was acidified to pH 2.5, passed through C_{18} Sep Paks, eluted with 2 mL of MeOH, and diluted to 4 mL with 0.01 M H_3PO_4 for subsequent HPLC–UV. For ELISA the media required no treatment except dilution 5000–20000 times with TBST to place it in the proper range of the immunoassay.

Chromatography. Preparative HPLC was performed on a Whatman Mag 20 ODS-3 column with a 35-min linear gradient from MeOH–0.002 M H_3PO_4 (50:50) to 100% MeOH. The volume corresponding to the R_t for ABA was collected. After evaporation, the aqueous phase was passed through a C_{18} Sep Pak and ABA eluted with 5 mL of MeOH. After evaporation again, the residue was dissolved in 1 mL of MeOH and 100 μL separated on a Waters Bondapak NH_2 analytical column with a 20-min gradient from 0.05% HOAc in MeOH to 0.4% HOAc in MeOH. Another portion was methylated with ethereal CH_2N_2 for GC-FID and GC–MS. Samples were injected by the splitless injection mode onto a fused silica capillary column (30 m × 0.25 mm i.d.) coated with SPB-1 bonded phase (0.25-μm thickness): He, 0.7 mL/min; initial 60 °C for 2 min and then increased to 160 °C at 20 °C/min and to 290 °C at 5 °C/min; injector and detector temperature (FID), 300 °C. GC/EIMS (70 eV) used the same GC conditions with the column connected directly into the ion source of a VG 7070F.

Immunoassay Equipment. Titertek Multiskan MC plate reader and Titertek microplate incubator were from Flow Laboratories, Inc., McLean, VA. Immunolon II microtiter plates (96 well, flat bottom) were from Dynatech Laboratories, Alexandria, VA.

Buffers. TBST: 6 g of Tris [tris(hydroxymethyl)-aminomethane]; 0.2 g of $MgCl_2$, 8.8 g of NaCl, 0.5 mL of Tween 20, and 0.1 g of sodium azide/L, pH 7.5. Bicarb: 50 mM $NaHCO_3$ with sodium azide, 0.1 g/L, pH 9.6.

Standards. (+)-ABA (99%) used for standards was isolated from *C. rosicola* cultures, purified by HPLC and crystallized from $CHCl_3$–hexane (50:50). A stock solution contained 0.01 g in 100 mL of MeOH, which was further diluted with TBST to contain 5 ng and 10–500 pg/100 μL.

ABA–TH–BSA Conjugate. (±)-ABA–4′-tyrosylhydrazone and *p*-aminohippuric acid substituted BSA were prepared and coupled to form an ABA–4′-TH–BSA conjugate according to Weiler (1980) except that the ABA–4′-tyrosylhydrazone was used without purification by TLC. The resulting solution contained about 1.4 mg of conjugate/mL and was stored in 1-mL aliquots at −14 °C. A 1-mL aliquot was diluted to 5 μg/mL with bicarb for coating microtiter plates. Sealed plates were stored 24 h or up to 1 month at 4 °C.

Monoclonal Antibody. MAb (2 mg) was dissolved in 2.0 mL of 50% TBST–glycerol, and 50-μL aliquots were stored at −14 °C. In this solution the MAb was stable for 2–3 months. A 50-μL portion was diluted to 0.8 μg/mL with TBST just before use.

Antimouse Alkaline Phosphatase Conjugate. Just prior to use 0.85 g of PEG 8000 was added to 21 mL of

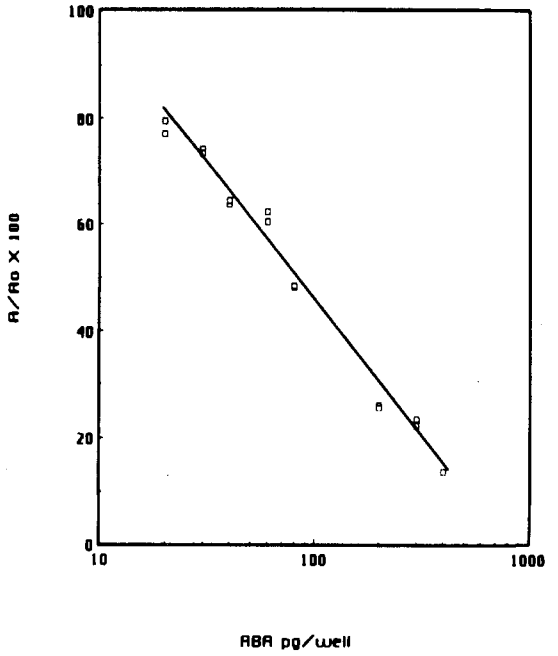

Figure 1. Typical ELISA standard curve for (+)-ABA.

TBST and then 27 μL of antimouse conjugate added.

Substrate. Five tablets of *p*-nitrophenyl phosphate were dissolved in 25 mL of bicarb previously warmed to 37 °C to minimize edge effects on the plate.

ELISA Procedures

1. A coated plate was emptied and washed six times with distilled H_2O.
2. Each well was filled with 100 μL of standard, TBST, or leaf tissue extract. A standard curve in duplicate and three different dilutions in triplicate for each leaf tissue sample were used. One column contained excess ABA (5 ng) for nonspecific binding and one column contained TBST only for a maximum absorbance reading (A_0).
3. Dilute MAb (100 μL) was added to each sample or standard and the plate incubated overnight at 4 °C.
4. The plate was emptied and washed six times with H_2O.
5. Dilute antimouse alkaline phosphatase conjugate (200 μL) containing PEG was added to each well and the plate incubated 1.5 h at room temperature in the dark.
6. The plate was emptied and washed six times with H_2O.
7. Substrate (200 μL) was placed in each well and the plate incubated 30 min at 37 °C or until a reading of 1 AU or more was obtained.
8. The enzyme was inactivated with 50 μL of 5 M KOH and the absorbance of each well measured at 405 nm. Absorbance of the standards was divided by A_0 and multiplied by 100. This value was plotted against the log of ABA concentration in picograms. The quantity of ABA in the sample was calculated on the basis of the calibration curve of (+)-ABA for each plate.

RESULTS AND DISCUSSION

The amounts of ABA–TH–BSA and MAb required for optimum conditions were determined by checkerboard titrations without ABA to obtain absorbance readings near 1. At first, the MAb and ABA or tissue were incubated in a test tube overnight and then incubated 2–3 h in the microtiter plate. Later it was found that much more consistent results were obtained by incubating the MAb and ABA or sample overnight directly in the microtiter plate. Under these conditions the optimum amounts of coating and MAb were 5 and 0.8 μg/mL, respectively. The relative absorbance was linear for the log of ABA standards between about 10 and 500 pg (Figure 1).

Table I. Specificity of the Monoclonal Antibody for (+)-ABA Compared with an Equal Molar Amount of Some Structurally Similar Compounds Determined by the Indirect ELISA Method

compound[a]	cross-reactn, %
2-*cis*-(+)-ABA	100
2-*cis*-(±)-ABA methyl ester	0
2-*cis*-(±)-ABA, racemic	48
2-*cis*-(+)-ABA(59.4%) + 2-*trans*-(+)-ABA(40.6%)	42
phaseic acid	0
trans,trans-farnesol	0
dehydrovomifoliol	0
(+)-*cis*-4′-keto-α-ionylideneacetic acid	0
(±)-*cis*-α-ionylideneacetic acid	0
4′-keto-α-ionone	0
1′-hydroxy-α-ionone	0

[a] Source, isolation, and synthesis of compounds tested are described in Materials and Methods.

Various other aspects of the assay were also optimized. It was found that distilled H_2O was just as effective as TBST for washing the plates. PEG accelerates immune reactions (Tijssen, 1985). PEG had little effect when added to the coating phase. Addition of 4% PEG to the antimouse alkaline phosphatase conjugate shortened the incubation time of the antimouse alklaine phosphatase from 4 to 1.5 h and shortened the subsequent incubation with the substrate from 1 to 0.5 h. (PEG had no effect when added to the substrate incubation.) Addition of PEG to the antimouse alkaline phosphatase doubled the absorbance readings and, thus, reduced the optimum amount of MAb from 2 to 0.8 μg/mL. In addition, well to well variation was reduced when PEG was used. The ABA content of 11 Valencia leaf extracts measured with and without PEG gave ABA values of 0.28 ± 0.16 and 0.27 ± 0.14 μg/g dry weight, respectively.

Mertens et al. (1983) found no cross-reactivity of the MAb for the ABA methyl ester, glucose ester, *cis*-(−)-ABA, *trans*-(+)-ABA, xanthoxin, nor *trans,trans*-farnesol. They obtained a slight cross-reactivity with PA and dihydrophaseic acid, but this was 1000-fold less than the case of ABA. We confirmed some of these findings using our indirect ELISA assay and also verified the lack of cross-reactivity of several closely related compounds (Table I). (+)-*cis*-4′-Keto-α-ionylideneacetic acid was of particular interest because *C. rosicola* produces, in addition to large amounts of the optically active (+)-ABA, minor amounts of (+)-*cis*-4′-keto-α-ionylideneacetic acid as a secondary metabolite. None of the compounds we tested showed immunoreactivity except for the optically active (+)-ABA. A racemic mixture of 2-*cis*-(±)-ABA and a mixture of *cis*- and *trans*-(+)-ABA gave cross-reactions of 48% and 42%, respectively. Dehydrovomifoliol was tested because it is a naturally occurring constituent of several higher plants (Stuart and Coke, 1975).

C. rosicola produces up to 50 μg of (+)-ABA/mL of media in liquid shake culture. The high sensitivity of ELISA is not needed for such large amounts of ABA, but it provided a good model system to compare results. The ABA content of 63 cultures of *C. rosicola* media was determined by HPLC–UV and by ELISA. A correlation between the two methods was observed (Figure 2). ELISA values averaged 7.9% higher than those determined by HPLC–UV and were consistent ($r^2 = 0.94$) with them. Our results using the indirect ELISA are in contrast to those of Wang et al. (1986) who measured the ABA content of different dilutions of *C. rosicola* media by a RIA method

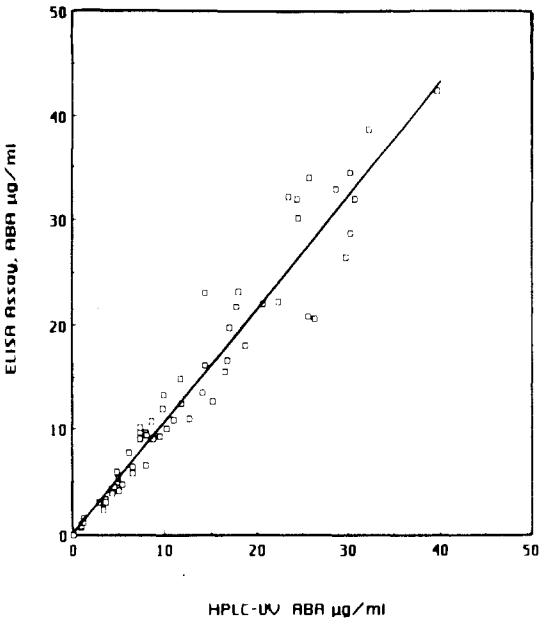

Figure 2. Correlation of ABA content of *C. rosicola* culture media determined by HPLC–UV and ELISA assay.

Table II. Comparison of ABA Content[a] of *Citrus* Leaves Determined by HPLC–UV and the Indirect ELISA Assay Using Various Cleanup Procedures Described in Materials and Methods

procedure[b]	grapefruit leaves	navel leaves
HPLC–UV: C_{18} prep col, followed by Bondapak NH_2 col	0.98 ± 0.08 (2)	4.95 ± 0.24 (2)
ELISA:		
no cleanup	0.82 ± 0.11 (17)	2.89 ± 0.33 (15)
from eluate of HPLC prep col	1.68 ± 0.27 (5)	5.07 ± 0.66 (3)
0.45-µm Lid/x	0.88 ± 0.12 (7)	2.89 ± 0.47 (7)
0.2-µm Lid/x	1.08 ± 0.18 (6)	4.68 ± 0.62 (12)
0.2-µm Acrodisc	0.79 ± 0.10 (8)	2.83 ± 0.40 (6)
centrifuging	0.84 ± 0.00 (2)	2.86 ± 0.06 (3)
centrifree tube in centrifuging	0.64 ± 0.01 (2)	3.11 ± 0.38 (3)
silica Sep Pak	0.66 ± 0.06 (4)	2.68 ± 0.32 (8)

[a] Mean ABA: microgram/gram dry weight ± SD (number of determinations). Percent dry weight was 33.58 and 27.68 for grapefruit and navel leaves, respectively. [b] TBST solutions without plant tissue extract passed through these procedures as a reagent blank gave 0 values for ABS.

using a different MAb. They encountered significant interference (e.g., twice the quantity of culture medium assayed did not give twice the value for ABA).

It was not known whether the aqueous residue after evaporation of the acetone from the plant extraction solvent made to volume with TBST could be analyzed directly or if cleanup procedures would be required. Several different filters and cleanup procedures were compared for *Citrus* leaves (Table II). Centrifree tubes, silica Sep Paks, centrifugation, and filters other than the 0.2-µm Lid/x consistently gave low values on the ELISA assay. Particulate material insoluble in the aqueous residue when the acetone is removed from the extracts appears to interfere. High ABA values, which were closer to the HPLC values, were obtained when the samples were filtered through a 0.2-µm Lid/x nylon 66 filter. Similar results were obtained with use of a Centrex 0.2-µm nylon 66 filter. Thus, a nylon 66 filter was chosen for routine use.

Crude plant extracts often contain components that interfere with most ELISA methods, particularly the direct assays, which must be removed. Established cleanup procedures for plant hormone extraction and extract purification were devised to suit the needs of physiochemical assays and may not be optimal for immunoassays although they are used extensively. We found that some cleanup procedures commonly used in purification of plant extracts for HPLC–UV and/or ELISA analyses gave high false ABA values using ELISA when TBST buffer without plant extract was passed through the procedure (Table III). We did not, however, determine whether the interferences were caused by particulate matter from the charcoal, polyamide, or PVPP, which might explain the variable results, or whether they were caused by contaminants that eluted from the powders. We also found high false ABA values on the ELISA using C_{18} Sep Paks. Prewashing C_{18} Sep Paks with $CHCl_3$ reduced the false value by 40%. We found that a material that eluted from C_{18} Sep Paks with MeOH would, after concentration, crystallize out of the MeOH solution when held at 4 °C for a few days. This material seems to be mainly dimethyloctadecylsilanol: GC–MS, *m/z* (rel intens) 328 $[M]^+$ (0), 313 $[M - CH_3]^+$ (21), 75 $[M - C_{18}H_{37}]^+$ (100), 61 (9); high-resolution EIMS (probe) $[M - CH_3]^+$ ($C_{19}H_{41}OSi$, found 313.2922, calcd 313.2927), $[M - C_{18}H_{37}]^+$ (C_2H_7OSi, found 75.0242, calcd 75.0266). It also contained a smaller amount of methyl dimethyloctadecylsilyl ether: GC–MS, 342 $[M]^+$ (0), 327 $[M - CH_3]^+$ (19), 89 $[M - C_{18}H_{37}]^+$ (100), 75 (12); high-resolution EIMS (probe) $[M - CH_3]^+$ ($C_{20}H_{43}OSi$, found 327.3123, calcd 327.3083), $[M - C_{18}H_{37}]^+$ (C_3H_9OSi, found 89.0400, calcd 89.0423). This material gave a false value equivalent to 0.5 µg of ABA/g dry weight of plant tissue when a crystal was dissolved in TBST and assayed on the ELISA. Careful attention should be paid to analyzing reagent blanks as well as samples when a cleanup procedure is evaluated for ELISA measurements. All of the procedures listed in Table II gave 0 ABA values when TBST without plant extract was processed.

Table III. Cleanup Procedures[a] That Gave False ABA Values When TBST without Leaf Tissue Extract Processed and Assayed with the Indirect ELISA

reagent blank treatment	false ABA[b]
charcoal	6.02 ± 1.06 (2)
C_{18} Sep Pak	1.58 ± 0.48 (7)
C_{18} Sep Pak, $CHCl_3$ prewash	0.65 ± 0.15 (4)
polyamide	5.90 ± 3.77 (3)
PVPP	6.25 ± 3.81 (3)
Millex-GS 0.22-µm filter	1.61 ± 0.03 (2)

[a] Procedures are described in Materials and Methods. [b] Mean false ABA values (µg/g) ± SD (number of determinations) were calculated on the basis that 0.01 g dry weight plant tissue normally used for ELISA assay was present in 1 mL of TBST.

Estimates of the ABA content by ELISA were made following the addition of 10–200 pg of authentic ABA to crude Valencia orange leaf extracts (equivalent to 0.1 g dry weight tissue) filtered through a 0.2-µm nylon 66 filter (Figure 3). We obtained parallel lines offset by the ABA content of the plant tissue. Additionally, the ELISA values were consistent with the appropriate dilution.

The ABA content determined by HPLC–UV and ELISA for fresh and wilted leaves of several different plant materials was similar (Table IV). The mass spectra were identical with those of authentic ABA methyl ester when some of the ABA-containing fractions from preparative HPLC were methylated and analyzed by GC–MS. The approximate quantity of ABA was confirmed by GC-FID. ELISA values were slightly higher for wilted *Xanthium* and fresh *Ricinus* and Valencia orange leaves and slightly lower for navel orange and fresh *Xanthium* leaves when

Table IV. Comparison of ABA Content as Determined by HPLC-UV or the Indirect ELISA Assay for Fresh and Wilted Leaves

leaf tissue	treatment	confirmed[a] GC-MS	ABA content[b] HPLC	ABA content[b] ELISA
		Detached		
R. cummunis L.	fresh	GC-MS	3.10 ± 0.07 (4)	3.37 ± 0.56 (3)
C. sinensis L. Osb.				
navel orange, new growth[c]	fresh		4.95 ± 0.24 (2)	4.68 ± 0.62 (12)
Valencia orange	fresh		0.08	0.28 ± 0.02 (3)
	wilted	GC-MS	1.06	0.95 ± 0.08 (5)
X. Strumarium l.	fresh		0.85	0.61 ± 0.07 (4)
	wilted	GC-MS	2.59	2.81 ± 0.44 (3)
		Trees Severely Water-Stressed and Recovered		
C. sinensis L. Osb.				
Valencia orange	fresh		0.36 ± 0.02 (2)	0.35 ± 0.02 (3)
C. paradisi Macfad.				
grapefruit[c]	fresh		0.98 ± 0.08 (2)	1.08 ± 0.18 (6)

[a] ABA fraction from preparative HPLC was methylated and analyzed by GC-MS, which validated that the compound being measured was ABA. [b] Mean ABA (μg/g dry weight leaf tissue) ± SD (number of determinations). [c] The same navel and grapefruit extracts were used for comparison of cleanup procedures in Table II.

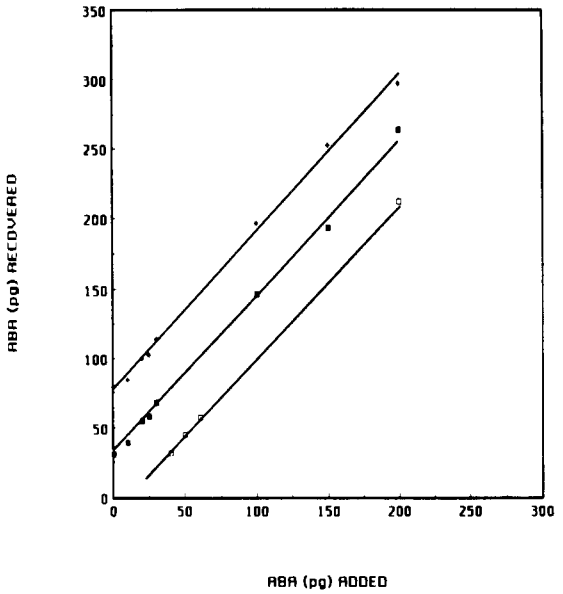

Figure 3. Effect of sample dilution on ELISA estimate of crude Valencia leaf extracts after addition of from 10 to 200 pg of exogenous ABA: (□) no leaf tissue extract added; (♦) leaf tissue extract diluted 1:5; (■) leaf tissue extract diluted 1:10. Data are the mean of three sample dilutions. ABA content of the leaf tissue was 0.68 μg/g dry weight.

compared with HPLC-UV values. The ABA content (using the ELISA values) increased about 3- and 5-fold for Valencia orange and *Xanthium* leaves, respectively, when detached leaves were wilted. The water-stressed and recovered pot-grown Valencia orange tree leaves contained considerably less ABA than detached, wilted leaves. Stressed mature grapefruit tree leaves contained about 3 times more ABA than the similarly stressed mature Valencia orange tree leaves.

Ricinus and *Xanthium* leaves were chosen for this work because the ABA content of fresh and wilted leaves is well documented (Cornish and Zeevaart, 1984, 1985; Zeevaart, 1980). Zeevaart (1980) reported that the ABA content of detached *Xanthium* leaves increased from 0.2–0.4 to 2–3 μg/g fresh weight with water stress.

Little information is available concerning the ABA content of citrus leaves. Wheaton and Baushe (1977) reported that the ABA content of stems and expanding leaves of Valencia oranges ranged in concentration from 0.09 to 0.25 μg/g fresh weight by HPLC-UV. Weill et al. (1979) reported that ABA-like inhibitors, measured by bioassay, in fresh leaves of *C. sinensis* cv. Shamouti and *Citrus aurantium* L. were about 0.1 μg/g dry weight. They reported that ABA-like inhibitors in water-stressed detached leaves increased from 0.1 to 0.4 μg/g dry weight during the first 4 h and continued to increase to 1.1 μg/g in 24 h. These inhibitors increased slowly to about 0.4 μg/g dry weight during 13 days in water-stressed pot-grown *Citrus* seedlings. They found that field-grown *Citrus* trees were extremely difficult to water-stress and that ABA-like inhibitors did not increase in field-stressed trees. Goldschmidt (1984) found the ABA content of "on" (fruiting) and "off" (nonfruiting) Wilking mandarin tree leaves to be about 0.59 and 0.14 μg/g fresh weight, respectively, by GC-ECD. Recently, Harris and Dugger (1986) found the ABA content of leaves subtending the navel fruit and newly expanding leaves to be about 1.4 and 1.19 μg/g fresh weight, respectively, using their ELISA assay. The ABA content we found for young new growth of navel orange leaves (4.83 μg/g dry weight; 1.34 μg/g fresh weight) is comparable to that found by Harris and Dugger (1986). Generally, the ABA content per unit weight is highest in young leaves and declines as the leaves expand (Zeevaart, 1979).

Our indirect ELISA offers several advantages over direct ELISA methods: (1) only one step is required for coating; (2) only microgram quantities of MAb per plate are required compared with the 2 mg required when the MAb is coated directly on the plate; (3) sample extracts are incubated with the MAb rather than an ABA–enzyme conjugate, which may result in interaction between the enzyme and potential enzyme inhibitors in the plant tissue; (4) PEG shortens the time required for two incubation steps; (5) the aqueous residue from 80% acetone extracts of *Xanthium*, *Ricinus*, and *Citrus* leaf tissue can be assayed using only 0.2-μm nylon 66 filtration. The assay excludes the simultaneous determination of ABA conjugates. If conjugates are of interest, free and conjugated ABA can be determined simultaneously after hydrolysis of the tissue extract.

Our indirect ELISA method coupled with the discriminatory power of the MAb offers an inexpensive method for investigation of the physiological functions of ABA, its intercellular distribution, and its mode of action in very small amounts of tissue. The assay should be equally applicable to other types of plants and different tissues. However, because of the wide variation of endogeneous constituents of different plants, our procedure should be

validated, both quantitatively and qualitatively, by definitive methods to ensure that certain endogeneous plant constituents do not interfere with the ELISA response. If cleanup procedures are required, then reagent blanks should always be used to ensure that the procedure per se is not giving erroneous (high) values.

ABBREVIATIONS USED

Abscisic acid, ABA; phaseic acid, PA; antibody, Ab; monoclonal antibody, MAb; polyclonal antibody, PAb; enzyme-linked immunosorbent assay, ELISA; radioimmunoassay, RIA; (±)-ABA-4′-tyrosylhydrazone–BSA conjugate, ABA-TH–BSA; retention time, R_t; high-pressure liquid chromatography–UV detection, HPLC–UV; gas chromatography flame ionization detection, GC-FID; gas chromatography electron capture detection, GC-ECD; gas chromatography–mass spectrometry, GC–MS; gas chromatography–electron impact mass spectrometry, GC–EIMS; methanol, MeOH; ethyl acetate, EtOAc; TBST, Tris-buffered saline with Tween 20; acetic acid, HOAc.

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REVIEW

Approaches to the Citrus Browning Problem. A Review

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When subjected to nonrefrigerated storage, citrus juices rapidly develop objectionable flavor and color. Sugar breakdown through the Maillard reaction is initiated by formation of hexose amines from amino acids and sugars present in citrus juice and proceeds through the Amadori (or Heyns) rearrangement to produce deoxy amino hexoses. These deoxy compounds go through a series of dehydrations, deaminations, and enolizations to produce either 1-deoxy-2,3-dicarbonyls or 3-deoxy-1,2-dicarbonyls. The pathway depends upon a relationship between the pH of the juice and the basicity of the amine. The 1-deoxy compounds further react to produce furanones and pyrones, whereas the 3-deoxy compounds produce furfurals and pyrroles. Both pathways are known to be operable because all four types of compounds are found in citrus juice and some of these have been shown to have taste significance. Ascorbic and dehydroascorbic acids may enter into the browning scheme as highly reactive α-dicarbonyls similar to those derived from the sugars.

Browning of citrus products has been a problem throughout the history of the processing industry. Maintaining the product at low temperature has been and still is (*Citrus World*, 1984) the only means to avoid color and flavor deterioration of processed citrus fruit juices, concentrates, and dehydrated products in long-term storage (Van Loesecke et al., 1934; Loeffler, 1941; Curl, 1947). Although color changes are still a concern in citrus products (Karakasides and Marousakis, 1981; *Citrus World*, 1984), the series of reactions culminating in formation of brown pigment are preceded or accompanied by other reactions that produce smaller molecules having taste significance. Thus, flavor usually becomes unacceptable before there is an objectionable color change (Shaw et al., 1977).

For 50 years compositional changes that relate to taste change have been the subject of studies with orange juice and juice simulation models. From this review of these studies it is concluded that adverse flavor changes associated with browning result from formation of compounds that impart objectionable flavor at low concentrations.

ROLE OF AMINO ACIDS

The early work of Curl (1949), Joslyn (1957), Wolfrom (1974), and others showed that amino acids accelerate sugar breakdown to produce brown color, and the model studies of Jurch and Tatum (1970) and Shaw and Berry (1977) show that amine catalysis produced compounds of taste significance. Specifically, Huffman (1974) and Varsel (1980) made direct claims that naturally occurring amino acids cause or accelerate flavor damage in orange juice. It is also noteworthy that a great number of studies with products other than citrus and models have demonstrated that amine-assisted sugar breakdown is the source of most cooked, baked, or otherwise heated food aromas (Reynolds, 1970; Fors, 1983). It should be noted, however, that there is a great similarity between acid-catalyzed and amine-catalyzed degradation of sugars. The major difference is that milder conditions of heat and acidity are needed in the latter case.

Isotope exchange experiments (Hicks and Feather, 1975) show that the same compounds, such as the deoxy sugars, the dicarbonyls, and their enols, and other browning intermediates are formed in either case. The basis for the milder conditions is that the Amadori compounds apparently enolize and convert to reactive browning intermediates much faster, because of the amine substituent in the sugar molecule (Feather, 1982). In models simulating orange juice, many end products of browning are found both in the presence and in the absence of amines (Shaw et al., 1977). However, quantitative data are not available so we have no estimate of the extent to which amines cause or enhance sugar breakdown and formation of compounds of taste significance.

Although there is still very little known about the brown polymers produced in nonenzymic browning, it is interesting to note that a recent study of polymers produced from the interaction of glycine with D-glucose, D-fructose, and (hydroxymethyl)furfural were similar and suggest that the amino acid is incorporated into the material. This interaction was confirmed by elemental analyses. Calculations for the polymers derived from D-glucose and D-

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