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Communication

Comparison of a Commercial ELISA Assay for Indole-3-Acetic Acid at Several Stages of Purification and Analysis by Gas Chromatography-Selected Ion Monitoring-Mass Spectrometry Using a $^{13}C_6$-Labeled Internal Standard[1]

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ABSTRACT

Quantitative analysis of indole-3-acetic acid (IAA) using selected ion monitoring gas chromatography-mass spectrometry (GC-MS) with $^{13}C_6$[benzene ring]-IAA as the internal standard was used to compare the quantitative accuracy of commercial enzyme-linked immunoabsorbent assay (ELISA) kits. Plant materials differed in the amount of purification required prior to use of ELISA for reliable estimates to be made. Purification similar to that obtained by at least one high performance liquid chromatographic (HPLC) step was generally necessary prior to ELISA analysis of plant materials. Additional levels of purification appeared to be required for some plant materials prior to HPLC in order to obtain an accurate estimate by ELISA techniques. In no case was it possible to obtain reasonable estimates of IAA from crude extracts or even from acidic fractions of extracts of plant tissues. GC-MS techniques provide a rapid and simple method for checking the validity of ELISA techniques. Quantitative GC-MS, or a similar technique that provides an independent quantitative validation, should, whenever possible, be applied to each new plant material under study if use of the ELISA is planned.

Accurate and precise quantitative analysis of phytohormones is an important aspect of the study of these compounds and their roles in plant growth and development. Methods of phytohormone analysis are invariably time consuming and frequently require highly specialized techniques and advanced instrumentation. The application of immunological methods to the analysis of phytohormones has been described as "the most promising development to occur since the application of GC-MS to structure elucidation more than a decade ago" (6). Although immunological techniques (8) have been utilized by a number of investigators for phytohormone analysis (5–8, 11–22), only a few workers have taken detailed precautions to independently validate the quantitative values obtained by immunoassay in their studies (7, 14, 16, 18, 19).

The recent introduction of commercial ELISA[2] kits[3,4] for analysis of several phytohormones has made this technology widely available. These kits are based on use of monoclonal antibodies developed by Weiler (12, 22). However, the initial studies reporting their development provided little information on (a) how to apply this technique to various plant materials so as to obtain accurate and reproducible results, (b) the level of sample purification necessary for determinations, or (c) the quantitative validity of the assay when applied to plant extracts (21, 22) (see also Refs. 12 and 13). A rapid and exact method for analysis of the plant hormone IAA using GC-SIM-MS analysis with [$^{13}C_6$]IAA as an internal standard has recently been described (4). We report herein our direct comparison of the two methods on a variety of plant materials. These studies point out

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[2] Abbreviations: ELISA: enzyme-linked immunoabsorbent assay; FW: fresh weight; GC-SIM-MS: selected ion current monitoring gas chromatography-mass spectrometry analysis; R_t: retention time; TBS: tris-buffered saline.

[3] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

[4] Phytodetek kits; Idetek, Inc., 1057 Sneath Lane, San Bruno, CA 94066.

the necessity of quantitative validation and, also, provide information on the precautions necessary for the successful use of this new technology for research applications.

MATERIALS AND METHODS

The general scheme for the analysis is shown in Figure 1. These studies were conducted at three different laboratories in North America. Specific techniques and equipment variations are thus detailed below by location and plant species. GC-MS-SIM analysis using [$^{13}C_6$]IAA (99% enrichment) as an internal standard were as described (4), except as noted.

Beltsville, Maryland. Procedures utilized for analysis of leaves of field-grown mature apple trees, and whole plants of duckweed (*Lemna gibba* G-3) grown in sterile culture were essentially as outlined in Figure 1 and as previously detailed (4). Approximately 15 g FW of apple leaves and 30 g FW of *Lemna* were used for each set of analyses and all experiments were repeated twice. Following extraction and acidic diethyl ether partitioning, samples were processed in four different ways. The sample for GC-MS analysis was redissolved in 100 μl 50% methanol-water and injected onto a 250 mm × 4.6 mm Whatman 5 μm Partisil ODS-3 column with a Co:Pell guard column. Elution was with 35% methanol/water plus 1% acetic acid at a flow rate of 1 ml/min. Fractions (1 ml) were collected and elution was monitored on-line by absorption at 282 nm. Aliquots of fractions were counted by liquid scintillation spectrometry. The fraction or fractions containing significant radioactivity at the R_t of [^{14}C] IAA were dried *in vacuo*, redissolved in methanol and treated with ethereal diazomethane (3). The methanol-ether was removed under a N_2 stream at 37°C and the sample was redissolved in 30 μl ethyl acetate and analyzed by GC-MS-SIM as described (4). ELISA was performed using kits provided by Idetek (21). Kits were shipped using wet ice packs and 1 d delivery. Upon arrival the ELISA plates were stored at −20°C and the other reagents were stored at 4°C. The procedure utilized was essentially as described in the protocol provided by the supplier. Since the antibody used in these kits was produced against IAA conjugated to a protein carrier through the carboxyl (21), the antibody shows a much higher affinity for methyl-IAA than for the free acid. Thus, the samples were methylated (3) prior to assay and a standard curve (10 points, 0–57 ng/well) was constructed from a methyl-IAA (ICN Biomedicals, K and K Labs) solution, the concentration of which was confirmed by its UV absorption at 282 nm (A_{282}) (using $E = 6060$ [1]). A similar procedure was used for standardization of the [$^{13}C_6$]IAA. Thus the A_{282} served as the primary analytical reference for both methodologies.

Sample location on the ELISA plate was randomized and designed to minimize edge effects. The developed plate was analyzed by an automatic plate reader and the average of three readings was used. Each sample was analyzed at two or more dilutions. Values reported are from the samples where B/B° readings corresponded only to the log-linear part of the standard

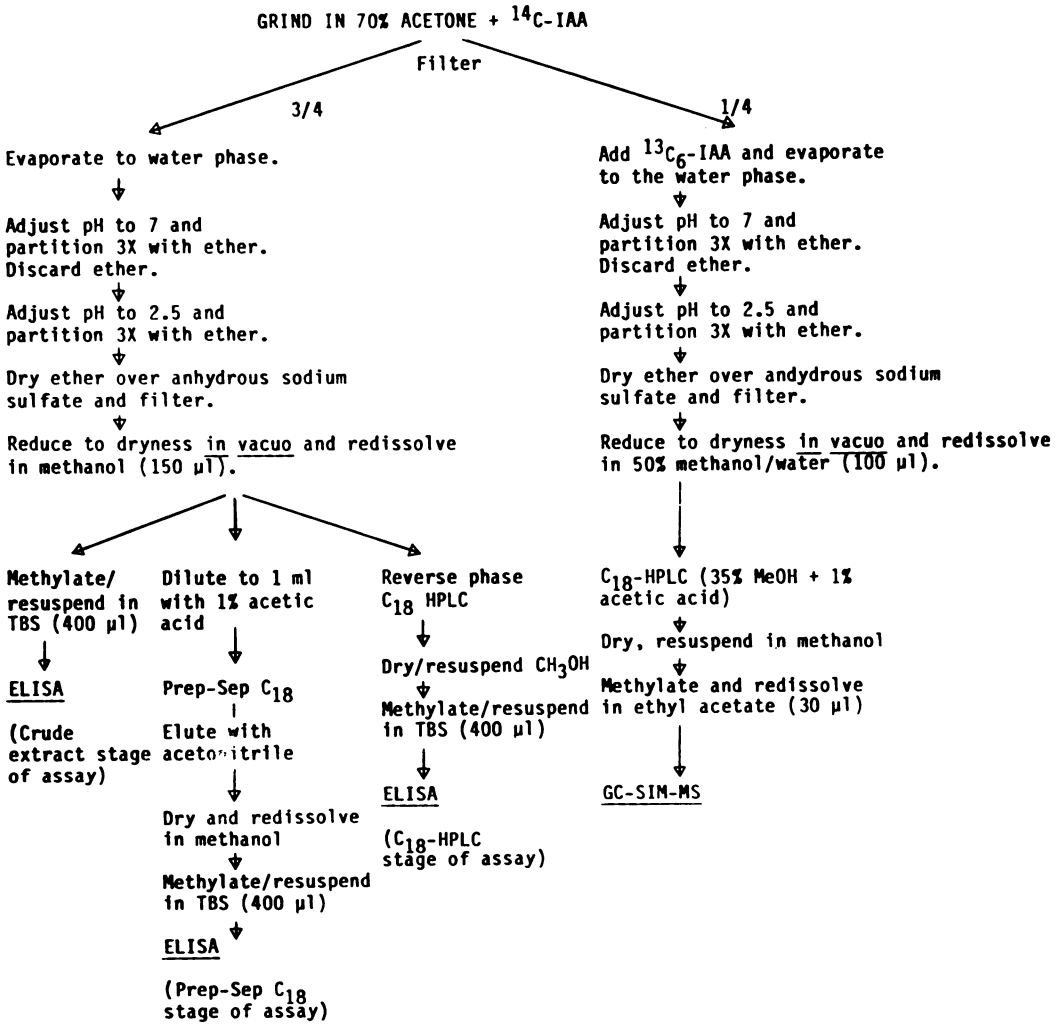

FIG. 1. The basic extraction and purification procedure used for ELISA and GC-SIM-MS comparison for quantitative analysis of IAA.

curve. In all cases the data calculated from the log-linear portion of the standard curve are closer to the values determined by GC-MS than were values calculated from dilutions falling on curvilinear portions of the standard curve.

Seed powder of *Phaseolus vulgaris* was prepared as previously described (2). Bean seeds contain mainly IAA in amide linkage to peptides (2). Thus, the bean IAA-conjugates are poorly soluble in the aqueous acetone usually employed for extraction. In order to measure total IAA in bean seed powder, strong alkaline hydrolysis conditions were used directly with the seed powder. After incubation in 7 N NaOH at 100°C for 3 or 4 h under N_2, the hydrolysates were neutralized with HCl and acetone added to a final concentration of 70%. The sample was left for 12 h at 4°C, filtered, and subsequently treated as the apple and duckweed samples. The only difference was that the [$^{13}C_6$]IAA internal standard was added after ether partitioning; the [^{14}C]IAA internal standard served for loss correction up to that point.

Orlando, Florida. The basic extraction and analysis scheme as outlined in Figure 1 for HPLC purification was followed except that replicate leaf samples were collected rather than dividing one pooled sample. Plant material consisted of leaf samples (10 g) of healthy 15-year-old Hamlin orange trees (*Citrus sinensis* L. Osbed.) growing on lemon rootstocks (*Citrus limon* [L.] Burm.). Young terminal leaves (15–20 d after shoot expansion) from the same growth period, and collected at the same time of day, were used. After washing in H_2O, the leaves were fractured in liquid N_2 and immediately extracted with solvent according to the previously described methods for apple leaf tissue. Following purification by solvent partitioning and HPLC on a Waters 5 μm C_{18} Bondapak (250 × 4.5 nm) column, as described above, the samples were methylated and analyzed by ELISA, or resuspended in ethyl acetate for GC-MS analysis.

Calgary, Alberta. The protocol used for analysis of samples was generally as given in Figure 1, or detailed above, except as noted below. Tissue was obtained from arrested lateral buds of intact plants of approximately 3-week-old *Ph. vulgaris* cv Kentucky Wonder, and the two most rapidly elongating (uppermost) internodes of approximately 3-week-old *Pisum sativum* cv Alaska. The extraction/purification protocol was designed to compare values obtained by ELISA at two levels of purification. One consisted of a crude extract, and the other consisted of the IAA-containing fractions obtained following purification by a C_{18} preparative column step (9) to remove nonpolar substances, including chlorophyll, then purification on a preparative SiO_2 partition column designed to separate *n*-hexane:ethyl acetate (5:95)-soluble compounds (including IAA) from highly water-soluble substances (9). The IAA-containing fraction was then subjected to reversed phase C_{18} HPLC (10) using a Waters analytical μ-Bondapak column, eluted with a gradient of 10 to 73% aqueous MeOH:1% acetic acid (*Pisum*), or isocratically using 24.4% aqueous MeOH:0.86% acetic acid (*Phaseolus*). Aliquots were taken in serial dilution for assay by ELISA at each of the two stages (see Table III footnotes for details). Samples for HPLC were spiked with either 9 nCi of [^{3}H]IAA (*Phaseolus*) (Amersham, 16 Ci/mmol) or with [^{3}H]GA_1 (*Pisum*) (Amersham, 32.6 Ci/mmol). IAA and GA_1 co-elute under the gradient HPLC conditions used.

RESULTS AND DISCUSSION

Estimation of IAA levels by ELISA showed good agreement with GC-MS analysis only following HPLC purification for samples from *Lemna* and bean seeds (Table I). With consideration for the variation between trees and in replicate samples, it was not possible to determine if the agreement was also reasonable in the *Citrus* samples (Table II). Both techniques, however, gave values in the same broad range and in agreement with other reports using immunoassays with *Citrus* (17). These results also demonstrated the problem of high variability we have often seen in field-grown plant material (JD Cehen, KH Cohen, AN Miller, unpublished data). Samples from IAA-containing HPLC fractions of extracts of apple leaf tissue gave ELISA estimates more than twice the GC-MS value. Samples with less purification, such as a simple solvent partitioning or partitioning followed by C_{18}-Prep-Sep (Fisher) mini-column, gave ELISA values that were consistently higher than those obtained after HPLC. As the samples were progressively purified the values for ELISA were in better agreement with GC-MS values (Table I). Tables II and III illustrate the extreme differences in values obtained for crude extracts as compared to more purified samples. Table III also notes that the range in values obtained from different serial dilutions at the crude extract stage is much greater than after HPLC, even when all B/B° values lay within the log linear portion

Table I. *Isotope Dilution Analysis of IAA in Various Tissues of Selected Plant Species using ELISA or GC-SIM-MS for Quantitation*

Plant Material	Hydrolysis	Stage of Assay by ELISA or GC-SIM-MS	IAA Amount	Percentage above GC-MS Value
			ng/g FW	%
Lemna gibba G-3	None	ELISA (crude acidic fraction)	92	557
		ELISA (C_{18}-Prep-Sep)	33	136
		ELISA (C_{18}-HPLC)	17	21
		GC-SIM-MS	14	
Apple leaf tissue	None	ELISA (crude acidic fraction)	65	983
		ELISA (C_{18}-Prep-Sep)	23	283
		ELISA (C_{18}-HPLC)	17	183
		GC-SIM-MS	6	
Phaseolus dry seed (ground)	7 N/3 h	ELISA (crude acidic fraction)	990	60
		ELISA (C_{18}-Prep-Sep)	800	29
		ELISA (C_{18}-HPLC)	690	11
		GC-SIM-MS	620	
	7 N/4 h	ELISA (crude acidic fraction)	2630	80
		ELISA (C_{18}-Prep-Sep)	2100	44
		ELISA (C_{18}-HPLC)	1870	28
		GC-SIM-MS	1460	

Table II. *IAA Values from Repetitive Field Sampling of 15-Year-Old Hamlin Citrus (Orange) Trees Growing on Lemon Rootstocks*

All samples were purified by HPLC (as outlined in Fig. 1) prior to assay.

Tree No.	Sample No.	Amount of IAA by ELISA Assay	Amount of IAA by GC-MS Assay
		ng FW	
1	1	350	
	2	289	
	3	942	
	4		200
2	1	109	
	2	254	
	3	840	
	4		1149
3	1	192	
	2	200	
	3	578	
	4		200

of the IAA standard curve. The very great difference between crude extract and HPLC ELISA estimates for tissue of *Pisum* internodes may be due to presence of a cross reactive IAA conjugate such as IAA-glycine (12). From these data it would appear that for most tissues purification and/or chromatographic separation from potentially cross-reactive substances by a method such as HPLC is necessary for most quantitative work. However, even HPLC techniques cannot give assurance of a reliable determination on all material, as is illustrated with samples of apple leaves (Table I). In no case, with any of the materials tested, was it possible to estimate IAA levels in crude extracts or in acidic fractions derived from solvent partitioning. For some material, however, such as bean hydrolysate, the C_{18}-Prep-Sep type purification may be adequate to give an accurate estimate of IAA. Even so, validation of the ELISA by a definitive technique is obviously required.

Although, as discussed in several reviews (5, 6, 13, 20), the most rigorous validation of phytohormone immunoassays is by direct comparison to mass spectral techniques, only a few studies of this type have been done and most of these have involved antibodies of highly limited availability (7, 14, 19). Techniques for internal validation of immunoassays have been discussed by a number of authors as have methods of "successive approximation" for validation, and although these methods are time consuming, laborious and do not offer the advantages of absolute methods such as GC-SIM-MS, they may be useful for tracking sources of error in these assays. However, because of its speed, sensitivity and reliability, GC-SIM-MS quantitative validation of IAA immunoassays by the methods we have described (4) seems preferable to these other techniques. In fact the ease of using [^{13}C]IAA as a quantitative tool, its very good sensitivity (20 pg or less of injected sample), and its 'foolproof' character (*e.g.* use of molar ratios of 2 or 3 characteristic ions) make GC-SIM-MS the technique of choice for routine assay of IAA unless the number of samples is inordinately large, or GC-MS facilities are not routinely available.

CONCLUSIONS

1. ELISA techniques can give a reasonable estimate of IAA in plant materials if proper care is given to sample purification and chromatography (*e.g.* separation from cross-reactive IAA-conjugates and other sources of interference).

2. It is mandatory to include radioactive internal standards, ideally [^{3}H]IAA of high specific activity, in the extracts to determine loss during the extraction, purification, and chromatographic procedures. The efficiency of methylation is also a variable which may need to be ascertained by an HPLC system which can separate free IAA from methyl-IAA.

3. Plant materials differ in the amount of purification required prior to use of ELISA for reliable estimates to be made. Our results indicate that at least one HPLC step is necessary using

Table III. *Comparison of IAA Amounts Obtained by ELISA from Aliquots Taken at the Crude Methanol Extract Stage,[a] or from Extracts after Further Purification and Reversed Phase C_{18} HPLC[b]*

Losses during the workup were estimated by use of an internal standard (9 nCi) of [^{3}H]IAA (16 Ci/mmol).

	Crude Extract		Purified Extract after HPLC	
	Average	Range[c]	Average	Range
		μg/g FW		
Phaseolus lateral buds:				
Most apical, 0.1155 g FW	1.62	(0.95 to 3.52)	0.37	(0.35 to 0.41)
Larger of the lower buds, 0.149 g FW	2.40	(1.41 to 3.52)	0.33	
Smaller of the lower buds, 0.64 g FW	2.45	(0.88 to 3.91)	1.08	
Pisum elongating internodes: uppermost two, from 80 plants, 14.67 g FW	0.38	(0.27 to 0.48)	0.0037	(0.0033 to 0.0042)

[a] For *Phaseolus* bud tissue aliquots were taken from the 80% aqueous methanol extract in serial dilution (ranging from 0.6 to 7.8 mg FW tissue equivalents), methylated with diazomethane, and assayed by ELISA. For *Pisum* internode tissue a similar approach was used, with aliquots ranging from 14.7 to 29 mg FW tissue equivalents. [b] The residual crude 80% methanol extract was then passed through a C_{18} preparative column to remove nonpolar compounds, including chlorophylls, the eluate being further purified on a SiO_2 partition column eluted with formic acid saturated *n*-hexane:ethyl acetate (5:95), to yield free IAA and remove highly water-soluble substances. The fraction containing free IAA was then subjected to reversed phase C_{18} HPLC, and the fraction eluting with the [^{3}H]IAA internal standard was methylated with diazomethane and assayed by ELISA in aliquots equivalent to 1.3 to 7.7 mg FW tissue (*Phaseolus*) or 23 to 226 mg FW tissue (*Pisum*). [c] Only B/B° values in the ELISA assay that fell within the log linear portion of the IAA standard curve were used to estimate IAA amount in the tissue sample.

these procedures prior to analysis of most materials. Additional levels of purification may be required for some plant materials prior to the HPLC (step(s) in order to assume an accurate and precise quantitation by ELISA techniques.

4. In no case was it possible to obtain reasonable estimates of IAA from crude extracts by ELISA or even from acidic (solvent partitioned) fractions of extracts of plant tissues.

5. Randomizing all samples, including replicate samples within the plate (and elimination of the outer rows is recommended) is important and should be a part of any protocol using this ELISA system.

6. GC-SIM-MS techniques provide a rapid and simple method for checking the validity of ELISA techniques and should, whenever possible, be applied to each new plant material under study. Alternatively, other less rigorous, but still appropriate methods (*e.g.* definitive bioassay after HPLC; electron capture or nitrogen thermionic detection GC, with appropriate internal standards) should be used for quantitative *and* qualitative validation of the ELISA technique for phytohormones.

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