This method may be extended to include quantification of the vulgaxanthines, the yellow pigments of the beet root. Singer and von Elbe (1980) have resolved the vulgaxanthines from interfering components using a strong anion-exchange (SAX) LC column. The chromatographer may envision the quantification of all betalain pigments, by coupling a C_{18} reverse-phase column with an SAX column. This method may then be employed to analyze many commodities after sufficient sample cleanup, such as commercial colorant concentrates, fresh beet cultivars, or betalain colored food products.

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Received for review October 11, 1979. Accepted January 28, 1980. Research supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, and with funds made available by General Foods Corporation, White Plains, NY.

Radioimmunoassay of Limonin Using a Tritiated Tracer

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A radioimmunoassay is described for the determination of nanogram quantities of limonin in crude tissue extracts or juice. The detection limit of this assay, using tritium tracer, is 0.22 ng or 2.2 parts per billion of limonin and the processing capacity is several hundred samples per working day. Standard curves are linear over the wide range from 0.5 to 100 ng of limonin. No prior purification of extracts is necessary.

Limonin is the intensely bitter triterpenoid lactone present throughout the Rutaceae (Goodwin and Goad, 1970, and references therein) but is of major economic concern in certain citrus fruits and their processed products. The major contribution of limonin to deterioration of fruit and juice quality has been observed in California Navel Oranges, grapefruit, and certain other oranges (Maier and Dreyer, 1965; Wilson and Crutchfield, 1968; Barton et al., 1961). In addition, there are a large number of other limonoid compounds present in citrus (Dreyer, 1968; Bennett, 1971).

There have been a number of analytical methods developed for limonin (Fisher, 1978, 1973 and references therein) but in general they are comparatively slow, some are based on subjective evaluation and none are sensitive below the microgram range (ppm).

Recently we have developed and described a radioimmunoassay (RIA) which has a high specificity for limonin and a detection level of 0.071 ng which corresponds to 150 fmol or less than 1 ppb. This original test system was developed using an ^{125}I tracer which was stable for about 10–12 weeks (Mansell and Weiler, 1979). The object of the present work was to develop an alternative assay utilizing tritium tracer which has a much longer half-life. This alternative method would lessen the number of radioactive syntheses necessary but would retain the sensitivity and accuracy of the test system.

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MATERIALS AND METHODS

Apparatus. A Model BF 5000 scintillation counter (Berthold Co., Wildbad) with 200 sample capacity and punched tape output was used for radioactivity determinations. All calculations were done on a HP 9825/9871 desk-top calculator/printer-plotter (Hewlett-Packard). Centrifugations were performed in a cooled Heraeus Cryofuge 6-6 with Analmatic 100 centrifuge head (Heraeus Co., Osterode). Pipetting was done using Eppendorf pipets.

Materials. Radioimmunoassays were performed in 63 × 12 mm glass tubes. For radioactivity determinations, standard scintillation vials modified to accomodate the 63 × 12 mm tubes were used.

Chemicals. Sodium [^{3}H]borohydride, sp act. 11.1 Ci/mmol, was purchased from New England Nuclear. The scintillator used was PCS (Amersham-Searle). Bovine serum was supplied by Mediapharm, Aschaffenburg (cat. no. SER Bo 1000). Phosphate-buffered saline (PBS, 0.1 M potassium phosphate, 0.15 M NaCl, pH 7.4) was used as RIA buffer. Concentrated ammonium–sulfate solution was prepared by stirring 800 g of $(NH_4)_2SO_4$ and 1000 mL of water overnight. After filtration, the pH was adjusted to 7.0 with concentrated NaOH. Standard limonin was a gift from Dr. James Fisher, Florida, Department of Citrus. Ten milligrams of limonin was dissolved in 1 mL

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of acetonitrile and then diluted to 10 mL with absolute MeOH. From the stock solution, serial dilutions were made with water. Standards were stored at 4 °C between uses. Under these conditions, solutions in the standard curve range were stable for at least 14 days. Limonol was synthesized by $NaBH_4$ reduction (see tracer), purified by TLC, and identified by MS.

Sample Preparation. Fresh plant tissues (100–400 mg) were extracted with 5 mL of acetone for 90 min at 55 °C. After decanting, the tissues were reextracted with a second portion for an additional 30 min. The acetone fractions were combined and brought to a total volume of 10 mL. Two-milliliter aliquots were removed and diluted to 5 mL with 0.01 N HCl. All subsequent dilutions were done with distilled water. Aliquots of 0.1 mL were immunoassayed.

Antiserum and Tracer. Antiserum was obtained from rabbits as previously described (Mansell and Weiler, 1979). The animals were immunized against a conjugate of limonin and bovine serum albumin which had a limonin/protein ratio of 16:1. $[^{125}I]$Limonin was prepared with a specific activity of approximately 600–800 Ci/mmol.

Limonin tracer was synthesized as follows: 2.74 mg of limonin was dissolved in 0.1 mL of pyridine and 0.4 mL of MeOH was added. This solution was added to $NaB(^3H)_4$ and shaken for 10 min. The entire reaction mixture was streaked on silica gel TLC plates and developed in $EtOAc/CHCl_3/HOAc$ (50:10:1). The major immunoreactive band was located at R_f 0.55 and cochromatographed with an authentic sample of limonol. The tracer was chromatographed in benzene/HOAc/H_2O (6:7:3) and again in 50:10:1. After final elution with MeOH, the tracer was stored at 4 °C. The specific activity of the final product was ca. 20 Ci/mmol (calculated).

Immunoassay Procedure. All samples and standards were assayed in triplicates. To 0.1 mL of sample or limonin standard, the following reagents were added successively: 0.5 mL of PBS, 0.1 mL of tenfold diluted bovine serum, 0.1 mL of dilute tracer (10 000 cpm, 1.1 pmol of $[^3H]$limonin). After mixing, 0.1 mL of diluted antiserum was added, and the tubes were shaken again. After incubation for 60 min at room temperature (23 °C), 1 mL of a freshly prepared solution of ten volumes of saturated ammonium sulfate and one volume of water was added. After mixing and incubating for 15 min at room temperature, the precipitated protein was centrifuged at 4000 rpm for 15 min, and the supernatant was decanted. The pellets were washed once with 1 mL of half-saturated ammonium sulfate and then dissolved in 0.15 mL of water. Finally, 1 mL of PCS scintillator was added to each tube and thoroughly mixed to obtain a clear solution. The samples were then inserted into the modified counting vials and counted for 2 min each. Counting efficiencies for standards and unknowns were 19.1 ± 1.1%. Result calculation was performed using the spline approximation method (Marschner et al., 1974). The immunoassay procedure which employs $[^{125}I]$limonin was followed as described.

RESULTS

Assay Specificity. An antiserum of known specificity in combination with $[^{125}I]$limonin was used for this study. However, to test for any changes in the properties of the system using ^3H tracer, the antibody was reevaluated for cross-reactivity (Table I). The values obtained are in close agreement to those calculated for the same antibody using the iodinated tracer (Mansell and Weiler, 1979). Thus, antibody specificity in this system is not influenced by the tracer material used. Structural models of the $[^3H]$limonin tracer showed little structural change relative to limonin and this is confirmed by the good immunoreactivity of the material and the complete reversibility of binding of the tracer when limonin is added in excess. Deoxylimonin cross-reacted with the antibody very strongly, probably as a result of the structural similarities between this compound and the tracer. Deacetylnomilin reacted only to a low extent and the other limonoids tested did not react at all. Deoxylimonin and deacetylnomilin are present in very low amounts in citrus tissue (Barton et al., 1961; Dreyer, 1965), but since tissues must be highly diluted for limonin determination, any other limonoids present in the raw extracts are not likely to interfere with the assay. In addition, extract dilution curves for fruit and leaf tissues paralleled standard curves. This further provides evidence for the absence of unspecific interfering material in biological samples. For this assay, extracts can be used without any previous purification.

Table I. Cross-Reactivities of Limonin Antibody Using Tritiated Tracer

compound	nmol of compound required to displace 50% of $[^3H]$limonin	cross-reactivity, %
limonin	6.3	100
deoxylimonin	6.3	100
deacetylnomilin	185	3.4
nomilin	1012	0.6
obacunone	1454	0.4
nomilinic acid	2000[a]	0
isoobacunoic acid	2000[a]	0

[a] Highest concentration assayed.

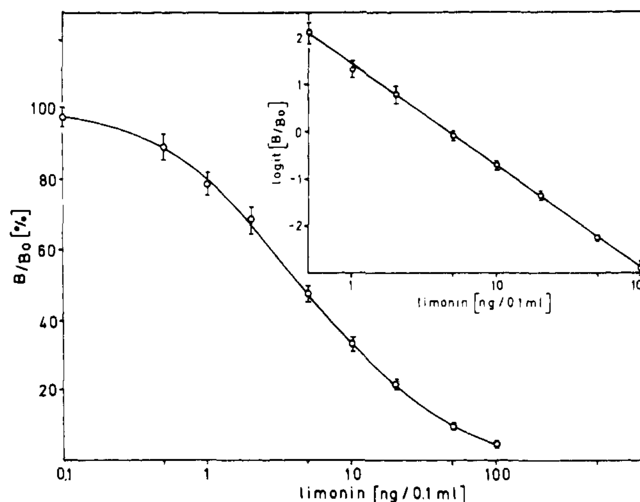

Figure 1. Limonin standard curve in lin/log and logit/log plot. (O) Mean ± SD for n = 10 consecutive assays.

Assay Sensitivity and Variability. The limonin standard curve is shown in Figure 1 using two different plots. Standard curves from ten consecutive assays were used for this figure. As the bars representing ±SD indicate, the day-to-day variation of the assay is very small. Coefficients of variation for triplicate determinations (B/B_0 values) throughout the measuring range are 3.0 ± 1.8%, and the measuring range extends from 0.5 to 100 ng of limonin/0.1 mL of sample. This corresponds to 0.005–1 ppm. The detection limit of this assay, defined as that amount which can be safely distinguished from a zero sample (99.5% confidence limit), is 220 pg (2.2 ppb).

To further test for reproducibility, various extracts were divided into five aliquots, and each portion was stepwise diluted 1000-fold. Each sample was assayed in triplicate. The variability for these replicate determinations ex-

Table II. Comparison of Assay Parameters as a Function of Tracer Material Used

assay parameter	[^{125}I]-limonin	[^{3}H]-limonin
antiserum titre	1:27 000	1:1350
measuring range, ng	0.1–100	0.5–100
detection limit, pg/ppb	71/0.7	220/2.2
unspecific binding, %	27	1.1
solvent blanks, ng	0	0
recovery of limonin, %	93	97
coeff. of variation for triplicates (B/B_0), %	2.5 ± 1.8	3.0 ± 1.7
processing capacity per day and worker	400–600	200–400

pressed as coefficients of variation ranged from 2.7 to 4.5%. Recovery of limonin added to extracts prior to dilution was 97%.

Processing Capacity. Using the assay performance described herein, one person can easily complete about 200–400 individual analyses per working day. Thus, the average time per analysis is only 1–2 min. Radioactivity counting (2 min/sample) is done overnight and data calculation requires about 45 min/400 samples using a programmable calculator. Thus, radioimmunoassay by far exceeds the processing capacity of any other determination method for limonin currently available.

Comparison of Methods Using [^{125}I]Limonin and [^{3}H]Limonin as Tracers. In Table II, various assay parameters are compared as a function of the tracer used. Although with iodinated limonin, lower amounts of limonin may be detected, both assays have essentially comparable measuring ranges from 100 ng to well below 1 ng and possess comparable precision and recovery. However, serum titres were much higher with iodinated tracer and this assay also has higher processing capacities due to shorter work-up procedures. In contrast, ^{125}I-labeled limonin has to be prepared several times a year due to the short half-life of the radionuclide, and this material exhibits considerably higher, though constant, unspecific binding effects.

Results obtained with both methods are completely equivalent as can be seen from the correlation experiment shown in Figure 2. Sixty-seven extracts from various plant tissues were analyzed with both techniques, and a high degree of correlation was obtained (r = 0.95, regression equation: $y = 0.96x + 0.03$).

DISCUSSION

Recently, we introduced radioimmunoassay for the determination of the *Citrus* constituent limonin (Mansell and Weiler, 1979) by using an [^{125}I]limonin derivative as tracer. In the present paper we show that it is also possible to use a tritiated limonin of high specific activity to establish a very sensitive and precise assay for this compound. The use of tritium in this system is advantageous since this isotope has a very long half-life compared with ^{125}I and thus one tracer batch should be usable for several years. In addition, β counting equipment is more readily available than γ counters. Using these immunoassay techniques, it has now become possible to detect and quantitate this intensely bitter principle in the parts per billion range. Thus, this increases the detection limits over other existing analytical techniques available for this compound by a factor of more than 1000. In addition, whereas only very few samples can presently be processed for limonin quantitation per day (e.g., TLC or LC), RIA provides the possibility of assaying several hundred samples per day with a precision equal to the other analytical methods available. Thus, this technique offers new potentials in

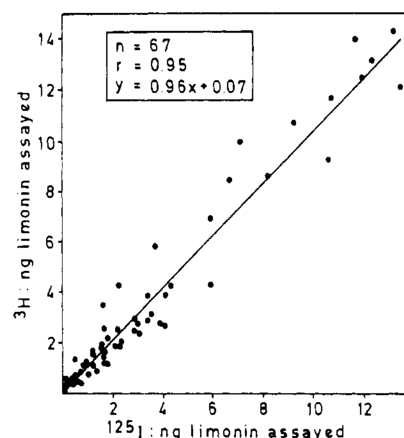

Figure 2. Scatter diagram for various plant tissue extracts assayed by both methods.

citrus research and also can be used as the basis for searching for new citrus varieties with reduced levels or lacking this bitter principle. The screening of citrus trees and selection of cell culture strains now becomes a real possibility and physiological experiments can be done on a mass scale.

It is important to note that no purification of extracts prior to analysis is required for the RIA technique. Acetone extracts of tissues or juice, acidified to convert the limonoate A-ring lactone to limonin, have only to be diluted to the measuring range and can be measured directly. More importantly, since the other limonoids known to be present in substantial amounts in *Citrus* do not cross-react to any extent, they will not interfere in the assay. Since RIA consists mainly of pipetting steps, the technique is especially adapted to be automatically run using continuous or discontinuous pipetting devices. The fact that the RIA of limonin could be established using tritiated and iodinated tracers of different structure demonstrates the versatility of the immunoassay system. It is now planned to extend this assay to develop alternative methods for the quantitation of limonin which avoid the use of radioisotopes and require less expensive and more widely distributed equipment, e.g., spectrophotometers.

ACKNOWLEDGMENT

We thank R. Bennett, USDA, Pasadena, CA, for a gift of the limonoids.

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Received for review October 10, 1978. Accepted February 2, 1979. This paper is part 9 in the series: "Use of Immunoassay in Plant Science". This work was supported in part by a grant from the Florida Citrus Commission, Lakeland, FL, and in part by a grant of the BMFT, West Germany. One of us (R.L.M.) was the recipient of a Senior Scientist Award of the Alexander von Humboldt Foundation, Bonn, West Germany.