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RADIOIMMUNOASSAY FOR THE DETERMINATION OF LIMONIN IN *CITRUS**

RICHARD L. MANSELL† and ELMAR W. WEILER

Lehrstuhl für Pflanzenphysiologie, Ruhr-Universität Bochum, D-4630 Bochum, W. Germany

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Key Word Index—*Citrus*; radioimmunoassay; limonin; limonin content.

Abstract—An ^{125}I-radioimmunoassay technique has been developed for the triterpenoid bitter principle, limonin. Synthesis of the iodinated tracer and the limonin–bovine serum albumin conjugate are described. The antibody has a high affinity (K_a 1.1×10^9 l/mol) and specificity for limonin and the detection limit of the assay is 0.07 ng or 0.7 ppb. Standard curves are linear over a range of 0.5–100 ng limonin, assays can be performed in crude extracts, and several hundred samples can be processed per day. The distribution of limonin in fruits and vegetative parts of *Citrus paradisi* has been determined, highest values (0.92%) being found in the seeds, lowest (0.0007%) in the juice vesicles of ripe fruits. The potential of this assay method in citrus research is discussed.

INTRODUCTION

The tetracyclic triterpenoid dilactone, limonin (**1**), is most widely distributed in the Rutaceae and has been found in greatest abundance in *Citrus* and related genera [1]. The presence of limonin is responsible for bitterness in some processed citrus products, namely California Navel oranges, Australian Valencia oranges and grapefruit [2]. The bitterness of these processed fruits is of economic importance and has affected the public acceptance of especially the juice products.

Limonin arises from a limonoate A-ring lactone precursor [3] and the bitterness characteristic develops only after acidification or heating. More recent studies have revealed that the A-ring lactone is synthesized in the young leaves of citrus and is subsequently transported to the fruit [4] where it is ultimately deposited in the seeds, carpellary membranes and central conductive tissue.

There have been a number of quantitative methods developed for the detection of limonin but at present most of these techniques are either susceptible to subjective evaluation [5, 6], require extensive prepurification [7, 8] or have other limiting characteristics [9]. In addition, the maximum sensitivity is in the microgram range. Furthermore, the present methods are slow and usually less than 20 samples can be processed within an 8 hr working period.

Radioimmunoassay (RIA) is presently the most powerful analytical tool for the quantitation of very diverse compounds and only recently has this technique been introduced into phytochemistry and plant physiology [10]. RIA possesses the unique features of high specificity, extreme sensitivity (usually <10 ng) and a wide measuring range (from a few picograms to 100 ng). The assay can be almost fully automated and more than 1000 samples can be processed in a single day. This extremely versatile procedure is becoming increasingly useful in plant science and has been used for enzyme kinetic studies [11], intact plant and cell culture sceening [12, 13], as well as for routine monitoring.

In this paper, we report on the characteristics of a RIA developed for limonin, using an ^{125}I-tracer and an antiserum fraction from rabbits. In addition, the distribution of limonin in various tissues of *Citrus paradisi* (grapefruit) is described.

RESULTS

Properties of antiserum

The antiserum fraction used for the present study had a high affinity for limonin. Under the assay conditions employed, the antiserum titre was 1:27 000 (defined as that dilution which bound 50% of 10 fmol of ^{125}I-TME-limonin). From a Scatchard plot [14] of standard curve data, a maximum affinity constant of K_a 1.1×10^9 l/mol has been calculated. This K_a value is in the higher range for RIA suitable antibodies [15].

Assay sensitivity

In Fig. 1 a typical standard curve is shown in two different plots. The measuring range of this assay, using the linear form of the standard curve, extends from 0.5 to 100 ng, however, the non-linear plot, as calculated by spline approximation, permits the use of

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† To whom correspondence should be addressed. Permanent address: Department of Biology, University of South Florida, Tampa, FL 33620, U.S.A. Recipient of a Senior Scientist award of the Alexander von Humboldt Stiftung, Bonn-Bad-Godesberg.

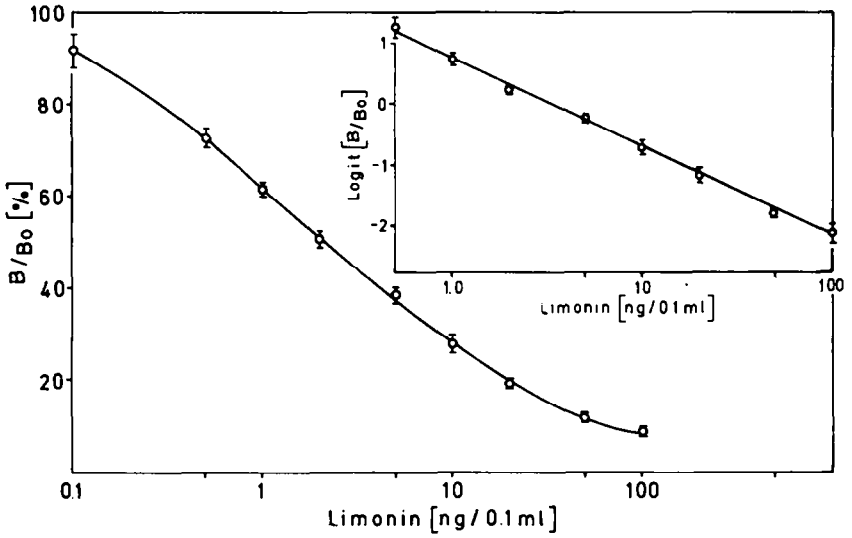


Fig. 1. Typical limonin standard curves shown in two different plots. —○— Mean ±s.d. of triplicate determinations.

an even wider range from 0.1 to 100 ng of limonin per assay, for result calculation.

The detection limit of this method, defined as that amount of limonin which can be distinguished from a zero sample at the 99.5% confidence limit, is 71 pg (0.15 pmol) of limonin/0.1 ml sample. Thus, less than 1 ppb (2×10^{-13} mol) of limonin can be detected and quantitated with this assay.

Specificity of assay

Various experiments were performed to test for antibody specificity. Per cent cross reactivities were determined on a molar basis with pure compounds of similar structure or biogenesis (Table 1) and calculated according to ref. [16].

Furthermore, to test for unknown but possibly interfering material in plant extracts, seeds from a grapefruit variety (White Marsh) were extracted in acetone, diluted ten-fold with water, acidified, and an aliquot spotted on a TLC plate. The developed plate was cut into 0.5 mm strips which were eluted with MeOH. After a ten-fold dilution, aliquots of these eluates were immunoassayed. As can be seen from Fig. 2, only one immunoreactive band could be detected on the chromatograms and this band co-chromatographed with authentic limonin (R_f 0.4). Similar results were obtained with leaf extracts.

Table 1. Cross reactivities of limonin antibodies

Compound	Amount of compound required to displace 50% of ^{125}I-TME-limonin (nmol)	Cross-reactivity (%)
Limonin (**1**)	4.2	100
Deoxylimonin (**2**)	15.5	27
Deacetyl-nomilin (**3**)	63.7	6.6
Nomilin (**4**)	447.5	0.9
Obacunone (**5**)	1101	0.4
Nomilinic acid (**6**)	»2000*	0
Iso-obacunoic acid (**7**)	»2000*	0

* Highest concentration assayed.

Assay variability and recovery

Coefficients of variation of triplicate B/B_0 values throughout the measuring range were 2.5 ± 1.8%, and the recovery of limonin added to extracts prior to dilution was found to be 93%. This demonstrates the high reproducibility of this assay.

Tracer stability

In order to be useful as a routine procedure, the stability of the iodinated derivative should extend at least over a period of 2 months (1 half-life). In order to test for this, repeated assays were performed with one batch of tracer over a period of 3 months (Table 2).

As can be seen, immunoreactivity (tracer binding in excess of antibody), unspecific binding as well as the position of the standard curve (as fixed by its mid-range value at $B/B_0 = 50\%$) did not change significantly over a period of 72 days, and one batch of tracer could be used for 3 months. Chromatographic analysis

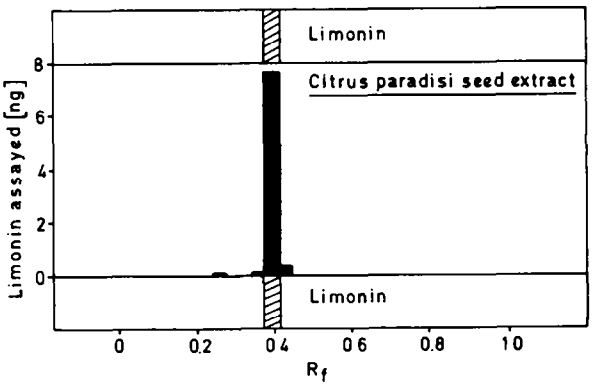


Fig. 2. Distribution of immunoreactive material on a TLC of a *Citrus paradisi* (White Marsh) seed extract after acidification. Solvent system: C_6H_6–EtOH–HOAc–H_2O (200:47:1:15). Adsorbent: Si gel.

Table 2. Stability of ^{125}I-TME-limonin

Age of tracer preparation (days)	UB* (%)	B† (%)	Mid-range‡ (ng)	Immunoreactivity§ (%)
2	31.5	46	2.0	65.5
10	24.5	51	2.1	—
72	27.5	44	2.1	66.3
88	27.5	47	2.0	—

* Unspecific binding.
† Specific binding.
‡ Value giving $B/B_0 = 50\%$.
§ Tracer binding in excess of antibody.

also revealed the stability of ^{125}I-TME-limonin over this time period. Thus, only four labelling experiments, which can be performed within a few hours, are required per year.

Distribution of limonin in plant tissues

The distribution of limonin within the fruit, leaves and twigs of *Citrus paradisi*, as measured by radioimmunoassay, is shown in Fig. 3. Within the fruit, highest concentrations were found in the embryo and the seed coat. A pronounced gradient in limonin concentrations could be seen with values decreasing from the central conductive tissue to segmentary membranes, albedo and flavedo. The juice vesicles were almost free of limonin. In the green, vegetative parts of *C. paradisi*, relatively high concentrations of limonin were also detected.

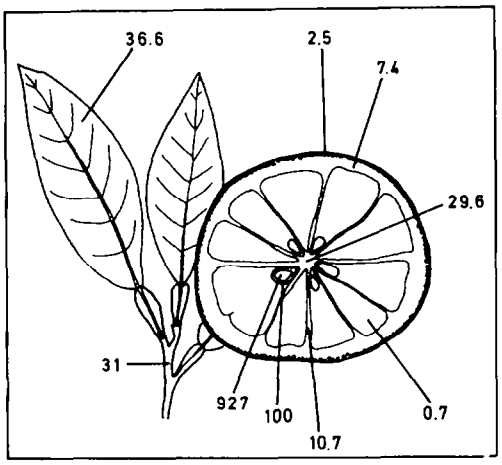
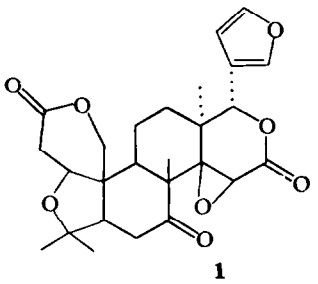


Fig. 3. Distribution of limonin in a fruit and vegetative parts of *Citrus paradisi* (White Marsh). Limonin concentration values are given as μg/100 mg fr. wt.



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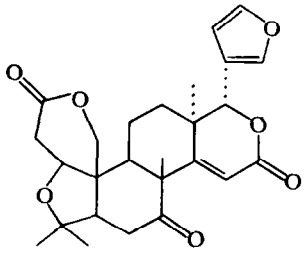
advantages and potentials over conventional analytical methods.

In the specificity tests, it was observed that only deoxylimonin (**2**) and deacetyl nomilin (**3**) cross-reacted with the antibody. However, from previous studies on the relative concentrations of limonin and deoxylimonin in citrus tissues [16, 17], it was found



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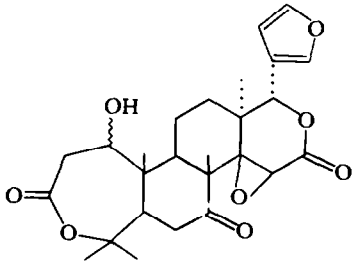

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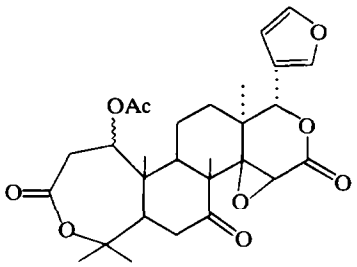
DISCUSSION

In this study we have described a radioimmunoassay system for the analysis of the triterpenoid, limonin (**1**). The antibody produced against this compound is characterized by a high titre which will permit the analysis of 30 000 samples per ml. The sensitivity of the assay is by far the highest yet developed for this compound and corresponds to <1 ppb. This sensitivity is characteristic for the RIA and offers a series of that deoxylimonin is present at only 0.47% that of limonin and therefore should not be detectable in diluted samples. Deacetyl nomilin, also, is one of the minor constituents of the total limonid fraction present in tissues and will not contribute to the limonin values.

The occurrence of only a single immunoreactive band on TLC of different tissue extracts clearly demonstrates again the monospecificity of the antibody



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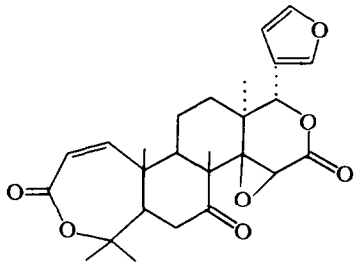

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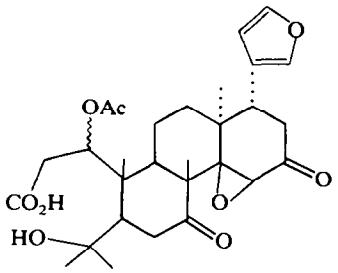

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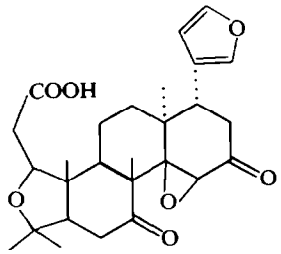

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used. Compounds **2–7** are, under these conditions, well separated from **1**.

In the study of the distribution of limonin within various grapefruit tissues, the values obtained were in good agreement with values available in the literature [5, 16] and the gradient from growing leaves into the fruit conductive tissue and ultimately the seed supports the recent results obtained by Hasegawa and Hoagland [4]. However, an extensive quantitative analysis of both fruit and vegetative tissue has not yet been done nor have the kinetics of accumulation been investigated. With the development of a RIA for limonin, these studies are now possible.

It is also important to note that the analysis of tissues were performed in unpurified extracts and radioimmunoassay thereby provides a major improvement over current methods. The potential uses of the RIA can now be extended to other areas of citrus research. With this system the mass screening of plant populations for low limonin-producing trees now becomes possible and the same can be applied to cell culture screening and enzyme kinetic studies. With modification, the immunoassay can also be used in facilities such as fruit processing plants, where the use of radioactivity would be undesirable.

EXPERIMENTAL

Materials. Bovine serum albumin was purchased from Serva, bovine serum from Mediapharm (SER Bo 1000), and $Na^{125}I$ was purchased from NEN (NEZ 033-L). Limonin was a gift from Dr. James Fisher, Florida Department of Citrus. Freund's complete adjuvant was supplied by Difco. Aminooxyacetic acid was purchased from Sigma.

Plant extracts. Fruit tissue was purchased from local markets; other plant material was obtained from a *Citrus paradisi* tree growing in the University botanical garden. Fresh plant material (100–400 mg) was extracted in 15 ml Me_2CO (90 min at 55°), followed by a ten-fold dilution of the extracts in 0.01 N HCl to convert all the limonoate A-ring lactone to limonin. These extracts were then further diluted with H_2O and aliquots of 0.1 ml taken for radioimmunoassay.

Synthesis of limonin-7-(O-carboxymethyl)oxime. Limonin (300 mg) and aminooxyacetic acid (600 mg) were refluxed for 2 hr in 24 ml Py–EtOH (1:1) and the solvents evapd *in vacuo* to give an oily residue. This was extracted with MeCN and then chromatographed on Si gel 0.5 mm, solvent system C_6H_6–EtOH–HOAc–H_2O (200:47:1:15). The band at R_f 0.19 was eluted with MeCN and upon concn 200 mg crystalline material was obtained. Needles, mp 256° (decomp.), from MeOH. Limonin-7-(*O*-carboxymethyl)oxime in CH_2Cl_2 was treated with etheral CH_2N_2 to give the methyl ester (R_f 0.47 in the above TLC system; limonin R_f 0.37). Needles, mp 124–127° (CH_2Cl_2–Et_2O); IR cm^{-1}: δ-lactone 1735; C=N 1626 (limonin lactone 1750; C=O 1706). MS:M⁺ *m/e* 558 (calc. for $C_{29}H_{35}O_{10}N$:557.6). (Found: C, 56.17; H, 6.36; N, 2.37. $C_{28}H_{33}O_{10}N{\cdot}3H_2O$ requires: C, 56.27; H, 6.58; N, 2.34%).

Coupling of limonin-7-(O-carboxymethyl)oxime to bovine serum albumin. Limonin-7-(*O*-carboxymethyl)oxime 33.3 μmol (18.1 mg) was dissolved in 2 ml DMF and 54 μmol (10 μl) tri-*n*-butylamine added. The soln was cooled to 0° and 36.5 μmol (5 μl) isobutylchlorocarbonate added. After stirring for 20 min, the mixture was added to an ice-cold soln of 420 mg bovine serum albumin in 7 ml DMF–H_2O (1:1) containing 0.14 ml 1 N NaOH. DMF (0.3 ml) was added to clear the soln and the reaction mixture was stirred for 60 min. 1 N NaOH (0.07 ml) was added and stirring was continued for 3 hr. Finally, the conjugate was dialysed against 2 l. 10% DMF in H_2O overnight, against H_2O for 3 days and then lyophilized. Coupling ratios were determined from spectral data (490 nm) in conc H_2SO_4 and the limonin/protein ratio was found to be 16:1.

Immunization procedure. Randomly bred rabbits were used for immunization. After a pre-immunization with 4 weekly intradermal injections of 0.5 mg of the conjugate applied as a buffered emulsion in Freund's complete adjuvant, intramuscular booster injections (1 mg) were given monthly and blood collected at various times thereafter. After removal of the whole cells by centrifugation, a fraction of the antiserum was used for the present study.

Preparation of limonin-7-(O-carboxymethyl)oxime-tyrosine methylester. Limonin-7-(*O*-carboxymethyl)oxime 37 μmol (20.1 mg) was dissolved in 1 ml DMF and chilled to 0°. 10%

triethylamine (52 μl) in DMF was added and the reaction mixture cooled to −10°. 10% Isobutylchlorocarbonate (50 μl) in DMF was added and after 3 min, 37 μmol (8.6 mg) tyrosine methylester and 0.1 ml 10% triethylamine in DMF were added and the reaction mixture stirred for 3 hr. The limonin-7-(*O*-carboxymethyl)oxime-tyrosine methylester, R_f 0.13 in $CHCl_3$–EtOAc (1:1), gave a positive reaction with diazotized sulphanilic acid for tyrosine and the characteristic H_2SO_4 colour for limonoids. It was purified by TLC and was obtained chromatographically pure.

Preparation of ^{125}I-*TME-limonin.* Limonin-7-(*O*-carboxymethyl)oxime-tyrosine methylester (TME-limonin) was iodinated to specific activities of at least 600–800 Ci/mmol (calculated) by a modified chloramin-T procedure [18]. The ^{125}I-TME-limonin was purified by TLC, R_f 0.23 in C_6H_6–EtOH–HOAc–H_2O (200:47:1:15), and eluted and stored in MeOH.

Immunoassay procedure and data calculation. Triplicate determinations were performed throughout; 0.1 ml of diluted sample or limonin standard was added to glass tubes. Then 0.5 ml buffer (0.01 M KPi, 0.15 M NaCl, pH 7.4), 0.1 ml tenfold diluted bovine serum and 0.1 ml of dilute tracer (10 000 cpm, *ca* 10 fmol ^{125}I-TME-limonin) were added. After mixing, 0.1 ml dil antiserum fraction was added (0.1 ml H_2O replacing antiserum for the determination of unspecific binding) and the samples were incubated 2 hr at room temp. Then, 1 ml of a freshly prepared soln of 10 vols satd $(NH_4)_2SO_4$ and 1 vol. H_2O were added to the tubes to precipitate the antigen-antibody complexe. After 15 min the samples were centriguged, the supernatants decanted, and the pellets dissolved in 0.5 ml H_2O. Radioactivity determinations were done in an automatic gamma counter (MAG 510, Berthold Co.) with punched tape output. The counting time per sample was 2 min. Calcns were performed on a HP 9825/9871 desk top computer/printer-plotter (Hewlett–Packard) using the spline approximation method [19].

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