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RADIOIMMUNOASSAY FOR THE QUANTITATIVE DETERMINATION OF HESPERIDIN AND ANALYSIS OF ITS DISTRIBUTION IN *CITRUS SINENSIS*

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Key Word Index—*Citrus sinensis*; Rutaceae; radioimmunoassay; hesperidin; flavonoidglycosides; rutinosides.

Abstract—A simple and sensitive radioimmunoassay (RIA) for the citrus flavanone hesperidin and other flavonoid 7-rutinosides is described. The assay utilizes antibodies raised against a hesperidin 4-*O*-carboxymethyl-oxime hapten and a tritiated radiotracer prepared by direct reduction of hesperidin with NaB[3-H]4. The detection limit of the assay is 0.2 ng/0.1 ml (0.3 pmol/0.1 ml) and the measuring range extends to 10 ng/0.1 ml (16.4 pmol/0.1 ml). This assay is specific for flavonoid rutinosides, is characterized by a low coefficient of variation, and shows good correlation with HPLC for the quantification of hesperidin in oranges. The application of this method in determining the distribution of hesperidin in leaves, fruit, seeds, and seedlings of *Citrus sinensis* is also reported.

INTRODUCTION

Hesperidin, 5,7,3′-trihydroxy-4-methoxyflavanone 7-*O*-rutinoside is widespread in *Citrus* and the major flavonoid in the sweet orange and lemon [1]. In young immature oranges it can account for up to 14% of the fresh weight [1]. Some years ago, there was a great deal of interest in hesperidin because of its possible physiological and biochemical activity as part of the 'bioflavonoids' or 'vitamin P complex' [1] Other interest in hesperidin centered on its organoleptic properties and those of its isomer, neohesperidin; in addition, flavanone 7-neohesperidosides are extremely bitter, whereas the rutinosides are tasteless [2]. The biosynthesis and metabolism of hesperidin in *Citrus* have received limited attention and then only as they relate to fruit development and maturation [3]. The bitter flavanone neohesperidosides (mainly naringin) accumulate in grapefruit whereas the non-bitter rutinosides of these flavonoids predominate in orange and lemon [4,5].

Studies on the biosynthesis and metabolism of hesperidin have been very limited [3–5], in part because of a lack of simple and accurate methods for its quantification. Although several methods have been developed, none combines both simplicity and specificity [1,6,7]. Simple colorimetric methods (e.g. Davis test) are still widely used, but these methods are not sufficiently accurate [1,8,9]. Unfortunately, the more specific methods such as TLC or HPLC [5,10] are either laborious, insensitive, or require highly sophisticated instruments.

Radioimmunoassay methods have been shown to combine sensitivity, specificity, high sample capacity, speed and relative simplicity and therefore are becoming widely used for the quantification of plant secondary compounds. We have previously demonstrated that RIAs can be developed for the determination of flavanone 7-neohesperidosides [11,12] and report here on the development and application of a RIA for the quantitative determination of hesperidin.

RESULTS

General properties of the antiserum

Antisera produced against an hesperidin-oxime-BSA conjugate was able to reversibly bind a [3-H]-hesperidinol tracer. The synthesis of these derivatives is schematically shown on Fig. 1. At a 1600-fold dilution of the antiserum, 30% of the tracer was bound within 30 min of incubation at 25°; this binding was independent of pH over a range of 5.5–10.0. Unspecific binding of the tracer (ie., in the absence of anti-hesperidin antiserum) was 3%. From Scatchard plots [13] of tracer binding curves, a *Ka* of 3.9×10^7 L/mol has been calculated.

Sensitivity

Binding of the tracer to the antiserum was inhibited by increasing concentrations of hesperidin. Figure 2 shows a typical standard curve. The detection limit of this method, defined as that amount of hesperidin which can be distinguished from a zero sample at the 99.5% confidence limit, is 0.2 ng (0.3 pmol) of hesperidin/0.1 ml sample (0.3 nM). Thus, less than 2 ppb of hesperidin can be detected. The measuring range for the assay is 0.2 to 10 ng/0.1 ml sample.

Specificity

The specificity of the assay was tested by measuring the ability of various flavonoids to compete with the [3-H]-hesperidinol tracer for antibody binding sites. Per cent cross-reactivities were determined on a molar basis with pure compounds (Table 1) and calculated as in ref. [14].

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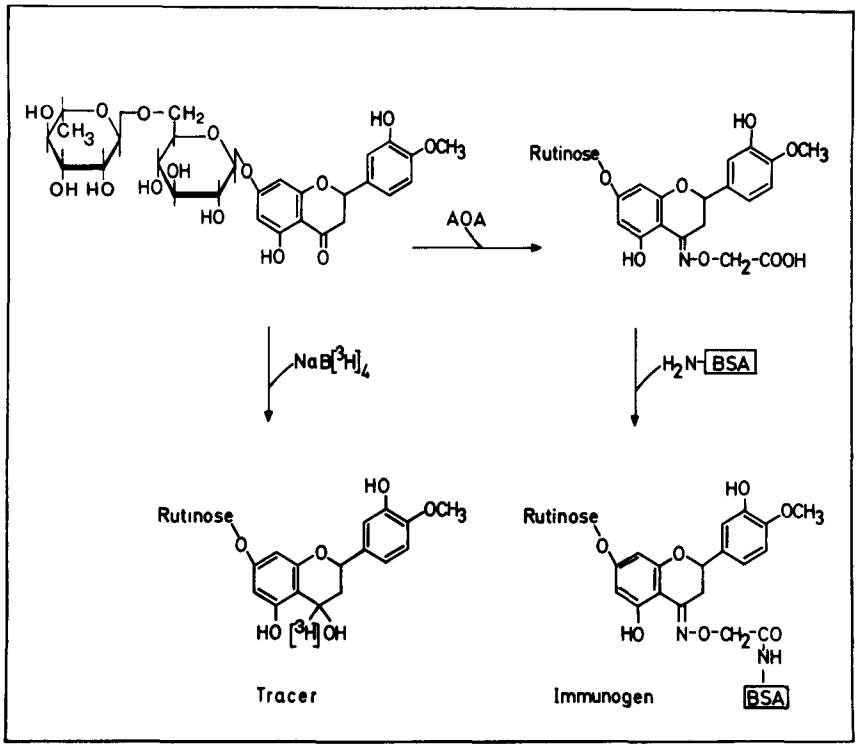

Fig. 1. Preparation of hesperidin-BSA conjugate and tritiated hesperidin tracer.

To demonstrate the applicability of this assay for the detection of hesperidin in crude extracts of orange leaves, albedo, juice, and seeds, these extracts were chromatographed by one-dimensional TLC. The chromatograms were cut into 5 mm strips, eluted with methanol, and aliquots diluted for immunoassay. In all cases, the immunoreactive material was confined to the area of the chromatogram corresponding to the R_f of hesperidin (Fig. 3). In addition, assay of serial dilutions of crude samples generated tracer displacement curves that were parallel to the hesperidin standard curve, further validat-

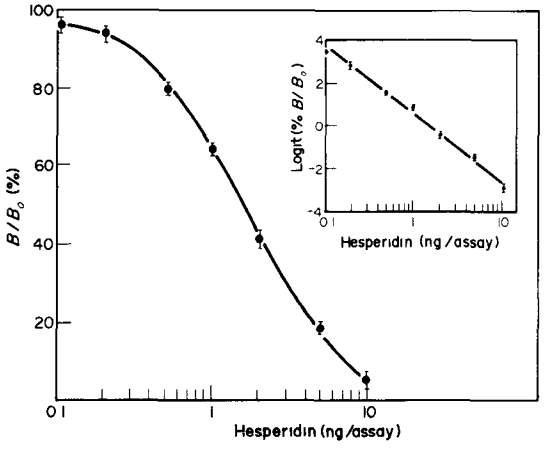

Fig. 2. Typical standard curve of hesperidin radioimmunoassay. Points are the average of triplicate determinations ± standard deviation. The insert shows the linear transformation of the standard curve using the logit plot. Logit $(\%B/B_0) = \ln [\% B/B_0)/100 - (\%B/B_0)]$.

Table 1. Comparison of cross-reactivities

Compound	% Cross-reactivity*
Hesperidin	100
Diosmin	48
Narirutin	29
Naringenin 7-robinobioside	24
Eriocitrin	20
Naringin	< 0.1
Hesperitin	< 0.1
Naringenin	< 0.1
Poncirin	< 0.1
Neohesperidin	< 0.1
Rhoifolin	< 0.1
Prunin	< 0.1
Rutin	< 0.1
Phloroacetophenone 4'-neohesperidoside	< 0.1
5,7,4'-Trihydroxyflavonone 7-(2-α-D mannopyranosyl-1-β-D-glucopyranoside	< 0.1
5,7,4'-Trihydroxyflavanone 7-(6'-*O*-methyl-*O*-α-D-mannopyranosyl-1-β-D-glucopyranoside)	< 0.1
Apiin	< 0.1
Quercetinn 3-rhamnoside	< 0.1
Morin	< 0.1

*Cross-reactivities are on a molar basis with hesperidin assigned as 100%.

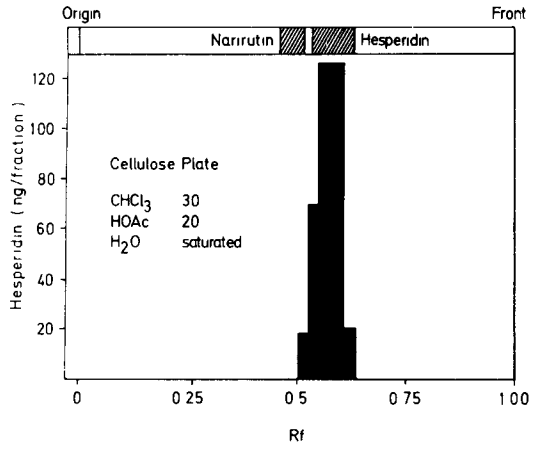

Fig. 3. Distribution of immunoreactive material on a thin-layer chromatogram of a Parson Brown orange leaf extract.

ing the use of this assay for the analysis of hesperidin in crude orange samples (Fig. 4).

Variability and recovery

Coefficients of variation of triplicate determination of each sample throughout the measuring range were 5.0 ± 2.1(standard deviation) and the inter-assay %cv (for 5 separate assays) was 10.8 ± 4.8. The recovery of hesperidin added to extracts prior to dilution was 95%.

Comparison of assay with HPLC

Analysis of 10 orange juice samples by HPLC and RIA showed good correlation [Y(HPLC) = 1.04 X(RIA) − 5 ppm; $r = 0.988$] indicating that there is no real difference in the measurement of hesperidin by these methods. However, during analysis of crude grapefruit (*Citrus paradisi*) samples, a poor correlation was found between HPLC and RIA values. This discrepancy was due to the high narirutin levels (5–10 fold higher than hesperidin) and the cross-reactivity of narirutin (naringenin 7-rutinoside) with the antibody (CR % = 29). The assay was therefore modified to measure both narirutin and hesperidin in these samples. The procedure required an initial chromatographic separation on cellulose TLC plates using chloroform–acetic acid–water (3:2: sat'd) as solvent. Crude methanol extracts of grapefruit were applied to a 5 × 20 cm plate and after development the plate was cut into 5 mm strips and eluted in methanol. The samples were then diluted with water and assayed. The values from the strips corresponding to authentic narirutin (R_f 0.4–0.5) were corrected for the % cross reactivity to obtain the actual levels of this flavanone.

Distribution of hesperidin in Citrus sinensis

The distribution and concentration of hesperidin within the different tissues of mature fruit, as measured by immunoassay, is shown in Table 2. The flavanone was present in high levels in the albedo, membranes, and pith whereas the concentration was much lower in the juice vesicles and seeds.

Analysis of leaves from a branch of a Parson Brown orange tree showed no significant difference between the total amount of hesperidin per young leaf (new flush) and the amount in older leaves (previous flush). The average hesperidin concentration, however, was 35% higher in the

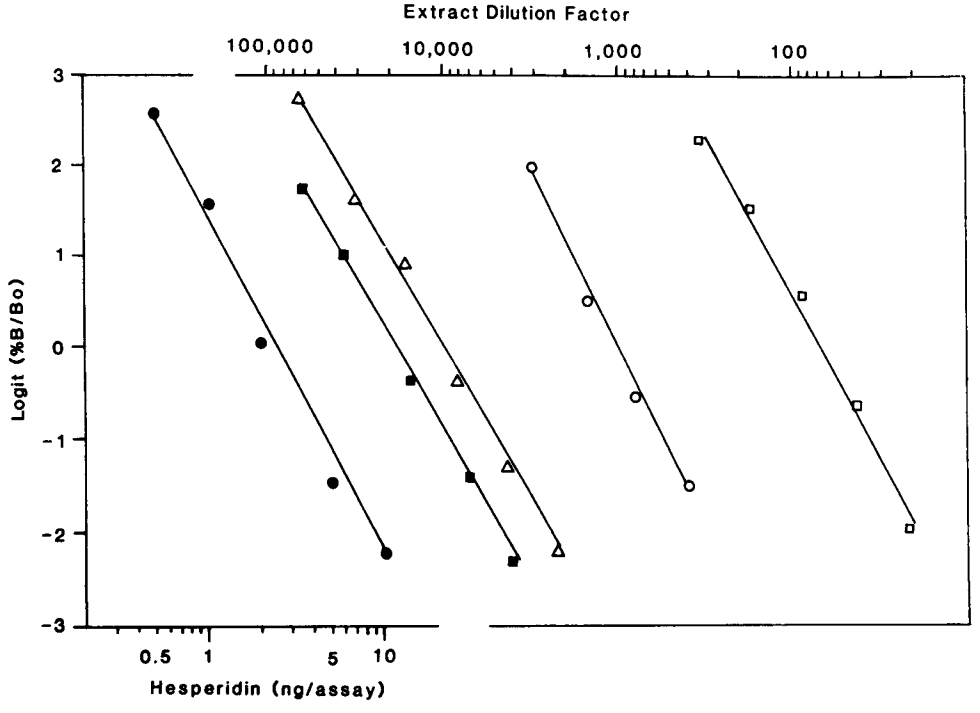

Fig. 4. Comparison of the immunoreactivity of serial dilutions from a standard hesperidin solution (●) and from various Parson Brown orange tissue extracts: (O) seed; (■) albedo; (□) outer seed integument; (Δ) leaf. All serial dilution curves from each of the different tissues and organs extracted were parallel to the dilution curve of the hesperidin standard. Logit $(\%B/B_0) = \ln [(\%B/B_0)/100 - (\%B/B_0)]$.

Table 2 Distribution of hesperidin in fruit and vegetative tissue of Parson Brown orange compared to the distribution of naringin in Duncan grapefruit

Tissue	X̄ PPM* Hesperidin†	s.d.	X̄ PPM naringin‡
Flavedo	4,177	523	4,882
Albedo	15,114	2,752	11,647
Juice vesicles	942	282	471
Membrane	8,005	1,719	7,412
Pith	5,924	1,911	9,647
Seeds	355	78	411
Young leaves	8,957	3,475	
Old leaves	5,776	3,386	

*PPM = μg/g fresh weight.
†Each value represents the average of three samples.
‡Calculated from ref. [14]

younger leaves (8957 ppm versus 5776 ppm in older leaves) due to their smaller size (Fig. 5).

In orange seeds the average hesperidin content per seed was 18.2 μg ± 2.6 (s.d.). The seed coat contained 93% of the total hesperidin and the remainder was found in the cotyledons and embryo (Table 3). The outer seed coat had both the highest amount and the highest concentration of hesperidin.

In light-grown seedlings, the average hesperidin content per seedling was 132.2 μg ± 26.4 (s.d.), a seven-fold increase over the ungerminated seed. The highest levels and concentration of hesperidin were found in the primary and young foliage leaves. After germination, the hesperidin content in the seed coats had decreased 21-fold while in the cotyledons it remained essentially unchanged. A representative seedling is shown in Fig. 6. In dark-grown seedlings, however, there was a net decrease in the total hesperidin when compared to the ungerminated seed (from 18.2 to 10.1 μg). The hesperidin content in the seed coat showed a decrease of 95%. Approximately 70% of the total hesperidin in the etiolated seedlings was found in the shoot and the plumules had the highest content. The distribution in a representative seedling is shown in Fig. 6.

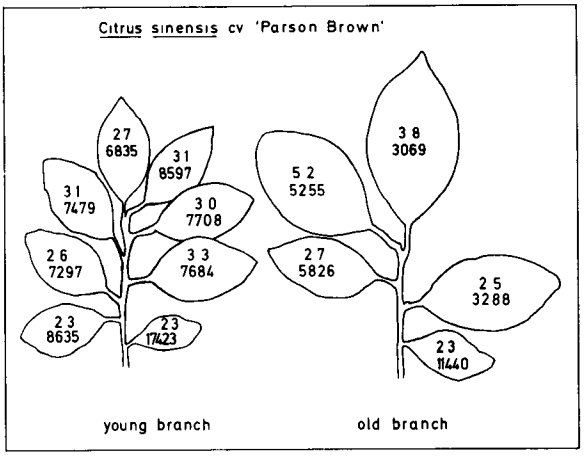

Fig. 5. Distribution of hesperidin in leaves of a new (flushing) branch compared to an old (previous flush) branch of a Parson Brown orange tree. The upper number is the mg hesperidin/leaf and the lower number represents the concentration of hesperidin in the leaves (μg/g fr. wt).

DISCUSSION

In this study we have described a radioimmunoassay system for the analysis of the flavanone glycoside, hesperidin and have demonstrated its applicability to the quantification of this compound in *C. sinensis*. The antibody produced against this compound is characterized by a titre which will permit the analysis of 2000 samples per ml of serum. The sensitivity of the assay (2 ppb) is by far the highest yet developed for this compound. This sensitivity is characteristic for the RIA and offers a series of advantages and potentials over conventional analytical methods.

The antiserum shows a primary specificity for the type of linkage in the disaccharide at position 7 of the flavonoid nucleus (Table 1). The 7-*O*-rutinosides (1 → 6 rhamnoglucosides) cross-react with the antiserum (e.g. hesperidin, 100%) whereas the 7-*O*-neohesperidosides (1 → 2 rhamnoglucosides) do not (e.g. naringin, <0.1%). In contrast, the antiserum does not differentiate between 1 → 6 rhamnoglucosides and 1 → 6 rhamnogalactosides as can be seen by the similar cross-reactivities for narirutin (naringenin 7-*O*-rutinoside, 29%) and naringenin 7-*O*-robinobioside (24%). The antiserum also shows some specificity towards the flavonoid nucleus. Changes in the substitution of the heterocycle (e.g. narirutin and eriocitrin) or in the oxidation state of the heterocycle (e.g. the flavone diosmin) cause a reduction in the ability of the compound to compete with the tracer for antiserum binding sites. Thus, extracts of tissues in which hesperidin is the major 7-rutinoside present (e.g. sweet orange) can be assayed directly. However, when more than one immunoreactive compound is present an initial separation step would be required for quantitative analysis of the different immunoreactive compounds. The major citrus rutinosides can be fractionated in a polyamide TLC system [5] and the rutinoside fractions quantitated with the RIA. By correcting the measured amounts with the appropriate cross-reactivity value, the concentration of all the major cross-reacting compounds can be determined.

The distribution of hesperidin in tissues of *Citrus sinensis* determined in this study is remarkably similar to that of naringin in *Citrus paradisi* [14, 15]. In seeds, the hesperidin content increased after germination suggesting that there is a net production of this compound in the developing seedling which is either directly or indirectly stimulated by light (cf. Table 3, Fig. 6). In leaves, the levels of hesperidin were similar in young and old leaves, however the concentration was higher in the younger ones due to the smaller size of the leaves. In fruit tissues, the distribution of hesperidin determined by RIA is in good agreement with that reported in the literature with the highest levels found in the albedo tissue [1]. It appears that the flavanone glycoside distribution may be almost identical in these two species of *Citrus* although the occurrence of the two compounds is for the most part mutually exclusive.

EXPERIMENTAL

Chemicals. Hesperidin (98% pure; Sigma) was recrystallized × 3 from 50% dimethylformamide (DMF). Aminooxyacetic acid, isobutyl chloroformate, tri-*n*-butylamine and bovine serum

Table 3. Distribution of hesperidin in Parson Brown orange seeds

Tissue	PPM	s.d.	Total μg	s.d.
Outer coat	296	57	14.5	2.5
Inner coat	178	12	2.4	0.1
Cotyledon and embryo	6	0.8	1.3	0.1

Each value is the average of five samples; PPM = μg/g fresh weight.

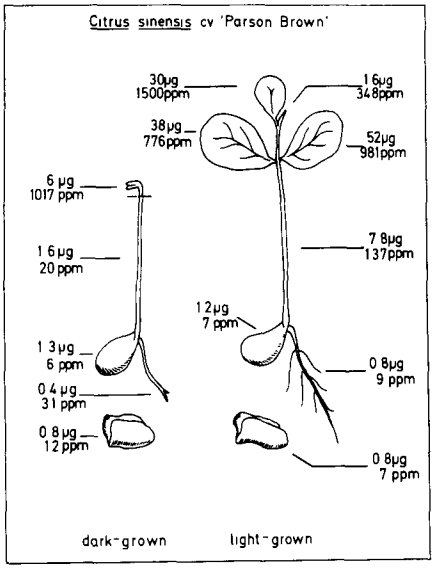

Fig. 6. Distribution of hesperidin in 4-week old dark-grown and light-grown orange seedlings. (PPM = μg/g fr. wt).

albumin (BSA) were also purchased from Sigma. NaB[3-H]$_4$ (no TRK. 45–109; sp. act. 25 Ci/mmol) was obtained from Amersham. Saturated $(NH_4)_2SO_4$ was prepared by stirring 800 g $(NH4)_2SO_4$ in 1 l H_2O overnight then filtering and adjusting to pH 7.0 Phosphate-buffered saline (PBS, 0 01 M K-Pi, 0.15 M NaCl, pH 7.4) was used as the RIA buffer The scintillation cocktail consisted of 5.5 g PPO, 0.1 g POPOP, 333 ml Triton X-100 (Sigma) and 667 ml xylenes (Mallinckrodt).

Synthesis of hesperidin 4-O-*carboxymethyl oxime.* 1.2 mmol (760 mg) hesperidin were refluxed with 3.9 mmol (500 mg) aminooxyacetic acid in EtOH–pyridine (1 : 1, 80 ml) for 2 hr. The solvent was removed *in vacuo* and the residue taken up in a minimum vol of MeOH. The sample was chromatographed on Whatman (no. 3) paper and developed in *t*-butyl alcohol–HOAc–HOH (3 : 1 : 1). A dark purple band visible under UV (366 nm), R_f 0.5 (hesperidin R_f 0.65) was eluted with MeOH. The MeOH was evaporated to an oily residue (*ca* 380 mg) was taken up in 5 ml dry DMF and stored over P_2O_5 under red. pres Acid hydrolysis of the hesperidin derivative yielded rhamnose and glucose and the UV spectrum of the aglycone in MeOH showed absorption maxima of 345 and 290 nm. Treatment of the sample with NaOH did not give the characteristic flavanone shift in absorption due to chalcone formation. The IR spectrum of the product contained a major band 1730 cm^{-1} typical of ester carbonyls.

Synthesis of hesperidin oxime-BSA conjugate. 0.2 ml (15 mg) of the hesperidin 4-*O*-carboxymethyl oxime in DMF were mixed with 10 μl (40 μmol) tri-*n*-butylamine. After the soln was cooled to $-10°$ on a salt-ice bath, 10 μl (73 μmol), isobutylchloroformate added and the reaction mixture stirred for 20 min. 100 μl aliquots of the activated oxime solution were added over a period of 2 min to a stirred soln of 140 mg BSA dissolved in 7 ml H_2O–DMF (1 : 1), containing 150 μl 1 N NaOH. The mixture was stirred for 3 h, dialysed against 4 × 5 l H_2O and finally lyophilized giving 110 mg of conjugate. From a spectrophotometric analysis in conc H_2SO_4, a coupling ratio of 15 mol hesperidin per mol of protein was estimated.

Immunization. One randomly bred rabbit (10 wk old) was immunized with an emulsified mixture of the conjugate and Freund's complete adjuvant (1 : 1) after the procedure of ref [12]. The rabbit was bled twice monthly; the serum was separated from the whole cells by centrifugation and stored at $-18°$.

Preparation of 3H-*hesperidinol.* Hesperidin 5 μmol (3.1 mg) was dissolved in 0.5 ml EtOH–DMF (2 : 3) and 3 μmol (0.1 mg) of NaB[3H]4 was added. After 30 min room temp. the reaction was stopped by the addition of 0.5 ml 10% HOAc. This preparation was stored $-18°$. An aliquot of this stock was purified as needed by TLC (silica gel; solvent system $CHCl_3$–HOAc–H_2O, 3 : 2 : sat'd). The major radioactive band (R_f 0.22) was scraped, eluted with MeOH, and stored at $-18°$. The stock tracer is stable for at least 2 yr and the purified tracer is stable for at least 2 months.

Plant material. Leaves and mature fruit of a *Citrus sinensis* Osbeck cv 'Parson Brown' were obtained from a tree grown at Florida Southern College, Lakeland, Florida. One cm thick slices from the equatorial section of 5 fruit were cut and sepd into the various tissue components (c.f. Table 2) for extraction Seeds removed from five other fruit were pooled and 5 seeds were analysed for total hesperidin while others were separated into inner and outer seed coats and cotyledons/embryo. The remaining seeds were sown in soil with half kept in darkness and half under continuous illumination for one month. After 4 weeks, 10 seedlings from each light condition were analysed for hesperidin. Each plant part was extracted and assayed separately. Samples were refluxed in MeOH (1 ml for each 50 mg fr. wt of tissue) for 2 hr at 55°. Extracts were diluted 1 : 1 with DMF and stored at $-18°$ until assayed. Appropriate dilutions were made with H_2O (5–5000 fold) and assayed directly.

Radioimmunoassay procedure and data calculation The procedure followed for the hesperidin RIA was identical to that for the naringin RIA reported elsewhere [11]. Briefly, each assay tube consisted of the following components added in order: 0 1 ml standard hesperidin or unknown sample; 0.7 ml RIA mix (0.5 ml PBS, 0.1 ml 10-fold diluted calf serum, 0.1 ml (10000 cpm) [3-H]-hexperidinol); and 0.1 ml 200-fold diluted antiserum (or 0.1 ml HOH for unspecific binding). Hesperidin standards were assayed in triplicate while unknown samples were assayed either in duplicate or triplicate. After mixing, the tubes were incubated at room temp for 30 min and the immune complex was pptd by

addition of 1 ml 91% sat. $(NH_4)_2SO_4$ pH 7.0. The ppts were sedimented by centrifugation, washed with 1 ml 50% sat. $(NH_4)_2SO_4$, centrifuged, and the pellets dissolved in 0.15 ml H_2O. After addition of 1 ml scintillation cocktail, the tubes were counted 2 min. Calculations were performed on a North-Star Horizon computer system using a cubic polynomial least squares approximation (Mansell and K. Schweiker, unpublished).

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