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# Radioimmunoassay for Naringin and Related Flavanone 7-Neohesperidosides Using a Tritiated Tracer

Pablo S. Jourdan,[1] Elmar W. Weiler, and Richard L. Mansell*

A radioimmunoassay (RIA) that can be used for the quantification of naringin in grapefruit tissues is described. The assay utilizes antisera raised against a naringin 4-*O*-(carboxymethyl)oxime hapten. The tracer used was a $^3H$ derivative of naringin that was stable for at least 8–12 months. The detection limit of the assay is 0.2 ng or 2 ppb of naringin. Only flavanone 7-neohesperidosides are detected by the assay and naringin is the most reactive compound; the isomeric 7-rutinosides do not interfere. The RIA has a high sample throughput and requires only dilution of crude extracts. The reliability and reproducibility of the assay are demonstrated by intra- and interassay variability of 2.3% and 5.0% cv, respectively.

Naringin (5,7,4′-trihydroxyflavanone 7-*O*-β-neohesperidoside) is an intensely bitter compound that occurs in the Rutaceae, primarily in *Citrus paradisi* Macf. (grapefruit), *Citrus grandis* Osbeck (pummelo), *Citrus aurantium* L. (sour orange), *Poncirus trifoliata* L. (Raf.) (trifoliate orange), and *Fortunella margarita* Swing (kumquat) (Horowitz and Gentili, 1977). In the processing of citrus fruits, various juice parameters are routinely measured in order to maintain proper quality control (McAllister, 1980), and it is therefore important to be able to accurately monitor the levels of bitter principles (Attaway, 1977). The most widely used method for naringin quantification is the Davis test (McAllister, 1980), which is a very simple and inexpensive technique. Unfortunately, this test is highly unspecific since it does not discriminate between other flavanone 7-neohesperidosides and the nonbitter, isomeric flavanone 7-rutinosides. A highly specific HPLC method for the detection and quantification of naringin and related bitter flavanone 7-neohesperidosides has been developed (Fisher and Wheaton, 1976), but this technique is limited because of high cost, complexity of instrumentation, and, most importantly, small sample throughput—making it impractical for routine analysis of large numbers of samples. Thus, a rapid, simple, and specific procedure to measure naringin would be of potential importance to citrus processors.

Recently we reported the development of a $^{125}I$-based radioimmunoassay (RIA) for naringin and related flavonoid neohesperidosides (Jourdan et al., 1982). In the present paper we report on an improved RIA for naringin that uses a tritiated tracer of long shelf life and is characterized by greater selectivity and sensitivity than the $^{125}I$ RIA. The assay is being used routinely in our laboratory for the analysis of naringin in citrus juices and fruits.

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## MATERIALS AND METHODS

**Chemicals.** Naringin and narirutin (naringenin 7-*O*-rutinoside) were a gift from Dr. James Fisher, Florida Department of Citrus. Fortunellin, poncirin, and neohesperidin were kindly provided by Prof. Dr. H. Wagner, Munich. Phloroacetophenone 4-neohesperidoside was prepared by base hydrolysis of naringin after the method of Horowitz and Gentili (1961). Prunin was prepared by specific hydrolysis of naringin using naringinase (Sigma), following the methods of Versteeg et al. (1977). Other compounds tested for cross-reactivity (cf. Table I) were purchased from Roth, Karlsruhe, FRG. (Aminooxy)acetic acid and 5-aminolevulinic acid were purchased from Sigma, St. Louis, MO. Calf serum was supplied by Mediapharm, Aschaffenburg, FRG, and bovine serum albumin (BSA) was purchased from Serva, Heildeberg, FRG. Sodium [$^3H$]borohydride, specific activity 50–60 Ci/mmol, came from New England Nuclear, Boston, MA. Phosphate-buffered saline (PBS, 0.01 M phosphate, 0.15 M NaCl, pH 7.4) was used as the RIA buffer. Saturated ammonium sulfate solution was prepared by stirring 800 g of ammonium sulfate and 1000 mL of water overnight, filtering, and adjusting the pH to 7.0 with 4 N NaOH. Naringin standards were prepared from a stock solution of 1 g/mL in MeOH which was stored at −18 °C; serial dilutions were done with water and the standards were stored at 4 °C. Under these conditions, they remained stable for 2 weeks. The scintillation cocktail was Minisolve (Zinsser), and all other solvents were of the highest purity available.

**Synthesis of Naringin 4-*O*-(Carboxymethyl)oxime.** The oxime of naringin was prepared by refluxing 500 mg

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of naringin and 300 mg of (aminooxy)acetic acid for 3 h in 20 mL of absolute EtOH and 20 mL of dry pyridine. The solution was subsequently dried repeatedly in vacuo at 35 °C to remove the pyridine. The yellow oily residue was dissolved in a minimum volume of warm water and the solution was stored at 4 °C. After 3 days, white needles formed, and these were collected by filtration, washed in cold water, and dried over $P_2O_5$. An aliquot of the derivative was dissolved in warm dimethylformamide (DMF) and chromatographed on silica gel plates (Polygram Sil G/$UV_{254}$, Macherey-Nagel & Co., Duren) by using chloroform–acetic acid–water (3:2:saturated, CAW) as the solvent system; a single band of $R_f$ = 0.25 (naringin $R_f$ = 0.36) that fluoresced light blue under 366-nm light was detected. A total of 400 mg of the oxime was recovered. To determine that a free reactive carboxyl group was present, [$^{14}$C]tyramine was reacted with the oxime by the mixed anhydride procedure (see below). A new radioactive band of $R_f$ = 0.33 (naringin oxime $R_f$ = 0.25; tyramine $R_f$ = 0.49) was detected on silica gel chromatograms developed in CAW. The new product showed quenching of the fluorescence indicator on the plates.

Treatment of naringin 4-*O*-(carboxymethyl)oxime with naringinase (Sigma) after the method of Versteeg et al. (1977) resulted in a compound with properties identical to a naringenin 4-*O*-(carboxymethyl)oxime prepared in the same way as the naringin derivative. Acid hydrolysis of naringin 4-*O*-(carboxymethyl)oxime yielded glucose and rhamnose. The naringenin 4-*O*-(carboxymethyl)oxime methyl ester was prepared from the free acid by treatment with ethereal diazomethane: MS $M^+$ *m/e* for the methyl ester 359 (calculated for $C_{18}H_{17}O_7N_1$, 358.55). Naringin 4-*O*-(carboxymethyl)oxime: white needles; mp 199–201 °C dec; IR (C=N) 1630 $cm^{-1}$.

**Coupling of Naringin 4-*O*-(Carboxymethyl)oxime to BSA.** A total of 100 mg of naringin oxime was dissolved in 2 mL of DMF; 30 μL (120 μmol) of tri-*n*-butylamine and 30 μL (220 μmol) of isobutyl chlorocarbonate were added, and the solution was cooled on ice. After 20 min, this solution was added to a rapidly stirring mixture of 140 mg of BSA, 3.5 mL of water, 3.5 mL of DMF, and 0.14 mL of 1 N NaOH. The stirring was continued for 1 h and then 70 μL of 1 N NaOH was added. After 3 more h, the solution was dialyzed against 5 × 10 L of water for 5 days and then lyophylized. From spectrophotometric analysis, a coupling ratio of 21 mol of naringin oxime/mol of BSA was determined.

**Preparation of Anti-Naringin Antibodies.** Three randomly bred rabbits received weekly intradermal injections of an emulsified mixture of Freund's Complete Adjuvant (FCA, Difco) and 500 μg of naringin oxime–BSA for 1 month. This preimmunization was followed by a monthly intramuscular boost with 1 mg of the naringin conjugate emulsified in FCA. Blood was collected for 2 weeks after the boost; the serum was isolated and stored at −18 °C.

**Synthesis of 5-[Naringin-4-ylidine(aminooxy)acetamido]levulinic Acid (I) [CA Nomenclature for I = 5-[[[[(2*S*)-7-[[[2-*O*-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxyl]oxy]-2,3-dihydro-5-hydroxy-2-(*p*-hydroxyphenyl)-4*H*-1-benzopyran-4-ylidene]-amino]oxy]acetamido]levulinic Acid].** The 5-aminolevulinic acid derivative of naringin was prepared by a modification of the mixed anhydride procedure: 25 mg (38 μmol) of naringin 4-*O*-(carboxymethyl)oxime was first dissolved in 0.4 mL of DMF. After 10 μL (40 μmol) of tri-*n*-butylamine was added and this was cooled to 0 °C, activation of the carboxyl groups was achieved by reaction with 5 μL (37 μmol) of isobutyl chlorocarbonate for 30 min. The activated oxime solution was then mixed with 20 mg (120 μmol) of 5-aminolevulinic acid dissolved in a solution of tri-*n*-butylamine–DMF (10% v/v). The coupling of carboxyl and amino groups was allowed to proceed at 4 °C for 24 h, and the major product was isolated by chromatography on silica gel plates developed in CAW [$R_f$ of naringin 4-*O*-(carboxymethyl)oxime = 0.28; $R_f$ of major product = 0.38]. The product, I, was eluted from the plates with methanol and dried thoroughly in vacuo over $P_2O_5$. A total of 5 mg of I was recovered.

**Reduction of I To Yield II [CA Nomenclature for II = 5-[[[[(2*S*)-7-[[2-*O*-6-Hydroxyl-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-2,3-dihydro-5-hydroxy-2-(*p*-hydroxyphenyl)-4*H*-1-benzopyran-4-ylidene]amino]oxy]acetamido]-4-hydroxyvaleric Acid.** A total of 1.5 mg (ca. 2 μmol) of I was dissolved in 300 μL of absolute EtOH that had been redistilled over $NaBH_4$. To this solution, 200 μL of Na[$^3$H]$_4$ (ca. 80 mCi, specific activity 50–60 Ci/mmol) was added; the mixture was allowed to react on ice for 30 min. The reaction was stopped by addition of 3 drops of 10% HOAc in MeOH. The entire solution was chromatographed on silica gel plates developed twice in $CHCl_3$–acetone–MeOH (20:6:5). The major radioactivity peak ($R_f$ = 0.15; $R_f$ of unreduced compound = 0.20) was cut out and eluted with MeOH. The solution was stored at −18 °C under a nitrogen atmosphere. The specific activity of the product was estimated at 10 Ci/mmol by the self-displacement method (Chervu and Murty, 1975).

**Radioimmunoassay.** The immunoassay procedure was essentially as described previously (Weiler and Mansell, 1980). Briefly, each assay tube consisted of 0.5 mL of PBS, 0.1 mL of tracer (4.9 pmol of [$^3$H]naringin derivative, ca. 15000 cpm at 14% counting efficiency), 0.1 mL of 5-fold diluted calf serum, 0.1 mL of sample, and 0.1 mL of antiserum (or 0.1 mL of PBS for unspecific binding determination). Samples were assayed in either duplicate or triplicate. The tubes were incubated at 4 °C for 2 h or alternatively at room temperature for 1 h, and the antigen–antibody complexes precipitated by addition of 1 mL of 91% saturated ammonium sulfate. After 30 min at room temperature, the tubes were centrifuged at 4000*g* for 15 min; the supernatants were decanted and the pellets were washed once with 49% ammonium sulfate. The tubes were again centrifuged and the resulting pellets were dissolved in 0.25 mL of water and then mixed with 1 mL of scintillation cocktail. All radioactivity determinations were done in a refrigerated counter (Berthold BF 5000) and each tube was counted for 2 min.

**Extraction of Plant Material.** A total of 100–800 mg of tissue was extracted in 5 mL of either 0.1 M Tris, pH 8.0, or MeOH and boiled for 30 min. The tissue was then macerated with a glass rod and boiled for another 30 min. The extracts were decanted and the solutions brought to a known volume; dilutions were then done in water. For the thin-layer chromatographic analysis of juices and extracts, the undiluted samples were applied to 20 × 20 cm precoated plastic sheets of silica gel and developed in the indicated solvent systems. After development, 0.5-cm sections of the entire plate were cut out and extracted in 1 mL of MeOH overnight. The samples were then diluted with water and assayed.

## RESULTS

**Properties of the Antiserum and Conditions for Assay.** The synthetic scheme for both hapten and tritiated tracer is summarized in Figure 1. The derivatization procedure of naringin was chosen to maintain a stereo-

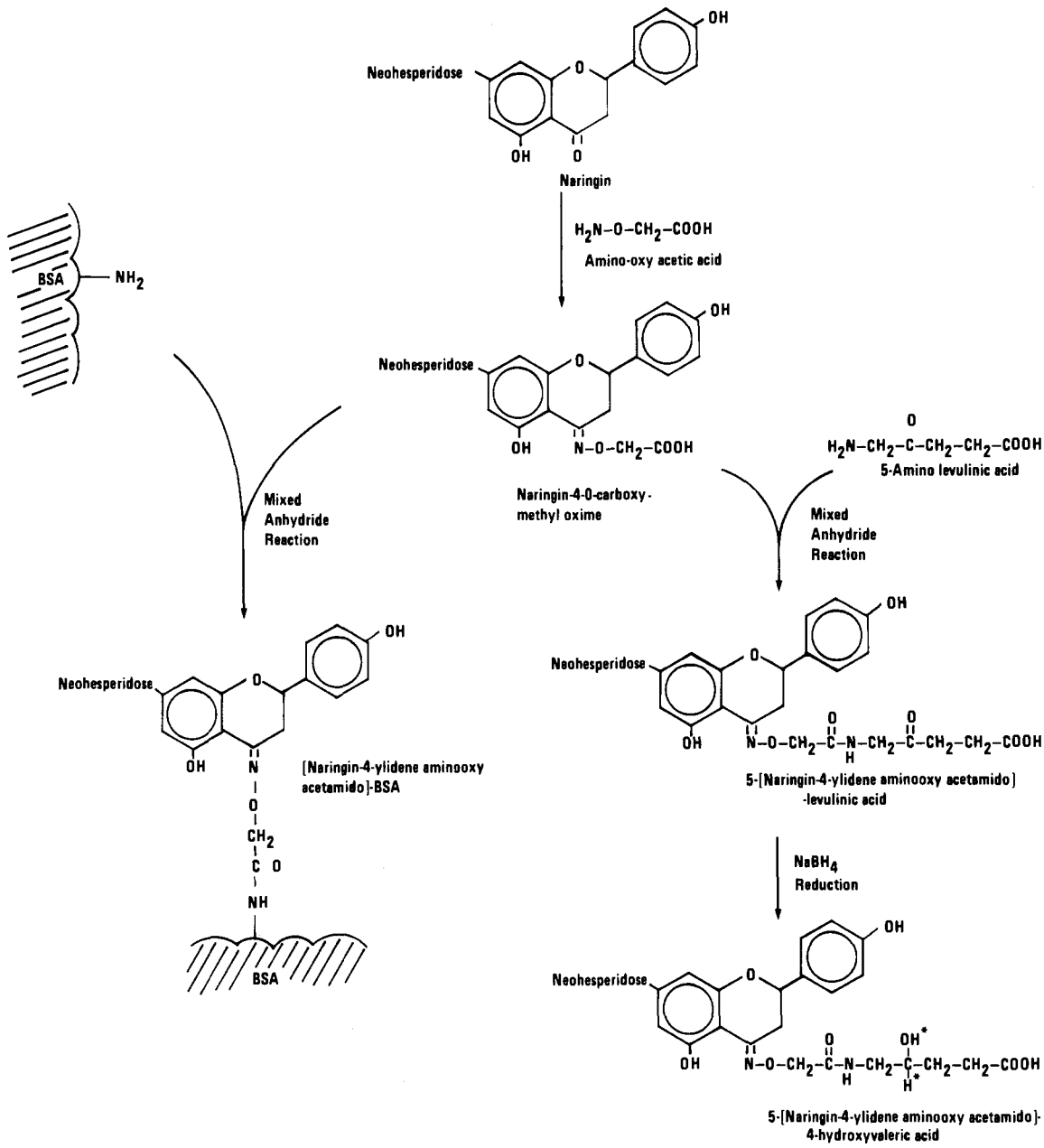



**Figure 1.** Synthetic scheme and structures of the naringin 4-*O*-(carboxymethyl)oxime–BSA conjugate used for immunization and the [$^3$H]naringin derivative used as a tracer. The asterisks denote possible sites of label incorporation in the reduction step (see Materials and Methods).

chemistry of the new compound similar to that of the parent molecule. The immunogen, naringin 4-*O*-(carboxymethyl)oxime–BSA, when administered to rabbits, elicited antibody production. Antisera from a single animal was characterized in this study; however, all rabbits immunized produced sera that bound the tritiated naringin derivative. Under the assay conditions employed, the antiserum bound 2.4 pmol (ca. 7500 cpm) of [$^3$H]naringin derivative at a final dilution of 1:9000. From a Scatchard plot of binding data, a maximum affinity constant of $K_a = 2.4 \times 10^9$ L/mol was calculated.

The antigen–antibody reaction reached maximum association within 15–20 min at room temperature at 30–40 min at 4 °C. The binding of tritiated tracer was independent of pH in the range of pH 6.0–9.5. For all subsequent work, phosphate-buffered saline at pH 7.4 was used. Complete precipitation of the antigen–antibody complex was achieved at 49% saturation of ammonium sulfate. Under these conditions, unspecific binding was 1.5%.

**Assay Sensitivity and Specificity.** The binding of tritiated tracer to the antibodies was inhibited by the addition of increasing amounts of naringin standard. A typical standard curve is shown in Figure 2. The assay has a measuring range of 5–500 ppb (0.9–90 pmol) as determined from the logit linear transformation of the standard curve data. The detection limit of the assay (at the 99.5% confidence limit) is 2 ppb (0.3 pmol).

The specificity of the naringin antibodies was determined by measuring the inhibition of tracer binding in the presence of increasing amounts of potential cross-reacting compound. Various flavonoid compounds were tested for cross-reactivity, and the results are summarized in Table I. It is evident that the antiserum shows marked specificity for the dissaccharide linkage at position C7 of flavonoids. Neohesperidosides of flavanones, flavones, and phloroacetophenone were highly immunoreactive while rutinosides, glucosides, apiosides, and aglycons were insignificantly, if at all, immunoreactive (see Figure 3). The antiserum also shows a limited specificity for the aromatic ring structure of the flavonoid since flavanone neohesperidosides reacted more strongly than the corresponding flavone neohesperidosides.

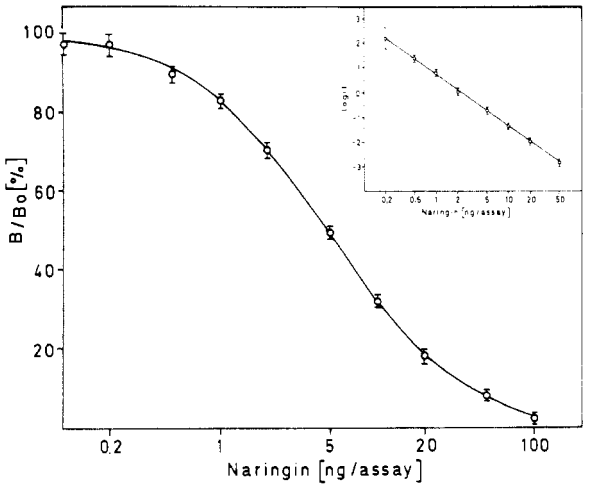



**Figure 2.** Typical standard curve for naringin radioimmunoassay using the [$^3$H]naringin derivative as the tracer. The bars indicate standard deviations for triplicate samples. $B$ = binding of the [$^3$H]naringin derivative to antibodies in the presence of unlabeled naringin; $B_0$ = binding in the absence of unlabeled naringin. 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 I. Cross-Reactivity of Various Compounds Structurally Related to Naringin with the Antiserum

| compound | pmol needed for 50% displacement of the tracer | % cross-reactivity |
|---|---|---|
| naringin | 4.8 | 100 |
| poncirin | 6.7 | 71 |
| neohesperidin | 8.4 | 57 |
| 4-phloroacetophenone neohesperidoside | 5.9 | 82 |
| fortunellin | 34.0 | 8 |
| rhoifolin | 18.0 | 27 |
| narirutin | 2400 | 0.2 |
| hesperidin | >>2500 | <0.1 |
| apiin | >>2500 | <0.1 |
| prunin | 230 | 2.0 |
| apigenin 7-glucoside | >>2500 | <0.1 |
| rutin | >>2500 | <0.1 |
| naringenin | 300 | 1.6 |
| dihydroquercetin | >>2500 | <0.1 |

Further evidence for the specificity of the antiserum is afforded by the distribution of immunoreactive compounds on a thin-layer chromatogram of a grapefruit juice. Figure 4 shows that the bulk of immunoreactive material comigrates with naringin. The other flavanone 7-neohesperidosides generally present in grapefruit (Rouseff, 1980) are either absent or in very small amounts in this sample. Similar analysis of grapefruit leaf, seedling, seed, and whole fruit extracts also showed that the only immunoreactive peak is associated with the area of the plate having $R_f$ values identical with that of the naringin reference. In addition, acid hydrolysis of crude extracts of leaf and fruit, which would cleave off the saccharide linkage in flavonoids, resulted in complete loss of immunoreactivity in the extracts. However, base hydrolysis did not result in a significant reduction of immunoreactive material. It is known that treatment of flavanone 7-*O*-neohesperidosides with base yields the corresponding phloroacetophenone 4′-neohesperidoside (Horowitz and Gentili, 1961). This compound can react with the antinaringin antibodies. The presence of an immunoreactive product distinct from naringin in base-hydrolyzed extracts was confirmed by cellulose thin-layer chromatography (using CAW as the solvent system), which showed a new band at $R_f$ = 0.38 (naringin $R_f$ = 0.51). Furthermore, it was evident that the hydrolysis had been complete since all the immunoreactive material in the extracts was confined to the TLC band of lower $R_f$.

Table II. Summary of Assay Characteristics

| | |
|---|---|
| measuring range | |
| ng | 0.5–50 |
| pmol | 0.9–90 |
| ppb | 5–500 |
| detection limit | |
| ng | 0.2 |
| pmol | 0.3 |
| ppb | 2 |
| precision | |
| within-assay cv, %, ± SD | 2.3 ± 1.3 |
| between-assay cv, %, ± SD | 5.0 ± 1.4 |
| unspecific binding, % of total act. ± SD | 1.5 ± 0.8 |
| recovery, % ± SD | 96 ± 2.3 |
| antiserum dilution for 50% $B$ (titer)[a] | 1:9000 |
| optimum pH range | pH 6.5–9.0 assays at pH 7.4 |

[a] The titer is the dilution of antiserum that will bind 50% of the tracer under the specified assay condition.

Table III. Comparison of the Basic Parameters Characteristic of the $^{125}$I and $^3$H RIA's

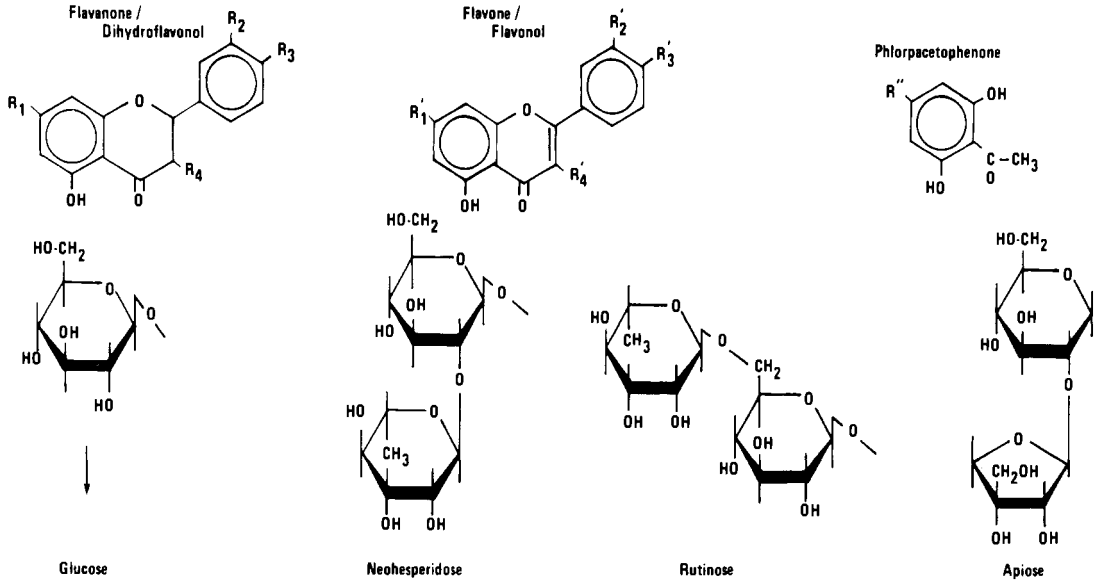
| assay parameter | [$^{125}$I]naringin | [$^3$H]naringin |
|---|---|---|
| antiserum titer[a] | 1:900 (30% $B$) | 1:9000 (50% $B$) |
| measuring range, pmol/0.1 mL | 34–340 | 0.9–90 |
| detection limit, pmol/0.1 mL | 34 | 0.5 |
| unspecific binding, % | 7 | 1.5 |
| naringin recovery | 98 | 96 |
| specificity | flavonoid 7-neohesperidoside | flavanone 7-neohesperidoside |

[a] The titer is the dilution of antiserum required to bind a given percentage (30% or 50%) of the tracer under defined reaction conditions.

**Assay Variability and Recovery.** The precision and reproducibility of the naringin radioimmunoassay are characterized by a within-assay coefficient of variation for triplicate values of 2.3 ± 1.3% SD ($n$ = 11) and by between assay variations of 5.0 ± 1.4% SD (for 15 samples assayed 5 times). Naringin added to juices or fruit extracts was recovered at approximately 96%. Serial dilutions of extracts or juices resulted in an inhibition of a tracer binding curve that was parallel to the naringin standard curve. Table II summarizes the various assay characteristics.

**Comparison of $^{125}$I Radioimmunoassay with $^3$H Radioimmunoassay.** The first reported immunoassay for naringin utilized a [$^{125}$I]naringin as a tracer and antibodies that had been raised against a naringin hapten coupled to BSA via the B ring (Jourdan et al., 1982). The modifications of the naringin immunoassay presented in this paper resulted in a significant improvement in the titer of antiserum, limits of assay detection, and measuring range. The basic parameters of both immunoassay systems are summarized in Table III.

**Naringin Content of Grapefruit Juice and Distribution in Fruit.** Fifteen commercial grapefruit juices obtained from local markets in Germany were analyzed for naringin content. The juices consisted of a variety of grapefruits and were from concentrate as well as single strength. The juices were diluted 5000–10000-fold before assay. The concentration of naringin ranged from 200 to 700 ppm with the average being 446 ± 135 ppm. This range coincides with the "slightly bitter" to "bitter" taste classification for naringin concentration (Maier et al., 1977).




Flavanones and Dihydroflavonols

| Compound | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| Naringin | neohesperidose | H | OH | H |
| Poncirin | neohesperidose | H | $OCH_3$ | H |
| Neohesperidin | neohesperidose | OH | $OCH_3$ | H |
| Narirutin | rutinose | H | OH | H |
| Hesperidin | rutinose | OH | $OCH_3$ | H |
| Prunin | glucose | H | OH | H |
| Naringenin | OH | H | OH | H |
| Dihydroquercetin | OH | OH | OH | OH |

Flavones and Flavonols

| Compound | $R_1'$ | $R_2'$ | $R_3'$ | $R_4'$ |
|---|---|---|---|---|
| Fortunellin | neohesperidose | H | $OCH_3$ | H |
| Rhoifolin | neohesperidose | H | OH | H |
| Apiin | apiose | H | OH | H |
| Apigenin-7-glucoside | glucose | H | OH | H |
| Rutin | OH | OH | OH | rutinose |

Phloroacetophenones

| Compound | $R''$ |
|---|---|
| Phloroacetophenone 4-neohesperidoside | neohesperidose |

**Figure 3.** Structures of the compounds used to test for specificity of the antiserum.

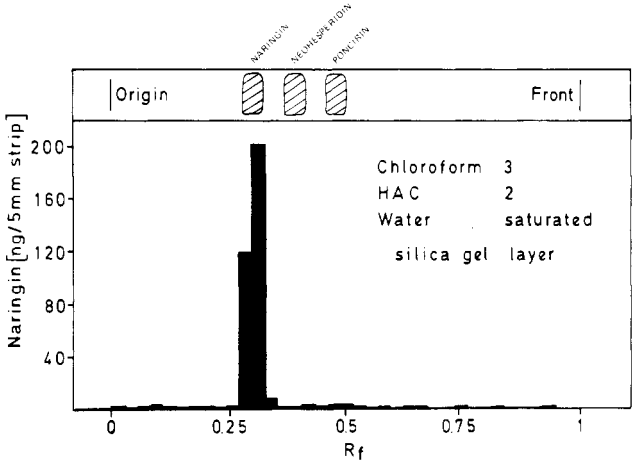



**Figure 4.** Distribution of immunoreactive material on a thin-layer chromatogram of a grapefruit juice.

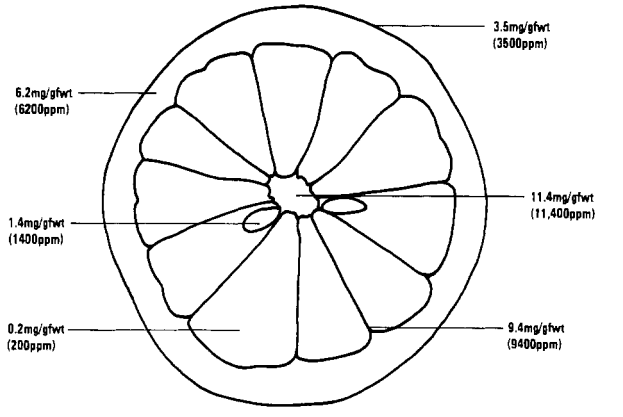



**Figure 5.** Distribution of naringin in a 1 cm thick cross section of a Ruby Red grapefruit. gfwt = gram fresh weight.

During a large-scale analysis of naringin levels in grapefruit juice samples from three Florida juice processing plants, it was found that the naringin levels had a range of 6–2115 ppm. The average value was 412 ± 149 ppm SD ($n$ = 6685) (Mansell et al., 1983).

To determine if the measurement of naringin by RIA compared favorably with another method of naringin analysis, 19 grapefruit juices were analyzed for naringin content by both RIA and HPLC. The determinations showed good correlation ($r$ = 0.92, $y$ = 0.8$x$ + 123 ppm) although RIA values were on the average 15% higher than HPLC values. Studies are under way to further analyze this difference.

The distribution of naringin within grapefruit tissues was also examined (Figure 5). A 1 cm thick cross section was cut from the middle of a Ruby Red grapefruit, and the individual tissues were dissected, extracted, and analyzed for naringin content. It was found that the pith contained the highest amount of naringin (11.9 μg/mg fresh weight), followed by the membranes, albedo, flavedo,

seeds, and juice vesicles (0.2 μg/mg fresh weight). Leaves from a grapefruit tree were analyzed as well, and the highest levels, up to 27.3 μg of naringin/mg fresh weight (2.7% g fresh weight) were found in young tissues. In contrast, older leaves contained only one-seventh as much naringin (4 μg/mg fresh weight). Other citrus fruits such as lemon and orange were virtually devoid of immunoreactive material.

## DISCUSSION

In a recent publication (Jourdan et al., 1982) we reported the development of a radioimmunoassay for naringin and related flavonoid neohesperidosides. This previous assay utilized antisera directed against a naringin hapten that had been coupled to protein through the flavonoid B ring and a tracer molecule of naringin that had been directly iodinated. The $^{125}I$ RIA proved to be very applicable to the analysis of flavonoid 7-neohesperidosides in citrus tissues; however, a few limitations were noted: (1) The tracer had a relatively short shelf life (2–3 months), which required frequent synthesis and sophisticated handling facilities. (2) The strong radioactive emission of $^{125}I$ presents some potential health hazards and requires highly trained personnel for handling. (3) The sensitivity of the assay did not permit the routine analysis of flavonoid 7-neohesperidosides in tissues with low levels of the compounds (e.g., tissue cultures). (4) The antibody specificity was too broad since it was unable to distinguish between flavanone and flavone neohesperidosides.

We have now developed a RIA for naringin that utilizes new derivatives for the production of antiserum and tracer. The position on the naringin molecule chosen for derivatization was the carbonyl group at C4. The synthesis of a 4-*O*-(carboxymethyl)oxime derivative introduced a carboxyl group that could be coupled to amino residues on proteins as well as the 5-amino group of 5-aminolevulinic acid. The latter derivative can serve as a tracer when the keto group at γ-carbon of levulinic acid is reduced with tritiated sodium borohydride (cf. Figure 1). Moreover, the naringin residues of the new derivatives maintained a stereochemistry similar to that of the naturally occurring naringin.

The production of a [$^3H$]naringin derivative resulted in a tracer of long shelf life (at least 8–12 months). The antiserum raised against the 4-*O*-(carboxymethyl)oxime had a 10-fold higher titer than the previous antisera, and the sensitivity of the assay was increased by approximately 70-fold (cf. Table III). The specificity of the assay was also enhanced with the new combination of antiserum and tracer. Although all 7-neohesperidosides of flavonoids tested reacted to some extent, there was a significantly lower cross-reactivity with flavone 7-neohesperidosides. Some selectivity for the flavanone ring structure is evident since poncirin and neohesperidin are less reactive than naringin. Since the RIA does not have absolute specificity for naringin, samples containing significant amounts of other flavanone 7-neohesperidosides may yield unreliable results. Thus, if the flavanone composition of the tissues being analyzed is not already known, a preliminary chromatographic analysis may be required. A simple chromatogram as shown on Figure 4 would indicate the presence of other flavanone 7-neohesperidosides. However, the major varieties of grapefruit grown for commercial purposes (e.g., White Marsh, Duncan, Thompson Pink, and Ruby Red) all have naringin as the major flavanone 7-neohesperidoside (Dr. R. L. Rouseff, personal communication). The 4′-neohesperiodoside of phloroacetophenone was highly reactive, but this compound does not occur naturally in citrus tissues (Horowitz and Gentili, 1977) and would, thus, not affect the measurement of naringin in tissue extracts and juices.

The specificity of the $^3H$ RIA toward naringin is also demonstrated in the distribution of immunoreactive material on a thin-layer chromatogram of extracts from various plant parts and of commercial grapefruit juice. All the immunoreactivity was confined to the naringin band. In this chromatographic system, the flavanone 7-rutinosides do not separate from the isomeric 7-neohesperidosides; however, since specificity studies showed that 7-rutinosides cross-react to less than 1%, they would not interfere. Studies on the flavonoid content of grapefruit juice have shown that narirutin is present in the fruit, but its concentration is generally about 30–40% that of naringin (Hagen et al., 1965). Assuming 1% cross-reactivity, a flavanone 7-rutinoside would have to be present at a 100-fold higher level than naringin to increase the estimate of naringin concentration by 10% using the $^3H$ RIA. In addition, although other flavanone 7-neohesperidosides, if present, would contribute to the estimation of naringin in juices, their presence can still be used as a measure of juice bitterness since all flavanone 7-neohesperidosides thus far tested are bitter (Horowitz and Gentili, 1977; Rouseff, 1980). However, as stated earlier, grapefruit juice samples have to be diluted 5000–10 000-fold for assay; with such high dilutions, the only way flavanone 7-neohesperidosides would significantly contribute to the estimation of naringin itself would be for the concentration of these compounds to equal or exceed that of naringin. In the more important commercial varieties of grapefruit, the level of naringin is much higher than that of poncirin, neohesperidin, and naringin 4′-glucoside (Rouseff, 1980).

The general applicability of the naringin $^3H$ RIA is demonstrated by the analyses of naringin in juices and citrus tissues. In a 7-month study of grapefruit juices from three Florida processing plants, an average of 250 samples were analyzed per week by a single individual. A total of 6685 samples—representing an identical number of truckloads of fruit—were tested. For the given season, this is 6% of the entire grapefruit crop in Florida (Mansell et al., 1983). Such large-scale studies would facilitate the careful analysis of changes in naringin content of fruits during the growing season. Furthermore, studies with such numerous samples would give a more reliable assessment of the effect of environmental factors on naringin levels in grapefruit.

In the analysis of 15 grapefruit juices obtained from local markets, the level of naringin was found to agree closely with the values reported for the freshly processed juices in the 7-month study. The distribution of naringin within the fruit tissues of a grapefruit also corroborates previous findings that the pith, membranes, and albedo are the major sources of naringin (Kesterson and Hendrickson, 1953). This is an important fact to be considered in the processing of fruits since an acceptable balance must be established between juice yield (determined in part by the pressure used in squeezing) and the naringin levels, which would increase as the albedo, membranes, and pith are damaged.

The ability of the antiserum to measure other flavanone 7-neohesperidosides besides naringin means that this serum can be used to quantify each of these compounds in crude samples containing mixtures of the neohesperidosides. In such tissues, a thin-layer chromatographic system [e.g., Hagen et al. (1965)] should be used to separate the flavanones, and after elution, they can be quantified by the RIA. Thus, the broad selectivity of the

antiserum is an asset that provides the experimenter with great flexibility.

The increased sensitivity, selectivity, and tracer stability of the assay reported here permits studies to be done on the biosynthesis and regulation of naringin and other flavanone 7-neohesperidosides, particularly in controlled systems such as tissue culture. The assay is well suited for the analysis of numerous samples such as in monitoring programs during juice processing and for screening of whole fruits, seedlings, individual callus cultures, and even entire groves. Thus, it is now possible to analyze the effects of nutrients, geographic distribution, root stocks, cultivation methods, and freezes on the production and accumulation of naringin and related flavanone 7-neohesperidosides in citrus fruits at the population level.

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# Contents of Detergent-Extracted Dietary Fibers and Composition of Hulls, Shells, and Teguments of Almonds (*Prunus amygdalus*)

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The contents of acid and neutral detergent fibers (ADF and NDF) and their fractions—hemicellulose, cellulose, and lignin—in hulls, shells, and teguments of almond fruit were determined. Pectins, protein, tannins, and acid polysaccharides were found in ADF and NDF residues. Some nutritional aspects of these subproducts in animal nutrition are considered. Determinations of polyphenols, pectins, gums and mucilages, mineral elements, oil, soluble sugars, and nitrogen were also carried out on hulls, shells, and teguments.

The fruit of the almond is made up of four parts. The mesocarp (hull), which opens when the fruit ripens, is the most external. The endcarp (shell) is hard and woody and contains one or two kernels covered by a thin brown skin called the tegument. Using average values determined by the authors for 14 almond varieties cultivated on the island of Mallorca, Spain, these parts represent the following percentages of weight for the whole fruit: hulls, 32.5; shells, 47.9; teguments, 1.2; kernels, 18.4.

According to 1981 data (El Campo, 1982) Spanish production was about 75 000 tons of almond kernels, which represent approximately 124 000 tons of hulls, 183 000 tons of shells, and 4600 tons of teguments. These subproducts have very few practical applications today.

Aside from obtaining furfural from the shells, which is a well-known process, Lopez-Gonzalez et al. (1976), Berenguer et al. (1977), and Linares-Solano et al. (1980) have obtained active carbons from this subproduct and amply studied their properties. Shells have also been used for preparing several other products. Among them are xylose and xylitol (Nobile, 1971), a stop-leak for automobile cooling systems (Lasswell and Monier, 1967), a fluid with bactericidal properties (Martín et al., 1965), fuel gas and charcoal (Sachdev, 1974), and a substance to increase microbial growth in soil (Choroszy et al., 1981).

Velasco et al. (1965), Ohanesian et al. (1973), Sanchez-Vizcaino and Moreno-Rios (1978), and Alibés et al. (1979) have studied the application of almond subproducts for animal feeding. Farmers of Mallorca have traditionally employed hulls for animal nutrition and used shells as a fuel source.

Copious information concerning the composition of almond kernels exists. In previous papers, Saura-Calixto et al. (Saura-Calixto et al., 1980, 1981, 1982; Saura-Calixto and Cañellas, 1982a) studied the soluble sugars, general composition, mineral elements, protein, and amino acids of the kernels, referring to data of other authors and comparing results. Few references in the literature con-

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