value of LPC. The results also show the indispensability of evaluation with animals for LPC from unknown plant sources as a conclusive evidence.

ACKNOWLEDGMENT

I am thankful to Dr. Narendra Singh for suggesting the problem and arranging the facilities. I am also grateful to Harendra Nath and S. Yunus Ahmed for their excellent technical help in the preparation of LPCs and Dr. M. S. N. Rao for many meaningful discussions. I also thank the Director, CFTRI, Mysore, for his permission for submission of this work as part of my doctoral thesis to the University of Mysore.

Registry No. Lysine, 56-87-1; histidine, 71-00-1; tryptophan, 73-22-3; pepsin, 9001-75-6; pancreatin, 8049-47-6.

LITERATURE CITED

Akeson, W. R.; Stahman M. A. *J. Nutr.* **1964**, *83*, 257.
Allison, R. M.; Laird, W. M.; Synge, R. L. M. *Br. J. Nutr.* **1973**, *29*, 51.
AOAC "Official Methods of Analysis", 11th ed.; Association of Official Agricultural Chemists: Washington, DC, 1970.
Bender, A. E.; Doell, B. H. *Br. J. Nutr.* **1957**, *11*, 140.
Buchanan, R. A. *Br. J. Nutr.* 1969, *23*, 633.
Byers, M. *J. Sci. Food Agric.* **1967**, *18*, 33.
Byers, M. *J. Sci. Food Agric.* **1971**, *22*, 242.
Carpenter, K. J. *Nutr. Abstr. Rev.* **1973**, *43*, 424.
Cranwell, P. A.; Haworth, R. D. *Tetrahedron* **1971**, *27*, 1831.
Dubois, M.; Yilles, R. A.; Hamilton, J. K.; Rebers, P. A.; Smith, R. *Anal. Chem.* **1956**, *28*, 350.
Feeny, R. E.; Blankenhorn, G.; Dixon, H. B. F. *Adv. Protein Chem.* **1975**, *29*, 135.
Hall, R. J.; Trinder, N.; Givens, D. I. *Analyst (London)* **1973**, *98* 737.
Henry, K. M.; Ford, J. E. *J. Sci. Food Agric.* **1965**, *16*, 425.
Lexander, K.; Carlsson, R.; Chalen, V.; Simonsson, A.; Lundborg, T. *Ann. Appl. Biol.* **1970**, *66*, 193.
Maliwal, B. P. *Nutr. Rep. Int.* **1981**, *23*, 419.
McCready, R. M.; Guggolz, J.; Silviera, V.; Owens, H. S. *Anal. Chem.* **1950**, *22*, 1156.
Morrison, J. E.; Pirie, N. W. *J. Sci. Food Agric.* **1961**, *12*, 1.
Pierpoint, W. S. *Rep. Rothamstead Exp. Stn., 1970* **1971**, 199.
Pirie, N. W. In "Leaf Proteins: It's Agronomy, Preparation, Quality and Use"; Pirie, N. W., Ed.; Blackwell Scientific Publications: Oxford, U.K., 1971.
Saunders, R. M.; Connor, M. A.; Booth, A. N.; Bickoff, E. M.; Kohler, G. O. *J. Nutr.* **1973**, *103*, 530.
Smith, E. B.; Pena, P. M. *J. Food Sci.* **1977**, *42*, 674.
Subba Rau, B. H.; Ramana, K. V. R.; Singh, N. *J. Sci. Food Agric.* **1972**, *23*, 233.
Swain, R.; Hillis, W. E. *J. Sci. Food Agric.* **1959**, *10*, 63.
Van Sumere, C. F.; Aebrecht, J.; Dedonder, A.; dePooter, H.; Pe, I. In "Chemistry and Biochemistry of Plant Proteins"; Harborne, J. B.; Van Sumere, C. V., Eds.; Academic Press: London, 1975; Chapter 8.
Walker, H. G.; Kohler, G. O.; Kuzmicky, D. D.; Witt, S. C. In "Protein Nutritional Quality of Feeds and Foods"; Friedman, M., Ed.; Marcel Dekker: New York, 1975; Part I.
Wolzak, A.; Elias, L. G.; Bressani, R. *J. Agric. Food Chem.* **1981**, *29*, 1063.

Received for review September 28, 1981. Revised manuscript received July 1, 1982. Accepted November 5, 1982.

Distribution of Limonin during the Growth and Development of Leaves and Branches of *Citrus paradisi*

Cecilia A. McIntosh and Richard L. Mansell*

This report analyzes the distribution of limonin within a leaf, within a branch, and between branches of an individual *Citrus paradisi* tree. Limonin was evenly distributed within single leaves and the concentration in the leaves on a given branch was constant, regardless of branch age. The total amount of limonin per leaf varied with leaf size. Not all branches within the tree exhibited the same potential for limonin metabolism as reflected in their limonin and limonoate A-ring monolactone levels. The limonin content showed increasing levels as the leaves bud and grow and then decreasing levels as they mature and turn dark green. Preliminary information on the levels of limonoate A-ring monolactone and limonin shows that the ratio of these compounds changes as the leaves develop, thus indicating the dynamic nature of the metabolic pools of these compounds.

Limonoids are a group of compounds which are widely distributed in *Citrus* sp. and other members of the Rutaceae. The naturally occurring liminoids are triterpenoid in origin and are diverse in their chemistry (Maier et al., 1977). The intensely bitter dilactone limonin ($C_{26}H_{30}O_8$) has been studied most extensively since it occurs in abundant amounts (Maier et al., 1977) and is of major importance in processed citrus fruit, especially grapefruit and navel oranges. Most of the research related to processing has focused on discovering or developing debittering methods to improve the quality of the end product, whereas relatively few studies have focused on improvement of the citrus crop itself.

Chandler et al. (1976) attempted to correlate limonin levels in Navel and Valencia oranges with rootstock but found that species and cultivar seemed to be the most important criteria. Seasonal effects have also been studied and it is now well established that fruits harvested later in the season yield juice which has a lower limonin content than juice from early season fruit (Marsh, 1953; Kefford and Chandler, 1961; Wilson and Crutchfield, 1968; Scott, 1970; Albach et al., 1974; Levi, 1974). However, due to simple logistics it is not possible to wait until the end of the growing season and then harvest all fruits at once.

Little is known about the potential for production, transport, or storage of limonin in an individual tree nor about the environmental, geographic, nutritional, or genetic factors which control limonin synthesis and accumulation.

Department of Biology, University of South Florida, Tampa, Florida 33620.

0021-8561/83/1431-0319$01.50/0

In addition, an argument can be made that all trees in a population of cultivars (e.g., grapefruit) are not homozygous and that although all trees within a population may be derived from a common grafting source (scion), there will be an inherent variation due to spontaneous mutations which occur frequently in citrus (Speigel-Roy, 1978). Thus, an analysis of a population of trees for the identification of "sports" which may exhibit altered limonin metabolism and subsequent use of these specimens in the development of new strains of citrus could be a focal point for improving this crop.

Also, little is known about the actual site of limonin synthesis or about the distribution in the plant itself. It has recently been demonstrated that citrus leaves can synthesize both limonoate A-ring monolactone, the putative nonbitter precursor of limonin, and limonin (Hasegawa and Hoagland, 1977). In addition, it has been shown that limonin can be transported from the leaves to the fruit and into the seeds (Hasegawa and Hoagland, 1977; Hasegawa et al., 1980). All attempts to show that the fruits themselves can synthesize limonin have been negative. Thus, if the leaves are the real biosynthetic site, the possibility of using the concentration (ppm) of limonin in leaves as an index of limonin levels in fruits could prove to be invaluable. However, at present there is no information concerning the variation in concentration of limonin within a leaf, between leaves within a branch, or between branches of individual trees or populations. As a prerequisite for mass screening, inherent variation must first be determined and enough basic information amassed before large and well-designed field studies to identify either genetic "sports" or individuals exhibiting environmentally induced altered limonin metabolism can be conducted. Such studies have not been possible in the past due to limitations in assay methods; however, with the development of a sensitive, accurate, reproducible radioimmunoassay (RIA) for limonin (Mansell and Weiler, 1980) studies of this nature are now possible.

In this paper we present the results of a study conducted to determine the distribution of limonin within a leaf, within a branch, and between branches of an individual *Citrus paradisi* tree (cultivar Ruby Red). In addition, preliminary studies on the limonoate A-ring monolactone/limonin levels in leaf tissue were performed. Thus, not only the bitter dilactone was measured but also the nonbitter limonin precursor.

EXPERIMENTAL SECTION

Materials. Tris(hydroxymethyl)aminomethane was purchased from Sigma Chemical Co. The limonin antiserum, limonin standard, and radiolabeled antigen, [^{3}H]limonol, were from a pool produced during the original limonin RIA development (Weiler and Mansell, 1980). *C. paradisi* (cultivar Ruby Red) samples were obtained from a tree located at 13508 Little Lake Place, Tampa, FL.

Methods. *Extraction of Limonin from Leaf Tissue.* The frozen samples were extracted with 0.1 M Tris-HCl buffer, pH 8.0, by heating in a boiling water bath for 30 min; the tissue was crushed with a glass rod and reextracted for another 30 min. Earlier studies had shown that these extraction conditions did not alter the limonin molecule (McIntosh, 1981). However, it was found that limonin was not stable when stored in this buffer; therefore, samples were diluted with water (nonacidified) or HCl/water to pH 2.0 (acidified) and assayed within 1 day.

Radioimmunoassay for Limonin. The basic procedure of Weiler and Mansell (1980) as modified by McIntosh (1981) was used for the quantitative determination of limonin in citrus leaf tissue. The limonin measured in the buffer extracts diluted with water is the limonin extracted from the tissue (*a*). The limonin detected in the extracts diluted with 0.01 N HCl is equal to the limonin and the A-ring monolactone in the extract (*b*). For determination of the amount of A-ring monolactone in the tissue, the value *a* was subtracted from *b*, thus giving the amount of limonoate A-ring monolactone in limonin equivalents.

Leaf Disk Analysis. One branch was chosen at random and harvested. The position of each leaf on the branch was mapped and then each leaf weighed. The entire leaf was sampled by using a 5.0-mm diameter paper punch to make the leaf disks, and each disk was weighed and mapped. All samples were stored at −20 °C until extraction and assay. This experiment was used to suggest a leaf gridding pattern for the following experiment.

Distribution of Limonin within Leaves. An 11-part grid system was used to determine the distribution of limonin within leaves. The petiole was used as the first section and the tip (top 10% of the leaf by length) was the second. The remaining nine sections were taken by dividing the leaf into thirds by length and by width. Forty leaves from a single tree were chosen at random, weighed, mapped, and gridded. Each grid section was labeled, weighed, and stored frozen. Samples were extracted and assayed as previously described.

Concentration (ppm) of Limonin in Old vs. Young Leaves. Two dark green, mature branches and five flushing branches were selected at random and harvested. The leaves were mapped, weighed, and stored frozen. Samples were extracted and assayed as previously described.

Distribution of Limonin in the Leaves and Branches of the September Flush. All flushing branches of the tree were tagged (Aug 31–Sept 2, 1979), and the branch length, which should be related to branch age, and number of leaves of each branch were recorded. The 171 branches were then categorized into five groups based upon branch length at time of tagging. Group 1 contained those branches less than 5.0 cm in length, group 2 5–10 cm, group 3 10–15 cm, group 4 15–21 cm, and group 5 all those over 21 cm in length. Four branches from each group were harvested at random after 7, 14, 21, 28, and 56 days. Upon harvest, each branch was traced and measured. The position of each stem section and leaf was recorded before the weights were measured, and the samples were frozen until extracted and assayed.

RESULTS

Leaf Disk Analysis. The results of the analysis of the total limonin content in leaf disk samples taken from a single branch are shown in Figure 1. It would appear, based upon the data from this single branch, that the limonin in the leaves tends to be more concentrated toward the center of the leaf and toward the proximal end of the leaf although this was not observed in every leaf of this branch. Therefore, while these preliminary results in themselves are inconclusive, they do show that a leaf sectioning system of 11 pieces should be sufficient to determine the pattern of distribution of limonin within individual leaves.

Leaf Grid Analysis. A completely random design analysis of variance (CRD ANOVA) (Zar, 1974) was done to test the hypothesis that all grids had the same concentration of limonin (ppm) against the alternate hypothesis that there was an inequality somewhere (α = 0.05). A data transformation of log (ppm + 1) was utilized to meet the basic assumptions of the model. The results of the CRD ANOVA showed that there were no statistically significant differences in the distribution of limonin

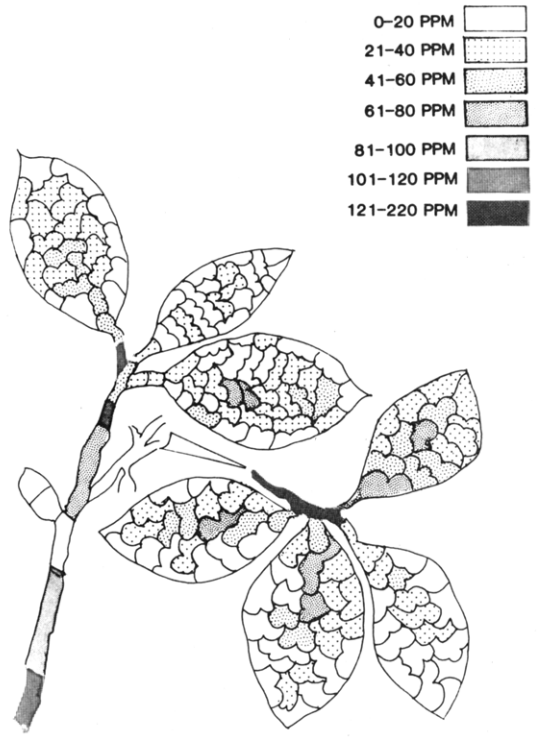
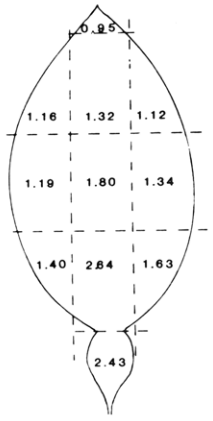


Figure 1. Concentration of limonin in grapefruit leaf disk samples.



CRD ANOVA

H_0: all grids have same ppm limonin
H_1: there is an inequality somewhere

Source	SS	DF	MS	F
Total	77.0779	439		
Groups	2.9763	10	0.2976	1.8749
Error	68.1016	429	0.1587	

Critical F 2.07, accept H_0

Figure 2. Distribution of limonin within grapefruit leaves (data are average ppm of limonin) and CRD ANOVA results (SS = sum of squares, DF = degrees of freedom, and MS = mean square).

within a leaf; however, a difference between the total limonin content in dark green, mature leaves and the total limonin content of flushing leaves was observed (Figure 2).

Concentration of Limonin in Mature vs. Flushing Leaves. Figure 3 is a scatter plot of the limonin concentration measured in flushing leaves plotted against the weight of the leaves. The limonin content of these flushing leaves ranged from 40 to 784 ppm, but limonin levels did

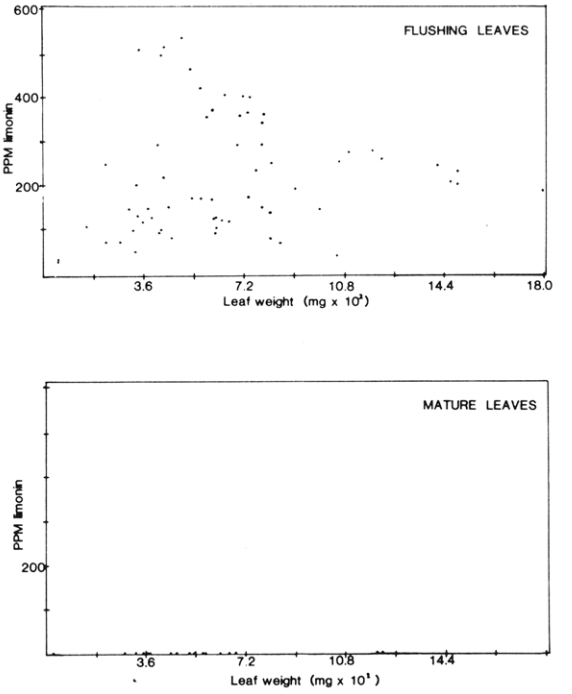


Figure 3. Scatter plot of the concentration of limonin in flushing and mature grapefruit leaves.

Table I. Summary of Results of Position CRD ANOVAs and Student-Neuman-Keuls Test for Location of Statistically Significant Differences

group at harvest	total ng/leaf	ppm	n^a
1	P = M = D[b]	P = M = D	2, 11, 50
2	P = M = D	P = M = D	7, 40, 88
3	P M D	P = M = D	30, 83, 136
4	P M D	P = M = D	28, 89, 136
5	P ≠ M = D	P = M = D	79, 177, 221

[a] n is respective of the number of leaves in each branch position. [b] Positions: P = proximal third; M = middle third; D = distal third.

not appear to follow any relationship with leaf weight. In contrast, the limonin concentration of older, mature leaves ranged from only 2–4 ppm (Figure 3) and was independent of leaf weight. This confirmed the earlier observations that flushing leaves and dark green, mature leaves differ in their limonin content and that it is in the development and aging of flushing leaves where the limonin content is most dynamic.

Distribution of Limonin in Developing Leaves and Branches of the September Flush. In the initial analysis, data from leaves were grouped according to leaf position on the branch. Position P consisted of those leaves on the proximal third of the branch, position M the middle third, and position D the distal third of the branch. A CRD ANOVA ($\alpha = 0.05$) testing the hypothesis that the total amount of limonin per leaf was equivalent in each of the three branch positions vs. the alternate hypothesis that there was an inequality somewhere was performed for each length group. The results of the ANOVAs, summarized in Table I, showed that in groups 1 and 2, where the leaves are youngest and approximately the same age, the amount of limonin per leaf was not significantly different throughout the branch. In groups 3 and 4 the proximal and distal portions of the branch were significantly dif-

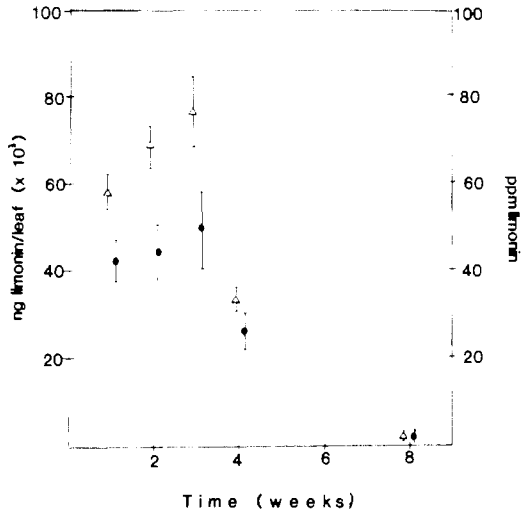


Figure 4. Limonin content of September flush leaves during development. (●) = nanograms of limonin per leaf and (△) = ppm of limonin. Data presented are average values ± SE.

ferent from each other in limonin content per leaf. In these groups, however, the midsection (position M) was intermediate and not significantly different from either the proximal or distal portions of the branch. In group 5, the total nanograms of limonin in the leaves in position P of the branch was significantly different from the total nanograms of limonin in the leaves in the rest of the branch.

An ANOVA for each group also was performed to test the hypothesis that the concentration of limonin in the leaves in each of the three positions was equal vs. the alternate hypothesis that there was an inequality somewhere ($\alpha = 0.05$). These ANOVAs showed that there was no statistically significant difference in the ppm of limonin of the leaves within a branch at any stage of growth (Table I).

Figure 4 illustrates the limonin levels of the flushing leaves during development. The limonin content of these leaves increased from an average of 58 ppm in week 1 to an average of 76 ppm by week 3. A sharp decrease to 33 ppm of limonin occurs at week 4, and this decline continues through the eighth week when the leaves, now dark green, averaged only 2 ppm of limonin. In addition, the concentration of limonin in the individual leaves was plotted against the leaf weight (Figure 5). The scattering of the points in this plot illustrates the lack of an obvious relationship between these two parameters. This figure gives an indication of the variability in the limonin content of leaves within this grapefruit tree where the values ranged from 0.2 to 509 ppm.

Figure 6 illustrates the developmental changes in limonin content after the leaves were segregated according to the branch group. Leaves from groups 1 and 2 follow the same general pattern of increasing limonin concentration for 3 weeks, from a mean of 1 and 10 ppm to that of 80 and 120 ppm, respectively. This increase is followed by a decrease in the fourth week to 45 and 36 ppm of limonin and a drop to 2–4 ppm by the eighth week. The leaves of group 3 branches were at their maximum measured limonin on the first week (85 ppm) and showed a steady decrease from that time to 74 ppm in the second week to 43 ppm in the third week to 15 ppm in the fourth week and to a minimum of 1.5 ppm by week 8. Group 4 leaves showed the same general trend as group 1 and 2 leaves: an increase in the ppm of limonin over the first 3 weeks (from 69 to 86 ppm) followed by a decrease at week 4 to 46 ppm and a drop to 3 ppm of limonin by the eighth week. The leaves from group 5 branches contained their maximum limonin of 103 ppm on the first week followed by a steady decrease over the next 3 weeks (68, 57, and 25 ppm of limonin) to a minimum of 2 ppm by week 8.

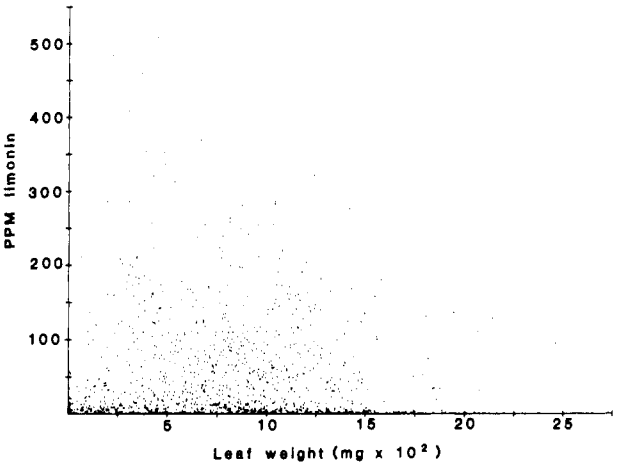


Figure 5. Scatter plot of the ppm of limonin of all September flush leaves tested vs. the leaf weight (milligrams × 10^2).

Figure 7 is a three-dimensional graph of the total amount of limonin per leaf vs. the total weight of all leaves on the branch vs. time (weeks past tagging). This graph illustrates an overall increase in the limonin content with increasing leaf weight as well as the initial increase (cf. Figure 4) and subsequent decrease of total limonin per leaf over time.

It also was found that the concentration of limonin (ppm) in the leaves of each branch tends to remain constant throughout most of the growth of the leaf. When the leaves reach maturity, however, the concentration of limonin is greatly decreased (Figure 8).

Figure 9 shows the limonin content of the flushing branches during development. The concentration of limonin increased for the first 3 weeks from 75 to 80 ppm followed by a decrease to 40 ppm by the fourth week and to a minimum of 10 ppm by the eighth week. These kinetics paralleled the same pattern of change as observed in the leaves (Figure 4).

The ppm of limonin in the branches was also plotted against the weight of the branch (Figure 10). The data have been segregated according to the branch group. It is evident that not all the flushing branches on the tree, not even those within the same group, have the same limonin levels (ppm). For example, group 1 leaves had a range of 1.4–196 ppm of limonin and group 5 a range of 4.9–306 ppm, and the other groups showed similar ranges. By the eighth week, however, it was apparent that whole branches, as well as leaves, exhibited decreased limonin levels (2–20 ppm) with maturity regardless of final branch weight or length (Figure 11).

Limonoate A-Ring Monolactone Concentrations. Results from the preliminary determinations of the A-ring monolactone/limonin levels indicate that both limonin and its A-ring monolactone were present in varying amounts in the September flush branches of the tree used in this study. The averaged data indicate that the A-ring monolactone to limonin ratio was 1:1 at week 1, 2:1 at week 2, 9:1 at week 3, 2:1 at week 4, and 1.5:1 at week 8. However, there were branches whose leaves contained only A-ring monolactone and also some whose leaves contained only limonin.

DISCUSSION

In this study, the concentration and distribution of limonin within leaves, within branches, and between

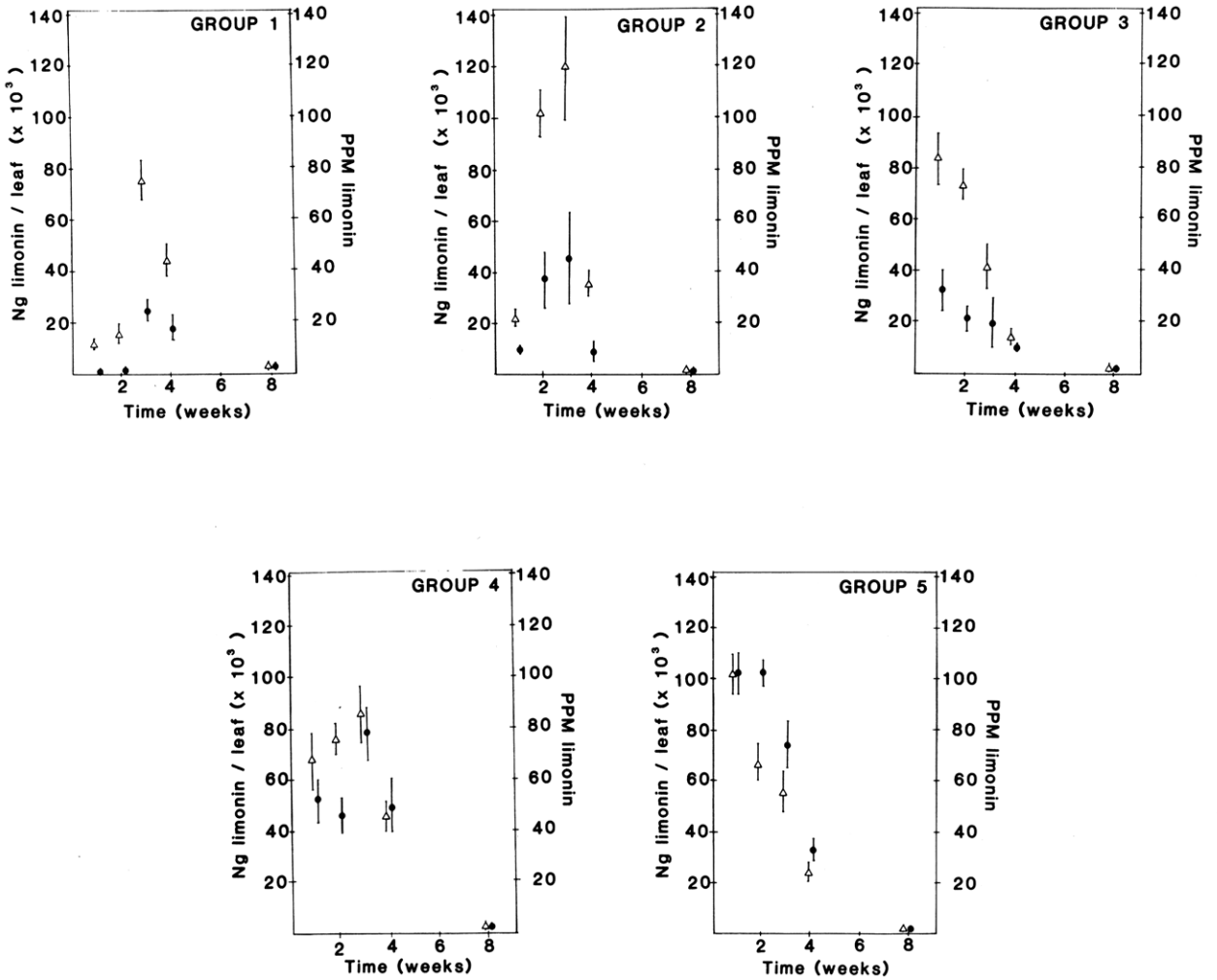


Figure 6. Concentration of limonin in the leaves of each branch group through branch development and growth. (●) = nanograms of limonin per leaf and (△) = ppm of limonin. Data presented are average values ± SE.

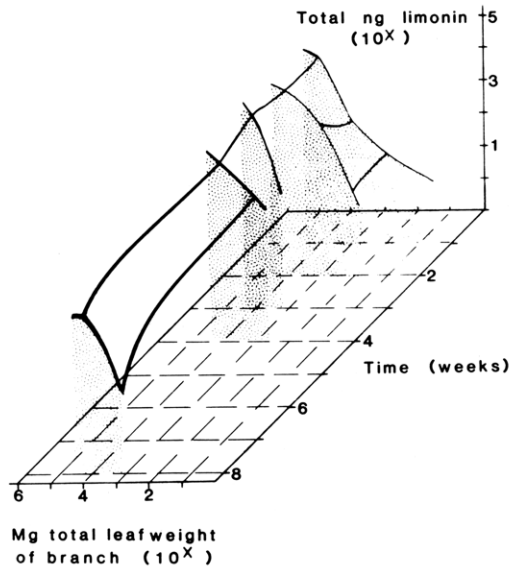


Figure 7. Three-dimensional graph of the total limonin content per leaf vs. leaf weight over time. Curves represent the best fit of scattered points.

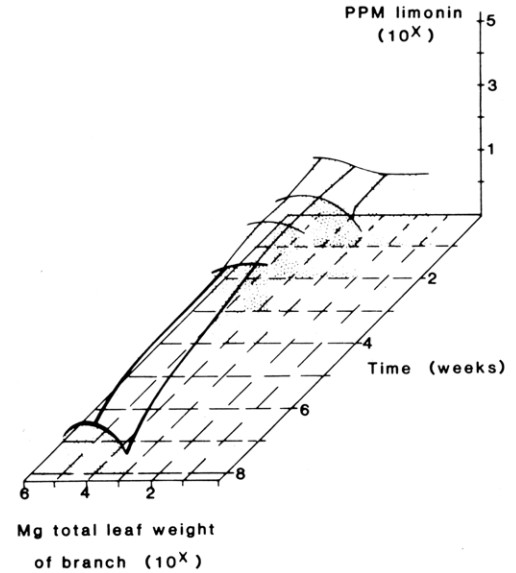


Figure 8. Three-dimensional graph of the ppm of limonin of leaves vs. leaf weight over time. Curves represent the best fit of scattered points.

branches of a single grapefruit tree (Ruby Red) were determined, and the results clearly demonstrate the dynamic nature of limonin metabolism during leaf growth and maturation.

The ANOVA testing the hypothesis that limonin is evenly distributed within a leaf showed that the limonin concentration is constant within a leaf. Thus, a single punched disk taken from a given location on a leaf would be a sufficient sample to use as an index to determine the ppm of limonin in that leaf.

The ppm of limonin of leaves was also shown to be statistically the same within a branch. However, scatter

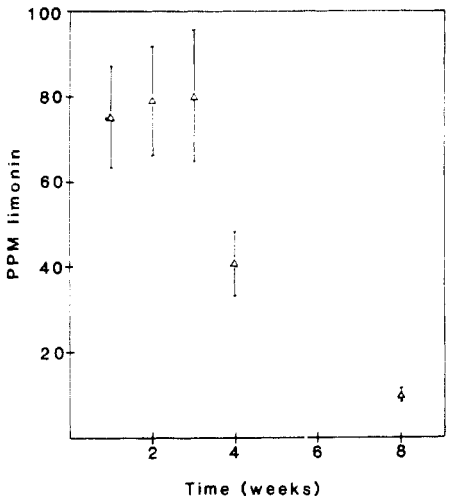


Figure 9. Concentration of limonin in whole branches during development. Data represent average values of 20 branches per week ± SE.

diagrams of the limonin concentration in the leaves and branches show the broad range in the limonin content throughout the tree even though the distribution within a branch was not as variable. Thus, while the amount of limonin in a single leaf is representative of the branch from which it was taken, no single branch accurately reflects the amount of limonin within the tree. Therefore, future studies to determine the distribution of limonin within a tree and within a population of trees could be greatly simplified by sampling a single leaf disk from each branch.

The uniformity of the limonin levels within a branch and the variation between branches is important since this indicates that not all branches on the tree exhibit the same limonin metabolism. The flushing leaves do not bud or develop at the same time or rate and the effect of age on the limonin content is significant. This is evidenced by the comparison of the ppm of limonin in flushing leaves vs. mature leaves where flushing leaves had 40–784 ppm of limonin and mature leaves contained only 2–4 ppm. Thus, it is perhaps predictable that limonin metabolism does not appear to be turned on and off synchronously throughout the tree.

In the study of the limonin content of the September flush leaves and branches of this tree, the branches were categorized into five groups according to their length on the first day of the study. It was anticipated that the branch length would be somewhat indicative of branch age and that by comparing data obtained from a given group, any effect that leaf and branch age had upon limonin levels would be standardized. The results indicate that the developmental process tends to follow a logical pattern of age as inferred from branch length, although group 4 deviates from this pattern; it is expected that the graph shape would be similar to that of groups 3 or 5. It is also possible that group 4 contained enough aberrant data so that the points showing averages are not truly representative.

Hasegawa and Hoagland (1977) measured the limonin content of lemon leaves and found that the average amount of limonin per leaf increased with increasing leaf weight whereas the ppm of limonin of the leaf decreased with increasing leaf weight. We did not find this to be the case in the grapefruit tree used in our study, and in fact no correlation with leaf weight was observed. Only leaf age appeared to be correlated to limonin content in any way. In addition, in the present study individual leaves were assayed and plotted whereas the lemon leaf analyses were done on sets of leaves where all leaves of a size (no indication if all were mature, immature, or some of both) were picked, grouped, extracted, and measured. This may explain some of the discrepancy between these two studies. Hasegawa also tested some grapefruit leaves and found flushing 70-mg leaves had 480 ppm of limonin and dark green, 480-mg leaves contained 52 ppm of limonin. This observation follows the trend of decreasing limonin levels with leaf maturity observed in the grapefruit leaves analyzed here. In a recent study by Casa and Rodrigo (1981) on navel orange leaves, a decrease in both the microgram limonin/leaf and the ppm of limonin over time was ob-

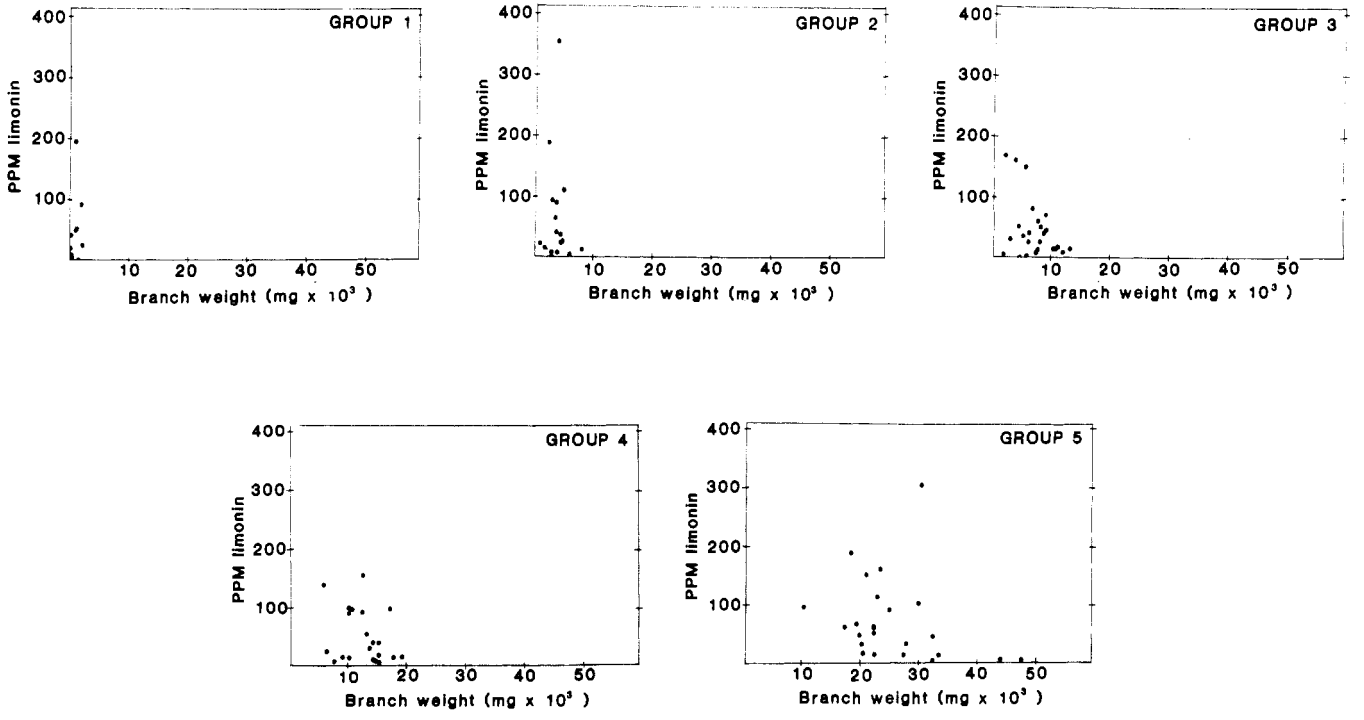


Figure 10. Scatter plot of ppm of limonin in whole branches vs. branch weight for each branch group. Data represent individual branches.

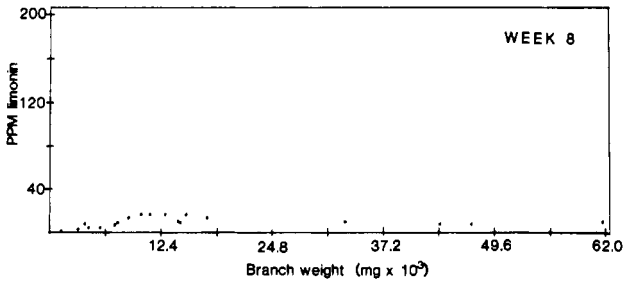


Figure 11. Concentration of limonin in branches harvested in the eighth week. All branches were dark green and mature. Data represent individual branches.

served. This trend was also evidenced in the present study, when average data rather than individual leaves are considered, after the initial increase in both of these parameters during the first few weeks of sampling.

In another study (Hasegawa and Hoagland, 1977) it was shown that young leaves of navel orange could synthesize limonin and that the limonin was subsequently translocated to the fruit. Casa and Rodrigo (1981) observed that the accumulation of limonin in Washington navel oranges ceased when limonin "disappeared" from the leaves; thus, it appears that the endogenous synthesis of limonin in citrus takes place in the young developing leaves. As the leaves begin to mature this limonin is translocated out of the leaves into the fruit where it ultimately accumulates. The mature leaves are found to contain only small limonin levels; however, it has not been clearly established whether this is endogenous residual limonin which was not translocated or whether these older leaves continue to synthesize and maintain low amounts of limonin which is either metabolized or translocated out of the leaf.

From the early studies of limonin it was concluded that the dilactone limonin was the major naturally occurring liminoid in certain citrus fruits. Later studies which established the presence of the A-ring monolactone as the precursor of limonin also showed that limonin was not present in significant amounts (Maier and Beverly, 1968; Maier and Margileth, 1969). Since this time, a general conclusion has been reached that only the A-ring monolactone is naturally occurring in leaf and fruit tissues whereas limonin is the natural component of seeds. In our studies, however, we have found that 10 leaves from different branches and groups contained only limonin and 175 contained only the A-ring monolactone. The remainder, 979 leaves, were found to contain both compounds. In addition, the change in the A-ring monolactone to limonin ratio over time indicates that the metabolic pool of this compound is also dynamic and may have endogenous kinetics quite different from that of limonin. The relative amounts of limonoate A-ring monolactone and limonin changed during leaf maturation as evidenced by the ratios which start at 1:1 in the youngest leaves, rise to 9:1 at a leaf age of 3 weeks, and fall to 1:5:1 after 8 weeks. This could be due to a coordinated relationship between these compounds during maturation or possibly due to the existence of an equilibrium condition which shifts to limonin accumulation after the monolactone reaches a certain concentration level either by a decrease in the synthesis or by translocation of the A-ring monolactone. At this point it is not really possible to explain the significance of the presence of the mono- or dilactone forms nor of the changing ratios which were observed. Since, however, the immunoassay technique which we used to do the quantitative measurements is at least 10 times as sensitive as existing procedures (Mansell and Weiler, 1980), it is possible that limonin does exist in tissues in which it was not previously detected. Thus, until more determinations of these compounds can be made and until the nature of the change in ratios is more fully understood, a firm conclusion about the nature of endogenous limonoids cannot be drawn.

From these current studies other interesting areas for research have been revealed. It would be important to determine whether there is a correlation between limonin content in the leaves of an individual flushing branch and that in the fruit on that branch. In addition, a comparison of the rates and levels of limonin biosynthesis during the four or five flushes which occur in a tree during a single year might make it possible to determine whether limonin production is a function of or can be influenced by seasonality or whether this metabolic process is simply endogenous to the growth and development of new leaf and branch tissue. With further clarification of the dynamics of limonin metabolism it might also be possible to predict the transport activities into the fruit, thereby making possible the selection of a lower limonin containing fruit.

ACKNOWLEDGMENT

We gratefully acknowledge the assistance of Kevin S. Schweiker in computer programming and Dr. Bruce Cowell for statistical discussions.

Registry No. Limonin, 1180-71-8; limonoate A-ring monolactone, 22149-49-1.

LITERATURE CITED

Albach, R. F.; Redman, G. H.; Lime, B. J. Citrus Chemistry and Technology Conference, Winter Haven, FL, 1974.
Casa, A.; Rodrigo, M. I. *J. Sci. Food Agric.* **1981**, *32*, 252.
Chandler, B. V.; Nicol, K. J.; von Biedermann, C. *J. Sci. Food Agric.* **1976**, *27*, 866.
Hasegawa, S.; Bennett, R. D.; Verdon, C. P. *J. Agric. Food Chem.* **1980**, *28*, 922.
Hasegawa, S.; Hoagland, J. E. *Phytochemistry* **1977**, *16*, 469.
Kefford, J. F.; Chandler, B. V. *Aust. J. Agric. Res.* **1961**, *12*, 56.
Levi, A. *Lebensm.-Wiss. Technol.* **1974**, *7*, 234.
Maier, V. P.; Bennett, R. D.; Hasegawa, S. "Citrus Science and Technology"; Nagy, S.; Shaw, P. E.; Veldhius, M. K., Eds.; Avi Publishing Co.: Westport, CT, 1977; Vol. 1, Chapter 9.
Maier, V. P.; Beverly, G. D. *J. Food Sci.* **1968**, *33*, 488.
Maier, V. P.; Margileth, D. A. *Phytochemistry* **1969**, *8*, 243.
Mansell, R. L.; Weiler, E. W. *Phytochemistry* **1980**, *19*, 1403.
Marsh, G. L. *Food Sci.* **1953**, *7*, 145.
McIntosh, C. A. M.A. Dissertation, University of South Florida, Tampa, 1981.
Scott, W. C. *Proc. Fla. State Hortic. Soc.* **1970**, *83*, 270.
Speigel-Roy, P. "Citrus genetics and breeding; in Production of natural compounds by cell culture methods-a symposium"; Alferman, A. W.; Reinhard, E., Eds.; Gesell Strehlen- and Umweltforschung: Munich, 1978.
Weiler, E. W.; Mansell, R. L. *J. Agric. Food Chem.* **1980**, *28*, 543.
Wilson, K. W.; Crutchfield, C. A. *J. Agric. Food Chem.* **1968**, *16*, 118.
Zar, J. H. "Biostatistical Analysis"; Prentice-Hall: Englewood Cliffs, NJ, 1974.

Received February 10, 1982. Revised manuscript received September 20, 1982. Accepted October 10, 1982. This research was supported in part by a Sigma Xi Grant-in-Aid of Research awarded to C.A.M. and by funds from a USDA/SEA grant as well as funds from the Florida Citrus Commission awarded to R.L.M.