Distribution of Limonin in the Fruit Tissues of Nine Grapefruit Cultivars

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This report presents the results of a study conducted to determine the distribution of limonin within the fruit tissues of nine grapefruit cultivars. It was found that the highest limonin levels within the fruit tissues were found in the cotyledon, followed by inner seed coat, outer seed coat, central pith, segment membrane, albedo and flavedo, and juice vesicles, in decreasing amounts. The concentration of limonin in the tissues of the individual cultivars is also presented, and the variation in limonin content within a single tissue and within single fruit is discussed. The limonin content of the fruit of the nine cultivars was also compared. The overall results showed that Davis Krome was significantly higher in limonin content followed by Triumph, Duncan, White Marsh, and Foster Pink (which were statistically equivalent to each other) and then by Thompson Pink, Wheeney, and Mott. Leonardy contained the lowest ppm of limonin. The ranking of the cultivars according to the limonin content of each tissue is also presented.

Limonin is the intensely bitter triterpenoid dilactone derivative which is widely distributed throughout the Rutaceae. This compound is of major importance in certain citrus fruits, grapefruits and navel oranges, as well as their processed products. In the past, limonin localization and distribution in citrus fruit and plant parts has received only minimal attention since an assay procedure that is rapid, is sensitive, is objective, and permits a high sample throughout has not existed. We have reported on the recent development of a radioimmunoassay (RIA) for limonin (Weiler and Mansell, 1980; Mansell and Weiler, 1980) which has made it possible to do studies which heretofore were impractical.

Recently we have presented the first in a series of reports on the limonin levels in grapefruit cultivars (Manesell and McIntosh, 1980). In this study samples were taken from truckloads of grapefruit being brought into processing plants. This sampling tended to minimize variation within and between fruits and trees but not between different varieties, groves, and trees of different age, nutritional status, and geographical distribution. Statistically significant differences were observed between the various cultivars sampled and between the test houses of the various processing plants.

Since it is generally accepted that grapefruit quality is variable from one season to another, it was important to conduct a series of studies on the production, concentration, and distribution of limonin within the fruit tissues of different grapefruit cultivars. In this report the results of a study to determine the distribution of limonin within fruit and between fruits, harvested from a single tree, of each of nine grapefruit cultivars are reported. A comparison of the limonin content and distribution of the nine cultivars will also be presented.

MATERIALS AND METHODS

For this study samples of nine different varieties of grapefruit (Davis Krome, Thompson Pink, Duncan, White Marsh, Foster Pink, Triumph, Wheeney, Mott, and Leonardy) on either Milam or Estes rootstock were obtained from the variety block at the University of Florida AREC in Lake Alfred. In March of 1980, 10 fruit of each variety were randomly harvested from a single tree on the same day and dissected into pieces of flavedo, albedo, juice vesicles, segment membranes, central pith, outer seed coat, inner seed coat, and cotyledon with no regard to the location on the fruit. Ten samples per tissue per fruit for a total of 7200 individual samples (3–1200 mg) were weighed, labeled according to variety, fruit number, and tissue and were frozen (−20 °C) until ready for extraction. (All fruit were dissected within 1 week from harvest.) Samples were extracted with 5.0 mL of 0.1 M Tris-HCl, pH 8.0, for 30 min in a boiling water bath after which the tissue was crushed with a glass rod and reextracted for an additional 30 min. The extracts were adjusted to pH 2.0 with HCl and stored at 4 °C. Each sample was diluted from 50- to 5000-fold with water (in order to be within the range of the standard curve), and after being assayed in duplicate by the tritium RIA method (Weiler and Mansell, 1980), an average value was calculated as parts per million (ppm) on a fresh weight basis.

Statistical analyses were done on the IBM-370 mainframe computer located at the University of South Florida. Statistical programs were taken from the compatible SAS pack (SAS Institute, Inc., 1979) and the standard critical values were obtained from Zar (1974).

So that possible erratic results which might skew the calculated means could be eliminated the data were edited by using the following criteria: When the number of samples (n) assayed for each tissue of each fruit was greater than or equal to 6, the highest and lowest ppm values were excluded from statistical analysis. In 37 of the 720 sets of samples, n was less than or equal to 5 and the data were subjectively evaluated. Of the samples subjectively evaluated, 24 were not changed, 6 had both the high and low values deleted, and 7 had only the high or low value deleted. Reported ranges are the ranges of means of the edited data.

RESULTS AND DISCUSSION

Intrafruit Variation. Since tissue samples were taken randomly, i.e., without regard to location within the fruit, it was not possible to correlate the observed range in limonin values with a distribution pattern. It can be seen from the segment membrane data in Table I that there is an approximate 10-fold concentration range within each fruit. Similar ranges and variation were observed for all other fruit tissues, but for illustrative purposes only the segment membrane data are presented here. It is unlikely that the observed range of limonin values is due to random error since it has been shown that the variation of this method is less than 5% (Weiler and Mansell, 1980).

Ting (1969) found that the distribution of components such as vitamin C, soluble solids, etc. in both Duncan and Marsh grapefruit was distinctly different with respect to

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Table I. Concentration of Limonin in Segment Membranes[a]

cultivar		fruit no. 1	2	3	4	5	6	7	8	9	10
Davis Krome	mean:	81 (7)	147 (8)	279 (8)	263 (8)	240 (5)	338 (8)	517 (8)	302 (8)	584 (7)	
	range:	9-192	68-243	11-631	82-719	90-427	73-861	93-1100	69-721	72-2088	
Duncan	mean:	195 (7)	181 (8)	19 (8)	146 (8)	87 (8)	106 (8)	83 (8)	103 (7)	116 (8)	75 (7)
	range:	43-311	56-424	2-27	43-276	35-172	26-237	27-169	69-235	11-227	44-126
Foster Pink	mean:	156 (8)	389 (8)	60 (6)	85 (8)	251 (8)	98 (8)	122 (7)	128 (8)	199 (8)	113 (8)
	range:	35-411	194-563	25-98	43-135	7-591	15-224	52-223	70-353	67-407	36-248
Leonardy	mean:	144 (7)	157 (7)	189 (8)	84 (8)	103 (8)	156 (8)	168 (8)	76 (8)	51 (7)	64 (6)
	range:	59-454	46-352	72-277	41-180	34-210	61-306	33-499	46-121	21-126	30-111
Mott	mean:	617 (6)	355 (8)	72 (8)	203 (5)	271 (4)	187 (6)	143 (8)	488 (8)	177 (8)	35 (8)
	range:	318-1188	232-612	33-108	124-317	94-391	48-340	12-517	229-836	32-388	8-137
Thompson Pink	mean:	178 (7)	96 (8)	31 (8)	194 (8)	34 (7)	227 (8)	465 (8)	65 (8)	148 (7)	180 (8)
	range:	12-372	2-225	5-78	54-388	3-85	28-579	149-985	17-112	19-316	46-292
Triumph	mean:	35 (8)	39 (7)	84 (7)	18 (8)	34 (8)	75 (8)	51 (8)	7 (6)	3 (8)	93 (8)
	range:	2-80	5-109	4-217	2-44	3-148	4-186	1-385	2-31	1-4	25-187
Wheeny	mean:	162 (8)	139 (7)	160 (8)	114 (6)	164 (8)	144 (8)	57 (8)	99 (7)	174 (6)	
	range:	81-384	64-242	75-256	44-186	85-244	65-217	40-72	20-207	20-480	
White Marsh	mean:	187 (8)	156 (8)	244 (6)	158 (8)	209 (8)	156 (7)	82 (8)	177 (8)	298 (8)	270 (8)
	range:	38-435	29-312	102-510	28-388	1-598	33-372	17-200	19-437	51-667	58-493

[a] Data are in ppm of limonin; numbers in parentheses represent n.

Table II. Parts per Million of Limonin in Fruit Tissues[a]

cultivar[a]		flavedo	albedo	segment membrane	juice vesicle	outer seed coat	inner seed coat	cotyledon	central pith
Davis Krome	mean:	34 (72)	66 (70)	306 (67)	17 (71)	2058 (56)	7473 (36)	8999 (72)	324 (65)
	range:	5-92	14-150	81-584	4-35	6-4056	555-12 121	1824-13 034	134-584
Duncan	mean:	12 (74)	11 (75)	111 (78)	6 (79)	1169 (80)	4658 (73)	6227 (78)	180 (79)
	range:	5-35	6-34	19-195	2-9	425-2468	152-10 585	3695-8662	43-338
Foster Pink	mean:	27 (78)	26 (76)	163 (77)	8 (79)	578 (54)	3145 (78)	6820 (77)	307 (76)
	range:	5-113	7-58	60-389	3-16	213-1259	659-9156	4957-9755	92-970
Leonardy	mean:	172 (78)	101 (80)	121 (75)	24 (78)	595 (79)	2035 (22)	4851 (74)	482 (77)
	range:	67-287	69-164	51-189	18-34	501-759	812-2843	3990-6202	214-1358
Mott	mean:	142 (70)	132 (75)	247 (69)	23 (73)	711 (73)	2821 (50)	4283 (64)	827 (52)
	range:	64-308	19-429	35-617	5-40	397-1025	1873-5866	1779-6963	136-1387
Thompson Pink	mean:	11 (77)	24 (79)	163 (77)	5 (78)	726 (48)	2802 (38)	6842 (67)	206 (79)
	range:	2-28	3-102	30-465	1-12	98-1692	79-5874	843-10 631	57-400
Triumph	mean:	95 (78)	66 (74)	44 (76)	13 (78)	779 (74)	2820 (60)	8381 (78)	322 (75)
	range:	13-219	19-183	3-93	3-45	381-1207	1704-5176	5909-10 316	63-649
Wheeney	mean:	31 (66)	35 (64)	135 (66)	8 (71)	284 (44)	3032 (46)	7789 (47)	43 (27)
	range:	4-58	11-66	57-174	4-16	202-401	2138-4459	6972-9274	12-89
White Marsh	mean:	52 (78)	78 (78)	193 (77)	8 (73)	1157 (63)	4366 (39)	7180 (73)	372 (77)
	range:	6-134	13-204	82-298	2-16	277-2333	1378-7239	4852-11 391	209-615

[a] Mean and range values of 10 fruits; numbers in parentheses represent n.

the core and periphery. In a recent study of the limonin content in the albedo of developing Navel and Valencia oranges, Chandler et al. (1976) found that both limonin and soluble solids accumulated in the distal end of the fruit. It was also found that this "distribution factor" was greater than the rootstock factor. Thus it appears that the observed range of limonin values is primarily due to the specific location within the fruit from which each sample was taken.

Intracultivar Variation. Table II shows the mean and range of the ppm limonin in each tissue of each cultivar. These values represent the mean edited values of all samples from among the 10 fruits picked at random from a single tree. Whereas the data expressed in Table I reflect the variation between individual samples of a specific tissue, Table II shows the composite means and range values of each cultivar.

Juice vesicle limonin content is of greatest commerical concern because this is the tissue that is usually consumed. It can be seen in Table II that as a group the so-called nonbitter grapefruit (i.e., Leonardy, Mott, and Triumph) have the greatest average juice vesicle limonin content. This group of grapefruit is classified nonbitter because they lack the bitterness due to the flavanone glycoside naringin. The high limonin content (24, 23, and 13 ppm, respectively) is not immediately perceived in the fresh fruit because limonin is probably present as a tasteless precursor (Maier and Beverly, 1968). However, the high limonin contents found in this study indicate that these cultivars would be less desirable for a pressed product such as juice. In a processed product the limonin would be completely converted to bitter form and the resulting bitterness might be judged excessive.

For flavedo tissues and juice vesicles, the highest limonin values were found in Leonardy and Mott whereas the lowest limonin content was in Duncan and Thompson Pink. In a comparison of the high-value cultivars, the mean values and ranges appeared quite similar. This was also observed in the low-value cultivars. Leonardy had the lowest inner seed coat value, and Leonardy and Mott had the lowest cotyledon values. Mott also had the highest limonin content of the albedo, segment membranes, and central pith tissue whereas Davis Krome was the highest of the inner seed coat, outer seed coat, and cotyledon tissues. The analysis of the albedo tissue shows that the limonin level was lowest in Duncan. The cultivar with the lowest limonin content in the segment membranes was triumph, and for central pith and outer seed coat Wheeney gave the lowest value.

In addition, it was observed that within a cultivar a fruit that contained a low concentration of limonin in one tissue also tended to have low limonin concentrations in other

Table III. Parts per Million of Limonin in Tissues of Duncan Grapefruit[a]

fruit no.	flavedo	albedo	segment membrane	juice vesicles	outer seed coat	inner seed coat	cotyledon	central pith
1	10	10	195	8	2468	5401	5423	261
2	35	34	181	5	2252	4568	3695	181
3	15	4	19	2	1083	10586	3932	43
4	7	10	146	5	2422	152	5175	104
5	14	11	87	7	494	4722	8662	332
6	5	6	106	6	460	3818	7546	71
7	5	10	83	4	425	3878	6342	275
8	8	6	103	4	439	2574	7748	130
9	10	8	116	9	1056	4598	7253	83
10	6	8	75	9	591	4975	6582	338

[a] Data are average values from five to eight observations (after data editing).

Table IV. Cultivar Analysis: Summary of Anova[a] and SNK[b] Results

tissue	conclusions[c]								
flavedo	LE	MO	TR	WM	DK	WH	FP	DS	TP
albedo	MO	LE	WM	DK	TR	WH	FP	TP	DS
segment membrane	DK	MO	WM	TP	FP	WH	LE	DS	TR
juice vesicles	LE	MO	DK	TR	FP	WH	WM	DS	TP
outer seed coat	DK	DS	WM	TR	TP	MO	LE	FP	WH
inner seed coat	DK	DS	WM	FP	WH	MO	TR	TP	LE
cotyledon	DK	TR	WH	WM	TP	FP	DS	LE	MO
central pith	MO	LE	WM	DK	TR	FP	TP	DS	WH
overall[d]	DK	TR	DS	WM	FP	TP	WH	MO	LE

[a] General linear model analysis of variance ($\alpha = 0.05$) testing H_0 (all cultivars have the same ppm of limonin) vs. H_1 (there is an inequality somewhere). In all cases, H_1 was accepted. [b] Student Neuman Keuls test for location of statistically significant differences. [c] Where appropriate, cultivars are arranged in order of descending limonin content: DK = Davis Krome, DS = Duncan, FP = Foster Pink, LE = Leonardy, MO = Mott, TP = Thompson Pink, TR = Triumph, WH = Wheeney, and WM = White Marsh. [d] In this ANOVA, differences due to tissue were partitioned out of the error term; results showed that this was the correct model to use when testing for differences between cultivars.

Table V. Tissue Analysis: Summary of ANOVA[a] and SNK[b] Results

cultivar	conclusions[c]							
Davis Krome	C	I	O	P	M	A	F	J
Duncan	C	I	O	P	M	F	A	J
Foster Pink	C	I	O	P	M	F	A	J
Leonardy	C	I	O	P	F	M	A	J
Mott	C	I	P	O	M	F	A	J
Thompson Pink	C	I	O	P	M	A	F	J
Triumph	C	I	O	P	F	A	M	J
Wheeney	C	I	O	M	P	A	F	J
White Marsh	C	I	O	P	M	A	F	J
overall[d]	C	I	O	P	M	A	F	J

[a] General linear model analysis of variance [data transformation log (ppm + 1), $\alpha = 0.05$] testing H_0 (all tissues have the same ppm of limonin) vs. H_1 (there is an inequality somewhere). In all cases, H_1 was accepted. [b] Student Neuman Keuls test for location of statistically significant differences. [c] Where appropriate, tissues are arranged in order of descending limonin content; F = flavedo, A = albedo, J = juice vesicles, M = segment membranes, P = central pith, O = outer seed coat, I = inner seed coat, and C = cotyledon. [d] In this ANOVA, differences due to cultivar were partitioned out of the error term; results showed that this was the correct model to use when testing for differences between tissues.

tissues. This is illustrated by the data in Table III where the average limonin concentration of Duncan grapefruit no. 3 was the lowest in five of the eight tissues analyzed (albedo, segment membrane, juice vesicles, cotyledon, and pith). Grapefruit no. 8 of Duncan also was among the lowest in limonin concentration for four of the eight tissues studied (albedo, juice vesicles, outer seed coats, and inner seed coat). This same trend was noted in the other cultivars but for illustrative purposes only the data from the Duncan grapefruit is presented here.

A general linear model analysis of variance (GLM ANOVA) (SAS Institute, Inc., 1979) testing the hypothesis that all cultivars contained the same ppm of limonin vs. the alternate hypothesis that there was an inequality somewhere was performed ($\alpha = 0.05$) by utilizing the data of Table II. Differences due to tissues were partitioned out of the error term in order to test only the differences due to cultivar, and results showed that this was the correct design. In this ANOVA, the alternate hypothesis was accepted, and a Student Neuman Keuls (SNK) test (Zar, 1974) for the location of statistically significant differences was performed. The overall results showed that Davis Krome was significantly higher in limonin content than all other varieties. Triumph, Duncan, White Marsh, and Foster Pink were statistically equivalent and were ranked next. These varieties were followed by Thompson Pink, Wheeney, Mott, and Leonardy, the latter of which contained the lowest ppm of limonin (Table IV). It must be emphasized that this study was done on fruit harvested at one time from a single tree in a single season; therefore we cannot predict what the effect of seasonality, weather, nutrition,and other parameters has on both limonin concentration and distribution in each of the cultivars studied.

In addition, for each tissue a GLM ANOVA ($\alpha = 0.05$) was performed, testing H_0 (all cultivars contained the same ppm of limonin in that tissue) vs. H_1 (there was an inequality somewhere). For all tissues H_1 was accepted and a SNK test for the location of statistically significant differences was performed for each tissue. The ranking of each of the varieties as a function of fruit tissue is presented in Table IV.

Distribution within Fruit. In the overall analysis, all tissues were significantly different in their limonin content except for albedo and flavedo which were not significantly different from each other. In decreasing order of concentration the ranking of fruit tissues was cotyledon, inner seed coat, outer seed coat, central pith, segment membranes, albedo and flavedo, and juice vesicles (Table V).

This was determined by utilizing a GLM ANOVA, testing the hypothesis that all tissues in a grapefruit contained the same ppm of limonin vs. the alternate hypothesis that there was an inequality somewhere ($\alpha = 0.05$). A data transformation of log $(x + 1)$ was used to meet the basic assumptions of the model. Differences due to cultivar were partitioned out of the error term in order to test only the differences due to tissue, and the results showed that this was the correct model. The alternate hypothesis was accepted, and a SNK test was performed to locate the statistically significant differences.

The distribution of limonin within the fruit tissue of each cultivar was determined by performing a GLM ANOVA [data transformation of log $(x + 1)$ and $\alpha = 0.05$], testing the hypothesis that all tissues of that cultivar had the same ppm of limonin levels vs. the alternate hypothesis that there was an inequality somewhere. For all cultivars, the alternate hypothesis was accepted, and a SNK test was performed to locate the statistically significant differences. The ranking of the various tissues from each cultivar is presented in Table V.

In all the cultivars studied the highest concentration of limonin was found in the cotyledons and the lowest was in the juice vesicles. With few exceptions the distribution pattern was similar in the other tissues; thus it would appear that the distribution factor showed a similar and quite unifirm pattern of limonin content for all cultivars. This distribution pattern was similar to that already reported for grapefruit (Mansell and Weiler, 1980), and it would tend to support the theory that citrus seeds are the major depository for limonin. If this is indeed the case, then it will be important to determine whether there is a correlation between the number of seeds in a fruit and the percentage of the total limonin found in those seeds. In other words, fruit with fewer seeds would possibly have a greater percentage of the total limonin in the fleshy fruit parts and heavy seeded varieties a lesser percentage. It would also be interesting to determine whether the seeds of "seedless" varieties have more limonin per seed than "seedy" varieties simply because there are fewer storage structures. In future studies it will be important to do a complete three-dimensional analysis of individual fruit from the same branch on a single tree and then compare the results of similar analyses for each of the cultivars.

From the results presented in this study it is clear that a very complex pattern of variation exists with regard to the limonin content in grapefruit. The intrafruit variation pattern suggests that there is a strong distribution factor which results in a wide variation in limonin content even within a single tissue or a single fruit. This variation is somewhat based upon the specific location from which the tissue is sampled.

The intracultivar variation pattern revealed that there is a large variation between the fruit of a given tree, and since the fruit were sampled at random, it cannot be determined whether there is a positional effect, that is, whether the actual location plays no role in determining the final limonin content.

In a related study which has just been comleted in our laboratory (McIntosh and Mansell, 1982), it was found that although the amount of limonin in a single leaf is representative of the branch it was taken from, there was a wide variation in the ppm of the flushing leaves throughout a single tree. This means that each branch is unique in its limonin-producing potential and it might be that the final limonin content in a given fruit is a function of the branch from which it was taken. In this regard it would then be important to test the variation between fruit clusters of a single branch against the fruit of neighboring branches.

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Leaf Hydrocarbons in the Genus *Citrus*

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The composition of whole leaf alkanes for 71 *Citrus* and related biotypes has been determined. No biosystematically consistent grouping was found, and we propose that leaf alkanes seem to function as a noncritical sealing agent in the leaf cuticle.

Long-chain alkanes are believed to be chemically stable terminal products from a sequence of reactions. Their external deposition makes it unlikely that they are part of an active metabolic pool. Since they are ubiquitous in the plant kingdom, their study has elicited much interest in biosystematic investigations.

Early workers (Francis et al., 1930; Malkin, 1930; Garner et al., 1931; Piper et al., 1931; Pollard et al., 1931, 1933; Sahai and Chibnall, 1932) suggested that the *n*-paraffins were made up exclusively of the odd-numbered members of the series, but later work by Waldron et al. (1961) showed that even-numbered members are present as minor

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