Contents of Limonoids and Limonin 17-β-D-Glucopyranoside in Fruit Tissue of Valencia Orange during Fruit Growth and Maturation

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The amounts of limonoate A-ring lactone (LARL), nomilin, deacetylnomilin, and limonin 17-β-D-glucopyranoside (LG) in the fruit tissue of Valencia oranges were measured during fruit growth and maturation. The LARL content (milligrams per fruit) in fruit tissue (flesh plus peel) increased sharply from June to September and then decreased gradually thereafter. The LARL concentration in the flesh dropped below 35 ppm when the fruit reached its normal maturity and its harvest season began. LG in the flesh began to appear in September of the fruit-set year, and its content increased until July of the following year. The amount of total limonin (LARL + LG) in fruit tissue continued to increase from July of the fruit-set year until around March of the following year and was fairly constant thereafter. The ratios of LG to total limonoid glucosides in fruit tissue ranged from 0.15 to 0.55. The accumulation patterns of nomilin and deacetylnomilin differed from those of limonin.

Bitterness due to limonoids in a variety of citrus juices is a major problem in the citrus industry with bitter juices having a lower market value for producers. Limonin is the major cause of the bitterness problem. In general, bitterness occurs in juices extracted from early to mid season fruits of winter citrus, such as the navel orange. The concentration of limonoate A-ring lactone (LARL), which is a precursor of limonin and the predominant limonoid in the fruit tissue, decreases as fruit matures (Maier et al., 1980); subsequently, bitterness is greatly reduced in juices extracted from fruits harvested in late in the season. This natural limonoid debittering process occurring in citrus fruit has been known for many years, but how limonoids are metabolized in the fruit tissue during the late stages of fruit growth and maturation was not understood until recently when Hasegawa et al. (1989) discovered that limonoids are also present in citrus as nonbitter glucoside derivatives, such as limonin 17-β-D-glucopyranoside (LG).

Hasegawa et al. (1991) have also found that in navel orange the initial appearance of LG and the sudden decrease of LARL took place at the same time. Such correlation demonstrated that in navel oranges the decrease in LARL in the later stages of fruit growth and maturation is due to the conversion of LARL to LG. Radioactive tracer work also supported the above, showing that citrus fruit tissues are capable of converting limonoids to their glucoside derivatives (Fong et al., 1991).

Unlike the navel orange, the Valencia orange is not a winter citrus and it generally does not have the limonoid bitterness problem. In this study, changes in the amounts of LARL and its glucoside in Valencia orange fruit tissue during fruit growth and maturation were determined. In addition, changes in the amount of other limonoid aglycons were studied. The results were compared with those from navel oranges to see why juice obtained from Valencia oranges does not have the bitterness problem, whereas juices from navel oranges, particularly early to mid season fruits, do.

EXPERIMENTAL PROCEDURES

Materials. Samples were taken nine times between November 4, 1988, when the fruits averaged 103.6 g and were still green in color, and September 6, 1989, when the fruits were mature and had been on the tree for over a year, from three Valencia orange trees grown at the University of California, Riverside. Each time, a set of eight fruits were picked from each of three trees. Samples were also taken monthly between June 1990, when the fruits averaged 1.35 g, and December 1990, when the fruits averaged 126.2 g and were still growing, from five randomly chosen Valencia orange trees. The trees were located at the University of California's Lindcove Field Station in the San Joaquin Valley. Figure 1 shows changes in fruit weights during the experiment. Silica gel HLF plates were obtained from Analtech, Newark, DE. C-18 Sep-Paks were purchased from Waters, Milford, MA. For HPLC analyses, a Waters 6000A pump system with a Shimadzu (Kyoto, Japan) SIL-6A autoinjector was connected to a Perkin-Elmer (Norwalk, CT) LC-75 spectrophotometric detector. The HPLC column used was a C-18 reversed-phase, Spherisorb ODS-2, 5 μm (250 × 4.6 mm) column (Analtech Inc., Deerfield, IL).

Preparation of Samples. For 1988–1989 samples, each set of eight fruits harvested was peeled. Peel and flesh were analyzed separately. Each set of the flesh tissue was blended, without H_2O added, in a Waring blender for 90 s after the seeds were removed. For peel analysis, the whole peel from each orange was used, except from those sampled on July 28, 1989, and September 6, 1989, of which one-fourth of the peel was used. Each set of peel was blended for 90 s with H_2O. A sufficient volume of H_2O was used to obtain a thick slurry. The ratio of volume of H_2O (milliliters) to fruit weight (grams) was 2.5:1 for samples taken between November 4, 1988, and March 10, 1989. The ratio was 3:1 for peel sampled on April 21, 1989, and thereafter.

For 1990 samples, each set of eight fruits harvested in June was sliced and blended with 0.5 M Tris buffer at pH 8.0 using a Brinkman Polytron tissue homogenizer. A 2:1 [buffer volume (milliliters) to fruit weight (grams)] ratio was used. For oranges sampled on July 9, 1990, and thereafter, fruits were peeled and seeds were removed. Peel and flesh were analyzed separately. Flesh tissue and peel were blended in a Waring blender, except those sampled on July 9, for which the Polytron tissue homogenizer was used. Tris buffer (0.5 M) at pH 8.0 was added for blending for some samples. Buffer volume to flesh tissue weight ratios of 1:1, 1:1, and 0.5:1, were used for July 9, August 14, and

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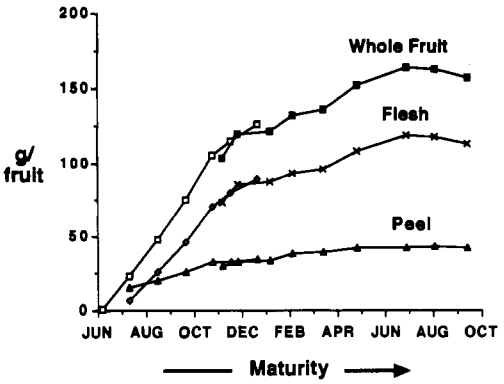

Figure 1. Increase in weight of Valencia oranges during fruit growth and maturation. Whole fruit includes fruit tissues and seeds. Solid symbols and (×) represent 1988–1989 samples. Open symbols represent 1990 samples.

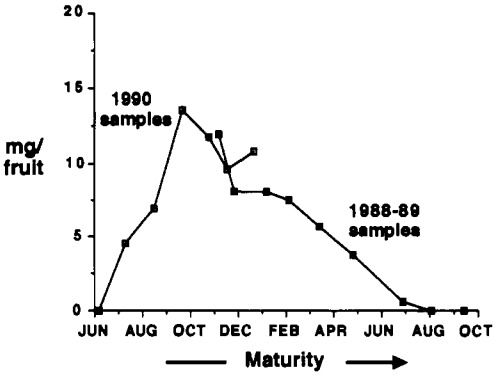

Figure 2. Changes in the limonoate A-ring lactone (LARL) content of Valencia orange fruit tissue during fruit growth and maturation.

September 18 samples, respectively. No buffer was added for October, November, and December flesh tissue samples. For peel analysis, half of the peel from each orange was used for those sampled on or after October 22. The whole peel was used for those sampled between July 9 and September 18. A buffer volume to peel weight ratio of 1.5:1 was used for July 9 samples. The ratio for samples taken on or after August 14 was 2:1.

Three weighed samples were taken from each slurry for limonoid aglycon analysis. One sample was taken from each slurry in 1988–1989 and June 1990 for limonin 17-β-D-glucopyranoside (LG) and total limonoid glucoside analyses. From slurries of July 1990 and thereafter, three samples were taken from slurry for LG analysis.

Aglycon Analysis. LARL was converted to and analyzed as limonin following modified procedures of Hasegawa et al. (1991), except for December 17, 1990, samples. The sample was transferred to a 3.5 × 20 cm test tube, and the volume was brought to a final of 35 mL with H_2O or Tris buffer. The samples were ground for 1 min with the Polytron. The homogenate was acidified to pH 2.0 with HCl to convert LARL to limonin and extracted twice with 70 mL of EtOAc, which contained an antioxidant (2,6-di-*tert*-butyl-*p*-cresol). For each extraction, the mixture was centrifuged at 10000*g* for 8 min, and the EtOAc fraction was filtered through Whatman No. 1 filter paper. The combined extracts were evaporated to dryness and dissolved in 1 or 2 mL of MeOH.

For December 17, 1990, samples, Tris buffer was added to the flesh and H_2O was added for peel. After transfer, grinding, and acidification similar to the aforementioned procedure, the mixture was centrifuged at 27000*g* for 10 min and the supernatant was filtered through Whatman No. 1 filter paper. The filtrate was then passed through a reversed-phase C-18 Sep-Pak, washed with H_2O, and eluted with MeOH. The MeOH fraction was collected in a volumetric flask, and the level was brought to 5 mL.

For the 1988–1989 samples, the extracts obtained above were used for the HPLC analyses of limonin, and the procedures of Hasegawa et al. (1991) were followed. For the 1990 samples, the extracts were used for the analyses of limonin, nomilin, and deacetylnomilin. Standards and extracts were spotted on silica gel TLC plates and developed with the following solvent systems: (a) EtOAc/cyclohexane (3:2); (b) EtOAc/CH_2Cl_2 (2:3); (c) CH_2Cl_2/MeOH (97:3). System a was used for the analysis of limonin and nomilin in flesh extracts. System b was used for the analysis of deacetylnomilin in flesh extracts, and system c was used for analysis of peel extracts. A separate plate was spotted for the analysis of each limonoid. The plate was then sprayed with Ehrlich's reagent, and the color was developed in a chamber of HCl gas. Two judges provided estimates of the limonoids by comparing the size and color intensity of spots with those of the standards. The concentration was expressed as average values obtained by two judges. Standards ranging from 0.2 to 2 μg at increments of 0.2 μg were used. A preliminary analysis was needed to determine the approximate concentration of the sample to be used on TLC.

Limonoid Glucoside Analyses. The extraction was performed according to the modified procedures of Fong et al. (1989) except for November 14, 1990, and December 17, 1990, samples. Water and MeOH were added to the sample to make an approximately 70% MeOH mixture. The mixture was blended with the Polytron and centrifuged at 13000*g* for 10 min. The residue was blended again with 70% MeOH and filtered. The combined extract was evaporated to remove most of the MeOH. For November and December 1990 samples, enough water was added to the sample to dilute the gelation effect of pectic substances. No MeOH was added. The mixture was blended and centrifuged similarly to other samples. The residue was blended again with water and filtered.

For all of the samples, a known portion of the extract was then passed through a C-18 Sep-Pak, washed with H_2O, and eluted with MeOH. Also, for both sets of samples, a known portion of the MeOH fraction was spotted on a silica gel TLC plate and developed with EtOAc/methyl ethyl ketone/formic acid (88%): H_2O (5:3:1:1). To estimate the total amount of limonoid glucosides for the 1988–1989 samples, the TLC procedure of Fong et al. (1989) was followed.

In both sample sets, HPLC was used to analyze for LG. In preparation of the analysis, the TLC spot, whose R_f was identical to that of LG, was scraped from the plate. The scraping was then extracted with water three times. To eliminate formic acid, the water extract was passed through a C-18 Sep-Pak, washed with H_2O, and eluted with MeOH. A portion of the MeOH fraction was then evaporated to dryness and dissolved in the initial HPLC mobile phase. For HPLC analysis, injection of 100 μL was made. The column was eluted by using a linear gradient starting with 15% CH_3CN in 3 mM H_3PO_4 and ending with 30% CH_3CN at 45 min for the 1988–1989 samples. For the 1990 samples, a linear gradient starting with 12% CH_3CN in 3 mM H_3PO_4 and ending with 15% CH_3CN at 30 min was used. LG was detected by UV absorption at 210 nm and quantified by peak area.

RESULTS AND DISCUSSION

The 1988–1989 samples and the 1990 samples were collected during different parts of the season. The combination of the two sets of results provides an overall picture for the entire season. We originally planned to collect samples throughout the entire 1990–1991 season, but freezing weather in late December damaged nearly all of the fruits on the five trees in the experiment, and the sample collection was terminated.

The weight of the fruit increased steadily and reached its maximal level in late June. During maturation, the increase was mainly due to increase in flesh tissue weight. In contrast, the weight of navel oranges reached its maximal level in November (Hasegawa et al., 1991).

For the 1990 samples, limonoate A-ring lactone (LARL) content (milligrams per fruit) in fruit tissue (peel plus flesh) increased steadily and sharply during June, July, and August when the fruits were small (Figure 2). It reached a maximal level, 13.6 mg/fruit, in September, and

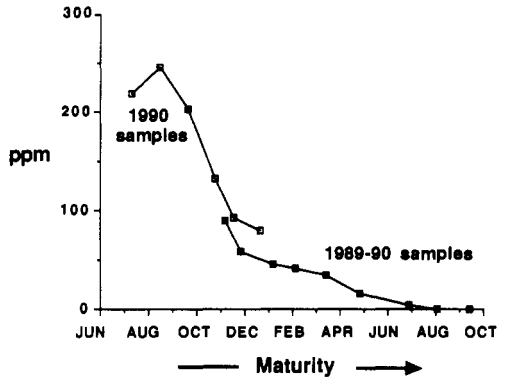

Figure 3. Changes in the limonoate A-ring lactone (LARL) concentration of Valencia orange flesh tissue during fruit growth and maturation.

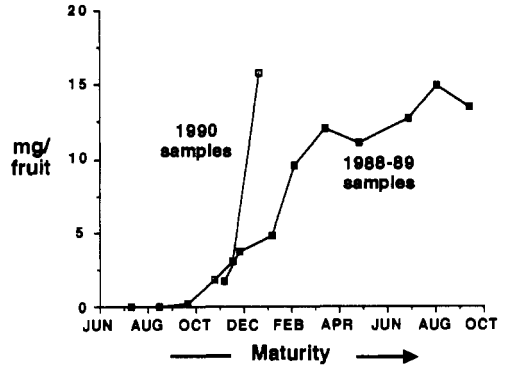

Figure 4. Changes in the limonin 17-β-D-glucopyranoside (LG) content of Valencia orange flesh tissue during fruit growth and maturation.

the content then decreased thereafter. For the 1988–1989 samples, the LARL content gradually decreased as fruit maturation progressed after remaining the same between November 4 and Nov 23. The content reached nondetectable levels in the July and August fruits, which had been on the tree for over a year and were at their maximal weight level. In comparison with the data reported by Hasegawa et al. (1991), LARL content in navel oranges reached the maximum level of 37.6 mg/fruit around the same time as in Valencia oranges. Due in part to its larger size, the LARL content of navel oranges was higher than that of Valencia oranges throughout fruit growth.

The LARL concentration in the flesh tissue is important in juice processing since LARL that enters the juice during processing is converted to bitter limonin. For the 1990 samples, the LARL concentration in the flesh peaked in August at 246 ppm and then decreased gradually (Figure 3). For the 1988–1989 samples, the concentration decreased over the whole period. Concentrations of LARL in Valencia oranges closely resembled those of navel oranges from October to April. The concentration of LARL in navel oranges was 208 ppm on October 5 and decreased to 127 ppm on November 2, 1988 (Hasegawa et al., 1991), while the concentrations in Valencia oranges were 202 and 132 ppm on September 18 and October 22, 1990, respectively. The concentration in navel and Valencia oranges fell below 40 ppm by March. The decrease in LARL concentration during the latter part of the season confirms results obtained previously (Kefford, 1959).

Limonin 17-β-D-glucopyranoside (LG) began to appear in the flesh portion of Valencia oranges during September (Figure 4). The content increased from September to July. The first appearance of LG in the flesh corresponded to the initial decrease of LARL content in Valencia orange

Table I. Changes in the Limonin 17-β-D-Glucopyranoside Concentration of Valencia Oranges during Fruit Growth and Maturation[a]

date	flesh	peel	total
1990			
July 9	0	0	0
Aug 14	0	0	0
Sept 18	5	0	3
Oct 22	25	0	17
Nov 14	39	0	28
Dec 17	59	25	49
1988–1989			
Nov 4	23	12	20
Nov 23	43	32	40
Jan 3	54	94	65
Jan 31	102	157	117
March 10	125	220	153
April 21	101	200	129
June 22	106	313	161
July 28	127	280	168
Sept 6	119	270	160

[a] Unit, ppm.

fruit tissue. This is expected since LG is formed by the glycosylation of LARL as demonstrated by Hasegawa et al. (1991). This natural debittering process started to take place in September for both navel (Hasegawa et al., 1991) and Valencia oranges when both oranges were still green.

For navel oranges, the conversion of LARL to LG had taken place for only 2 months prior to its harvest season, which began around November, when the flesh LARL concentration was still over 100 ppm. In contrast, in Valencia oranges, the conversion had taken place for 6 months before the fruit reached its normal maturity and harvest season around March, at which time the LARL concentration in the flesh portion had declined to 34 ppm. These differences clearly explain why Valencia oranges generally do not have the bitterness problem while navel oranges do.

The concentration of LG in peel increased with advanced maturity (Table I). It reached over 300 ppm, which is similar to the concentration in navel oranges (Hasegawa et al., 1991). The concentration of LG in the flesh also increased with advanced growth and maturity. It reached a maximum of only 127 ppm, which is much lower than the 328 ppm reported for navel orange flesh. From October to April, LG concentrations in the flesh of Valencia oranges were much lower than those in the navel orange flesh, while their flesh LARL concentrations were similar to one another during the same period. This indicates that the conversion of LARL to LG was more active in the navel orange flesh.

The changes in total limonin (LARL + LG) content in fruit tissue as a function of fruit growth are showed in Figure 5. To evaluate stoichiometric changes in total limonin content, LG values were expressed as limonin by multiplying by a correction factor of 0.723. The figure shows that total limonin content increased from June to September, remained fairly constant in October and November, increased again between December and March, and remained fairly constant thereafter. The biosynthesis of limonoids occurred continuously even after the glucosidation had begun. This phenomenon was also observed in navel oranges (Hasegawa et al., 1991).

The ratios of LG to total limonoid glucosides in the 1988–1989 fruit tissue samples increased sharply from 0.15 in November 1988 to 0.44 at the end of January 1989 (Table II). The ratio reached its maximum (0.55) in June 1989. These results differ from the navel orange ratios, which stayed fairly constant at around 0.7 (Hasegawa et al., 1991).

Table II. Changes in the Total Limonoid Glucoside Content of Valencia Oranges during 1988–1989 Season[a]

	dates of samplings								
	Nov 4	Nov 23	Jan 3	Jan 31	March 10	April 21	June 22	July 28	Sept 6
flesh	8.2	12.9	13.8	24.2	31.1	30.5	25.3	52.6	38.1
peel	5.6	7.2	13.4	12.2	16.4	18.3	22.0	31.8	20.5
total	13.9	20.1	27.2	36.4	47.5	48.8	47.3	84.5	58.6
LG/total G	0.15	0.25	0.29	0.44	0.43	0.40	0.55	0.32	0.42

[a] Unit, mg/fruit.

Table III. Changes in the Limonoid Aglycon Content of Valencia Orange Flesh Tissue during 1990 Season[a]

	dates of samplings					
	July 9	Aug 14	Sept 18	Oct 22	Nov 14	Dec 17
limonin	1.40	6.43	9.31	9.27	7.41	7.13
nomilin	0.93	3.27	5.44	5.53	5.92	3.80
deacetyl-nomilin	0.16	0.66	0.86	1.04	1.02	0.48

[a] Unit, mg/fruit.

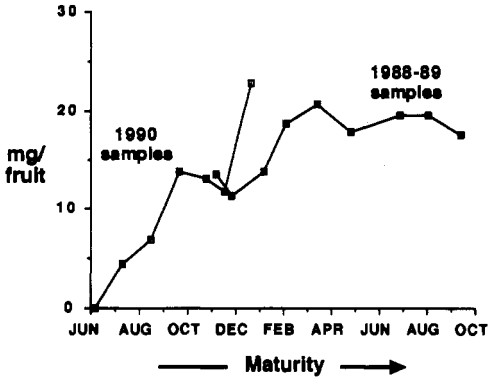

Figure 5. Changes in the total limonin (LARL + LG) content of Valencia orange fruit tissue during fruit growth and maturation.

The ratio increase indicates that not all limonoid aglycons were equally converted to their glucosides during fruit growth and maturation. The lower ratios in Valencia oranges also means that a higher percentage of limonoid glucosides other than LG was formed in Valencia oranges than in navel oranges.

Furthermore, the accumulation patterns of nomilin and deacetylnomilin in the flesh differed from the one of limonin (Table III). For the 1990 samples, nomilin content in the flesh increased sharply between July and August and remained fairly constant for several months before it declined. Deacetylnomilin content in the flesh increased gradually from July to October, when it reached a maximum of 1.04 g/fruit. On the other hand, limonin reached its maximal content in September, the earliest among the aglycons analyzed.

There were significant differences in limonoid compositions between the peel and the flesh tissue. Nomilin and deacetylnomilin were present in the flesh samples collected throughout the experiment, whereas in the peel nomilin was detected only in July and August samples. Surprisingly, no deacetylnomilin was detected (the detectable level is 5 ppm) in any of the peel samples. The glucosidation of limonoids appeared to initiate in the flesh portion of the fruit in September, 3 months earlier than that in the peel portion. Similarly, in navel oranges, glucoside formation began in the flesh in September, a month earlier than in the peel (Hasegawa et al., 1991). These results suggest that limonoids and their glucosides in peel may be biosynthesized independently from that of the flesh. Further research is needed to confirm the above.

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