Changes in the Limonoate A-Ring Lactone and Limonin 17-β-D-Glucopyranoside Content of Navel Oranges during Fruit Growth and Maturation

Shin Hasegawa,*,† Peter Ou,† Chi H. Fong,† Zareb Herman,† Charles W. Coggins, Jr.,‡ and Darwin R. Atkin‡

Fruit and Vegetable Chemistry Laboratory, Agricultural Research Service, U.S. Department of Agriculture, 263 South Chester Avenue, Pasadena, California 91106, and Department of Botany and Plant Sciences, University of California, Riverside, California 92521

The amounts of limonoate A-ring lactone (LARL) and limonin 17-β-D-glucopyranoside (LG) in navel oranges were measured during fruit growth and maturation. The LARL content (grams/fruit) increased sharply from June to August, reached a maximal level at the end of August, and then decreased gradually thereafter. LG began to appear in the flesh portion in September and in the peel 1 month later. Its content increased sharply from September to November and then slowly thereafter. The decrease in LARL and the increase in LG content occurred simultaneously at late stages of fruit growth and maturation, suggesting that LG is a metabolite of LARL. The amounts of total limonin (LARL + LG) continued to increase until the end of November and then remained fairly constant thereafter. This showed that the biosynthesis of LARL and its further conversion to LG appeared to continue in the fruit until the end of November.

Bitterness due to limonin in a variety of citrus juices is major problem of the citrus industry, worldwide, and has significant negative economic impact. In general, bitterness occurs in juices extracted from early-season to mid-season fruits of winter citrus, such as navel orange, Shamouti orange, grapefruit, and Natsudaidai. Bitterness is greatly reduced in juice extracted from fruits harvested later in the season. The concentration of limonoate A-ring lactone (LARL), a precursor of limonin, decreases as fruit maturation progresses (Maier et al., 1980). However, how LARL is degraded in fruit during late stages of fruit growth and maturation is not well understood.

Recently, we found that limonods are also present as glucoside derivatives, such as limonin 17-β-D-glucopyranoside (LG), in citrus (Hasegawa et al., 1989). Ten such glucoside derivatives have been isolated from citrus and identified (Bennett et al., 1989). More importantly, citrus fruit and juices were found to contain very high concentrations of the glucosides (Fong et al., 1989). For example, commercial orange juices contain an average of 320 ppm of total limonoid glucosides. The discovery of limonoid glucosides strongly suggests that LARL is metabolized to its glucoside during late stages of fruit growth and maturation.

Our primary research objective is to develop a preharvest method to reduce LARL in the fruit so that extracted juice will not be bitter. The biosynthetic pathways of LARL have been well established (Hasegawa et al., 1984; Hasegawa and Herman, 1985, 1986; Herman and Hasegawa, 1985), but the fate of the LARL that disappears during maturation is not known. To proceed toward our goal, we needed to determine whether LARL and its LG are inversely correlated during late stages of fruit growth and maturation in citrus.

In this study, we determined changes in the amounts of LARL and its glucoside in navel orange fruit tissue during fruit growth and maturation of navel oranges.

† U.S. Department of Agriculture.
‡ University of California.

EXPERIMENTAL PROCEDURES

Materials. Five Washington navel orange trees were used. The trees were randomly chosen from an orchard located in the San Joaquin Valley, near University of California's Lindcove Field Station. Samples were taken 10 times between June 2, 1988, and April 10, 1989. At each sampling date, one sample of eight fruits was harvested from each of the five data trees. Each sample consisted of two fruits from each of the four quadrants of a given tree. At each sampling date, fruits that were of average size were harvested. Figure 1 shows changes in fruit weights during the experiment. Silica gel HLF plates were purchased from Analtech, Newark, DE. C-18 Sep-Paks were purchased from Waters Associates, Milford, MA. The HPLC column used was a C-18 reverse-phase, Spherisorb ODS-11, 5 μm (4.6 × 250 mm) (Analtech Inc., Deerfield, IL).

Preparation of Samples. Each set of eight fruits harvested on June 2 was weighed and blended in a Waring blender for 3 min with 0.5 M Tris buffer at pH 8.0. A sufficient volume of buffer was used to obtain a thick slurry. A 3:1 [buffer volume, (mL) to fruit weight (g)] ratio was used for June 2 and July 14 fruits. Half of each fruit was used for the July 14 analyses. For the August 31 sample, one-fourth of each fruit was used and the buffer volume to fruit weight ratio was 1.5:1. For oranges sampled on October 5, 1988, and thereafter, fruits were peeled, and peel and flesh were analyzed separately. Each set of flesh tissue was blended for 90 s, without H_2O added. For peel analysis, one-fourth of the peel from each orange was used, except from those sampled on October 5 and November 2, of which the whole peel was used. Each set of peel was blended for 90 s with H_2O. The ratio of volume of H_2O to fruit weight was 2.5:1. Three weighed samples (6–8 g) were taken from each slurry for limonoate A-ring lactone (LARL) analysis, and one 10–15-g sample was taken for limonin 17-β-D-glucopyranoside (LG) and total limonoid glucoside analysis.

LARL Analysis. LARL was converted to and analyzed as limonin as follows. 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. All samples were ground for 2 min with a Brinkman Polytron tissue homogenizer, except the flesh samples which were ground for 1 min. 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). The aqueous layer was extracted a third time with 10–30 mL of EtOAc. For each extraction, the mixture was centrifuged

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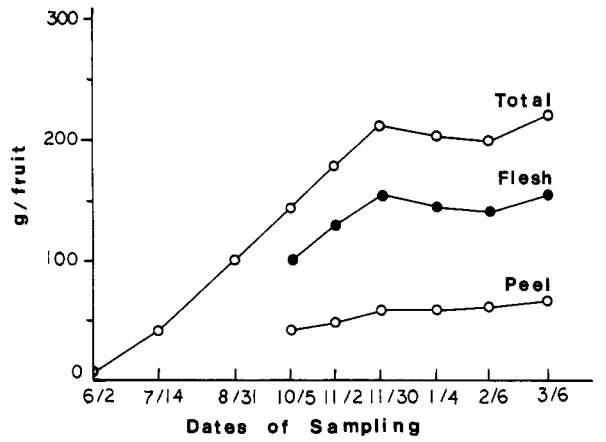

Figure 1. Increase in weights of navel oranges during fruit growth and maturation.

Table I. Changes in the Limonoate A-Ring Lactone and Limonin 17-β-D-Glucopyranoside Content of Navel Oranges during Fruit Growth and Maturation[a]

	dates of samplings									
	6/2	7/14	8/31	10/5	11/2	11/30	1/4	2/6	3/16	4/10
Limonoate A-Ring Lactone (LARL)										
peel				10.0	10.2	9.2	6.4	6.2	5.8	3.0
flesh				21.0	16.4	10.9	6.8	5.4	3.2	2.2
total	1.8[b]	19.8[b]	37.6[b]	31.0	26.6	20.1	13.2	11.6	9.0	5.2
Limonin 17-β-D-Glucopyranoside (LG)										
peel				0.	3.2	11.8	17.4	15.5	18.8	24.4
flesh				12.7	26.5	45.3	47.2	43.3	46.9	48.9
total	0[b]	0[b]	0[b]	12.7	29.7	57.1	64.6	58.8	65.7	73.3
LARL + LG										
peel				10.0	13.4	21.0	23.8	21.7	24.6	27.4
flesh				33.7	42.9	56.2	54.0	48.7	50.1	51.1
total	1.8[b]	19.8[b]	37.6[b]	43.7	56.3	77.2	77.8	70.4	74.7	78.5

[a] Average content (mg/fruit) of five trees. [b] Whole fruit analysis.

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 2 mL of MeOH and stored at −88 °C until used.

For HPLC analyses, a Waters 6000A pump system connected to a Shimazu SIL-6A autoinjector was used. For whole fruit and peel analyses, the column was eluted with a linear gradient system starting with 10% CH_3CN in 0.003 M H_3PO_4, and the gradient ended with 50% CH_3CN in 40 min. Limonin eluted at 41 min. For flesh samples, the column was eluted with a linear gradient starting with 10% CH_3CN in 0.003 M H_3PO_4 and concluding with 50% CH_3CN in 60 min. Limonin eluted at 55 min.

LG Analysis. The extraction was performed by the modified procedures of Fong et al. (1989a). To the 10–15-g sample were added 15–20 mL of H_2O and 70 mL of MeOH to make an approximately 70% MeOH mixture. The mixture was blended with a Polytron and centrifuged at 13000*g* for 10 min. The residue was blended again with 50 mL of 70% MeOH and filtered. The combined extract was evaporated to remove most of the MeOH. A known portion of the extract was then passed through a C-18 Sep-Pak, washed with H_2O, and eluted with MeOH. The MeOH fraction was brought to dryness, dissolved in 1.0 mL of 70% MeOH, and used for analysis. LG and total limonoid glucosides were estimated with the TLC method described previously (Fong et al., 1989a).

RESULTS AND DISCUSSION

Limonoate A-ring lactone (LARL) (Figure 4) content (grams/fruit) increased steadily and sharply during June, July, and August and reached a maximum level, 37.6 mg/fruit, at the end of August (Table I). The content then decreased gradually as fruit maturation progressed. The final samples harvested on April 10 contained 5.2 mg/fruit. The flesh portion contained only 2.2 mg, and the concentration was 14 ppm (Table II). On the other hand, the LARL concentration reached its highest level (478 ppm) on July 14 and then decreased gradually. The August 31 sample contained 375 ppm. The concentration decreased more noticeably in the flesh portion than in the peel portion. In the March 16 samples, the concentration in the flesh was 21 ppm, while that in the peel was 88 ppm. The decrease in LARL concentration in the flesh portion is important from a juice-processing viewpoint because LARL that enters the juice during processing is converted to limonin, which is largely responsible for juice bitterness.

Table II. Changes in the Limonoate A-Ring Lactone and Limonin 17-β-D-Glucopyranoside Concentration of Navel Oranges during Fruit Growth and Maturation[a]

	dates of samplings									
	6/2	7/17	8/31	10/5	11/2	11/30	1/4	2/6	3/16	4/10
Limonoate A-Ring Lactone (LARL)										
peel				244	211	158	110	103	88	44
flesh				208	127	71	47	39	21	14
total	362[b]	478[b]	375[b]	219	150	96	66	58	41	23
Limonin 17-β-D-Glucopyranoside (LG)										
peel				0	66	202	298	258	286	362
flesh				126	206	296	328	312	304	325
total	0[b]	0[b]	0[b]	90	168	269	319	295	296	336
LARL + LG										
peel				244	277	360	408	361	374	406
flesh				333	333	367	375	351	325	339
total	362[b]	478[b]	375[b]	307	318	365	385	353	339	359

[a] Average concentration (ppm) of five trees. [b] Whole fruit analysis.

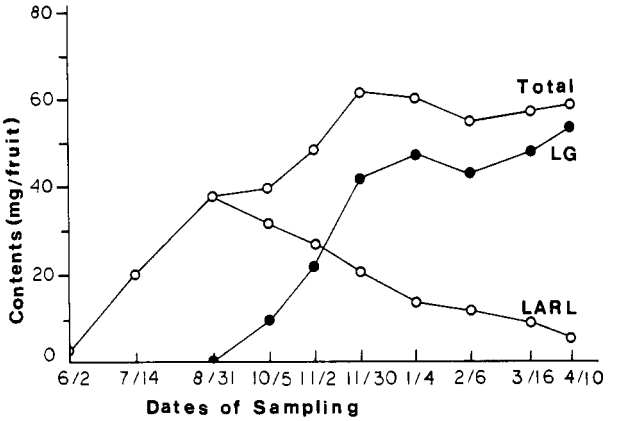

Figure 2. Changes in the limonate A-ring lactone (LARL) and limonin 17-β-D-glucopyranoside (LG) content of navel oranges during fruit growth and maturation. LG values are expressed as LARL by multiplying by 0.723.

During September when the fruits were still green (68 mm in horizontal diameter and 142 g), limonin 17-β-D-glucopyranoside (LG) (Figure 4) began to appear in the flesh portion, and the content increased sharply thereafter (Figure 2). In the peel, glucoside formation began in October when the fruit had turned slightly yellow (Tables I and II; Figure 2). The rate of increase thereafter was much slower than that of the flesh. If glucosidation of limonoids is considered to be a ripening-related process, these data suggest that ripening in navel oranges appears to initiate in the flesh portion of the fruit about 1 month earlier than that of the peel portion.

Previous radioactive tracer work demonstrated that ^{14}C-labeled nomilin was converted to its glucoside, nomilin 17-β-D-glucopyranoside, in the mature tissue of lemon fruit (Fong et al., 1989b), showing that nomilin aglycon is converted to its glucoside derivatives in the mature fruit. In the present study the sudden increase in the LG content

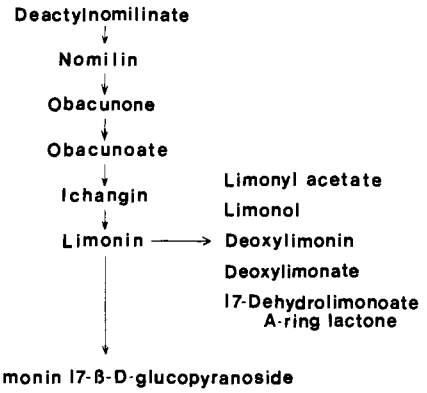

Figure 3. Biosynthetic and catabolic pathways of limonin in citrus.

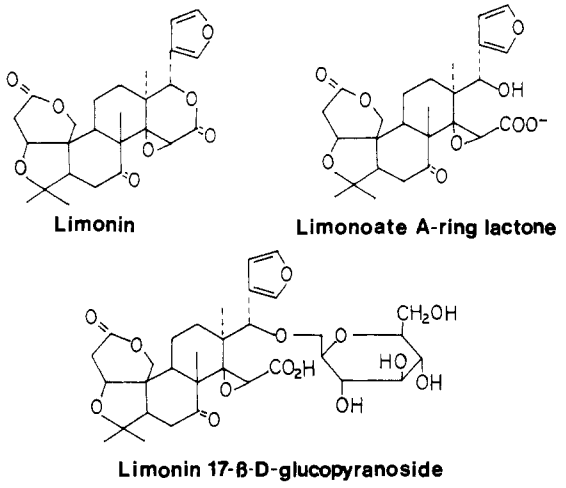

Figure 4. Structures of limonoids.

of navel oranges during September simultaneous with a sudden decrease in LARL confirmed that LG is formed by the glycosylation of LARL.

Since LG is practically nonbitter (Koski, 1988), the glucosidation of LARL to form LG would be a natural debittering process. Since limonin bitterness is a problem in early-season to midseason fruits and not in late-season fruits, the general belief is that LARL concentration decreases as fruit ripening progresses (Maier et al., 1980). This work confirmed the above by showing that LARL does decrease during fruit maturation (Tables I and II; Figure 2).

Although LARL has been shown to be converted to minor limonoids such as 17-dehydrolimonoate A-ring lactone (Hasegawa et al., 1974), deoxylimonin and deoxylimonate (Hasegawa et al., (1980), and possibly others such as limonol and limonyl acetate (Figure 3), these conversions are very minor and alone cannot explain the debittering process. The discovery of LG and the formation of LG finally explain how LARL disappears at late stages of fruit growth and maturation.

Figure 2 shows the changes in LARL, LG, and total limonin (LARL + LG) contents (grams/fruit) as a function of time of fruit growth. To evaluate stoichiometric changes in total limonin content, LG values were expressed as limonin by multiplying by 0.723 (MW of limonin/MW of LG). The figure, even after molecular weight differences were corrected for, still shows that the total limonin content increased until the end of November. It is of interest to note that the total weight of the fruit increased also until the end of November (Figure 1).

The total limonin (LARL + LG) content continued to increase until the end of November (Figure 2; Tables I and II). The increase after September is obviously due to the increase in LG. The continuous biosynthesis of LG at such a rapid rate after September was totally unexpected. We expected that the total limonin content would reach its maximal level when the LARL content reached its maximal level and that the level would remain fairly constant thereafter. Only the conversion from LARL to LG was expected during late stages of fruit growth and maturation. However, this was not the case. In addition to increases in quantity/fruit, the concentration of total limonin increased from 307 to 365 ppm between October 5 and November 30 (Table II). This increase could be either due to newly biosynthesized LARL, which was then further converted to LG, or due to conversion of LARL precursors such as nomilin, obacunone, and obacunoate converting to LG via LARL at late stages of maturation. However, ratios of LG to total limonoid glucosides at different stages of fruit growth and maturation were fairly constant, 0.7 (Table III). These data indicate that all limonoid aglycons were equally converted to their glucosides simultaneously during fruit growth and maturation. Thus, the increase in LG at late stages of fruit growth and maturation is most likely due to newly biosynthesized LARL which is further converted to LG.

Table III. Changes in the Total Limonoid Glucoside Content of Navel Oranges during Fruit Growth and Maturation[a]

	dates of samplings						
	10/5	11/2	11/30	1/4	2/6	3/16	4/10
peel	0	6.1	14.3	24.2	21.0	25.2	31.6
flesh	17.5	36.6	63.9	70.6	63.9	69.8	72.4
total	17.5	42.7	78.2	94.8	84.9	95.0	104.0
LG[b]/total G	0.72	0.70	0.73	0.69	0.69	0.69	0.70

[a] Unit, mg/fruit. [b] LG, see Table I.

There have been 38 limonoids isolated from citrus and its hybrids. Therefore, there could be 38 limonoid glucosides in citrus and its hybrids. Ten limonoid glucosides have thus far been isolated from citrus and identified (Hasegawa et al., 1989; Bennett et al., 1989). Analysis of individual limonoid aglycons and their glucosides as a function of time of fruit growth could clarify the whole biosynthetic system of limonoids in citrus.

The biosynthetic pathways of limonin and LG in citrus are shown in Figure 3, where the limonoids are shown in the dilactone form. The dilactones, such as limonin, are the predominant limonoids present in seeds, and the monolactones, such as LARL, are the predominant limonoids present in leaves, stems, and fruit tissues (Maier et al., 1980). Until recently, limonin was thought to be the major end product of limonoid biosynthesis (Maier et al., 1980; Hasegawa and Herman, 1986). Although the conversion of limonin to minor limonoids such as deoxylimonin, deoxylimonate, and 17-dehydrolimonate A-ring lactone has been shown to occur in citrus, these conversions are quite minor as compared to that of glucosides.

Since the conversion of LARL to LG is a natural debittering process, the stimulation of this conversion by bioregulation could reduce the content of LARL and subsequently reduce the limonoid bitterness in citrus juices. Along with studies on the inhibition of limonoid biosynthesis by auxins (Hasegawa et al., 1986) and the alteration of biosynthetic pathways of limonoids by genetic engineering or breeding (Herman et al., 1989), we are currently studying this new approach to develop a preharvest method to reduce the LARL content of citrus fruit.

ACKNOWLEDGMENT

We gratefully acknowledge Jere and Jon Runciman, Runciman Ranches, Inc., for their cooperation and for providing the experimental site.

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Received for review December 14, 1989. Revised manuscript received April 16, 1990. Accepted August 7, 1990.

Registry No. LARL, 22149-49-1; LG, 123564-61-4.