Limonoids and Their Glucosides in Valencia Orange Seeds during Fruit Growth and Development

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The amounts of major citrus limonoids and their corresponding glucosides in Valencia orange seeds were measured during fruit growth and development. Limonin content, including limonoate A-ring lactone (LARL), increased steadily during the whole sampling period, July–December of the fruit-set year. Other limonoid aglycons also increased but not steadily. Limonoid glucosides began to appear in either September or October, and their contents steadily increased thereafter with the exception of obacunone 17-β-D-glucopyranoside. Citrus seeds, demonstrated in Valencia orange, were shown to have no significant effect on the concentrations of LARL and total limonoid glucosides in fruit tissue. Data suggested that seeds possess limonoid biosynthetic systems independent from the biosynthesis occurring in the fruit tissue.

Citrus seeds are known to contain high concentrations of limonoids (Maier et al., 1977), and most limonoid isolation work has been done on seeds. Hasegawa et al. (1989) discovered the presence of limonoid glucosides, and they were first isolated from grapefruit seeds. Analyses of citrus seeds (Ozaki et al., 1991a,b) showed that citrus seed is a rich source of limonoid glucosides. Among the seeds analyzed, Valencia orange was found to contain the highest content, 0.87% of dry weight, of total limonoid glucosides.

Valencia orange generally does not have the limonoid bitterness problem associated with the juice extracted from early- to mid-season fruits of winter citrus such as the navel orange. Since the navel orange is seedless, the seed in Valencia orange had been suspected to play an important role in the absence of the limonoid bitterness problem.

In this study, changes in the amount of limonoids and their glucosides in Valencia orange seeds during fruit growth and maturation were determined. In addition, the correlation between seed number and accumulation of limonoids and their glucosides in the fruit tissues were studied.

EXPERIMENTAL PROCEDURES

Materials. For the study of fruit growth and development versus the limonoid and limonoid glucoside content of seeds, samples were taken monthly between July and December 1990 from five Valencia orange trees located at the University of California's Lindcove Field Station in the San Joaquin Valley. Each time, a set of eight orange fruits was picked from each of the five trees and seeds were taken from the fruits and analyzed for limonoid and limonoid glucoside contents. To determine whether there is a correlation between Valencia seed number and the concentration of limonoate A-ring lactone (LARL) and/or total limonoid glucosides, 65 fruits with a diameter between 5.5 and 6.0 cm were harvested on December 22, 1989, from a Valencia orange tree grown at the University of California, Riverside. The fruits were divided into eight groups according to the number of seeds inside the fruit. Flesh and peel tissue was analyzed for LARL and total limonoid glucosides.

C-18 reversed-phase and Accell QMA Sep-Pak cartridges were purchased from Waters Associates, Milford, MA. For HPLC analysis, a Waters 6000A pump system connected to a Shimadzu SIL-6A autoinjector was used. Compounds were detected by UV absorption at 210 nm, using a LC-75 spectrophotometric detector (Perkin-Elmer, Norwalk, CT). The 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 the 1989 samples, each group of fruits was peeled and the seeds were removed. Peel and flesh were analyzed separately. Each group of flesh tissue was blended in a Waring blender for 90 s. For peel analysis, one-fourth of the peel from each orange was used. Each group of peel was blended for 90 s with 0.5 M Tris buffer at pH 8.0. The ratio of volume of buffer (milliliters) to fruit weight (grams) was 2.5:1, except for group F, for which a ratio of 2.65:1 was used. Three weighed samples (7-8 g) were taken from each slurry for limonoate A-ring lactone (LARL) analysis. One 11-17-g peel sample and three 11-15-g flesh samples were taken for limonin 17-β-D-glycopyranoside (LG) analysis.

For the 1990 samples, each set of seeds was blended with a Brinkman Polytron tissue homogenizer for 2 min with H_2O. The ratio of volume of water (milliliters) to seed weight (grams) was 2:1. Three weighed samples (about 1 g) were taken from each thick slurry for limonoid analysis. Three 1.0-1.5-g samples were taken for limonoid glucoside analysis, except those sampled on September 18 and before. For the seeds of July 9, a 0.7-1.2-g sample was used, while two 2-5-g samples were taken from the slurries of August 14 and September 18.

Limonoid Analysis. For the 1989 samples, the extraction procedure of Hasegawa et al. (1991) was followed to extract LARL. The procedure converted LARL into limonin. For HPLC analysis, a linear gradient starting with 10% CH_3CN in 3 mM H_3PO_4 and concluding with 50% CH_3CN in 3 mM H_2PO_4 in 40 min was used for both flesh and peel samples. Limonin eluted at 38 min.

For the 1990 seed samples, 0.15 M Tris buffer at pH 8.0 was added to each sample. The mixture was stirred for 20 h and then acidified to pH 2 with concentrated HCl. The acidification converted LARL to limonin. An equal volume of EtOAc containing an antioxidant (2,6-di-*tert*-butyl-*p*-cresol) was added to the mixture and mixed well with a Super-mixer or a separating funnel. It was then centrifuged at 10000g for 10 min to separate out the two layers. The top EtOAc layer was pipetted and filtered

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Table I. Changes in the Weight of Valencia Orange Seeds during Fruit Growth and Development

date	g/fruit	seed/fruit	g/seed
July 9	0.09	6.3	0.014
Aug 14	0.44	7.4	0.059
Sept 18	1.09	7.1	0.155
Oct 22	1.45	6.6	0.222
Nov 14	1.38	6.2	0.225
Dec 17	1.61	6.1	0.263

through a Whatman No. 1 filter paper. The aqueous layer was extracted again with EtOAc. The combined extract was evaporated to dryness and dissolved in a small known quantity of MeOH. For HPLC analyses, an isocratic system with CH_3CN–MeOH–H_2O (10:41:49) was used. The flow rate was 1 mL/min. The retention times for limonin, deacetylnomilin, nomilin, and obacunone were 16, 18, 26, and 45 min, respectively.

Glucoside Analysis. The extraction procedure of Hasegawa et al. (1991) was followed to extract limonoid glucosides for the 1989 flesh samples. For the peel samples, a modified procedure was used. After extraction with 70% MeOH twice, the combined peel extract was evaporated to dryness. The sample was then redissolved in pH ~ 6.5 H_2O. A known portion of the aqueous extract was loaded onto a Sep-Pak Accell QMA cartridge, which was washed with 1 M acetic acid and water prior to the loading. The cartridge was then eluted with 1 M NaCl solution. The eluate was collected and passed through a C-18 reversed-phase Sep-Pak cartridge to remove the salt. The C-18 cartridge was washed with water and eluted with MeOH. The MeOH fraction was brought to dryness, dissolved in 1.0 mL of 70% MeOH, and used for analysis. The total limonoid glucosides were estimated with the TLC method described by Fong et al. (1989).
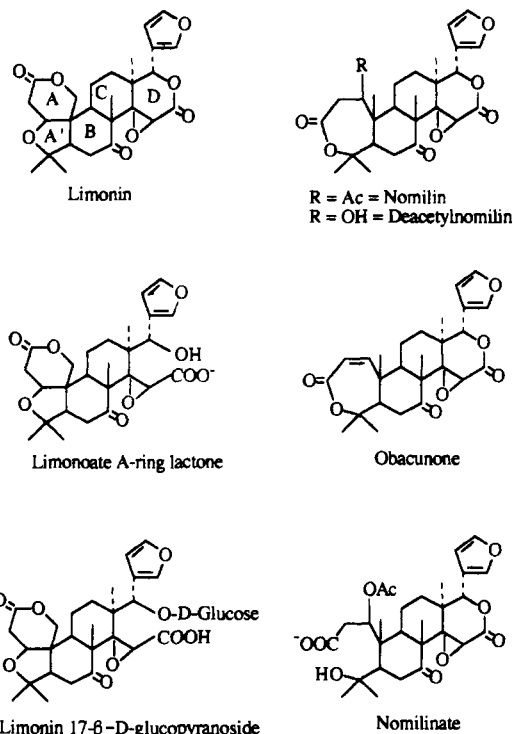
For the 1990 seed samples, two comparable methods were used for extraction. For August–October samples, 15 mL of MeOH was added and the mixture was homogenized with the Polytron. The homogenate was centrifuged at 13000g for 10 min and filtered. The residue was extracted again with 15 mL of 70% MeOH. Most of the MeOH in the combined extract was evaporated. A known portion of the extract was then passed through a C-18 reversed-phase Sep-Pak cartridge, washed with H_2O, and eluted with MeOH. For the July, November, and December samples, a sufficient amount of H_2O was added to prevent gelation from pectic substance. The mixture was homogenized, centrifuged, and filtered similarly to the above method. The residue was extracted again with H_2O. The combined aqueous extract was passed through a C-18 reversed-phase Sep-Pak cartridge, washed with H_2O, and eluted with MeOH.

A portion of the MeOH fraction from both extraction methods was evaporated to dryness. The aqueous residue was treated with hesperidinase in 0.1 M sodium formate buffer at pH 3.8 for 20 h at room temperature. The treated mixture was passed through a C-18 reversed-phase Sep-Pak cartridge. The MeOH fraction from the C-18 cartridge was then evaporated to dryness and redissolved in the starting HPLC mobile phase. For HPLC analysis, a linear gradient system starting with 10% CH_3CN in 3 mM H_3PO_4 and concluding with 26% CH_3CN in 3 mM H_3PO_4 in 56 min was used. The retention times for the glucosides of limonin, deacetylnomilin, nomilin, nomilinic acid, and obacunone were 29, 40, 48, 49, and 54 min, respectively.

RESULTS AND DISCUSSION

The plan was to collect samples throughout the entire 1990–1991 season for this study, but freezing temperatures damaged the fruit on the experimental trees during December; consequently, the sample collection was terminated. Average seed weight increased sharply from July to October and only slightly thereafter (Table I). The number of seeds per fruit was fairly constant throughout the experiment, averaging 6.5 seeds per fruit.

Limonoids are present in citrus seeds in two forms, dilactones (closed D-ring) and monolactones (open D-ring) (Figure 1). The predominant form in mature seeds is dilactones, such as limonin, while in leaves, stems, and

Limonin

R = Ac = Nomilin
R = OH = Deacetylnomilin

Limonoate A-ring lactone

Obacunone

Limonin 17-β-D-glucopyranoside

Nomilinate

Figure 1. Structures of limonoids.

Table II. Changes in the Limonoid and Limonoid Glucoside Contents in Seeds of Valencia Orange during Fruit Growth and Development[a]

date	L	N	O	D	LG	NG	OG	DG	NAG
July 9	0.06	0.008	0.012	0.008	0	0	0	0	0
Aug 14	0.77	0	0.059	0.069	0	0	0	0	0
Sept 18	2.68	0	0.149	0.064	0.228	b	b	b	b
Oct 22	4.59	0.687	0.143	0.647	0.495	1.66	0.082	0.85	0.140
Nov 14	4.85	0.729	0.131	0.820	0.699	2.33	0.069	1.24	0.156
Dec 17	6.04	1.001	0.243	0.962	0.948	3.69	0.122	1.96	0.314

[a] Unit, mg/fruit. L, limonin; N, nomilin; O, obacunone; D, deacetylnomilin; NA, nomilinic acid; G, glucoside. [b] Samples lost.

fruit tissues only monolactones, such as limonoate A-ring lactone (LARL), are present. For the purpose of this paper, it is not necessary to make a distinction between these two forms. Therefore, all analyses of limonoids in seeds included both forms.

The amount of limonin, nomilin, obacunone, and deacetylnomilin in seeds increased throughout the experiment (Table II). The increase was not steady but reached the highest levels in December. In fruit tissues, however, limonin content decreased steadily after it reached its maximal level in September (Fong et al., 1992).

Limonin was the predominant limonoid in the seeds throughout the whole sampling period, confirming the results obtained previously (Dryer, 1966; Ozaki et al., 1991a). Nomilin, which is considered to be the most active limonoid biologically, did not accumulate to significant amounts until October. By December, however, nomilin was the second most abundant limonoid, followed by deacetylnomilin and obacunone. This concentration order is similar to that reported by Ozaki et al. (1991a) for mature Valencia seeds.

Limonin 17-β-D-glucopyranoside (LG) (Figure 1) began to appear in the seeds in September and increased steadily through December (Table II). Other limonoid glucosides, including the 17-β-D-glucopyranosides of nomilin (NG), obacunone (OG), deacetylnomilin (DG), and nomilinic acid (NAG), were first detected in October but probably started to appear in September and increased thereafter. NG

was the predominant glucoside in December samples followed by DG, LG, NAG, and OG in order of decreasing concentration. This trend was very similar to those of other citrus seeds (Ozaki et al., 1991a). In contrast, LG was the major glucoside in flesh tissues of navel orange (Hasegawa et al., 1991) and in commercial juices of orange, grapefruit, and lemon (Fong et al., 1989). The LG content was lower than that of its corresponding aglycon over the whole sampling period. However, the contents of NG and DG were higher than the contents of their corresponding aglycons between October and December. The higher amounts of limonin, NG, and DG were also observed previously in mature Valencia orange seeds (Ozaki et al., 1991a).

The initial appearance of limonoid glucosides takes place at the same time in both seed and flesh tissue (Fong et al., 1992). However, unlike the fruit tissue, where the increase in LG corresponds to the decrease in limonin content (Fong et al., 1992), both aglycons and glucosides in the seed increased together through December.

The biosynthesis of limonoids and limonoid glucosides in citrus seeds is catalyzed by three types of enzyme. The first class of enzymes is involved in the biosynthesis of other limonoids from nomilin in stem tissue. Then these limonoids are translocated to other parts of the plant (Hasegawa et al., 1986). The second class contains limonin D-ring lactone hydrolase, which is involved in the lactonization of the open D-ring of monolactones to form dilactones. This enzyme has been isolated from citrus (Maier et al., 1969). The third class contains UDP-D-glucose transferase, which converts the open D-ring aglycons into their corresponding glucosides during late stages of fruit growth and maturation (Hasegawa et al., 1991; Fong et al., 1992).

The accumulation of high concentrations of both limonoid aglycons and glucosides in citrus seeds is due to the following. Newly synthesized monolactones are converted to dilactones by the action of limonoid D-ring lactone hydrolase during fruit growth. The closed D-ring limonoids formed are not capable of forming glucoside derivatives. This lactonization continues to take place in seeds concurrently with the glucosidation of monolactones during later stages of fruit growth and maturation. In fact, during maturation, two enzymes, limonin D-ring lactone hydrolase and UDP-D-glucose transferase, are competing with each other for newly biosynthesized monolactones. Thus, mature seeds accumulate high concentrations of both limonoid aglycons and glucosides.

In contrast, in the fruit tissue there are only two types of those enzymes directly involved in the biosynthesis and metabolism of limonoids. No lactonization of monolactones appears to occur in fruit tissues, and monolactones remain biologically active during fruit growth. These compounds are converted to their glucoside derivatives during late stages of fruit growth and maturation. This is why the fruit tissue accumulates high concentrations of the limonoid glucosides instead of the aglycons. The ratio of glucosides to aglycons in commercial orange juices is about 150:1 (Fong et al., 1989), whereas in the seeds the ratio is about 1:2 (Hasegawa et al., 1989).

It has been generally believed that citrus fruits containing seeds do not have a limonoid bitterness problem because the limonoid concentration is reduced by migration of the limonoids from the fruit tissue to the seeds. However, our data so far suggest that the limonoids accumulated in the seeds are biosynthesized independently and are not translocated from the fruit tissue. To test the above hypothesis, Valencia orange fruit tissue was studied

Table III. Eight Groups Separated According to Different Number of Seeds

group	seed/fruit	no. of fruit	fruit	flesh	peel
			wt/fruit, g		
A	1, 2	4	89.0	59.9	28.4
B	3	9	92.7	62.4	29.3
C	4	12	92.9	63.3	28.5
D	5	11	92.9	63.4	28.1
E	6	8	91.4	61.9	28.0
F	7	11	93.6	63.6	28.3
G	8	4	91.1	63.2	25.8
H	9, 10	6	99.0	66.6	29.8

Table IV. Effects of Seeds on the Concentrations of Limonoate A-Ring Lactone (LARL) and Total Limonoid Glucosides in Fruit Tissue of Valencia Oranges

group	LARL, ppm flesh	LARL, ppm peel	total lim glu, ppm flesh	total lim glu, ppm peel
A	65.6	73.1	150	a
B	56.6	101.3	130	110
C	67.4	84.1	180	59
D	53.2	33.7	160	53
E	57.1	78.1	140	77
F	51.4	100.6	150	52
G	60.8	73.8	170	100
H	67.6	199.5	170	140

a Sample lost.

by analyzing eight groups of fruits separated according to the number of seeds per fruit (Table III). There was no significant difference in LARL and total limonoid glucoside concentrations among the eight groups in the flesh (Table IV). This shows that there is no correlation between the number of seeds and LARL content, and that the seeds do not play an important role in the removal of limonoids from the edible flesh in citrus fruit. The limonoids that accumulate in the seeds are most likely biosynthesized in the seeds, supporting our hypothesis that seeds possess their own limonoid metabolic and catabolic systems. In fact, radioactive tracer work has shown previously that citrus seeds are capable of converting [^{14}C]nomilin to other limonoids (Herman et al., 1991).

The ability of fruit tissue to metabolize limonoid glucosides into other limonoid glucosides or back to aglycons was investigated by Herman et al. (1991). Their results showed that [^{14}C]NG was not further metabolized in the mature albedo tissue of navel orange. Limonoid glucosides are most likely accumulated as end products. Thus, citrus tissues accumulate high concentrations of limonoid glucosides steadily without any decrease during late stages of fruit growth and maturation.

LITERATURE CITED

Dreyer, D. L. Citrus bitter principles. V. Botanical distribution and chemotaxonomy in the *Rutaceae*. *Phytochemistry* **1966**, *5*, 367.

Fong, C. H.; Hasegawa, S.; Herman, Z.; Ou, P. Limonoid Glucosides in Commercial Citrus Juices. *J. Food Sci.* **1989**, *54*, 1505.

Fong, C. H.; Hasegawa, S.; Coggins, C. W., Jr.; Atkin, D. R.; Miyake, M. Contents of limonoids and limonin 17-β-D-glucopyranoside in fruit tissue of Valencia orange during fruit growth and maturation. *J. Agric. Food Chem.* **1992**, *40*, 1178.

Hasegawa, S.; Herman, Z.; Orme, E.; Ou, P. Biosynthesis of limonoids in *Citrus*: sites and translocation. *Phytochemistry* **1986**, *25*, 2783.

Hasegawa, S.; Bennett, R. D.; Herman, Z.; Fong, C. H.; Ou, P. Limonoid Glucosides in *Citrus*. *Phytochemistry* **1989**, *28*, 1717.

Hasegawa, S.; Ou, P.; Fong, C. H.; Herman, Z.; Coggins, C. W., Jr.; Atkin, D. R. Changes in the limonoate A-ring lactone and

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limonin 17-β-D-glucopyranoside content of navel oranges during fruit growth and maturation. *J. Agric. Food Chem.* **1991**, *39*, 262.

Herman, Z.; Fong, C. H.; Hasegawa, S. Biosynthesis of Limonoid Glucosides in Navel Orange. *Phytochemistry* **1991**, *30*, 1487.

Maier, V. P.; Hasegawa, S.; Hera, E. Limonin D-ring-lactone hydrolase. A new enzyme from citrus seeds. *Phytochemistry* **1969**, *8*, 405.

Maier, V. P.; Bennett, R. D.; Hasegawa, S. Limonin and other limonoids. In *Citrus Science and Technology*; Nagy, S., Shaw, P. E., Veldhuis, M. K., Eds.; AVI Publishing: Westport, CT, 1977; Vol. 1, p 355.

Ozaki, Y.; Fong, C. H.; Herman, Z.; Maeda, H.; Miyake, M.; Ifuku, Y.; Hasegawa, S. Limonoid Glucosides in Citrus Seeds. *Agric. Biol. Chem.* **1991a**, *55*, 137.

Ozaki, Y.; Miyake, M.; Maeda, H.; Ifuku, Y.; Bennett, R. D.; Herman, Z.; Fong, C. H.; Hasegawa, S. Ichangensin Glucoside in *Citrus Junos, Citrus Sudachi and Citrus Sphaerocarpa*. *Phytochemistry* **1991b**, *30*, 2659.