Limonoid Glucosides in Orange Juices by HPLC

Zareb Herman,* Chi H. Fong, Peter Ou, and Shin Hasegawa

Fruit and Vegetable Chemistry Laboratory, Agricultural Research Service, U.S. Department of Agriculture, 263 South Chester Avenue, Pasadena, California 91106

A simple HPLC method was developed to quantify the major limonoid glucosides in orange juice. This method measures the concentrations of the glucosides of limonin, nomilin, nomilinic acid, deacetylnomilinic acid, and obacunone. Fourteen commercial orange juices were found to contain 331 ± 51 ppm of total limonoid glucosides. Replicate and recovery studies demonstrate that the method is both reproducible and accurate.

INTRODUCTION

Limonoid-caused bitterness in citrus juices, such as navel orange juice, is a major problem in the citrus industry with bitter juices having a lower market value for producers. Bitter juices are either blended with nonbitter juices to obtain an acceptable product or more often sold separately at a reduced price to drink manufacturers. Limonin is the primary limonoid that is responsible for citrus juice bitterness. This subject has been reviewed by Dekker (1988).

Recently it was discovered that limonoids are also present in glucoside form in citrus (Hasegawa et al., 1989; Bennett et al., 1989). Each of these compounds contains one glucose molecule attached via a β-glucosidic linkage to the 17-position of the limonoid A-ring lactone. Fong et al. (1989) have reported a TLC method for quantifying total limonoid glucosides in orange juices and an HPLC method for quantifying the glucoside of limonin (limonin 17-β-D-glucopyranoside). The concentrations of these glucosides in orange juices are roughly 100 times the concentrations of the aglycon forms.

Orange juices contain a number of different limonoid glucosides. This paper describes a simple HPLC method for quantifying the major limonoid glucosides in orange juices.

EXPERIMENTAL PROCEDURES

Materials. Fourteen commercial frozen concentrated orange juices were obtained from local food stores. Each juice was diluted to 11.8° Brix prior to analysis. Limonoid glucoside standards were purified in our own laboratory according to the method of Hasegawa et al. (1989).

Extraction of Limonoid Glucosides. Methanol (100 mL) was added to 50 mL of each orange juice sample. This mixture, containing approximately 70% MeOH, was homogenized with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). It was then centrifuged at 13000*g* for 10 min, and the supernatant was vacuum filtered by using Whatman No. 42 filter paper. The pellet was homogenized again with 100 mL of 70% MeOH, centrifuged, and filtered. The filtrates were combined, and the MeOH and water were evaporated with a rotary evaporator until a volume of approximately 5–10 mL of H_2O remained. This was then brought to a volume of exactly 20 mL of H_2O.

One milliliter of the above extract was loaded onto a C_{18} Sep-Pak column (Waters Associates, Milford, MA), washed with H_2O, and eluted with MeOH. This was evaporated to dryness in a test tube, 1 mL of MeOH was added, and it was sonicated for 10 min. Then 100 μL was placed in a test tube, evaporated, and treated with 10 mg of hesperidinase enzyme (Sigma Chemical Co., St. Louis, MO) in a total volume of 1.0 mL of 0.1 M sodium formate buffer, pH 3.8. This was incubated overnight at room temperature. It was then loaded onto a C_{18} Sep-Pak column, washed with H_2O, and eluted with MeOH. The MeOH was evaporated in a test tube; 1 mL of H_2O was added, and it was sonicated for 10 min. This was immediately analyzed by HPLC.

HPLC and TLC. For each sample extracted, duplicate 100-μL injections were made onto a C_{18} reverse-phase analytical HPLC column (4.6 × 250 mm, 5-μm particle size, Alltech Associates, Deerfield, IL). The flow rate was 1 mL/min. The column was eluted by using a linear gradient starting with 10% CH_3CN in 0.003 MH_3PO_4 and ending with 26% CH_3CN at 56 min. Compounds were detected by UV absorption at 210 nm. The spectrophotometer was connected to a Shimadzu CR3A integrator (Shimadzu Corp., Kyoto, Japan). Each compound was quantified by peak area. Average values from the two injections were used. Standard curves were run for each limonoid glucoside.

Three extracted juice samples were spiked with 1 μg of each of the limonoid glucosides being quantified. This was analyzed by HPLC as above. In addition, three concentrated juice samples were injected onto the HPLC, and each glucoside peak was collected. A UV scan was performed on each peak fraction collected as well as on each limonoid glucoside standard. This was done by using a Gilford response spectrophotometer (Gilford Corp., Oberlin, OH). Each peak fraction was then loaded onto a C_{18} Sep-Pak column, eluted with MeOH, evaporated, and spotted onto two silica gel TLC plates. One plate was developed with EtOAc–methyl ethyl ketone–formic acid–H_2O (5:3:1:1). The other plate was developed with CH_2Cl_2–MeOH–HOAc–H_2O (40:20:3:1). Limonoids were visualized by spraying with Ehrlich's reagent followed by exposure to HCl gas. The plates were then sprayed with 50% H_2SO_4 and heated to reveal any other compounds that might have coeluted with each peak during HPLC.

Replicate Study. Four 50-mL identical orange juice samples, 11.8° Brix, were analyzed as above by HPLC for limonoid glucosides.

Recovery Study. Four 50-mL identical orange juice samples, 11.8° Brix, were each spiked with 100 ppm of limonin glucoside (LG), nomilinic acid glucoside (NAG), nomilin glucoside (NG), deacetylnomilinic acid glucoside (DAG), and obacunone glucoside (OG). These were analyzed as above by HPLC.

RESULTS AND DISCUSSION

Figure 1 shows the HPLC profile of one of the commercial frozen concentrated orange juices that was analyzed. As the figure shows, this method produced good peak resolution for the major limonoid glucosides in orange juice. LG eluted at 29.2 min (peak 1). DAG eluted at 34.6 min (peak 2). NG eluted at 48.0 min (peak 3). NAG eluted at 49.1 min (peak 4). OG eluted at 53.8 min (peak 5).

When extracted juice samples were spiked with 1 μg of each limonoid glucoside standard, each standard peak coeluted with the corresponding peak in the juice. When each peak was collected and spotted on TLC and the plates were sprayed with H_2SO_4 and heated, no additional compounds were visualized on either of the two plates. This suggests that each peak collected is pure. In addition, the UV scan of each collected peak matched perfectly the scan of the corresponding limonoid glucoside standard.

Treatment of samples with hesperidinase proved helpful in obtaining good peak resolution. Hesperidinase cleaves the sugars off several of the flavonoid glucosides present

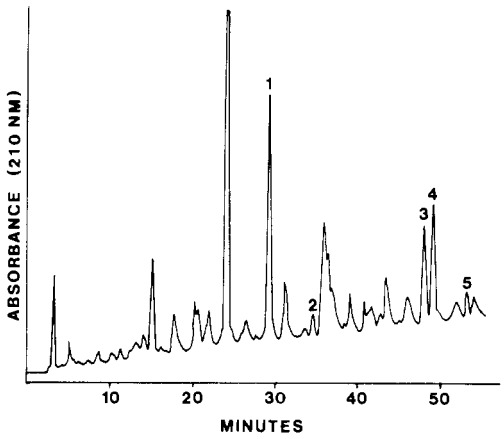

Figure 1. HPLC chromatogram of orange juice. Peak 1, limonin glucoside; peak 2, deacetylnomilinic acid glucoside; peak 3, nomilin glucoside; peak 4, nomilinic acid glucoside; peak 5, obacunone glucoside.

Table I. Limonoid Glucosides in Orange Juices

	limonoid glucoside,[a] ppm					
sample	LG	NAG	NG	DAG	OG	total
1	194	90	76	25	4	389
2	183	81	52	26	3	346
3	152	67	33	20	1	273
4	199	75	64	14	2	363
5	161	75	38	19	3	295
6	163	71	41	16	3	294
7	145	55	36	14	1	252
8	131	58	49	16	2	256
9	192	113	48	25	4	382
10	162	93	39	22	2	318
11	190	74	37	24	3	328
12	211	101	54	24	5	396
13	209	108	41	26	5	390
14	168	101	53	26	4	353
mean	176	84	47	21	3	331
SD	25	18	12	5	1	51

[a] LG, limonin glucoside; NAG, nomilinic acid glucoside; NG, nomilin glucoside; DAG, deacetylnomilinic acid glucoside; OG, obacunone glucoside.

in orange juice, such as rutin, narirutin, and hesperidin. Before enzyme treatment, hesperidin eluted at 46.3 min. This interfered with the quantification of NG. After enzyme treatment, this was no longer a problem. Hesperidinase exhibited no glucosidase activity on control solutions of purified limonoid glucosides.

One compound not included in our analysis is deacetylnomilin glucoside (DG). DG is present in orange juice at concentrations of approximately 15 ppm, but was not included because of poor peak resolution. It elutes at 40.6 min.

Table I gives the results of the HPLC analysis of limonoid glucosides in orange juices. LG was the predominant limonoid glucoside present in all juice samples, wtih concentrations in the 130–210 ppm range. This was followed by NAG, NG, DAG, and OG in order of decreasing concentration.

The high concentration of nomilin glucoside (NG) in orange juice is notable. The aglycon nomilin has been shown in mice to be a potent inducer of glutathione *S*-transferase (GST), an enzyme that detoxifies certain carcinogens (Lam et al., 1989). In addition, limonin has exhibited anticarcinogenic properties in hamsters (Miller et al., 1989). Experiments are currently underway to determine if NG, LG, or other limonoid glucosides also induce GST or prevent tumor formation.

As Table I shows, the relative concentration of each limonoid glucoside was fairly constant in the 14 juices analyzed. However, the total concentration showed greater variation. Possible sources of this variation include orange variety, cultivar, maturity, and commercial processing methods used.

Table II. Replicate Study[a]

	limonoid glucoside,[b] ppm					
LG	NAG	NG	DAG	OG	total
168 ± 5	102 ± 4	35 ± 2	24 ± 2	4 ± 0.5	335 ± 8

[a] $N = 4$. [b] Values are mean ± SD.

Table III. Recovery Study[a]

	percentages/recovered[b]			
LG	NAG	NG	DAG	OG
97 ± 3	95 ± 2	97 ± 2	98 ± 4	98 ± 2

[a] $N = 4$ identical samples spiked with 100 ppm of each limonoid glucoside. [b] Values are mean ± SD.

Table II gives the results of the replicate study. The limonoid glucoside values obtained for the four identical samples were quite similar. This is evidenced by the small standard deviations and shows that the extraction and quantification methods produce consistent results.

Table III gives the results of the recovery study. In the spiked samples, the recoveries ranged from 95 to 98%, suggesting that roughly 2–5% of the compounds are being lost during the extraction procedure. These recoveries are certainly within acceptable limits.

The values for total limonoid glucosides in orange juice by HPLC averaged 331 ppm (Table I). This agrees closely with the value of 320 ppm by TLC reported by Fong et al. (1989). The method reported here by HPLC not only gives a reliable method for total limonoid glucosides but quantifies the individual glucosides as well. This method is useful to those wishing to quantify the compounds in citrus products or those attempting to purify specific limonoid glucosides for cancer research. The method will also be of interest to those doing flavor research, as the flavor of the individual limonoid glucosides has yet to be determined.

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Registry No. Limonin glucoside, 123564-61-4; nomilinic acid glucoside, 125107-15-5; nomilin glucoside, 123564-62-5; deacetylnomilinic acid glucoside, 125107-16-6; obacunone glucoside, 123564-64-7.