Spectrophotometric Determination of Limonin in Orange Juice

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Limonin in navel orange juice was extracted twice with dichloromethane. The combined extracts were treated with acid-washed alumina and filtered, and the alumina was washed with chloroform. Combined dichloromethane and chloroform extracts were evaporated and the residue was partitioned between acetonitrile and petroleum ether. Limonin in the acetonitrile layer was determined by treatment with alkaline hydroxylamine followed by addition of acid ferric perchlorate solution and measurement of the absorbance of the solution at 510 mμ. This method was useful for determining limonin in the 5- to 40-p.p.m. range, and the results correlated well with the bitterness of navel orange juice. It was not satisfactory for orange peel, twigs, leaves, roots, or for grapefruit juice, because of interference from coumarins.

Navel oranges are not generally used for making processed orange juice because a bitter flavor develops that is unacceptable to the consumer. This bitter flavor is caused by limonin, primarily in the albedo and segment walls, which passes into the juice during processing. This bitterness level varies greatly from year to year. Investigations of bitterness in orange products have been hampered because a satisfactory method for the quantitative determination of limonin has been lacking; in fact, the structure of limonin was not known completely until comparatively recently (Arigoni *et al.*, 1960).

Limonin (see Figure 1) is a triterpenoid oxidation product containing one furan ring, one ketone group, one epoxide group, and two lactone rings. Chandler and Kefford (1966) developed an analytical procedure using the keto group based on conversion of limonin to the dinitrophenylhydrazone, purification of this derivative by thin-layer chromatography, and colorimetric analysis of the eluted limonin dinitrophenylhydrazone spot. Because the dinitrophenylhydrazone forms slowly, the procedure is lengthy. It is straightforward, however, and where sufficient time is available, it might be used for routine analyses. Nomura and Santo (1965), utilizing the furan ring, describe a direct colorimetric determination of limonin in the benzene extract of citrus juice, using *p*-dimethylaminobenzaldehyde and sulfuric acid. This method has not given the authors reproducible results nor has it proved specific for limonin, since colors were produced from nonbitter juice. The interfering substances were not identified. A titrimetric method for limonin assay has been developed (Emerson, 1952) in which limonin is oxidized to limonilic acid by alkaline hypoiodite. This reaction is not sufficiently sensitive for the determination of limonin in the p.p.m. range, and the low specificity of the reaction requires an almost perfect isolation and purification procedure. A thin-layer chromatographic method has been developed by Dreyer (1965) using Erlich's reagent to detect limonin; however, the color intensities of the spots are so difficult to reproduce that the method gives only semiquantitative results.

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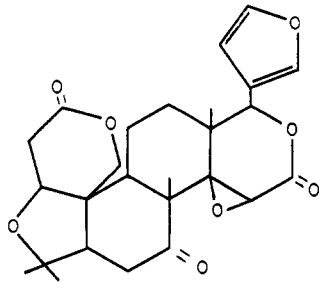

Figure 1. Limonin

The authors have tried to develop a simple, rapid, analytical method for determination of limonin for routine use. In the course of this work, several methods were investigated and found to be unsuitable. Reaction with *m*-dinitrobenzene in alcoholic base, useful for some ketones (Beher and Gaebler, 1951; Nathanson and Wilson, 1943), gave colors that were not sufficiently intense and that faded rapidly. Direct polarographic reduction of limonin in acidic or basic methanol or in aqueous solutions gave no wave characteristic of the ketone group. Concentrated sulfuric acid reacted with limonin at room temperature to form a red-brown product with intensity proportional to limonin concentration; however, the absorption spectrum showed only a monotonic absorbance that increased toward the shorter wavelengths. This lack of a definite peak indicated a complex reaction with the formation of many products, which was unsuitable for an analytical method.

A satisfactory method was developed based on formation of the colored ferric complex of the hydroxamic acid derivative of limonin. Although lactone rings in limonin are considered to be rather nonreactive (Geissman and Tulagin, 1946), both lactone groups can be opened by strong base (Emerson, 1952). An alkaline hydroxamation method (Gutnikov and Schenk, 1962) proved satisfactory for the conversion of limonin to the hydroxamic acid. Dichloromethane was a satisfactory solvent for extraction of limonin from orange juice; however, it also extracted some interfering substances. These substances were removed by passage of the extract through alumina, evaporation of the solvent, and partitioning of the residue between acetonitrile and petroleum ether (30° to 60° C.).

The acetonitrile layer contained the limonin and was used directly for colorimetric analysis. The presence of possible interfering substances is discussed later.

APPARATUS

Bausch & Lomb Spectronic 20 colorimeter with 1-inch tubes or similar instrument. Centrifuge capable of accommodating 125-ml. separatory funnels and 250-ml. bottles.

REAGENTS

Standard Limonin Solution. A solution of 0.100% limonin (K and K Laboratories, Inc.) and 0.05% butylated hydroxytoluene in acetonitrile was used as standard.

Alumina. Certain lots of acid-washed alumina (Merck) performed satisfactorily as received; others held limonin so tenaciously that it could not be removed by elution with chloroform. In the latter case, the alumina should be washed with a solution of 5.9% sodium acetate, 18.5% distilled water, 2.6% glacial acetic acid, and 73% ethanol; dried for 2 hours at 105° C.; and cooled.

Acetonitrile, Dichloromethane, Acetone, and Petroleum Ether (30° to 60° C.). Reagent or ACS grades.

Hydroxamation Reagent. For Solution A, sodium hydroxide, 167 grams, was dissolved in sufficient methanol to make 1 liter of solution. For Solution B, 96% hydroxylamine hydrochloride, 65 grams, was dissolved in sufficient methanol to make 1 liter of solution. Equal volumes of Solutions A and B were mixed and the precipitated sodium chloride was removed by filtration through Whatman No. 1 filter paper or its equivalent. The reagent should be prepared immediately before use, as it gives high blank readings if it stands more than 2 hours.

Acid Ferric Perchlorate Reagent. Ice-cold methanol, 80 ml., was placed in a 1000-ml. volumetric flask; 70% perchloric acid, 72 ml., was added slowly, and 2% ethanolic ferric perchlorate solution, 40 ml., was then added. The solution was warmed to room temperature and diluted to volume with methanol. (This solution will be stable for at least a week.)

Testing Acidity of Reagents. Each new batch of reagents was tested before use as follows: Solutions A and B, 5.00 ml. each, were pipetted into a flask, mixed but not filtered, and titrated with standard acid to the phenolphthalein end point. The concentration of Solution A was adjusted until the mixture was 1.60N in sodium hydroxide.

Acid ferric perchlorate reagent, 10.00 ml., was titrated with standard base to the bromphenol blue end point. The reagent should be 0.875N in acid.

The following relationship should hold for the two reagents: (N ferric reagent) $-$ $\frac{(N \text{ solutions A} + \text{B})}{2}$ = between 0.025 and 0.150

Butylated Hydroxytoluene (2,6-di-*tert*-butyl-*p*-cresol). Reagent of purified grade was used as received.

ISOLATION OF LIMONIN FROM ORANGE JUICE

Step 1. Approximately 100 ml. of juice (200 ml. if the limonin content was expected to be less than 10 p.p.m.) were centrifuged at 2000 r.p.m. for 5 minutes, and the upper layer was decanted.

Step 2. Centrifuged juice, 65 grams, was placed in a 125-ml. separatory funnel that could be centrifuged. (If the limonin content was expected to be less than 10 p.p.m., two 65-gram portions of juice were extracted, Step 2, and each portion chromatographed, Step 3, but solutions were combined before solvents were evaporated.) Dichloromethane, 20 ml., was added; the mixture was then shaken thoroughly and centrifuged at 1200 r.p.m. for 10 minutes to separate the layers. The lower organic phase containing limonin was removed and the aqueous phase again extracted with 10 ml. of dichloromethane and again centrifuged to separate the layers. The dichloromethane extracts were combined (the juice layer and precipitated solids were discarded), and a few milligrams of butylated hydroxy toluene were added.

Step 3. Acid-washed alumina (Merck), 3 ml., was added to the combined dichloromethane extracts; the solution was mixed and then filtered through a fritted funnel. The alumina was washed with 10 ml. of chloroform and the chloroform was added to the dichloromethane solution. Solvents were evaporated on a steam bath.

Step 4. Acetonitrile, 9.0 ml., and petroleum ether, 50 ml., were added to the residue (30° to 60° C.). The solution was mixed and transferred to a separatory funnel; the acetonitrile layer was removed to a 25-ml. volumetric flask.

COLORIMETRIC DETERMINATION OF LIMONIN

Hydroxamation reagent, 5.00 ml., was added, by pipet, to the acetonitrile solution of limonin in the 25-ml. volumetric flask, mixed well, and allowed to stand 30 minutes at room temperature. Acetone, 1 ml., was added, the solution mixed, and acid ferric perchlorate reagent, 10.00 ml., added by pipet. After stirring, volume was adjusted to 25.00 ml. with acetonitrile and the sample was allowed to stand for 30 minutes. Two standards, prepared from 1.00 ml. of standard limonin solution and 7.5 ml. of acetonitrile, and a blank of 8.5 ml. of acetonitrile containing no limonin were treated with hydroxamation reagent, acetone, and ferric perchlorate reagent and then diluted to volume according to the procedure used for the samples. The contents of the volumetric flasks were transferred to 1-inch colorimeter tubes and the absorbance at 510 mμ was measured against that of the reagent blank. Milligrams of limonin in each sample were then calculated by comparing the absorbancies of the samples and standards as follows:

$$\text{Mg. of limonin} = (\text{mg. of limonin in standard}) \frac{(\text{absorbance of sample})}{(\text{absorbance of standard})}$$

For maximum accuracy, a standard aqueous solution of limonin should be taken through the entire analytical procedure, as small losses of limonin occur in Steps 2, 3, and 4 of the isolation procedure. It was necessary to do this only when new lots of dichloromethane, alumina, petroleum ether, or acetonitrile were used. In a typical instance, 88% recovery of limonin was found. This value was used as a correction factor for all analyses using the same lots of reagents. To calculate p.p.m. of limonin in juice, the following relationship was used, which included

an empirically determined correction factor to compensate for small losses of limonin:

$$\text{P.p.m.} = \frac{\text{mg. of limonin}}{\text{grams of juice}} \times \frac{1000}{0.88}$$

Table I shows results of typical limonin analyses on known mixtures of limonin and a reconstituted commercial non-bitter juice concentrate. These results indicate the variability to be expected from this method. Without added limonin, the three replicate determinations gave an average value of 3.0 ± 1.2 p.p.m.; with 18.5 p.p.m. of added limonin, the results were 21.4 ± 0.6 p.p.m. (expected value 21.5 ± 1.2 p.p.m.); and with 37.0 p.p.m. of added limonin, the results were 40.3 ± 1.1 p.p.m. (expected value 40.0 ± 1.2 p.p.m.). The standard deviation of the results was of the order of 1 p.p.m. regardless of the magnitude of the limonin content.

ORGANOLEPTIC TESTING

Members of an expert taste panel were selected from the laboratory staff on the basis of the consistency of their judgments in rating standard juice samples that were presented to them at intervals over a period of several weeks. Three persons proved to make consistent and reliable judgments. These were selected as members of the panel. Because of the problems presented by the lingering bitter aftertaste of limonin, only three juice samples (cooled to 10° to 15° C.) were presented to the panel at any one time, and no control samples were tasted along with the test samples. Each panel member rated a juice on a 0 to 3 scale, and the sum of the members' ratings for each sample was used to indicate a bitterness score.

Table I. Recovery of Limonin Added to Reconstituted Orange Juice Concentrate

Limonin Added, P.P.M.	Limonin Found, P.P.M.	Recovery,[a] %
0	1.9	
0	2.3	
0	4.7	
18.5	21.7	101
18.5	20.5	95
18.5	21.9	102
37.0	38.8	97
37.0	41.5	104
37.0	40.6	101

[a] Assuming 3.0 p.p.m. limonin initially present.

DISCUSSION OF METHOD

Hydroxamation and Ferric Complex Formation. Neutral methanolic hydroxylamine was unreactive toward limonin at room temperature and at reflux. An alkaline hydroxamation reagent is required to react with the stable lactone rings of limonin. With the alkaline reagent, the hydroxamation of limonin was complete at ambient temperature in about 10 minutes; thus, the 30-minute reaction period specified in the analytical procedure includes a considerable safety factor.

Other authors (Goddu *et al.*, 1955; Gutnikov and Schenk, 1962) recommend that colored ferric hydroxamates be formed at acidities of 0.08*M* and 0.10*M*; however, as shown by data in Figure 2, these acidities are too great for the satisfactory development of the limonin–ferric hydroxamate color. The most intense color was developed at the lowest acidity investigated (0.01*M*). Because the final acidity of the colored solution is determined by a comparatively small difference between equivalents of base added in the hydroxamation reagent and equivalents of acid added in the color development reagent, a very low acidity might be difficult to maintain reproducibly. A value of 0.03*M* for the final acidity was chosen as a compromise, inasmuch as increasing the acidity to this value caused only a slight drop in color intensity and could be

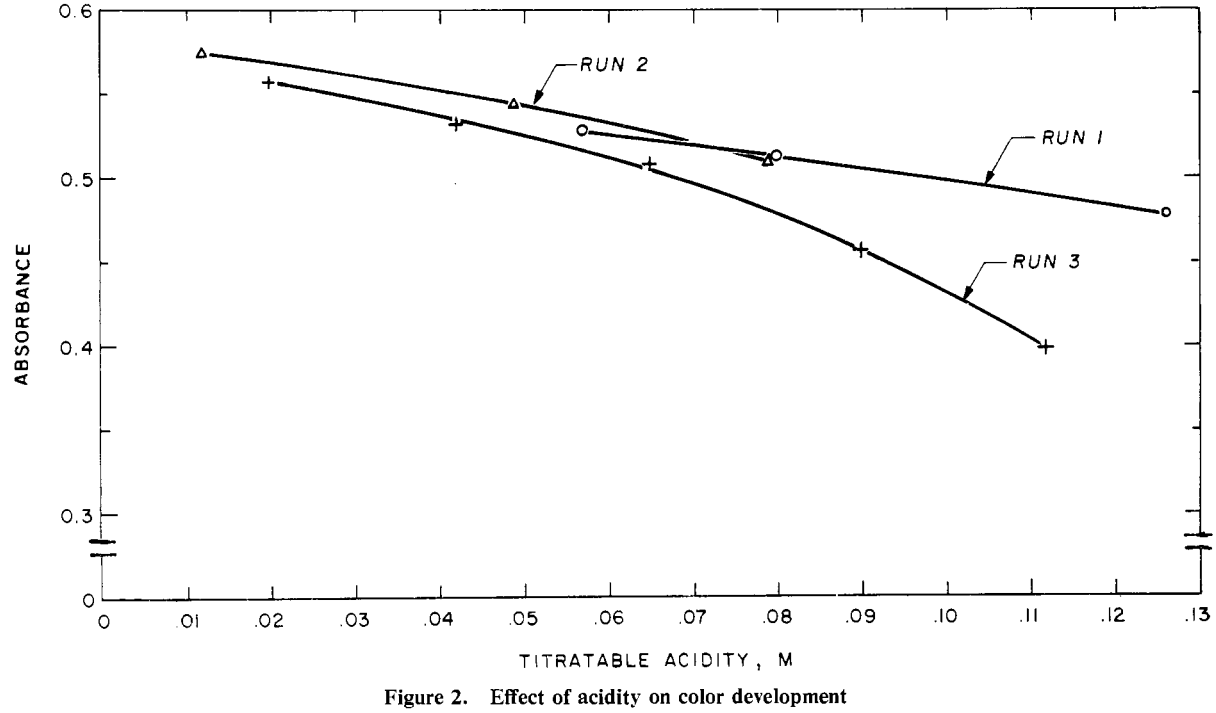

Figure 2. Effect of acidity on color development

reproduced more easily than 0.01*M* acidity. Because acidity of final solution was important, the analytical procedure includes a test for acidities of reagents before they were used on actual samples.

The developed color from most hydroxamate procedures is not stable, presumably owing to reduction of the ferric iron by excess hydroxylamine (Goddu *et al.*, 1955; Gutnikov and Schenk, 1962). The addition of acetone, to combine with the hydroxylamine, has been recommended (Gutnikov and Schenk, 1962) as a means of stabilizing the color but, as shown by data in Figure 3, acetone did not prevent a slow decrease in the color intensity of limonin ferric hydroxamate. After 15 minutes, the change in color was so slow as to be insignificant, if readings of sample and standard colors were within 5 minutes of each other.

The absorption maximum of the limonin ferric hydroxamate varied from 490 to 520 mμ., depending on the acidity —the higher acidity moving the maximum to longer wavelengths. At the suggested acidity of 0.03*M*, the maximum was at 510 mμ, but the peak was quite broad, so slight shifts in the maximum caused no errors. For standard limonin solutions, Beer's law was obeyed at 510 mμ and molar absorptivity was about 1200. Because absorptivity varies slightly from one batch of reagents to the next, due to slight differences in final acidity, standards should always be analyzed with each set of samples.

Interfering Substances in Orange Juice. The hydroxamation reaction is not specific for limonin; rather, it measures total lactone and ester content of the juice. The success of the method depends on the removal of interfering esters or lactones by appropriate steps. Experiments in this laboratory with known compounds indicated that the following limonoids all gave colors similar to limonin: nomilin, obacunone, limonilic acid, obacunoic acid, and limonin oxime. The following coumarins were tested and found to give interfering colors: limettin, bergapten, and isopimpinellin.

During experiments to develop the isolation procedure, at least three classes of material in orange juice that interfere with limonin analysis were isolated. The structures of these materials were not determined, but they can be characterized as follows:

A material that can be eluted from an alumina column with petroleum ether or benzene and that can be separated from limonin by partitioning between acetonitrile and petroleum ether (present in amounts equivalent to about 20 p.p.m. of limonin in early-season juice and about 80 p.p.m. of limonin in late-season juice).

A material that can be eluted from an alumina column with chloroform along with limonin and that can be separated from limonin by partitioning between acetonitrile and petroleum ether (present in small amounts in early-season juice and in amounts equivalent to about 12 p.p.m. of limonin in late-season juice).

A material that is retained on an alumina column after limonin is eluted with chloroform (present in late-season juice in an amount equivalent to about 25 p.p.m. of limonin; no information was obtained on typical concentrations in early-season juice).

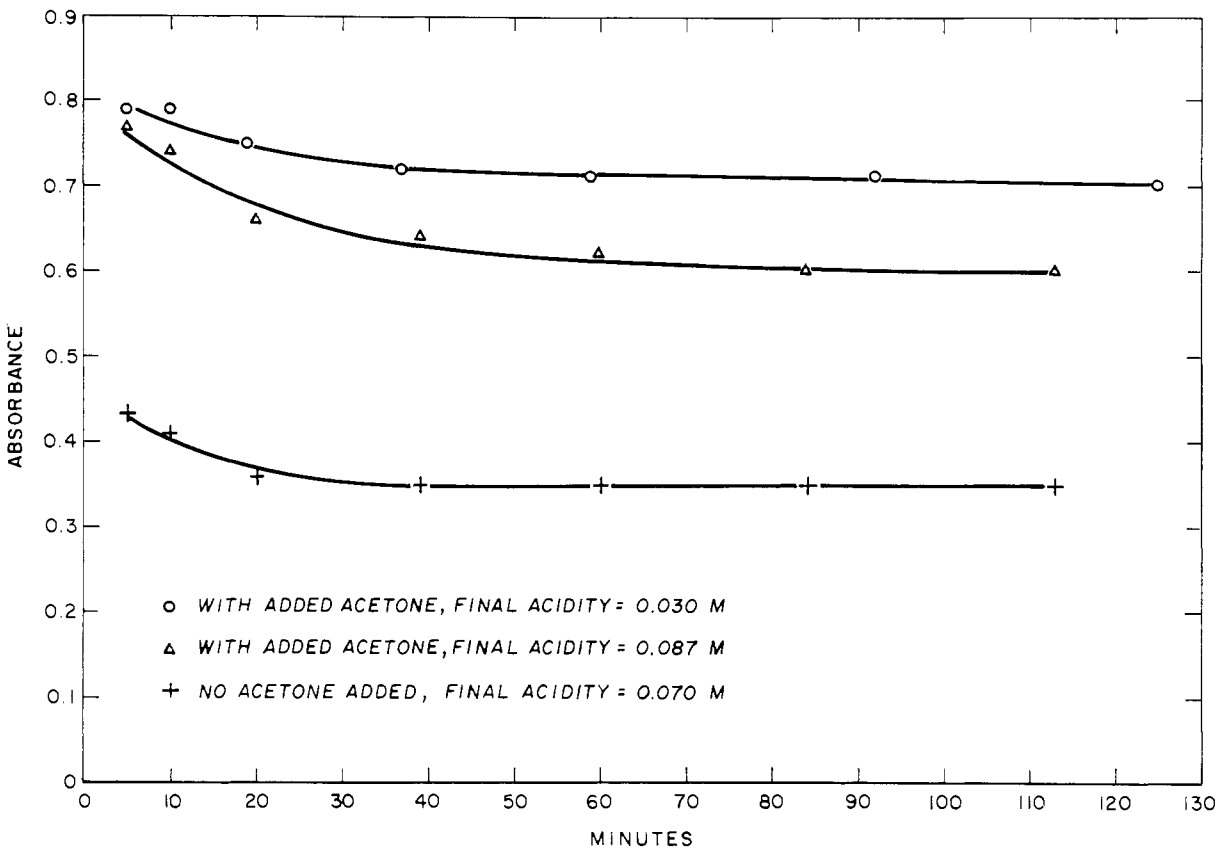

Figure 3. Stability of formed color

When the prescribed analytical method is followed, these interferences are eliminated, and the low limonin values obtained for nonbitter juice actually indicate traces of limonin and probably some limonilic acid (determined by thin-layer chromatography).

The amount of interfering material extracted from the juice can be minimized by centrifuging the juice and extracting only the resulting semiclear liquid. Because the analytical method was designed to give an objective measure of bitterness, and because the taste of the juice depends primarily on the concentration of limonin in solution, it was desirable to remove the solid material by centrifugation before extracting limonin. Chloroform extracted more interfering substances than did dichloromethane and therefore is not recommended as an extractant.

As limonin is sensitive to air oxidation, an antioxidant was added to the juice extract to prevent serious losses during the limonin isolation procedure (Chandler and Kefford, 1966). Three antioxidants were examined for suitability. Butylated hydroxyanisole and 2-mercaptobenzothiazole caused considerable interference in the colorimetric determination of limonin, and therefore were unusable; however, butylated hydroxytoluene caused no interference.

ANALYSES OF JUICE AND OTHER ORANGE PRODUCTS

Canned Juice and Concentrate. Samples of commercial juice and juice concentrate were supplied by Sunkist Growers, Orange Products Division, Ontario, Calif. The material was sealed in 6-oz. cans and kept frozen until the limonin analyses were made. In no case did more than 1 month elapse between the time of processing and the time of the limonin analysis. The analytical values for limonin content of these juices were compared with the bitterness ratings indicated by the three-member taste panel. These data are presented in Table II. Bitterness and limonin content tended to decrease directly with the length of the growing season, and the correlation between p.p.m. of limonin and degree of bitterness was reasonably good except for the concentrate sample dated 1/3/66, which clearly had too high a limonin content to be rated so low on the bitterness scale. Presumably, the limonin contents of single-strength juices and the reconstituted concentrates should be nearly identical for any given date of sampling. Only rarely, however, did the two values agree. Whether these differences represent uncertainties in the analytical method or variations in the composition of juice and concentrate on a given day at the Sunkist processing plant is not known.

Fresh Juice. Fresh navel oranges were obtained from the Citrus Experiment Station of the University of California at Riverside. Fruit was picked from three trees, each on a different rootstock, having similar growing conditions. Fruit was picked at monthly intervals to obtain increasingly mature fruit. Juice was squeezed by hand, and analyses were made on the fresh juice and on the juice after it had been heated to 70° C. for 2 hours and cooled. The results of these analyses are given in Table III. The limonin contents of unheated fresh juices were low and none of the juices was bitter. The taste threshold for limonin was between 4 and 5 p.p.m. The early-season juices definitely were more bitter than the late-season ones. In each case, heating increased the concentration of limonin and the bitterness of the juice.

Juice from the tree on Trifoliate rootstock was definitely less bitter than juices from trees on Troyer or Cleo rootstocks (Table III); however, it is questionable whether conclusions about the effect of rootstock on bitterness should be drawn from data obtained from a single tree of each rootstock. Other workers (Chandler, Kefford, and Lenz, 1966; Marsh, 1953) have also found juice from trees on Trifoliate rootstock to be less bitter than juice from trees on other rootstocks, so one may attribute the observed variations to rootstock rather than to growing conditions with some confidence.

Some lots of different clonal varieties of early-season navel oranges on different rootstocks were obtained from orchards in the vicinity of the Kearney Horticultural Field Station at Parlier, Calif. Fresh and heated juices from these oranges were analyzed as above, and data are shown in Table IV. There was little limonin in the fresh juices, and the juices were not bitter. Even heated juices contained only moderate amounts of limonin and were of low to intermediate bitterness. Because only small differences in bitterness were observed, and only one sample of each type of orange was available, only tentative

Table II. Limonin Content of Commercial Juice and Reconstituted Concentrate

Sampling Date	Single-Strength Juice					Reconstituted Juice Concentrate				
	Brix[a]	Acid,[a] %	Limonin, p.p.m.	Bitterness		Brix[a]	Acid,[a] %	Limonin, p.p.m.	Bitterness	
				Judgments	Score				Judgments	Score
12/13/65	10.8	1.11	42	2,2,2	6	10.8	1.07	25	2,2,2	6
12/28/65	12.2	0.81	13	1,1,0	2	10.9	0.82	18	0,2,2	4
1/3/66	11.4	0.92	23	1,2,2	5	10.9	0.98	33	1,1,2	4
1/13/66	12.2	0.95	25	2,2,2	6	10.9	0.92	35	2,2,3	7
1/18/66	11.0	0.90	20	1,1,1	3	10.9	0.88	28	2,2,2	6
1/25/66	10.9	0.85	23	1,2,2	5	10.9	0.91	29	2,2,2	6
2/15/66	9.8	0.70	18	1,2,2	5	10.8	0.84	25	2,2,2	6
3/1/66	12.8	0.95	14	2,1,1	4	10.9	0.79	18	1,1,1	3
3/7/66	11.6	0.92	14	2,2,1	5	10.8	0.77	14	1,1,1	3
4/1/66	13.0	0.83	8	1,1,0	2	10.9	0.71	10	1,1,1	3
4/6/66	14.2	0.90	2.3	0,0,0	0	[b]	[b]	[b]		[b]
4/18/66	13.8	0.76	5.6	0,0,0	0	13.8	0.77	6.1	0,0,0	0

[a] Information supplied by Sunkist Growers, Orange Products Division.
[b] Sample was not supplied.

Table III. Limonin Content as Influenced by Rootstock

Picking Date	Rootstock	Fresh Juice		Heated Juice		
		Limonin, p.p.m.	Bitterness score	Limonin, p.p.m.	Bitterness Judgments	Bitterness Score
12/15/65	Troyer	2.0	0	26	2,2,1	5
12/15/65	Cleo	4.0	0	19	1,1,1	3
12/15/65	Trifoliate	1.3	0	11	1,0,1	2
1/19/66	Troyer	3.0	0	16	1,2	4½
1/19/66	Cleo	8.0	0	12	1,1,1	3
1/19/66	Trifoliate	1.5	0	5.5	1,1,1	3
2/16/66	Troyer	1.3	0	2.7	0,0,0	0
2/16/66	Cleo	2.1	0	4.9	0,1	1½
2/16/66	Trifoliate	1.3	0	1.6	0,0,0	0
3/17/66	Troyer	0.5	0	5.3	0,1,0	1
3/17/66	Cleo	0.8	0	2.0	0,0,0	0
3/17/66	Trifoliate	0	0	1.3	0,0,0	0

Table IV. Limonin Content of Miscellaneous Navel Orange Juices

Description of Fruit and Rootstock	Age of Trees, Yr.	Fresh Juice		Heated Juice		
		Limonin, p.p.m.	Bitterness score	Limonin, p.p.m.	Bitterness Judgments	Bitterness Score
Tulegold on Troyer[a]	2	1	0	14	1,1,1	3
Tulegold on Trifoliate[a]	2	1	0	16	1,1,2	4
Frost Nucellar on Trifoliate[a]	2	0	0	14	1,1,1	3
Frost Nucellar on Trifoliate[b]	12	1	0	9	1,0,1	2
Atwood on Rough Lemon[b]	20	0	1	17	2,2,1	5
Washington on Rough Lemon[b]	20	2	0	12	1,0,1	2
Atwood on Trifoliate[b]	12	0	0	11	1,1,2	4
Atwood on Troyer[b]	12	2	0	18	1,1,1	3

[a] From orchards of A. Mabs.
[b] From orchards of Russ Davis.

conclusions on the effects of variety or rootstock can be drawn from these data.

The results obtained by the method reported in this paper are similar to those of Chandler and Kefford (1966) and Chandler, Kefford, and Lenz (1966) with regard to limonin contents and bitterness. They found 3 to 6 p.p.m. of limonin in nonbitter juice, more than 9 p.p.m. in definitely bitter juice, and 24 to 30 p.p.m. in strongly bitter juice. No other quantitative determinations of limonin in navel orange juice have been reported. The present authors found general agreement between the thin-layer chromatographic results (run as occasional checks) and the method described herein; however, the thin-layer method gives only order-of-magnitude estimates of limonin content.

Grapefruit Juice. Limonin has been isolated from grapefruit juice (Maier and Dreyer, 1965) in concentrations up to 9.5 p.p.m. and is believed to contribute to the bitterness of that juice. Grapefuit juice was analyzed by the same procedure used for limonin, and about 25 p.p.m. of limonin were found; however, when the limonin content was estimated by a thin-layer chromatographic method (Dreyer, 1965), values of about 5 p.p.m. were obtained. Some interfering substance is probably present in grapefruit juice (perhaps a coumarin) that is not removed by the limonin isolation procedure described for orange juice. The presence of interfering substances was confirmed by altering Step 3 of the limonin isolation procedure so that limonin was removed from the alumina by washing with a 1 to 1 mixture of chloroform and benzene and collecting several separate 10-ml. fractions of the eluate. Limonin was mostly eluted in the first 10-ml. fraction, with a small amount in the second 10-ml. fraction. Subsequent fractions contained no limonin but did react with the analytical reagent to form colored ferric hydroxamate derivatives that interfered in the limonin analysis. When the limonin content was estimated by analyzing the first two 10-ml. fractions from the chloroform-benzene elution, values of about 4 p.p.m. instead of 25 p.p.m. were obtained. Before the method is used on grapefruit juice, the applicability of the modified method to juices from fruit of different varieties and maturities should be checked. The original version of the method described here for use with orange juice obviously is inapplicable to determination of limonin in grapefruit juice.

Peel, Leaves, Twigs, and Roots. Limonin has been stated to be in the seeds, albedo, central vascular bundle, and veins of the section covering of navel oranges (Higby, 1938) and has been found in the peel, endocarp, and seeds of grapefruit (Maier and Dreyer, 1965). Little is known about the occurrence of limonin in navel orange peel or in other parts of the orange tree. Some semiquantitative studies were carried out to determine whether the ferric hydroxamate method for orange juice could be applied to peel, leaves, twigs, and roots.

Limonin was estimated crudely by mixing the material with 1% citric acid in a Waring Blendor, heating the mixture to 70° C. for 2 hours, cooling, and then following the

analytical procedure for orange juice. The acetonitrile layer was removed (Step 4), the solvent was evaporated, and the residue was dissolved in 1.00 ml. of chloroform and chromatographed in 5-, 10-, and 20-μl. portions on a silica gel thin-layer plate using benzene and chloroform in a 1:1 ratio. Limonoids were detected by using Ehrlich's reagent (Dreyer, 1965) and the amounts estimated by comparison with standards. The results of the thin-layer chromatographic analysis were compared with those obtained by the ferric hydroxamate method as given for orange juice. In every case, the ferric hydroxamate method gave values for limonin content that were higher than those estimated from the thin-layer plates. The thin-layer plates showed spots that fluoresced blue under ultraviolet light and spots that gave blue or violet colors with Ehrlich's reagent, indicating the probable presence of indoles (Reio, 1960) and coumarins.

The indole derivatives should not interfere with the ferric hydroxamate analysis for limonin unless they are esters. Comparatively little is known about the exact structures of these indole derivatives, but some esters may be present along with many indoles that are not esters (Stowe, 1959). Coumarins have been isolated from roots and leaves of citrus (Feldman and Hanks, 1965) and from sweet orange essence (D'Amore and Calapaj, 1965) in amounts up to 200 p.p.m. Representative coumarins (limettin, bergapten, and isopimpinellin) were analyzed in this laboratory by the ferric hydroxamate procedure for limonin and found to produce comparable colors, indicating definite positive interferences in the limonin procedure. The procedure described here for limonin in orange juice clearly should not be used for peel, leaves, twigs, or roots. Suitable procedures possibly could be worked out for these products—particularly for peel, which seems to contain the fewest interfering substances.

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