Specific Thin-Layer Chromatography Assay of Limonin, a Citrus Bitter Principle

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Limonin, once it has formed in citrus juices or in acidified tissue suspensions, is removed using two chloroform extractions. The chloroform extracts are evaporated, and the residue is dissolved in a measured volume of acetonitrile. The acetonitrile solution is quantitatively spotted on a silica gel thin-layer chromatogram along with authentic limonin; the chromatogram is developed in benzene:ethanol:water:acetic acid (200:47:15:1, upper phase), dried, sprayed with Ehrlich's reagent, and treated with gaseous HCl. Readout is by visual or spectrodensitometric comparison of the unknown and known limonin spot densities. This method is specific for limonin, is sensitive to at least 0.5 p.p.m., and has been used on a wide variety of citrus fruit tissues and juices.

An analytical method is reported for limonin (Figure 1) the intensely bitter triterpenoid dilactone formed during the delayed bittering of citrus juices (Maier and Beverly, 1968). The method can also be used to determine indirectly the naturally occurring nonbitter limonoate A-ring lactone (potential limonin) of citrus fruit tissues (Maier and Margileth, 1969) by acid catalyzed conversion to limonin (Figure 1). Of the two current methods for determining limonin, one is limited primarily to orange juice because of interfering substances in other orange tissues and in the juices and tissues of other citrus fruits (Wilson and Crutchfield, 1968), and the other is quite lengthy (Chandler and Kefford, 1966). In addition, both methods are only moderately sensitive. The recent discovery of limonin in commercial grapefruit juice (Maier and Dreyer, 1965) and lemon juice (Maier, 1968) along with its previously known presence in certain orange juices prompted the development of this simple, more rapid, and specific method which is suitable for industrial quality control purposes as well as for research studies. This new method is similar in some respects to an approach used by Wilson and Crutchfield (1968) in semiquantitative tests for the presence of limonin in nonjuice tissues.

We have previously shown that limonin does not occur to any significant extent in normal, intact citrus fruits (with the exception of seeds). Rather limonoate A-ring lactone, a nonbitter limonoid, is present. When juice is extracted from citrus fruits, the acidic environment of the juice results in the eventual conversion of limonoate A-ring lactone (which enters the juice from the disrupted tissues) to limonin (Figure 1), and the juice becomes bitter. When analyzing freshly prepared juice for total limonin, conversion of limonoate A-ring lactone to limonin is speeded by boiling the sample before chloroform extraction. When analyzing the nonjuice tissues which are only mildly acidic, acid and heat are used to ensure total conversion of limonoate A-ring lactone to limonin. BHT is added to prevent possible oxidative loss of limonin during boiling (Chandler and Kefford, 1966).

Limonin, once it is formed in the juice or tissue suspensions, is extracted with chloroform, and quantitatively spotted on TLC. Final determination is then made directly on the chromatogram by visual or spectrodensitometric comparison with knowns, after color formation with a spray of Ehrlich's reagent. The method is specific for limonin since the TLC system separates limonin from other limonoids (Maier and Beverly, 1968) and Ehrlich's reagent gives a characteristic color with limonoids (Dreyer, 1965). Total time for the analysis of a group of four samples is about four hours.

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MATERIALS

Reagents. Reagent grade benzene, acetic acid, methanol, ethanol, acetic acid, chloroform, and magnesium sulfate, crystal ($MgSO_4 \cdot 7H_2O$) and practical grade acetonitrile and petroleum ether (30° to 60° C.) were used. Butylated hydroxy-toluene (BHT), Eastman Chemical Products, Inc., food grade, was added to chloroform to give a 0.05% solution.

Ehrlich's Reagent. An 0.5% solution of *p*-*N*-dimethylaminobenzaldehyde (Eastman Organic Chemicals, practical grade) was used.

Hydrogen Chloride. A lecture bottle of the anhydrous gas connected to a glass desiccator was used to treat the sprayed chromatograms.

Limonin Standard. A solution of 0.0100% limonin and 0.0050% BHT in acetonitrile was used. The limonin was extracted from citrus seeds and recrystallized from methylene chloride-2-propanol, m.p. 296°. It was chromatographically pure.

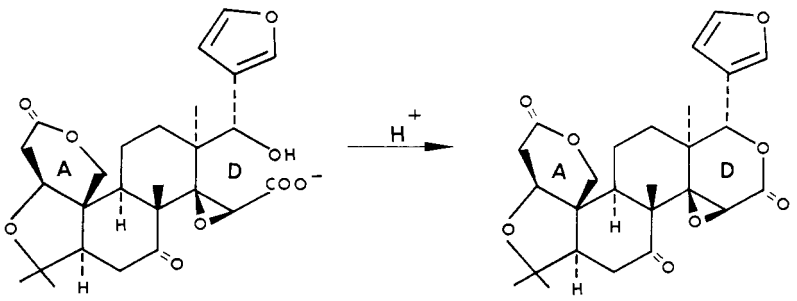
Thin-Layer Chromatography. Silica gel thin-layer plates or sheets with 0.25-mm. layer thickness, 20 cm. long and 5 to 20 cm. wide were used. The developing solvent was the upper phase of benzene, ethanol, water, acetic acid (200:47:15:1).

PROCEDURE

Step 1. Sample Preparation. For soluble limonin in juice, approximately 25 grams of juice or reconstituted concentrate was centrifuged at top speed in a clinical centrifuge for 10 minutes, and 20 grams of the supernatant weighed to the nearest 0.05 gram was used as the analytical sample.

For total limonin in juice, 20 grams of juice or reconstituted concentrate was weighed into a 100-ml. beaker, BHT added (0.01% of sample weight) and the mixture boiled for 5 minutes. The boiled mixture was used as the analytical sample. Boiling ensured complete conversion of limonoate A-ring lactone to limonin (particularly with raw juice samples), aided extraction of limonin from suspended tissue particles (pulp), and improved the handling of pulp in the subsequent extraction steps.

For total potential limonin (or indirectly and nonspecifically, limonoate A-ring lactone content) in whole fruit or specific fruit tissues (flavedo, albedo, section membranes, juice sac walls, etc.), a weighed aliquot of the finely macerated fruit tissues made to about 20 ml. with water was adjusted to pH 3 with 1*N* HCl, BHT added, and the mixture boiled for 5 minutes. The boiled mixture was used as the analytical sample.



LIMONOATE A-RING LACTONE (nonbitter) LIMONIN (bitter)

Figure 1. Conversion of limonoate A-ring lactone to limonin

Step 2. Extraction. The analytical sample was quantitatively transferred to a 50-ml. stoppered centrifuge tube and 1 gram of magnesium sulfate was added. The resulting solution was vigorously shaken with 2 × 10 ml. of chloroform and centrifuged for 5 minutes at top speed in a clinical centrifuge. The chloroform layers were transferred to a 250-ml. separatory funnel with a disposable pipet. Care was taken to exclude as much solid or emulsified material as possible. The combined chloroform extract in the separatory funnel was washed with 100 ml. of water by gently swirling (shaking gave an emulsion). The organic phase was transferred with 2 × 10 ml. of chloroform washings to a 100-ml. round-bottomed flask and evaporated to dryness at 40° C. on a rotary vacuum evaporator. The flask was cooled and then quantitatively diluted with 0.50 to 2.00 ml. of acetonitrile (to give approximately 0.1–0.2 μg. limonin/μl. solution). The flask was immediately stoppered to prevent evaporative loss and rotated to ensure solubilization of the limonin. This acetonitrile analytical sample was stored in a tightly closed screw-cap vial at 10° C. until ready for TLC limonin determination in Step 3.

In the case of orange juice extracts, the chloroform residue was partitioned between acetonitrile (5 ml.) and petroleum ether (5 ml.) to remove acetonitrile-insoluble substances. The acetonitrile phase (lower) was transferred to another 100-ml. round-bottomed flask with a pipet and combined with 2 × 5 ml. of subsequent acetonitrile washings of the petroleum ether. The acetonitrile extract was then evaporated to dryness and quantitatively diluted as above. This partitioning step should be employed with all samples having large amounts of acetonitrile-insoluble substances.

Step 3. Thin-Layer Chromatographic Determination of Limonin. The acetonitrile analytical sample was spotted on pre-coated silica gel TLC plates with a microsyringe. Spotting volumes were usually 5–25 μl. to give a spot density comparable to 1–3 μg. of standard limonin. A preliminary analysis was generally made to establish the approximate limonin concentration by spotting 5, 10, and 20 μl. of the sample spaced between standards of 1.0, 2.0, 3.0, and 4.0 μg. of limonin. The plate was developed in benzene, ethanol, water, acetic acid (200:47:15:1, upper phase) (Maier and Beverly, 1968) until the solvent front had advanced approximately one third of the plate height (about 20 minutes). The plate was then dried, sprayed moderately with Ehrlich's reagent, and the color was developed in a chamber of HCl gas for eight minutes. The intensities of the brown limonin spots ($R_f \sim 0.48$) were estimated by visual (or spectrodensitometric) comparison with the knowns. When the approximate limonin concentration was established, the sample was respotted at two different volumes (preferably not direct multiples of each other to aid objectivity in the readout) in the 1–3 μg. range and bracketed by appropriate standards (0.5-μg. increments). After development, the intensities were estimated independently and the limonin concentrations averaged:

$$\text{P.P.M. limonin} = \frac{\mu\text{g. limonin} \times \mu\text{l. acetonitrile dilution}}{\mu\text{l. spotted} \times \text{grams of juice or tissue}}$$

Precision and Accuracy. Replication experiments were performed on single-strength lemon juice and on navel orange and grapefruit juice concentrates, Table I. In each case aliquots of each juice were analyzed for total limonin. Accuracy of the procedure was tested in a series of recovery experiments outlined in Table II in which known amounts of limonin were added to the juices. The total native limonin was determined in triplicate analysis of each juice without added limonin.

RESULTS AND DISCUSSION

Individual steps in the procedure were tested for their contribution to experimental error. Recoveries of limonin from aqueous model systems carried through the extraction procedure for total limonin were 99–100%. No residual limonin was detected in juice samples after the two chloroform extractions and the aqueous wash solution was also limonin free. The quantitative dilution step with acetonitrile gave 98–100% recoveries when tested by adding known amounts of limonin to chloroform, evaporating to dryness, and adding 0.50 ml. of acetonitrile. When this experiment was repeated with

Table I. Replication of Limonin in Aliquots of Citrus Juices

	Sample Weight, Grams	Limonin Content P.P.M.	Limonin Content Average P.P.M.	Standard Deviation, %
Lemon juice (single strength)	20.0	3.8		
	20.0	3.4		
	20.0	3.8		
	20.0	3.4		
	20.0	3.9		
	20.0	4.0	3.7	±3.0
Grapefruit juice (5:1 concentrate)	1.19	75.5		
	1.53	65.5		
	2.98	77.2		
	3.51	82.0		
	4.95	78.8		
	4.95	82.0	76.8	±2.8
Navel orange juice (6:1 concentrate)	2.00	69.5		
	2.18	68.9		
	2.18	69.4		
	2.21	70.0		
	3.42	65.7	68.7	±1.1

Table II. Recovery of Limonin Added to Citrus Juices

	Sample Weight, Grams	Limonin Added, μg.	Limonin Added + Natural, μg.	Limonin Found, μg.	Recovery, %
Lemon juice		0		(2.9)[a]	
	20.0	45.0	103	100	97
	20.0	90.0	148	154	104
	20.0	135	193	200	104
	20.0	400	458	485	106
Grapefruit juice		0		(70)[a]	
(5:1 concentrate)	1.73	400	521	510	98
	1.73	800	921	890	97
	1.53	1200	1307	1315	101
Navel orange juice		0		(56)[a]	
(6:1 concentrate)	4.91	200	475	460	97
	3.69	200	406	400	98
	3.50	400	596	600	101
	6.17	800	1146	1070	93
	3.70	800	1007	1015	101

[a] μg./g. average of triplicate determinations.

Table III. Limonin and Potential Limonin[a] Content of Miscellaneous Citrus Fruit Juices and Tissues

Description of Material	Limonin, P.P.M.[b]
Commercial lemon juice concentrates	
Sample A (6% acidity basis)	4.2
Sample B (6% acidity basis)	6.0
Sample C (6% acidity basis)	14.2
Lemon peel (yellow-ripe)	219[a]
Grapefruit juice (mature, late-season Marsh)	2.2
Valencia orange section membranes (mature, mid-season)	50[a]
	2.3
Valencia orange juice (mature, early-season)	4.6
Commercial Valencia orange juice concentrate (66° Brix)	
Navel orange whole fruit (immature, 47-g. size)	850[a]

[a] Limonin formed from limonoate A-ring lactone by acidification and boiling. [b] Wet weight basis.

the addition of some petroleum ether soluble materials (limonin free) from orange juice, the limonin additions showed a positive error. This indicated that the presence of appreciable amounts of acetonitrile-insoluble materials in the final dilution step was a source of error. Accordingly, care was taken to prevent the transfer of aqueous or solid material to the flask with the chloroform extract. When contamination in this step was apparent, a partitioning of the residue between petroleum ether and acetonitrile (as in the procedure for orange juice) followed by evaporation of the latter, was helpful.

Various types of precoated silica gel systems for thin-layer chromatography were tested and found to be satisfactory, including glass plates, aluminum sheets, and plastic sheets. Spotting volumes between 5 and 25 μl. were most convenient. By adjusting sample weights, quantitative dilution volumes and spotting volumes, samples containing from 10 to 10,000 μg. of total limonin (0.05 to 500 p.p.m. for a 20-g. sample) have been routinely determined. By increasing sample size and spotting volume, the lower limit of sensitivity may be reduced to less than 0.25 p.p.m.

The spraying level of Ehrlich's reagent had an influence on the sensitivity of the method. Very light spraying resulted in incomplete and inconsistent color development whereas very heavy spraying resulted in faint spots even at high limonin levels. Several passes with a fine spray of 0.5% reagent in methanol gave the best results. Also, Ehrlich's reagent which was allowed to stand one day or longer after preparation gave more intense limonin spots than freshly prepared reagent.

On chromatograms of orange extracts, a yellow spot slightly behind but overlapping the limonin spot was present. The yellow spot, however, faded rapidly (1–2 hr.) when the plate was placed in the draft of a fume hood out of direct light (the limonin spot fades slightly on continued exposure to light). The fading of the yellow spot was hastened considerably by using warm air; however, a yellow background color developed if heating was excessive.

In replication experiments the highest standard deviation was ±3.0%, obtained with lemon juice, the sample with the lowest limonin content, 3.7 p.p.m. (Table I). The grapefruit and orange juice concentrate samples, which had considerably higher limonin contents, gave standard deviations of ±2.8% and ±1.1%, respectively. Average recoveries of added limonin covering the range 45 to 800 μg. varied from 99% in grapefruit concentrate and 98% in navel orange concentrate to 103% in lemon juice (Table II).

The results shown in Tables I and II were obtained by visual evaluation of spot size and density using indirect reflected light. A spectrodensitometer, if available, could be used to measure limonin spot density and thereby increase objectivity and probably the accuracy. Nevertheless, the visual technique gives a measure of limonin concentration that is specific, relatively accurate and rapid, and very sensitive and inexpensive.

The broad usefulness of the method is demonstrated by the data in Table III. Listed are a variety of individual citrus juices and fruit tissues analyzed for limonin or potential limonin. The concentration range covered is 2.2 to 850 p.p.m. In general, the method should also prove to be applicable to other plant parts and species and should be useful for the determination of other Ehrlich's reagent positive limonoids, as well.

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