# Fluorometric Determination of Limonin in Grapefruit and Orange Juice

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Microgram quantities of limonin were determined after separating it from citrus juice by solvent extraction and column chromatography. Further purification was accomplished by converting the crude limonin into water-extractable sodium limonoate, which was reconverted to chloroform-extractable limonin. Isolated limonin was measured fluorometrically in concentrated sulfuric acid.

Several analytical methods for limonin, a bitter constituent of grapefruit juice (Maier and Dreyer, 1965), appear in the literature. However, one of them (Wilson and Crutchfield, 1968) is not recommended for grapefruit juice. Others (Chandler, 1971; Maier and Beverly, 1968; Maier and Grant, 1970) employ procedures susceptible to possible subjective evaluations. A spectrophotometric procedure (Chandler and Kefford, 1966) has been reported for oranges and orange juice.

The role limonin plays in citrus juice bitterness in general and grapefruit juice in particular has required the development of a practical objective method of analysis for limonin in grapefruit and orange juice.

The object of this work was to design an assay which would obviate visual comparisons of color intensities and be suitable for determining microgram levels of limonin in citrus juice. The following procedure was developed and has been used in this laboratory.

## MATERIALS

**Column Chromatography.** A glass column 300 × 6 mm containing 1.0 g of W 200 neutral aluminum oxide (Waters Associates Inc.) with a small layer of chloroform-washed glass wool at both the bottom and top of the aluminum oxide column was used. Elution was performed with two systems: A, chloroform–petroleum ether (30–60°), 25:75; and B, chloroform–petroleum ether, 70:30.

**Apparatus.** A G. K. Turner Associates fluorometer (Model 111) and an International Clinical Centrifuge (Model CL) were used.

**Thin-Layer Chromatography.** Silica gel LQD plates (Quantum Industries, Fairfield, N. J.) were used. The solvent and detecting systems employed were those reported by Chandler (1971), Maier and Grant (1970), and Maier and Margileth (1969). Visualization of the spots by fluorescence was accomplished by spraying with 10% sulfuric acid in ethyl alcohol, heating at 100° for 10 min, and observing the plates under 360 nm light.

## PROCEDURE

**Sample Preparation.** A 5-g sample of processed single-strength grapefruit juice or reconstituted concentrate or a 10- to 20-g sample of processed orange juice or reconstituted concentrate was vigorously shaken for 1 min with 3 × 10 ml of chloroform and centrifuged for 5 min at top speed. Freshly expressed juice that has not been heated may be boiled for 5 min to convert all limonoate A-ring lactone to limonin (Maier and Grant, 1970). The chloroform layers were transferred with a disposable pipet to a 100-ml beaker. The beaker was placed in a water bath maintained at 30 to 40° and the chloroform was evaporated under a gentle flow of nitrogen. The resulting residue of citrus extract was dissolved in 3 × 1 ml of eluting solvent A and the 3 ml was pipetted onto the column of alumina. This solution was allowed to percolate into the aluminum oxide. Eluate collection was started at the same time. When the extract, except enough to keep the surface of the column wet, had penetrated the aluminum oxide, an additional 1.0 ml of eluting solvent A was used to rinse the beaker, pipet, and walls of the column. This rinse was also allowed to pass into the alumina. The column was then filled with elutant A to 22 cm above the surface of the alumina. A 35-ml eluate was collected while maintaining the 22-cm head. This 35-ml eluate contained most of the citrus material less polar than limonin and was discarded. The eluting system remaining in the column was siphoned out to just above the alumina surface and eluting system B was added and maintained at the 22-cm level until 10 ml of eluate was collected. This eluate containing the limonin (most of the more polar material, such as the phenolics, remained on the column) was evaporated as above and the residue redissolved in 3 ml of chloroform. This chloroform solution was placed in a 15-ml centrifuge tube and shaken for 1 min with 3 × 1 ml of 10% sodium hydroxide. The combined alkaline layers containing sodium limonoate were adjusted to pH 1 with about 2 ml of 6 *N* hydrochloric acid. This acidic aqueous system containing limonin was extracted with 3 × 3 ml of chloroform. The combined chloroform layers were washed with 1 × 3 ml of water. All washings and extractions were in glass-stoppered 15-ml centrifuge tubes. The chloroform layer containing limonin was evaporated to dryness in a 50-ml beaker, as previously described.

**Fluorometric Determination of Limonin.** Into the above dry 50-ml beaker containing the limonin residue was weighed 18.4 g of concentrated sulfuric acid (specific gravity 1.84). The beaker was covered and held at 25° for 1.0 hr with occasional gentle swirling to bring the sulfuric acid into contact with any limonin on the beaker walls.

The fluorescence of the sulfuric acid system was measured at 460 nm when excited at 405 nm at a range setting of 30× and 25° against a sample blank prepared by carrying a citrus juice sample, the limonin content of which was below our level of detection, through the procedure.

The micrograms of limonin were read from a standard curve and converted to ppm.

**Preparation of Standard Curve.** The curve was prepared from a 1.0 μg of limonin/ml of chloroform solution using 5.0-μg increments of limonin from 5.0 to 40.0 μg. Each aliquot was evaporated to dryness and treated with concentrated sulfuric acid, and the fluorescence was measured as previously described. Each increment of limonin was measured against its own blank of an equal volume of limonin-free chloroform treated exactly as the increment. The average fluorometer reading from triplicate determinations of each increment was plotted along the ordinate *vs.* the corresponding μg of limonin/18.4 g of concentrated sulfuric acid along the abscissa. The curve was linear over the range plotted and passed through the origin.

**Identification of Limonin.** The authenticity and purity

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of the limonin recovered by the isolation procedure, prior to the sulfuric acid treatment, was established by thin-layer chromatography in four different solvent systems. Chandler's (1971) solvent system together with his detection method is very selective and sensitive for limonoids. Two other systems, Maier and Grant (1970) and Maier and Margileth (1969), are both specific for limonin since they also separate limonin from other limonoids. The fourth system was ethyl ether–acetic acid–water, 15:3:1 (Maier and Margileth, 1969). The last three systems employed Ehrlich's reagent and hydrogen chloride gas, which form a characteristic color with limonoids (Dreyer, 1965). Silica gel plates and authentic limonin as a reference were used with all systems.

**Error and Precision.** The error of the procedure was determined by a series of recovery experiments in which base samples of citrus juices were fortified with known amounts of limonin. The native limonin in each base sample was previously determined in triplicate and found to be in the order of 0.5 ppm. Recoveries of limonin from triplicate assays of the individual fortified base samples, ranging from 1 to 10 ppm of total limonin in 1-ppm increments, were all within ±10% of the total limonin.

The precision of the method was determined by carrying five 5-g portions from the same sample of grapefruit juice through the procedure by different investigators. The range was 3.8–4.3 ppm, with a mean of 4.0 and a standard deviation of ±0.2. Similar precision was found with orange juice.

## RESULTS AND DISCUSSION

Each step of the procedure was examined, by thin-layer chromatography and fluorometry, for its contribution to experimental error. Thin-layer chromatography is not a part of the assay.

In order to minimize fluorescing impurities, an antioxidant such as butylated hydroxytoluene (Chandler and Kefford, 1966) was not employed. All evaporations were performed under nitrogen to minimize oxidation. Evaporation of the chloroform extracts to dryness under water pump vacuum at 30 to 40° with the Büchi rotary evaporator resulted in low and erratic limonin values.

Neutral alumina was found to be the most satisfactory adsorbent. Both acidic and basic aluminas retained prohibitive amounts of limonin. The basic alumina displayed the greatest affinity for limonin.

The sodium hydroxide converted the limonin into water-soluble sodium limonate which was extracted into the aqueous phase. Acidification of this aqueous layer converted the sodium limonate into chloroform-extractable limonin. The water wash of the final chloroform extract removed possible interfering inorganic materials.

The quantity of sulfuric acid used, to develop the fluorescence, was more consistent when added gravimetrically. Precision was improved when the same sulfuric acid stock was used for the preparation of the standard curve and the samples.

The final chloroform evaporate (prior to the sulfuric acid treatment) showed, by tlc, only limonin except for an occasional small, less polar fluorescing spot which was shown to be due to some chloroform samples. However, this fluorescence was cancelled with the blank.

Samples which afforded values beyond 40 μg on the standard curve were diluted with concentrated sulfuric acid and reevaluated.

The citrus juice sample blank containing limonin below our level of detection refers to both late season Florida "Valencia" orange juice and "Duncan" grapefruit juice. Late season fruit contains less limonin than early fruit. These samples were obtained by very mild juice extraction, thus avoiding the higher concentration of limonin in the segment membranes and seeds. These samples were carried through the procedure and consistently gave the same fluorescence values of 4 units on the fluorometer dial. This was the same value as observed with our reagent blank (the method conducted without citrus juice). Therefore, the procedure does not allow citrus material which contributes to fluorescence, other than limonin, to reach the sulfuric acid step.

In a multicomponent mixture such as citrus juice, many of the constituents have similar properties; therefore, the purification of any one fraction is a delicate and tedious process. A quantitative analysis for a specific constituent is virtually unobtainable because both purity and recovery with this type system are incompatible. A ±10% error for this analysis is, in the author's estimation, acceptable. The procedure can detect 0.5 ppm of limonin. However, part of the price one must pay for assaying quantities in this low range are extreme cleanliness and care. While this limit of detection and range of error are realized in the research laboratory, assay on a routine basis in commercial plant laboratories may not be as analytical. Six samples are processed in our laboratory by one individual in an 8-hr period.

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