the experiment with water described above. The possibility that the remaining naphthalenes, which appear here in trace amounts, are also artifacts cannot be ruled out.

As can be seen from Figure 1, a number of trace components have remained unidentified. However, it is believed that the aroma compounds identified in the present work in tamarind adequately account for the citrus, the warm spice-like flavors, and the roasted notes which are characteristic of tamarind.

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Quantitative Determination of Limonin in Grapefruit Juice by High-Pressure Liquid Chromatography

James F. Fisher

Microgram quantities of limonin were resolved from a chloroform extract of grapefruit juice by high-pressure liquid chromatography using a microporasil column and eluting with a chloroform–acetonitrile system. The limonin was detected with a refractometer.

Several analytical methods (Chandler and Kefford, 1966; Wilson and Crutchfield, 1968; Maier and Beverly, 1968; Maier and Grant, 1970; Chandler, 1971; Kruger and Colter, 1972; Tatum and Berry, 1973; Fisher, 1973; Beisel, 1974) for limonin, a bitter constituent of grapefruit juice (Maier and Dreyer, 1965), appear in the literature. However, a simplification of the parameters associated with these methods such as time, sample preparation, detection, and objectivity would be advantageous.

The important role limonin plays in grapefruit juice bitterness and its correlation with grapefruit flavor (Fisher, 1975) required the development of an improved objective method for the quantitation of limonin in grapefruit juice.

The object of this work was to design such an assay. The following procedure was developed and has been used in this laboratory.

MATERIALS

Apparatus. A Model ALC202 high-pressure liquid chromatograph (HPLC) with a Model 6000 pump, U6K injector, and R401 differential refractometer (Waters Associates, Milford, Mass.) was used. A Precision Scientific Company constant temperature water circulating system (Model 66600) was used. The recorder was a Texas Instrument servo/riter II 2-pen. A Model BB Burrell wrist action shaker (Burrell Corp., Pittsburgh, Pa.) was used. A Waters Associates Sample Clarification Kit with 1.0 or 0.5 μm Millipore–fluoropore filter was used for filtering.

Column. A Waters Associates 30 cm × 4 mm i.d. μ porasil column (10 μ porous silica packing) was used. The column was contained within an insulated water jacket.

Reagents. The eluting system was chloroform–acetonitrile (95:5) (Burdick and Jackson Laboratories, Muskegon, Mich.).

PROCEDURE

Sample Preparation. A 10-g sample of processed single strength grapefruit juice or reconstituted concentrate was extracted with 3 × 10 ml of chloroform for 2 min on the Burrell shaker set for maximum agitation. A centrifuge was used to separate the layers. The combined chloroform layers were evaporated to approximately 1.0 ml under nitrogen at 40°C. The resulting solution was filtered and the filtering system well rinsed. The filtrate was evaporated to dryness under nitrogen and redissolved in 50 to 100 μl of the eluting system, depending upon the expected limonin concentration.

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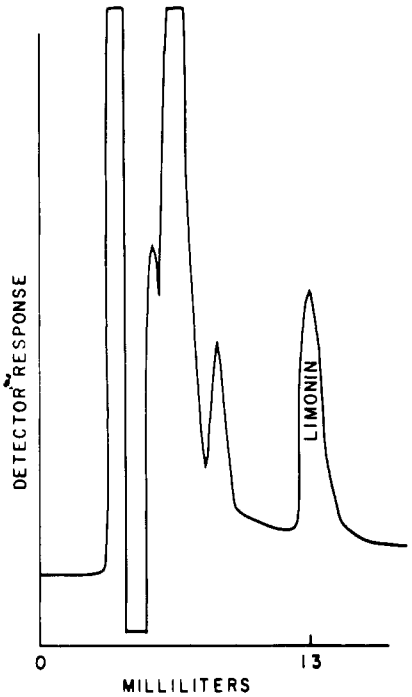

Figure 1. Separation of limonin in grapefruit juice. For experimental details, see text.

High-Pressure Liquid Chromatographic Determination of Limonin. An aliquot of the above filtered citrus chloroform–acetonitrile solution was injected onto the column with a flow rate of 2.5 ml/min. The limonin was eluted isocratically in about 5.5 min and detected with the refractometer at an attenuation of 2 × (full scale signal, 4.8 × 10^{-5} RI). The temperature of both the column and refractometer was maintained at 35°C, which was about 10°C above ambient, with the circulating water bath. The limonin peak was observed at a retention volume of 13 ml, with a recorder chart speed of 0.75 in./min (Figure 1). The column capacity factor, k', was 2.54.

The peak areas were determined by the height × width at half-height method.

The amount of limonin in unknown samples was determined from a standard plot of peak areas vs. micrograms of limonin. The plot was linear over the range of 5.0 to 50 μg of limonin (r = 0.999). The standard limonin samples were eluted and detected under the same conditions as above.

Identification of Limonin. The eluate corresponding to the peak area labeled limonin in Figure 1 was collected and examined by thin-layer chromatography as previously reported (Fisher, 1973). The results confirmed limonin as the compound responsible for this peak. The identity was also supported by peak enrichment.

Percent Recovery and Precision. The reliability of the procedure was determined by a series of recovery experiments in which base samples of grapefruit juice were fortified with known amounts of limonin. The native limonin in each base sample was previously determined by this liquid chromatographic procedure. Individual samples were fortified with sufficient limonin to provide a concentration of 2 to 5 ppm of limonin in 1-ppm increments.

The repeatability of the method was determined by analyzing five 10-g aliquots from a grapefruit juice sample.

RESULTS AND DISCUSSION

The refractometer was found to be the most suitable means of detection. The ultraviolet (uv) absorption of limonin, at any practical wavelength, is not sufficient to be of use. When working with the refractometer at high sensitivities such as an attenuation of 2×, it was necessary to maintain the same constant temperature on both the column and refractometer; otherwise the base line was erratic. Also, the 1% ethyl alcohol present in chloroform can react with silica gel columns in a manner which is temperature influenced. This can cause base-line difficulties, when using the refractometer at high sensitivities (Conroe, 1975).

The amount of native limonin in each base sample was found to be on the order of 1.0 ppm. The recoveries of limonin from the fortified samples were all within ±10% of the total limonin. The range of limonin found in the repeatability experiments was 6.5 to 7.1 ppm, with a mean of 6.8 and a standard deviation of ±0.2.

Injected samples containing more than 50 μg as found from the standard plot were reevaluated at lower sample injection volumes. For samples low in limonin, larger aliquots were injected. As much as 1000 μl of sample was injected without peak broadening. This was because of the low diffusion on the column used.

The range of from 5 to 50 μg of limonin on the standard plot was sufficient to accommodate our samples. The plot was extended to 60 μg with linearity. The shape of the plot beyond 60 μg is unknown. We consistently found that the lower detection limit of limonin with good reliability was 1.0 ppm.

The number of theoretical plates for the column, using limonin as the reference peak, was 1067, equivalent to a plate height of 0.28 mm.

Filtering was necessary to minimize clogging of the 5-μ stainless steel frit in the column end fitting. Obstruction of this frit resulted in pronounced pressure increase and erratic base-line behavior. With an unclogged frit, under optimized operating conditions our pressure was about 1700 psi. When the pressure reached 3000 psi, the frit was cleaned with 6 N nitric acid followed by an ultrasonic cleaner. Also, the Whatman no. 50 filter paper disk in the end fitting was replaced. Washing the column periodically with methyl alcohol was helpful in maintaining the lower pressure and preventing a buildup on the column of highly polar grapefruit constituents. Column activity was regenerated with the eluting system. The number of samples chromatographed prior to cleaning varied with the degree of filtering, type of sample, and amount injected.

The procedure's major time consumption was the removal of polar constituents, which required about 9.5 min/sample, after limonin elution and prior to a subsequent sample injection. This time can be shortened by increasing the flow rate after elution of limonin. The use of a more polar mobile phase such as chloroform–acetonitrile (90:10) saves time but sacrifices resolution. The average time required for six complete analyses was about 4.0 hr compared to 8 hr by our fluorometric method (Fisher, 1973). Sample preparation and time have been simplified and shortened with respect to most of the published procedures. The time distribution between sample preparation and LC analysis was about 1:1. Objectivity was enhanced by the use of instrumental detection and display as opposed to visual evaluation.

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Nuclear Magnetic Resonance Spectroscopic Determination of α- and β-Acid Homolog Composition in Hops

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Hexane extracts of hops have been analyzed by nuclear magnetic resonance spectroscopy for percentages of the three major homologs present in the α- and β-acid fractions. A range of hop varieties, including commercial and experimental types, was examined and the homolog composition was utilized in classification of these hops as Continental or Domestic types. Analysis of Cascade hops grown at different locations indicated that the proportions of each homolog are genetically fixed and less prone to environmental variation than α- and β-acid levels. The technique provides a simple, rapid, and accurate means for measuring homolog composition.

The major resin constituents of hops are the α-acid homologs humulone, cohumulone, and adhumulone (**1a, b,** and **c**), together with the corresponding β-acids lupulone, colupulone, and adlupulone (**2a, b,** and **c**). The relative proportions of these homologs are characteristic for each particular hop variety (Rigby, 1956), with the Continental types having lower levels of cohumulone (**1b**) in comparison with the Domestic types. It has been postulated (Rigby, 1972) that high levels of cohumulone are responsible for the unpleasant, harsh bitter flavor imparted to beers brewed with certain varieties of hops and it is known that the three α-acid homologs are utilized to different extents in the brewing process (Howard and Slater, 1957).

Although the heritability of homolog composition has not been definitely established, it is nevertheless essential that such information be obtained for both the male and female parents as well as their progeny produced through a directed hop breeding program. Experimental varieties can thus be screened and appropriate selections made for desirable flavor characteristics and optimum utilization. In addition, it may be desirable to evaluate and identify commercial hop samples, pellets, and extracts using the same criteria.

The large number of samples which would have to be analyzed in such a program requires that a rapid, accurate technique be available for measurement of homolog percentages, preferably needing only a small sample of hop material, since the quantity of hops available from new crosses in the first year of production is strictly limited. Counter-current distribution (Rigby and Bethune, 1953) is accurate, but too time consuming for this purpose. A similar objection applies to GLC analysis of the isopropyl esters of isovaleric, isobutyric, and 2-methylbutyric acids produced by oxidative cleavage of the acyl side chains of the α- and β-acids (Rigby et al., 1960). A rapid method has been developed (Likens and Nickerson, 1971) whereby the acids produced on pyrolysis of a lupulin sample are analyzed directly by GLC, but the adhumulone homologs are not separated from the corresponding humulone or lupulone homologs and the co-fraction value obtained is a combination of contributions from both α- and β-acids. Since the homolog ratios are not constant for both α- and β-acids in a given hop, the analysis provides only an estimate of the relative contribution of the co-homolog in different hop varieties. High-pressure liquid chromatography offers considerable promise as a rapid method for hop analysis but sufficiently well-resolved separations, which would allow determination of individual homolog proportions, have not yet been achieved (Molyneux and Wong, 1973).

We have now developed a rapid, small-scale analytical method which provides a measurement of the homolog composition of both α- and β-acids from a single determination. The technique has been routinely applied to evaluation of new genotypes produced by selective crossing of parents having characteristics desirable in commercial hops.

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EXPERIMENTAL SECTION

Apparatus. Nuclear magnetic resonance (NMR) spectra were obtained using a Varian HA-100 spectrometer. Samples were run as 20% solutions in $CDCl_3$, dried over 4A molecular sieves. Tetramethylsilane (Me_4Si) was used as an internal standard.

Sample Preparation. (*A*) *Lead Salt Method.* The hops (30–40 g) were ground in a Waring Blendor and extracted with benzene (400 ml) for 1 hr. The solvent was removed under reduced pressure and the residue redissolved in methanol (50 ml). α-Acids were precipitated as their lead salts on addition of 4% methanolic lead acetate, leaving β-acids in the methanol solution. The lead salts were collected and washed several times with methanol. α-Acids were regenerated on addition of 6 *N* H_2SO_4 (<2 ml) to a suspension of the lead salts in methanol (100 ml) and isolated by extraction with isooctane. The solution was dried over Na_2SO_4 and the isooctane evaporated to yield the α-acids.

To regenerate the β-acids, the methanol filtrate was acidified with 6 *N* HCl and extracted with hexane. The hexane solution was washed with water and dried over Na_2SO_4. After removal of the solvent, the residue was dissolved in benzene and chromatographed on a silicic acid column (100 mesh) with ethyl acetate–hexane (15:1, v/