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Nomilin, a New Bitter Component in Grapefruit Juice

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A new bitter component in grapefruit juice has been separated and conclusively identified as nomilin from chromatographic, chemical, and mass spectral evidence. Nomilin was shown to be a natural component of grapefruit juice vesicles. In commercial grapefruit juice, produced during the 1978-1979 season, nomilin concentrations ranged from 1.6 to less than 0.1 ppm. Nomilin concentrations were found to be greatest in early season juices and decreased rapidly with increasing fruit maturity. Nomilin concentrations fell more rapidly than that of limonin during the 1978-1979 season. In November the nomilin/limonin ratio was 0.125 and by May it was 0.04. Nomilin concentrations increase with increasing extractor pressure up to a point but unlike limonin increase very little under very heavy squeeze conditions. For fruit harvested on the same day, juice nomilin and limonin contents were lower in the Duncan cultivar than the Marsh seedless cultivar.

Nomilin is a limonoid first isolated from the seeds of oranges and lemons (Emerson, 1948). It is bitter (Emerson, 1948, 1951, Dreyer, 1965) and is reported to be about twice as bitter as limonin (Hashinaga et al., 1977). Emerson (1949) reported that limonin was the sole bitter principle isolated from the juice of navel oranges. In some unpublished data, Bennett (1972) reported finding minor amounts of deacetylnomilin, nomilin, obacunone, deacetylnomilinic acid, and nomilinic acid in the peel of navel oranges. Limonoids such as limonin and nomilin are actively synthesized in orange and lemon leaves, particularly in young, immature leaves according to Hasegawa and Hoagland (1977). Hashinaga et al. (1977) found both limonin and nomilin in the seeds, segment membrane, peel, and flesh in ponkan mandarins. Hasegawa et al. (1980) found the ratio of nomilin to limonin was considerably lower in mature compared to immature leaves and fruit tissue of Eureka lemons.

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While limonin was thought to be the sole bitter component in orange juice, the bitterness of grapefruit juice was ascribed to flavanone neohesperidosides, primarily naringin. Later Maier and Dreyer (1965) found the bitter limonin present in grapefruit juice as well.

In a recently developed high-pressure liquid chromatographic separation for citrus limonoids (Rouseff and Fisher, 1980), a peak with the same retention time as nomilin was observed in grapefruit juice samples. Therefore, the primary purpose of this study was to determine if yet another bitter limonoid (nomilin) is present in grapefruit juice. Since grapefruit seeds are known to contain nomilin in concentrations greater than 800 ppm (Hasegawa et al., 1980; Rouseff and Nagy, 1982), experiments were conducted to determine if nomilin is a natural component of grapefruit juice vesicels or is found in juice as a result of seeds ruptured during juice manufacture. Another goal was to determine how juice nomilin concentrations change with increasing fruit maturity and what concentration ranges are likely to be found in commercial juices from a given processing season. A final goal was to determine how juice nomilin concentrations are affected

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by different juice extractor pressures.

EXPERIMENTAL SECTION

Materials. LiChrosolv-grade chloroform was used to extract limonoids from juice. HPLC-grade (LiChrosolv) heptane, hexane, 2-propanol, and methanol from E. Merck, Darmstadt, West Germany, were used to prepare the chromatographic mobile phase.

Limonon was obtained from James Fisher of the Florida Department of Citrus, Lake Alfred, FL. It was extracted from ground, defatted grapefruit seeds similar to the method employed by Emerson (1948). Crude limonin was purified by repeatedly dissolving it in methylene chloride and then recrystallizing it from methanol, mp 295–299 °C (with decomposition). purified nomilin was supplied by Shin Hasegawa of the U.S. Fruit and Vegetable Chemistry Laboratory in Pasadena, CA. Chromatographic analysis of these standards indicated that they could be used without further purification. Stock solutions were prepared in acetonitrile because the limonoids could not be readily dissolved directly into the mobile phase. Standard solutions of 100 ppm were prepared by making the appropriate dilutions with mobile phase. All standards were refrigerated when not in use.

Apparatus. A Waters Associates (Milford, MA) M-6000A pump with a WISP programmable sample injector and a Tracor (Austin, TX) Model 970A variable-wavelength UV–vis detector was used as the chromatographic system. The detector had a 6-nm band-pass and an 8-μL sapphire window flow cell. A 0.1 V/AU output was used with a 3-s time constant. Chromatograms were recorded and peak areas determined by integration with a Spectra-Physica (Santa Clara, CA) SP-4000 chromatographic data system. Samples were dissolved and solvents degassed with the use of a Branson (Shelton, CT) Model B-220, 125-W ultrasonic cleaner.

Liquid Chromatographic Conditions. A Du Pont (Wilmington, DE) Zorbax CN column, 25 cm × 4.6 mm i.d., was used to separate citrus limonoids by the procedure of Rouseff and Fisher (1980). The mobile phase consisted of heptane, 2-propanol (IPA), and methanol in a ratio of 11:12:2 (v/v). The flow rate was 1.0 mL/min. The column was heated to 40 °C to improve resolution. Column head pressures under these conditions were typically 800 psi. Each solvent was degassed separately by applying an aspirator vacuum while it was placed in an ultrasonic bath for about 3 min.

Thin-Layer Chromatographic Conditions. Silica gel G thin-layer chromatographic plates manufactured by Analtech, Newark, DE, were used. Of the two solvent systems used by Maier and Beverly (1968), the developing solvent system of ethyl ether–acetic acid–water (15:3:1 v/v) was chosen because limonin and nomilin were more effectively separated. Collected chromatographic peaks were reduced under a stream of nitrogen to a volume of approximately 50 μL and spotted on the TLC plates. Standard solutions of limonin and nomilin were spotted on either side of the collected peak for comparitive purposes. After air-drying, the plates were placed into an equilibrated solvent tank and removed after the solvent front had traveled two-thirds of the plate height. Limonoids were visually detected and R_f values calculated after the plates had been sprayed with Ehrlich's reagent [5% *p*-(dimethylamino)benzaldehyde in ethanol] and exposed to HCl gas for about 10 min in a glass chamber.

Sample Preparation. Commercial grapefruit juice samples were extracted and prepared according to the procedure of Rouseff and Fisher (1980). Grapefruit juice vesicles were obtained by peeling the fruit and separating individual fruit segments. Each segment was then slit with a scalpel and the juice vesicles were carefully removed. Ten to twenty-five grams of juice vesicles were ground in a Sorvall Omni Mixer. The sample was filtered with cheesecloth and prepared in the same manner as the juices.

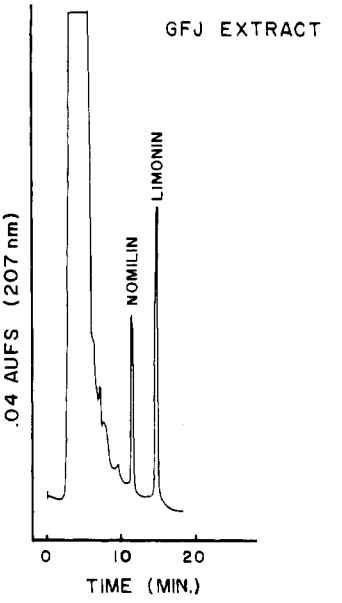

Figure 1. A 10-μL injection of a Florida grapefruit juice extract. This juice was packed as a canned single-strength grapefruit juice in Nov 1978.

Limonoid Concentration Calculations. Limonoid concentrations were determined for each sample by dividing the respective peak areas by the concentration factor determined from the averages of three 1-μg injections of standard limonin and nomilin. Sample weight and dilution factors were then used to determine the original concentration in micrograms per milliliter (ppm). Standard solutions of nomilin were used to establish that nomilin peak area and concentration were linear between 0.05 and 10 μg.

RESULTS AND DISCUSSION

Identification of the Nomilin Peak. Since nomilin has not been conclusively identified as a component of citrus juices, most of the emphasis in this study was directed toward the identification and quantification of the nomilin peak. Shown in Figure 1 is a chromatogram of an early season commercial grapefruit extract. The peak at 11 min occurs at the same retention time as nomilin. It is very sharp and symmetrical. There are no visible shoulders to indicate that more than one compound might be present. Solutions of standard nomilin and grapefruit juice extract were stack injected, resulting in the expected increase in peak height for only the nomilin peak. Retention time for the spiked nomilin peak was identical with that for standard nomilin or the nomilin peak in the grapefruit juice extract. Peak shape for the spiked peak remained symmetrical. These experiments indicate that the compound in the grapefruit juice extract is either nomilin or some compound with very similar retention characteristics in this solvent system.

To determine if the peak from the grapefruit juice extract was composed of something other than nomilin, we cochromatographed the extract and standard nomilin in the recycle mode. The nomilin peak was cycled 5 times through the column, thus greatly multiplying the separation power of the chromatographic system. If the peak of interest contained a substance other than nomilin, one

would normally see a distortion of the single peak and, after several passes through the column, indications of two or more peaks. However, after five passes through the column the nomilin peak remained perfectly symmetrical. This suggests that there is no difference between nomilin in the grapefruit juice extract and standard nomilin.

Alternative Chromatographic Systems. For further identification of the peak from the grapefruit juice extract, its chromatographic characteristics were determined by using a completely different solid support and solvent system. Collected peaks were spotted on a TLC plate along with standard solutions of limonin and nomilin. By use of ethyl ether–acetic acid and water to develop the plate, only a single spot with an R_f of 0.67 was observed for the collected peak. R_f's for standard nomilin and limonin were 0.65 and 0.74, respectfully. Again the chromatographic behavior of the peak of interest was the same as that of standard nomilin. Also, since the spots were developed with Ehrlich's reagent, a reagent that is indicative of compounds containing a furan ring (Dreyer, 1965), the compound probably contains a furan ring. Furthermore, since limonoids give a characteristic color with this reagent (Maier and Beverly, 1968) and since the color of the collected peak spot was the same as that of standard nomilin, the compound in the collected peak is also a limonoid. This additional chromatographic and chemical evidence strengthens the identification of nomilin in grapefruit juice.

Mass Spectral Identification. The literature apparently contains limited information on the mass spectra of limonoids. Baldwin et al. (1967) obtained the mass spectra of limonoid-like compounds using electron impact (EI) ionization. Dreyer (1967) found that limonin does not show a molecular ion. No mass spectral information on nomilin was found. By use of a solid probe and EI, the spectra of the collected peak and standard nomilin were essentially identical. Both contained a small molecular ion (M + 1) peak at m/e 515 (>1%) and major m/e peaks at 331 (100%) and 391 (67%). For better definition of the molecular weight of the collected peak and standard nomilin, chemical ionization (CI) using methane gas was employed. In these experiments the major positive ion peak from the collected chromatographic data was at m/e 515. The positive ion fragmentation pattern for standard nomilin was again essentially identical with a molecular ion at m/e 515 (100%) and major peaks at m/e 455 (47%) and 316 (24%).

Since the substance from the grapefruit juice extract peak and standard nomilin have the same chromatographic behavior in two dissimilar chromatographic systems, chemically react with Ehrlich's reagent in the same manner, and have the same molecular weight and fragmentation pattern by both CI and EI, the compound from the grapefruit juice extract must be nomilin.

Nomilin Concentration Levels and Fruit Maturity. Nomilin concentrations are greatest in early season grapefruit juice. As shown in Figure 2 highest average nomilin concentrations were found in November grapefruit juices and decreased rapidly thereafter. Nomilin concentrations decreased from 1.11 ppm in November to 0.56 ppm in January. Thus, the average nomilin concentration was halved in just 2 months. Individual juice samples ranged from as high as 1.6 ppm for a November juice to less than 0.1 ppm for some May juices. In every case the nomilin concentration was less than that of limonin. While it is widely recognized that limonin concentrations decrease with increasing fruit maturity, the concurrent decrease in nomilin concentration is much more rapid. For example,

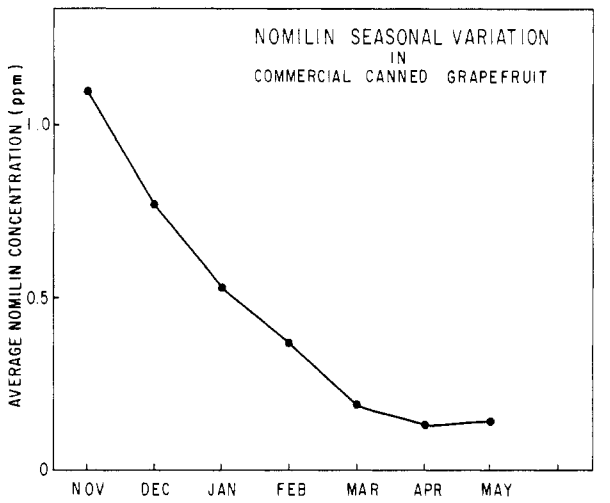

Figure 2. Average nomilin concentrations of Florida canned single-strength grapefruit juice from Nov 1978 to May 1979. Average values were computed from the results of 113 juice samples collected from the 12 major processing plants in Florida.

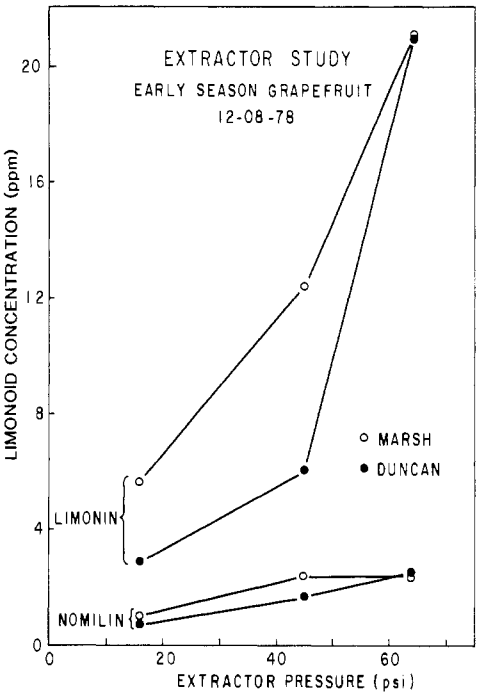

Figure 3. Effect of extraction pressure on juice limonoid concentrations from Duncan and Marsh grapefruit.

in the 1978–1979 juices the ratio of average nomilin concentration/average limonin concentration was 0.125 for November juices. By January the level had fallen to 0.100 and the ratio for the April juices was 0.038. Thus it appears that nomilin is found in significant concentrations only during the first few months of the grapefruit season.

Nomilin in Grapefruit Juice Vesicles. Since nomilin is known to exist in grapefruit seeds in high concentrations, there was some question as in the source of nomilin found in the commercial grapefruit juice samples. Therefore, to determine if nomilin was a natural component of grapefruit juice vesicles as opposed to an artifact produced from seed rupture when the juice is mechanically extracted from the fruit, we carefully removed juice vesicles from intact fruit segments and analyzed them for limonoids. Both limonin and nomilin were found. Since the sample was prepared from a fruit harvested in November, the amount of nomilin was substantial, amounting to approximately 40% of the

total limonoids. However, even in this sample the major limonoid was still limonin.

Extractor Pressure Effects. As previously reported by Attaway (1977) and shown in Figure 3, juice limonin concentrations increase rapidly with increasing extractor pressure. As the fruit was squeezed harder, the amount of juice recovered is increased, but the juice was higher in limonoid content and, thus, more bitter. The increase in bitterness has generally been attributed to extraction of limonoids from other fruit parts such as segment membrane, central core, and seeds in which limonoid concentrations are much greater. The three extractor pressures in Figure 3 correspond to a soft, hard, and very hard squeeze. It is interesting to note that in the soft and hard squeezes the seedy Duncan cultivar had less limonin and nomilin than the seedless Marsh cultivar. Under very heavy extractor pressure there was no cultivar difference in limonin or nomilin contents of the juice. Both nomilin and limonin concentrations doubled as extractor pressure was increased from 14 to 45 psi. As extractor pressure was increased to 64 psi the limonin content of Duncan grapefruit juice more than tripled and Marsh doubled, whereas the nomilin concentration of Marsh grapefruit juice remained unchanged and of Duncan increased only slightly. Therefore, under soft and hard squeeze conditions both limonin and nomilin concentrations increase in roughly the same proportion. Under very harsh squeeze conditions limonin concentrations continue to increase whereas nomilin concentrations are relatively unaffected.

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Quantitative Analysis of Cold-Pressed Lemon Oil by Glass Capillary Gas Chromatography

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Cold-pressed lemon oil, derived from California and Arizona fruit, was analyzed by glass capillary gas chromatography. Thirty-seven components were determined in a single chromatographic run by using the internal standard method and a computing integrator. Six of these components, α-thujene, 3-carene, octanol, nerol, geraniol, and nonyl acetate, had not previously been quantitated in lemon oil. Because of the much higher resolution of capillary columns, compositional data from the present study should be more accurate than those previously obtained by packed column techniques.

The advantages of glass capillary gas chromatography for analysis of complex mixtures of volatiles have been discussed extensively in the recent literature. The unparalleled resolution, inertness, and speed of analysis offered by glass capillary columns have prompted their use in place of packed columns and more than compensate for the somewhat greater care and dexterity required for their proper installation. The commercial availability of excellent capillary columns has made high-resolution gas chromatography available to most laboratories.

Recently, Jennings (1979) has surveyed some applications of glass capillary columns for food and essential oil analysis. The inability of packed columns to adequately separate such samples was clearly illustrated, and the benefits of analysis by capillary techniques were summarized.

Citrus oils are ideal candidates for analysis by such techniques. Their components display a wide range of functionality, polarity, and volatility, yet certain groups of components, such as the mono- and sesquiterpene hydrocarbons, require high column efficiency for complete separation. Although incompletely understood, sensory properties are known to depend critically on the presence of several minor components in the correct proportion (Shaw, 1977).

The economic importance of citrus oils and their widespread use in the flavor and fragrance industries make the acquisition of accurate compositional data highly desirable.

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