RNA content of fruit slightly increased and then decreased with length of storage (Table 4). Fruit exposed to ethylene had a faster rate of RNA decrease than control fruit. These data support the concept that ethylene seems to hasten senescence.

It has been shown by Schinneller (11) that 5'GMP and 5'ADP will modify the flavor of specific compounds present in citrus juice, particularly octanal. Only 10 ppm was required to enhance the detection of octanal. These data and a previous report demonstrated tissue levels of nucleotides in citrus juice that could modify flavor. Luh and Chen (9) have reported isolating nucleotides from other fruits and vegetables and have suggested that nucleotides in combination with other compounds could be related to their taste appeal. The modifying of flavor by nucleotides is well known, particularly on meat products (7, 8).

References
has hampered investigation of acceptable levels of Limonin in citrus juice.

The method currently in use (7) involves direct TLC of an acetonitrile extract containing Limonin, spraying with Ehrlich's Reagent, and treatment with gaseous hydrogen chloride to reveal the Limonin spots. Quantitation is made by visual comparison with Limonin of known concentration. Although this method suffers from reproducibility, since the color intensities of the spots are so difficult to reproduce, it is quite adequate where lower limits of Limonin have been set (e.g. below 5 ppm). A new approach to quantitation was therefore sought.

Fales and Luukkainen (5) had found that steroids with high melting points (up to 254°C) could be successfully analyzed by GC on a 1% SE-30 column at 223°C. Fales and Pisano (6) also found that physiologically important amines that were highly polar and only slightly volatile could be chromatographed on a 4% SE-30 column at 205°C. Martin et al. (8) did a study using a 5% SE-30 column at 270°C on the relationship between the log of the retention time and the boiling point of high boiling phenothiazine drugs. He was able to elute the compounds having high boiling points of over 430°C in a time span of 10 min. Limonin has a melting point of 296°C and was, therefore, analyzed without derivative formation.

The sample extraction in the proposed method follows to some extent the Maier and Grant (7) TCL procedure, with some minor deviations. It was found that 2 x 10-ml chloroform does not extract all the Limonin from the sample. This was remedied by using 3 x 10-ml chloroform and adding 3 g of magnesium sulfate as a salting out and de-emulsifying agent. Also, to aid in recovery of the Limonin in the final step of the proposed method, a 50-ml round bottom flask was used.

This proposed method has several advantages. Eight juice samples can be analyzed in a day and its sensitivity and precision are at the 0.25 ppm level.

**Experimental Methods**

**Apparatus and Reagents.** A Varian Aerograph Model 1860-4 Gas Chromatograph, equipped with dual on-column injection capabilities, attached to a Varian Model 20 Strip Chart Recorder, were employed. The chromatographic column was a stainless steel tube of 1/8" O.D., 6 feet in length and coiled to a 6" diameter. It was hand packed with 100 - to 120 - mesh Varaport 30, coated with SE-30 5% by weight. A Gas Chrom Q solid support also gave comparable results to the Varaport 30. The low, 5% SE-30, loading is necessary to elute the high molecular weight Limonin in a reasonable time. In turn, this low liquid loading demands an extremely inert support to help avoid adsorption. Before operation, the GC temperature settings (i.e. injector, column, and detector) were correctly calibrated using an external pyrometer. It was found that the GC suffered a decrease between 5° and 10° temperature change in each mode after each shutdown. Therefore, the GC was re-calibrated following each shutdown.

Reagent grade acetonitrile, hexane, chloroform and practical grade magnesium sulfate were used.

Standard solutions of Limonin were made by dissolving 10 mg of TLC and GC pure, m.p. 294 - 296°C Limonin in acetonitrile to make 10.0 ml of 1 µg/1 ml Limonin solution. Then, a series of dilutions were made to give a range of 0.1 µg to 1.0 µg Limonin per 1 µl.

**Column preparation.** The column was temperature pre-conditioned for eight hours at 310°C with a 10 ml/min. helium flow rate. Since this is on column injection, the injector temperature was also set at 315°C during the conditioning. It was found that the positioning of the column as close to the front of the injector port, but not subject to closure from the septum, was all important. If the column is too far away, Limonin analysis is impossible because the sample was not injected onto the column packing. It was also found that a glass wool plug at the column inlet sometimes resulted in the syringe being plugged by glass wool. Subsequent columns were made with no glass wool plug at the column inlet. If not enough time is given for pressure decrease on the column, there will be column packing blow out when changing septums. If too much column packing is blown out, this will result in decomposition of Limonin, presumably because of too great an exposure to the hot stainless steel. This can be remedied by repacking the column inlet.

At the end of column conditioning, the column was attached to the detector inlet and checked for leaks. This was done by applying 80 psi of helium full-open GC flow regulator to the column for one hour and watching for any pressure decrease. In order to assure sharp elution of peaks for analysis, the column must be leak-proof. After the leak check, the GC settings were made:- 300°C column, 310°C injector, 330°C detector. After GC temperature equilibrium, usually in 5 hours, the flow rates were:- 40 ml/min. helium,
26 ml/min. hydrogen, and 350 ml/min. air. The same column was in operation daily for one month, with no significant changes in performance. Build-up of SiO₂ “bleed” from the column during the one month operation did not result in any appreciable baseline drift or noise to interfere in analysis. Build-up of SiO₂ on detector parts was removed by ultra-sonic bath wash and wiping of each part with acetone.

Injection reproducibility. To aid in reproducibility, a GC septum needle guide (to give “same-hole” penetration) and a 1 in. syringe spacer (to give same depth of penetration) on a 7105-N 5 μl Hamilton Syringe were used.

Before a sample is drawn in the syringe, the syringe is filled with acetonitrile and then brought to the 0.5-μl mark. Then, 0.5-μl of air is drawn, bringing the syringe to the 1-μl mark. The syringe needle is placed into the sample and the plunger pumped between the 1-μl and 5-μl mark several times. The syringe is then left at the 5-μl mark, withdrawn from the sample and a soft tissue pierced with the needle. The plunger is depressed to the 2-μl mark, the needle gently withdrawn from the tissue, catching the excess expelled sample, and air is then drawn to the 3.5-μl mark. The needle is then wiped with the tissue. This, in effect, sandwiches the sample between air with a solvent wash at the tail end to allow for complete injection of the sample onto the column. The syringe is placed into the GC septum needle guide, with the syringe μl scale facing to the left of the operator. With repeated usage, a syringe needle may develop a slight curvature and if the needle is not placed into the septum needle guide in the same manner for each injection, this will result in non-reproducibility of same hole penetration. (i.e. shorter septum life).

With a quick motion, pierce the septum, press the piston, hold for one second, then quickly withdraw the syringe. Duplicate injections can be reproduced to within 5% limits.

Loading effect. The first injection of any sample (unknown or standard) gave a smaller response than injections that followed. This problem was also observed by Bloomfield (2) and he attributed it to an adsorptive phenomenon of the column packing. Therefore, before daily analysis 5 x 5-μl injections of Freon 113 were made. This seemed to stabilize the column, especially if repeated each day following overnight operation of the GC.

Standard curve. A series of 1-μl samples of Limonin standard solutions were injected into the chromatograph operated under conditions described. The resulting chromatograms were measured for height of Limonin peak and plotted against amounts injected. The regression line of best fit for these points did not pass through the origin since the points actually fall on a curve. The results of a statistical analysis of data obtained from 30 injections of standard solution are shown in Table 1.

Analysis of juice. For total available Limonin, not including potential Limonin, (i.e. Limonin A-ring lactone), 25.0 g of well-shaken juice is weighed into a 50-ml stoppered centrifuge tube containing 8 g of magnesium sulfate. After shaking well to dissolve the magnesium sulfate, the solution is extracted with 3 x 10-ml of chloroform. Each 10-ml portion of chloroform is shaken for 30 sec. then centrifuged 5 min. at top speed in a clinical centrifuge. The chloroform layers are transferred to a 125-ml separatory funnel using a 10-ml volumetric pipette. The combined chloroform extract in the separatory funnel is transferred to a 125-ml separatory funnel with 1 x 10-ml hexane followed by 2 x 10-ml acetonitrile. Each 10-ml portion of acetonitrile is partitioned with the hexane. This partitioning reduces the quantity of extraneous compounds, presenting a cleaner chromatograph. The combined acetonitrile phase (lower) is transferred to a 50-ml round bottom flask and evaporated to dryness at 30°C. The flask is cooled and 0.250-ml...
of acetonitrile is quantitatively added. The flask is quickly stopped to prevent evaporative loss of acetonitrile and rotated several times to thoroughly wet the sides of the flask. This insures leaching of Limonin from the residue. The flask is allowed to stand several minutes before an acetonitrile aliquot is transferred to a tightly closed screw cap vial. This sample is used for GC Limonin determination.

A reproduction of a chromatograph (Fig. 2) illustrates the results obtained from a grapefruit sample having a 3.5 ppm Limonin (2 a) and when 5 ppm Limonin were added (2 b). Although the base line is not achieved in each instance, the Limonin shows a characteristic response which allows for identification and quantitation. The height of the Limonin peak measured from a drawn base line was the simplest method of quantitative analysis. Since the Limonin peak was a tall, narrow peak, the peak height gave a concentration relationship which was linear over the 0.1 μg, 1.0 μg range. A standard solution of Limonin in acetonitrile should be injected each day to check stability of instrument conditions.

Limonin concentration was calculated by the equation:

\[
\text{ppm Limonin} = \left( \frac{\mu g \text{ Limonin from sd. curve}}{\mu l \text{ sample injected}} \times \frac{\mu l \text{ acetonitrile dilution}}{(g \text{ weight of juice})} \right)
\]

Results and Discussion

Identification of Limonin. Ten injections of 5 μg Limonin each were collected from the GC, combined, and spotted on two Silica gel-G TLC plates (Chromatogram a & b), along with known Limonin. Chromatogram a was developed in Maier’s system (7) benzene, ethanol, water, and acetic acid (200: 47: 15: 1). Chromatogram b was developed in Chandler’s system (3) acetic acid, acetone, hexane, benzene (3: 10: 22: 65). The Limonin spots were then made visible by spraying the TLC plate with a 10% solution of concentrated sulfuric acid in alcohol and heating it at 100°C for 10 min. (4). The GC Limonin had identical Rf with the standard Limonin in each system. Although the GC Limonin showed some decomposition products, this amounted to less than 0.1% of the total Limonin.

Two silica gel-G TLC plates were streaked with grapefruit juice containing 20 ppm Limonin and developed in each above TLC system. The Limonin was scraped from each plate, eluted with acetonitrile, evaporated to a small volume, and injected separately on the GC. Limonin obtained from each TLC system had identical retention times (12.2 min.) as that of a known Limonin standard.

A grapefruit sample containing 3.5 ppm Limonin (determined by the GC standard curve) was chromatographed and the Limonin peak identified at 12.2 min. To this sample, Limonin was added to give a final concentration of 13.5 ppm. This was injected on the GC and the GC graph of the sample showed a corresponding increase in only the Limonin peak at 12.2 min.

Recovery.—Recovery of added Limonin from two grapefruit samples (in three replicate analyses carried through the extraction procedure) is shown in Table 2.

<table>
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<th>No.</th>
<th>Initial, μg</th>
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<th>Found, μg</th>
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<th>%</th>
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<td>0.28</td>
<td>1.0</td>
<td>1.24</td>
<td>97</td>
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</tr>
<tr>
<td>6</td>
<td>0.28</td>
<td>1.0</td>
<td>1.30</td>
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</table>
Each step in the procedure was tested for possible contribution to experimental error. A fourth chloroform extraction of each (6 samples) were combined and analyzed for Limonin. The Limonin value obtained was less than 0.5 ppm (or < 0.08 ppm per sample).

The quantitative acetonitrile dilution step was tested by adding known amounts of Limonin to chloroform, evaporating to dryness and adding 0.250 ml of acetonitrile. Recovery ranged between 98 - 103%.

A check of the combined (6 samples) of hexane showed no Limonin.

Reproducibility.—Seven replicate aliquots of a sample of commercial canned grapefruit juice were run through the procedure. The acetonitrile samples were injected in triplicate. Standard deviation on the twenty-one results was calculated.

<table>
<thead>
<tr>
<th>No.</th>
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<th>Ave. ppm</th>
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</tr>
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<td>2.6 Standard</td>
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<td>1.9 Deviation:- 0.31</td>
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</tr>
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Acknowledgement

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Literature Reference