differences ranged from −7.0 to +6.6% (in 9 of these runs the differences were less than 1%). Peak areas due to added lysine were about equal to the areas under the phenanthrene internal standard peaks (these reached a height of *ca.* 0.15–0.30 mV, with 1 µl injected volume). To make the phenanthrene peak larger (*ca.* 0.7–0.9 mV) and the determination more accurate we recommend the use of 1.8 ml of dry ethanol containing 2.0 mg/ml of phenanthrene, making the total phenanthrene per sample 3.6 mg (step 9).

Column lengths of 12 ft provided better resolution. Twelve-foot columns, lower carrier gas rates, and a column temperature of 190° extended the time for lysine emergence to 10–11 min, providing a better base line for lysine and better separation from the small peak immediately preceding lysine. Carrier gas pressure was varied to make lysine passage time equal for two columns. We also adapted a weekly treatment of columns with 15 µl of Freon R11 to clean the flame detectors. Ten runs on wheat over 6 weeks on one pair of columns had differences of −2.0 to +5.2%, a considerable decrease from the earlier group. Likewise, ten runs on rice over 8 weeks on one pair of columns had differences of −1.0 to +5.0%. Columns have performed satisfactorily for 200 samples. Usually the first few inches of column next to the injector become caked or discolored and only this portion needs repacking.

Since the small pre-lysine peak was sometimes not completely separate on the sloping base line, we employed a slow program of temperature change to resolve these peaks more completely. A rice sample gave ratios of lysine/phenanthrene of 1.022 and 1.025 on column A and 1.001 and 1.032 on column B by the short isothermal method. It was then programmed, after 5 min at 100°, at 2°/min to 210°. Lysine emerged at 45 min with a horizontal base line and phenanthrene at 51 min; after the phenanthrene peak, five other small peaks appeared, which would normally emerge while the alternate column was in use. Two such programmed runs gave ratios of 1.071 on column A and 1.060 on column B; thus, ratios were higher by 4.5% on column A and 4.3% on column B. We, therefore, consider our results on rice by the usual short method to be *ca.* 4.4% low because of failure to integrate lysine completely with no correction for the sloping base line. Wheat often has a larger pre-lysine peak than rice; a similar study on a wheat with a large pre-lysine peak (0.06 mV) gave an estimated 6% low value for the rapid method.

When short analysis time is not of prime importance, we employ 12-ft columns (isothermal column at 190°, with injector and detector temperatures of 245°) with carrier gas pressures adjusted to deliver lysine to the detector at 10 min and phenanthrene at 18–20 min for complete chromatography. The base line is nearly horizontal for lysine. With 0.5-g samples of seed meal, 15 ml of the 25-ml hydrolysate (step 4) gives good quantitative peaks. For calibration with known lysine (step 5) a 1.0-g sample of meal is hydrolyzed with 20 ml of 6 $N$ HCl and the lysine is added to a second 15-ml aliquot from the 50-ml hydrolysate (step 4).

Dried leaves and immature heads of wheat produced curves similar to those of grain and were analyzed in the same manner.

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# A Modern Approach to the Isolation and Characterization of L-Ascorbic Acid (Vitamin C)

Vitamin C was isolated from lemons in 55% yield by a new method utilizing ion exchange techniques. The substance was characterized by modern physical chemical methods.

Classical methods (Zilva, 1924, 1930; Szent-Györgyi, 1928; Waugh and King, 1932) of isolating L-ascorbic acid involve cumbersome fractional precipitations and employ conditions toward which the compound is not stable. As a result, the recovery of pure material from most natural sources is often low. We have, therefore, devised a general isolation procedure in which ion exchange techniques are employed. When specifically applied to the isolation of vitamin C from lemons, an overall recovery of at least 55% of crystalline material was obtained.

In essence the isolation procedure involves precipitation of citric acid and other strong organic acids as $Pb^{2+}$ salts, removal of the excess $Pb^{2+}$ from the supernatant by cation exchange chromatography, removal of acetic acid by evaporation, and separation of ascorbic acid by displacement chromatography.

A somewhat similar anion exchange method was used (Klose *et al.*, 1950) to obtain ascorbic acid from immature walnut hulls in which the vitamin is stated to be present to the extent of 16–20% of the dry weight. The anion exchange procedure was not, however, used in conjunction with Pb precipitation and cation exchange techniques. Ion exchange was also used for the partial purification of vitamin C from citrus rinds (Mottern and Buck, 1948).

## METHODS

Juice was expressed from 40 lemons and particulate matter was removed by filtration through glass wool. The filtrate, 1.4 l., contained about 600 mg of ascorbic acid in 180 g of nonvolatile material. The filtrate (pH 2.40) was passed through 100 ml of AG 50W-X4 (50–100 mesh, H+ form) cation exchange resin and the resin was washed with 300 ml of $H_2O$. This step was included to give generality to the procedure. It is designed to remove heavy metal ions, convert all anions to free acids, and inactivate ascorbic acid oxidase. It probably could be omitted with lemon juice. The combined effluent and wash (pH 2.15) contained 600 mg (3.4 mequiv) of ascorbic acid in 1200

mequiv of total acids (determined by titration of a sample to pH 7.0). Strong organic acids were precipitated by the addition, with good stirring, of 270 g (1400 mequiv) of $Pb(OAc)_2 \cdot 3H_2O$ previously dissolved in 700 ml of $H_2O$. In the process the pH of the suspension rose to 3.2. The precipitate was removed by filtration and the bulky cake was washed with 600 ml of $H_2O$. At this stage classical procedures would precipitate lead ascorbate by raising the pH to 7.6. We obtained recoveries of only 10–30% in this precipitate and noted rapid decomposition of the nonprecipitated ascorbic acid. The above filtrate and wash which contained 500 mg of ascorbic acid were passed through 100 ml of AG 50W-X4 (50–100 mesh, $H^+$ form) cation exchange resin and the column was washed with 200 ml of $H_2O$. The combined effluent and wash which contained 460 mg of ascorbic acid and 22 g of nonvolatile material was evaporated at reduced pressure to a syrup. The syrup was taken up in 50 ml of $H_2O$ and the pH was adjusted to 6.3 with 26 ml of 1 $N$ $NH_4OH$. The solution was immediately applied to a column (25 cm × 1.9 cm i.d.) which contained 70 ml of AG 1-X8 (100–200 mesh, $Cl^-$ form) anion exchange resin. The resin was then eluted with 400 ml of 0.05 $M$ HCl. The last 60 ml of eluent contained the ascorbic acid. It was concentrated under reduced pressure to 1 g of syrup and 140 mg of crystalline ascorbic acid, mp 188–189°, was obtained after adding 1 ml of ethanol and 2 ml of ethyl acetate.

Experimental data are: nmr (100 MHz, 20 mg, $D_2O$ solution, tetramethylsilane as external reference) $\delta$ 4.20 (d, 2, $J$ = 7 Hz, $CH_2$), 4.53 (d of t, 1, $J$ = 2 and 7 Hz, $H_5$), 5.43 (d, 1, $J$ = 2 Hz, $H_4$); ir (1 mg, KBr) 3525, 3410, 3310, 3215, 3030 (br), 2730 (br), 1750, 1665 (br), 1315, 1270, 1120 (br), 1020, 985, 865, 820, 755 cm$^{-1}$; ORD ($c$ 0.176%, $CH_3OH$; 25°) $[\phi]700$ +54°; $[\phi]589$ +75°; $[\phi]259$ +1000°; $[\phi]250$ 0° (int); $[\phi]235$ −2500°; $[\phi]224$ 0° (int); $[\phi]215$ +1370°; $[\phi]211$ 0° (int); $[\phi]202$ −6750°; $[\phi]200$ −4250° (last); CD ($c$ 0.176%, $CH_3OH$; 25°) $[\theta]300$ 0; $[\theta]268$ −200; $[\theta]260$ 0; $[\theta]245$ +900; $[\theta]237$ 0; $[\theta]224$ −2400; $[\theta]212$ 0; $[\theta]200$ −7500. $Anal.$ Calcd for $C_6H_8O_6$: C, 40.92; H, 4.58. Found: C, 40.76; H, 4.86.

A second crop, 197 mg, mp 186–187°, was obtained from the mother liquor ($Anal.$ Found: C, 41.02; H, 4.68). Thus, the overall purification of 300-fold was obtained with about 55% recovery of activity. It is noteworthy that ascorbic acid crystallizes cleanly from a very concentrated highly impure solution apparently because none of the contaminants are structurally similar.

Synthetic sodium ascorbate, produced by Hoffmann-La Roche, lot 36112, 99.9% pure, was converted to the free acid by passage through an $H^+$ cation exchange resin. Ascorbic acid was assayed by reduction of 2,6-dichlorophenolindophenol (Bessey and King, 1933). Assays on crude materials may be falsely high due to the presence of other reducing substances and colored substances which obscure the end point of the titration.

Vapor phase chromatography was performed with 12 ft × 0.25 in. glass columns packed with Gas-Chromosorb Q. The initial temperature was 120° for SE-30 and 150° for OV-17 with a gradient of 2°/min in both cases. Retention values (methylene units) are determined by running the sample with a series of normal hydrocarbons (Horning et al., 1967). The mass spectra were obtained on a Hitachi

## Table I. Comparison of Properties of Synthetic Ascorbic Acid and Natural Vitamin C

| Property | Synthetic | Natural |
|---|---|---|
| Mp (corr) | 189–190° | 188–189° |
| $[\alpha]^{25}D$ ($c$ 1, $CH_3OH$) | +46.7° | +47.1° |
| $pK_a$ (spectrophotometric) | 4.15 ± 0.05 | 4.18 ± 0.05 |
| Ultraviolet spectrum | | |
| pH 1, $\lambda_{max}$ 243 nm | $\epsilon$ 10,000 | $\epsilon$ 9500 |
| pH 6, $\lambda_{max}$ 265 nm | $\epsilon$ 13,600 | $\epsilon$ 13,750 |
| Vpc (TMS derivative)[a] | | |
| 5% SE-30 (methylene units) | 19.76 | 19.76 |
| 3% OV-17 (methylene units) | 20.17 | 20.16 |
| Mass spectrum (TMS derivative) | | |
| $m/e$ 464 (M$^+$) | 2.5% | 2.5% |
| $m/e$ 449 | 7% | 8% |
| $m/e$ 333 | 100% | 100% |
| $m/e$ 205 | 75% | 64% |

[a] The vpc analyses indicated that both preparations were essentially free of volatile contaminants detectable by flame ionization.

RMU-6L gas chromatographic–mass spectral system at 70 eV. The relative intensities are based upon $m/e$ 333 = 100%. A Durrum-Jasco recording spectropolarimeter and circular dichroism recorder, Model ORD/UV/CD-5, was used. The curves were very noisy due to the high absorption; therefore, the experimental error is large.

## RESULTS

The properties of isolated vitamin C and those of synthetic L-ascorbic acid are presented in Table I. The data do not differ in any significant respect.

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