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Improved 2,4-Dinitrophenylhydrazine, Thin Layer Chromatography Methods for the Determination of Micro- and Macroamounts of Ascorbic Acid

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Ascorbate Determination, 2,4-Dinitrophenylhydrazine

Microamounts of vitamin C could be readily determined in 20 μl-samples using the 2,4-dinitrophenylhydrazine method together with separation by thin layer chromatography. The condensation reaction was carried out for 5 min at 100 °C on a glass fibre disc. Purification of vitamin C hydrazones was accomplished by repeated separation on TLC plates. An aqueous solution of 65% acetic acid was employed to dissolve the vitamin C hydrazones, providing maximal absorbance at 500 nm. The minimum amount detectable by this method is 0.4 μg of dehydroascorbic acid. The macrodetermination of vitamin C was improved by simplifying a previous method and employing 65% aqueous acetic acid as a solvent for the hydrazones.

The determination of ascorbic acid by means of 2,4-dinitrophenylhydrazine [1] is frequently used, in spite of the fact that osazone-forming carbohydrates [2] cause some errors, particularly in presence of high concentrations of the latter. Such errors may be successfully eliminated by combining the above dinitrophenylhydrazine method with thin layer chromatography [3, 4]. This technique was modified in order to determine vitamin C in small volumes of body fluids or tissues of insects; it was also improved for the macrodetermination of vitamin C, as described below.

Materials and Methods

Microdetermination. The following reagents were used: 0.5% oxalic acid, 0.5% 2,6-dichlorophenol-indophenol dissolved in boiled distilled water (stored in a refrigerator and renewed weekly), 2% 2,4-dinitrophenylhydrazine in 9 N sulfuric acid (stored in refrigerator and renewed every second week), 0.25% thiourea dissolved in the 2,4-dinitrophenylhydrazine-sulfuric acid solution (renewed for each set of determinations), 0.02% standard L[+]-ascorbic acid solution in 50% oxalic acid. TLC glass plates (20 × 20 cm) were coated with a layer (thickness = 0.25 mm) of a mixture of 40 g Kieselgel H (Stahl) and 80 ml distilled water (sufficient for 5 plates) and air-dried overnight prior to use. Glass fibre discs were of Whatman grade GF/A 23 mm, TLC development solvents were 35 ml ethyl acetate, 25 ml chloroform, 3.5 ml glacial acetic acid and 3.0 ml acetone. The solvent for the vitamin C hydrazones was 65% aqueous acetic acid.

The reagents for the vitamin determination were pipetted together with a sample into the cavity (diameter 2 cm) of a porcelain spotting plate. The mixture usually included a 100 μl aliquot of centrifuged sample extract prepared with 0.5% oxalic acid, 20 μl indophenol solution (until the sample mixture remains pink), and 20 μl 2,4-dinitrophenylhydrazine-thiourea solution. The same procedure was carried out with 100 μl standard solution containing 4 μg of L[+]-ascorbic acid. The samples and reagents were mixed by gentle shaking of the plate. A glass fibre disc was then placed into each cavity until the reaction mixture was completely adsorbed. The disc was deposited at the bottom of a test tube, whose opening was tightly covered with aluminium foil. Subsequently these test tubes were kept for five minutes in boiling water and left overnight at room temperature. The discs were dried by the air current of a ventilator. Each disc was put in a microtest tube containing 400 μl acetone. Gentle shaking of the tube and pressing the disc with a glass rod facilitated the extraction of the hydrazones by the acetone. An amount of 200 μl of the hydrazone solution as well as 2 reference samples of ascorbic acid hydrazone were spotted on the TLC plate. After full development, the red band containing the purified dehydroascorbic acid hydrazones was scraped off, dissolved in 600 μl of 65% acetic acid in a microtest tube and centrifuged for 5 min at 3000 rpm. The transparent supernatant liquid was transferred to another test tube and an aliquot thereof delivered into the microcell of a spectrophotometer (Bausch and Lomb, Spectronic 20), whereupon the absorbance was determined at 500 nm using 65% acetic acid as the blanc.

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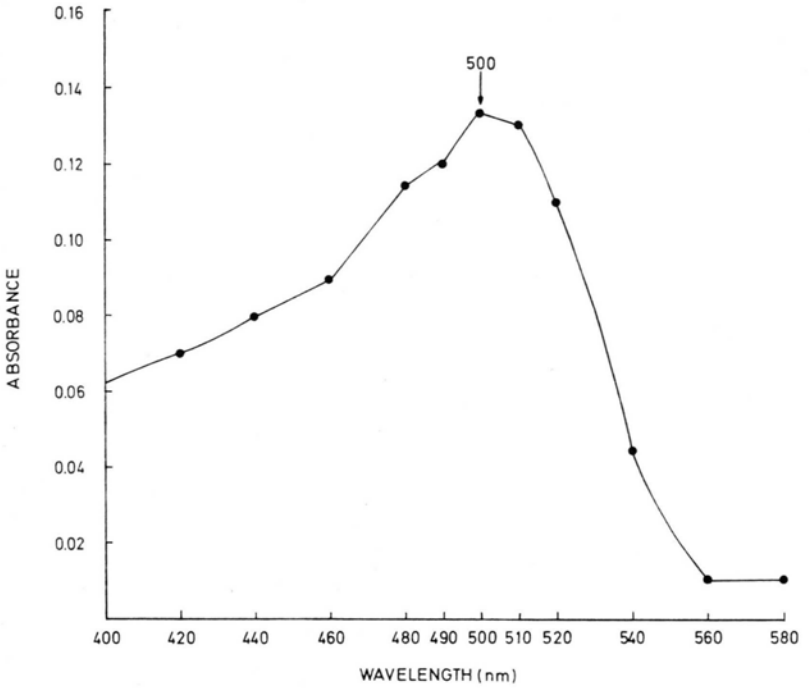

Fig. 1. Absorption curve of L[+]-ascorbic acid; microdetermination.

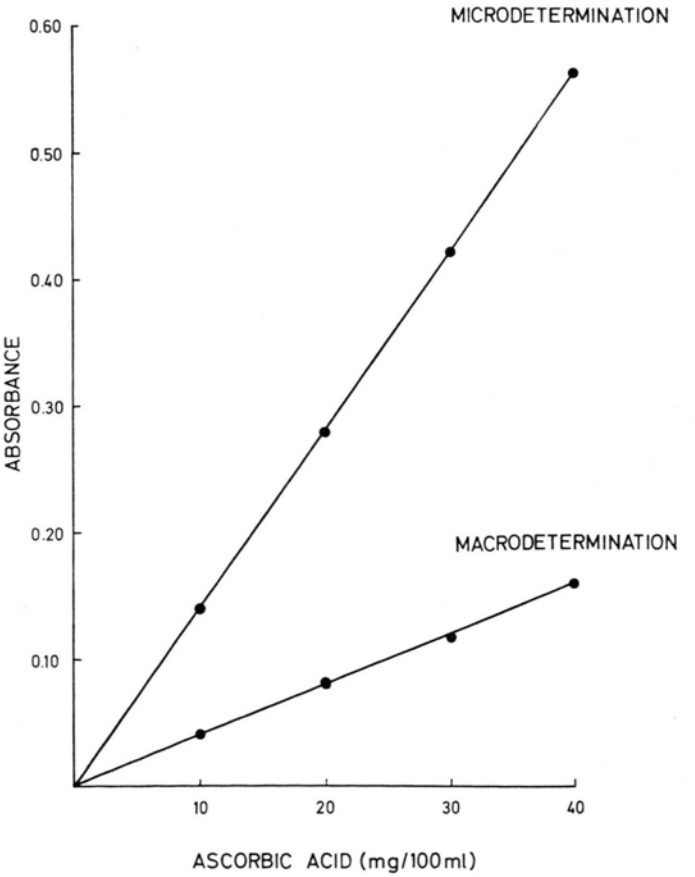

Fig. 2. Relationship between concentration of dehydroascorbic acid hydrazones in 65% aqueous acetic acid and absorbance at a wave length of 500 nm; microdetermination and macrodetermination.

Macrodetermination. The determination was carried out according to the former method [4] with modifications. The condensation was performed at 100 °C for 10 min, and the samples were kept overnight at room temperature. The reddish precipitate of hydrazones was collected on a Whatman GF/C glass fiber disc (thickness = 25 mm) which was adjusted on the supporting screen of a stainless-steel millipore filter.

The precipitate was washed with equal volumes of 0.2 M sodium bicarbonate, 0.1 N sulfuric acid and distilled water, and finally dried by an air current. It was dissolved in 5 ml of acetone and a 100 µl-aliquot of this solution employed for chromatographic separation. The orange-coloured band of vitamin C was scraped from the plate, dissolved in 5 ml of 65% acetic acid and centrifuged for 5 min at 2500 rpm. The supernatant liquid was collected and an aliquot part of the latter measured in the spectrophotometer at 500 nm, using an aqueous solution of 65% acetic acid as the blanc.

Results and Discussion

Microdetermination. Oxalic acid was found more suitable than trichloroacetic acid for the extraction of ascorbic acid, mainly because the latter is relatively hygroscopic and interferes with the drying of hydrazones. Condensation with 2,4-dinitrophenylhydrazine was carried out after adsorption of the ascorbate solution on glass fibre, which increased the surface of the reaction mixture, improved handling and prevented loss of the hydrazone solution. Since the 2,4-dinitrophenylhydrazones of vitamin C can be readily separated from those of concomitant carbohydrates in solution, the condensation temperature [3] does not deserve particular attention. Moreover the TLC removes the chromogens usually found in samples of biological origin, whereby the previously employed clearing with active carbon [6] becomes superfluous. Maximal absorbance was obtained at 500 nm when the vitamin C hydrazones were dissolved in 65% acetic acid, as evident from Figs 1 and 2. Furthermore a linear correlation between absorbance and concentration of ascorbic acid within the range of 10 – 40 mg/100 ml was obtained (Fig. 2). The minimum amount of vitamin C detected was 0.4 µg, and the experimental error was not more than 3% beyond the vitamin concentration.

Macrodetermination. The previous method involved rinsing the hydrazones with water in a sintered glass funnel, dissolving the former in

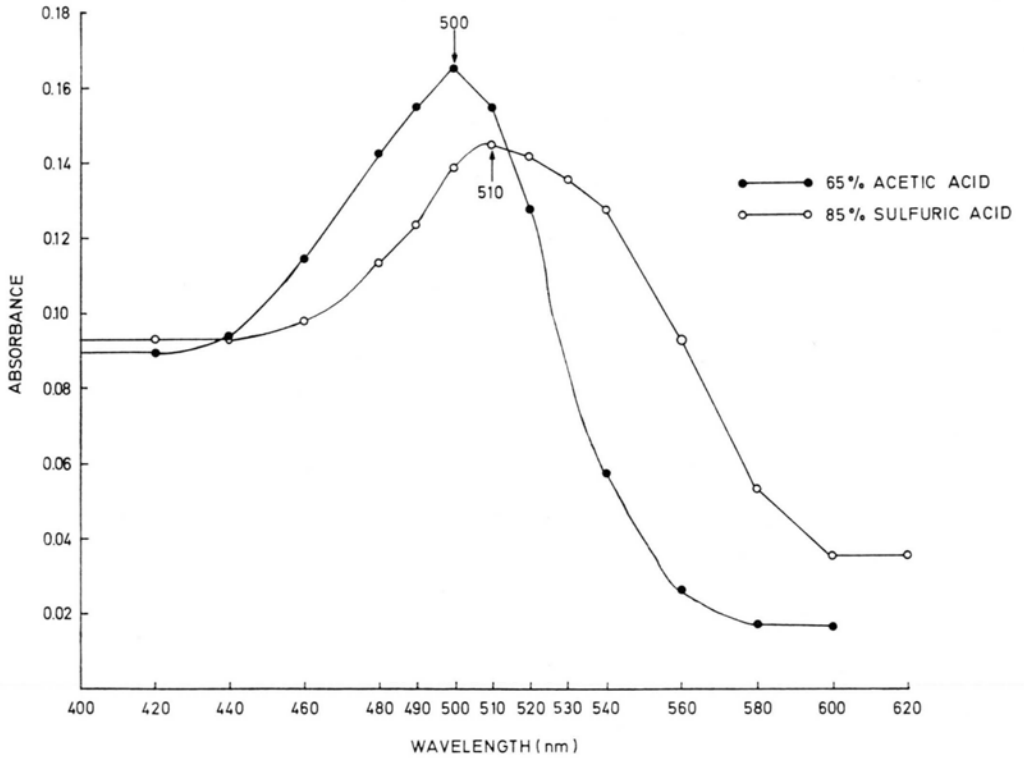

Fig. 3. Absorbance of ascorbic acid dissolved in sulfuric acid and acetic acid; macrodetermination.

acetone or warm ethyl acetate, and evaporation of the solvent under reduced pressure [3]. In the present study, this procedure was simplified and improved. The hydrazone solution was kept overnight to obtain a maximal yield of hydrazones. Washing of the hydrazones with bicarbonate and sulfuric acid [6] promoted the elimination of unchanged 2,4-dinitrophenylhydrazine and α-oxohydrazones. Figs 2 and 3 reveal results resembling those obtained by the microdetermination. Since the vitamin C hydrazones are distinctly separated from interfering derivatives by thin layer chromatography, acetic acid can be substituted for sulfuric acid [7] without any risk. With this technique, sodium ascorbate, calcium ascorbate, D-isoascorbic acid and dehydroascorbic acid could also be determined, but not D-glucoascorbic acid.

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