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Determination of ornithine, lysine, arginine, citrulline and histidine

Separation of the common amino acids in plant material with good resolution on a single sheet of filter paper cannot be accomplished with present procedures. MCFARREN[1] and MCFARREN AND MILLS[2] used a series of buffers and solvents, but these procedures have been found to be long and tedious. THOMPSON *et al.*[3] partially overcame this problem by separating the amino acids on columns of Dowex 50X4 resin into a basic fraction and an acidic and neutral fraction. This preliminary separation also purified the acids from extraneous materials which was necessary for high resolution. In

TABLE I

R_F VALUES OF AMINO ACIDS AND AMINES SEPARATED ON EDTA BUFFERED PAPER

Compound	R_F
Hydroxylysine	0.03
Ornithine	0.07
Lysine	0.12
Arginine	0.24
Citrulline	0.31
Histidine	0.40
Homocitrulline	0.52
Tyramine	0.65
Ethionine	0.76

order to obtain good separation of the basic amino acids it is necessary that the reagents and paper be free of metal ions. THOMPSON AND MORRIS[4] accomplished this by washing the papers and repurifying the reagents. In addition, 84 h was required to run the basic acids. More recently SIBALIC AND RADEJ[5] reported a rapid method using high temperature separation for lysine, arginine, and histidine.

The present procedure will not only separate the three acids listed above, but will also resolve ornithine, citrulline, hydroxylysine, homocitrulline, tyramine and ethionine (Table I). These separations can be accomplished in 12 h and without prior washing of the paper or purifying the reagents.

Materials

Filter paper. Whatman No. 52, size 18 1/4 × 22 1/2 in.

Buffer No. 1. 29.2 g ethylenediaminetetraacetic acid (EDTA) in deionized water. Add NaOH to dissolve the acid and adjust to pH 7.0 and make to 1 l.

Buffer No. 2. 3.944 g boric acid in deionized water. Add NaOH (about 1.45 g) and adjust to pH 9.3 and make to 1 l.

Phenol–cresol solvent. 190 ml 88 % phenol, Fisher liquified, Cat. No. A-931, 165 ml *m*-cresol, Eastman practical grade, Cat. No. P-369 and 45 ml pH 9.3 buffer.

Ninhydrin 1 % and triethylamine 0.25 % in 95 % ethanol.

Standard amino acids. (Nutritional Biochemicals Corporation, Cleveland, Ohio.) DL-Ornithine monohydrochloride, L-lysine, L-arginine, DL-citrulline and L-histidine. These were made to concentrations of 5, 10, 15 and 20 mg of amino nitrogen per 100 ml of 10 % isopropanol.

Procedure

Approximately 10 g of fresh leaves are extracted four times in a Lourdes mixer with 80 % ethanol; the extract is passed through 2 Dowex 50X4 columns[3] and the basic and acidic fractions recovered separately and taken to dryness. Each fraction is then taken up in 2 ml of 10 % isopropanol.

Sheets of Whatman No. 52 filter paper are dipped in EDTA buffer and hung on glass rods to drain for about 15 min. The damp papers are dipped in acetone, allowed to drain for 5 min, and then dried in an oven (Reco) at 65° for 1 h.

The samples are spotted on a Reco Model S-1250 sample applicator using a Hamilton syringe No. 701 with a guide and with the needle cut blunt to 3/4 in. The syringe is held in position by the holder on the applicator. The samples are spotted 1 in. apart with a set of standards on each paper. Two μl of each standard are applied to each spot in 0.4 μl increments, allowing each application to dry. The amount of sample applied may vary but it is necessary that all spots be the same size and the amount applied between dryings, that is 0.4 μl, be kept constant for both samples and standards.

The papers are placed in a chromatogram cabinet between two liners of Whatman No. 4 papers and with one sheet of paper in the bottom of the cabinet. These papers serve to increase the saturation of the cabinet. The liners are moistened with a mixture of 200 ml of pH 9.3 buffer and 8 ml of the phenol–cresol solvent. The remainder of the mixture is put into the trough holding the two liners. The troughs containing the spotted papers are filled with about 100 ml of the phenol–cresol solvent and allowed to run overnight or about 12 h. The temperature is maintained at 27° ± 2°.

After development the papers are removed from the cabinet and dried in an oven at 65° for 1 h. They are then dipped in ninhydrin and after the excess alcohol has dried placed in an oven at 65° for 30 min.

Following development of the color the papers are kept in the dark for 30 min and then read in subdued light on a Photovolt Model 501A Densitometer using a 570 mμ filter or the spots may be cut out of the paper, placed in tubes of 50 % ethanol, and read on a colorimeter.

If the latter procedure is used, the concentration of the standards and samples spotted on the papers should be increased 10 times. Also the concentration of the ninhydrin should be 2 g per 100 ml.

Results and discussion

The use of this method has given excellent separation of basic amino acids in plants (Fig. 1). Whatman No. 52 paper was selected rather than Whatman No. 1 because the former has greater wet strength and better resolution. The wet strength is important for dipping the paper in buffer.

In preparing the EDTA buffer one lot of EDTA was found unsatisfactory because of impurities that reacted with ninhydrin. However, three other lots, all by different manufacturers, have been satisfactory. The concentration of the buffer has been studied using 0.01, 0.05, 0.1, and 0.15 *M* EDTA. At the two lowest concentrations citrulline and histidine did not separate while with 0.15 *M* EDTA arginine and citrulline remained unresolved. The pH of the buffer was also compared. There was good separation between pH 6.0 and pH 7.0 with slightly more compact spots using the buffer adjusted to the higher pH. The papers are dipped in acetone following dipping

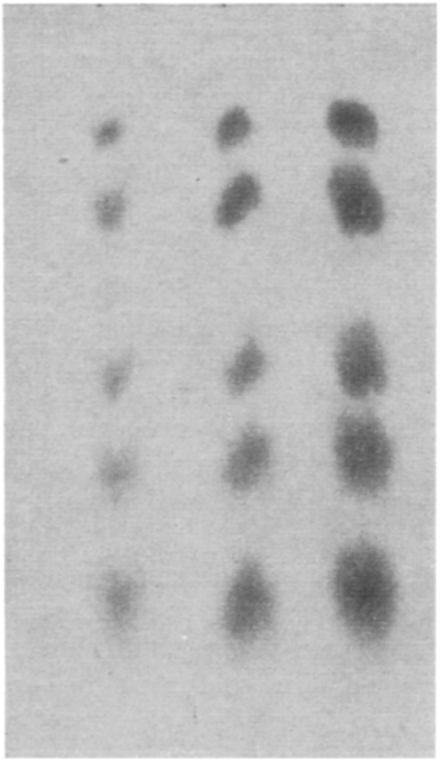

Fig. 1. One-dimensional paper chromatogram of ornithine, lysine, arginine, citrulline, and histidine.

in buffer to prevent an accumulation of salt on the surface which will interfere with the final reading.

The solvent used for developing the papers is essentially that of LEVY AND CHUNG[6]. Redistilling the phenol and cresol has not been found to improve the procedure. A high degree of saturation of the cabinet is important during the time the separations are being made. A lack of vapor can prevent good resolution, particularly of ornithine and lysine. This was the case when only a single narrow strip of paper was run. It is desirable to run at least two papers each time.

The development of color with ninhydrin causes the entire paper to have a light pink color. This color will darken slowly with time and the papers should be kept in subdued light. The ninhydrin color is sufficiently stable to obtain satisfactory determinations (Table II). Comparisons were made of developing the color in a ventilated

TABLE II

VARIATION OF DENSITY FOLLOWING TIME OF DEVELOPMENT WITH NINHYDRIN

Compound	Densitometer*		Spectrophotometer**	
	30 min	2 h	30 min	24 h
Ornithine	0.39	0.42	0.20	0.20
Lysine	0.39	0.39	0.22	0.20
Arginine	0.31	0.28	0.25	0.25
Citrulline	0.27	0.27	0.30	0.30
Histidine	0.20	0.22	0.25	0.23

* The density of the spots was read through the paper.
** The spots were eluted and the density of the solvent was determined.

oven as compared with using a closed box heated to 65° and filled with CO_2. The latter gave less color, especially with arginine, citrulline and histidine.

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