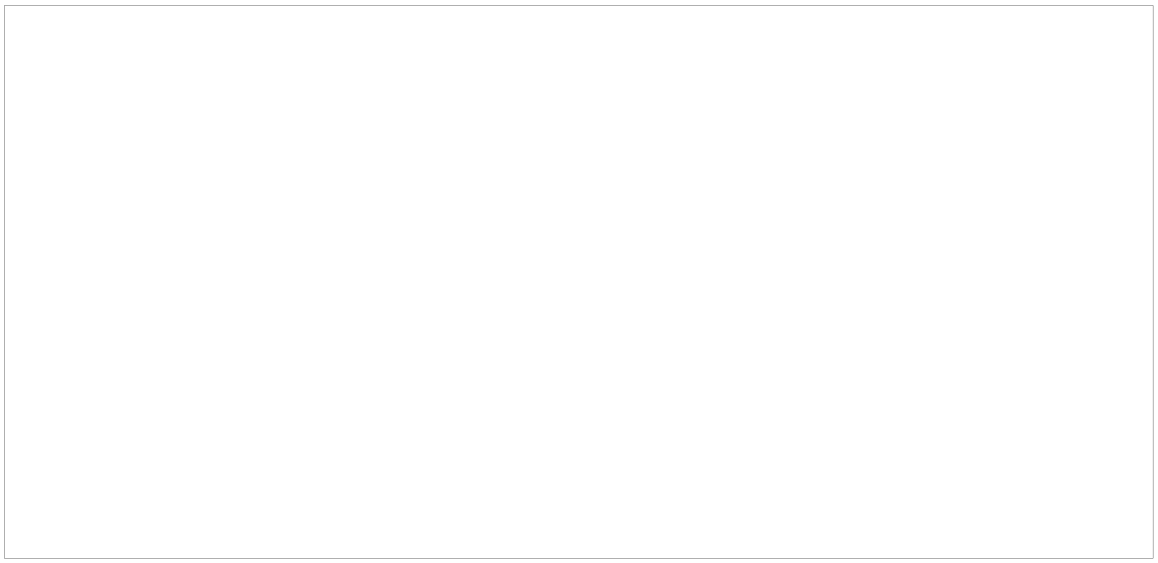

Title: CHROMATOGRAPHIC DETERMINATION OF AMINO DICARBOXYLIC ACIDS IN PLANTS by V. L. Krotovich and A. A. Bundel' (USSR)

Source: Doklady Akademii Nauk SSSR, LXI, 5, 861-4, 11 Aug 1948 (Thrice monthly periodical)

CONFIDENTIAL

CONFIDENTIAL

CHROMATOGRAPHIC DETERMINATION OF AMINO DICARBOXYLIC ACIDS IN PLANTS

V. L. Kretovich and A. A. Bundel'

The role of dicarboxylic amino acids in plants is very great. From them asparagine and glutamine are formed; they form a necessary link in the enzymatic transamination reaction; and they are of singular importance in the synthesis of amino acids [1,2]. For these reasons, their quantitiative determination is of very great significance in investigating the metabolism of the plant cell. However, the Foreman method currently in use for this determination is very difficult and very inaccurate.

The classical investigations of M. Tsvet [3] for the first time initiated the wide-spread application of the chromatographic method in organic and biological chemistry. Wieland then [4] established the possibility of separating dicarboxylic amino acids from other amino acids by exchange adsorption on aluminum oxide. Later on, he used this principle to investigate protein hydrolyzates [5,6].

Kretovich and Bundel' used the chromatographic method with plants to determine amounts of amino dicarboxylic acids. Their procedure consisted in passing an aqueous extract of plant material (inactivated with boiling ethyl alcohol) through a column of Al_2O_3, which had been treated with weak hydrochloric acid, and the subsequent elution of the adsorbed dicarboxylic amino acids with a solution of alkali.

The aluminum oxide, standarized according to Brokmann (Russian spelling), has an alkaline reaction and does not adsorb amino dicarboxylic acids from a neutral solution. As a result of treating it with a aqueous solution of hydrochloric acid it becomes anionotropic and acquires the ability to combine with acids to form salts. The hydrochloric acid used for preliminary treatment of the adsorbent is displaced by the amino dicarboxylic acids. The neutral (with the

exception of cystine) and basic amino acids pass freely through the "acid" column, but aspartic and glutamic acids, as the aqueous solutions and sodium salts pass through such a column, are retained by the Al_2O_3. Elution of amino dicarboxylic acids from the adsorbate is accomplished with a weak caustic potash solution. Cystine, although it does not possess acid properties, apparently forms an aluminum salt which is dissolved only with difficulty.

Determination of amino dicarboxylic acids in an eluate can be done by different methods: Wieland and Wirth [7] determined them colorimetrically with ninhydrin. Their method, however, is not accurate. Schramm and Princeigh [8] used the more accurate Kjeldahl micro method, which was also employed by Darling [9]. The latter scientist, however, did not at all consider the possibility of cystine being adsorbed in the column, and it also appears that he used a quantity of adsorbent which is insufficient for adsorption of the amino dicarboxylic acids from plant extracts.

Kretovich and Bundel' first attempted their determination in 6 ml of a pure solution containing 2 mg of glutamic acid and 4 mg of aspartic acid (containing 0.38 mg and 0.21 mg of nitrogen, respectively). In the eluate from a column containing 2 g of Al_2O_3, 0.60 mg of nitrogen were found after making a correction for the reagents. Nevertheless, checking the "detection ability" for amino dicarboxylic acids added to plant extracts showed that 2 g of Al_2O_3 is not sufficient, and for this reason the authors used 4 g in all subsequent experiments. Moreover, Kretovich and Bundel' took issue with Darling's use of trichloroacetic acid for the precipitation of proteins, since the acid reaction induces a pronounced hydrolysis of glutamine and the formation of free glutamic acid.

Experiments to investigate the "detection ability" of amino dicarboxylic acids added to plant materials were conducted with sugar beets, wheat and lupine sprouts, and the young leaves of beans and willows. Results of these experiments are shown in Table 1, which appears in the Appendix.

In view of the considerable amounts of glutamine and asparagine in plants, Kretovich and Bundel' interested themselves in the behavior of amides in the chromatographic determination of amino dicarboxylic acids, and were able to establish that asparagine is not adsorbed by Al_2O_3, but rather that it passed completely through the column along with the wash water. Since there is evidence of the presence of free cystine in plants [10] and since cystine is retained by the Al_2O_3 column, they found it necessary to wash the substances adsorbed in the column with a saturated hydrogen sulfide solution. As a result of this, the cystine was reduced into cysteine which by a subsequent water rinsing was completely removed from the column. Results of such procedure are given in Table 2 (in the Appendix) which shows that washing with an aqueous hydrogen sulfide solution does not affect the ability of the amino dicarboxylid acids to be adsorbed. As confirmation of this fact, these authors prepared an extract from young willow leaves (fixed by boiling with ethyl alcohol) and added to them a mixture of glutamic and aspartic acids containing 0.66 mg of nitrogen; they found 0.68 mg of nitrogen of amino dicarboxylid acids.

In another test, they washed the adsorbed amino dicarboxylic acids (which had not been added but had been present in the plant extract in the natural state) with a hydrogen sulfide solution. Lupine sprouts were used, and the adsorbed extract washed the first time with 100 ml of a saturated H_2S solution, and then with 50 ml of distilled water. In both instances, 0.37 mg of nitrogen of dicarboxylic acids was detected per 2 ml of extract.

Foregoing experiments were instrumental in establishing the following procedure in this research:

Fresh dried plant material (2 g) is inactivated by boiling with 96% ethyl alcohol in a porcelain dish for 5 minutes, during which time part of the alcohol evaporates. The remainder of the alcohol is removed with benzene, and the dried material then pulverized in a mortar, after which

it is transferred quantitatively into a porcelain dish and covered with 30 ml of distilled water. The material is well mixed with the water and allowed to stand for a half hour at 20°, before being passed through the paper filter of a Buechner funnel. To a fiven quantity of the filtrate (usually 2-4 ml) is added a drop of an alcohol solution of phenolphthalein. It is then neutralized with 0.05 N KOH to a faint pink coloration and introduced into the adsorption tube containing Al_2O_3, which is prepared in the following manner:

4 g of Al_2O_3, standardized according to Brokmann [Russian spelling], are treated with 12 ml N HCl for 5 minutes, during which time it is continuously agitated. Then the mixture is permitted to stand for 10-15 minutes, after which the cloudy liquid is poured off and 40-50 of distilled water added. This mixture is well shaken, and then decanted after a precipitate settles. The precipitate is washed by decantation until there is no acid reaction with litmus. The adsorbent prepared in this way is kept in the same flask in which it was treated, under water.

A glass adsorption tube, 50 cm in length and 7-8 mm in internal diameter with a constriction 12 cm from the lower end, is used for chromatographic determination. A small piece of adsorbent cotton is placed in the constricted part. The tube itself is fastened with a rubber stopper into a Bunsen flask and clamped to a rack. The adsorbent is poured into the tube, and the water in which it was contained passes off entirely (either because of the force of gravity or aided by a small amount of suction). The solution to be tested is then sucked through the adsorbent (taking care that the adsorbent is at all times covered by the solution) at the rate of one drop in 2 seconds. Next, the column is washed with 50 ml of distilled water. As a result, all of the neutral amino acids (including cystine, reduced by the H_2S to cysteine) pass through the adsorption column. The wash water is then poured out of the receptacle which is rinsed. For the elution of the adsorbed dicarboxylic amino acids, 3 ml 3 N KOH and then 30 ml of 0.05 N KOH are introduced into the

tube and sucked through into the receptacle. The liquid from the column is drawn off until the residue is dry, so that the salts of dicarboxylic amino acids are transferred to the receptacle. The solution is quantitatively transferred into a Kjeldahl flask, and the nitrogen determined with a Parnas-Vagner [Russian spelling] micro apparatus.

Results are expressed in milligrams of nitrogen of amino dicarboxylic acids per gram of dry substance. A control solution is prepared by substituting an equal quantity of pure water for the extract.

Kretovich and Bundel' attempted the determination of free amino dicarboxylic acids occurring in the natural state in plants, and prepared the data shown in Table 3.

Their method is described as accurate, quick, and not requiring complicated apparatus. They say it can be used, too, for determination of the amino dicarboxylic acids in hydrolzates of proteins.

The authors of this paper, which was submitted 15 June 1948 and presented the same day by Academician A. I. Oparin, are affiliated with the Institute of Biochemistry imeni A. N. Bakh, Academy of Sciences USSR.

Appendix

Table 1

Results in the Determination of Dicarboxylic Amino Acids which were Added to Extracts of Various Plant Specimens

	N of dicarboxylic amino acids in mg				
	sugar beets	wheat sprouts	lupine sprouts	bean leaves	willow leaves
Amount added to the extract	0.53	0.55	0.65	0.63	0.66
Amount detected	0.55	0.49	0.61	0.56	0.63

Table 2

Adsorption of Cystine by Al_2O_3 under Different Washing Conditions

How Washed	N of cystine in mg		
	Given	Found in the column	Found in the wash water
With water	0.42	0.16	0.30
With hydrogensulfide solution	0.43	0.002	0.41

Table 3

Amounts of Free Dicarboxylic Amino Acids in Plant Materials (in terms of 1 mg % per 1 g of the dried specimen)

Sugar beets	Wheat sprouts	Lupine sprouts	Bean leaves	Willow leaves
0.69	2.76	5.5	7.85	0.75

REFERENCES

1. A. Braunshteyn, Uspekhi sovremennoy biokhimi, I, 40, 1947.
2. Kretovich and Bundel', Doklady Akademii Nauk SSSR, LIX, 9, 1947.
3. M. Tsvet, Chromophyllae in the Plant and Animal World, Warsaw, 1910.
4. T. Wieland, Hoppe-Seyler's Z, CCLXXIII, 24, 1942.
5. Wieland, Naturwissenschaften, XXX, 333, 1942.
6. Wieland, Ber., LXXV, 1001, 1942.
7. Wieland and L. Wirth, Ber., LXXVI, 823, 1943.
8. G. Schramm and J. Primosigh, Ber., LXXVI, 373, 1943.
9. S. Darling, Acta physiologica scandinavica, X, fasc. 1, 91, 1945.
10. C. Dent, W. Stepka, and F. Steward, Nature, CLX, 682, 1947.

- E N D -