A QUANTITATIVE ASSAY FOR APPLE LEAF AND TWIG PROTEIN INVOLVING THE USE OF NINHYDRIN

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Quantitative colorimetric determinations of apple leaf and twig protein by standard methods are complicated by the presence of brownish-colored substances. The brown color begins to develop immediately in the liquid plant extract and when first noticeable, it gives a positive ferric chloride test which indicates the presence of phenolic compounds. After 15-20 minutes a negative ferric chloride test is observed which suggests that the phenolic materials is degraded, probably by auto-oxidation or by phenolase activity.

A search of the literature revealed no techniques available for the direct analysis of apple leaf and twig protein. The method most frequently used involves the determination of nitrogen by the Kjeldahl method, with the conversion to per cent protein by multiplying by a nitrogen-protein conversion factor of 6.25. This method has the disadvantage of basing the amount of protein upon the amount of total nitrogen present, which includes nonprotein compounds and, therefore, results in slightly higher values. An added disadvantage is the calculated ratio of protein to nitrogen; a relationship which does not always hold true. Since a true relationship between nitrogen and protein was desired, a new method of plant protein analysis was devised. The techniques used and the values obtained are given in this report.

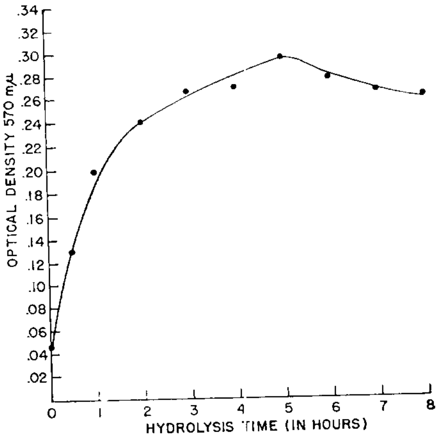
MATERIALS AND METHODS

In the initial determinations of protein, the following preparatory techniques were used. Fresh plant material was ground with deionized water in a Waring Blendor and the homogenate was strained through cheesecloth. Three ml of the extract were added to 3 ml of 10 per cent trichloroacetic acid and the mixture was centrifuged at 1800 x g for 20 minutes. The pellet obtained was suspended in 8 ml of 0.1M sodium hydroxide and allowed to stand overnight. Biuret analysis of the suspension could not be made because of the formation of brown substances which interfered with the determination. The suspension was reprecipitated and resuspended 3 times in trichloroacetic acid and sodium hydroxide, respectively. However, the brown color persisted. Washing the pellet in acetone, carbon tetrachloride or water did not remove the color, nor did treatment with activated charcoal. In further studies, the grinding of plant tissue at reduced temperatures in 0.1M phosphate buffer at pH 7.0 in the presence of sodium bisulfite did not prevent the formation of the brown materials.

The method of analysis used was based upon the hydrolysis of known quantities of protein and plant material, with the subsequent assay of the amino acids by the use of ninhydrin solution (triketohydrindene hydrate) (Moore and Stein, 1948, 1954). The ninhydrin solution was prepared as follows: (a) 400 mg reagent grade $SnCl_2 \cdot 2H_2O$ was dissolved in 250 ml of 0.2M citrate buffer at pH 5.0; (b) the above was added to 250 ml of methyl cellosolve which contained 10.0 g of dissolved ninhydrin.

It was necessary to determine a protein hydrolysis time at which the liberation of amino acids was the greatest. Known quantities of bovine serum albumin were hydrolyzed for various lengths of time, after which the samples were allowed to react with ninhydrin and assayed colorimetrically. The hour at which the highest optical density was recorded was considered to be the optimum hydrolysis period. In each case, hydrolysis was carried out by adding 10 mg of bovine serum albumin to 0.5 ml of 6N hydrochloric acid in a test tube. The tubes were covered with glass marbles and placed in an autoclave for the designated periods of hydrolysis (0, ½, 1, 2, 3, 4, 5, 6, 7, and 8 hours). The autoclave was operated at 15 lbs pressure at 120° C. After cooling, the samples were dried *in vacuo* over sodium hydroxide pellets for 20 hours and dissolved in 10 ml of sodium citrate buffer of pH 2.2 (Moore, *et al.*, 1958). The buffer used was prepared as follows: (quantities expressed per liter of water), citric acid ($1H_2O$)-21.0 g; sodium hydroxide (95 per cent pure)-8.5 g; concentrated hydrochloric acid-16 ml; caprylic acid-0.1 ml; thiodiglycol-5 ml; Brij 35 (polyoxyethylene lauryl alcohol)-2 ml of a solution prepared by dissolving 50 g of Brij 35 in 100 ml hot water.

Fig. 1.—Determination of the optimum acidic hydrolysis period of bovine serum albumin by use of ninhydrin.



The hydrolysate was filtered under vacuum. An aliquot of the filtrate was allowed to react with 1.5 ml of ninhydrin solution for 20 minutes in a boiling water bath. The sample was diluted to 10 ml with 1:1 *n*-propanol-water and read within ½ hour at 570 mμ on a Model 6A Coleman spectrophotometer. Optical density was plotted versus hydrolysis time. The optimum hydrolysis period occurred at 5 hours. Hydrolysis periods of less than 5 hours were incomplete, whereas, periods greater than 5 hours apparently resulted in the destruction of certain amino acids. These data are presented in Figure 1.

Succulent shoots from dwarf Jonathan apple trees were thoroughly washed in 8 rinses of deionized water and placed in paper bags and dried at 65° C for 48 hours in a forced-air oven. The dried material was ground by the use of a Wiley Intermediate Mill to pass a number 40 mesh sieve. Forty mg of the plant powder were used for each ninhydrin analysis, and 700 mg were taken for each Kjeldahl nitrogen determination. Plant samples to be analyzed using ninhydrin were hydrolyzed 5 hours as concluded from the optimum hydrolysis data. A 10-30 μl aliquot of the buffered hydrolysate was used for the analysis. Analyses of samples before hydrolysis indicated that prehydrolysis ninhydrin-positive material was negligible.

A standard curve using 0-50 μg of 5-hour-hydrolyzed bovine serum albumin was constructed and repeated each time a fresh ninhydrin solution was prepared. Ninhydrin solutions were prepared each month and stored under gaseous nitrogen in tightly-stoppered, light-proof, refrigerated bottles.

Kjeldahl nitrogen was determined by standard methods, A.O.A.C. (1960).

RESULTS

As a criterion of accuracy, average protein values obtained by the use of ninhydrin are compared with values calculated by the Kjeldahl method (Table 1).

DISCUSSION

Comparisons of the 2 methods of protein determination showed similar plant protein levels during the spring and summer. In the fall, the ninhydrin-determined protein content decreased and eventually reached the level found at dormancy (April 12). Protein titers, as determined by the Kjeldahl method, dropped very slightly in the fall. It would appear that the ninhydrin test best depicts the true protein content of the plants in the fall, and it is suggested that the high Kjeldahl figures found late in the season are due to an accumulation of non-ninhydrin-positive, nonprotein, nitrogenous compounds.

A disadvantage of the ninhydrin technique, as cited by Moore and

TABLE 1.—Average Per Cent Protein of Jonathan Apple Tree Shoots.[1]

Average per cent protein				
April 12	June 28	August 2	September 11	October 10
		Ninhydrin		
4.2	11.5	11.7	7.3	4.4
		Kjeldahl		
6.5	12.7	11.6	11.0	10.5

[1] Values determined by the ninhydrin technique are listed in addition to values calculated using the Kjeldahl nitrogen procedure. Each figure represents the average of 80 analyses.

Stein (1948), concerns the capacity of this compound to react with any substance having a free amino group, including amino sugars, peptides, amines and ammonia. Using the capacity of alanine to react with ninhydrin as 1 ninhydrin-positive unit, NH_3 gives a value of 0.98 and glucosamine a value of 1.00. The occurrence of these compounds is known in plants, but their concentrations are considered to be low.

SUMMARY

Attempts to determine protein content of succulent Jonathan apple tree shoots by standard colorimetric procedures were hampered by the formation of brownish phenolic substances. A method of analysis was devised by which the use of ninhydrin and new techniques eliminated this interference. The greatest liberation of amino acids from bovine serum albumin occurred at 5 hours, as determined by acidic hydrolysis in the autoclave. Dried plant material was hydrolyzed for 5 hours, redried, neutralized and dissolved in sodium citrate buffer. An aliquot was reacted with ninhydrin and the protein content determined as compared to standard bovine serum albumin hydrolysates A comparison of values obtained by this method to Kjeldahl protein values suggests that the new procedure is more accurate than the latter method.

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