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METHODS OF DETERMINING PROTEID NITROGEN IN VEGETABLE MATTER.

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THE method usually used for the determination of proteid nitrogen, is that modification of the Ritthausen method proposed by A. Stutzer,[1] which consists in precipitating the proteids with copper hydroxide, and determining the nitrogen in the precipitate. This method has been adopted by the Association of Official Agricultural Chemists, and is in use by chemists generally.

Mallet[2] has recently proposed that the proteids be precipitated with phosphotungstic acid at 90° C., using in addition tannic acid when peptones are present.

Wiley[3] determines the proteids in animal materials by precipitating them with bromine.

The work here to be described is a study of the applicability of phosphotungstic acid and bromine as precipitants for the proteids of vegetable materials.

[1] *Jour. f. Landw.*, 28, 103.

[2] Bull. 54, Division of Chemistry, U. S. Dept. of Agr.

[3] Bulletin 54, Division of Chemistry, U. S. Dept. of Agr.

THE PHOSPHOTUNGSTIC ACID METHOD.

The method as proposed by Mallet is, briefly, as follows: The sample is digested with hot water, filtered on a nitrogen-free filter, and washed with hot water containing a little free acid so long as it gives up soluble matter in sensible amount. It is not advisable to use hot water at first when much starch is present. The filtrate is made slightly acid with acetic acid, heated to about 90°, and filtered. To the second filtrate an acidified solution of phosphotungstic acid is added so long as a precipitate continues to be formed, avoiding any large excess of reagent, the liquid heated to about 90°, filtered, and the precipitate washed with water of about the same temperature. The nitrogen in the precipitates is determined by the Gunning-Kjeldahl method, and calculated to proteids. When peptones are present they are precipitated with tannic acid from the solution which has been acidified with acetic acid. When proteoses are present it may be well to make a check determination of their amount by saturation of the aqueous solution, after acidification with acetic acid, heating and subsequent cooling, with zinc sulphate. and determining nitrogen in the precipitate. It may be well to remove fat when it is present in large quantity.

METHOD MODIFIED.

The method above described involves three, or, if peptones are present, four filtrations, which make it very long and tedious. The object of the precipitation with acetic acid is to reduce the bulk of the subsequent phosphotungstic acid precipitate, out of which the amides are to be dissolved by hot water. Its use is unnecessary. When the above method was followed it was found impossible to get a clear filtrate from most of the vegetable materials tested, although various modifications were tried. At 60°, however, no such difficulty was encountered, although at a slightly higher temperature—depending on the material—turbidity would begin to appear. It was also found that between 60° and 100°, a considerable portion of the nitrogen goes into solution. For this reason the temperatures of 60° and 100° were selected for further tests, 100° giving the maximum turbidity and minimum amount of nitrogen, 60° the minimum of

turbidity, *i. e.*, a clear filtrate. The method after much experimentation was simplified as follows: 1.4 grams of the substance in a beaker were stirred well with 100 cc. water, the phosphotungstic acid reagent added and the liquid heated. The liquip was kept at the desired temperature (60° or 100°) for fifteen minutes, filtered, the precipitate washed with water at the same temperature, and nitrogen determined in it.

The reagent used was a 5 per cent. solution of phosphotungstic acid in 2.5 per cent. hydrochloric acid; 5 cc. were used for 3 per cent. or less of nitrogen, 10 cc. for 6 per cent., and 15 cc. for 9 per cent.

EFFECT OF REAGENT.

In dealing with vegetable materials it was found impossible to tell when a slight excess of the phosphotungstic acid reagent had been added. The following results were obtained by using different quantities of the reagent, and heating to 60°. The figures are means of two closely agreeing determinations.

No. 1. COTTONSEED MEAL.

	Nitrogen. Per cent.
10 cc. reagent	6.61
30 " "	6.64
50 " "	6.27

No. 2. WHEAT BRAN.

5 cc. reagent	2.07
10 " "	2.08
25 " "	2.11
45 " "	2.09

No. 3. COWPEA MEAL.

5 cc. reagent	3.18
10 " "	3.11
25 " "	3.22
45 " "	3.17

A small excess of reagent does not affect the results. A very large excess affects them in the case of cottonseed meal. It seems that 5 cc. of the reagent are sufficient for any vegetable material containing less than 3 per cent. of nitrogen, 10 cc. for 6.5 per cent., and 15 cc. for 9 per cent.

EFFECT OF TEMPERATURE.

As has already been stated, a clear filtrate could not, as a rule,

be obtained at a temperature much over 60°, and nitrogen goes into solution between 60° and 100°. In cottonseed meal and wheat bran, the greatest amount of solution takes place between 75° and 90°; cottonseed meal gave at 60°, 6.61 per cent. nitrogen; at 75°, 6.58 per cent.; at 90°, 4.94 per cent.; at 100°, 4.22 per cent. Wheat bran at 60° gave 2.08 per cent. nitrogen; at 75°, 2.01 per cent.; at 100°, 1.75 per cent. The amount of nitrogen dissolved between 60° and 75° is very small.

In order to show the solvent action between 60° and 100°, the following figures are given. Of the nitrogen precipitated at 60°, in cottonseed meal (No. 1.), 36 per cent. goes into solution at 100°; another sample (No. 2.), 24 per cent.; still another (No. 3.), 21 per cent.; cowpea meal, 9 per cent.; green peas, 7 per cent.; soy beans, 18 per cent.; horn meal, 9 per cent.; linseed meal, 12 per cent.; and dried blood, 47 per cent.

Working with blood at 100°, it was found that 5.97 per cent. (37.31 per cent. proteids) goes into solution between 60° and 100°. This sample of blood contained 13.66 per cent. nitrogen, 13.39 per cent. of it being insoluble in water. The filtrate from 100° phosphotungstic acid method was clear, and remained clear although 5.97 per cent. of proteid nitrogen was therein dissolved. This observation threw doubt upon the basis upon which the phosphotungstic acid method is founded; namely, that the phosphotungstic acid precipitate with proteids is not soluble in hot water. This statement is based upon the observation that "the supernatant liquid remained clear on being heated along with the precipitate and subsequently cooled". The following experiments confirmed this suspicion, and proved that phosphotungstic acid does not completely precipitate proteids at 90° or 100°.

0.35 gram of the materials named below were placed in a Kjeldahl flask, 100 cc.of water and 5 cc. of the phosphotungstic acid reagent added, and the solution heated to 60°. The solution was kept at this temperature for fifteen minutes, filtered, washed with water at the same temperature, and nitrogen determined in the precipitate by the Gunning method. Determinations were also conducted at 90° and 100°. Those at 90° were conducted as the others except that the filtration was proceeded with as soon as that temperature was attained.

Name of material.	Total nitrogen. Per cent.	Nitrogen. 60° phosphotungstic acid method. Per cent.	Nitrogen. 90° phosphotungstic acid method. Per cent.	Nitrogen. 100° phosphotungstic acid method. Per cent.
Casein	14.12	14.24	13.24	13.34
Blood albumen	11.82	11.74	11.26	9.92
Egg albumen	12.42	12.58	12.38	11.90
Haemoglobin	13.40	12.82	11.28	9.20
Blood fibrin	13.94	13.62	....	12.60
Gelatin	14.98	....	12.90	11.52

It will be noted that in every case the proteid precipitate was partially dissolved when it was heated. The phosphotungstic acid reagent therefore is of no value for precipitating proteids at 90° or 100°. At 60°—excepting gelatin, and perhaps haemoglobin,—the proteids seem to be completely precipitated.

COMPARISON OF THE MODIFIED PHOSPHOTUNGSTIC ACID AND THE STUTZER METHODS.

The 60° phosphotungstic acid method has already been described. The following table contains some results obtained by this method together with determinations made by the Stutzer method. The figures are means of two determinations:

Name of material.	Total nitrogen. Per cent.	Nitrogen. 60° phosphotungstic acid method. Per cent.	Nitrogen. Stutzer method. Per cent.
Wheat bran	2.20	2.07	1.79
Corn bran	1.54	1.48	1.38
Waste rape	3.57	2.32	2.62
Green peas	1.76	1.57	1.45
Linseed meal	2.86	2.74	2.62
Cottonseed meal No. I	6.80	6.61	6.51
" " No. II	6.18	6.24	6.17
" " No. III	6.62	6.48	6.42
Dried blood	13.66	12.82	13.00
Soy beans	6.15	5.90	5.97
Horn meal	14.70	13.50	13.00
Cowpea meal	3.32	3.18	3.19

It will be noted that this method gives results which are, as a rule, almost identical with those by the Stutzer method, although slightly higher. The mean difference is +0.06 per cent., with a maximum of +0.50 per cent., and a minimum of —0.30 per cent. Neither method gives good results with blood; the filtrate from blood by the 60° phosphotungstic acid method

contained 0.57 per cent. water-insoluble nitrogen, and gave a reaction (with copper sulphate and caustic soda) for proteids, and the copper hydroxide dissolved 0.39 per cent. of water-insoluble protein. With regard to the other materials it is impossible to say which method is correct. The 60° method promises, however, to be of value. It is possible that the determination might be carried on at a slightly higher temperature than this, but hardly over 80° in any case.

PRECIPITATION WITH ZINC SULPHATE.

A comparison was made between the nitrogen precipitated by zinc sulphate and that by the other methods on a few materials. The method was as follows: 1.4 grams of the substance were heated with 100 cc. of water to boiling, allowed to cool, 2 cc. of dilute sulphuric acid (1:4) and 140 grams crystallized zinc sulphate added. It was allowed to stand a day or more, with frequent stirring, filtered, and washed with a saturated solution of zinc sulphate containing 1 cc. of the dilute acid in 50 cc.

Name of material.	Nitrogen insoluble in zinc sulphate.	Nitrogen. Stutzer method.	Nitrogen. 60° phosphotungstic acid method.
	Per cent.	Per cent.	Per cent.
Cottonseed meal	6.45	6.51	6.61
Soy beans	6.07	5.97	5.90
Blood	13.48	13.00	12.82
Cowpea meal	3.26	3.19	3.18

The results are higher than by the other methods in three of the four cases, and seem to point to the Stutzer method as being more nearly correct.

EXTRACTION OF WATER-SOLUBLE NITROGEN.

Extraction of a vegetable material with hot water sometimes gives discordant results, as was the case with cottonseed meal. The extraction was performed as follows: 1.4 grams were placed in a beaker with 50 cc. water, stirred well, and allowed to stand one hour. The liquid was decanted through a filter, 50 cc. water added to the residue in the beaker, heated to boiling, filtered, and the residue washed with boiling water. The undissolved nitrogen was, in case (*a*) 4.54 and 4.68 per cent., mean 4.61 per cent.; in case (*b*) (6 months later) 4.12 and 4.22 per cent., mean 4.17 per cent.; and case (*c*) (volume of filtrate

less than in case (*b*)) 5.55 and 5.67 per cent., mean 5.61 per cent. There is thus a variation of 1.44 per cent. of nitrogen, or 9 per cent. of protein. It is quite possible that had the extracting water been slightly acid the results would have been more uniform.

THE BROMINE METHOD.

Rideal and Stewart[1] have proposed to use bromine as a precipitant for gelatin. Allen and Searle[2] applied the method to the analysis of meat extracts. Wiley[3] has proposed the following method for the determination of proteids in animal matters. About 1 gram of the dried animal matter is washed with ether by decantation, using from 50 cc. to 100 cc. ether, and decanting through a filter which is to receive the portion insoluble in hot water. After allowing the ether to evaporate, the sample is washed by decantation, first with cold water and then with hot water, the total filtrate being from 300 cc. to 400 cc. The undissolved residues are brought on the filter with the last portions of water and the nitrogen in the residues determined by the Gunning-Kjeldahl method. The filtrate from the insoluble portions is received in Kjeldahl flasks, acidulated with 2 or 3 drops of strong hydrochloric acid, and then about 2 cc. of liquid bromine are added, and the contents of the flask shaken vigorously. Bromine is added until about 0.5 cc. remains undissolved and the supernatant liquid is thoroughly saturated. After standing over night, it is filtered and washed by decantation, the globule of bromine serving to saturate the wash-water. The filter with the precipitate is returned to the flask in which precipitation has taken place, and the nitrogen therein determined by the Gunning method.

METHOD MODIFIED.

After some experimentation the method above described was modified for vegetable materials as follows: 200 cc. of water were added to 1.4 grams of the substance in a Kjeldahl flask, heated to boiling, and allowed to cool. It was then acidified with hydrochloric acid, and bromine added until a small globule remained undissolved, the liquid allowed to stand over night,

[1] *Analyst*, 22, 228.
[2] *Ibid.*, 22, 258.
[3] Bulletin 54, Division of Chemistry, U. S. Dept. of Agr.

filtered, and the precipitate washed by decantation, keeping the wash-water saturated with bromine. The filter and precipitate were returned to the flask, and the nitrogen therein determined by the Kjeldahl method.

COMPARISON OF THE BROMINE AND STUTZER METHOD.

Proteid nitrogen was determined in a number of materials by the method just described, and the results compared with those obtained by the Stutzer method. The following table contains the results:

Name of material.	Nitrogen. Stutzer method. Per cent.	Nitrogen. Bromine method. Per cent.
Corn silage	0.68	0.67
Crabgrass hay	1.38	1.51
Green peas	1.45	1.25
Cattail millet	1.34	1.10
Linseed meal	2.62	2.37
Corn bran	1.38	1.11
Wheat bran	1.79	1.51
Dried blood	13.00	12.53
Cowpea meal	3.19	2.53
Sheep excrement	2.79	2.13
Soy beans	5.97	5.23
Green rape	2.62	1.84

The bromine method is not applicable in the case of cottonseed meal. In one case the meal was extracted with water, and the extract gave a precipitate which settled almost immediately and contained 0.51 per cent. nitrogen. A duplicate determination gave a turbid liquid, which would not filter clear after standing over night, and when the precipitate finally settled it yielded only 0.10 per cent. nitrogen. When the meal was treated directly with bromine a turbid liquid was formed which refused to filter clear and the precipitate contained varying amounts of nitrogen. The results by the bromine method with two exceptions, are lower than with the Stutzer or the phosphotungstic acid methods. Until it has been proved that bromine precipitates all vegetable proteids quantitatively, which is doubtful, this method must be condemned.

THE STUTZER METHOD.

The Stutzer method used in this work is as follows: place 0.7

gram of the substance in a beaker, add 100 cc. water, heat to boiling, or, in case of substances rich in starch, heat on the water-bath ten minutes; add a quantity of copper hydroxide mixture containing about 0.5 gram of the hydroxide; stir thoroughly, filter when cold, wash with cold water and without removing the precipitate from the filter, determine nitrogen adding sufficient potassium sulphide solution to completely precipitate all copper and mercury. If the substance examined consists of seeds, or seed residues, or anything else rich in alkaline phosphates, add a few cubic centimeters of a concentrated solution of alum before adding the copper hydroxide and mix well by stirring.

Several objections have been made to this method. It has been stated[1] that, in some cases, working with a proteid alone, the copper compound underwent partial solution, a blue liquid being formed, although care had been taken to avoid the presence of free alkali. The proteids acting in this way were not named. Another objection has been founded on "the very slight solubility of the copper salts of some of the simpler amido-acids, especially leucin and glutamic acid; in a less degree the same statement applies to aspartic acid. Even at the temperature of boiling water the copper compounds of these substances are but very sparingly soluble, and if the liquid after digestion with cupric hydroxide, be filtered cold,[2] the compounds in question will, if present, be almost certainly left on the filter along with the proteid material."

Laszczynski[3] also states that copper hydroxide precipitates the albumen of wort and beer completely, but also partly precipitates the albumoses and amides.

The copper salt of leucin is soluble in 3,045 parts of cold water and 1,460 parts of boiling water (Beilstein). When 0.7 gram of the substance and 100 cc. of water are used, for any of this salt to remain on the filter, 0.033 gram must be present, or 0.026 gram of leucin (since it contains 19.5 per cent. copper) which would be 3.7 per cent. The copper salt of glutamic acid is soluble in 3,400 parts of cold water and 400 parts of boiling water, and 0.029 gram must be present before any will separate

[1] Bulletin 54, Division of Chemistry, U. S. Dept. of Agr.
[2] Bulletin 46, Division of Chemistry, U. S. Dept. of Agr. (1895), p. 25.
[3] *Analyst* (Abs.), **24**, 184.

from 100 cc. water, equal to 0.022 gram glutamic acid, or 3.1 per cent. The solvent action of wash-water is left out of consideration.

When the material contains less than 3.7 per cent. leucin, or 3.1 per cent. of glutamic acid, there is no danger of the amide separating in the cold. But the limits are much higher than these. The solubilities above noted are for the pure salts in pure water. E. Schulze[1] emphasizes the fact that the copper salts of these amides are much more soluble when impurities are present. While the copper salts of aspartic acid and glutamic acid separate quickly from a pure solution, from a mixture of the two they separate very slowly, or not until the liquid has been evaporated.

It is probably better, however, to conduct the determination in a hot solution.

It has also been objected to this method, that albumoses are not precipitated. This objection might be overcome by the use of tannic acid to precipitate them. The tannic acid should be used after the precipitation with copper hydroxide. It is only in rare cases that its use would be necessary. Qualitative tests with tannic acid were made on the filtrates from cottonseed meal, wheat bran, cowpea meal, corn bran, cattail millet, excrement from crabgrass hay, waste rape, excrement from waste rape, and corn silage. A small precipitate was formed in all cases except with cattail millet and corn silage. The precipitate from cowpea meal and corn bran dissolved when the liquid was heated, reappearing on cooling. A repetition of the experiment showed that the precipitate did not always appear with the same material. A determination of nitrogen in the precipitate from cottonseed meal gave 0.03 per cent.—practically none.

CONCLUSIONS.

Phosphotungstic acid does not precipitate proteids completely at 90° or 100°.

Phosphotungstic acid at 60° precipitates very nearly the same quantity of nitrogen (with vegetable materials) as copper hydroxide.

Extraction of proteids with hot water does not always give concordant results.

[1] *Landw. Versuch-Stat.*, 6, 220 (1880).

Bromine is not a suitable precipitant for proteids in vegetable materials.

The Stutzer method seems to be the method open to the fewest objections.

Acknowledgment is due Mr. H. W. Primrose, formerly assistant chemist, for assistance in the analytical work.

The above investigation was carried out in the laboratory of the North Carolina Agricultural Experiment Station with the permission of Professor W. A. Withers, chemist.

DETERMINATION OF CARBON IN FERROCHROME.

By A. A. Blair.

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THE method in general use for the determination of carbon in ferrochrome may be briefly described as follows: Place 1 gram of the finely ground ferrochrome in a porcelain or platinum boat with 25 grams of fused potassium bisulphate and insert the boat in a porcelain tube in a gas furnace. Fit each end of the tube with a rubber stopper carrying a glass tube, and fill the forward part of the tube with lumps of cupric oxide. Connect the tube in the forward stopper with a U-tube containing strong sulphuric acid and chromic acid, a second U-tube containing dry pumice, a third containing dried, not fused, calcium chloride, the weighed absorption apparatus, and a guard tube. Connect the tube in the rear stopper with sources of purified oxygen and air. Start the oxygen through the apparatus and heat the tube carefully, beginning at the forward end which contains the oxide of copper, until the entire length of the tube inside the furnace is at a dull red heat in order to fuse the contents of the boat. Replace the oxygen with air, detach and weigh the absorption apparatus.

This does not seem very troublesome, but in practice several difficulties arise that make the method not only unsatisfactory, but very unreliable.

The sulphuric acid, both that evolved from the potassium bisulphate as sulphuric acid, and that evolved as sulphurous acid and oxidized to sulphuric acid by the oxide of copper and oxygen, acts on the rubber stoppers and sometimes carbonizes them