Studies in Mineral Metabolism XXVII.

Modifications of the Methods Used at Onderstepoort for the Determination of (A) Magnesium and Calcium; (B) Potassium, in Grass-Extracts.

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(A) THE DETERMINATION OF MAGNESIUM AND CALCIUM.

IN the course of work on the mineral analysis of grass-extracts, it was observed that the method for the determination of magnesium as described by Malan and Van der Lingen (1931), was not quite satisfactory. Even after changing the washing mixture and washing the final precipitate of magnesium ammonium phosphate with a solution containing ammonia and not sodium hydroxide as described in the original procedure, the method in as far as it was indiscriminately applied to all grass-extracts, still sometimes led to erroneous results. As will be seen later, the chief factor responsible for these errors was shown to be the presence of iron in varying amounts. It should be pointed out here that the concentration of iron in a grass-extract is not necessarily directly proportional to the total amount of iron in the grass, but would appear to be appreciably affected by almost unavoidable fluctuations in ignition temperature and duration, ash composition, etc. It therefore became necessary to modify the magnesium method in such a manner that it would, within reasonable limits, be free of the influence of an unknown and fluctuating iron concentration.

As already indicated, the first step in the present study of the method was directed towards the washing of the final precipitate of magnesium ammonium phosphate. From theoretical considerations, the presence of sodium hydroxide in the washing mixture would tend to decompose the magnesium ammonium phosphate, liberating ammonia, redissolving some of the phosphate and precipitating the magnesium as tri-magnesium phosphate, according to the equation:—

$$3 \ MgNH_4PO_4 + 2 \ NaOH \rightarrow Na_2NH_4PO_4 + Mg_3 \ (PO_4)_2 + 2 \ NH_4OH.$$

It can be readily shown that this reaction is practically complete under these conditions of washing. The phosphate equivalent of a magnesium standard, which should read at 29·4 m.m. on the colorimeter against a phosphate standard of 0·2 mgm. P_2O_5 at 30, when all the magnesium was precipitated as magnesium ammonium phos-

phate, was found to give a reading of 42. On the basis of a 100 per cent. transformation to tri-magnesium phosphate according to the above equation, the theoretical reading would be 44. The washing mixture was therefore changed accordingly, 30 c.c. concentrated ammonia (30 per cent.) being added to 1,000 c.c. of the mixture described by Malan and Van der Lingen (1931).

The results obtained with 0·07 mgm. magnesium as read against a 0·2 mgm. phosphate standard at 30 m.m., have been tabulated in Table I. The mixture used for washing the final precipitate was that described above.

TABLE I.

Mg. standard.	Actual colorimetric reading, (average of three readings).	Theoretical reading.
0·07 mgm..........................	29·7	29·4
,,	30·0	,,
,,	29·9	,,
,,	29·4	,,
,,	30·0	,,
,,	30·1	,,
,,	29·5	,,
,,	30·2	,,
,,	29·7	,,
,,	29·7	,,
,,	29·3	,,
,,	30·1	,,

In the method under review, the magnesium is determined by first precipitating the calcium as oxalate in a solution just alkalinized with ammonia. Without removing this precipitate, the magnesium is then precipitated as magnesium ammonium phosphate by adding excess phosphate and ammonia. The magnesium in this precipitate is determined indirectly by determining its phosphate equivalent colorimetrically. The calcium on the other hand is determined as its phosphate in a second aliquot by directly precipitating the calcium as calcium phosphate from a solution made alkaline with sodium hydroxide.

Since the grass-extract itself contains some phosphate and in addition contains various other metallic ions such as iron, copper, aluminium, manganese, etc., it is clear that a very complex system is being dealt with. With micro-quantities it is possible to choose conditions in such a way that the precipitation of an undesired compound is avoided, either by keeping the product of its ionic concentrations below their solubility product or by utilizing the possibility of complex salts specially characteristic of the micro-concentration range. It follows, however, that any such method must be restricted in its application, since its correctness must be limited to a definite range of concentrations for every individual ion capable of influencing the system.

In dealing with grass-extracts the position is greatly simplified in as much as such metals as aluminium, copper, manganese, etc., are present in such relatively low concentrations that their presence does not in any way appreciably affect the analytical values for calcium and magnesium. In the case of iron the position is different, since grass-extracts frequently contain iron in such quantities, as to lead to serious errors.

Before considering the possible influence of a third element such as iron, it would seem advisable to consider the possible effect of calcium on the magnesium determination. In a neutral solution, containing calcium, magnesium and phosphate with a considerable excess of ammonium oxalate it may be anticipated that only at a relatively high level of phosphate and calcium concentration would some calcium be precipitated as calcium phosphate and thus cause too high magnesium values. In actual experiment it was found that the calcium and initial phosphate had to rise as high as 10 mgm. per aliquot volume before a distinctly measurable error was introduced. This is about five times the amount usually present in grass-extracts.

EFFECT OF IRON ON MAGNESIUM DETERMINATION.

Grass-extracts have been examined with a ferric iron content as high as 24 mgm. Fe per 100 c.c. extract ($+10$ gm. grass), and possibly higher. Such iron concentrations are sufficiently high to cause at least some of the iron to be precipitated as ferric phosphate under favourable conditions, thereby causing errors in the magnesium values obtained. In the following table the relevant data obtained on standard solutions containing known amounts of magnesium, iron and " initial phosphate " have been compiled, the presence of calcium being maintained merely because it is always present in actual grass extracts.

TABLE II.

No.	Mgm. Mg. present.	Mgm. Ca present.	Mgm. Fe present.	Mgm. (initial) P_2O_5 present.	Product $[Fe] \times [P_2O_5]$.	Colorimetric reading.
1................	0·10	0·50	0·10	0·40	0·04	20·8
2................	0·10	0·50	0·20	0·40	0·08	17·4
3................	0·10	1·00	0·10	0·80	0·08	17·0
4................	0·10	1·00	0·20	0·40	0·08	16·9
5................	0·10	0·50	0·20	0·40	0·08	17·3
6................	0·10	0·50	0·30	0·40	0·12	15·4
7................	0·10	0·50	0·40	0·40	0·16	13·8
8................	0·10	1·00	0·50	0·40	0·20	13·2

According to the quantity of magnesium present, the above colorimetric readings, compared with a 0·2 mgm. P_2O_5 standard at 30 m.m., should all have been in the neighbourhood of 21 m.m. It will be seen that only No. 1 gives a correct reading, all the other readings giving too high values. It will also be noticed that as the product $[Fe] \times [P_2O_5]$ increases, the error increases. It may thus be concluded that the presence of iron must either be excluded or

limited to such amounts that the product $[Fe] \times [\text{``initial''}$ $P_2O_5]$ is kept well below $0 \cdot 04$ per aliquot volume. The above further suggests that at least some iron is precipitated as ferric phosphate. This conclusion may be confirmed by excluding the magnesium and calcium from the above experiment.

TABLE III.

Mgm. Fe present.	Mgm. (initial) P_2O_5 present.	Product (Fe) $\times$ [`` initial '' P_2O_5.]	Colorimetric reading.
$0 \cdot 05$	$1 \cdot 0$	$0 \cdot 05$	Slight colouration.
$0 \cdot 10$	$1 \cdot 0$	$0 \cdot 10$	$\div 80$
$0 \cdot 15$	$1 \cdot 0$	$0 \cdot 15$	± 60
$0 \cdot 20$	$1 \cdot 0$	$0 \cdot 20$	48
$0 \cdot 25$	$1 \cdot 0$	$0 \cdot 25$	44
$0 \cdot 45$	$0 \cdot 8$	$0 \cdot 36$	$25 \cdot 5$
$0 \cdot 55$	$0 \cdot 8$	$0 \cdot 44$	$21 \cdot 6$

Obtaining such readings as the above against a $0 \cdot 2$ mgm. P_2O_5 standard at 30 clearly illustrates that at least some iron is precipitated as phosphate since in the above case both magnesium and calcium were absent. The table again suggests that a figure of approximately $0 \cdot 04$ for the product $[Fe] \times [\text{`` initial ''} P_2O_5]$ is a maximum value for approximately correct readings. If it is allowed to rise to about $0 \cdot 4$ the error on $0 \cdot 1$ mgm. magnesium would at least be 100 per cent. In solutions containing both iron and phosphate the only safe procedure would thus be either to exclude or remove the iron in some way. It was observed that the addition of oxalate under certain conditions prevented the precipitation of iron, probably in a similar manner as the better known action of citrate on iron in solution. However, the results obtained by exploiting the use of oxalate for preventing the iron from being precipitated were somewhat erratic. The use of citrate proved efficacious as far as keeping the iron in solution, but apparently prevented complete precipitation of the magnesium.

It was therefore decided, first to precipitate the iron as hydroxide (phosphate) by adding ammonia, then precipitating the calcium by adding oxalate, centrifuging off, and determining the magnesium in the supernatant liquid.

The results thus obtained on some standard solutions have been tabulated in Table IV.

TABLE IV.

Mgm. Mg. present.	Mgm. Ca. present.	Mgm. Fe. present.	Mgm. P_2O_5 present (initial).	Product $[Fe] \times$ [`` initial'' P_2O_5.]	Mgm. Mg. recovered.	% Recovery
$0 \cdot 07$	$0 \cdot 5$	$0 \cdot 3$	$0 \cdot 6$	$0 \cdot 18$	$0 \cdot 067$	96
$0 \cdot 07$	$0 \cdot 5$	$0 \cdot 3$	$0 \cdot 6$	$0 \cdot 18$	$0 \cdot 067$	96
$0 \cdot 10$	$1 \cdot 0$	$0 \cdot 1$	$0 \cdot 4$	$0 \cdot 04$	$0 \cdot 095$	95
$0 \cdot 10$	$0 \cdot 5$	$0 \cdot 3$	$0 \cdot 6$	$0 \cdot 18$	$0 \cdot 098$	98
$0 \cdot 10$	$0 \cdot 5$	$0 \cdot 3$	$0 \cdot 6$	$0 \cdot 18$	$0 \cdot 097$	97
$0 \cdot 15$	$0 \cdot 5$	$0 \cdot 3$	$0 \cdot 6$	$0 \cdot 18$	$0 \cdot 146$	97

All these values are satisfactory, since some slight loss was inevitably incurred by the decantation of the supernatant fluid. Such losses, however, are mechanical, and may easily be avoided by taking an aliquot of the supernatant.

To confirm the correctness of this procedure further, and to test its applicability to the analysis of grass-extracts, six such extracts were selected on the basis of their iron content. Using 1 c.c. aliquots, the magnesium was first (A) determined on the calcium oxalate plus ferric hydroxide-phosphate precipitate, then (B) in the supernatant liquid, and finally (C) in a second aliquot on the total precipitate obtained by the original method. These values have been tabulated in Table V.

TABLE V.

Specimen.	A. Mgm. MgO per 100 gm. grass.	B. Mgm. MgO per 100 gm. grass.	C. Mgm. MgO per 100 gm. grass.	Mgm. Fe per 100 c.c. grass-extract.	Mgm. P_2O_5 per 100 c.c. grass-extract.
2...............	0·08	0·22	0·31	23·5	13·9
13...............	0·04	0·13	0·18	15·6	9·5
6C (1)...........	0·00	0·17	0·18	10·3	8·6
44...............	0·03	0·12	0·15	9·1	9·5
51...............	0·00	0·20	0·20	7·8	10·3
27...............	0·00	0·19	0·20	7·7	11·7

In the above table, the values under column B must be considered as correct, those under column A increasing with increase in iron content. When the iron and incidentally the phosphate content is low as in samples 6 C (1), 44, 51 and 27 the difference between columns B and C is either zero or insignificantly small. Where the iron contents are higher, as for example in sample 2, the error is appreciable. The difference between columns C and B, however, more or less exactly equals the figures under column A, whose values were shown to be due to the precipitation of ferric phosphate under the conditions of the original procedure.

After the modification of the method for the determination of magnesium was found to be satisfactory, it seemed possible to determine both calcium and magnesium in the same aliquot of grass-extract, provided the method for calcium determination could be modified accordingly. The method of procedure suggesting itself at this stage was to utilize the precipitate of calcium oxalate for the calcium determination. The precipitate, consisting of calcium oxalate, ferric hydroxide and possibly some ferric phosphate was re-dissolved in dilute hydrocloric acid, and the calcium in this solution directly determined by precipitation as phosphate according to the original method for calcium. The procedure thus resembles very closely that of Kramer and Tisdall (1921) for the determination of calcium and magnesium in serum, except that the calcium is here determined as phosphate, while according to the method of Kramer and Tisdall the calcium is titrated as oxalate with permanganate. Malan and Van der Lingen (1931) state that the presence of iron,

up to four times the quantity usually present in grass-extracts, has no appreciable effect on the calcium figures obtained. As the following table will show, this statement has been confirmed, correct values being obtained even with iron as high as 0·8 mgm. Fe per c.c. " extract ".

TABLE VI.

Mgm. Mg. present.	Mgm. Ca. present.	Mgm. Fe. present.	Mgm (initial) P_2O_5 present.	Mgm. Mg recovered.	Mgm. Ca recovered.
0·07	0·20	0·10	0·40	0·070	0·200
0·07	0·20	0·10	0·60	0·068	0·198
0·07	0·20	0·20	0·40	0·068	0·203
0·07	0·20	0·20	0·60	0·069	0·203
0·07	0·20	0·30	0·40	0·070	0·204
0·07	0·20	0·30	0·60	0·067	0·203
0·07	0·20	0·50	0·40	0·068	0·204
0·07	0·20	0·60	0·40	0·069	0·200
0·07	0·20	0·80	0·20	0·067	0·194
0·07	0·20	0·80	0·40	0·067	0·192

Incidentally it should be noted that in this procedure the effect of magnesium on calcium determination as stressed by Roe and Kahn (1929) is obviated in as much as magnesium is absent when the calcium is precipitated as phosphate.

As a final test for the applicability of the modified method to grass-extracts, the method has been used for the analysis of grass-extracts to which known amounts of calcium and magnesium have been added. These results have been tabulated in Table VII.

TABLE VII.

Mg present from grass-extract, mgm.	Mgm. Mg added.	Ca present from grass-extract, mgm.	Mgm. Ca added.	Mgm. Mg recovered.	Mgm. Ca recovered.
0·041	0·036	0·074	0·10	0·076	0·174
0·031	0·036	0·101	0·10	0·069	0·198
0·045	0·036	0·148	0·10	0·079	0·232
0·034	0·036	0·114	0·10	0·070	0·214
0·044	0·036	0·158	0·10	0·081	0·246
0·036	0·036	0·072	0·10	0·073	0·172

DETAILED DESCRIPTION OF TECHNIQUE FOR THE DETERMINATION OF MAGNESIUM AND CALCIUM.

1 c.c. of grass-extract is pipetted into a 15 c.c. centrifuge tube. 4 c.c. of distilled water are added and then a minute drop of methyl red. A further drop of strong ammonia (35 per cent.) is sufficient to make the solution just alkaline. After shaking the tube, 1 c.c. of saturated ammonium oxalate solution is added to precipitate the calcium. The tube is then well shaken and left for at least four hours for complete precipitation of calcium. It is then centrifuged

for 10 minutes at about 2,000 r.p.m. The supernatant liquid is then carefully decanted and a 5 c.c. aliquot pipetted into another 15 c.c. centrifuge tube. In this solution the magnesium is determined by precipitating as magnesium ammonium phosphate by adding 1 c.c. of a 1 per cent. mono-potassium phosphate, 2 c.c. of strong ammonia (35 per cent.) and 1 c.c. of a 2 per cent. ammonium chloride. The inside of the tubes are then rubbed with a rubber-tipped glass rod until the precipitate of magnesium ammonium phosphate begins to form. The rest of the procedure is the same as described by Malan and Van der Lingen (1931), employing, however, the new washing mixture described at the beginning of this paper.

The standard to be used in this method, containing appropriate amounts of magnesium and calcium, would be the following:— 0·8 c.c. of a pure solution containing 0·1 mgm. magnesium per c.c. is taken and 1·7 c.c. of a pure solution containing 0·1 mgm. calcium per c.c. is added. The solution is made up to 5 c.c. and proceeded as described above. The magnesium standard obtained in this manner should then theoretically read at 30·7 against a 0·2 mgm. P_2O_5 standard at 30, and the calcium standard at 29·9.

The calculation for the magnesium values of grass would be the following:—

$$\text{gm. Mg. per 100 gm. grass} = \frac{0 \cdot 067 \times 30 \times 6 \times 100 \times 100}{R \times 5 \times v \times g \times 1000}$$

when the 0·067 mgm. magnesium standard is set at 30, R the reading of the unknown, v the aliquot volume of the 100 c.c. grass-extract taken, and g the weight of grass taken for the extract.

The calcium oxalate precipitate in the original tube is then dissolved in 2 to 3 c.c. of N HCl, and the solution made up to 10 c.c. In this solution the calcium is determined as its phosphate, as described by Malan and Van der Lingen (1931). The calcium standard mentioned above is the same as in the original method.

The procedure described above, of determining both calcium and magnesium on the same aliquot of grass-extract is undoubtedly time saving, as it enables one under present conditions, to determine both calcium and magnesium in approximately the same time devoted previously to magnesium only.

SUMMARY.

A modified method for determining calcium and magnesium in grass-extracts is described, and the effect of iron on the original magnesium determination illustrated. Also, a modification of the washing mixture substituting ammonia for sodium hydroxide, for washing the precipitate of magnesium ammonium phosphate was found necessary.

REFERENCES.

KRAMER, B., AND TISDALL, F. F. (1921). A Simple Technique for the Determination of Calcium and Magnesium in Small Amounts of Serum. *Jnl. Biol. Chem.*, Vol. 47, p. 475.

MALAN, A. I., AND VAN DER LINGEN, G. W. B. (1931). Studies in Mineral Metabolism, XVI. The Micro-Determination of Some In-organic Elements in Blood and Vegetation. *17th Rept., Dir. Vet. Serv.*, Un. of S.A., pp. 443-452.

ROE, J. H., AND KAHN, B. S. (1929). The Colorimetric Determination of Blood Calcium. *Jnl. Biol. Chem.*, Vol. 81, p. 1.

(B) **THE DETERMINATION OF POTASSIUM.**

The alterations adopted in the procedure of this method, as described by Malan and Van der Lingen (1931) resulted from a publication by Roger S. Hubbard (1933). The author advises the standardization of conditions under which the precipitation of potassium is carried out, because potassium and sodium may form a series of double nitrites with cobalt, due to chemical and physical variations.

One c.c. of buffer solution (100 gm. of crystalline sodium acetate made up to 250 c.c. with distilled water) is added to the solution prior to the precipitating agent sodium cobalti nitrite. During the period of precipitation (one hour) the tubes are kept in ice-water, thereby maintaining a constant temperature for precipitation independent of room temperature variations.

Instead of washing the potassium precipitate with distilled water an organic solvent is used (acetone diluted with three parts of water for the first two washings, and pure acetone for the subsequent washing). With this washing fluid the precipitate may be disturbed and easily centrifuged down again, where with pure distilled water some of it would invariably remain on the water surface in spite of centrifuging. These alterations have been applied to the potassium determination with advantage.

REFERENCES.

HUBBARD, R. S. (1933). Note on the Precipitation of Small Amounts of Potassium as Potassium Sodium Cobalti-Nitrite. *Jnl. Biol. Chem.,* Vol. 100, pp. 557-559.

MALAN, A. I., and VAN DER LINGEN, G. W. B. (1931). Studies in Mineral Metabolism, XVI. The Micro-Determination of some Inorganic Elements in Blood and Vegetation. *17th Rept., Dir. Vet. Serv.,* Un. of S.A., pp. 446-447.

APPENDIX.

Since the above was written, it was found necessary to determine the calcium and magnesium contents of not only grasses but of other feeding stuffs like maize, beans, etc., in which the limits of variation of these elements were wider than those given. Under such conditions the method could not be applied wholly as given and slight modifications had to be introduced.

First of all, in the combined determination of calcium and magnesium, the calcium concentration in the aliquot should not exceed 0·25 mgm. (see Table VII) but should rather be kept between 0·1 mgm. and 0·2 mgm. As described above, the calcium oxalate precipitate is dissolved in HCl and then the calcium is again precipitated as calcium phosphate in an alkaline medium. Under these conditions, with a higher calcium concentration than that given, viz., 0·1 mgm. to 0·2 mgm. per aliquot, and an equivalent concentration of oxalate present, some calcium oxalate may again be precipitated in the alkaline medium when the calcium is precipitated as phosphate.

In other cases, when both magnesium and phosphate are exceptionally high, some magnesium may be precipitated as $Mg\ NH_4\ PO_4$ when precipitating the calcium oxalate. This difficulty may be overcome by taking a very small aliquot of the extract, or by precipitating the calcium oxalate at a pH between 3 and 4 when the magnesium remains in solution. Under these conditions, however, the iron concentration in the aliquot should be very low or nil, else the iron would eventually be precipitated as iron phosphate along with the $Mg\ NH_4\ PO_4$.

In the event of it being impossible to take an alquot to suit the determination of both calcium and magnesium, the determinations should be carried out on separate aliquots, the calcium being determined directly as calcium phosphate as described by Malan and van der Lingen, and the magnesium as $Mg\ NH_4\ PO_4$ according to the procedure described in the article given above.