A STATISTICAL STUDY OF THE ERRORS INVOLVED IN THE SAMPLING AND CHEMICAL ANALYSIS OF SOILS AND PLANTS, WITH PARTICULAR REFERENCE TO CITRUS AND PINEAPPLES.

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SUMMARY.

1. Accurate and reproducible methods for the determination of N and acid-extractable P in soils are described.

2. The variations in N, P, and K found by intensive sampling of three different soil types are presented.

3. Suggestions for the adequate sampling of soils for various purposes are given.

4. A study is presented of the preparative stages in leaf analysis (washing, drying, grinding and storage) as applied to citrus and pineapple leaf material. Likely errors are pointed out and procedures whereby these may be minimised are described.

5. A reliable wet-ashing procedure for obtaining leaf solutions is described.

6. Accurate and reproducible methods for the determination of total N, P, K, Ca, Mg, Na, Fe, Mn, Zn and Cu in citrus and pineapple leaves are presented.

7. It is shown that diurnal variations in nutrient concentrations occur in citrus and pineapple leaves.

8. The variations in N, P, K, Ca, Mg, Na, Fe, Mn, Zn and Cu, occurring from tree to tree in 3 different Navel orange blocks are presented. It is shown that if ALL these elements are to be represented to within 10% of the mean values on a 19 : 1 probability level, all the trees in the blocks would have to be sampled. Reasonable sampling procedures for various purposes are suggested.

9. The variations in N, P, K, Ca, Mg, Fe, Mn, Zn and Cu, occurring in 3 different Cayenne pineapple plantations are presented and described. Suitable sampling procedures are suggested.

C O N T E N T S.

INTRODUCTION.

In view of the extent to which soil and plant studies have been pursued, it is surprising that more attention has not been paid to the problem of the errors involved in a particular sampling procedure. The validity and usefulness of the chemical analysis of soils and plants hinge upon an intelligent and realistic approach to the problem of obtaining a reliable sample. If the sample taken is not representative of the commodity or of the plot from which it was obtained, all the careful and costly work put into the subsequent analysis will be wasted because the results will not be valid. Literature on soils and plants contains very little quantitative information on what constitutes a truly representative sample. It was mainly in order to throw some light on this important problem that the work reported here was carried out.

The object of sampling biological material is to obtain a quantity as nearly as possible identical in properties with the bulk material over the original area in question. The true value could only be obtained with certainty, by taking all the material in the area as constituting the sample. For practical reasons, however, there is a limit to the number of samples which can be taken from an area, and it is important to realise that the true values for the material are unknown.

The collection of a representative sample is influenced by a number of variables which have to be taken into account before a sampling procedure can be established. In the case of soils and plants, these variables include, among others: The soil type and its uniformity in the sampling area; farming practices, such as fertilisation, cultivation and irrigation; the presence of grazing animals on the sampling area; and the age, variety, and uniformity of the plant material. Of critical importance in obtaining a representative sample is the design of the experiment; rarely is the sampling technique evaluated as to reliability or variability. Lack of attention to and understanding of the sampling problem are likely to result in analytical data of little or no value to anyone.

A question which arises when considering sampling problems, is the relation between precision in sampling and in analysis. It is clear that the sampling error for biological materials is commonly much greater than the analytical error, and the limit of accuracy generally is determined by possible variability of the sample rather than by errors in the laboratory. A value obtained by chemical analysis of a sample of biological material defines only a characteristic of a small subsample of the area in question, and only approaches an accurate description of the material to the extent that;

(a) the sample from which it was taken accurately represents the material in question;

(b) the subsample analysed accurately represents the gross sample, and;

(c) the analysis determines the true value of the characteristic in the subsample under investigation.

A survey of the literature on soil sampling reveals probable errors which are apparently three to six times greater for sampling than for analysis (1). In the case of plant analysis it has been stated that with standard chemical methods the analytical errors will generally be negligible compared with the errors from other sources, such as sampling (2). Yet, most published data record analytical figures for soil and plant analysis to an accuracy of at least 1% and often much better. The question now arises as to how far this degree of accuracy is justifiable in view of the sampling procedures usually adopted. An extensive survey of the literature could not answer this question satisfactorily.

Another question which is important when considering sampling error in relation to chemical analysis is the following:- If the analyst knows that the sampling of biological material is subject to relatively large errors, would he be justified in devising and adopting rapid and approximate methods of analysis, rather than in using the more lengthy, accurate procedures? It will be shown, during the course of this dissertation, that the answer to this question should be in the negative, particularly with respect to leaf analysis and the determination of the trace elements. There is too great a tendency for an analyst, if he knows that a procedure gives approximate results only, to assume that the loss of a small part of a solution, precipitate or residue, does not matter, or that less care need be taken in carrying out manipulations. It is easy to visualise a state of affairs, eventually, in which the analytical error may be larger than the sampling error. All analytical operations should be carried out with the greatest care and exactness that the individual is capable of, while ways and means of reducing sampling and other errors should be sought, rather than looking for short-cuts in the analytical procedure.

The nutrient elements of plants were selected as the statistics for investigating sampling variations. In consequence, the major part of the research consisted in the study and accurate standardisation of analytical procedures in order that any variations found could confidently be ascribed to sampling errors rather than to inherent analytical errors. It is claimed that the accuracy and precision obtainable from most of the analytical procedures described in this thesis, are better than those hitherto reported in the literature.

The soil sampling studies were carried out during the period 1945-1947. The work on the variation of potassium in soils is embodied in an M.Sc. thesis which was submitted by the author in 1945 (3).

A study of the variations of nitrogen and phosphorus in soils is presented in Part I of this dissertation. A summary of these results was published in 1956 (4).

The work reported in Parts II & III, on the chemical analysis and the sampling of citrus trees and pineapple plants, was carried out during the period 1954-1957. This research was stimulated by the establishment in 1952 at Rhodes University of a Research Team on the Mineral Status of Plants and Soils, and financed to a considerable extent by the Council for Scientific and Industrial Research. The members of the Team are Professor W.F. Barker (Chemistry), Professor E.S. Twyman (Botany), Professor J.A. Gledhill (Physics), Dr. E.E.A. Gledhill (late Union of S.A. Dept. of Agriculture) and the author, who is mainly responsible for the analytical section of the work. Summaries of some of the analytical procedures have been published (5,6).

Where numerical results are quoted in this thesis, the emphasis is always on their accuracy and on the methods by which they were obtained, and never on their interpretation from a physiological point of view.

PART I.

THE SAMPLING OF

SOILS

FOR CHEMICAL ANALYSIS.

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1. REVIEW OF LITERATURE ON SOIL SAMPLING STUDIES.

Instructions given in the literature for taking soil samples for chemical analysis appear to be more or less similar (see for example (7) and (8)). It is always clearly stated that it is of the utmost importance that the samples should be truly representative of the soil conditions to be investigated in view of possible differences in fertility within the same field. It is usually recommended to take samples from 4 or more localities within the field, and to mix such samples to form a composite sample, representative of the whole area in question. Stress is laid upon the depth to which the sample is to be taken, and upon the possible sources of contamination, such as from the surface portion of the soil, or from the possible mixing of various horizons in the profile. The actual procedure for taking the samples varies from the use of soil augers and tubes to the digging of a profile pit. The importance of adopting different procedures for different types of investigation is also usually stressed in the literature (8).

The most useful information regarding soil sampling procedure was obtained from a paper by Cline (9), who gave a theoretical discussion of the problem. The paper appeared a considerable time after the commencement of the author's studies.

Cline described methods for sampling soils, (a) to represent an area for estimates of mean values only, estimates of variability and estimates of significance and fiducial limits, and, (b) to represent a soil type.

Instructions for subdivision of areas and selection of sampling sites were included. The relative merits of general types of sampling tools were also pointed out.

Cline stated that the accuracy with which a soil sample represents the population sampled depends upon the soil variability, the number of sampling units contributing, and the way in which the sample is drawn. The number of samples to be drawn from an area should never be decided upon arbitrarily, but an estimate of the required number should be based on valid statistical argument.

Cline also stressed that horizontal subdivision to give sampling areas homogeneous with respect to soil type, plant growth, and treatment, and vertical subdivision into recognizable horizons of the same soil type, should be observed. Only a layer in the middle of each horizon should be sampled.

Three general principles should be kept in mind when selecting sampling units:-

(a) A sample composed of few sampling units scattered at random throughout a homogeneous population contains information up to the limits of its size, but even a large sample, confined to a part of the population, contains no information about the excluded parts.
(b) An unbiased estimate of the mean requires that every sampling unit should have an equal chance of being drawn.
(c) An unbiased estimate of significance and fiducial limits requires that every sample of n sampling units should have an equal chance of being drawn.

Complete randomisation is necessary to meet requirement (c). Numbers required for this kind of test are generally great enough for restriction (a) to be met automatically by a random sample. For objectives that require only an unbiased estimate of the mean, incomplete randomisation by means of a grid superimposed at random, satisfies principle (b). A grid gives every sampling unit, but not every possible combination of n sampling units, an equal chance of being drawn. This method requires special precautions to avoid bias from superimposing the grid parallel to some systematic variation in the soil.

Chemical properties of soil also vary with time and precautions should be taken to meet this problem.

Compositing of samples is important because it saves the time and expense of analysing each sample separately. Compositing is valid only if :-

(a) the sampling volume represents a homogeneous population,
(b) equal amounts of each sampling unit contribute to the subsample analysed,
(c) no interactions that would affect the results materially occur, and
(d) an unbiased estimate of the mean is the only objective.

A sampling tool should provide a sampling unit which is uncontaminated, approximately uniform in cross-section to the desired depth, and reproducible. Cline preferred a sampling tube or completely sheathed auger.

Unfortunately, Cline's paper was only a theoretical treatment of the problem and no numerical data were given to indicate the probable variability of soil properties over a given area.

Some other good reviews on different sampling procedures have been published (10, 11, 12, 13). These were adequately reviewed in the author's M.Sc. Thesis (3).

The only published works which recorded intensive studies of soil properties were a paper by Youden and Mehlich (14), who applied statistical treatment to studies of variations in pH of soil samples over a large area, and by Iyengar and Tamhane (15), who determined the statistical variance in salt content (conductometrically) on irrigated soils in India. No reference could be found to statistical studies of the variance in plant nutrient content over a given area of soil.

Since the completion of the author's research on soil sampling, one other paper of importance appeared in the literature, by McKenzie in 1955 (16). He determined copper, magnesium, vanaduim, molybdenum and manganese by spectrographic methods in 68 samples taken from the surface horizon of an apparently uniform virgin soil, at sites uniformly distributed over a small area of 8 x 3½ feet, i.e. over a total area about 50 times smaller than the 1/40 acre plots studied by the author. McKenzie reported the average coefficients of variation for these elements to be about 10%, with the highest concentration for each element about twice the lowest. It is interesting to note that these variations for the trace elements are considerably greater than the variations for N, P and K as found by the author, though one wonders what effect the magnitude of the spectrographic error had on McKenzie's results.

2. METHOD OF TESTING SAMPLING EFFICIENCY.

An investigator who aims to do research on soil sampling problems is faced at the outset with the difficulty of choosing a suitable mode of investigation from among the many soil properties which can be determined in a laboratory. It should have the following vitally important characteristics:-

(a) The results, obtained by this method, should be reproducible as well as accurate so that, when treating them statistically, no doubt will exist as to their analytical precision, and any variance occurring can be confidently ascribed to soil irregularities.

(b) In view of the fact that sampling research involves the analysis of hundreds of samples, time is a very important factor when considering a suitable method of analysis. In addition to being reliable, then, the method must be relatively short and specially adaptable for routine work.

(c) The constituent to be determined should have significantly different values for different soil types.

It is obvious when considering these requirements that no physical determination will meet the specification. Although, for example, reliable results can be obtained by pH measurements, the pH value is an intensity factor and therefore not suitable. Work already done on sampling using pH as the method of investigation (14), shows that for intensive sampling in small areas, soil pH variations are too small to be regarded as significant.

In a preliminary study carried out by the author during the early part of this work, it was found that larger variations occurred in the nutrient element concentrations from sample to sample over a small area than were shown by any other chemical or physical property determined. In consequence it was decided to use the determination of nitrogen, phosphorus and potassium, the "Big Three" in the fertiliser industry, as the method of investigating soil variability.

Potassium was chosen as the first constituent to be determined, because preliminary research showed it to be the most variable of the three, and also, because it was present in much larger proportions than either of the other two, it was thought that it would yield the best data for the preliminary treatment presented in the M.Sc. Thesis (3).

Once the method of investigation was selected it became necessary to determine the variation of the analytical method itself, before it could be applied. In the case of potassium (3), a modified volumetric cobaltinitrite method was developed which proved to be both accurate and precise. A reproducible extraction procedure, in which the soil was digested for 24 hours in constant boiling-point hydrochloric acid at 100°C, was used.

3. THE DETERMINATION OF TOTAL NITROGEN IN SOILS.

3.1: AVAILABLE METHODS.

The standard method for determining nitrogen in biological materials was then (1946) and still is, the Kjeldahl procedure. There were, however, many different variations in the digestion technique, mainly as regards the catalyst used to promote oxidation of the organic matter. There were also a number of variations in the distillation technique. Without going into the details of these various modifications, it is sufficient to say that a satisfactory degree of accuracy or reproducibility could not be obtained with any of the methods tested, and it became clear that a suitable technique had to be developed.

Of all the methods tried that developed by P.H. Kitto (17) gave the most reproducible results. The essential feature of this method was the use of a catalytic mixture of anhydrous sodium sulphate, crystalline copper sulphate and sodium selenate in the proportions of 9:4:1. However, the author invariably obtained incomplete recovery when testing the method on standardised nitrogenous substances, the results being about 2% too low. The technique of the method also left much to be desired. Because of the large amount of copper sulphate in the catalyst a very bulky precipitate appeared on adding excess sodium hydroxide prior to distillation. This increased the tendency for the mixture to bump. Moreover, the expensive nature of the method counted against its usefulness as a routine procedure.

It was eventually decided to investigate the possibilities of hydrogen peroxide as an oxidant in the destruction of soil organic matter. Promising results were reported from the use of this reagent on different organic materials (18,19,20).

After an exhaustive investigation the procedure described below was finally developed for soil nitrogen.

3.2: MODIFIED PERHYDROL METHOD FOR SOIL NITROGEN.

Reagents:-

(a) Concentrated H_2SO_4, A.R.

(b) Hydrogen peroxide solution, 15%.

(c) Standard HCl, 0.05 N: Standardise against borax.

(d) Standard NaOH, 0.05N: Standardise against (c).

(e) Screened Indicator: Mix 2 volumes of 0.2% methyl red in 95% ethanol with 1 volume of 0.2% methylene blue in 95% ethanol just before use. This mixture is very sensitive to light.

(f) Commercial grade NaOH solution, 50%.

(g) Pure granulated zinc.

Apparatus:-

Digestion and distillation racks to accomodate 8 Kjeldahl flasks (500 ml.) were built according to the conventional design for these

multiple units. Gas heating was employed while steam distillation was dispensed with.

Procedure:-

Weigh out accurately 5 - 15 g. (depending on the N content) air-dry soil (1 m.m.) into a 500 ml. Kjeldahl flask. Add 15 ml. of a 15% H_2O_2 solution, rinsing down any soil sticking to the neck of the flask. Swirl to mix. Heat gently at first, with constant swirling to prevent loss by frothing and then more strongly until the mixture has the consistency of a thick paste (DO NOT HEAT TO DRYNESS). Cool and carefully run in 20 ml. of conc. H_2SO_4 from an automatic pipette, swirling constantly to prevent loss. Place on the digestion rack in the fume chamber and heat gently until all the water has been driven off. Then boil until the mixture has become light-brown or grey and continue for a further 5 minutes (about 25 - 30 minutes in all). Cool, dilute to about 150 ml. with water, add a piece of granulated zinc and connect to the distillation apparatus. Run in 70 ml. 50% NaOH, raise to boil and absorb the ammonia in 25.00 ml. N/20 HCl. Keep boiling vigorously for 15 minutes. To the absorption flask add 5 drops of screened indicator and titrate with N/20 NaOH to a permanent green colour. Calculate the % N in the soil from the amount of N/20 HCl consumed.

1 ml. N HCl = 14.01 mg. N.

Remarks:-

(i) If the soil organic matter content is high it is sometimes necessary to carry out a double evaporation with H_2O_2.
(ii) Less H_2SO_4 and NaOH are used than in the Kitto method.
(iii) No catalyst is necessary, H_2O_2 and H_2SO_4 being the only reagents used during digestion.
(iv) The oxidation of the organic matter proceeds very rapidly and digestion is complete within a relatively short period.
(v) On addition of a piece of granulated zinc the distillation proceeds smoothly, the bubbles of liberated hydrogen apparently aiding in this respect.
(vi) All the ammonia was distilled by 15 minutes of vigorous boiling.
(vii) The screened indicator gave very satisfactory and sensitive endpoints, despite the use of dilute NaOH.
(viii) The method is rapid. Up to 24 determinations could be carried out per day with the apparatus available.

3.3: ACCURACY AND PRECISION OF THE MODIFIED METHOD.

For lack of space, the experimental results obtained during the testing of such factors as time of digestion for complete recovery, amount of H_2O_2 necessary, duration of distillation period, suitable indicator, etc., are not recorded here. It is sufficient to state that every step of the procedure was thoroughly tested and the method, as described, was the final result of these tests. The accuracy and precision recorded below provide further clear proof of the reproducibility of the various steps.

3.31: TESTS ON THE NITROGEN RECOVERY FROM DIFFERENT SOIL TYPES:-

There are two possible ways whereby an investigator may determine whether a particular method yields the absolute or true value of a particular constituent in biological materials. The most reliable procedure is to use another independent method of analysis and to carry out a set of statistical comparisons, side by side, on the same sample. It is extremely unlikely that the same constant error will recur in both sets of figures if the two methods are truly independent. The other method is to add accurately known amounts of the constituent to the sample and then to determine the percentage recovery of the constituent by analysis, having previously determined the average amount of the constituent in the sample as yielded by the method to be tested. If any interfering factor is present incomplete recovery of the added constituent will result. It is, of course, of little use to test the method on standard chemical compounds containing the constituent, as the real difficulty lies in the many different elements and foreign substances present in the biological material which may influence the recovery.

Since no satisfactory independent method for soil nitrogen could be found, the latter method was resorted to. The results are recorded in Table 1. Known amounts of nitrogen were added by employing an accurately standardised ammonium sulphate solution.

TABLE 1.

TESTS ON THE RECOVERY OF NITROGEN.

SOIL TYPE	mg. N found (100g. soil)	Mean mg. N	mg. N added.	mg. N recovered.	Mean % Recovery.
1. Reddish brown sandy loam. (Karoo).	12.48 12.48 12.48 12.43 12.40	12.45	5.00 5.00 5.00	17.40 17.42 17.45	99.8
2. Black, heavy clay soil. (Drakensberg).	16.66 16.65 16.74 16.65 16.68	16.67	2.50 2.50 2.50	19.05 19.10 19.10	99.5
3. Grey sandy loam. (Grahamstown).	11.06 11.12 11.37 11.17 11.15	11.17	5.00 5.00 5.00	16.10 16.09 16.08	99.4
4. Light-grey sandy coastal soil. (Robhoek).	6.57 6.53 6.51 6.57 6.50	6.54	5.00 5.00 5.00	11.50 11.48 11.50	99.5
5. Grey sandy loam. (Orange Free State).	10.15 10.06 10.13 10.12 10.10	10.11	5.00 5.00 5.00	15.00 14.95 14.94	98.9

From the figures in Table I it can be concluded that the method yielded precise results on totally different soil types and that the values were a true reflection of the total nitrogen content in these soils.

3.32: STATISTICAL COMPARISON OF THE PERHYDROL AND KITTO METHODS.

The method of Kitto, employing a catalytic mixture of sodium selenate, copper sulphate and soduim sulphate, was compared statistically with the new perhydrol method. Twentyfive parallel determinations were carried out on a standard soil sample. Extreme care was exercised to keep conditions throughout exactly the same for both methods. The results are recorded in Table 2. A brief summary of the statistical terms used is given below, obtained from standard texts (21,22):-

Standard Deviation = $d = \sqrt{\frac{\text{Sum of the squares of the deviations}}{\text{Degrees of Freedom}}}$

Coefficient of Variation = $C.V. = \frac{d \times 100}{\text{Mean}}$

Error Variance = $E.V. = d^2$

Standard Error of the Mean = $S_D = \frac{d}{\sqrt{n}}$ (Where n = no. of replicates)

Difference between two means = D

Standard Error of the Difference between two Means = E_D

$$= \sqrt{S_{D1}^2 + S_{D_2}^2}$$

Fisher's $t = D/E_D$

If the calculated value of t is greater than the t value from the t Table at a probability level of 5% (P = 0.05) the difference between two means is considered to be significant, and if the value is greater than the theoretical t value at a probability level of 1%, the difference is considered to be highly significant.

The Degree of Precision of $Method_1$ relative to $Method_2$ is given by $Error\ Variance_2/Error\ Variance_1$. These statistical terms have been used throughout this thesis.

TABLE 2.

STATISTICAL COMPARISON OF TWO METHODS FOR SOIL NITROGEN.

Number	KITTO METHOD			PERHYDROL METHOD		
	mg. N/ 100 g.	Dev. from M x 100	Dev.^{2}	mg. N/ 100 g.	Dev. from M x 100	Dev.^{2}
1	14.85	9	81	15.09	12	144
2	14.63	13	169	15.27	6	36
3	14.75	1	1	15.29	8	64
4	14.48	28	784	15.26	5	25
5	14.87	11	121	15.15	6	36
6	14.82	6	36	15.11	10	100
7	14.65	11	121	15.07	14	196
8	14.94	18	324	15.11	10	100
9	14.63	13	169	15.19	2	4
10	14.78	2	4	15.07	14	196
11	14.86	10	100	15.29	8	64
12	14.83	7	49	15.35	14	196
13	14.95	19	361	15.31	10	100
14	14.91	15	225	15.25	4	16
15	14.61	15	225	15.27	6	36
16	14.54	22	484	15.19	2	4
17	14.86	10	100	15.29	8	64
18	14.63	13	169	15.25	4	16
19	14.48	28	784	15.27	6	36
20	14.94	18	324	15.11	10	100
21	14.87	11	121	15.21	0	0
22	14.61	15	225	15.21	0	0
23	14.78	2	4	15.27	6	36
24	14.82	6	36	15.19	2	4
25	14.91	15	225	15.28	7	49
Mean	14.76	Sum	5242	15.21	Sum	1622
Standard Deviation	0.148			0.082		
Coeff. of Variation	1.0%			0.5%		
Error Variance	0.022			0.007		
Standard Error of M	0.030			0.016		

Standard Error of D = E_D = 0.034

$t = D / E_D$ = 13.3

t from Table at P = 0.01 & 24 D.F. = 2.797

Therefore the difference between means is highly significant. Degree of Precision of Perhydrol Method to Kitto Method

= Error Variance$_K$/Error Variance$_P$

= 3

From the Analysis of Variance in Table 2 it is quite clear that the Kitto method gave incomplete recovery of soil nitrogen, despite the relatively good precision of 1%. When these two methods were tested side by side on the soil samples described in Table 1, the Kitto method invariably returned lower nitrogen values than the perhydrol method.

The author was quite satisfied that the modified perhydrol method would be eminently suitable for determining the variability of soil nitrogen.

4: THE DETERMINATION OF ACID-EXTRACTABLE PHOSPHORUS IN SOILS.

Because of the uncertainty as regards the extraction technique for "available" P in soils, it was decided to develop a method whereby the total amount of inorganic P in the soil could be extracted. It was considered that a greater degree of reproducibility would be obtained from the latter technique, besides the fact that a larger P value would be yielded. The author was not concerned with the "available" fractions of the nutrients in soils, but rather in developing methods of absolute reproducibility and accuracy in order to study soil variations.

4.1: AVAILABLE METHODS.

At that time (1946), three methods were commonly used for the determination of P in soils:-

(a) The gravimetric method of Lorenz (8).
(b) A volumetric phosphomolybdate method (8, 23).
(c) Various modifications of the Denigès colorimetric method (23).

The last method, although more rapid than the other two, was eventually discarded owing to the fact that satisfactory reproducibility could not be obtained with the instruments available to the author at that time. Method (a) was considered to be too cumbersome and laborious to be used for routine analysis, and so it was decided to concentrate on the volumetric determination of P.

Although many variations of the volumetric method were in use, the essential features were the precipitation of the phosphorus as ammonium phosphomolybdate in acid medium, the solution of the precipitate in excess standard NaOH and subsequent back-titration with standard HCl. It was assumed that the following reaction occurred.

$$(NH_4)_3\,PO_4 \cdot 12MoO_3 + 23NaOH \rightarrow 11Na_2\,MoO_4 + (NH_4)_2\,MoO_4 + Na(NH_4)\,HPO_4 + 11H_2O$$

Whence 1 ml. N NaOH = 3.088 mg. P_2O_5.

An accurately standardised phosphate solution (by the gravimetric pyrophosphate procedure) was used to test the accuracy of the volumetric method. The results were consistently low, a figure of 2.84 mg. P_2O_5/ml. N NaOH being obtained instead of the theoretical equivalent of 3.088. It was concluded, therefore, that the ammonium phosphomolybdate precipitate obtained did not bear a stoichiometric relationship to the NaOH used.

Eventually, entirely satisfactory results were obtained by modifying the precipitation and titration techniques of a method described by Harrison and Parratt (24) for the determination of small amounts of phosphorus in steel. The method, as finally modified and applied to soils, is described below.

4.2: THE VOLUMETRIC DETERMINATION OF HYDROCHLORIC ACID-EXTRACTABLE P IN SOILS.

Reagents:-

(a) Ammonia-nitric Acid Mixture:- Add 420 ml. conc. ammonia (S.G. 0.88) slowly with stirring to 1575 ml. HNO_3 (S.G. 1.20), prepared by diluting 598 ml. conc. HNO_3 (1.42) to 1575 ml. Keep in a pyrex bottle.

(b) Nitro-molybdate Reagent:- Dissolve 50g. A.R. molybdic acid in 100 ml. water and 50 ml. conc. ammonia (0.88). Pour this solution slowly with stirring and cooling into 625 ml. HNO_3 solution (S.G. 1.20). Leave to stand for several days at $\pm$ 25°C, filter and store in a pyrex bottle in the dark.

(c) Washing Solution:- 1% KNO_3 in water.

(d) Gooch Asbestos:- Boil a suitable quantity of good quality Gooch asbestos in 1 N NaOH for 1 hour. Add an excess of 6N HCl, digest for a further half-hour and finally wash the asbestos free of acid, employing a Buchner funnel.

(e) Indicator:- 1% phenolphthalein in 50% ethanol.

(f) NaOH, N/25, CO_2-Free:- Standardise against succinic acid.

(g) HCl, N/25:- Standardise against (f).

(h) Soil-Extracting Solution:- 600 ml. conc. C.P. HCl + 400 ml. H_2O.

Apparatus:-

(a) The Kjeldahl digestion unit mentioned in 3.2.
(b) Multiple evaporation waterbath to take 12 units.

Procedure:-

Weigh out accurately 20g. of air-dry soil (1mm.) into a 500 ml. Kjeldahl flask. Add 150 ml. of soil-extracting solution, cover the mouth of the flask by inserting a short-stemmed thistle funnel and boil for 1½ hours on the digestion rack. At the end of the digestion period about 60 ml. of liquid should be left in the flask. Cool, filter through Whatman No. 40 filter paper and wash the soil twice by decantation with 10 ml. portions of hot water containing 10 drops conc. HCl per litre. Finally transfer the soil to the filter and wash 6 times with 5 ml. portions of the hot dilute HCl solution. Cool and make up to 250 ml. in a volumetric flask.

Into a 10 cm. porcelain evaporating basin, pipette out a suitable aliquot of the HCl extract (50 or 100 ml.), and evaporate on a waterbath. When the residue is just moist carefully add 3-5 ml. conc. HNO_3 in order to destroy the organic matter. Gently swirl the basin so as to dissolve the residue sticking to the sides. Finally evaporate to complete dryness and heat for a further half-hour in order to dehydrate the silica. Add 5 ml. hot ammonia-nitric acid mixture and stir to a thin paste, employing a glass rod fitted with a small rubber stopper at the end. Rinse the stopper with water and raise the contents of the basin to the boiling point on a hotplate to ensure complete solution of the phosphate. Filter into a 150 ml. beaker and wash 6 times with 3 ml. portions of hot ammonia-nitric acid mixture. Evaporate the filtrate to

about 25 ml.

Cool the filtrate to about 70°C (thermometer) and, from an automatic pipette, run in 10 ml. nitro-molybdate reagent, slowly and with constant stirring. Leave to stand for 20 minutes and filter through a Gooch crucible well-packed with the specially treated asbestos (d). Wash the glass-rod and precipitation beaker with 1% KNO_3 solution until acid-free, pouring the washings through the filter. Finally wash the precipitate 8 times with 1% KNO_3. Transfer the precipitate and asbestos to the original beaker with the aid of a glass-rod. From a burette, run standard N/25 NaOH through the crucible into the beaker, manipulating the crucible so as to rinse off the precipitate sticking to its walls. Run in about 25 ml. N/25 NaOH, depending on the amount of phosphorus in the soil (about 10 ml. in excess). Stir the contents of the beaker so as to dissolve the precipitate, whilst still holding the crucible above the beaker. When solution is complete (no more yellow particles visible), rinse the crucible well with distilled water. Dilute the solution in the beaker to about 60 ml. and boil gently on hotplate for 1-2 minutes. Without cooling, run in N/25 HCl, from a burette, to about 5 ml. in excess. Add 5 drops 1% phenolphthalein and titrate to faint pink with N/25 NaOH from a micro-burette.

Calculate the % P_2O_5 in the soil from the volume of NaOH consumed.

1 ml. N NaOH = 3.088 mg. P_2O_5.

Remarks:-

(i) The HCl-extraction technique removed virtually all the acid-soluble P from the soil. It was found that after initial extraction, less than 0.000 3% P_2O_5 was extracted in a subsequent digestion with 200 ml. HCl for 2 hours.

(ii) The treatment of the HCl-extract prior to precipitation was found to yield reproducible results.

(iii) The results clearly indicated that if the precipitation was carried out as above, the precipitate had the standard composition of $(NH_4)_3\ PO_4,\ 12\ MoO_3$.

(iv) The recommended procedures for the washing of the soil and of the precipitate were the final results of thorough testing.

(v) By boiling the mixture after addition of NaOH, the last traces of precipitate were dissolved. It was also found that the endpoint was much more stable and reproducible after boiling, probably due to expulsion of CO_2 which seriously influences phenolphthalein endpoints.

(vi) The pH transition interval of phenolphtalein is shortened from 8.3 - 10.0 at room temperature to 8.1 - 9.0 at 100°C (25). By titrating the hot solution a sharper endpoint was obtained. This was found to be essential in view of the dilute NaOH used.

(vii) Despite the seemingly complex nature of the procedure, the method was relatively short and the author could comfortably carry out 24 determinations per day with the apparatus at his disposal.

4.3: ACCURACY AND PRECISION OF THE MODIFIED METHOD FOR SOIL PHOSPHORUS.

When the modified method was tested on a phosphate solution, accurately standardised according to the gravimetric pyrophosphate procedure (23), quantitative recoveries were found over the range 1-10 mg. P_2O_5.

The completeness of recovery of added phosphorus was studied on the three different soil types used in the sampling studies, in a similar manner as for nitrogen. These results are given in Table 3.

In Table 4 the precision of the modified method is exhibited, as tested on a sample of the Venterstad soil.

TABLE 3.

TESTS ON THE RECOVERY OF PHOSPHORUS FROM DIFFERENT SOIL TYPES.

Soil Type	mg.P_2O_5/ 100 g. Found	Mean mg. P_2O_5	mg.P_2O_5 Added	mg.P_2O_5/ 100 g. Recovered	Mean % Recovery
1. ROBHOEK. Grey sandy coastal soil.	8.91 8.94 8.90 8.96 8.93	8.93	10.00 10.00 10.00	18.90 18.85 18.86	99.7
2. GRAHAMSTOWN. Grey sandy loam.	14.70 14.60 14.70 14.60 14.70	14.66	5.00 5.00 5.00	19.55 19.54 19.58	99.4
3. VENTERSTAD. Reddish brown loam.	44.2 44.3 44.3 44.4 44.2	44.3	5.00 5.00 5.00	49.2 49.3 49.2	99.9

TABLE 4.

PRECISION OF THE MODIFIED PHOSPHORUS METHOD.

Sample No.	% P_2O_5	Dev. from Mean x 10,000	Dev.^{2}
1	0.0440	1	1
2	0.0438	3	9
3	0.0443	2	4
4	0.0443	2	4
5	0.0441	0	0
6	0.0442	1	1
7	0.0444	3	9
8	0.0443	2	4
9	0.0441	0	0
10	0.0442	1	1
11	0.0440	1	1
12	0.0437	4	16
13	0.0440	1	1
14	0.0445	4	16
15	0.0442	1	1
Mean	0.0441		Sum = 68

Standard Deviation = 0.00021

Coefficient of Variation = 0.5%

There is no doubt that the method yielded remarkably accurate and reproducible results, provided extreme care was taken during the manipulative procedures. It was considered that the method would be very suitable to test sampling variations in soil phosphorus.

5. SOIL SAMPLING STUDIES.

5.1: SAMPLING PROCEDURE AND LAY-OUT OF PLOTS.

As has been pointed out earlier, an investigator can never obtain the absolute value of a constituent in a soil to represent a certain area by sampling - only a close approach to the value can be made in practice by taking a large number of samples. For comparative purposes when testing soil sampling efficacy, it is essential to approach this value as closely as possible, and an investigator should sample the chosen area almost completely. Furthermore, all possible interfering factors must be eliminated and the area to be sampled must be homogeneous and fully representative of the soil type under which it falls. Special attention should be given to the visible physical characteristics like vegetation, slope, degree of erosion and, for cultivated fields, time of fertilisation.

It was clear that, in order to rule out as far as possible all interfering factors and to ensure that the absolute values for the whole soil in the given area were approached as closely as possible, large numbers of samples had to be taken over small areas only. It was decided to limit the size of the sampling area to 1/40 acre, a commonly-used size for experimental plots. It was felt that such small areas could be treated much more efficiently and economically, and that conclusions applicable to larger areas might be drawn from the results. Areas of three distinctly different soil types were sampled:-

(a) Robhoek:- A grey, sandy coastal soil derived from Table Mountain Sandstone was selected in the Winter Rainfall Area of the Union, on the farm Robhoek in the Humansdorp district; average rainfall = 35 inches per annum; predominant vegetation, sour grasses and coastal shrub. Acid, poor soils with no physical structure. The profile characteristics were:-

Horizon A:- 0-9": Dark grey, sandy soil.
Horizon B:- 9-14": Yellow clay, mixed with sand.
Horizon C:- 14"- : Red gravel and stones.

(b) Grahamstown:- A grey, podsolic type soil of the Eastern Province semi-coastal belt, derived from quartzite of the Witteberg Series and Lower Dwyka Shale was selected on the Grahamstown Commonage; average rainfall = 25 inches per annum; predominant vegetation, grass. Slightly acid to neutral sandy loam.

Horizon A:- 0-12": Grey, sandy loam.
Horizon B:- 12-16": Clay, mixed with gravel.
Horizon C:- 16"- : Gravel.

(c) Venterstad:- A reddish brown, loamy Karoo soil, alkaline in reaction, derived from mudstone and sandstone of the Beaufort Series and falling in the Summer Rainfall Area was selected on the farm Broedersbank, Venterstad district in the North Eastern Cape; average rainfall 18 inches; predominant vegetation, Karoo shrub; fertile alkaline loamy soil.

Horizon A:- 0-13": Reddish brown loam.
Horizon B:- 13-18": Sand mixed with gravel.
Horizon C:- 18" - : Gravel and stones.

The sampling procedure finally adopted was as follows:- An outwardly homogeneous area was selected on virgin land. On this a plot 32 feet square (1/40 acre) was marked off and divided into small plots each 4 feet square according to a grid system, giving 64 plotlets. From each of these small plots soil samples were taken at symmetrically placed points 1 foot in from the four corners. So as to minimise any fortuitous surface contamination, all vegetation and about half-inch of the topsoil was carefully removed with a sharp spade before taking a 6 inch boring with a 1 inch auger at each of the points. The four borings from each small plot were placed on a clean metal tray, well mixed and placed in a labelled bottle. This procedure resulted in 64 composite samples representing 256 borings taken at 2 feet intervals over 1/40 acre.

5.2: EXPERIMENTAL RESULTS:-

After collection, the soil samples were spread out on drying paper and air-dried in the laboratory, away from fumes and dust, for 3 days. Each sample was passed through a 1 mm. sieve with round holes, the larger particles being broken with a wooden pestle and mortar. Stones and plant-roots were discarded. Each sample was replaced in the original bottle, well-shaken, and put aside for analysis. The samples weighed approximately 1 kilogram each.

Complete duplicate determinations of nitrogen and phosphorus were made on each of the 64 samples collected from each of the 3 areas. Whenever the duplicates differed by more than 1%, the determinations were repeated, a procedure which was seldom necessary. The mean percentages of the elements for the 3 soil types are given in Tables 5, 6 and 7. The values for K, published in the author's M.Sc. thesis (3) are included for comparative purposes. The values for P and K are given as P_2O_5 and K_2O.

The results were treated and assessed on a statistical basis in order to determine the degree of significance to be attributed to the variance of the elements. The statistics are given in Table 8.

It is necessary to explain how the values for the number of samples to be taken for different levels of representation were arrived at. The formula used was

$$n = \frac{2t^2 d^2}{D^2}$$

which can be derived as follows:-

Tests of significance depend on the estimation of the appropriate standard errors of the means, obtained by calculating $d/\sqrt{n}$, where d = the standard deviation and n = the number of replicates. In other words, for any particular experiment, the magnitude of the standard error

varies inversely with the square root of the number of variates from which it was computed. To halve the significant difference or double the experimental precision would theoretically entail the multiplication of the number of replicates by 4. More generally, if it is desired to reduce the significant difference in a given experiment to x/y of its former value, the number of replicates would have to be increased y^2/x^2 times. On this basis, to effect a reduction in the significant difference from 15 to 10% of the mean would necessitate the multiplication of the number of replicates by $15^2/10^2$ or 2.25. In practice this tends to exaggerate the number of replicates required for any stipulated increase in precision, as any addition to the number of replicates increases the number of degrees of freedom of the error variance, which in turn will tend to reduce the estimate of the significant difference. This formula, therefore, errs on the safe side.

If now, from previous experiments, the approximate value of the error variance likely to be provided by future observations of the same kind, is known, it is possible to arrive at a satisfactory estimate of the number of replicates required for any specified level of precision. The standard test for a significant difference between two means is based on the value of $t = \frac{D,\ \text{the difference between the means}}{S_D,\ \text{the standard error of this difference.}}$

If n is the number of replicates of each mean and d^2 the anticipated error variance as shown by the analysis of variance of previous experiments, then

$$t = \frac{D}{(d/\sqrt{n} \times \sqrt{2})} = \frac{D \times \sqrt{n}}{d \times \sqrt{2}}$$

and

$$n = \left(\frac{t \times \sqrt{2} \times d}{D}\right)^2 = \frac{2t^2d^2}{D^2}$$

By substitution of the appropriate values in this equation, it is possible to calculate the number of replicates of each treatment necessary to prove significant any difference greater than D. In using the formula, it is advisable to express D and d as percentages of the general mean; d then becomes the average coefficient of variation of previous experiments. The value of t is the reading from the t Table (21,22) for any desired probability and the number of degrees of freedom from which d was originally estimated.

For example, if the expected coefficient of variation is about 6%, as evaluated from 24 degrees of freedom, the number of replicates required to show significance in treatment differences exceeding 5% of the general mean would be

$$n = \frac{2 \times (2.064)^2 \times 6^2}{5^2}$$

$$= 12.26$$

(t for 24 D.F. and P = 0.05) = 2.064

Thirteen replicates might therefore be taken as a reasonable estimate of the number required for the specified level of precision for future experiments. The accuracy of the test depends on the accuracy with which the coefficient of variation can be determined from previous research.

In applying this formula to the results of Table 8, where an accurate estimate of the coefficient of variation for each element is given, a reasonably reliable estimate of the number of samples to be taken from an area 1/40 acre in size to represent each element to within a specified percentage of the general mean, could be computed.

A graphical representation of the results is given in Fig. I.

TABLE 5.

ROBHOEK.

PLOTS	1	2	3	4	5	6	7	8
N	0.073	0.082	0.065	0.066	0.060	0.068	0.064	0.076
1 P	0.0086	0.0087	0.0083	0.0080	0.0075	0.0080	0.0080	0.0088
K	0.056	0.056	0.046	0.053	0.050	0.045	0.052	0.056
N	0.073	0.068	0.070	0.079	0.064	0.070	0.081	0.077
2 P	0.0083	0.0089	0.0060	0.0081	0.0075	0.0080	0.0083	0.0083
K	0.054	0.046	0.051	0.056	0.046	0.055	0.059	0.057
N	0.070	0.070	0.067	0.065	0.067	0.074	0.075	0.078
3 P	0.0092	0.0075	0.0076	0.0060	0.0079	0.0071	0.0080	0.0083
K	0.050	0.050	0.060	0.057	0.058	0.062	0.061	0.065
N	0.076	0.067	0.064	0.070	0.063	0.073	0.073	0.065
4 P	0.0076	0.0075	0.0078	0.0088	0.0061	0.0077	0.0079	0.0073
K	0.050	0.050	0.053	0.053	0.051	0.050	0.075	0.071
N	0.073	0.068	0.070	0.069	0.070	0.070	0.066	0.067
5 P	0.0078	0.0080	0.0092	0.0075	0.0080	0.0080	0.0090	0.0074
K	0.069	0.068	0.068	0.064	0.062	0.062	0.062	0.061
N	0.069	0.069	0.072	0.062	0.060	0.074	0.072	0.075
6 P	0.0081	0.0076	0.0086	0.0090	0.0078	0.0079	0.0080	0.0080
K	0.063	0.063	0.060	0.063	0.061	0.065	0.062	0.059
N	0.078	0.074	0.071	0.070	0.062	0.073	0.064	0.059
7 P	0.0074	0.0073	0.0079	0.0079	0.0077	0.0082	0.0081	0.0073
K	0.065	0.058	0.060	0.060	0.056	0.059	0.060	0.061
N	0.071	0.066	0.072	0.074	0.077	0.065	0.066	0.076
8 P	0.0082	0.0076	0.0061	0.0061	0.0081	0.0080	0.0079	0.0080
K	0.059	0.056	0.058	0.061	0.070	0.070	0.069	0.072

TABLE 6.

GRAHAMSTOWN.

PLOTS	1	2	3	4	5	6	7	8
N	0.114	0.106	0.111	0.105	0.105	0.113	0.113	0.111
1 P	0.0133	0.0133	0.0138	0.0132	0.0141	0.0141	0.0143	0.0140
K	0.135	0.132	0.148	0.134	0.130	0.138	0.150	0.137
N	0.099	0.107	0.101	0.100	0.106	0.106	0.106	0.101
2 P	0.0133	0.0134	0.0139	0.0135	0.0134	0.0134	0.0144	0.0135
K	0.133	0.133	0.137	0.138	0.137	0.136	0.144	0.147
N	0.105	0.103	0.099	0.103	0.109	0.104	0.106	0.100
3 P	0.0149	0.0137	0.0143	0.0138	0.0149	0.0149	0.0149	0.0149
K	0.139	0.131	0.131	0.122	0.130	0.139	0.150	0.137
N	0.098	0.101	0.099	0.099	0.097	0.099	0.101	0.105
4 P	0.0161	0.0143	0.0142	0.0164	0.0142	0.0144	0.0132	0.0162
K	0.126	0.130	0.124	0.134	0.127	0.128	0.144	0.142
N	0.100	0.097	0.101	0.103	0.098	0.094	0.111	0.100
5 P	0.0161	0.0152	0.0160	0.0161	0.0152	0.0142	0.0149	0.0152
K	0.122	0.132	0.139	0.129	0.132	0.136	0.142	0.137
N	0.102	0.101	0.098	0.097	0.100	0.095	0.098	0.096
6 P	0.0141	0.0139	0.0156	0.0144	0.0160	0.0161	0.0166	0.0164
K	0.129	0.140	0.133	0.126	0.125	0.148	0.129	0.131
N	0.098	0.098	0.097	0.095	0.095	0.093	0.092	0.105
7 P	0.0148	0.0149	0.0152	0.0144	0.0152	0.0148	0.0162	0.0158
K	0.123	0.133	0.136	0.133	0.129	0.135	0.139	0.140
N	0.096	0.097	0.102	0.100	0.102	0.096	0.097	0.109
8 P	0.0153	0.0164	0.0159	0.0154	0.0156	0.0152	0.0160	0.0155
K	0.199	0.131	0.133	0.125	0.117	0.124	0.128	0.133

TABLE 7.

VENTERSTAD.

PLOTS	1	2	3	4	5	6	7	8
N	0.056	0.055	0.053	0.054	0.051	0.054	0.056	0.059
1 P	0.052	0.050	0.044	0.044	0.043	0.044	0.044	0.044
K	0.387	0.382	0.379	0.379	0.371	0.397	0.429	0.417
N	0.065	0.061	0.055	0.052	0.053	0.053	0.055	0.057
2 P	0.036	0.035	0.045	0.044	0.046	0.044	0.044	0.045
K	0.486	0.456	0.388	0.399	0.365	0.376	0.395	0.421
N	0.055	0.056	0.056	0.060	0.056	0.059	0.057	0.061
3 P	0.040	0.050	0.046	0.047	0.045	0.047	0.046	0.047
K	0.423	0.390	0.410	0.408	0.429	0.449	0.461	0.459
N	0.056	0.057	0.060	0.063	0.055	0.046	0.055	0.058
4 P	0.037	0.038	0.048	0.047	0.046	0.046	0.044	0.044
K	0.460	0.452	0.448	0.448	0.432	0.448	0.406	0.409
N	0.060	0.059	0.057	0.056	0.053	0.054	0.055	0.057
5 P	0.047	0.046	0.046	0.047	0.045	0.043	0.044	0.048
K	0.444	0.426	0.397	0.393	0.384	0.395	0.403	0.400
N	0.055	0.053	0.053	0.053	0.051	0.058	0.051	0.055
6 P	0.046	0.045	0.046	0.045	0.044	0.045	0.044	0.045
K	0.401	0.411	0.412	0.405	0.408	0.429	0.417	0.437
N	0.057	0.053	0.056	0.054	0.059	0.054	0.053	0.052
7 P	0.046	0.053	0.047	0.046	0.046	0.045	0.046	0.044
K	0.381	0.347	0.409	0.408	0.423	0.399	0.466	0.447
N	0.056	0.057	0.055	0.054	0.052	0.052	0.052	0.055
8 P	0.046	0.047	0.046	0.046	0.046	0.045	0.046	0.046
K	0.451	0.441	0.445	0.440	0.383	0.379	0.379	0.385

TABLE 8.

MINIMUM NUMBER OF SAMPLES TO SHOW SIGNIFICANCE ON A 5% LEVEL IN SAMPLE DIFFERENCES EXCEEDING D% OF THE MEAN.

MEAN PERCENT	Coeff. of Variation	D = 20%	D = 10%	D = 5%
ROBHOEK.				
N = 0.070	7.5	2	5	18
P_2O_5 = 0.0079	8.8	2	6	24
K_2O = 0.058	16.1	5	20	80
GRAHAMSTOWN.				
N = 0.102	7.0	1	4	16
P_2O_5 = 0.0149	6.9	1	4	15
K_2O = 0.134	8.9	2	7	25
VENTERSTAD.				
N = 0.056	5.8	1	3	11
P_2O_5 = 0.045	7.0	1	4	16
K_2O = 0.414	7.4	2	5	17
AVERAGE		2	6	25

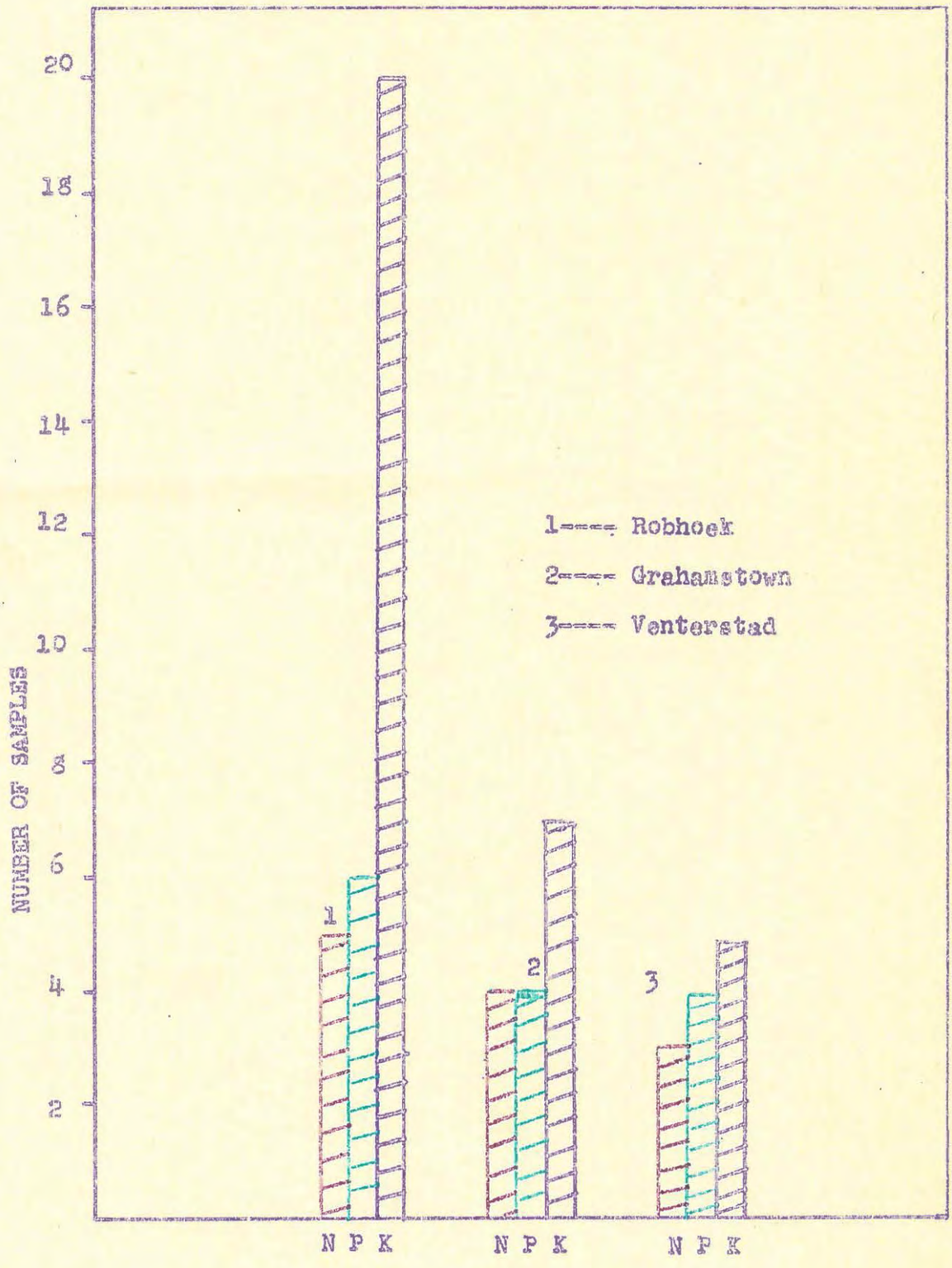


Fig. I.

MINIMUM NUMBER OF SAMPLES TO SHOW SIGNIFICANCE ON A 5% LEVEL IN SAMPLE DIFFERENCES EXCEEDING 10% OF THE MEAN.

5.3: DISCUSSION.

From Table 8 it is evident that an average of 2 sampling units (8 borings) would be necessary from an area 1/40 acre in size in order to represent N, P and K to within 20% of their mean values on a probability-level of 19 to 1; six sampling units (24 borings) would be necessary for 10% representation, while an average of 25 sampling units (100 borings) would be required for 5% representation. Of the three elements, potassium showed the greatest degree of variation, a phenomenon also reflected in the leaf sampling studies reported in Part III. For potassium determinations, therefore, more intensive sampling would be required than for the determination of nitrogen or phosphorus. It is also clear from Table 8 that when the element is present in relatively small amount it shows a greater degree of variation than when it appears in larger amount, for example the Robhoek soil as compared to the Venterstad soil.

Such intensive sampling has seldom been followed in fertility studies, even where the plots have not been larger than 1/40 acre. It seems, therefore, that in such studies little significance should be attached to differences of less than 10%, or even 20%in some cases, unless adequate attention has been paid to the sampling procedure.

The figures reported in Table 8 may be regarded as the normal variations which would occur in virgin soil on small areas of outwardly homogeneous appearance. It may be assumed that larger variations would occur on cultivated soils, since on such soils factors like fertiliser and cropping practices, irrigation, grazing by stock, etc, would tend to upset the normal distribution of the nutrients. It can be concluded, therefore, that cultivated soils will require more intensive sampling than that indicated in Table 8 for virgin soils.

Another factor which will seriously influence the variability of the nutrients is the size of the area to be sampled. In the introduction it was pointed out that soils often show great variation in the same field or orchard and the danger of including variable soil types in the same composite sample must be guarded against. The validity of taking a composite sample from an area depends to a large extent on the uniformity of the soil and the uniformity of the farming practices, such as fertiliser application for instance. Where such uniformity does not occur, it is obvious that the composite sample may contain sufficient material of high nutrient content to suppress any real deficiency occurring in a certain locality of the area sampled, or vice versa.

It appears essential that a preliminary survey of an area should be carried out before deciding upon a sampling procedure. The survey should be so designed as to furnish information about any large soil variations which may occur in the area. In fact, it is the opinion of the author that, unless an expert operator takes the soil samples, little importance should be attached to the analytical results. The practice of instructing the farmer concerned to take soil samples for advisory

purposes regarding fertiliser needs, must be strongly criticised. It is reasonable to assume that most farmers have little knowledge of the possible variations which may exist in a field. Very often such samples are analysed with great care and expense, and undue importance is attached to the results. If the inherent variations occurring in small plots, as indicated by the figures in Tables 5, 6 and 7, are studied it is clear that by taking a few scattered subsamples over a large field, the chances of arriving at correct conclusions as regards the nutrient status of the field must be small indeed.

In the light of the results reported above it must be concluded that the only satisfactory procedure for obtaining a truly representative soil sample is one obtained by intensive sampling. It seems that in the past investigators have not paid sufficient attention to sampling intensity, and the degree of accuracy with which soil nutrient values are often reported in the literature appears to be unjustified.

In view of the many possible factors which may influence the variability of the nutrient content of a particular soil in a given area, no standard sampling technique to cover all cases can be laid down. Each area has problems peculiar to itself and these must be determined and assessed before deciding upon a sampling procedure. However, as a result of this study, the following suggestions can be made.

5.4: SUGGESTIONS FOR TAKING SOIL SAMPLES.

(1) Only an expert operator, with a knowledge of soils, should take the samples.

(2) An intensive sampling procedure is essential for adequate representation of the nutrients. Some nutrients, like potassium, require more intensive sampling for adequate representation than others, like nitrogen and phosphorus.

(3) Differences in nutrient values of less than 20% should not be considered as significant at the 5% level of probability, unless the sampling variation has been determined.

(4) When soil nutrient studies are carried out for research purposes, a preliminary sampling study should be attempted in order to estimate the degree of variation in a given area before deciding upon a final sampling procedure.

(5) It would require more intensive sampling to establish and confirm a nutrient deficiency than would be necessary if the nutrient occurs in large amount.

(6) When large fields or orchards are sampled for advisory purposes, the operator should carry out a preliminary survey of the area. The field should be divided into a number of smaller sections, grouping together all similar factors such as soil type, slope, physical characteristics, drainage, etc. Each of these smaller sections should be sampled as intensively as circumstances will allow, and the composite samples so obtained must be analysed

separately. This should provide a fairly good estimate of conditions over the whole area, as well as indicating the need for a more intensive sampling procedure in a particular locality. Such a procedure will tend to minimise the danger of including material with a very high or very low nutrient content, which will happen if only one composite sample is taken to represent the whole area.

P A R T II.

THE CHEMICAL ANALYSIS OF

CITRUS AND PINEAPPLE LEAVES.

---

1. INTRODUCTION.

Ever since de Saussure showed in 1804 that the composition of the ash of plants varied with the part analysed, with the age of the plant, and with the soil upon which the plant grew, the concept of the chemical analysis of parts of the plant for diagnostic purposes slowly developed. It made particularly rapid strides during the past 25 years or so. Excellent reviews of the historical development of plant analysis have been published by Goodall & Gregory (2) and Ulrich (31), among others. In the wake of this development have followed chemists and physicists to provide more specific, more exact, and more convenient analytical devices, that were taken over by biochemists and modified to suit plant material. Many outstanding advances in modern biology have been made as a result of the invention and application of these efficient methods.

So voluminous has the literature in plant analysis technique become, that the newcomer to this field is completely bewildered and often has great difficulty in selecting efficient methods to suit his purpose. In consequence, almost every laboratory uses its own modifications or variations of existing techniques, and there are few applied analyses as "unstandardised" as leaf analysis. If one considers but one aspect of leaf analysis, viz. the drying of the material in order to stop enzymic reactions, the truth of the above statement is convincingly demonstrated. A careful survey of the literature has revealed that drying temperatures in use range all the way from 50°C to 130°C, the latter temperature maintained, admittedly, only for a short period. It is probable of course, that different materials behave differently during drying, but these limits of temperature have often been used for the same plant species. Differences in drying temperature are bound to influence the nutrient values, which are usually based on the "dry-weight" of the material. In any case, it must be perfectly apparent to workers, from the smell in a laboratory when plant material is dried at 105°C, that what is lost is not all moisture!

This inconsistency in technique is apparent throughout the whole complex process of plant analysis, from the early preparative stages to the final analytical method. If, to this variation in technique, the operative errors introduced, for example, by the use of different qualities of distilled water and reagents, different types of instruments, local laboratory conditions, and the personal equation of the analyst, are added, the combined error must be large. No wonder that workers find it extremely difficult to reproduce published nutrient values, found elsewhere in the same plant material. The analytical chemist is familiar with the difficulty of reproducing analytical figures obtained in a different laboratory on the same material, even where the methods and conditions are standardised. It follows, therefore, that where the methods are not similar or even standardised, the errors will be much larger. In view of this state of affairs, the general use of Tables of

Critical Nutrient Levels, carefully drawn up in one centre, must be seriously questioned.

That the authorities are fully aware of this unsatisfactory state of affairs is perfectly clear from the efforts of many to publish revised and standardised methods of analysis from time to time, for example the A.O.A.C. of America.

When the present analytical study of citrus and pineapple leaves was commenced, it soon became apparent that existing methods would have to be modified considerably to suit local conditions, as well as in order to yield the accuracy required for the ultimate studies on sampling.

Because of the lack of detailed instructions as to the preparation of the material for analysis, particularly as regards the pineapple plant, and also in order to study the reproducibility of this important step, it was decided to carry out a thorough investigation of the whole process, from the picking of the leaf to the final result.

A detailed description of the analytical procedures, as finally modified, is given at the end of each Section. Over the past two years laboratory assistants have used these methods to carry out routine determinations of nitrogen, phosphorus, potassium, calcium, magnesium, sodium, iron, manganese, zinc and copper in citrus and pineapple leaves. No serious difficulties have been encountered, while the results have always been reproducible. Once trained an assistant could complete the analysis of 6 leaf samples in four days.

2. EXPERIMENTAL STUDIES ON THE PREPARATION OF LEAF MATERIAL FOR ANALYSIS.

After harvesting, the plant material is usually subjected to four different preparative steps before the actual chemical analysis is carried out. These steps consist of washing the material in order to get rid of surface impurities; drying, in order to stop enzymic reactions from taking place and to prepare the material for grinding; mechanical grinding in order to reduce the material to a state of subdivision suitable for analysis; and final ignition to constant weight in order to obtain a standardised value on which to base the analytical figures. Two other stages, which are often necessary, may be added to these: storage of the material prior to washing and drying, and storage of the leaf powder after grinding prior to analysis.

The experimental studies on this preliminary phase of leaf analysis were carried out after the accurate standardisation of the analytical methods (Section 3), since it was necessary to use these methods in the study of some of the steps.

2.1. THE WASHING OF THE LEAF MATERIAL.

Citrus leaves are always covered with a thin film of dust which is very difficult to remove by mechanical wiping or brushing. In addition to dust, the leaves are often contaminated with spray residues, whether applied for nutritional, insecticidal or fungicidal purposes. These residues stick tenaciously to the leaf. Soil contamination is considerably more pronounced in the case of pineapple leaves, where the white basal tissue selected for analysis is practically in continuous direct contact with moist soil.

Whereas soil contamination may not affect the values for the major elements to any significant degree, it will certainly do so in the case of some trace elements, particularly of iron. As regards contamination from spray residues, the frequent presence of heavy metals in high concentration, even where the sprays were not applied for nutritional purposes, makes this a serious hazard. It is quite obvious that if a complete analysis, including the trace elements, is to be carried out accurately, surface contamination of the leaves must be removed as completely as possible. The only satisfactory process seems to be individual washing of the leaves.

Instructions in the literature for the removal of surface contamination vary considerably, though it is probable that different materials behave differently towards washing. Piper (8) maintains that owing to the loss, by solution, of some of the more soluble inorganic constituents, it is not permissible to wash plant samples in order to remove surface contamination; any soil particles should be removed by brushing the plant samples individually with a camel hair brush. Workers like Boynton et al (27), Goodall (28) and Roach (29) have wiped the surface of the leaves with cloth or cottonwool. On the other hand, Jacobson (30) found it necessary to rub leaves, intended for iron determinations, with 0.3N HCl, in order adequately to remove spray residues and dust. Ulrich (31) washed sugar beet samples in 0.2N HCl solution prior to drying. Mann and Wallace (32) showed that if apple leaves were leached long enough with distilled water, up to 99% of the total potassium may be lost. In the excellent authoritative text on Modern Methods of Plant Analysis (33), no mention is made of any cleaning technique.

Bathurst (34) washed citrus leaves in bulk in 2% acetic acid solution, rinsing afterwards with distilled water. Chapman and Brown (35) cleaned citrus leaf samples, intended for potassium analysis, by wiping each leaf on both sides with damp cheese cloth. Where trace elements were to be determined, each leaf was dipped into an Ivory soap solution, rubbed with the fingers, the soap solution washed off with tap water and the leaf finally rinsed with distilled water.

The Hawaiian Islands research workers on the foliar diagnosis of pineapples, Sideris et al (36,37) and Nightingale (38) did not give any

cleaning technique to be used for this material.

Early experiments by the author, in which the leaves were washed individually in distilled water, yielded erratic iron results, pointing to the fact that this technique was not satisfactory in removing dust. In view of the apparently satisfactory results obtained by Chapman and Brown with soap solution (35), it was decided to try the effectiveness of a synthetic detergent, Teepol. Excellent results were finally obtained by first washing each leaf individually in a 0.1% Teepol solution, then in distilled water followed by rinsing in deionised water. Nicholas et al (39) later on introduced 0.3% Teepol as washing liquid for leaf tissue testing at Long Ashton Research Station, England.

In order to get some idea of the efficiency of this washing procedure as well as to determine possible leaching losses, the following experiments were carried out:-

CITRUS LEAVES:-

A large sample of leaves was picked from a single tree according to the standard technique (34, 35). All the leaves were cleaned by wiping both sides thoroughly with clean pieces of damp cotton wool. The leaves were then well mixed and divided into 4 lots of 50 leaves each. Two samples were washed according to the Teepol technique, while the other two samples were not washed. All four samples were analysed carefully as described in Section 3. The duplicates agreed well in all cases.

PINEAPPLE LEAVES:-

A large sample of leaves, picked according to the standard technique (36), was treated in the same way as the citrus leaves. It must be mentioned that in this case the whole of the basal white portion was washed first before cutting out the middle third section for chemical analysis (See Part III). This procedure minimised the danger of soluble constituents like potassium from being leached out by way of the freshly-cut ends. It also made the washing procedure much more simple, since it was almost impossible to wash all the soil particles from ends cut in the field.

The results are recorded in Table 9.

TABLE 9.

LOSSES DURING THE WASHING STEP.

ELEMENT	CITRUS LEAVES			PINEAPPLE LEAVES		
	WIPED	WASHED	% DIFFERENCE	WIPED	WASHED	% DIFFERENCE
N%	2.67	2.70	+ 1%	1.27	1.26	- 1%
P%	0.113	0.111	- 2%	0.202	0.205	+ 1%
K%	0.44	0.45	+ 2%	3.13	3.11	- 1%
Ca%	3.53	3.47	- 2%	0.34	0.33	- 3%
Mg%				0.30	0.30	0%
Na%	0.43	0.38	- 10%			
Fe p.p.m	144	78	- 46%	139	53	- 60%
Mn p.p.m	23	23	0%	127	131	+ 3%
Cu p.p.m	5.6	4.6	- 20%	15.4	10.5	- 33%

The high iron and copper losses clearly indicate the inefficiency of the wiping procedure for removing dust and surface contamination. The loss of 10% in the case of soduim from citrus leaves must be considered as a possible leaching loss and where this element is of importance, for example in citrus growing in brack soils, the leaves should only be wiped (i.e. for the determination of this element only.) The other losses can be considered as normal sampling variations in view of the results reported in Part III.

The Teepol washing technique was adopted for both citrus and pineapple leaves in the subsequent studies. The procedure is described in Section 2.4.

2.2. THE DRYING AND STORAGE OF LEAF MATERIAL.

2.21. INTRODUCTORY:-

It is generally recognised that all plant samples should be dried as rapidly as possible after collection so as to reduce chemical and biological changes to a minimum. If drying is unduly delayed considerable loss in dry weight may occur, due to respiration, while proteins are also broken down to simpler nitrogenous compounds. Goodall (40) has shown that tomato plants may lose 5-10% of the dry weight in a period of 24 hours if not dried immediately. Before reviewing the various standard methods of drying as employed by other workers, it is necessary to consider some of the general aspects of oven-drying for the determination of moisture.

The determination of moisture in organic materials, whether they be synthetics or biological materials, is not a simple procedure. Even today a common concept of moisture analysis is "the loss in weight when a substance is heated to 105°C for a specified period". This simple method will give approximate moisture values, but cannot be depended upon to produce accurate results because it fails to recognise the complexity of the process of water removal. During the heating of a moist organic substance the following changes may be expected to occur:- Volatilisation of water as moisture; volatilisation of other adsorbed material; and volatilisation of the gaseous products formed by decomposition reactions. This decomposition does not begin at any particular temperature, but goes on at all temperatures at widely different rates, as was shown by Willits (41), for example, when he dried potato starch at different temperatures.

Many investigators have contended that naturally occurring organic substances, such as plant tissue, can not be dried without decomposition of the original moisture-free substance. The physical structure of plant tissue probably has a lot to do with errors in drying. This tissue often contains as much as 90% moisture and the removal of moisture from the cells and tubes presents a real problem. Cell moisture must diffuse through the cell wall before it can be volatilised and this becomes increasingly difficult as the drying proceeds. The moisture leaves the surface of the cells by diffusion and if the material is thick it requires considerable time for this to take place. Furthermore, as the cells lose water and the cell fluid becomes more concentrated, there is a lowering of the vapour pressure of the residual water. To drive the last traces of moisture out of the cells at a reasonable rate, the temperature must be increased, and this probably makes the cell walls less permeable to water. Heat also tends to seal the tubes in the tissue, making diffusion of deep-seated water vapour very slow. There is the real danger that the outer drier tissues may undergo thermal decomposition to a considerable extent before the deep -seated moisture is removed.

It must be concluded, therefore, that what is commonly termed the "dry-weight" of plant material, may not at all represent a completely

moisture-free condition of the original material in an undecomposed state. It is obvious that there are two quite separate requirements that must be satisfied when drying plant material for analysis:- A sufficiently high temperature to destroy the enzymes, and the optimum temperature for moisture removal without appreciable thermal decomposition.

2.22. REVIEW OF DRYING TEMPERATURES IN COMMON USE.

Piper (8) dried fresh cereals at 60-70°C in a forced draught oven prior to grinding, while the "dry weight" for analysis was obtained by drying the powdered material in a separate oven at 100°C. Ulrich (31) dried sugar beet samples at 70-80°C prior to grinding - no mention being made of any subsequent drying. Goodall & Gregory (2) stated that the exact temperature of drying is probably not a matter of great importance since there is little likelihood of appreciable loss of nutrients if the temperature does not exceed 100°C. On the other hand, Pucher et al (42), in determining nitrogen fractions in tobacco tissue, found a loss of 3-5% in amide nitrogen when the tissues were dried for 90 minutes at 68-69°C and losses of up to 20% with 2½ hours drying at 85-86°C.

Thomas (43), in reviewing leaf analysis up to 1945, stated that preference was given by most investigators to initial drying at a temperature of about 70°C. Both the texts, "Modern Methods of Plant Analysis (33), and "Official Methods of Analysis of the A.O.A.C. (44), recommend that the moisture content of plant samples should be determined by standard techniques other than oven-drying, such as drying in vacuo, by distillation with toluene, by freeze-drying, etc.

Bathurst (34) dried citrus leaves at 70°C prior to grinding. After grinding the powder was stored in tightly-stoppered bottles, no mention being made of further drying before analysis. At the Citrus Experiment Station of the University of California (45), Chapman and his coworkers dry leaves at 50-55°C for 24-48 hours, or as long as is necessary to enable grinding. Before analysis, a separate sample is dried for 5 hours at 105°C for determination of moisutre loss. At the Florida Citrus Experiment Station (46) leaves are dried at 70°C for 48 hours.

The Hawaiian research workers on pineapple leaves (36,37,38) reported nutrient figures on a fresh-weight basis. They reported the moisture content of the white basal tissue used for analysis to be consistent at 89-92%, determined by drying at 100°C. This procedure seems open to serious criticism, as will be seen below.

It is clear, therefore, that the oven-drying of plant material usually involves two definite phases, initial drying at a low temperature around 70°C to "kill" the material and to prepare it for grinding, and final drying at about 100°C in order to obtain the "dry-weight" basis for the analytical figures.

During the initial stages of this work it soon became apparent that an intensive study of this complex problem would have to be carried out in order to find a reproducible procedure to be used for the sampling

studies. It was found that perfectly satisfactory drying occurred at low temperatures in a forced-draught oven, but that as soon as the temperature was raised to 105°C in order to remove all the moisture, as recommended, severe browning of both citrus and pineapple leaf powder occurred. It was evident, too, from the failure of the material to reach constant weight, that decomposition was taking place.

A study of this nature would be incomplete if an investigation of the decomposition losses prior to drying were not included. It is also necessary to study the keeping quality of the material after drying, because it seems reasonable to assume that low temperature drying may only deactivate the enzymes and that if the material is not completely free of moisture, the enzymes may again become active on storage (47).

2.23. THE DECOMPOSITION OF LEAF MATERIAL PRIOR TO DRYING.

The washing of individual leaves is a laborious and lengthy process and because the author had to deal with large numbers of citrus and pineapple leaves during the sampling studies, it was necessary to know the degree of decomposition of the samples before they could be washed and dried. It was also necessary to devise a suitable method of storage.

A large composite sample of leaves (citrus and pineapple) was picked and transported to the laboratory as quickly as possible, where they were cleaned by wiping with dry cotton wool. In order to minimise sampling errors, the leaves were well mixed and each determination carried out in triplicate. The leaves were divided into equal lots, placed in stoppered silica weighing dishes (sealed tightly) and weighed. The fresh samples were stored as follows:-

(a) In sealed polythene bags for 2, 4, 7 and 14 days.
(b) In the open laboratory atmosphere for 2, 4, 7 and 14 days.
(c) In sealed polythene bags placed in a refrigerator set at - 5°C, for 7 and 14 days.

After the lapse of the appropriate period, each sample was placed in a silica weighing dish, dried for 72 hours in a forced draught oven at 65°C and then accurately weighed. One lot of samples was, of course, dried and weighed immediately after picking to act as reference. The percentage losses in dry weight of the samples are recorded graphically in Figs. II and III.

From Fig. II it is seen that citrus leaves decomposed fairly rapidly in open air up to the second day, while the losses were more gradual and constant thereafter, amounting to a total loss in dry weight of only 3% after 14 days. When the leaves were kept in a sealed polythene bag, however, the decomposition was very rapid and constant all the way, amounting to a loss in dry weight of 10% after 14 days. This must be ascribed to the fact that the moisture could not evaporate and the leaves turned mouldy after the fourth day, reaching a black colour after 14 days. The material stored in the refrigerator showed virtually

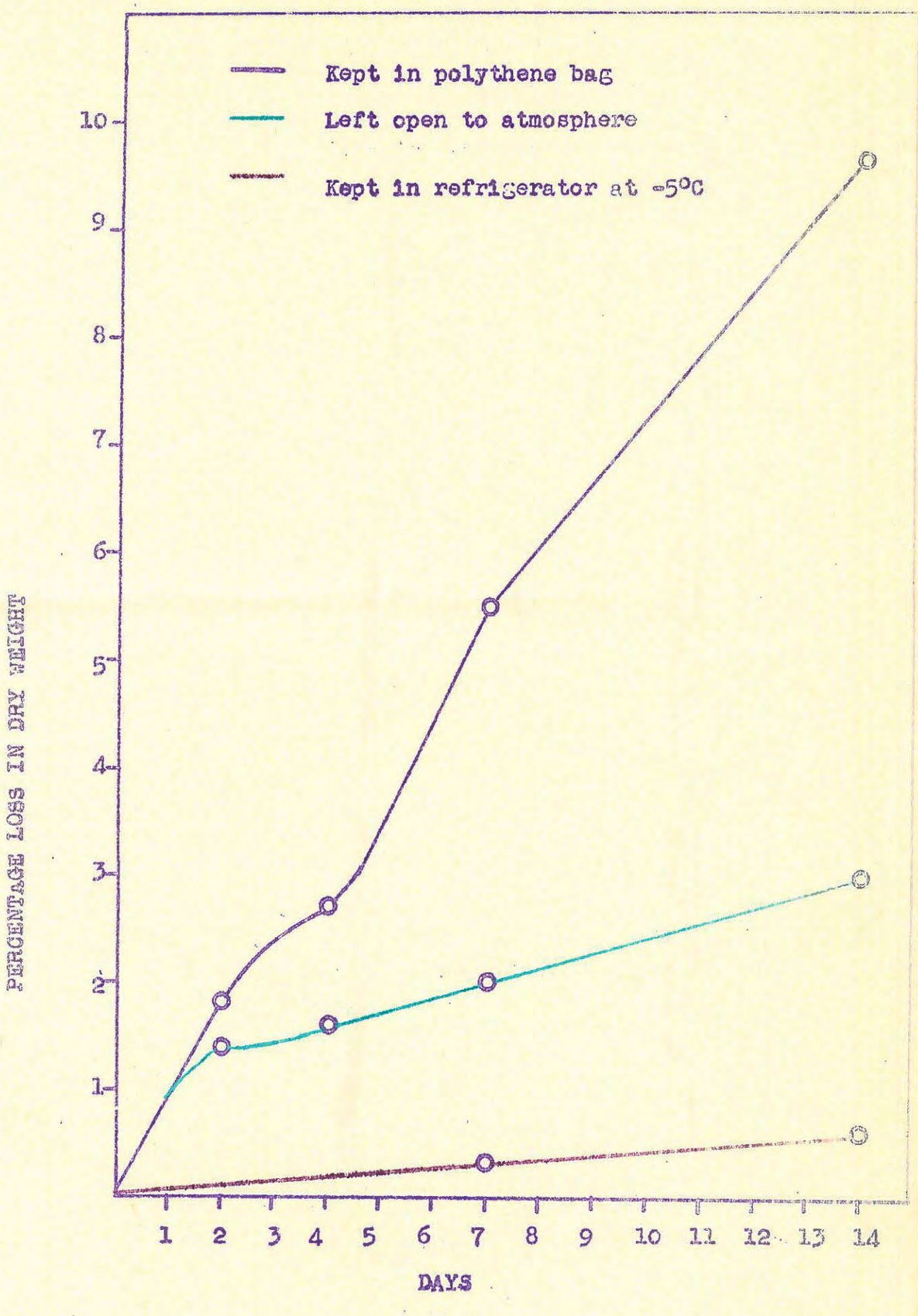


Fig. II.

DECOMPOSITION OF FRESH CITRUS LEAVES.

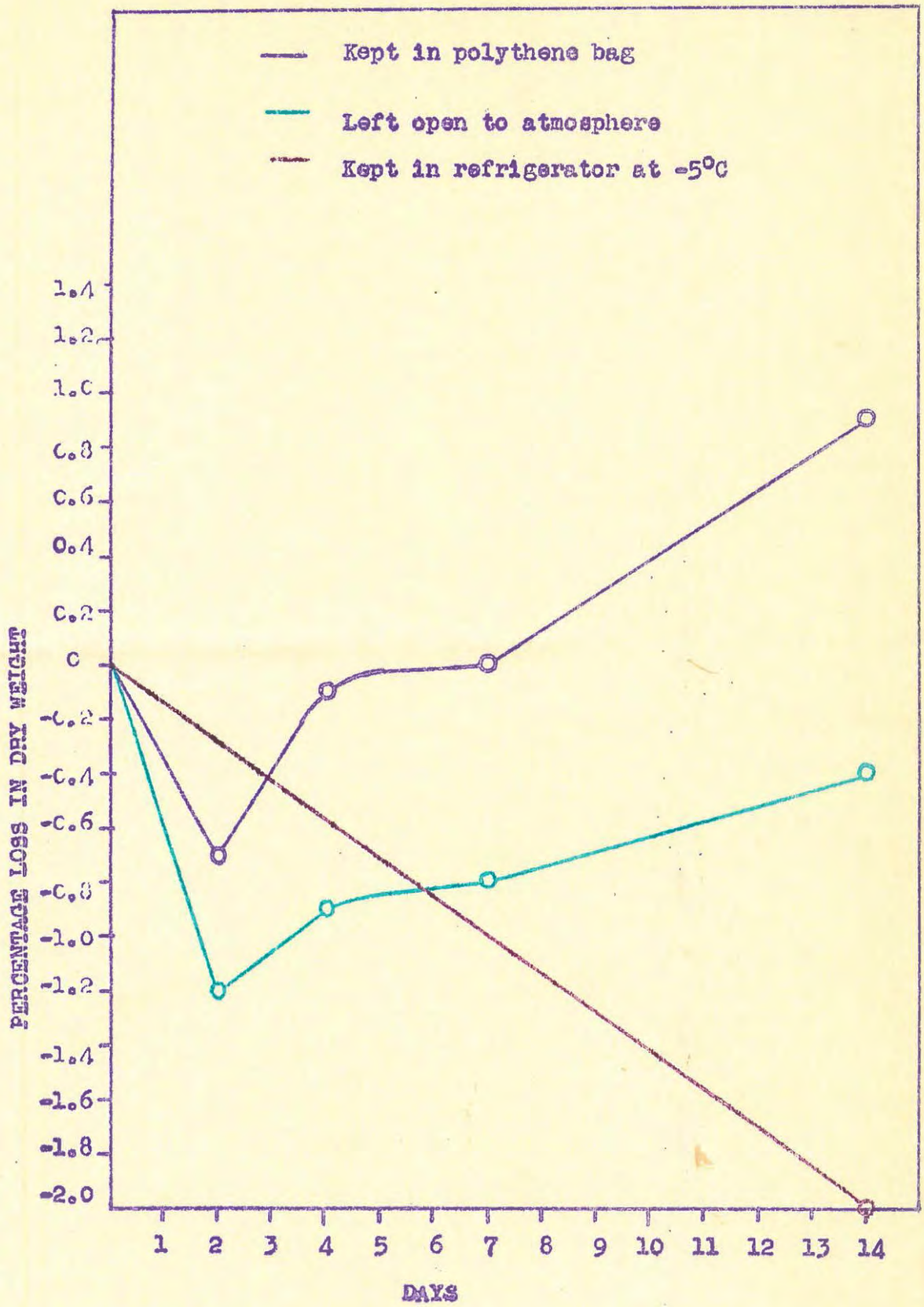


Fig. III.

DECOMPOSITION OF FRESH PINEAPPLE LEAVES.

no loss in dry weight. The problem of storing fresh citrus leaves prior to washing and drying was, therefore, solved.

The loss in dry weight would mean a corresponding increase in the nutrient percentages, that is, assuming that the decomposition is solely a combustion process. However, it is probable that nitrogen may be lost through the breakdown of the proteins.

The contrary behaviour of pineapple leaves as shown in Fig. III is rather interesting. Here the leaves did not lose, but apparently gained in dry weight at first. The reason for this was later found to be incomplete drying, although drying fresh leaves at 65°C for 72 hours has been proved previously to be entirely satisfactory. It seems, therefore, as if pineapple leaves rapidly "seal up" when they age after picking, and it becomes increasingly more difficult to remove the moisture. This was even more pronounced when the material was frozen. Nevertheless, the curves indicate the occurrence of rapid decomposition after the 2nd day, when the decomposition rate outstripped the difficulty of moisture removal. Again, the decomposition rate was more rapid when the leaves were kept in sealed polythene bags, than when they were left in the open. Mouldiness appeared after the 7th day in the leaves stored in polythene.

Both the citrus and pineapple leaves stored in the refrigerator had a perfectly fresh and healthy appearance when removed after 14 days. Henceforth, the leaves were always stored in polythene bags in the refrigerator as soon as possible after collection. It was necessary, however, to increase the drying period when dealing with frozen leaves.

It seems perfectly clear that leaves should not be transported from the field to the laboratory in sealed polythene packages - open-woven cloth bags would be more suitable. Neither should fresh leaves be sent through the post in sealed packages. Where the leaves cannot be sent packed in ice, they should first be oven dried at a low temperature, or air-dried for some time, when a correction factor can be applied.

2.24. THE DRYING OF LEAF MATERIAL PRIOR TO GRINDING.

In order to study the rate of moisture loss and possible thermal decomposition during the drying of fresh citrus and pineapple leaves, the following experimental procedure was adopted:- A large sample of fresh leaves was cleaned with dry cotton wool. In the case of citrus leaves, the midribs were cut out with stainless steel scissors (the usual procedure; see later), the leaf halves well mixed and equal portions placed in stoppered silica weighing dishes (tightly sealed). In pineapple leaves, the middle third portion of the white basal tissue was cut out and used for the experiment. During this experiment every effort was made to duplicate the conditions normally prevailing during bulk drying of the leaves.

After weighing the fresh samples, they were divided into triplicate lots and placed uncovered in three ovens, set at 50 ± 1°C, 65 ± 1°C and

105 ± 1°C, respectively. The samples were periodically removed for weighing. Unfortunately only one forced-draught oven was available, so that three convection-type ovens had to be used. However, the same trends were found when running controls in the forced-draught oven.

After the loss in weight had been investigated over a period of 7 days drying at constant temperature, the weighing dishes were tightly sealed and stored in desiccators. The leaf material was again dried at the respective temperatures for 24 hour periods after successive intervals of 14 days storage over a period of two months.

The results are recorded in Table 10, with graphical representations in Figs. IV and V.

Table 11 and Fig. VI record the loss in weight when leaf material was heated to successively greater temperatures. The fresh leaf material was first heated to constant weight at 40°C (96 hours in all) and then in stepwise increases of 10°C, 24 hours per step, to 100°C. Two ovens were used during this experiment, the one being set to the next rise in temperature while the samples were drying in the other oven.

It was reported to the author (private communication) that certain British institutions dry leaf material at an initial temperature of 130°C for a short while in order to stop enzyme reactions as quickly as possible. Thereafter the temperature is dropped to 80°C for the remainder of the drying period. This procedure was carried out in triplicate on a citrus leaf sample by drying at 130°C for 1 hour and then at 65°C for 96 hours. The results are also recorded in Table 11.

TABLE 10.

LOSS IN WEIGHT OF FRESH LEAVES AT CONSTANT TEMPERATURE.

Time in hours	PERCENTAGE LOSS IN WEIGHT					
	CITRUS LEAVES			PINEAPPLE LEAVES		
	50°C	65°C	105°C	50°C	65°C	105°C
5	41.9 *	57.7 x	58.5 x	24.0 *	34.1 x	90.5 xx
10	56.7	57.9	58.5	58.5	90.1	90.7
24	57.2	58.0	58.7 xx	86.4	90.2	90.7
48	57.5	58.2	58.9	88.7	90.3	90.9
72	57.6	58.2	59.0	89.0	90.4 xx	91.3
96	57.7	58.2	59.1	89.1	90.6	91.6
168	57.8 *	58.2 x	59.5 xx	89.5 x	90.9 xx	92.0 xx
14 days later	57.8	58.2	59.5	89.7	90.9	92.0
1 month later	58.3	58.2	59.5	91.0	90.9	92.1
2 months later	58.8	58.3	59.5	92.0	90.8	92.1

* indicates no scorching -- fresh leaf colour.

x indicates slight scorching -- dull-brown mottling.

xx indicates severe scorching -- brown throughout.

TABLE 11.

LOSS IN WEIGHT OF FRESH LEAVES WITH RISE IN TEMPERATURE.

TEMPERATURE (± 1°C)	PERCENTAGE LOSS IN WEIGHT	
	CITRUS LEAVES	PINEAPPLE LEAVES
40°C	60.8	89.5
50	61.1	89.6
60	62.1	89.7
70	62.5	89.8
80	62.8	89.9
90	63.0	90.0
100	63.5	90.2
Fresh leaves dried at 130°C for 1 hour then at 65°C for 96 hrs.	62.2 x	

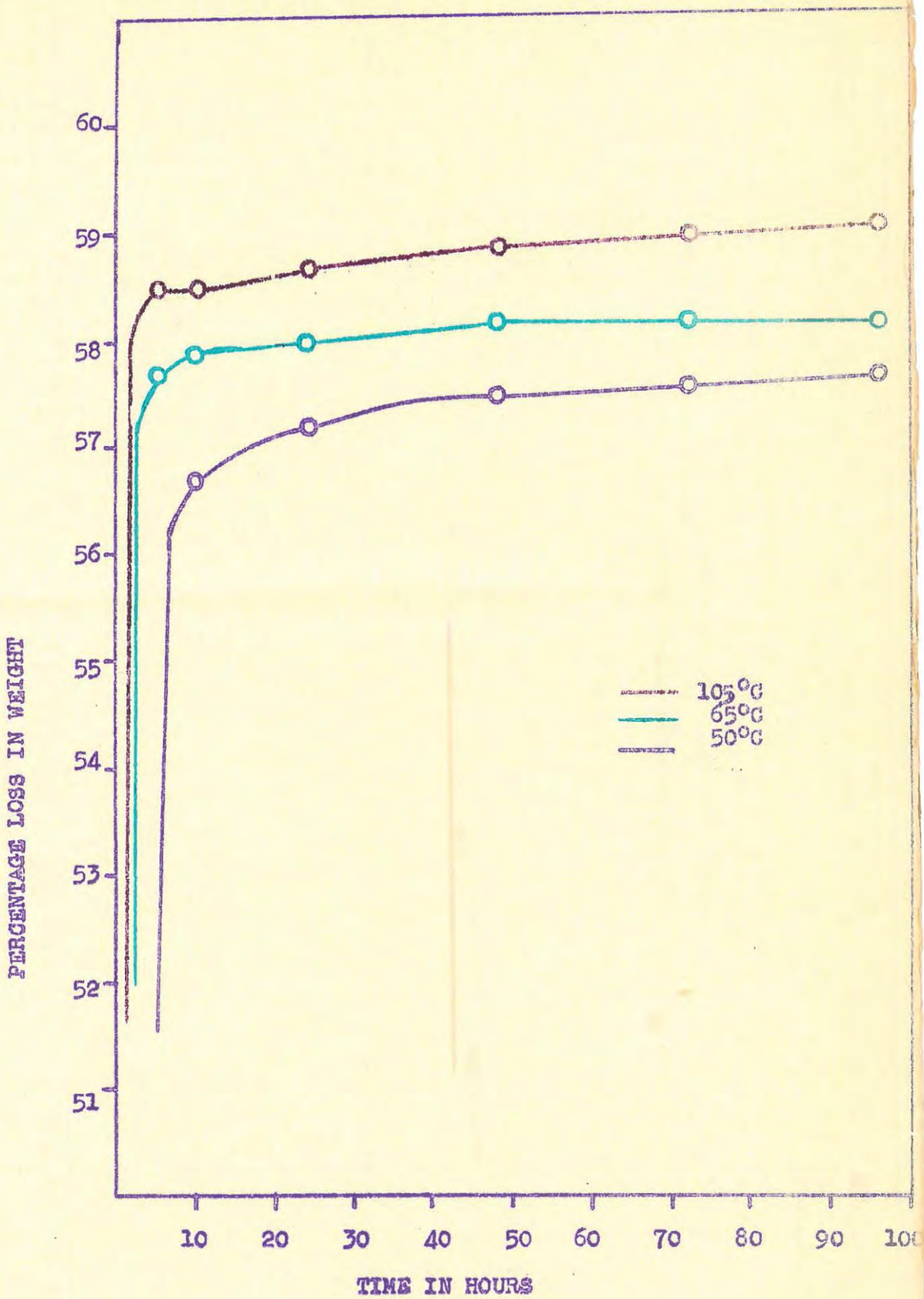


Fig. IV.

Fresh Citrus Leaves: Drying Isotherms.

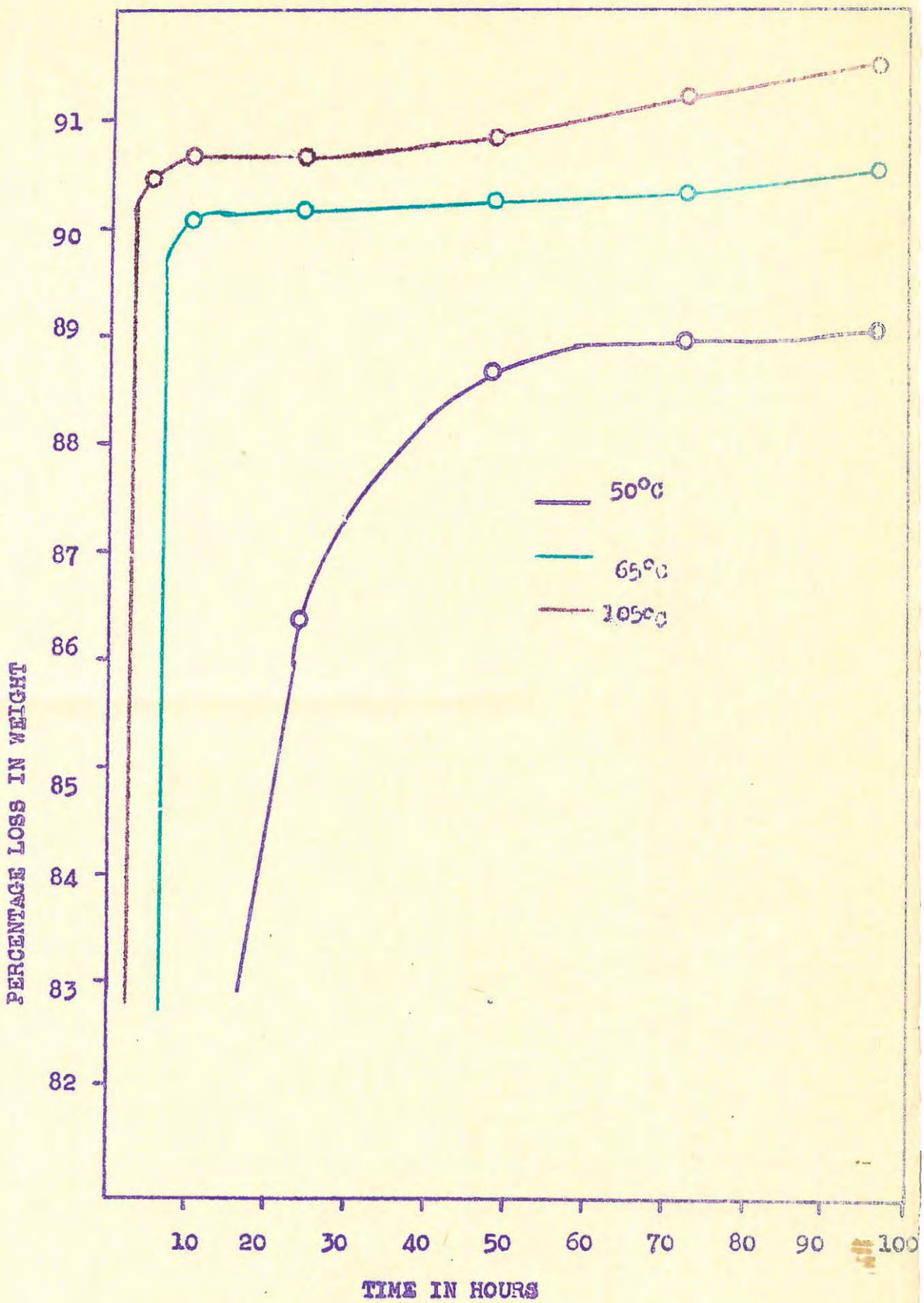


Fig. V.

Fresh Pineapple Leaves: Drying Isotherms.

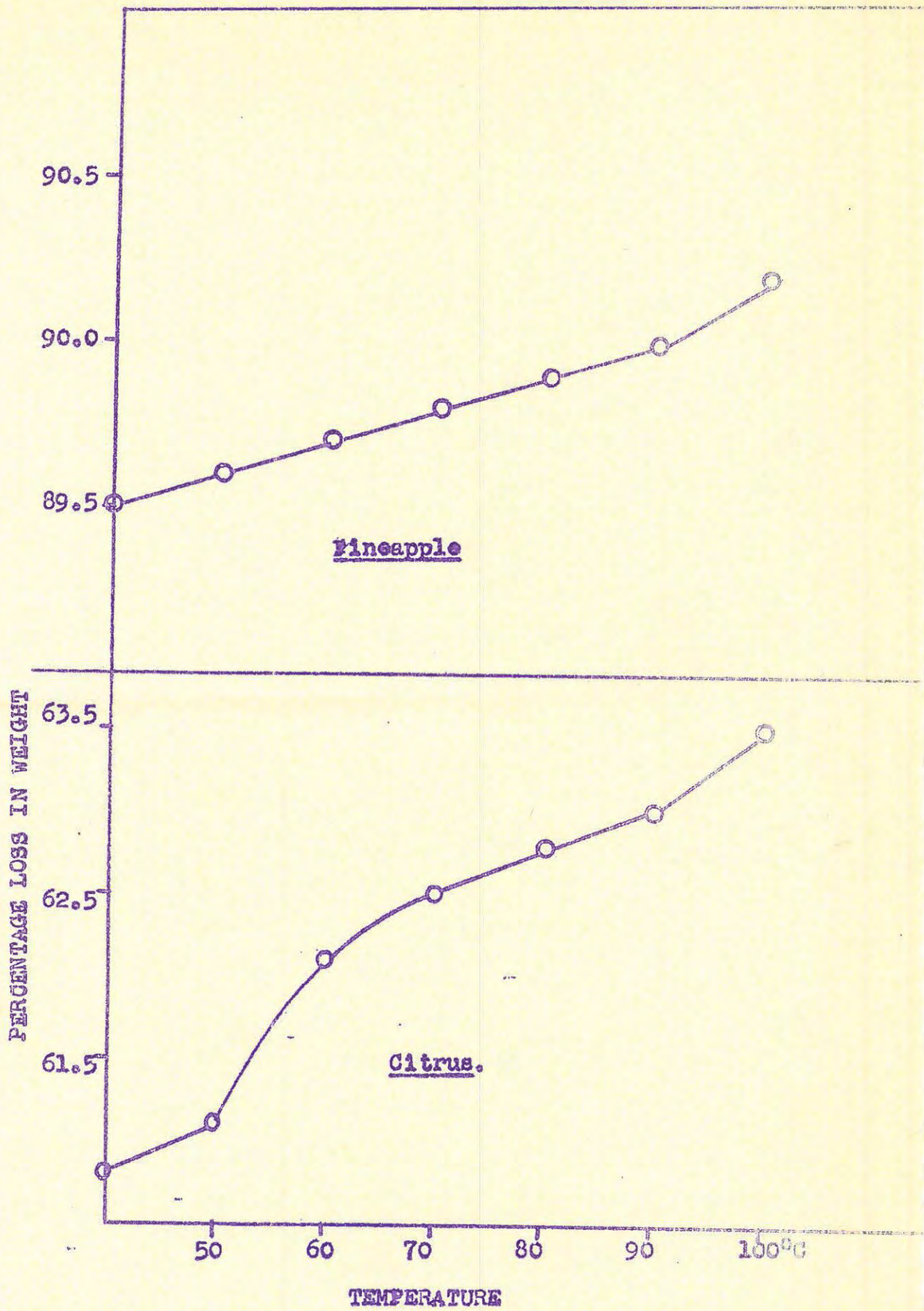


Fig. VI.

Fresh Leaves: Loss in weight with rising temperature.

DISCUSSION:

(a) CITRUS LEAVES: An examination of the drying isotherms for fresh citrus leaves (Fig. IV) reveals the attainment of a more or less constant weight after drying for 24 hours at each temperature, though the "dry weight" at 50°C is almost 2% greater than that at 105°C. The material dried at 105°C showed severe browning at an early stage while at 50°C the leaves maintained a healthy green, even after a period of 168 hours in the oven. It must be assumed, therefore, that drying at 105°C, and at 65°C, was accompanied by some thermal decomposition of the material. It seems reasonable, also, to assume that the material dried at 50°C was not completely moisture-free - the continuous slow loss in weight, even after 168 hours (7 days) in the oven testified to that. In fact, the same assumption probably holds true for material dried at 105°C; Willits (41), for example, in drying a sample of potato starch at increasing temperatures, found moisture still being evolved above 150°C. What then is the true moisture, if the one obtained by drying at 105°C is not an end value, and which curve shows the beginning of losses due to decomposition of the sample? In trying to answer these questions the author at first decided to determine the true moisture content of the material, using methods other than oven drying, such as distillation with toluene, vacuum drying, desiccation, the Karl Fischer titration method, etc. However, due to lack of suitable apparatus, this was finally abandoned. In any case, it is questionable whether these methods would have solved the problem, due to sampling errors and the lack of absolute reproducibility when trying to determine small differences

It would appear from the citrus curve at rising temperatures (Fig. VI) that a predominance of thermal decomposition over moisture losses occurred at the two breaks in the curve, the first between 50 and 60°C and the second between 90 and 100°C. The end result obtained at 100°C is of course cumulative in this case, and cannot be compared with the difference between 50°C and 105°C obtained in Fig. IV.

From these curves it would appear, therefore, that fresh citrus leaves should not be dried at a temperature above 50°C prior to grinding. However, this is not the end of the problem because from the literature (47) it seems quite clear that most of the enzymes will only be destroyed at a temperature above 60°C. It must, therefore, be assumed that at 50°C the enzymes will only be deactivated and that they will again become active in the presence of moisture. In order to test this theory the experiment summarised in Table 10 was carried on for a further 2 months as described. It was reasoned that the material dried at 50°C, even after 168 hours, would still contain some moisture which may be sufficient to activate decomposition reactions on storage. The figures recorded in Table 10 showed that this theory may be correct where, after 1 month storage under absolute moisture-free conditions the material dried at 50°C for 168 hours showed losses which surpassed the extent of thermal decomposition when the fresh material was dried at 65°C for the

same period. It seems reasonable to assume that the majority of the enzymes were destroyed at 65°C since no further decomposition could be detected in this material, even after 2 months storage, and it is certain that this material also had some moisture retained which could not be volatilised at 65°C.

There appears to be no advantage in going to the extra trouble of first heating the material at 130°C in order rapidly to destroy the enzymes, since the end result was the same as drying at 65°C. Furthermore, from the curves in Fig. IV it is clear that the material rapidly attained the temperature of the oven as most of the moisture was volatilised within 5 hours.

In drying fresh citrus leaves, then, the safest procedure is to dry at 50°C in a forced-drought oven. The thermal decomposition at this temperature appears to be negligible, while the amount of moisture retained by the material is small in comparison with the thermal decomposition which occurs above this temperature. Nevertheless, if this procedure is adopted, the leaf powder must again be dried after grinding and the analysis should be carried out shortly after grinding. However, the problem of storage was an acute one in the case of the sampling studies where large numbers of samples were handled, and it is propable that a similar lag in analysis will be experienced at most laboratories concerned with leaf analysis.

It was finally decided to adopt 65°C as the drying temperature for fresh citrus leaves. Before reaching this decision, similar experiments to those described were carried out between the r ange 50-70°C. Temperatures below or above 65°C never produced the same degree of constancy in the loss of weight of the material as was shown at 65°C; even when the material was left in the oven for 14 days at 65°C the same constant weight was obtained as shown in Table 10. The only explanation which the author can offer is that below 65°C there is a continuous slow loss of moisture combined with some thermal decomposition, while beyond this temperature thermal decomposition becomes increasingly more important, with loss in moisture taking a secondary position. The advantage of this loss in weight reaching a constant figure at 65°C is of great importance when handling large numbers of samples. The grinding of the material, which is a slow process, may be carried on over a period of a week or longer. The material may safely be left in the oven at 65°C over this period.

In selecting 65°C as the initial drying temperature it was realised that the material would undergo some thermal decomposition, probably less than 1%, and that this decomposition would be reflected by correspondingly higher nutrient values. However, because of the constancy of the decomposition and the safe storage of the material afterwards, drying at 65°C is in general to be recommended for citrus leaves.

After grinding, the leaf powder was placed in a clean bottle, dried for a further 24 hours and then sealed for storage in a cool, dark

cupboard. This extra heating was thought advisable in order to remove before storage the 3-5% moisture which the material picked up during grinding.

(b) PINEAPPLE LEAVES:- Figs. V and VI indicate that the drying of the fresh pineapple leaves followed much the same pattern as did citrus leaves, except that it was more difficult to remove the moisture at 50°C and that decomposition, although evident from the browning of the material, was more gradual with rising temperature (Fig. VI) until above 90°C, when the decomposition rate increased. The difficulty of drying at 50°C must be ascribed to the fact that the material was not cut up into small sections, since when this was done, rapid drying was achieved. On the other hand, it was noticed that the sap then showed a tendency to ooze out from the cut ends, leaving a brown stain in the weighing dish. This would have caused nutrient losses and for that reason the material was not cut up further than was necessary during the initial sampling step.

Unlike citrus leaves, pineapple leaves never attained a constant weight when dried at 65°C, even after a week in the oven. In fact the losses from thermal decomposition increased progressively, the material eventually being quite dark brown instead of white. Here again it was necessary to adopt a compromise procedure; because if the material is dried at 50°C, it will retain sufficient moisture to activate the enzymes soon after grinding and storage (See Table 10). On the other hand, drying the material at 65°C is an unsatisfactory procedure because of the continuous thermal decomposition taking place which may cause considerable variation when a large number of samples have to be ground. The following procedure was eventually adopted:-

Fresh pineapple leaves were dried for 48 - 72 hours at 50°C and then ground. Leaves that were stored in the refrigerator for some time were dried at 50°C for 4 or 5 days before grinding. After grinding, the leaf powder was placed in a clean bottle and dried for exactly 24 hours at 65°C. Although the material was slightly browned by this treatment, it could safely be stored under sterile conditions for 2 months prior to analysis (Table 10), and all the samples could at least be taken to the same state of thermal decomposition.

2.25. THE DRYING OF LEAF POWDER PRIOR TO ANALYSIS.

It is absolutely essential to dry the leaf powder before weighing out a sample for analysis. During the grinding step, as described in the next section, the material picked up between 3-5% moisture. This moisture must be removed, even though the material was heated after grinding prior to storage as done by the author. Because the leaf powder is in an entirely different physical condition than when the material was fresh, drying experiments similar to those described in the previous section were carried out on leaf powder.

The results obtained when citrus and pineapple leaf powders (after 2 weeks storage) were dried at 65°C and 105°C for different periods, are recorded in Table 12. The drying isotherms for these two materials are shown in Fig. VII.

TABLE 12.

LOSS IN WEIGHT OF LEAF POWDER AT CONSTANT TEMPERATURE.

TIME IN HOURS	PERCENTAGE LOSS IN WEIGHT			
	CITRUS POWDER		PINEAPPLE POWDER	
	65°C	105°C	65°C	105°C
2	4.09	5.79 x		
4	4.25	5.92	2.33	4.81 x
8	4.35	5.94	2.40	6.03
16	4.40	6.30	2.40	7.00
24	4.42	6.35	2.42	7.99
48	4.47	6.48	3.01 x	9.53 xx
96	4.64	6.68		
168	4.64	7.16 xx	3.08	12.30

x indicates severely scorched.
xx indicates blackish brown.

From these results it was quite evident that much greater thermal decomposition occurred when drying leaf powder than when drying the plant material in its original physical state. This is probably due to the finely divided state of the powder as compared to the original fresh material. Consequently the prevailing tendency of workers to dry citrus and pineapple leaf powder at 105°C prior to analysis, irrespective of the length of the drying period (45, 36, 37, 38), must be questioned.

From the drying isotherms in Fig. VII it is apparent that a difference of almost 2% would be obtained in the "dry weight" of citrus leaf powder between 65 and 105°C, the difference being already 1.9% soon after commencement of drying. In the case of pineapple leaf powder, this difference amounted to more than $5\frac{1}{2}$ % in a 24 hour period. In any analytical work, whether it be the analysis of a simple inorganic mixture or the analysis of complex plant material, an initial error of 2%, even before the analytical error itself has been taken into account, must be regarded most seriously. Whereas a certain degree of variation in temperature for the fresh material may still be permissible, the temperature must be strictly controlled when dealing with leaf powder which is so susceptible to thermal decomposition.

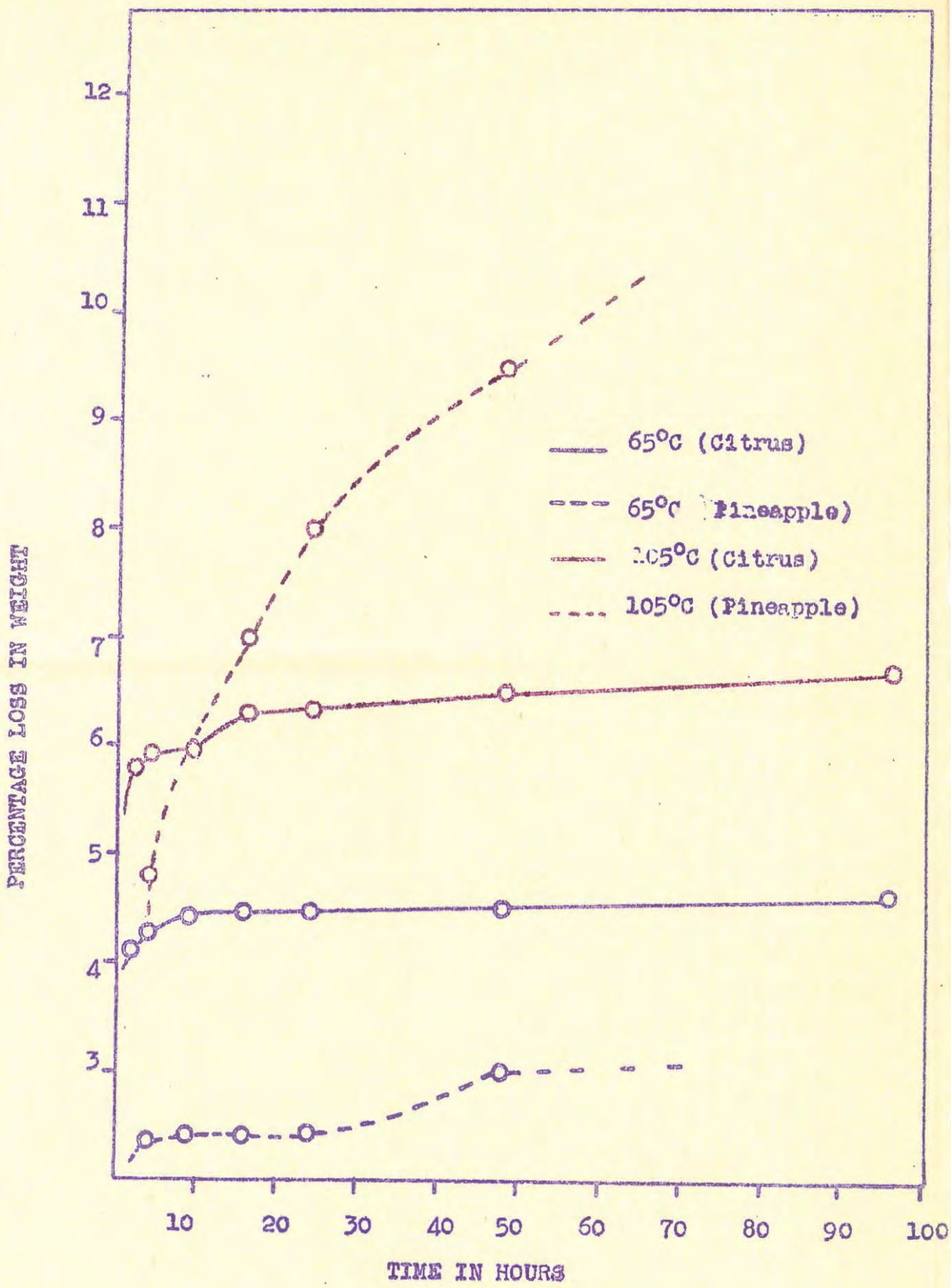


Fig. VII.

Leaf Powder: Drying Isotherms.

In view of the constancy of the "dry weight" obtained after the 24 hour period when drying at 65°C, for both materials, it was decided to adopt a drying period of 24 hours at 65°C prior to analysing citrus or pineapple leaf powder. It was considered that a negligible amount of moisture was retained by the material when treated in this way, whilst entailing the least possible amount of thermal decomposition under the circumstances.

The author cannot agree with the procedure adopted by the Hawaiian workers (36, 37, 38) by which pineapple nutrient values are based on the fresh weight of the leaf material, after determining the moisture content by ovendrying at 100°C. Besides the obvious conclusion that such a procedure will not give the true moisture content of the material, a more serious argument against it is the sampling error when selecting freshly cut plant material for moisutre analysis. It is obvious that with the leaves having a water content of 90% or more, an error of sampling which accounts for only a 1% error in moisture can cause a 10% error in the nonvolatile constituents reported on a moisture-free basis. The author feels that it is a much more accurate procedure to dry the material as described than to base nutrient figures on moisture determinations carried out on separate samples.

2.26. THE KEEPING QUALITY OF LEAF POWDER.

It was necessary, as a final step in the drying investigation, to know how long the leaf powder, dried as above, could safely be stored before chemical analysis was carried out. When dealing with a large set of samples several months may elapse before the last sample in a series can be analysed. The question is, how will the results of two similar samples compare when there is a considerable difference between the storage periods? Clearly the satisfactory storage of leaf powder is as important as any other step in the whole leaf analysis procedure.

Table 13 records the results obtained by carrying out periodic nitrogen determinations on citrus and pineapple leaf powder, dried and ground as described, and stored in sealed, sterile bottles in a cool dark place. The results are recorded graphically in Fig. VIII.

Nitrogen was selected as indicator element because it was felt that the nitrogenous compounds were the most likely to decompose on storage, and as this would be reflected in the "dry weight" of the material, all the other element values would be affected as well. Furthermore, the author wanted to disturb the material as little as possible when the bottles were unsealed for the removal of subsamples for analysis, and only a small subsample was necessary for triplicate N determinations. The material was, of course, thoroughly shaken before unsealing the bottle, because leaf powder, like other finely-divided materials, has a tendency to settle into definite layers on standing.

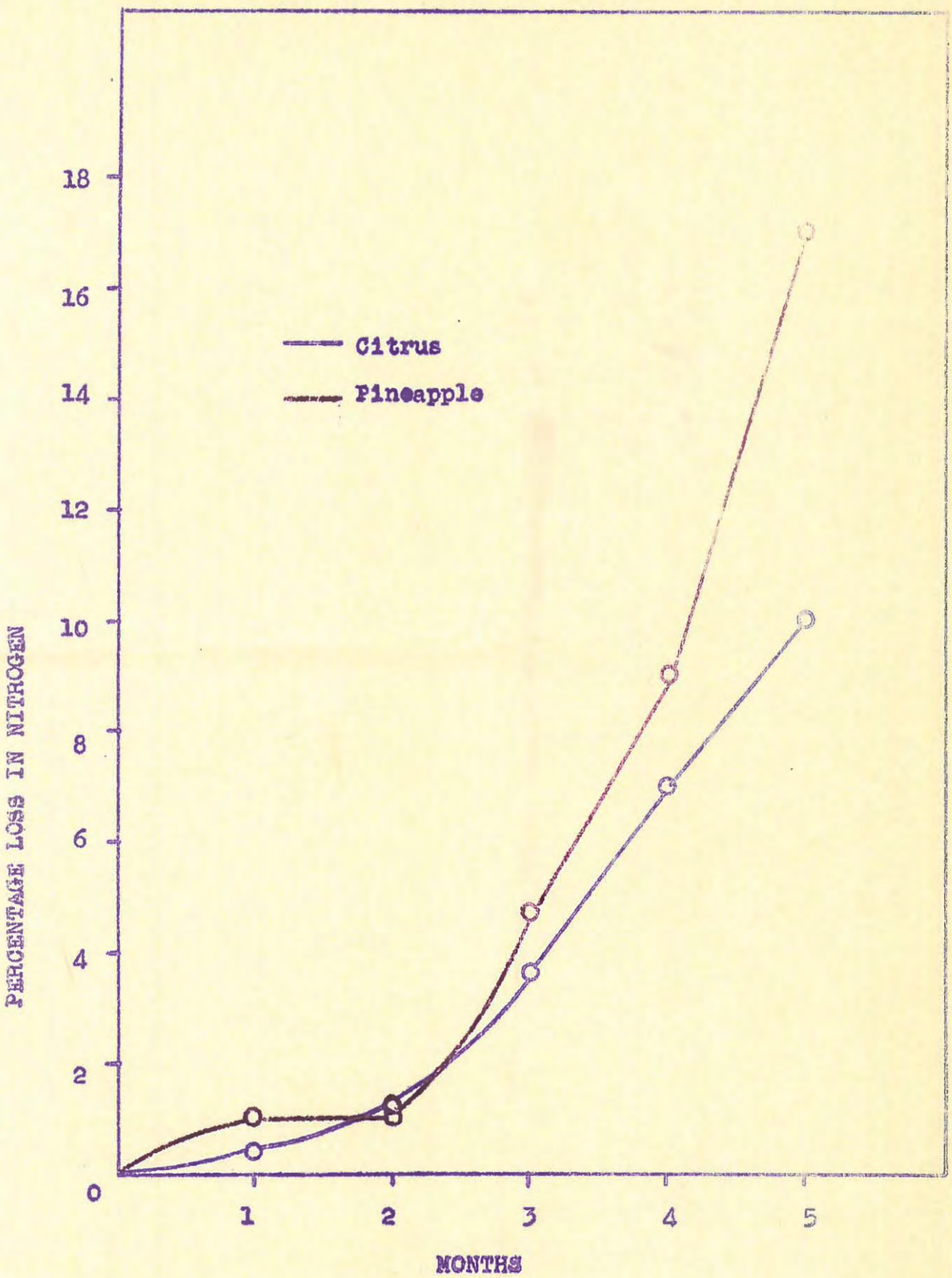


Fig. VIII.

Decomposition of Leaf Powder on Storage.

TABLE 13.

KEEPING QUALITY OF CITRUS & PINEAPPLE LEAF POWDER ON STORAGE.

MONTHS OF STORAGE	CITRUS POWDER		PINEAPPLE POWDER	
	% N	% Loss	% N	% Loss
0	2.59	0	1.36	0
1	2.60	0	1.35	1%
2	2.56	1.2%	1.35	1%
3	2.50	3.6%	1.30	4.7%
4	2.42	7%	1.25	9%
5	2.35	10%	1.15	17%

These losses were found when storing the material under the most careful conditions and much higher losses can be expected to occur with indifferent storage methods. An error of 1% is quite permissible and even common in this type of analysis (See Section 4), so that it appears as if the leaf powder can safely be stored for 2 months, provided the necessary precautions are taken viz. proper sterilisation and sealing of the container.

However, one other factor must be considered. The N figures show actual losses in this element and one would expect a certain amount of loss in "dry weight" as a result. As a loss in "dry weight" would tend to give high N values, one must assume that the true loss in nitrogen was actually greater than that recorded in Table 13. This would again emphasise the importance of carrying out the analysis with the shortest possible delay.

The author cannot agree with the statement, common in the literature, that once plant material has been dried it can be stored indefinitely. It may be that if it is "killed" to the point of excessive thermal decomposition and then stored under absolutely sterile conditions that no decomposition will take place.

2.3. THE GRINDING OF LEAF MATERIAL.

It is usual to grind the dried material before analysis partly for greater ease in manipulation, partly in order to ensure greater uniformity in composition. Because of the laborious nature of hand grinding, particularly when the samples are large, mechanical grinding in mills is favoured by most workers. When selecting a mill it is of the utmost importance to consider the possibility of contamination of the sample, particularly if the trace elements are to be determined.

Hood and his coworkers (48) carried out an intensive study on the mineral contamination during the grinding of plant samples. The types of grinding equipment tested were:- The Wiley mill, hammer mill, mortar and pestle, and a jar mill, with flint, porcelain and Mullite (a form of aluminium silicate)balls. Their results may be summarised as follows:- Grinding plant material in a Wiley mill and hammer mill led to contamination with iron and copper. Jar mill grinding with flint balls resulted in contamination with iron, zinc, copper, cobalt and sodium. The use of porcelain or mullite balls during grinding gave rise to appreciable contamination with iron, zinc, copper, cobalt, sodium, and in some cases calcium, sulphur and phosphorus. Flint, porcelain and Mullite balls all wear appreciably during jar mill grinding, the losses amounting to about 1% (10-15 g.) of the weight of the balls in the case of porcelain and mullite over a period of 11 days grinding. Flint balls lost only 0.3% in weight over the same period. Large increases in contamination resulted from decrease in size of plant samples ground in a jar mill. Hand grinding with a mortar and pestle resulted in no appreciable contamination. It is concluded that all the mechanical grinding methods tested resulted in serious contamination with one or more elements, and particularly marked errors would be involved in using the common mills for grinding plant tissue for micro element analyses.

The author carried out a similar investigation using a Vetter, all-agate, ball mill. A composite citrus leaf sample was washed and dried and divided into two sets of triplicate subsamples. One lot of samples was ground, using an agate pestle and mortar, and the other set ground in the agate ball mill for a period of 2 hours. Each sample was analysed separately. The means of the results are recorded in Table 14.

TABLE 14.

COMPARISON BETWEEN HANDGRINDING AND MECHANICAL GRINDING IN AGATE.

ELEMENT	HAND GRINDING	MECHANICAL GRINDING
Nitrogen, %	2.46	2.43
Phosphorus, %	0.121	0.122
Potassium, %	0.56	0.56
Calcium, %	3.67	3.66
Magnesium, %	0.58	0.57
Sodium, %	0.32	0.32
Iron, p.p.m.	65	64
Manganese, p.p.m.	32	33
Zinc, p.p.m.	15	15
Copper, p.p.m.	3.4	3.3

It was clear that no significant contamination occurred under these conditions and it must be concluded that agate grinding is better than other methods, particularly for trace element analysis.

An important point in using the ball mill is to make sure that the jar is at least $\frac{1}{2}$ - $\frac{3}{4}$ filled with plant material. It was noticed that if a very small sample was ground, the balls knocked unevenly against the walls of the jar and some chipping of the agate occurred, which would, of course, alter the weight of the material. The speed at which the motor is run is also important.

Citrus leaves could be ground to a fine powder within $\frac{1}{2}$ hour. Pineapple leaves, because of their fibrous nature, had first to be crushed into small fragments after drying. A sample could then be ground to a powder within 1 hour.

GENERAL REMARKS.

At the conclusion of this first phase in the investigation of the leaf analysis procedure a few remarks may be made in the light of the results presented.

It has been shown that each step in the preparative phase is subject to error; admittedly the errors are not large if careful control is exercised throughout, and it is probably true that from an agricultural point of view, the tendency would be to neglect these errors in view of the normally larger error associated with sampling.

However, these errors are cumulative and if they are neglected they may easily, as a whole, cause an error of 10% or more in the final result. In fact, the analytical error may even be larger than the sampling error. Admittedly the author had to carry out the analytical procedure with a high degree of accuracy in order to be sure of the sampling variations reported in Part III. But that does not justify a procedure in which less care need be taken when analysing leaf material for other purposes. The whole process is so highly susceptible to error that unless undue care is taken throughout, the final analytical results may be of little value.

It also seems clear that different plant materials react differently during the preparative phase, and no standard techniques can be laid down. Each type of material must be carefully studied and a satisfactory programme of treatment worked out.

The procedure described below was finally adopted during the preparative phase.

2.4. THE PREPARATION OF THE LEAVES FOR ANALYSIS.

CITRUS: After picking, the leaf samples should be transported to the laboratory in open-woven cloth bags as quickly as possible, where they are transferred to polythene bags and stored in a refrigerator. Removing one sample at a time from the refrigerator, they are then washed as follows in order to remove surface contamination:- Take five polythene dishes (about 6 inches deep and 12 inches diameter), and place about 750 ml. 0.1% Teepol solution in the first, 750 ml. distilled water in the second and third, and 750 ml. deionised water in the fourth. The fifth dish is used to collect the wet leaves. Sponge each leaf thoroughly with cotton wool in the Teepol solution, wiping both sides, and then rinse well in the three sets of water, rinsing last in deionised water. Shake off the drops of water and place in the fifth dish. When the complete sample has been washed, drain off the water as completely as possible and place the leaves on a sheet of Whatman drying paper. Using a pair of stainless steel scissors, carefully cut out the midrib of each leaf (as close to the midrib as possible) and place the leaf halves in a suitable sized clean muslin bag. The midribs are cut out in order to facilitate drying and grinding as well as to give a more representative sample of the lamina.

Close the muslin bag with a rubber band and suspend inside a stainless steel forced-draught oven, set at 65°C. Dry for 48 hours and then grind the sample in an agate ball mill, ensuring that the jar is at least half filled with material. After grinding, place the powder in a clean screw-capped bottle, leave open in the forced-draught oven for 24 hours at 65°C, and seal tightly whilst still warm. Store in a cool dark cupboard and carry out the analysis as soon as possible, in any case within 2 months.

PINEAPPLE LEAVES:- These are washed as described above. After washing, cut out the middle third (usually half-inch) of the white meristematic basal tissue and place in a clean muslin bag. Dry as above, but with the oven set at 50°C. Dry for 72 hours. After drying, place the sample on a piece of drying paper and crush into small fragments. Grind to a powder in an agate ball mill, place the powder in a clean screw-cap bottle, leave open in the forced draught oven at 65°C for 24 hours, and seal tightly whilst still warm. Store as above.

NOTES:-

(1) It is advisable to change the Teepol wash liquid after every two samples, and the water after washing 4 samples.

(2) The size of the storage bottle must conform closely to the size of the sample in order to have as little air inside as possible. These bottles must be sterilised properly.

(3) The utmost cleanliness is essential throughout the preparative phase.

3. EXPERIMENTAL STUDIES ON THE ANALYTICAL PROCEDURES.

The work carried out in developing and standardising accurate and precise methods for the determination of N, P, K, Ca, Mg, Na, Fe, Mn, Zn and Cu is described in this section. All of these elements, with the exception of nitrogen, were eventually determined from the same plant solution, a convenience for sampling studies.

3.1: REAGENTS, WATER AND APPARATUS.

The introduction of foreign substances during the preparation of the sample and in the course of the analysis may have very serious consequences, particularly for trace analysis, and special attention must be paid to this source of error.

Great care must be taken to prevent possible gross contamination of the sample through accidental introduction of metal from common articles of the laboratory - iron from ring stands and clamps, nickel from crucible tongs, copper and zinc from burners, zinc from rubber, etc. Small amounts of certain heavy metals may find their way into the sample from the glass ware and the platinum ware used in the analysis. Thus Pyrex glass may yield traces of arsenic, boron, zinc, lead and possibly other heavy metals. Platinum ware commonly contains iron, and will certainly yield some of it to acid solutions brought into contact with it. The glaze of porcelain may contain heavy metals such as lead. Fused silica ware is of great value in trace analysis because of its general freedom from heavy metals and its resistance to most acid solutions. Filter paper always contains appreciable quantities of certain metals, particularly Ca, Ba, Al, Zn, Pb, etc. When used, it should first be leached with a hot acid solution.

On the other hand, losses of a trace constituent may sometimes result from interaction with the substance of the containing vessel. For example, in ashing samples of organic nature, such as leaf material, in a silica or porcelain dish, an appreciable fraction of the constituents may be fixed at the surface of the dish, probably by the formation of silicates which may not be entirely decomposed by acids. Filter paper may adsorb such metals as lead and copper from solutions, especially if these are neutral or only slightly acid.

3.11: REAGENTS.

Common sources of contamination often neglected are the reagents used during the analysis. The author found that even the best quality reagents had to be purified for trace element work - in fact there are no short cuts, and workers in this field must be prepared to go to the extra trouble of these precautions or their results may be of little value.

The common acids of Analar Grade are as a rule very low in heavy metals, but when it is necessary to use them in comparatively large quantities, as in the destruction of organic matter, they have to be purified. It was found that by distilling ordinary grade acids through an all-borosilicate glass still, negligible traces of impurities were retained.

Alkalies, unlike strong acid solutions, continuously leach out metals from the walls of the glass containers in which they are stored. Ammonium hydroxide was the only alkaline solution used in the trace element work and this was purified by distillation of ammonia and absorption in deionised water. Alkaline solutions were always stored in polyethylene bottles.

Directions for the purification of the reagents are given with each method. One of the most sensitive tests to determine the degree of purity of a specific reagent, as regards heavy metal contamination, is to use a dithizone solution in carbon tetrachloride. When an alkaline solution of the reagent is extracted with dithizone, the organic layer should remain a pure green. If the layer acquires a purple or red colour, the reagent must be purified. It is also absolutely essential to run blanks during the actual analyses. If blanks are high, the source of contamination must be looked for and corrected.

Standard solutions play an extremely important role in plant analysis, since the accuracy with which a particular element is determined is directly dependent upon the purity and careful preparation of the standard solution used for calibration. The author used "specpure" chemicals throughout for the preparation of standards. These solutions were always stored in polyethylene bottles, since it was found that dilute standards stored in Pyrex bottles rapidly fell off in concentration, presumably due to ion-exchange with the constituents of the glass.

3.12: PURE WATER.

The author found that the ordinary distilled water of the laboratory frequently contained such relatively large amounts of certain metals, for example copper and iron, that its use in trace analysis was out of the question. It was also found that distilled water, stored in a Pyrex aspirator for some time, picked up sufficient calcium and sodium from the glass to upset the determination of these elements by flame photometer.

At first the very pure water obtained from a special automatic still, built by members of the Chemistry Department for conductivity work (49), was used, but it was soon realised that this still would not be able to supply something like 20 litres of pure water per day, the normal daily requirements needed for this study. Eventually,

water of a high purity was obtained by passing ordinary distilled water through a column containing the "mixed-bed" ion exchange resin, Amberlite MB 3. The deionised water had a consistent conductivity of between 0.09 - 0.10 micromhos per centimetre, which compared very favourably with the best water (0.062 micromhos per cm.) obtained from the Faure-Gledhill still (49).

A special automatic glass column was devised and constructed so that it needed the minimum amount of attention for operation. Distilled water is syphoned from an aspirator, passed through the resin bed (6" x 1") and collected in a 22 litre all-polythene aspirator. In this way a continuous supply of pure water was always at hand. By using distilled water as the influent, the "life" of the resin was considerably increased and up to 200 litres of deionised water could be produced before regeneration was necessary. The resin is of the self-indicating type, so that it is a simple matter to determine when the resin is exhausted.

It is an easy matter to determine whether water is of sufficient purity for the analytical procedure. When the water is sprayed into the flame of a flame-photometer it should produce no visible colour. Heavy metal contamination was determined by shaking the water with dithizone as previously described - the carbon tetrachloride layer should remain green. The sensitive molybenum - blue test was used for phosphate detection.

3.13: APPARATUS.

The use of apparatus of the best quality was found to be essential. Translucent silica Kjeldahl flasks were used during the digestion of the plant material in order to minimise contamination during this drastic treatment. Thoroughly leached "Pyrex" ware was used in all other operations where glass was necessary. Particular care was taken to keep a separate set of apparatus for specific use with each element. For example, if a set of volumetric flasks was used for the determination of manganese, in which phosphoric acid is one of the reagents, high results would be obtained if the flasks were subsequently used for the determination of phosphorus, it being extremely difficult to wash the last traces of phosphoric acid from the flask, as the author found by bitter experience. Similar precautions apply to the determination of the other elements.

Efficient washing of apparatus plays an extremely important role in accurate analytical work, and often serious error can be traced to this step. It was found good practice to rinse all glass apparatus at least once a week with chromic acid solution. This was followed by washing with tapwater and Teepol, using a suitable brush wherever possible, rinsing afterwards with distilled water and finally with deionised water. The glass apparatus was always stored

in an inverted position on sheets of drying paper inside a dust-free cupboard.

Volumetric measuring apparatus, like burettes and pipettes, both micro and macro, were of Grade A quality. These were always kept as clean as possible and stored in a special cupboard.

3.14. INSTRUMENTS.

The flame photometric work was carried out on the instrument manufactured by Evans Electroselenium Ltd., using "bottlegas" and compressed air. After suitable standardisation and by the use of specially prepared standards good results were obtained with this instrument. For accurate readings it is essential to have sufficient of the element to be determined present in the test solution to give a large deflection. With proper treatment, the instrument was found to be singularly free of the normal ailments associated with this type of apparatus.

The colorimetric work was carried out on a Hilger Spekker Absorptiometer of the latest design, Model H760. By using a special technique for calibration curves (described later) results of a high order of precision and accuracy were obtained, and it is doubtful if a much greater precision could have been obtained by using a spectrophotometer. The instrument was found to be extremely stable towards voltage fluctuations, easy and quick to operate, and to have a high degree of reproducibility.

Where the absorption characteristics of a particular solution had to be examined, a Beckman Model Du Spectrophotometer was used(6).

pH measurements were carried out on an Electrofact pH Meter, sensitive to 0.1 pH unit. Fresh Plant material was dried in an "ANALIS" forced-draught oven of large capacity and stainless steel interior ($\pm$ 1°C). The material was ground to a powder in a "VETTER" mechanical agate ball mill and the powder was dried in a "Gallenkamp" convection oven, with stainless steel interior.

Weighings were done on a "Sartorius" air-damped automatic balance, sensitive to 0.1 mg. Calibrated "Stanton" weights were used.

3.15: LABORATORY FACILITIES.

It is of the utmost importance that the laboratory where trace-element work is to be carried out, should be as dust-free as possible and that special attention should be given to the possible contaminating influence of extraneous factors. All colorimetric work, for example, was carried out in artificial light only. It is of particular importance to pay special attention to the fume-cupboard in which plant digestions are carried out, since this compartment is open to the outside atmosphere.

It was, for example, found that blank digestions carried out on windy days contained appreciable amounts of iron and copper, and special funnel-shaped cover glasses had to be designed to fit the Kjeldahl flasks in order to prevent similar contamination.

These are only a few examples in order to illustrate the exceptional care that must be taken in carrying out work of this nature. Only those workers with experience of trace element work will realise that one cannot be too fussy in precautionary measures; that even under the most stringent control, contamination will still occur from time to time until yet another source has been discovered; and so the process of modification in technique will go on till the end of time.

3.2: THE ASHING OF THE LEAF MATERIAL.

The object of ashing plant material is to remove all traces of organic matter which might otherwise interfere in an analytical determination. It is necessary to exercise considerable care in the process so that none of the inorganic constituents are lost either mechanically or by volatilisation. The two main methods of obtaining plant solutions are, by dry ashing in a muffle furnace at 450-500°C followed by subsequent solution of the ash in HCl and separation of the silica by filtration, or by wet digestion with strong oxidising acids like HNO_3, $HClO_4$ and H_2SO_4.

The disadvantages of the dry-ashing procedure appear to be as follows:- It has been shown conclusively that loss of potassium may occur if the temperature is too high and it is usual to avoid a temperature of over 480°C if all the potassium is to be retained (33). There is also the real danger that the silica residue, after digestion of the ash, will retain some of the elements. Thus it has been found that the following quantities of metals may be entrained by the silica on dry-ashing a 5 g. sample of plant material and extracting the ash with HCl (50):- Up to 60% of the total iron, up to 63% of the aluminium and up to 3% of the manganese. Piper (8) found that in many cases more than one-quarter of the total amounts of Mn, Cu and Zn may be retained by the insoluble residue. It is probable that many of the apparent losses are due to the formation of complex silicates at the high temperature used.

It seems that the wet ashing procedure is preferable to dry-ashing in many ways. Since the oxidation is carried out in solution in an acid medium, the temperature cannot exceed the boiling point of the mixture used; complex insoluble silicates are not formed and all bases are obtained in solution in the excess of acid. When sulphuric or perchloric acids are used the silica is completely dehydrated and left in a form in which adsorption is at a minimum. It has been found that negligible traces of the plant constituents are retained by the silica in this form (8). The oxidation of the material is also more rapid than in the case of dry ashing. Furthermore, the prodedure needs less manipulation and the possible introduction of error is correspondingly smaller.

The only disadvantage of the wet ashing procedure seems to be its costly nature as compared to dry ashing. Large quantities of acids are consumed during routine analysis. Also, many workers dislike perchloric acid because of its potential explosive nature. Piper (8) maintains that perchloric acid should only be used in the presence of H_2SO_4, in which case the possibility of an explosion is practically nil. However, the author found the use of sulphuric acid to be objectionable in many ways. For example, there is no simple method for purifying sulphuric acid; the introduction of

large amounts of sulphate may cause precipitation of calcium, an element that happens to be present in large amount in citrus leaves; sulphate interferes seriously with the removal of phosphate by ion-exchange as carried out by the author (5).

G. Frederick Smith (51) has the following to say about the perchloric acid explosion hysteria:- "Perchloric acid, beyond all reason, is one of the most feared reagents employed in widespread analytical applications. Perchloric acid on the other hand, in all justice to its scientific accomplishments, should be extolled for its remarkable virtue of exceptional stability." He maintains that the concentration of dilute aqueous solutions of perchloric acid by heating, beyond 72.5% strength, cannot result at ordinary atmospheric pressures because of the formation of the perchloric acid-water azeotrope. This fact insures the limitation of acid concentration by heating to concentrations which cannot, justifiably, be characterised as possible hazards.

It was decided, therefore, to use the wet ashing procedure, employing a mixture of nitric acid and perchloric acid only. During two years of constant usage, when several thousand wet digestions were carried out, there has never been a hint of an explosion, and the material was treated quite drastically, particularly during the early stages when the technique was developed and standardised. One important precaution was always taken, however. The fume cupboard, where the digestions were carried out, were periodically washed out completely with plenty of water, paying particular attention to the upper parts of the hood where perchloric acid fumes tend to collect. A case has been reported from overseas where a whole fume cupboard exploded spontaneously after becoming saturated with perchloric acid fumes. It may be that anhydrous perchloric acid was formed, which, of course, explodes spontaneously when in contact with organic material.

In the wet digestion method the greater part of the oxygen required for the oxidation is supplied by the nitric acid. For the most efficient use of this reagent, digestions must be carried out at a low temperature, thus avoiding excessive losses by evaporation in the early stages. Perchloric acid considerably assists the digestion since it appears to break down some of the more resistant organic compounds into simpler compounds, which are then more readily oxidised by the nitric acid. When the reaction of the nitric acid is complete, the high boiling perchloric acid volatilises and displaces the boiling nitric acid. With its elimination the perchloric acid concentrates slowly and progressively to the 72.5% water azeotrope, accompanied by a steady increase in oxidation potential. All but the most difficultly oxidisable organic matter is thus more intensely reacted upon and destroyed. In the presence of perchloric acid much smaller quantities of nitric acid suffice for

the digestion. Perchloric acid also prevents the excessive frothing which occurs when nitric acid alone is used. After thorough experimentation the author found a proportion of 4 HNO_3: 1 $HClO_4$ to be very suitable. This mixture was prepared in bulk from redistilled nitric acid and A.R. 70% $HClO_4$ and stored in a polythene bottle. Periodic blanks showed the mixture to contain negligible traces of impurities.

A great deal of experimentation was necessary before the technique of digestion was perfected to a stage where it could largely be left unattended. When a large amount of leaf powder was to be used, as in the complete analysis, a 300 ml. Kjeldahl flask was found to be most suitable. Translucent silica flasks were used to minimise contamination. One of the most serious difficulties which had to be overcome was the bumping of the mixture during digestion, resulting in loss by spurting. Different methods of heating were tried and the most satisfactory results were obtained from a sandbath. A sandbath rack to hold six flasks was constructed out of $\frac{1}{4}$" iron plate, while six Teclu type gas burners were installed to heat the base of the bath. Permanent hollows were made in the sand so that the flasks could be pushed well down, since it was found that if the sides of the flasks were heated as well, keeping the mixture at a more even temperature, less bumping occurred.

The rate of initial heating is also an important factor influencing bumping. After the initial vigorous reaction has subsided, the heating must be carried out at a very low temperature until most of the material is in solution, when danger of bumping is over. In this connection it was found that gas heating was far superior to electrical heating since the rate of heating could be altered at will to suit the conditions. It was found that no contamination of the samples occurred through the use of gas burners - this was to be expected, since the bath was so constructed as to shield the burners completely.

When danger of bumping was over, the mixture was boiled vigorously until all the nitric acid was boiled off. Thereafter, the perchloric acid mixture was given the full heat of the 6 burners until the solution became quite colourless. The whole digestion procedure could be carried out comfortably in 4-5 hours.

After digestion, the mixture was diluted and filtered through Whatman 40 filter paper, which had previously been washed with hot 1% HCl solution. It was found that sufficient amounts of Ca, Na and K were leached from the filter paper by acid solutions to cause an appreciable deflection on the flame photometer. After pretreatment with acid-washing solution leaching was found to be negligible. HCl was used as wash liquid, because it was found that greater amounts of the elements were leached out by this acid than by $HClO_4$. The

silica on the filter paper was washed 6 times with hot water. This was found by experiment to be sufficient. None of the elements determined could be detected in the silica, after the latter had been volatilised by the usual HF treatment and the residue analysed.

The reproducibility of the digestion procedure was found to be excellent. Proof of this will be found in the statistical tables recorded in Section 4, and the attached reprint on the determination of calcium (5), where the procedure for dealing with small amounts of plant material is also described.

The experimental details of the digestion procedure are described below.

3.21: THE PREPARATION OF THE LEAF SOLUTION.

APPARATUS:

(i) Six, 300 ml. translucent silica Kjeldahl flasks with lips (or well-leached Pyrex flasks).

(ii) Six cover glasses to fit the flasks (shaped like a small funnel with a tapered 4" glass rod fused on the apex).

(iii) Six, 30 ml. micro Kjeldahl flasks with lips (Pyrex).

(iv) A sandbath rack to take 6, 300 ml. Kjeldahl flasks (see above).

REAGENTS:

(i) Nitric Acid:- Distil C.P. HNO_3 in an all-glass Pyrex still, discarding the first and last fractions.

(ii) Perchloric Acid, 70% A.R.

(iii) Digestion Mixture: Mix 800 ml. HNO_3 (i) with 200 ml. $HClO_4$ (ii) and store in a polythene bottle.

(iv) Deionised Water: Store in a polythene aspirator, as deionised water takes up calcium and other ions when stored in Pyrex.

PROCEDURE:- Dry the leaf powder in open flat silica weighing dishes ($\frac{1}{4}$" powder per dish) in an oven at 65°C for exactly 24 hours. When removing the samples from the oven for cooling in a desiccator, stagger the removals so as to cool each sample for 30 minutes before weighing. After cooling for 30 minutes, weigh out accurately about 0.1 g. of the leaf powder into a 30 ml. micro Kjeldahl flask and set aside for the nitrogen determination (3.3). Use a glass weighing tube for this procedure so as to transfer the powder quantitatively to the bottom of the flask, employing a piece of wire hooked on at the end of the tube.

Next weigh out a suitable aliquot of the powder (3 g. for citrus, 2 g. for pineapple leaves) into a 300 ml. Kjeldahl flask. Pour 25 ml.

digestion mixture down the sides of the flask so as to wash all the leaf powder down to the bottom. Swirl to mix and heat on the sandbath rack until frothing starts. Remove the flask and swirl vigorously until the froth subsides, cooling under a tap if necessary. Proceed in this fashion until frothing ceases completely and the mixture starts boiling evenly. Place the cover glass on the flask and turn down the flame of the burner to about 1". Heat gently until most of the material is in solution (2 - 3 hours). If bumping occurs during this stage, the heat must be cut down further. On the other hand, the temperature must be maintained at a reasonable level otherwise the digestion takes too long. After some experience the correct temperature, just short of bumping, will be found.

When most of the material is in solution and danger of bumping is over, rinse the cover glass and remove it completely. Increase the heat to boil the mixture fairly vigorously. When most of the HNO_3 has boiled off the whole flask will suddenly fill with white perchloric acid fumes. Just prior to this stage, there may be a "whoosh" as the nitric acid is driven off rapidly when the boiling point of the mixture suddenly rises towards the end. However, there is no danger of loss. When all the nitric acid is driven off the mixture will stop boiling and the temperature must now be raised to the full heat of the burner. Boil the perchloric acid mixture vigorously for $\frac{1}{2}$ - 1 hour, when it should be perfectly colourless. If not, continue until it is.

Cool, dilute with water and filter through a 9 cm. Whatman No. 40 filter paper which has previously been well washed with boiling 1% HCl (A.R.) solution followed by deionised water. Collect the filtrate in a 100 ml. Pyrex beaker, wash the silica residue very well with hot deionised water, cool and make up to 100 ml. in a volumetric flask. Keep for the determination of P, K, Ca, Mg, Na, Fe, Mn, Zn, and Cu.

NOTES:-

(i) All weighings should be carried out rapidly as the leaf powder is very hygroscopic.

(ii) The sample bottles should be well shaken before removing a subsample for drying.

(iii) Each analysis must be carried out at least in duplicate (separate weighings).

(iv) The lower third of the Kjeldahl flasks should be covered with the sand in the bath.

(v) The complete digestion of 6 leaf samples, from weighing to the final solution, should not take longer than 5 - 6 hours.

(vi) The whole fume-cupboard should be washed down periodically with water in order to prevent accumulation of perchloric acid deposits.

(vii) The cover glass acts as a condenser and saves nitric acid.

3.3. THE DETERMINATION OF NITROGEN.

3.31: EXPERIMENTAL:-

In the procedure described below the total nitrogen in the leaf material, other than nitrate-nitrogen is determined.

The classical Kjeldahl method (52), in which the nitrogen of proteins and amino acids is converted quantitatively by sulphuric acid to ammonium sulphate, has undergone many modifications which have increased both speed and accuracy. In the literature of analytical chemistry there are several hundred papers on the Kjeldahl determination of nitrogen, and the lack of agreement between analysts on the optimum conditions for the determination is significant. One of the common objects of these studies has been the acceleration of the digestion. This has been done in two ways:- By addition of salts to raise the boiling point of the sulphuric acid, and by addition of oxidising agents and catalysts. Some of the more recent work is directly contradictory. Workers such as Jonnard (53) have found that digestion periods of up to 16 hours are needed to obtain maximum yields of nitrogen, whereas Miller et al (54), using only sulphuric acid and hydrogen peroxide, claim complete recovery of nitrogen from even the most refractory amino acids and proteins after about 15 minutes digestion (a claim which the author had already made in 1946 for soils (Part I)). In 1954, McKenzie and Wallace (55) carried out a critical study of digestion conditions, temperature, various catalysts and oxidising agents, and came to the conclusion that mercury is the only satisfactory catalyst for the quantitative recovery of nitrogen from the various organic nitrogen compounds which they used.

In the older methods the liberated ammonia was absorbed in standard hydrochloric acid and titrated with standard caustic soda, thus necessitating the use of two standard solutions. It is now more common to absorb the ammonia in boric acid solution, according to Winkler's method (56), and to titrate the ammonium borate with standard HCl and a mixed indicator.

Because of ease of operation and relatively lower cost of reagents, the micro-Kjeldahl technique has largely replaced the more cumbersome macro-method in modern routine laboratories.

It was decided to investigate the possibility of applying the McKenzie method (55) to plant material in view of its simplicity of manipulation as compared to the perhydrol method developed by the author for soils (Part I).

The method, which is essentially a micro technique, consists in digesting the organic compound (containing 0.3 - 1 mg. N) with a mixutre of 1.5 ml. 36 N H_2SO_4, 1.5 g. K_2SO_4 and 0.5 ml. $HgSO_4$ until 15 minutes after clearing. An excess of NaOH - $Na_2S_2O_3$ solution is added and the ammonia distilled and absorbed in 2% boric acid solution and then titrated with standard potassium biiodate ($KH(IO_3)_2$) using methyl red-methylene blue screened indicator. The investigators found that the high concentration of K_2SO_4 increased the temperature of the mixture sufficiently to allow very rapid digestion. They found that if the temperature of the boiling mixture rose much above 400°C virtually all the nitrogen was lost as a result of pyrolytic decomposition of the ammonium compounds. With the recommended mixture the temperature never exceeded 400°C. They showed that the use of mercury as catalyst gave excellent results, and definitely advised against the use of selenium compounds. The unpleasant features of distillation from mercury digests were avoided by using sodium thiosulphate dissolved with the sodium hydroxide used to make the digest alkaline, precipitating the mercury as black HgS. Success of this mixture was dependent on the ratio of thiosulphate to mercuric oxide, which should be 5 - if it was 3, some red HgO precipitated.

After introduction of a few minor modifications to suit plant material such as the use of 2 ml. concentrated H_2SO_4 instead of 1.5 ml., in order to lower the temperature somewhat, since losses of nitrogen occurred, the author found the method very satisfactory. Standard HCl was used to titrate the ammonium borate, while a 1% boric acid solution was used as recommended by Yuen and Pollard (57). A micro-distillation apparatus with a vacuum jacket (58) was built in the Chemistry Department workshop and found to be very suitable. The digestions were carried out on a Gallenkamp micro-Kjeldahl digestion rack.

The method, as finally modified, was tested on pure aminobenzoic acid (10.22% N). The results are recorded in Table 15.

TABLE 15.

NITROGEN RECOVERY.

NO.	% N	MEAN	THEORETICAL % N	MEAN % RECOVERY
1	10.28			
2	10.27			
3	10.27	10.26%	10.22%	100.4%
4	10.26			
5	10.24			
6	10.25			

The statistical precision and accuracy of the method as applied to citrus and pineapple leaves are recorded in Section 4.

The analytical procedure, as finally modified, is described below.

3.32: EXPERIMENTAL PROCEDURE FOR THE DETERMINATION OF NITROGEN.

APPARATUS:-

(i) A micro-distillation apparatus with vacuum jacket. (see above).

(ii) A micro-digestion rack to take 6 flasks.

(iii) A Grade A 10 ml. micro-burette.

REAGENTS:-

(i) Boric Acid, 1%:- Dissolve 5 g. A.R. boric acid in 500 ml. water. Store in a Pyrex bottle.

(ii) $NaOH - Na_2S_2O_3$ Solution: Dissolve 200 g. C.P. NaOH and 12.5 g. C.P. $Na_2S_2O_3, 5H_2O$ in 500 ml. water. Store in a polythene bottle.

(iii) $HgSO_4$ Solution:- Dissolve 10 g. of red A.R. HgO by warming in 100 ml. water containing 11 ml. conc. A.R. H_2SO_4.

(iv) Standard HCl, 0.05N: Dilute 10.5 ml. conc. A.R. HCl to 2 $\frac{1}{2}$ litres. Keep in a Pyrex aspirator. Standardise against recrystallised borax using the screened indicator (v).

(v) Screened Indicator: Prepare a 0.2% methyl red solution and a 0.2% methylene blue solution each in 95% ethanol. Mix in the ratio of 2 volumes of methyl red to 1 volume of methylene blue. Store in an amber bottle in the dark. Prepare fresh every month.

(vi) K_2SO_4, anhydrous powder, A.R.

(vii) H_2SO_4, A.R. conc.

PROCEDURE:-

To the leaf powder, weighed out into a micro-Kjeldahl flask (Section 3.21), add 1.5 g. K_2SO_4 and swirl to mix. By means of a 5 ml. graduated pipette, add 2.0 ml. conc. A.R. H_2SO_4 and 0.5 ml. $HgSO_4$ solution. Mix well and digest with a medium flame on the micro-digestion rack. If frothing is excessive initially, cut down the micro-jets to a smaller flame. Finally boil vigorously until the mixture is completely colourless without black particles anywhere in the flask. Boil for a further 15 minutes after the colourless stage (in all about $\frac{3}{4}$ - 1 hour).

While the digestion is proceeding, steam out the distillation apparatus for a full 30 minutes.

Cool the digest, add about 5 ml. water and warm to dissolve the cake completely. Transfer by washing through the funnel into the distillation apparatus. Rinse the funnel with a few ml. of water and close the tap. The final volume of liquid should be about 25 ml. Place 10 ml. of 1% boric acid in a 100 ml. conical flask and push up underneath the condenser tube so that the tip of the latter is immersed to about 5 mm. Pour 10 ml. of NaOH mixture (ii) into the funnel, run in slowly, finally rinsing with about 5 ml. water, closing the tap just before all the water has run down. Close all the taps of the apparatus so as to allow steam to bubble through the mixture. With the steam bubbling through at a good rate, distil for 5 minutes, after the splash-head has become steam-hot. Lower the absorption flask about 1 inch and continue distillation for 2 minutes to rinse out the condenser tube. Rinse the outside of the condenser tube, add 4 drops of screened indicator to the absorption flask and titrate against 0.05N HCl to the lilac endpoint, using a 10 ml. micro-burette.

After removing the residual liquid in the distillation flask by reduced pressure and rinsing with some water, the apparatus is ready for the next distillation.

1 ml. N HCl = 14.01 mg. N.
Express the results as % N.

NOTES:

(i) The procedure is rapid. Six samples can be analysed in 2 hours.

(ii) All the tubing used for the connections should be tygon or similar inert plastic tubing.

(iii) The HgS sticking to the inside of the distillation flask may be cleaned out periodically by dismantling the apparatus and pouring in 10 ml. hot aqua regia.

(iv) The Kjeldahl flasks very soon acquire a white deposit of silica. This may be cleaned out with a brush and Teepol.

3.4. THE COLORIMETRIC DETERMINATION OF PHOSPHORUS.

There are numerous phosphorus compounds in fresh plant material, both organic and inorganic, and when the material is ashed all the phosphorus is converted to phosphate. In the dry-ashing procedure, a loss of phosphorus occurs unless magnesium nitrate or acetate is added (33).

Two colorimetric methods have most commonly been used for the determination of phosphorus in various substances, including plant materials:- Denige's method (59), involving the reduction of phosphomolybdic acid by stannous chloride - the so-called molybdenum-blue method; and the molybdivanado phosphoric acid method, originally introduced by Misson (60). Numerous modifications of both methods have been published, attention being largely directed to details of technique suitable for specified experimental conditions. The molybdenum - blue method appears to be the more sensitive of the two, though comparatively susceptible to error.

Both methods were investigated fully.

3.41: THE MOLYBDIVANADO PHOSPHORIC ACID METHOD.

The method depends on the yellow colour formed when excess molybdate and vanadate are added to a weakly acid orthophosphate solution, attributed to a molybdivanado phosphoric acid complex of uncertain composition. The solution shows maximum absorption at 400 mμ (61).

Kitson & Mellon (61) carried out an exhaustive spectrophotometric study of the method, investigating such factors as acidity, temperature, stability and the influence of some 60 diverse ions on the reaction. They found the colour to be stable for more than 7 weeks, while greatest interference occurred from chloride, fluoride, arsenate, bismuth and thorium. In 1948 Barton (62) simplified the method by the introduction of a mixed colour reagent. Accurate results were reported. In 1953 Bridger et al (63) reported the use of a calibration curve which remained constant for 2 years. In a statistical comparison of the method with the standard volumetric method they claimed that the former is no less precise then the volumetric method, while it only takes 1/8th the time to complete. Recent workers to study the method were Baghurst et al (64) and Quinlan et al (65), in 1955. The former showed the method to be very sensitive to changes in room temperature and suggested that it should be carried out at $25 \pm 1^{\circ}C$. The latter showed the method to be accurate provided the optimum concentrations of acid, vanadium and molybdenum were used. The optimum range of the method was found to be 3 - 20 p.p.m. P_2O_5.

Most of these workers used the method for determination of phosphorus in steel and phosphate rock.

Incorporating most of the modifications suggested in these papers, the author tested the method on standard phosphate solutions containing various concentrations of the ions normally present in a plant solution. No interferences were found with the exception of chloride. It was found that if more than 100 p.p.m. Cl^- were present in the test solution, the recovery of phosphorus was incomplete, reaching an error of 15% in the presence of 1000 p.p.m. Cl^-. However, this source of error was not serious, since all the chloride was volatilised by the digestion technique as used by the author. It was found that the method was not seriously influenced by normal variations in room temperature.

3.42: THE PHOSPHOMOLYBDENUM - BLUE METHOD.

The method depends essentially on the formation of phosphomolybdic acid which is subsequently reduced to the blue molybdenum complex. The most commonly used reducing agent is stannous chloride.

The elements which show greatest interference are Si, As and Fe, but it has been found that by carefully controlling the concentrations of acid, molybdate and reducing agents, the interferences can be minimised without sacrificing the sensitivity of the method. The method is very sensitive, the optimum range of P being 5 - 20 micrograms. The blue complex shows maximum absorption at 700 mμ.

So many modifications in procedure have appeared in the literature that it would be impossible to review these here. The most useful of the modifications appeared to be one published in 1955 by Yuen and Pollard (66), working on phosphorus in soil extracts. These workers carried out an exhaustive investigation of interferences, the influence of acid concentration, of ammonium molybdate and of stannous chloride on the formation of the phosphomolybdenum - blue complex. They introduced a modified method and found that the various ions commonly occurring in soil solutions and extracts did not interfere. Arsenate, however, must be absent, but ferric iron in amounts up to 15 p.p.m. had no ill effect.

Their findings regarding the conditions of the procedure for accurate results may be summarised as follows:-

Acidity:- The acidity of the final test solution should be strictly controlled to within limits of 0.008 N either way.

Ammonium molybdate:- The amount of molybdate added should be controlled to within $\pm$ 0.02 ml.

Temperature:- The temperature should lie between 20 - 30°C with preference given to 25 - 26°C.

Stability of the Colour:- A maximal reading was obtained at 15 minutes after development and the colour was relatively stable for a further 10 minutes.

Despite these limitations, the author found the method to be excellent when applied to plant solutions. By using a carefully standardised technique during the mixing of the reagents, the method was found to be very precise, accurate, rapid and inexpensive. After testing all the factors described above, the procedure was adopted as described in the reference, with only a few minor modifications to suit local conditions as applied to plant solutions.

3.43: COMPARISON OF THE TWO PHOTOMETRIC METHODS.

The phosphomolybdenum - blue method, as described in Section 3.44, was compared with a modified molybivanado phosphoric acid method, employing a citrus and pineapple leaf sample. The results are recorded in Table 16 and the working curves for the two methods are shown in Figs. IX & X.

TABLE 16.

COMPARISON OF TWO PHOTOMETRIC METHODS FOR PHOSPHORUS.

LEAF SAMPLE		MOLYBDENUM - BLUE		MOLYBDIVANADO	
		% P	Mean % P	% P	Mean % P
CITRUS	1	0.120		0.119	
	2	0.121		0.120	
	3	0.120		0.122	
	4	0.122	0.120	0.121	0.120
	5	0.120		0.120	
	6	0.120		0.121	
PINEAPPLE	1	0.164		0.163	
	2	0.165		0.164	
	3	0.167	0.165	0.162	0.164
	4	0.164		0.167	
	5	0.166		0.165	
	6	0.167		0.166	

From the Table it can be seen that there were virtually no differences in the results. Both methods yielded precise and accurate results; they are equally rapid; the vanado method has the advantage of producing a more stable coloured complex, but on the other hand it is less sensitive and more expensive than the molybdenum - blue method. It was eventually decided to adopt the latter method - being more sensitive, a much smaller aliquot of the

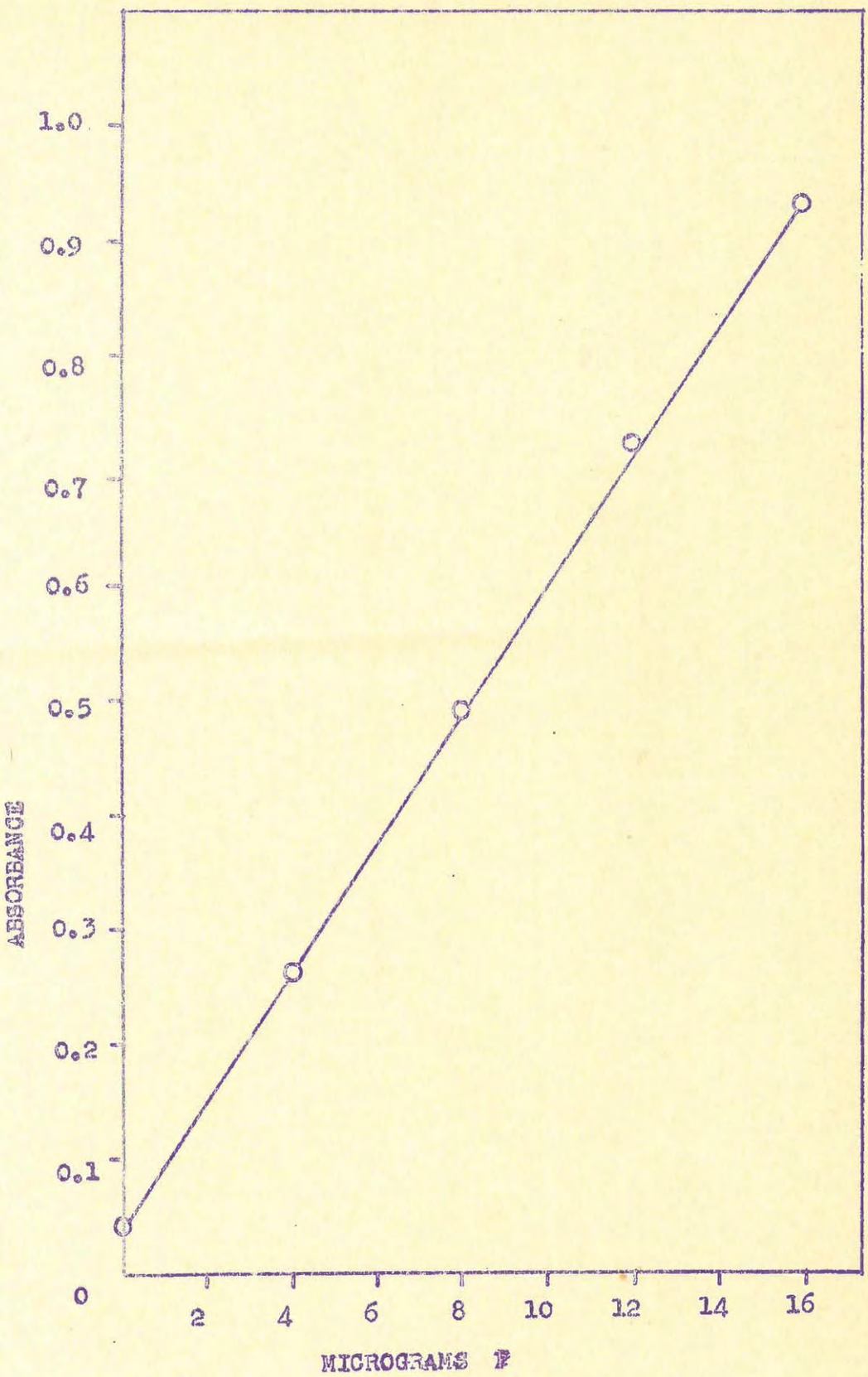


Fig. X

Working Curve: Molybdenum-blue Method.

plant solution is needed, leaving more for the other determinations. The statistical accuracy and precision of the method are recorded in Section 4, while the procedure is described below.

While this work was being written up (1957), Michelson (67) published a new modification of the molybdivanado method. It is claimed that by working at 315 mμ instead of 400 mμ, and by using more dilute reagents, accurate phosphorus determinations could be made at as low concentrations as 0.1 micrograms P per ml. Under these conditions, the molybdivanado method may very well be used in preference to the molybdenum blue method, because of the greater stability of the colour reaction.

3.44: EXPERIMENTAL PROCEDURE FOR THE DETERMINATION OF PHOSPHORUS.

REAGENTS:-

(i) Phosphorus Standard:- Dissolve 0.2197 g. "specpure" KH_2PO_4 in 1000 ml. water (1 ml. = 50 μg P). Store in polythene bottle. Dilute 40.00 ml. of this solution to 1000 ml. for use in preparing a calibration curve (1 ml. = 2 μg P). Store in a polythene bottle.

(ii) Ammonium Molybdate, 1.25%:- Carefully pour 105 ml. A.R. H_2SO_4 into 395 ml. water with stirring. Add 6.25 g. A.R. ammonium molybdate and stir to dissolve. Cool and store in a Pyrex bottle.

(iii) Stannous Chloride, 0.5%:- Dissolve 0.5 g. A.R. $SnCl_2$, $2H_2O$ in 2.5 ml. A.R. conc. HCl by warming, and dilute to 100 ml. This solution keeps for 6 hours.

Alternatively, a stock solution of stannous chloride may be prepared by dissolving 25 g. stannous chloride in 125 ml. conc. HCl and diluting to 500 ml. This solution is stored in a Pyrex bottle with a layer of liquid paraffin, 1½ cm. thick, on top, to protect it from the atmosphere. This solution will keep for several months. The working solution is prepared by diluting 5 ml. to 50 ml. just before use.

PROCEDURE:-

(a) Calibration Curve: Using a micro-burette, transfer 0, 2, 4, 6, 8 and 10 ml. of the diluted standard to 50 ml. volumetric flasks, dilute to about 45 ml. with water, add 2.0 ml. molybdate reagent (graduated 5 ml. pipette), and shake to mix. Then add 1.0 ml. stannous chloride (graduated pipette), make to the mark, shake well and leave to stand for 15 minutes to allow the blue colour to develop fully. Measure the absorbance at 700 mμ. The solutions

prepared as above contain 0, 4, 8, 12, 16 and 20 µg. P respectively.

(b) Plant Solution:- The plant solutions from both citrus and pineapple leaves, as prepared above, must be diluted before the phosphorus can be determined. Transfer 1.00 ml. of the plant solution (1 ml. micro pipette) to a 50 ml. volumetric flask, make to the mark, mix well and, using a 10.0 ml. aliquot, carry out the procedure as described in (a).

Express the results as % P.

NOTES:

(i) All volume additions must be measured accurately.

(ii) The colour reaches maximum intensity after 15 minutes and remains stable for another 15 minutes. It is best to stagger the additions of stannous chloride so as to measure each sample after about 15 minutes.

(iii) If room temperature fluctuates widely, it is best to leave the volumetric flasks in a bath of water at 25°C before adding the stannous chloride.

(iv) The original plant solutions are about 0.5 N with respect to $HClO_4$. As diluted above the final solution is only 0.002 N with respect to $HClO_4$ and has little effect on the total acidity of the solution.

(v) A new calibration curve must be prepared with every fresh batch of reagents.

3.5: THE DETERMINATION OF CALCIUM, MAGNESIUM POTASSIUM AND SODIUM.

Because of ease and simplicity of operation, potassium and sodium are most commonly determined by flame photometric procedures, while calcium and magnesium are determined complexometrically by titration with ethylene diamine tetra acetic acid (EDTA), or by flame photometry, or by colorimetric methods.

3.51: CALCIUM AND MAGNESIUM.

After an intensive investigation of the EDTA and flame - photometric methods, a modified procedure was developed involving the removal of interfering ions by ion-exchange. A summary of this work was submitted for publication in 1955 and is described in the attached reprint (5).

Only a few of the more important points that have been introduced since publication will be mentioned here. The method, as finally modified, is fully described in Section 3.53.

ANION EXCHANGE:- A side effect, which proved to be of great importance was discovered during the removal of interfering anions by ion exchange. It was found that all leachates contained virtually no iron or manganese, which, of course, was one of the reasons why the EDTA titrations were so reproducible since these two elements happen to interfere greatly, as is shown in the reprint (5). It was later found that iron and manganese were rendered insoluble when the perchloric acid digest was evaporated to dryness, the perchlorates probably being decomposed to the oxides, which were largely insoluble in the dilute HCl used to take up the residue. It was also found, by carrying out numerous tests, that if the residue was taken up in water, quantitative recoveries of Ca, Mg, Na and K were obtained. The solutions invariably had a pH of 2.5 - 3.0 and it was found that the addition of ammonium carbonate in order to raise the pH could safely be omitted. The final leachates had a pH of about 3.

This work further proved that on no account should perchloric acid solutions intended for iron and manganese determinations, be evaporated to dryness.

EDTA TITRATION OF CALCIUM:- Considerable difficulty was experienced in obtaining reproducible results when determining calcium in pineapple leaf solutions by flame photometer. This could mainly be ascribed to the small concentration of calcium in the solution as well as, partly, to the very high concentration of potassium which appeared to influence the emission of calcium. More work was carried out on the EDTA titration method for calcium, using a screened murexide indicator. Various blue, yellow and green dyes were tried

out, but the most successful screening results were obtained with Naphthol Green B. The screening quantities and the modified procedure are described in Section 3.53.

The method was found to be more accurate than determining Ca on the flame-photometer and was used exclusively for Ca in pineapple leaf material. The EDTA method for Ca also gave greater reproducibility in the magnesium values.

The statistical accuracy and precision of the procedures in their final forms, are recorded in Section 4.

3.52: POTASSIUM AND SODIUM.

Various workers have reported the interference of several ions in the flame photometric determination of potassium and sodium. Collins and Polkinhorne (68) carried out a thorough investigation of the interference from common anions in the determination of potassium and sodium with an "Eel" flame photometer. They reported the following limiting concentrations for zero interference in the determination of 10 p.p.m. Na and 10 p.p.m. K respectively:-
NO_3^- (40,000 & 100,000 p.p.m.); $SO_4^=$ (18,000 & 56,000 p.p.m.);
Cl^- (1400 & 1200 p.p.m.); Br^- (1000 & 2100 p.p.m.);
$PO_4^{\equiv}$ (600 & 400 p.p.m.).
An excess of these ions resulted in a suppression of emission of the element to be determined.

From these figures it was clear that none of the anions would interfere in the determination of potassium and sodium in citrus and pineapple leaves, as carried out by the author. Phosphate, sulphate, perchlorate and nitrate ions were removed by the resin, while equivalent amounts of chloride passed into the test solution. It may be reasoned that this procedure would push up the chloride concentration to a dangerous level. However, it was found that the final leachates never had a chloride concentration higher than 200 p.p.m. Moreover, the flame - photometric standards contained approximately the same chloride concentration as the test solutions.

Schrenk and Glendening (69), in studying the interference of Ca, Mg and Na on potassium determinations, reported that Na and Ca had little effect on the intensity of emission of K but that Mg had a depressing effect. In the determination of Na, K had no effect on the emission of Na, but Ca enhanced the reading for sodium while Mg caused a depressing effect.

A similar study was carried out on the "Eel." flame photometer, using standard solutions containing the various ions in concentrations

approximating their occurence in citrus and pineapple leaves. No significant interferences with the K or Na emission were found. In any case, by incorporating the various ions in the flame photometer standards as described below, the effect of interference by any ion would be largely nullified.

The statistical accuracy and precision of the methods are recorded in Section 4, while the procedure for the determination of Ca, Mg, K and Na is described below.

3.53: EXPERIMENTAL PROCEDURE FOR THE DETERMINATION OF CALCIUM, MAGNESIUM, POTASSIUM AND SODIUM.

Potassium and sodium are determined by flame photometer, calcium by flame photometer or EDTA titration and magnesium by EDTA titration, after phosphate, sulphate and perchlorate ions have been removed by ion exchange.

ION EXCHANGE COLUMN:-

A Pyrex glass column, 15 cms long and 2 cms internal diameter, with a reservoir on top, and a glass tap with 2 mm. delivery bore, has been found to operate very satisfactory. Before use, the resin is regenerated as follows:- About 50 g. of De-Acidite E resin (Hopkins & Williams) are shaken with 100 ml. 5% sodium carbonate in a conical flask, allowed to stand for $\frac{1}{2}$ hour and the liquid decanted. This treatment is repeated with two more portions of sodium carbonate. The resin is then washed free of alkali by decantation and treated as above with 4, 100 ml. portions of 5% HCl, leaving it to stand for 2 hours in contact with the last portion. The resin is finally washed with water by decantation until the supernatent liquid is free from any yellow colouration: It is now in the chloride form and ready for use.

The resin is packed into the column as follows:- A small glass wool plug is pushed to the bottom by means of a long glass rod. A slurry of the resin is poured into the column and tamped down into a firm bed with the glass rod. The resin bed should be about 5 cms. long. Another plug of glass wool is pushed down on top of this. The resin is washed with 200 ml. water and with the tap fully opened. The level of the liquid should never be allowed to drop below the top glass wool plug in order to prevent air bubbles from entering the resin bed. The column is now ready for use. Tests have shown that about 30-50 samples can be leached before the resin is exhausted, provided the procedure described below, is followed. However, the efficiency of the ion-exchange should be tested from time to time by passing through a solution containing 0.1 mg. P and testing the leachate by the molybdenum - blue method

(Section 3.4). After exhaustion, the resin is regenerated as above.

It was found that a battery of 6 columns is about the maximum number that can be handled conveniently by one operator.

REAGENTS:-

(i) FLAME PHOTOMETER STANDARD (CITRUS):- For accurate work, the flame-photometric standard solution should be prepared so as to contain the main elements in approximately the same proportion as they occur in the material to be analysed.

Prepare the following stock solutions from "specpure" chemicals and store in polythene bottles:-

(a) 1.248 g. $CaCO_3$ + 3 ml. 1 : 1 A.R. HCl diluted to 1000 ml. (1 ml. = 0.5 mg.Ca.).

(b) 2.535 g. $MgSO_4$, $7H_2O$ diluted to 1000 ml. (1 ml = 0.25 mg. Mg.)

(c) 1.907 g. KCl diluted to 1000 ml. (1 ml = 1 mg. K.).

(d) 1.271 g. NaCl diluted to 1000 ml. (1 ml = 0.5 mg. Na).

Pipette accurately 100 ml. of (a), 20 ml. of (b), 10 ml. of (c) and 10 ml. of (d) into a 1000 ml. volumetric flask, make to the mark and mix well. Pass through the ion-exchange column at a rate of 2 drops per second, discard the first 100 ml. of leachate, and collect the remainder in a clean, dry polythene bottle. This standard contains 50 p.p.m. Ca, 5p.p.m. Na, 10p.p.m. K, 5p.p.m. Mg with chloride the only anion present. It is used to determine Ca, K and Na in the citrus leaf solution.

This solution corresponds to a citrus leaf sample containing 5% Ca; 0.5% Na; 1% K; 0.5% Mg.

(ii) FLAME PHOTOMETER STANDARD (PINEAPPLE):- Into a 1000 ml. volumetric flask pipette accurately 2 ml. of (a), 4 ml. of (b) and 10 ml. of (c). Make up to the mark, mix well and pass through an ion-exchange column as described in (i). This solution contains 10 p.p.m. K and is used for determining K in pineapple leaves. The solution corresponds to a pineapple leaf sample containing 0.4% Ca; 0.4% Mg; 4% K.

(iii) EDTA SOLUTION, 0.01 N:- Dissolve 4 g. of the disodium salt of ethylene diamine tetraacetic acid in 1000 ml. water. Store in a Pyrex aspirator. Standardise against Ca standard (a) and Mg standard (b).

(iv) BUFFER SOLUTION, pH 10:- Dissolve 33.7 g. A.R. NH_4Cl in about 100 ml. water mixed with 285 ml. conc. A.R. NH_4OH. Dilute to 500 ml. and store in a polythene bottle.

(v) SODIUM HYDROXIDE, 10%:- Dissolve 50 g. A.R. NaOH in 500 ml. water and keep in a polythene bottle.

(vi) ERIOCHROME INDICATOR:- Grind 0.5 g. Eriochrome Black T and 100 g. A.R. NaCl to an intimate mixture. Store in a black-painted bottle.

(vii) SCREENED MUREXIDE INDICATOR:- Grind 0.5 g. ammonium purpurate + 3.5 g. Naphthol Green B (B.D.H.) + 60 g. powdered A.R. K_2SO_4 to an intimate mixture. Store in a black-painted bottle.

PROCEDURE:-

(a) CITRUS:-

Using a 2 ml. micro-pipette, transfer 2.00 ml. of the leaf solution to a 50 ml. Pyrex beaker and evaporate completely to dryness on a hotplate. Add 10 ml. water and simmer for 2-3 minutes to dissolve the residue. Cool and transfer to the ion-exchange column by washing. Leach into a 100 ml. volumetric flask at the rate of 2 drops per second. Wash the resin with small amounts of water until the flask is filled to the mark, waiting each time for the level of the wash liquid to reach the glass wool plug before adding the next portion. When the flask is $\frac{3}{4}$ full, the tap of the column can be opened fully.

Mix the leachate well by shaking, calibrate the flame-photometer with the citrus standard (i) and determine Ca, K and Na in the leachate in the usual way.

For the determination of magnesium, pipette 50 ml. of the leachate into a titration flask, dilute to 100 ml., add 5 ml. of the ammonia buffer (iv), a small portion of the eriochrome indicator, swirl to dissolve and titrate against EDTA to a permanent blue (without a trace of violet), using a 10 ml. micro-burette.

Express the results as % Ca, Mg, Na & K.

The EDTA titration gives Ca + Mg and Mg is found by difference.

1 ml. N EDTA = 24.32 mg. Mg. = 40.08 mg. Ca.

(b) PINEAPPLES:-

Evaporate and leach a 20.00 ml. aliquot of the leaf solution as described for citrus (a).

For the determination of K, dilute 5.00 ml. of the leachate to 100 ml. and determine on the flame photometer, using the standard (ii) .

For the determination of Ca, use a 50.0 ml aliquot of the leachate, dilute to 100 ml., add 5 ml. 10% NaOH, a small portion of the screened murexide, swirl to dissolve and titrate against EDTA to a permanent dark blue (without a trace of violet), using a micro-burette.

For the determination of Mg, use a 25 ml. aliquot of the leachate and proceed as in (a).

NOTES:-

(i) Once the resin columns have been prepared, the method is very rapid and 6 plant solutions can be analysed in $1\frac{1}{2}$ to 2 hours.

(ii) If preferred, the calcium in citrus may be determined against EDTA, using a 25.0 ml. aliquot of the leachate.

(iii) In the absence of all anions except chloride, and with the removal of Fe and Mn by the evaporation treatment, the EDTA endpoints are extremely sensitive and accurate. The same applies to the flame photometer readings.

(iv) Despite the manipulations, the method has been proved to be accurate, precise and rapid.

(v) It was found unnecessary to add the trace elements to the flame photometer standards.

3.6: THE COLORIMETRIC DETERMINATION OF IRON.

3.6: EXPERIMENTAL:-

Iron occurs in plants in widely varying amounts, though it appears as if some of the high values given in the literature may be due to soil or other contamination. The author usually found much lower concentrations of iron in citrus and pineapple leaves than reported from elsewhere, though in no instance were the characteristic chlorotic symptoms ascribed to iron deficiency noticed. Iron, of course, is the most common contaminating substance in trace element work, and it is always a difficult problem to keep it from being introduced during the preparative stages, during the ashing of the material and from the water and reagents used.

There is no lack of colorimetric reagents for iron, but comparatively few are well suited for the determination of traces of iron. Some reagents, like thiocyanate, react with ferric iron and this reaction is still extensively used for the colorimetric determination of iron. Its use, possibilities and shortcomings are reviewed by Woods and Mellon (70) and Sandell (50). Its chief disadvantages, besides its low sensitivity are that the colour fades rapidly and that pyrophosphate interferes.

Reagents which react with ferrous iron have found great favour amongst workers. Of these, O-phenanthroline and the similar alpha alpha'- bipyridyl are used extensively. The former is said to be slightly more sensitive. It was decided to investigate the O-phenanthroline method fully.

The method is based on the formation of an orange-red complex, $(C_{12}\ H_8\ N_2)_3\ Fe^{++}$, between O-phenanthroline and ferrous iron. The colour intensity is independent of the acidity in the pH range 2-9; below pH 2 the colour develops slowly and is much weaker. The complex is very stable and solutions show no change in colour after many months. Beer's law is closely followed. Ferric iron may be reduced to ferrous with hydroxylamine hydrochloride or hydroquinone. A great advantage in the use of O-phenanthroline lies in the possibility of using it in slightly acid solutions, so that the hydroxides, phosphates, etc. of many metals are not precipitated. The sensitivity of the colour reaction is 0.005 $\mu g.\ Fe/cm^2$ at 508 mμ (70).

Fortune and Mellon (71) carried out a spectrophotometric study of the method, including the study of various reducing agents, the pH range, the stability of the colour reaction and the influence of some 55 diverse ions. They reported the following maximum concentrations to be permissible for some of the ions in the presence

of 2 p.p.m. Fe:- Zn (10 p.p.m.); Ni (2 p.p.m.); Co (10 p.p.m.); P_2O_5 (20 p.p.m.). Chloride, sulphate and most other anions were without effect. If perchlorate was present in more than small amounts, a precipitate of the slightly soluble O-phenanthroline perchlorate may be produced. They found hydroxylamine hydrochloride to be the best reducing agent, 0.1 ml. of a 10% solution reducing 1 p.p.m. ferric iron quantitatively to ferrous iron.

Reitz et al (72) carried out a statistical comparison of the O-phenanthroline, the thiocyanate and the sulphide colorimetric methods and found the former to be the most accurate, reliable and reproducible.

Since the work carried out by Fortune and Mellon in 1938, no important modification has appeared in the literature, which speaks volumes for the generally satisfactory nature of this sensitive method.

However, in applying the method to plant material, it was necessary to investigate possible interference from foreign ions. More than 0.3% P was found in many pineapple leaf samples, giving P/Fe ratios of between 5 and 30 in the test solutions. Fortune and Mellon (71) reported interference from phosphate when the P/Fe ratio was greater than about 4. Even in citrus leaves, this ratio was always exceeded. Because the test solutions contained perchloric acid, it was also necessary to investigate the possible influence of perchlorate ions on the colour reaction.

The method, as described in Section 3.62, was tried out on standard solutions containing 20 µg. Fe plus the various ions in the concentrations normally present in citrus and pineapple leaves. To each test solution 0.25 ml. $HClO_4$ plus different amounts of a standard phosphate solution were added.

The results are recorded in Table 17.

TABLE 17.

INFLUENCE OF PHOSPHATE AND PERCHLORATE ON IRON DETERMINATION.

P/Fe Ratio	Fe Present µg.	Fe Found µg.	% Error
15	20	19.9	0.5
25	20	19.8	1.0
30	20	19.8	1.0
45	20.	19.0	5.0

The figures above correspond to 100 p.p.m. Fe in plant material containing 0.15, 0.25, 0.30 and 0.45% P.

These tests were repeated several times with the same end results. It was clear from these figures that only if the P/Fe ratio in the plant solution is greater than 30 will the phosphorus interfere with the colour reaction. The perchloric acid, when neutralised to pH 3 with sodium acetate, apparently had no effect. Should the P/Fe ratio be greater than 30, the phosphorus can be removed satisfactorily by neutralising the test solution to pH3 with a 10% solution of pure ammonium carbonate and passing it through the ion exchange column used for the calcium determination. Quantitative recovery of the iron was found when standard solutions were passed through the column. However, this procedure would seldom be necessary.

A very important point as regards colorimetric measurements, often neglected by workers, is the effect of the foreign ions in the test solution on the absorption spectrum of the coloured ion. The absorbance of these colourless ions is often slight, but when very dilute solutions in the microgram range are used, the error so introduced may be appreciable. When preparing a standard calibration curve, therefore, it is essential to add the various foreign ions in more or less the same concentrations as they have in the test solution, to the standard solutions. The medium of the test solution, in this case perchloric acid, must also be added to the standards. The author adopted this procedure throughout this work. The effect of the foreign ions can be seen from the working curves. (Figs. IX-XV), i.e. the difference between zero absorbance and the absorbance given by the blank standard. In the case of zinc, however, the big difference is due to the fact that the blank itself is coloured green by the dithizone reagent. By adopting this procedure interferences which may otherwise have been overlooked, were suppressed.

The working curve for iron is shown in Fig. XI. It will be noticed that much smaller concentrations were used than are normally recommended.

The statistical precision and accuracy of the method are recorded in Section 4, while the experimental procedure is described below.

3.62: EXPERIMENTAL PROCEDURE FOR THE DETERMINATION OF IRON.

REAGENTS:-

(i) Iron Standard:- Dissolve 0.1431 g. "specpure" Fe_2O_3 in 5 ml. constant boilingpoint HCl (Section 3.8), by warming in a small covered beaker on a hotplate. Dilute to 1000 ml.

and store in a polythene bottle (1 ml. = 100 µg. Fe.). Dilute 50 ml. of this solution to 1000 ml. and store in a polythene bottle (1 ml. = 5 µg. Fe.).

(ii) Hydroxylamine Hydrochloride, 10%: Dissolve 10 g. of the A.R. salt in 100 ml. water and store in a pyrex bottle.

(iii) Sodium Acetate, 1M: Dissolve 136 g. A.R. $Na\ C_2\ H_3\ O_2\ .\ 3H_2O$ in 1000 ml. of water. Add 2 drops formalin as preservative and store in Polythene.

(iv) O-Phenanthroline, 0.1%: Dissolve 0.25 g. of the A.R. salt in warm water ($\pm$ 80°C) and dilute to 250 ml. Store in a Pyrex bottle.

(v) Colorimetric Standard (Citrus): Weigh out the following "specpure" chemicals into a 680 ml. beaker:- 18.72 g. $CaCO_3$ + 7.60 g. $MgSO_4,\ 7H_2O$ + 1.27 g. NaCl + 0.95 g. KCl + 1.318 g. KH_2PO_4. Carefully add a solution containing 25 ml. $HClO_4$ in 100 ml. water, swirl to dissolve and evaporate to copious white fumes on a hotplate in order to volatilise the chloride. Cool, carefully add a solution containing 225 ml. $HClO_4$ in 200 ml. water, cool and finally make up to 1000 ml. in a volumetric flask. Store in a polythene bottle.
This solution contains 7.5 mg. Ca, 0.75 mg. Mg, 0.5 mg. Na, 0.9 mg. K, 0.3 mg. P and 0.25 ml. $HClO_4$ per ml.
It is used in preparing citrus calibration curves for Fe, Mn, Zn and Cu.

(vi) Colorimetric Standard (Pineapple): Weigh out the following "specpure" chemicals and carry on as described in (v) above:- 0.998 g. $CaCO_3$ + 4.06 g. $MgSO_4,\ 7H_2O$ + 7.25 g. KCl + 0.879 g. KH_2PO_4. This solution contains 0.4 mg. Ca, 0.4 mg. Mg, 4 mg. K, 0.2 mg. P and 0.25 ml. $HClO_4$ per ml.

PROCEDURE:-

(a) Calibration Curve (Citrus):- Into 50 ml. volumetric flasks burette (micro) 0, 0.5, 1.0, 2.0, 3.0 and 5.0 ml. of the dilute iron standard (1 ml. = 5 µg. Fe). Into each of the flasks pipette 1.0 ml. of citrus colorimetric standard (v), 0.3 ml. of Mn standard (Section 3.7), 0.6 ml. of dilute Zn standard (3.8) and 0.1 ml. of dilute Cu standard (3.9). Add 2.5 ml. of sodium acetate solution, to bring the pH to 3, 1 ml. 10% hydroxylamine hydrochloride and shake to reduce the ferric iron to the ferrous state. Dilute with water to about 40 ml., add 5 ml. 0.1% O-phenanthroline, make to the mark, shake well to mix and leave to stand for 30 minutes. Measure the absorbance at 510 mµ. The solutions, as prepared above, contain 0, 2.5, 5.0, 10.0, 15.0 and 25 µg. Fe respectively.

(b) Calibration Curve (Pineapple):- Proceed as above but add 1.0 ml. pineapple standard (vi), 0.6 ml. Mn standard, 0.8 ml. Zn standard and 0.2 ml. Cu standard to the iron standards.

(c) Plant Solution:- Into a 50 ml. volumetric flask pipette 5.0 ml. of leaf solution (citrus and pineapple), add 2.5 ml. sodium acetate and develop and measure the colour as described in (a).

Express the results as p.p.m. Fe.

NOTES:

(i) The colour is very stable and does not change over a period of 6 months.

(ii) All volume additions must be made by means of accurately graduated delivery pipettes.

(iii) A new calibration curve must be prepared when using a fresh batch of reagents.

3.7: THE COLORIMETRIC DETERMINATION OF MANGANESE.

Although other methods have been suggested from time to time, the colorimetric determination of manganese by oxidation to permanganate in acid solution is so sensitive and specific that it leaves little to be desired. A number of reagents such as lead dioxide, sodium bismuthate and persulphate (with silver as catalyst) have been used to oxidise the manganous ion to permanganate, but by far the best reagnet for the reaction is potassium or sodium periodate, suggested in 1917 by Willard and Greathouse (73).

The reaction

$$2Mn^{++} + 5\ IO_4^- + 3H_2O \rightarrow 2MnO_4^- + 5IO_3^- + 6H^+$$

proceeds rapidly in a hot acid solution, such as HNO_3, H_2SO_4 or $HClO_4$. Phosphoric acid should be present in order to decolourise ferric ions by complex formation as well as to prevent possible precipitation of periodates or iodates of manganese. If the manganese content is very small (as in the case of plant samples) the acidity of the solution should not be greater than 2N (74).

Reducing substances reacting with periodate or permanganate must be destroyed. Chloride should preferably be removed. These requirements are adequately met by the HNO_3 - $HClO_4$ oxidation as carried out by the author. It has been found that anions such as arsenate, borate, fluoride, perchlorate and pyrophosphate do not interfere (50). Most foreign metals, unless coloured, do not interfere. By adding copper to the calibration standards, manganese can be determined in copper alloys containing 50% or more Cu (50). Beer's law holds closely for concentrations of manganese up to 150 mg./litre at least, while the coloured solution shows maximum absorption at 540 mμ (50).

In 1953, Cooper (75) carried out an intensive investigation of the periodate method for manganese in steel. Using a Beckman Du spectrophotometer at 540 mμ, he found a precision of 0.2 - 0.4% for the method.

When the author investigated this method, it was soon apparent that, after introducing a few minor modifications to suit plant material, such as acid concentration and time of development of the colour (described below) the method was accurate, precise and specific.

Fig. XII shows the working curve for the Mn.

The statistical accuracy and precision of the method are recorded in Section 4, while the experimental procedure is described below.

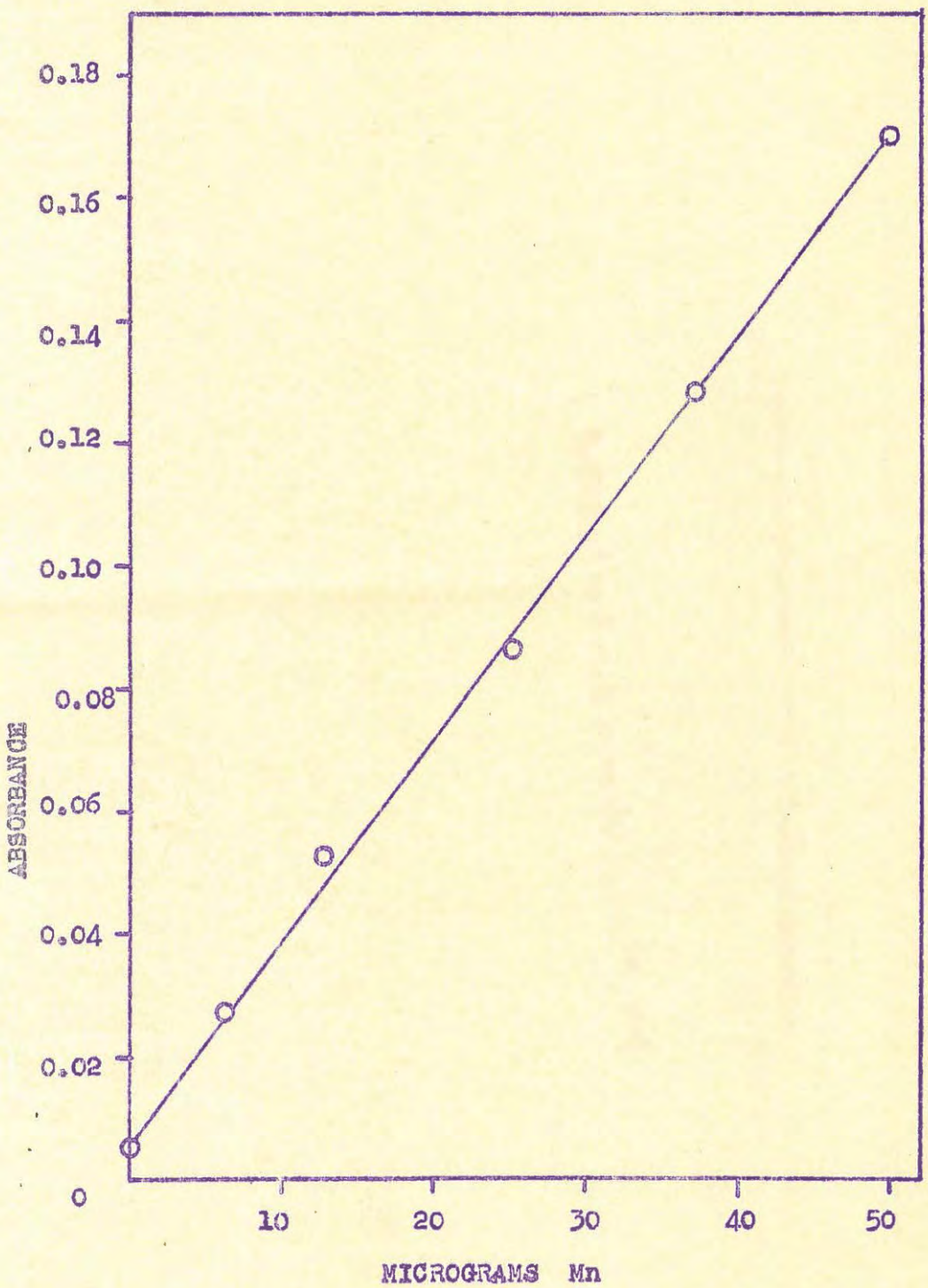


Fig. XII.

Working Curve for Manganese (Citrus).

In the presence of: 37 mg.Ca, 3.7 mg.Mg, 4 mg.K, 2.5 mg.Na, 75 µg Al, 75 µg.Fe, 7 µg.Cu, 15 µg.Zn, 2.5 mg.P, 1.25 ml. $HClO_4$.

3.71: EXPERIMENTAL PROCEDURE FOR THE DETERMINATION OF MANGANESE.

REAGENTS:-

(i) Manganese Standard:- Dissolve 0.1261 g. of "specpure" $MnSO_4$, $7H_2O$ in 1000 ml. water. Store in a polythene bottle. (1 ml. = 25 µg. Mn).

(ii) Phosphoric Acid, A.R. 85%.

(iii) Potassium Periodate, A.R.

PROCEDURE:-

(a) Calibration Curve (Citrus):- Into 50 ml. Pyrex beakers burette (micro) 0, 0.25, 0.50, 1.00, 1.50 and 2.00 ml. of the manganese standard, and to each beaker add:- 5 ml. citrus colorimetric standard ((v) Section 3.6), 15 ml. of dilute Fe standard (Section 3.6), 3 ml. of dilute Zn standard (Section 3.8) and 0.5 ml. of dilute Cu standard (3.9). Dilute to $\pm$ 25 ml. with water, add 0.3 g. potassium periodate, 2 ml. phosphoric acid, cover with a watch glass and boil gently for 30 minutes on the hotplate. Cool, transfer to 50 ml. volumetric flasks, make to mark, mix well and measure the absorbance at 525 mµ. The solutions as prepared above, contain 0, 6.25, 12.50, 25.00, 37.50 and 50 µg Mn respectively.

(b) Calibration Curve (Pineapple):- Proceed as above but add 2 ml. pineapple standard (vi), 6 ml. iron standard, 0.3 ml. Cu standard and 1.5 ml. zinc standard, to the manganese standards.

(c) Plant Solution:- Into 50 ml. Pyrex beakers pipette a suitable aliquot of the leaf solution (25.0 ml. for citrus and 10.0 ml for pineapples) and develop and measure the colour as described in (a).

Express the results as p.p.m. Mn.

NOTES:-

(i) The colour is stable.

(ii) All volume additions must be made accurately.

3.8: THE COLORIMETRIC DETERMINATION OF ZINC.

A modified dithizone (Dz) methodwas developed by D.J. Eve under supervision of the author. This work is described in the attached reprint (6).

A few minor modifications have been introduced in the general technique - these are described below.

Fig. XIII shows a working curve for zinc in citrus leaves.

The statistical accuracy and precision of the method as applied to citrus and pineapple leaves are recorded in Section 4.

3.81: EXPERIMENTAL PROCEDURE FOR THE DETERMINATION OF ZINC.

APPARATUS:-

A special rack was constructed to hold two sets of six Pyrex 100 ml. separating funnels, one set directly above the other. The stopcocks of these funnels were reground so as to avoid the use of grease. The funnels should always be kept in as clean a state as possible.

REAGENTS:-

(i) Zinc Standard: Dissolve 0.2488 g. "specpure" ZnO in 10 ml. 1N A.R. H_2SO_4 by warming on a hotplate. Cool, neutralise with redistilled ammonia, make up to 1000 ml. and store in a polythene bottle (1 ml = 200 µg. ZN). Dilute 25 ml. of this solution to 1000 ml. and store in a polythene bottle (1 ml. = 5 µg. Zn).

(ii) Carbon Tetrachloride:- Dry 5 litres of technical grade CCl_4 by shaking with granulated calcium chloride. Leave to stand for several hours, pour off and distil in an all-glass Pyrex still. Collect the first and last 100 ml. fractions separately and keep for redistillation. Collect and keep the pure distillate in an all-glass Pyrex aspirator. Used carbon tetrachloride is reclaimed by addition of a small volume of 6N NaOH containing a little sodium thiosulphate. Distil, wash the distillate with pure water, dry over $CaCl_2$ and distil as above.

(iii) Ammonium Hydroxide, IN: Distil C.P. NH_4OH in an all-glass Pyrex still and absorb in deionised water contained in a polythene bottle cooled in ice-water. Determine the normality (usually about 6N) and dilute part of the distillate to give 1 litre of exactly IN NH_4OH. Store in a polythene bottle.

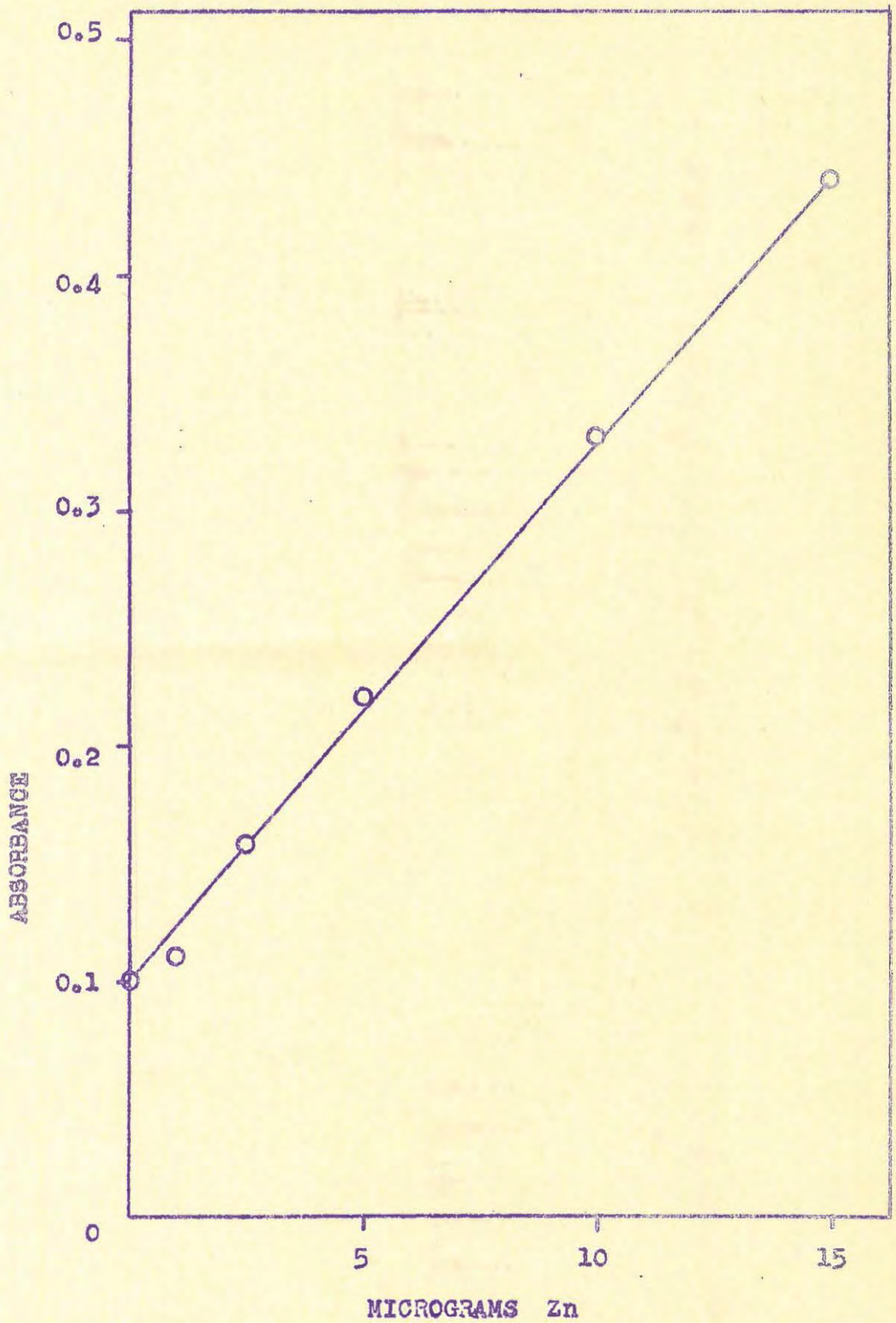


Fig. XIII.

Working Curve for Zinc (Citrus).

In the presence of: 25 mg.Ca, 2.5 mg.Mg, 3 mg.K, 30 µg.Al, 30 µg.Fe, 15 µg.Mn, 3 µg.Cu, 3 mg. P, 0.5 ml. $HClO_4$.

(iv) Hydrochloric Acid, IN: Distil a 1 : 1 mixture of C.P. HCl and water in an all-glass Pyrex still. Discard the first fraction and collect the distillate in a polythene bottle when the temperature has reached a steady value. This gives a constant-boiling point mixture of approximately 20% HCl. Determine the normality and dilute part of the distillate to give 1 litre of exactly IN HCl. Store in a polythene bottle.

(v) Dithizone Reagent: Dissolve 0.1 g. of A.R. diphenylthiocarbazone (Dz) in 500 ml. CCl_4 by shaking in a 2L separating funnel. Add 20 ml. N NH_4OH in 1 litre of water and shake to transfer the Dz to the aqueous phase. Wash with several small portions of CCl_4, discarding the washings. Add 500 ml. CCl_4 and 50 ml. of NHCl and shake well. The Dz is now once more in the CCl_4. Run this into a black-painted Pyrex bottle, dilute to 1 litre with CCl_4 and store in a refrigerator.

(vi) Ammonium Citrate, 0.5M: Dissolve 226 g. of A.R. dibasic ammonium citrate in 2 litres of water. Adjust the pH to 9 (pH meter) by adding redistilled ammonia. Extract the solution with 150 ml. Dz reagent and then wash with 100 ml. portions of CCl_4 until free of heavy metals, i.e. until the washings are pure green.

(vii) Solution A:- Dilute 1 litre of ammonium citrate plus 150 ml. of N NH_4OH to 4 litres. Store in polythene bottle.

(viii) Solution B:- Dilute 1 litre of ammonium citrate plus 320 ml. of N NH_4OH to 4.5 litres. Store in polythene bottle.

(ix) Solution C:- Make up 25 ml. of a freshly prepared 0.2% sodium diethyl dithiocarbamate solution to 250 ml. in a volumetric flask with Solution B, just before use.

(x) Hydrochloric Acid, 0.02N: Dilute 100 ml. of N HCl to 5 litres. Store in a polythene bottle.

PROCEDURE:

(a) Calibration Curve (Citrus):- Into 100 ml. Pyrex separating funnels burette (micro) 0, 0.25, 0.50, 1.00, 2.00 and 3.00 ml. of the dilute zinc standard, and to each funnel add:- 2 ml. of citrus colorimetric standard ((v), Section 3.6), 0.6 ml. of the Manganese standard (3.7), 6 ml. of the dilute Fe standard (3.6) and 0.2 ml. of the dilute Cu standard (3.9). Add 5 drops ammonium citrate solution, one small drop of 0.1% phenolphthalein indicator and titrate with redistilled ammonia until faintly pink. Then carry out the following extraction procedures:-

First Extraction:- (Separation of zinc and other dithizone complex-forming metals from iron, aluminium, calcium and

other substances). Pipette 40 ml. of Solution A and 10 ml. of Dz reagnet (automatic pipette) into the separating funnel and shake vigorously by hand for 2 minutes. The aqueous layer should now be yellow-orange, indicating excess of Dz. Allow the carbon tetrachloride layer to separate and run this phase into a second separating funnel, being careful not to let the aqueous layer enter the bore of the stopcock. Wash down with three 2 ml. portions of CCl_4, running each washing into the second funnel. The CCl_4 layer should now be pure green. Run this layer into the second funnel and flush with a small volume of CCl_4 as before. Discard the contents of the first funnel.

Second Extraction:- (Separation of zinc from other dithizone complex-forming metals). Pipette 40 ml. of 0.02 N HCl into the second funnel, shake vigorously for 2 minutes, allow layers to separate, and run off the CCl_4 phase (keep for reclaiming). The drops on the surface and the remaining coloured CCl_4 are flushed out as before. Then add 5 ml. of CCl_4, shake for about 15 seconds, and run out. This removes any impure CCl_4 which may stick to the sides of the funnel.

Final Extraction:- (To recomplex the zinc for photometric measurement). Pipette 40 ml. of Solution C and 10 ml. of Dz reagent into the separating funnel. Shake vigorously for 2 minutes, allow to separate, and run the organic phase into a 50 ml. volumetric flask. Flush out with three 2 ml. portions of CCl_4 to remove all of the coloured phase. Make up to the mark with CCl_4 and measure the absorbance of the solution at 520 mμ.

The solutions as prepared above contain 0, 1.25, 2.50, 5.0, 10.0 and 15.0 μg. Zn respectively.

(b) Calibration Curve (Pineapple):- Proceed as described above but add 2 ml. of pineapple standard ((vi) 3.6), 6 ml. of the dilute Fe standard, 1.2 ml. of the Mn standard and 0.4 ml. of the dilute Cu standard.

(c) Plant Solution:- Pipette 10.0 ml. of the leaf solution (citrus and pineapple) into the separatory funnel, add the ammonium citrate, phenolphthalein, ammonia and proceed as described above.

Express the results as p.p.m. Zn.

NOTE:-

The extraction procedures must be carried out in artificial light. Prepare a new calibration curve with each batch of freshly prepared reagents.

3.9: THE COLORIMETRIC DETERMINATION OF COPPER.

Copper is an important trace element, not only in the agricultural world, but in many other spheres as well and a great deal of work has been done on methods for its determination in minute amounts. Judging from the large numbers of analytical papers on copper, constantly appearing in the literature, all is not well with this determination. No doubt the minute concentration of copper in the presence of large amounts of interfering ions has a great deal to do with the constant search after improvements and new, more specific methods.

There are innumerable organic compounds that give sensitive colour reactions with copper and many colorimetric methods have been described. The only reagent absolutely specific to copper appears to be 2 - 2' - biquinoline, first introduced in 1939 by Breckenridge et al (76). Later, Hoste (77) reported this reagent to be superior to any other known reagent for copper. Biquinoline reacts with cuprous ions to give a purple colour. Cupric ions are reduced with hydroxylamine hydrochloride, the pH of the solution adjusted to 4 - 5 with sodium acetate, and the copper extracted with 10 ml. 0.02% biquinoline in isoamyl alcohol. The absorbance is measured at 540 mμ. In 1953, Cheng and Bray (78) reported interference from a number of anions, which must first be destroyed before the method can be used. The method is relatively expensive.

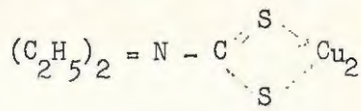
Other colorimetric reagents that have recently been suggested for copper are:- 2 - carboxy - 2'- hydroxy - 5' - sulphoformazylbenzene, suggested in 1954 by Rush and Yoe (79) for Cu and Zn; Biscyclohexanone - oxalyldehydrazone in 1954 by Williams (80) for Cu in plants; and 2, 9 - dimethyl - 1, 10 - phenanthroline (neo-cuproine) in 1954 by Gahler (81) for Cu in steels. All these methods appear to be sensitive, rapid and accurate.

However, the two reagents that have enjoyed most univeral use are sodium diethyl-dithiocarbamate (DDC) and dithizone (diphenylthiocarbazone). The latter method involves some lengthy extractions which may introduce error when working with minute concentrations. The method is described by Sandell (50).

The author decided to investigate the DDC method, mainly because the reagents used for this method are very similar to those used for zinc, which would simplify purification.

Callan and Henderson (82) first introduced the use of sodium diethyldithiocarbamate in 1929 for the colorimetric determination of copper. The reagent reacts with copper in slightly acid to alkaline medium to give a golden brown precipitate of copper DDC. This reaction has been considered one of the most sensitive for copper that has been developed - it can, for example, be used to detect

copper visibly in distilled water. The compound formed in this reaction has been assigned the following formula (83):-



In very dilute copper solutions a colloidal suspension suitable for colorimetric comparison is obtained, especially if it is stabilised by gum arabic or similar substances. However, in order to decrease the the effect of interfering ions and to increase the sensitivity of the reaction, it has become customary to extract the copper DDC with organic solvents such as isoamyl alcohol, isoamyl acetate and carbon tetrachloride, resulting in a brown solution more suitable for colorimetric measurement. The method has been criticised (76) because of the many interferences from different metals. The chief interfering metals are Fe, Mn and Ni, but in fact most metals, other than Ca and Mg, may interfere if present in sufficient amounts. Many complexing reagents such as pyrophosphate, citrate and ammonium hydroxide have been recommended for the elimination of interference by iron, provided the amount of iron is not too large (84). Interference by Ni and Co can be prevented by adding dimethylglyoxime to the sample solution before adding the ammonium hydroxide. The precipitate is separated by filtration, while the Co remains in the aqueous solution and is not extracted by CCl_4 (85). Manganese interferes to a considerable extent in the extraction procedure by imparting a pinkish colour to the organic layer. This colour is more or less unstable, and the solution quickly becomes colourless on standing if only small amounts of Mn are present.

In order to eliminate interferences, Piper (8) recommends extraction of Cu at pH 3 with dithizone in CCl_4. The CCl_4 is boiled off, the residue extracted with H_2SO_4 and $HClO_4$ and copper is determined by the usual DDC method. Cheng and Bray (78), and Forster (86), used a mixture of EDTA and ammonium citrate to eliminate interferences, and they claimed that by this procedure the DDC method is virtually specific for copper. Other metals, with the exception of bismuth, are prevented from reacting with DDC in the presence of EDTA.

It was decided to investigate the following aspects of the DDC method:- Completeness of extraction with CCl_4; the stability of the copper DDC complex; the effect of pH on the complexing of iron; and interferences in the range commonly occurring in citrus and pineapple leaves.

All reagents were specially purified by repeated extraction with dithizone in CCl_4; all acids and alkalies were redistilled in all-borosilicate glass stills and "specpure" chemicals were used for

the standards. The procedure as finally modified, is described in Section 3.95. Interference from iron was suppressed by the use of ammonium citrate at a pH of 9.0 - 9.5 . A 0.1% DDC solution was found to be very satisfactory, instead of the usual 1% solution (78, 86).

3.91: COMPLETENESS OF EXTRACTION.

Many workers have reported complete extraction of copper DDC by carbon tetrachloride only if the mixture was shaken for at least 5 minutes. As such a procedure is very laborious in routine analysis, it was decided to test the completeness of extraction, using 10 ml. CCl_4 and shaking vigorously by hand for 2 minutes. It was found that satisfactory extraction occurred in all cases between the range of 0 - 25 µg. Cu, a second extraction resulting in less than 0.3 µg. being recovered.

3.92: STABILITY OF THE COPPER CARBAMATE COMPLEX.

Previous workers have stated that solutions of copper DDC in carbon tetrachloride are stable in the dark but fade rather rapidly in diffuse light. Such a solution, containing 20 µg. Cu, was prepared and placed into two absorption cells. One cell was left in artificial light (normal fluorescent lighting) while the other cell was placed close to a window in diffuse daylight. The absorbance of both solutions was measured periodically. The results are shown in Fig. XIV.

It was clear that the solution left in artificial light did not show any deterioration in the 5 hours of the test, while the other solution deteriorated rapidly. In the laboratory in which the author worked, the blinds were permanently drawn as a precautionary measure. It is, of course, also essential to carry out zinc determinations in artificial light (6).

3.93. EFFECT OF pH AND COMPLEXING OF IRON.

Sandell (50) recommends that in order to complex iron with citrate and ammonium hydroxide, the pH should lie between 9 - 9.2. This pH is normally obtained by adding ammonium hydroxide to the test solution, containing the citrate and an indicator like thymol blue or phenolphthalein. Using 1 drop of 0.1% phenolphthalein in alcohol and measuring the pH of the test solution with a glass electrode, the author invariably found the pH to be just greater than 9, on adding ammonia to the first appearance of a pink colour.

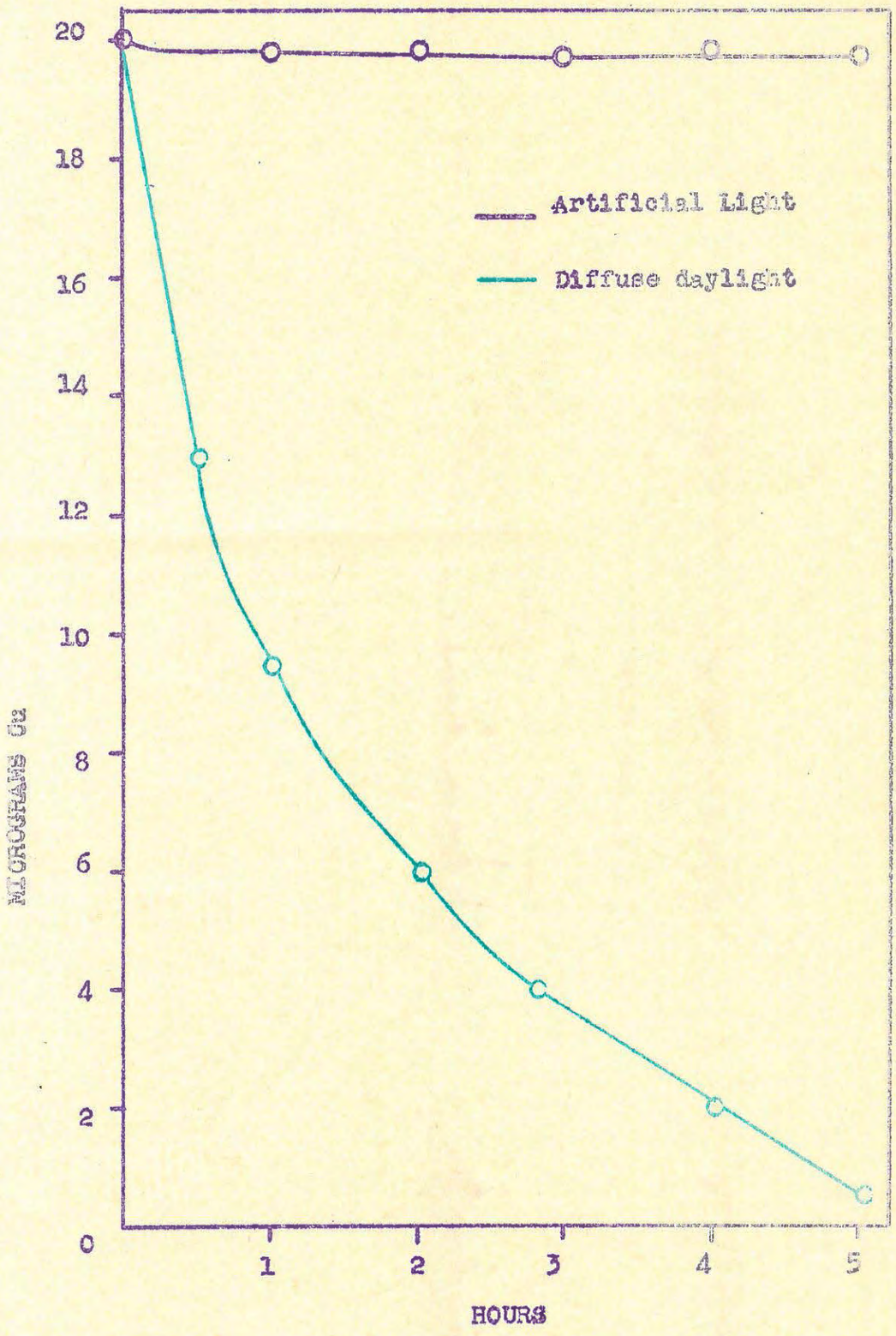


Fig. XIV.

Stability of Copper Carbamate.

3.94: INTERFERENCE FROM FOREIGN METAL IONS.

It was clear from previous work that greatest interference appears to be caused by iron, nickel and manganese. The concentration of nickel in all the citrus and pineapple samples studied was so low, that interference from this element could be considered as negligible. The addition of dimethylglyoxime made no difference to the amount of copper recovered in all the samples tested.

Without going into the experimental details of the study on other interferences, it can be stated that the author found none of the ions to interfere when present in the normal range of concentrations found in citrus and pineapple leaves. In the presence of 600 µg. of Fe and 600 µg. of Mn the copper reading was only 1 µg too high. It was concluded that the citrate successfully complexed the iron at pH 9, while no interference from Mn occurred if the colour was measured $\frac{1}{2}$ hour after development.

Clear proof of the smallness of the interference caused by several ions can be obtained from the working curve for Cu, Fig. XV. This curve was prepared from copper standards to which known amounts of the various ions (in pure form), in proportions normally present in citrus leaves were added. The iron and manganese correspond to 150 p.p.m. Fe and 75 p.p.m. Mn in the leaf. The small absorbance from the copper blank points to negligible interference.

It was concluded that it was not necessary to add other complexing agents (78, 86), if copper was determined in citrus and pineapple leaves as described below. It was felt that the number of reagents used should be kept to the bare minimum because of the laborious procedure of purifying them sufficiently for the determination of copper. The ammonium citrate, ammonium hydroxide and carbon tetrachloride used in the determination, are purified as described for the determination of zinc.

The statistical accuracy and precision of the method are recorded in Section 4, while the experimental procedure is described below.

3.95: EXPERIMENTAL PROCEDURE FOR THE DETERMINATION OF COPPER.

APPARATUS: Separating funnels and rack as in 3.8.

REAGENTS:

(i) Ammonium Citrate, 0.5M: Prepared as described for zinc, 4.8.

(ii) Carbon Tetrachloride: Prepared as described for zinc, 4.8.

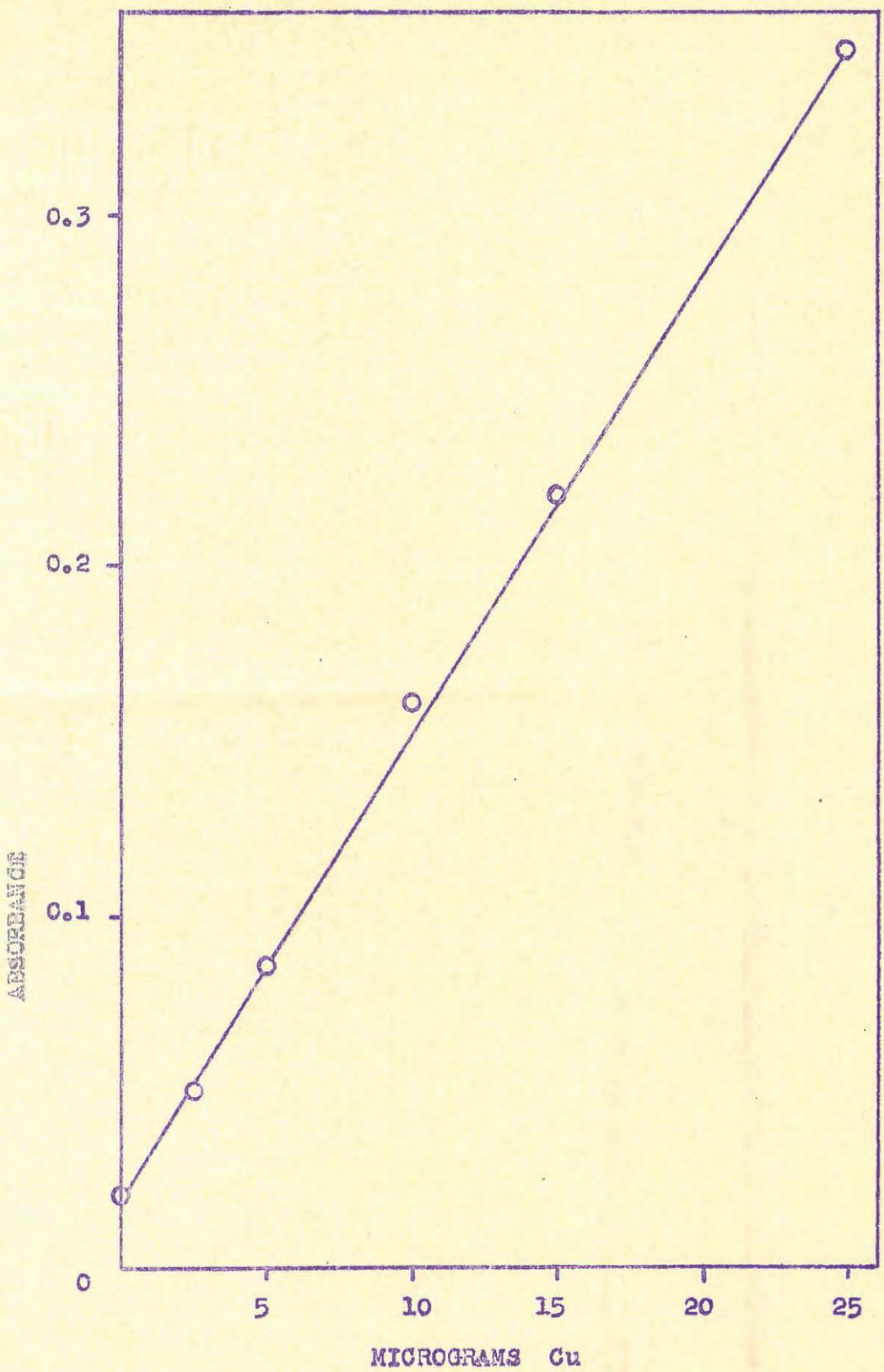


Fig. XV.

Working Curve for Copper (Citrus).

In the presence of: 60 mg.Ca, 6 mg.Mg, 7 mg.K, 4 mg.Na, 120 µg.Al
150 µg.Fe, 75 µg.Mn, 15 µg.Zn, 12 mg. P,
2 ml. $HClO_4$.

(iii) Sodium Diethyl Dithiocarbamate, 0.1%: Dissolve 0.1 g. of the A.R. salt in 100 ml. water. Prepare fresh before use.

(iv) Copper Standard: Dissolve 0.2502 g. "specpure" CuO in 5 ml. water + 1 ml. conc. A.R. H_2SO_4 by warming on the hotplate. Cool and make up to 1000 ml. (1 ml. = 200 µg. Cu). Dilute 50 ml. of this solution to 1000 ml. Store in polythene bottles (1 ml. = 10 µg. Cu).

(v) 0.1% Phenolphthalein in Ethanol (95%).

PROCEDURE:-

(a) Calibration Curve (Citrus):- Into 100 ml. separating funnels burette (micro) 0, 0.25, 0.50, 1.00, 1.50 and 2.50 ml. of the dilute copper standard and add to each funnel:- 8 ml. of citrus colorimetric standard ((v) 3.6), 24 ml. of the dilute Fe standard (3.6), 2.4 ml. of the Mn standard (3.7) and 4.8 of the dilute Zn standard (3.8).

Add 10 ml. of ammonium citrate, 1 drop of phenolphthalein and titrate with redistilled ammonia until faintly pink (pH 9-9.5). Add 1 ml. of 0.1% sodium diethyl dithiocarbamate reagent, swirl to mix well, add exactly 10.00 ml. of CCl_4 from an automatic pipette, stopper tightly and shake vigorously by hand for 2 minutes. Allow to settle for ½ hour. Dry the stem of the funnel with a piece of filter paper, insert a small plug of glass wool into the stem of the funnel to filter off any solid particles, and run the CCl_4 phase into a 1 cm. absorption cell until full. Measure the absorbance of the solution at 440 mµ.

The solutions, as prepared above, contain 0, 2.50, 5.00, 10.00, 15.00 and 25.00 µg Cu respectively.

(b) Calibration Curve (Pineapple):- Proceed as described above, but add to each of the funnels:- 8 ml. of pineapple standard ((vi) 3.6), 24 ml. of the dilute Fe standard, 5.0 ml. of the Mn standard (3.7), and 5 ml. of the dilute Zn standard (3.8).

(c) Plant Solution:- Pipette 40.0 ml. of the leaf solution (citrus and pineapple) into the separating funnel, add the ammonium citrate, phenolphthalein, ammonia and proceed as described above.

Express the results as p.p.m. Cu.

NOTES:-

(i) The experiment must be carried out in artificial light.

(ii) A new calibration curve must be prepared for each batch of fresh reagents.

GENERAL REMARKS.

The methods described above have been specially developed for citrus and pineapple leaves. They have been thoroughly tested and found reliable, accurate and reproducible, and they are especially suitable for routine work. Strict adherence to details is important if these procedures are to provide accurate and trustworthy results, and because methods, as described in the literature, often lack certain essential details of manipulation, the methods are described in full detail.

Most of the methods are on the micro or semi-micro scale. It is clear that any error introduced during some of the lengthy procedures will be greatly magnified under these conditions. All measuring apparatus such as burettes, pipettes, volumetric flasks, etc., should be of a reliable grade or be carefully standardised. In order to standardise conditions throughout, all additions of reagents, unless otherwise stated, should be made with graduated delivery pipettes.

Special attention must be paid to the purity of the water and reagents used, to the apparatus and to the laboratory conditions. It is absolutely essential to run blanks from time to time, particularly when a new batch of reagents are used. If blank values are high the source of error must be found before continuing with the determinations.

In order to keep in line with modern Analytical literature, the methods are described in the form of specific instructions.

4: THE STATISTICAL ACCURACY AND PRECISION OF THE ANALYTICAL METHODS.

Once the methods had been satisfactorily standardised as described in Section 3, their statistical accuracy and precision were determined on a standard citrus leaf sample and a standard pineapple leaf sample. The precision was tested by carrying out 16 parallel determinations on different portions of a well-mixed leaf sample. The accuracy of the methods was tested by carrying out 6 determinations on 2 g. samples of the citrus leaf powder to which known amounts of specpure chemicals were added prior to the digestion stage.

The final figures are grouped together in Tables 20, 21 and 22. Fig. XVI is a graphical representation of the precision of the methods.

The same statistical formulae described in Part I were used.

TABLE 20.

PRECISION ON CITRUS LEAF SAMPLE.

NUMBER	N %	P %	K %	Ca %	Mg %	Na %	Fe ppm	Mn ppm	Zn ppm	Cu ppm
1	2.26	0.120	0.497	4.97	0.589	0.271	123	52.0	12.9	3.4
2	2.29	0.121	0.500	5.05	0.570	0.273	125	52.9	13.5	3.3
3	2.30	0.120	0.501	4.99	0.596	0.274	123	52.0	13.6	3.2
4	2.30	0.122	0.492	5.06	0.589	0.277	122	52.7	13.5	3.1
5	2.27	0.120	0.500	4.98	0.559	0.271	123	52.3	13.5	3.5
6	2.29	0.120	0.467	4.96	0.570	0.275	123	53.8	13.1	3.3
7	2.28	0.120	0.473	5.00	0.596	0.273	124	53.1	12.4	3.2
8	2.26	0.123	0.522	5.01	0.601	0.276	123	52.6	13.5	3.0
9	2.30	0.121	0.496	4.96	0.580	0.276	127	53.0	13.1	3.4
10	2.29	0.121	0.491	4.99	0.560	0.275	124	52.4	13.6	3.5
11	2.27	0.120	0.494	4.97	0.603	0.277	123	52.4	13.3	3.0
12	2.29	0.125	0.500	5.06	0.589	0.272	124	53.0	12.3	3.3
13	2.28	0.120	0.501	4.99	0.596	0.274	123	52.0	13.6	3.2
14	2.30	0.121	0.490	5.00	0.560	0.275	124	52.4	13.6	3.5
15	2.29	0.121	0.479	5.02	0.596	0.273	124	53.1	12.4	3.2
16	2.30	0.122	0.518	5.01	0.601	0.276	123	52.6	13.5	3.0
Mean	2.29	0.121	0.495	5.00	0.588	0.274	124	52.6	13.2	3.2
Standard Dev.	0.015	0.001	0.014	0.04	0.016	0.002	1.5	0.49	0.47	0.17
Coeff. of Var. %	0.6	1.2	2.9	0.9	2.7	0.7	1.8	0.9	3.5	5.3

TABLE 21.

ACCURACY: PERCENTAGE RECOVERY OF ADDED ELEMENTS (CITRUS SAMPLE).

NUMBER	N	P	K	Ca	Mg	Na	Fe	Mn	Zn	Cu
1	99.1	98.5	98.4	99.5	99.1	99.7	99.6	99.3	99.5	98.7
2	99.4	97.0	98.9	99.0	98.6	100	98.6	101	99.8	99.3
3	100	96.0	101	98.9	98.6	99.5	98.4	98.2	99.5	97.6
4	99.7	98.5	99.6	98.9	99.8	99.3	97.3	97.1	99.3	98.7
5	99.8	98.8	99.1	99.5	99.3	99.9	98.7	98.5	99.3	98.2
6	99.5	97.6	98.6	99.3	98.5	98.9	99.7	98.0	99.3	97.3
Mean % recovery	99.6	97.7	99.3	99.2	98.9	99.5	98.7	98.6	99.4	98.3

TABLE 22.

PRECISION ON PINEAPPLE LEAF SAMPLE.

NUMBER	N %	P %	K %	Ca %	Mg %	Fe ppm	Mn ppm	Zn ppm	Cu ppm
1	1.48	0.164	3.28	0.376	0.356	53.0	127	17.7	8.2
2	1.47	0.165	3.27	0.380	0.343	52.5	128	18.7	9.2
3	1.48	0.167	3.25	0.379	0.337	54.0	130	16.8	8.1
4	1.49	0.168	3.24	0.379	0.348	52.8	125	16.6	8.7
5	1.48	0.169	3.22	0.375	0.350	53.0	126	17.8	8.1
6	1.47	0.169	3.20	0.377	0.352	54.5	124	18.8	8.9
7	1.47	0.164	3.29	0.376	0.348	54.0	127	16.4	7.9
8	1.48	0.167	3.27	0.380	0.337	54.8	128	17.1	8.1
9	1.48	0.169	3.27	0.379	0.350	54.0	128	17.4	8.2
10	1.47	0.167	3.31	0.379	0.348	53.5	127	16.7	8.3
11	1.49	0.164	3.29	0.376	0.345	54.0	127	16.4	7.9
12	1.49	0.167	3.27	0.380	0.350	54.8	128	17.1	8.1
Mean	1.48	0.167	3.26	0.378	0.347	53.7	127	17.3	8.3
Standard Dev.	0.007	0.002	0.03	0.002	0.005	0.78	1.6	0.82	0.29
Coeff. of Var. %	0.5	1.2	1.0	0.5	1.6	1.4	1.2	4.8	3.5

DISCUSSION:-

Sixteen replicate determinations were carried out on the citrus sample and 12 on the pineapple sample because these numbers conformed to the size of the blocks in the sampling studies (Part III).

From the figures recorded in Tables 20, 21 and 22, it is clear that the analytical methods, as finally modified, are both accurate and precise. The precision of the micro-nutrient methods is particularly good considering the small concentrations of these elements.

With a few exceptions, the reproducibility of the methods was more or less the same for both types of materials. The much greater precision for potassium on pineapples as compared to citrus leaves can partly be ascribed to the higher concentration of this element in the former case, giving a more accurate reading on the flame photometer. Calcium yielded a better precision in the case of pineapple leaves because the EDTA method, which was used, is generally a more precise procedure, being volumetric, than the flame photometric procedure, which was used for calcium in the citrus sample. When the calcium

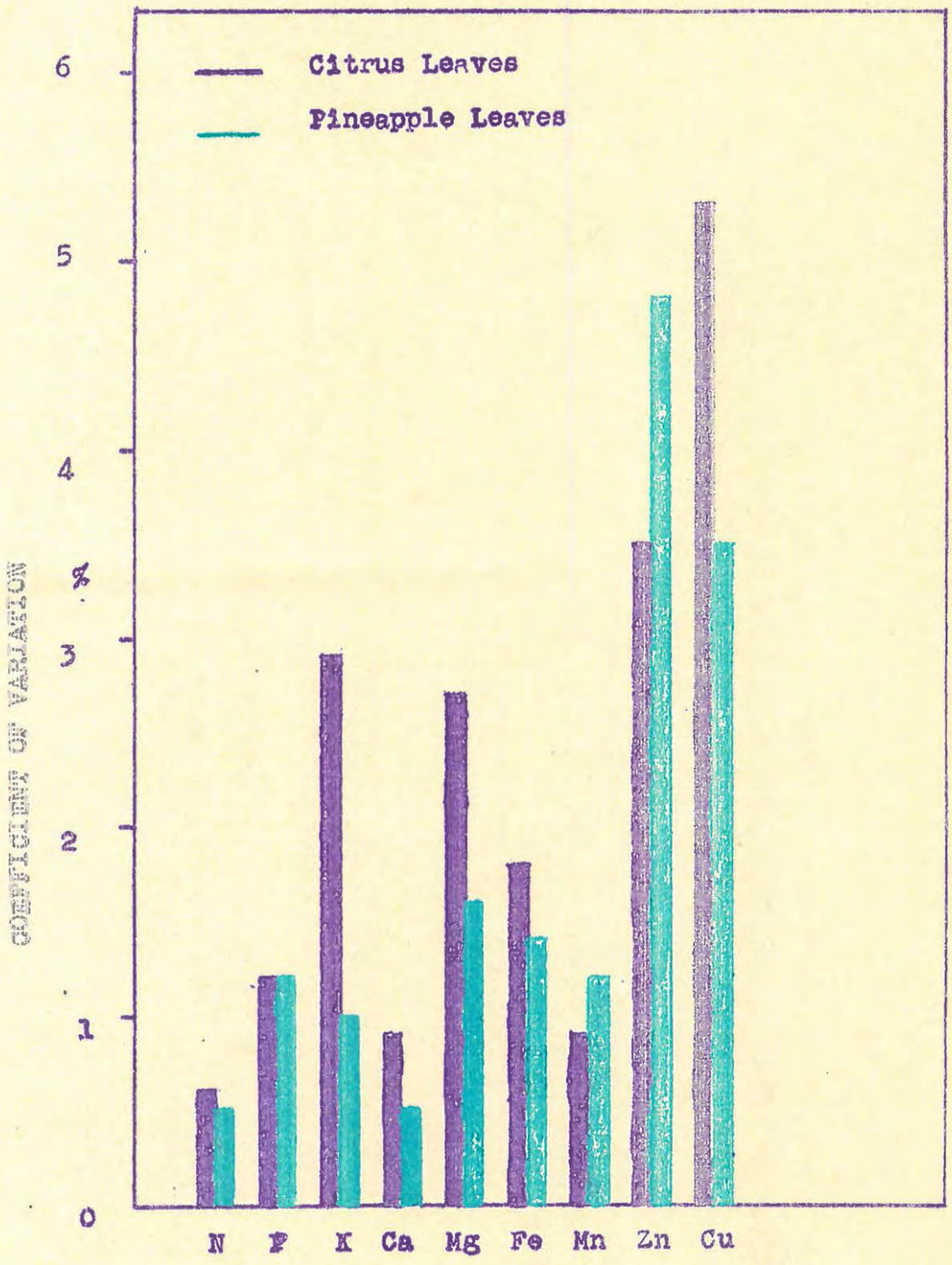


Fig. XVI.

Statistical Precision of the Analytical Methods.

concentration is small, as in the case of pineapple leaves, it is advisable to use the EDTA method. The use of EDTA for calcium yielded a more precise magnesium value in the case of pineapples.

A few words must be said about the form in which the results were expressed. Having determined the precision of the methods it was necessary to determine to what degree of accuracy the results should be reported in future. It would, for example, be foolish to continue in reporting the K values in citrus leaves as 0.495%, thus claiming a precision of 0.2%, when, under the most exact conditions, the method is only capable of yielding a precision of $\pm$ 3%. This important point is often lost sight of when nutrient figures are reported in the literature. In the subsequent studies, the author could be confident in reporting values for citrus, for example, as:- 2.29% N; 0.121% P; 0.49% K; 5.00% Ca; 0.59% Mg; 0.274% Na; 124 p.p.m. Fe; 52.6 p.p.m. Mn; 13 p.p.m. Zn; 3.2 p.p.m. Cu.

Having accurately determined the variations to be expected from the analytical procedures, the author could confidently carry out the studies on sampling variations as described in Part III.

P A R T III.

THE SAMPLING OF

CITRUS AND PINEAPPLE PLANTS

FOR CHEMICAL ANALYSIS.

---

1. REVIEW OF PREVIOUS SAMPLING STUDIES.

The proper collection of plant samples is quite as important as the use of reliable methods for their subsequent analysis. Unless a sample is truly typical of the material which it is intended to represent, the analytical results are of little or no value, however carefully they may be determined in the laboratory. The question is - what constitutes a representative sample for a particular species?

In many instances this question has been answered in a clear cut numerical fashion. Thus Lilleland and Brown (87) recommend the picking of 10 leaves from each of 10 trees in a plot when studying the fertiliser requirements of deciduous trees as regards potassium. Drosdoff (88), in sampling tung trees, picked a composite sample from 8 out of 15 trees in a plot, 6 or more leaves per tree. Thomas (89) recommends that when sampling herbaceous plants, all the plants in a row should be sampled, omitting only those that deviate from the average of the treatment. When a tree species is to be sampled, one leaf from each of 10 - 15 shoots should be taken. For grapes, Ulrich (31) found 80 petioles taken from plots varying from 16 to 160 vines to be adequate. In sugar beet studies, the same author found that 40 petioles from plots 20 feet wide and 600 feet long were adequate to reflect the fertilisers applied to the soil. On the other hand, Brown (90) found it necessary to pick 400 petioles from a plot of sugar beet, 125 x 500 feet to provide a sample with an error limit of 10%.

For the sampling of a citrus orchard, Bathurst (34) recommends picking leaves from 10 - 16 fruit stalks from each of 2% of the trees, when determining nitrogen and phosphorus. Chapman (35) states that the best way to make a detailed evaluation of the potassium status of commercial orchards is to secure composite samples of from 15 - 20 leaves each, from 5 or more representative trees in each of several parts of an orchard.

Nightingale (38), in sampling pineapples, selected 10 plants from each double-row bed, 100 yards long (approximately 600 plants), in several key locations in a field. This appears to be the standard practice for sampling pineapple plants in Hawaii.

Most of the recommendations listed above are based on the variations shown by two or three elements, mainly N, P and K. The question is, will this degree of sampling be adequate to represent all the nutrients, including the trace elements, in the particular plant species? There appears to be a complete lack of recommendations in the literature regarding the degree of sampling necessary for trace element determinations. Bathurst (34), in the case of citrus, assumed that a sample giving adequate representation for N and P will

also be a sufficiently large sample for the other constituents.

From the literature (2) it is quite clear that the mineral composition of a particular species is not a fixed entity. There is ample evidence that the compositions of plants vary from month to month, even from hour to hour on the same day, from soil type to soil type, as well as amongst the different parts of the plant itself. Rigorous quantitative comparision is possible only between leaves of the same physiological age taken from plants of the same species and variety grown under the same environmental conditions on a relatively uniform soil.

In the study reported here only variations occurring at a specific constant period of the day from plant to plant, employing sampling techniques which are in common use, were considered. In fact, as in the case of soils (Part I), every effort was made to keep possible interfering factors as constant as possible, in order to determine the inherent variations occuring in adjacent plants on small plots. It was felt that factors such as age of the part sampled, diurnal variations, etc., can be controlled to a certain extent, but there is no way of controlling the inherent variations from plant to plant, and this, therefore, is the essential factor to determine before recommending a sampling technique.

Despite the relatively small number of samples usually recommended for adequate representation, there is abundant evidence in the literature of large variations having been found. Thus Lilleland and Brown (91), when studying the phosphorus nutrition of peach trees, found that the composition of morphologically homologous leaves when taken from adjacent trees receiving the same fertiliser treatment, differed greatly. This was also the experience of Thomas (43) with apple trees, and he questioned the desirability of taking composite samples from several trees in an orchard. He stated that the decision with respect to the validity of compositing must rest upon the uniformity of nutrition. Thomas and Mack (92), in studying foliar diagnosis in relation to soil heterogeneity, found great variations in the yields from tomatoes between pairs of similarly treated plots, the differences in these adjacent plots ranging from -193 to + 82% in 15 out of 18 plots. Drosdoff (88), when studying the leaf analysis of tung trees, came to the conclusion that differences in leaf composition due to the nature of the soils on which the orchards were situated, were greater than those caused by any other factor which he studied. Ulrich (93) maintains that the procedure adopted for collecting plant samples from a field will modify the estimate of a critical level set for the crop. In a field with 50% of the plants at a deficient level, the remaining plants may increase the nutrient concentration of the composite sample above the critical range.

An extensive survey of the literature revealed only three papers in which a certain amount of statistical treatment was used in order to determine sampling efficiency, viz. by Brown (90), who studied sugar beets, and Bathurst (34) and Chapman (35) who studied the sampling of Valencia citrus trees.

Brown used a statistical formula in order to obtain an estimate of the number of petioles to sample from commercial sugar beet fields for an error limit of 10%, at the 19 to 1 probability level. He only studied variations in nitrogen and phosphorus. The following formula was used:-

$$N = V \left(\frac{t}{M - x} \right)^2$$

where N = the required number of sampling units;
V = the variance corresponding to this number;
M-x = the desired fiducial precision; and
t = the value from Fisher's t Table, corresponding to the required probability and number of degrees of freedom required.

By using this formula, Brown concluded that 400 properly chosen petioles from randomly selected beets in a plot 125 x 500 feet were required to provide a sample with an error limit of 10% at the 19 to 1 probability level.

Bathurst (34) used Brown's formula to study the variations in nitrogen shown by a citrus orchard. He selected two blocks, each of 192 full grown Valencia orange trees, the one in a healthy, vigorous orchard, and the other in a neglected poor orchard. Each of the two orchards were divided into 16 plots of 12 trees each. One tree in each of the 16 plots was sampled by picking leaves from 10 - 16 "fruit-stalks". The tree to be sampled in each plot was selected by numbering each of the 12 trees, placing the numbers in a hat, and drawing a number at random. This procedure yielded 16 samples, one for each plot of 12 trees, and consisting of 10 - 16 leaves per sample. The leaves were analysed for nitrogen. In the good orchard the highest and lowest N values differed by about 19%, while in the bad orchard the difference was about 24%. The coefficient of variation was 6.5% in the former case and 7.0% in the latter orchard. Using Brown's formula, Bathurst maintained that it was only necessary to sample 2 trees out of the whole block of 192 good trees, provided that they were chosen so as to be well separated from each other, in order to give odds of 19 to 1 that the estimate of N content was not more than 10% from the correct value. For the poor orchard three trees had to be sampled.

However, the author cannot agree with the way in which Bathurst set about in determining sampling efficiency. The most weighty

argument against his experiment is that he did not attempt to determine the "true" value of nitrogen in the block of 192 trees by intensive sampling. He selected, at random, a mere 16 trees in the block, determined the nitrogen in each and considered the mean value to be a true reflection of the mean nitrogen content of all 192 trees - at any rate that is what his final statistic seems to imply. It would have been more correct to say that 2 trees must be sampled in order to represent 16 trees to within 10%, because, unless the variance is determined more accurately, the use of this statistical formula is not valid. If the nitrogen values reported for the 16 trees are considered, it is quite evident that by only sampling 2 trees out of 192, a 20% error may very easily be arrived at, i.e. assuming that the mean nitrogen value for the 16 trees is indeed the same mean for all 192 trees.

It is also felt that the statistical formula used by Brown and Bathurst is not reliable. If expressed in the terms of the formula used by the author for soils in Part I, viz. $n = 2t^2d^2/D^2$, the Brown formula boils down to $n = t^2d^2/D^2$, in other words the very important 2 has been omitted. As this is the standard formula used to determine the significance of a difference between the means of two similar series, and as the standard error of the difference between the means is $\sqrt{2}$ x the standard error of any one, the author fails to see how this important factor could have been neglected by these workers. In applying the author's formula to the figures of Bathurst, 4 trees must be sampled to represent 16 trees to within 10% of the mean nitrogen value at the 5% point for the good orchard. With the relatively large variations shown by Bathurst's figures for N, it is surprising that he was satisfied with a 1 - 2% sampling of the trees.

Finally, having decided that a 1 - 2% sampling was needed to represent nitrogen to within 10% in an orchard, Bathurst made the following statement: "Healthy citrus trees may contain anything from 0.5 - 2% potassium in their leaves. The need for precision in the case of potassium analysis is therefore a good deal less than in the case of nitrogen, for example, where 1.6% N represents a fairly acute deficiency and 2.7% an excess. On the whole it is therefore assumed that a sample giving adequate precision in the case of nitrogen will also be a sufficiently large sample for the other constituents." The author disagrees emphatically with this statement, as will be seen from the results reported later on. But even accepting the assumption that the citrus tree will show no improvement if the potassium value in the leaf is progressively stepped up from 0.5% to 2%, provided all other growth factors are favourable (and it is very difficult to accept this), what happens, for example, if a value of 0.3% is obtained? Can such a figure be termed as deficient when Bathurst knows, as is quite clear from his work, that potassium shows a great

deal more variation than nitrogen and that 20 - 30% differences are quite common? Would such a low figure not merely represent a sampling error? The point is that, even if the tree shows an optimum range, surely there must be a critical level below which one can confidently say the trees are suffering from a deficiency, but how can one be confident if the sampling error is so large?

Chapman (35) studied the variation of potassium in 4 adjacent Valencia orange trees from two different fertiliser plots. Forty spring-cycle leaves from fruit bearing twigs were picked from each tree and analysed for K. He found a "moderate" degree of variability in the 4 trees from both plots. Chapman also studied the variation of K by taking random samples from experimental plots scattered over 50 acres. He found some great differences which were ascribed to differences in the soil. Apparently no attempt was made to use statistical treatment in order to determine the sampling efficiency. Chapman based his sampling recommendations, as outlined above, on the results of these studies.

As regards pineapples, no statistical work carried out on the variation in leaf composition from plant to plant in a field, could be traced in the literature. Nightingale (38) merely stated that no great variations were found in the samples as taken by him.

It appears that both in South Africa and in the U.S.A., citrus trees are sampled for complete chemical analysis according to the procedures as laid down by Bathurst and by Chapman, based on nitrogen and potassium variance respectively.

It is felt that a sampling procedure should be based on the variance shown by ALL the nutrient elements, and it was mainly in order to clarify the anomalies as outlined above, that the work reported below was carried out.

2. THE SAMPLING OF CITRUS TREES FOR CHEMICAL ANALYSIS.

2.1: THE SAMPLING TECHNIQUE.

In the study presented here the sampling technique as worked out for citrus trees by Bathurst (34) and also recommended by Chapman (35), was followed. In this procedure the spring cycle leaves on fruit-bearing twigs are picked. In Fig. XVII such a twig with 4 typical leaves is shown.

There appears to be no doubt that these spring-cycle leaves (1-4) give a good indication of the nutrient status of citrus trees (34, 35). A special advantage is the fact that these leaves are definitely dated and consequently it is a relatively simple matter to pick leaves of the same physiological age. Both Bathurst and Chapman found differences in leaves picked from the top part of a tree as compared with leaves picked lower down, as well as in leaves picked from different sides of the tree. Consequently they recommended that leaves should be picked in a band around the circumference of the tree from positions near to the ground to as high as one can reach, the object being to obtain leaves representative of this type all around the tree.

Since there are great variations in size amongst the typical leaves appearing on fruit-bearing twigs, those on the southerly side of a tree normally being smaller than on the northerly side, as well as variations in size on the same twig (Fig. XVII), Chapman recommends that leaves of average size only should be picked. The position on the stem is immaterial, provided the leaf is spring-cycle (between the fruit and the spring node). In some cases no spring-cycle leaves will be found on fruit-bearing twigs, though usually there are from one to seven such leaves per twig. Only one of these is picked.

Chapman recommends that 20-25 such leaves should be picked from each of 10 representative trees in a given sampling location. He prefers to pick the leaves (from Valencia oranges) when they are 3-7 months old, i.e. July - September in the U.S.A. Bathurst recommends that 16 such leaves should be picked from each of 1-2% of the trees in an orchard, provided the chosen trees are widely scattered at random throughout the block. He prefers to sample Valencia oranges in the winter months May - July, because it was found that the composition of the leaves was most constant during this period.

Fig. XVII.

Fruit-bearing twig showing typical sampling leaves.

1, 2, 3 and 4 are spring-flush leaves and correct for sampling. Leaves 5, 6 and 7 are from previous flushes and incorrect for sampling. N is the node from which the spring flush commenced.

2.2: EXPERIMENTAL PROCEDURE.

It was decided to study the variations in N, P, K, Ca, Mg, Na, Fe, Mn, Zn and Cu from tree to tree by intensive sampling according to the technique as described above. Navel oranges only were studied and because the fruit of this variety ripens earlier than the Valencia orange, the sampling studies were carried out during April and May, that is a few months before harvesting time in the Eastern Cape.

In deciding upon the experimental procedure to be followed, the following factors had to be considered:- From previous work it was quite clear that large soil variations usually occur in an orchard, a factor which greatly influences the composition of the leaves. It was decided to control this factor as far as possible by selecting small blocks of trees on outwardly homogeneous soil areas. As in the case of soils (Part I), it was thought essential to carry out an intensive sampling procedure from tree to tree in order to approach the "true" nutrient status of a tree as closely as possible. If a relatively accurate figure for the variance is available, the use of statistics in order to estimate the degree of sampling intensity for a particular level of precision, is more valid. Intensive sampling would involve the handling of large numbers of leaf samples, a very perishable commodity, as was shown in Part II. The only alternative was, again, to select small sampling blocks that could be handled safely and efficiently. It was, of course, impossible to use a helper during the sampling work, since Bathurst (34) proved conclusively that different samplers picked samples which were significantly different in nitrogen content. The same applies to the complete analytical procedure, which should be carried out by the same analyst in order to avoid the introduction of constant personal errors.

It was eventually decided to use square sampling blocks of 16 trees, i.e. 4 x 4, covering an approximate area of 1/20 acre. By careful selection, it was thought that soil variations could be kept at a minimum over such a small area. It was found that a block of 16 trees could be sampled intensively (100 leaves/tree) within a period of 2 hours, from 8 a.m. - 10 a.m. Three sampling areas, all within 12 miles of Grahamstown, were selected so that the samples could be transported to the laboratory with a minimum of delay.

The following studies were carried out in the order given:-

(a) Diurnal variations in leaf composition.

(b) Variations in the leaves of a single tree.

(c) Variations from tree to tree in an old orchard (40 year trees).

(d) Variations from tree to tree in a young orchard (18 year trees).

(e) Variations from tree to tree in a neglected orchard.

(f) Variations from sampling area to sampling area in a large orchard.

2.3: DIURNAL VARIATIONS.

Many investigators have reported variations in the nutrient content of plant tissues to occur during a single day. Thus Ingalls and Shive (94) found that the iron concentration in the sap of several species, especially succulents, increased during the day by as much as 40% of its mean value, and decreased again at night. Penston (95, 96) found diurnal variations in the potassium content of potato leaves of between 2.5 and 4.1%, and in maize leaves of between 3.7 and 4.9%. Chapman (97) stated that diurnal variations in mineral content occurred in rubber leaves. Bolas and Goodall (2) found variations of up to 30% of the mean value of the ash content of tomato leaves during a 24 hour period, while Woodward et al (98) found the ash content of lucerne hay to vary by as much as 40% of the mean with the time of day. Phillis and Mason (99) found that the percentage content in cotton leaves of most of the mineral elements they studied decreased by day, but increased at night. No published data on diurnal variations in citrus leaves could be found.

These results would suggest that some control of the time of day at which samples are taken should be exercised. The following experiment was carried out in order to determine whether such changes occurred in citrus leaves:-

Because it was necessary to have trees as close to the laboratory as possible in order to carry out periodic samplings throughout the day, the experiment was carried out on 3 year-old lemon seedlings grown in the gardens of the Botany Department of the University. Two uniform trees were selected and on each tree, 15 branches, situated more or less in similar positions all around the tree, were marked. Leaf samples consisting of 15 average-sized leaves, one from each branch, were picked from each tree at the following times on a bright, sunny day, early in April:- 7 a.m. (sunrise); 10 a.m.; 12 noon; 2 p.m.; and 4.30 p.m. (direct sunlight obscured by buildings). As it happened, the following day was completely overcast, with intermittent light rain early in the morning. Another sample was taken at 12 noon on this day. The samples were washed, dried, ground and analysed for N, P, K and Ca as described in Part II. There was not sufficient material per sample to include analysis for the trace elements. The determinations were carried out in duplicate on each sample. Exactly the same pattern was found for both trees. The means of the results are recorded in Table 23.

TABLE 23.

DIURNAL VARIATIONS IN CITRUS (LEMON) LEAVES.

ELEMENT	SAMPLES TAKEN ON BRIGHT SUNNY DAY					% Increase 7-12	CLOUDY HUMID DAY
	7a.m.	10a.m.	12noon	2p.m.	4.30p.m.		
N %	2.93	3.05	3.06	2.99	2.97	4.3	2.95
P %	0.166	0.167	0.170	0.168	0.167	2.5	0.161
K %	1.54	1.34	1.60	1.54	1.52	4.0	1.53
Ca %	2.78	2.82	2.85	2.84	2.78	2.5	2.65

From these figures there appeared to be a systematic increase in the nutrient content from sunrise to noon followed by a drop in the values towards sunset, reaching more or less the same figures at sunset as those prevailing at sunrise. These increases were relatively small and if the sampling error is taken into account, they may not be significant. The fact that similar results were obtained for both trees, though would indicate that the increases were real. Whether these values represent an actual increase in nutrient content, or whether they were due to some alteration in the dry weight of the material during the day, is difficult to prove conclusively. It is interesting to note that on the following day, when the sky was heavily overcast and the leaves received no direct sunlight, the nutrient content at noon was more or less the same as at sunset on the previous day, which would suggest that the changes may be due to the photosynthetic process.

At any rate, it seems clear that the safest procedure would be to take the leaf samples at more or less the same time of day; that all the leaf samples for one block should be collected on that day; and that future samples should be collected under the same climatic conditions.

All the samplings in the studies reported below were carried out between 8 - 10 a.m. on bright, sunny, calm mornings.

2.4: INTERNAL VARIATIONS AMONGST THE LEAVES.

From the studies of Bathurst (34) and Chapman (35) it seems clear that significant differences in composition occur in typical leaves taken from various parts of the tree. Consequently, particular care was taken to sample the leaves as recommended, namely all around a tree in a band from about 1 foot off the ground to a height of about 7 feet. However, two aspects of leaf variation had to be

investigated more fully before the sampling blocks could be tackled:- Differences in the composition of small and large leaves, and the number of leaves to be sampled per tree for adequate representation of all 10 elements studied.

2.41: VARIATIONS IN SMALL & LARGE LEAVES.

Big variations in the size of the spring-cycle leaves occur on the same tree, even on the same fruit-bearing twig, the leaves immediately behind the fruit often being smaller than those higher up the twig. Chapman (35) carried out analyses of the major elements on such leaves, made up of composite samples (large leaves and small leaves) collected from 8 trees. Although he found differences in the composition of small and large leaves, he described these differences as doubtful. The values for K differed by about 5%.

A similar experiment was carried out, except that only one tree was used in order to eliminate inherent tree variations. A large uniform tree was selected in a block of Navels (40 year old trees) and 100 fruit-bearing twigs were marked all around the tree in the 1-7 foot band. From each twig, one spring-cycle small leaf and one spring-cycle large leaf were picked, giving two composite samples of 100 leaves. Each sample was analysed in duplicate for the 10 elements. The means of the results are recorded in Table 24.

TABLE 24.

ANALYSIS OF SMALL AND LARGE LEAVES.

FACTOR	% N	% P	% K	% Ca	% Mg	% Na	ppm Fe	ppm Mn	ppm Zn	ppm Cu
Small Leaves	2.56	0.112	0.72	5.17	0.43	0.54	88	27	16	3.4
Large Leaves	2.56	0.114	0.56	5.17	0.43	0.51	95	31	19	4.1
% Dif.	0	-1.8	+22	0	0	+6	-8	-15	-19	-21

Since particular care was taken to keep sampling errors to a minimum by picking the two leaf types from the same fruit-bearing twigs on the same tree, it must be concluded that some of these large differences are significant. It would appear, therefore, that the small leaves contained a significantly higher potassium concentration, but significantly lower concentrations of the heavy trace metals, manganese, zinc and copper, than the large leaves.

Whether this fact has any physiological significance is immaterial. What is important is that it clearly confirms Chapman's recommendation that only leaves of average size should be picked. This procedure is not always so easy and simple as it appears to be and it must be emphasised that only a person thoroughly familiar with the procedure should pick leaves intended for analysis.

2.42: THE NUMBER OF LEAVES PER TREE TO CONSTITUTE A REPRESENTATIVE SAMPLE.

It was necessary to determine how many leaves must be picked from a single tree in order to approach the "true values" of the 10 elements in the typical leaves of a tree as closely as possible.

Bathurst (34) carried out the following experiment on nitrogen in the leaves:- Two trees were selected, one from a healthy orchard, the other from a poor one. Twenty separate samples, each consisting of all the spring-flush leaves from the "fruit-stalk" of a single in-season fruit, were picked from the healthy tree and 16 from the poor tree, each sample consisting of 4-6 leaves, 10 months old. Nitrogen was determined in each of the samples. By using the formula of Brown (90), Bathurst calculated that only 3 fruit-stalks need to be sampled in order to represent nitrogen to within 10% of the mean in the case of the healthy tree, while the sampling of 7 "fruit-stalks" was necessary in the case of the poor tree. He eventually recommended that 16 leaves should be picked per tree, while Chapman (35) recommends the picking of 20-25 leaves per tree.

The author carried out the following experiments:-

(a) Two large, healthy trees were selected in the heavy bearing orchard of 40 year old Navels. All the easily accessible, average-sized spring cycle leaves on fruit-bearing twigs were counted, numbering about 800 leaves on both trees. Three samples were picked from these leaves on both trees:- 25 leaves (all around the tree in the 1-7' band), 100 leaves (as above), and the remaining approximately 650-700 leaves. The 6 samples so obtained were analysed in duplicate for the 10 elements. The mean results are recorded in Table 25.

(b) Sixteen adjacent trees were selected in the 18 year old heavy bearing orchard (Section 2.5) and two composite samples were picked, the one consisting of 25 leaves from each of the 16 trees and the other 100 leaves from each of the 16. There were about 400 of the average-size spring-cycle leaves in the 1-7 foot band on each of these trees. The mean results for the 10 elements are recorded in Table 26.

Whether this fact has any physiological significance is immaterial. What is important is that it clearly confirms Chapman's recommendation that only leaves of average size should be picked. This procedure is not always so easy and simple as it appears to be and it must be emphasised that only a person thoroughly familiar with the procedure should pick leaves intended for analysis.

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TABLE 25.

SINGLE TREE SAMPLINGS.

(LARGE TREES)

ELEMENT	TREE NO. 1					TREE NO. 2				
	1 675/800 leaves 84%	2 100/800 leaves 12%	3 25/800 leaves 3%	% Age 1-2	Difference 1-3	1 675/800 84%	2 100/800 12%	3 25/800 3%	% Age 1-2	Difference 1-3
N %	2.59	2.62	2.68	1%	3%	2.78	2.76	2.70	1%	3%
P %	0.118	0.119	0.114	1	3	0.112	0.110	0.116	2	4
K %	0.56	0.55	0.60	2	8	0.50	0.49	0.52	2	4
Ca %	3.62	3.60	3.59	0.5	1	3.50	3.47	3.46	1	1
Mg %	0.52	0.58	0.47	12	10	0.51	0.51	0.52	0	2
Na %	0.32	0.36	0.30	13	7	0.34	0.36	0.32	6	6
Fe ppm	84	84	89	0	7	79	79	83	0	5
Mn ppm	27	24	23	10	12	21	20	20	5	5
Zn ppm	10	11	9	10	10	9	8	9	10	0
Cu ppm	5.2	5.7	5.0	10	4	5.7	5.8	6.0	2	6
	MEAN			6%	6%				3%	4%

TABLE 26.

COMPOSITE SAMPLES FROM 16 TREES.

(MEDIUM SIZE TREES)

ELEMENT	A 100 LEAVES FROM EACH OF 16 100/400 25%	B 25 LEAVES FROM EACH OF 16 25/400 6%	PERCENTAGE DIFFERENCE A - B
N %	2.82	2.77	2%
P %	0.128	0.131	2
K %	0.76	0.78	3
Ca %	5.06	5.12	1
Mg %	0.34	0.36	6
Na %	0.20	0.19	5
Fe ppm	183	188	3
Mn ppm	44	48	8
Zn ppm	19	17	10
Cu ppm	4.5	3.9	14
		MEAN	5%

By considering the figures in Tables 25 and 26, and comparing the differences with the coefficients of variation reported for the analytical methods in Part II, it is clear that no significant differences are obtained in leaf composition, by sampling all the typical accessible leaves, or by sampling 100 of them, or by sampling only 25, provided, of course, the leaves are selected with due care. Provided the leaves are thus carefully selected, a 25 leaf sample will represent most of the elements to within 10% of the "true values" in a tree. However, to be on the safe side, it is recommended that about 30 - 50 leaves per tree be picked when analysing for the trace elements.

In order to obtain a reliably reproducible sample, as well as to obtain sufficient material for accurate duplicate analysis, it was decided to pick 100 leaves per tree during the subsequent studies.

2.5: THE SAMPLING OF CITRUS ORCHARDS.

2.51:- EXPERIMENTAL:-

The reasons for selecting small blocks of trees have been outlined in Section 2.2.

Three Navel orange orchards on different soil types, situated around Grahamstown, were selected. A brief description of these orchards are given below:-

FACTOR	BLOCK A	BLOCK B	BLOCK C *
Variety	Navel	Navel	Navel
Age	40 years	18 years	25 years
Planting	23 x 23 ft.	15 x 25 ft.	23 x 23 ft.
Description	Large, healthy	Medium, healthy	Neglected, poor
Average yield	534 lbs/tree	250 lbs/tree	90 lbs/tree
Situation	Belmont Valley, Grahamstown.	Kariega Valley, Grahamstown.	Belmont Valley, Grahamstown.
Soil Type	Red-brown alluvial	Brown sandy loam	Grey sandy loam
Soil pH	8.0	6.8	6.2
Irrigation	Basin system	Basin system	Basin system
Cultivation	Clean	Clean	Clean
Fertiliser	N + Manure	N + P + Manure	None since 1948
Leaf symptoms **	Zn deficiency	Mg deficiency	Mg, Cu, Zn deficiency
Sampling block	16 trees, 4 x 4	16 trees, 4 x 4	8 trees, 2 x 4
No. of typical leaves per tree	± 800	± 400	± 100
Sampling date	April	May	April
Sampling time	8 - 10 a.m.	8 - 10 a.m.	8 - 10 a.m.

* -- Block C was an 8-tree control plot in an experimental orchard used by the Citrus Exchange in this area.

** -- Typical leaf symptoms were identified by means of the excellent manual by Bryan (100) on the subject.

The Blocks were carefully selected in order to contain outwardly homogeneous trees of average size. Special attention was paid to the physical characteristics of the soil, slope, etc., during the selection. Leaf samples (100 typical leaves per tree for Blocks A & B and 70 for C) were picked from each tree in a Block with the utmost care to limit any personal bias to the minimum. After complete sampling of a Block, the samples were transported to the laboratory as quickly as possible where they were placed in polythene containers inside a refrigerator. The samples were removed one by one for washing, this process being completed on the same day when the samples were collected. After drying and grinding the samples were analysed in duplicate for the 10 elements as described in Part II. Where duplicates differed by more than the average coefficient of variation (Part II), the analysis was repeated (a procedure which was seldom necessary). Every effort was made to analyse the samples as quickly as possible - by working 15 hours a day during this stage, 6 replicates (3 samples) could be analysed in 2 days. The complete procedure, washing, drying, grinding and analysis, for each Block was completed within 2 weeks. It was considered that during this short period no deterioration in the samples could have taken place, taking into account the careful way in which they were handled and stored. The differences recorded in the tables below may, therefore, confidently be ascribed to the internal variations which normally occur from tree to tree in a small block of citrus trees.

The results for the three Blocks are recorded in Tables 27, 29 and 31 while Tables 28, 30 and 32 contain the statistical treatment, using the formula $n = 2t^2d^2/D^2$ as described in Part I. In calculating the statistics recorded in the latter tables, the coefficient of variation for each element was adjusted by subtracting the analytical variance as determined in Part II from the variance as found in each block of trees. It was felt that this treatment would account for the analytical error and that the adjusted figure would be a true reflection of the variance of the elements in a block of trees.

The final statistics for the 3 orchards are summarised in Table 33, while Fig. XVIII shows the minimum number of trees to be sampled in the 3 orchards to represent the various elements to within 10% of the mean values at a probability level of 19 to 1.

The statistical formula was used in reverse in order to estimate the error in the nutrient values if only 1 tree was sampled in each of the 3 blocks.

In this case, $D = \sqrt{2t^2d^2/n}$

$= \sqrt{2t^2d^2}$

These results are recorded in Table 34.

In order to estimate the variations likely to occur in a large orchard, a block of 1000 trees was selected in the vigorous, young orchard B, i.e. the orchard which showed the least variation of the three. Five sampling spots were chosen at random and composite samples from 8 trees (100 leaves/tree) in each sampling spot were picked and analysed as before. The results are recorded in Table 35.

TABLE 27.

BLOCK A: HIGH-YIELDING 40-YEAR OLD NAVELS.

TREE NUMBER	N %	P %	K %	Ca %	Mg %	Na %	Fe ppm	Mn ppm	Zn ppm	Cu ppm
1	2.45	0.116	0.38	3.73	0.56	0.53	89	27.3	16	8.1
2	2.54	0.116	0.36	3.70	0.55	0.39	82	22.8	11	5.4
3	2.58	0.115	0.47	3.07	0.53	0.43	83	21.0	12	7.2
4	2.60	0.126	0.47	4.11	0.23	0.44	88	18.8	12	7.6
5	2.60	0.118	0.44	3.93	0.58	0.41	83	32.5	11	6.1
6	2.62	0.119	0.56	3.60	0.58	0.36	84	23.8	11	5.7
7	2.58	0.116	0.57	3.89	0.35	0.32	71	20.3	11	6.0
8	2.65	0.113	0.45	3.55	0.55	0.46	72	18.3	11	5.0
9	2.84	0.118	0.33	3.31	0.49	0.72	90	30.6	9	3.5
10	2.62	0.115	0.51	4.08	0.49	0.31	71	21.0	10	4.7
11	2.76	0.110	0.49	3.47	0.51	0.36	79	20.3	8	5.8
12	2.93	0.115	0.51	3.61	0.45	0.36	76	24.3	7	5.8
13	2.58	0.117	0.49	3.59	0.49	0.45	86	24.5	14	6.5
14	2.74	0.114	0.62	3.59	0.43	0.33	79	22.0	9	5.7
15	2.83	0.115	0.56	3.50	0.40	0.32	77	20.6	8	5.5
16	2.90	0.127	0.49	3.44	0.44	0.27	71	26.1	9	6.1
Mean	2.68	0.117	0.48	3.63	0.48	0.40	80	23.4	11	5.9
Standard Dev.	0.14	0.004	0.08	0.27	0.09	0.11	6	4.1	2.3	1.2
Coeff. of Var. %	5.3	3.7	16.4	7.5	19.3	27.1	8.1	17.4	21.5	18.7

TABLE 28.

BLOCK A: MINIMUM NUMBER OF TREES TO SAMPLE IN ORDER TO SHOW SIGNIFICANCE ON A 5% LEVEL IN DIFFERENCES EXCEEDING D% OF THE MEAN VALUES.

ELEMENT	COEFF. OF VARIATION (ADJUSTED)	D = 5%	D = 10%	D = 20%
Nitrogen	5.3	11	3	1
Phosphorus	3.6	5	2	1
Potassium	15.8	91	23	6
Calcium	7.2	19	5	2
Magnesium	18.3	122	31	8
Sodium	27.1	267	67	17
Iron	8.0	24	6	2
Manganese	16.9	104	26	7
Zinc	20.0	146	37	10
Copper	18.3	122	31	8

TABLE 29.

BLOCK B: HIGH-YIELDING 18-YEAR OLD NAVELS.

TREE NUMBER	N %	P %	K %	Ca %	Mg %	Na %	Fe ppm	Mn ppm	Zn ppm	Cu ppm
1	2.80	0.112	0.60	5.57	0.30	0.24	140	36	20	4.4
2.	2.84	0.120	0.71	5.32	0.22	0.22	140	46	19	4.7
3.	2.80	0.122	0.74	5.73	0.22	0.19	190	48	18	5.7
4	2.81	0.127	0.85	5.00	0.38	0.18	197	51	16	5.1
5	2.96	0.132	0.83	4.63	0.41	0.21	217	42	17	4.9
6	2.82	0.141	0.72	4.74	0.40	0.22	244	39	21	6.4
7	2.74	0.132	0.83	4.82	0.40	0.20	163	43	19	4.5
8	2.77	0.136	0.83	4.71	0.41	0.17	174	49	18	4.3
9	2.80	0.127	0.85	5.00	0.38	0.18	197	51	16	5.1
10	2.84	0.132	0.74	5.73	0.22	0.19	190	48	18	5.7
11	2.96	0.122	0.83	4.63	0.41	0.21	217	42	17	4.9
12	2.81	0.120	0.71	5.32	0.22	0.22	140	46	19	4.7
13	2.80	0.141	0.72	4.74	0.40	0.22	244	39	21	6.4
14	2.77	0.112	0.60	5.57	0.30	0.24	140	36	20	4.4
15	2.74	0.136	0.83	4.71	0.41	0.17	174	49	18	4.3
16	2.82	0.132	0.83	4.82	0.40	0.20	163	43	19	4.5
Mean	2.82	0.128	0.76	5.06	0.34	0.20	183	44	19	5.0
Standard Dev.	0.06	0.009	0.08	0.41	0.08	0.02	35	5	1.6	0.7
Coeff. of Var. %	2.2	7.1	11.0	8.1	23.8	11.4	19.2	11.4	8.6	14.0

TABLE 30.

BLOCK B: MINIMUM NUMBER OF TREES TO SAMPLE IN ORDER TO SHOW SIGNIFICANCE ON A 5% LEVEL IN DIFFERENCES EXCEEDING D% OF THE MEAN VALUES.

ELEMENT	COEFF. OF VARIATION (ADJUSTED)	D = 5%	D = 10%	D = 20%
Nitrogen	2.2	2	1	1
Phosphorus	6.8	17	5	2
Potassium	10.8	43	11	3
Calcium	8.1	24	6	2
Magnesium	23.5	201	51	13
Sodium	11.4	48	12	3
Iron	19.2	134	34	9
Manganese	11.4	47	12	3
Zinc	7.9	23	6	2
Copper	13.6	68	17	5

TABLE 31.

BLOCK C: LOW-YIELDING 25-YEAR OLD NAVELS.

TREE NUMBER	N %	P %	K %	Ca %	Mg %	Na %	Fe ppm	Mn ppm	Zn ppm	Cu ppm
1	2.63	0.143	0.42	5.83	0.02	0.28	128	31.5	9	3.0
2.	2.44	0.137	0.40	5.74	0.07	0.40	122	30.1	9	3.4
3.	2.17	0.154	0.47	5.23	0.15	0.38	119	31.5	15	2.9
4.	2.48	0.141	0.33	5.24	0.04	0.43	106	26.2	11	2.8
5	2.35	0.147	0.44	4.48	0.08	0.30	106	30.7	15	3.0
6	2.58	0.143	0.47	4.96	0.07	0.34	111	28.9	10	2.5
7	2.73	0.155	0.69	4.14	0.23	0.30	142	29.8	10	3.4
8	2.49	0.160	0.64	4.71	0.09	0.32	113	32.1	13	3.0
Mean	2.48	0.147	0.48	5.04	0.09	0.34	118	30.1	11	3.0
Standard Dev.	0.17	0.008	0.12	0.59	0.07	0.05	12	1.9	2.6	0.3
Coeff. of Var. %	7.0	5.4	25.4	11.7	71.4	15.9	10.4	6.3	23.6	9.9

TABLE 32.

BLOCK C: MINIMUM NUMBER OF TREES TO SAMPLE IN ORDER TO SHOW SIGNIFICANCE ON A 5% LEVEL IN DIFFERENCES EXCEEDING D% OF THE MEAN VALUES.

ELEMENT	COEFF. OF VARIATION (ADJUSTED)	D = 5%	D = 10%	D = 20%
Nitrogen	7.0	22	6	2
Phosphorus	5.4	14	4	1
Potassium	25.2	284	71	18
Calcium	11.6	61	16	4
Magnesium	71.4	2281	571	143
Sodium	15.8	107	28	7
Iron	10.3	48	12	3
Manganese	6.1	17	5	2
Zinc	23.2	241	61	16
Copper	8.5	33	9	3

TABLE 33.

MINIMUM NUMBER OF TREES TO SAMPLE IN A BLOCK FOR A GIVEN DEGREE OF REPRESENTATION AT THE 5% POINT.

ELEMENT	BLOCK A			BLOCK B			BLOCK C		
	Mean Value	10%	20%	Mean Value	10%	20%	Mean Value	10%	20%
Nitrogen	2.68	3	1	2.82	1	1	2.48	6	2
Phosphorus	0.117	2	1	0.128	5	2	0.147	4	1
Potassium	0.48	23	6	0.76	11	3	0.48	71	18
Calcium	3.63	5	2	5.06	6	2	5.04	16	4
Magnesium	0.48	31	8	0.34	51	13	0.09	574	143
Sodium	0.40	67	17	0.20	12	3	0.34	28	7
Iron	80	6	2	183	34	9	118	12	3
Manganese	23.4	26	7	44	12	3	30.1	5	2
Zinc	11	37	10	19	6	2	11	61	16
Copper	5.9	31	8	5.0	17	5	3.0	9	3
AVERAGE		24	7		16	5		79	20

TABLE 34.

PERCENTAGE DIFFERENCE FROM THE MEAN VALUE AT THE 5% POINT BY SAMPLING 1 TREE FROM EACH BLOCK.

ELEMENT	BLOCK A %	BLOCK B %	BLOCK C %
Nitrogen	16	7	24
Phosphorus	11	21	19
Potassium	48	33	85
Calcium	22	25	39
Magnesium	56	71	239
Iron	25	58	35
Manganese	51	35	21
Zinc	61	24	78
Copper	56	41	29

TABLE 35.

RANDOM SAMPLES IN A BLOCK OF 1000 TREES: 18-YEAR OLD NAVELS.

SAMPLE NUMBER	N %	P %	K %	Ca %	Mg %	Na %	Fe ppm	Mn ppm	Zn ppm	Cu ppm
1	2.81	0.120	0.72	5.40	0.28	0.21	167	45	18	5.0
2	3.02	0.137	0.67	3.70	0.46	0.24	159	37	14	3.2
3	2.84	0.145	0.49	4.03	0.50	0.28	168	40	18	4.5
4	3.00	0.128	0.55	4.50	0.36	0.20	167	41	14	3.6
5	2.95	0.130	0.60	4.00	0.45	0.20	160	35	15	3.2
Mean	2.92	0.132	0.61	4.33	0.41	0.21	164	40	16	3.9
Standard Dev.	0.09	0.009	0.09	0.67	0.08	0.04	4	4	2.1	0.8
Coeff.of Var. %	3.3	7.2	15.2	15.5	19.3	18.6	2.7	10.0	13.1	21.0
Min.No. of spots to sample for 5%	7	32	142	148	230	213	5	62	106	272
Number for 10%	2	8	36	37	58	54	2	16	27	68
Number for 20%	1	2	9	10	15	14	1	4	7	17

AVERAGE FOR 10% REPRESENTATION: 31 localities = 248 trees (± 25%)

AVERAGE FOR 20% REPRESENTATION: 8 localities = 64 trees (± 7 %)

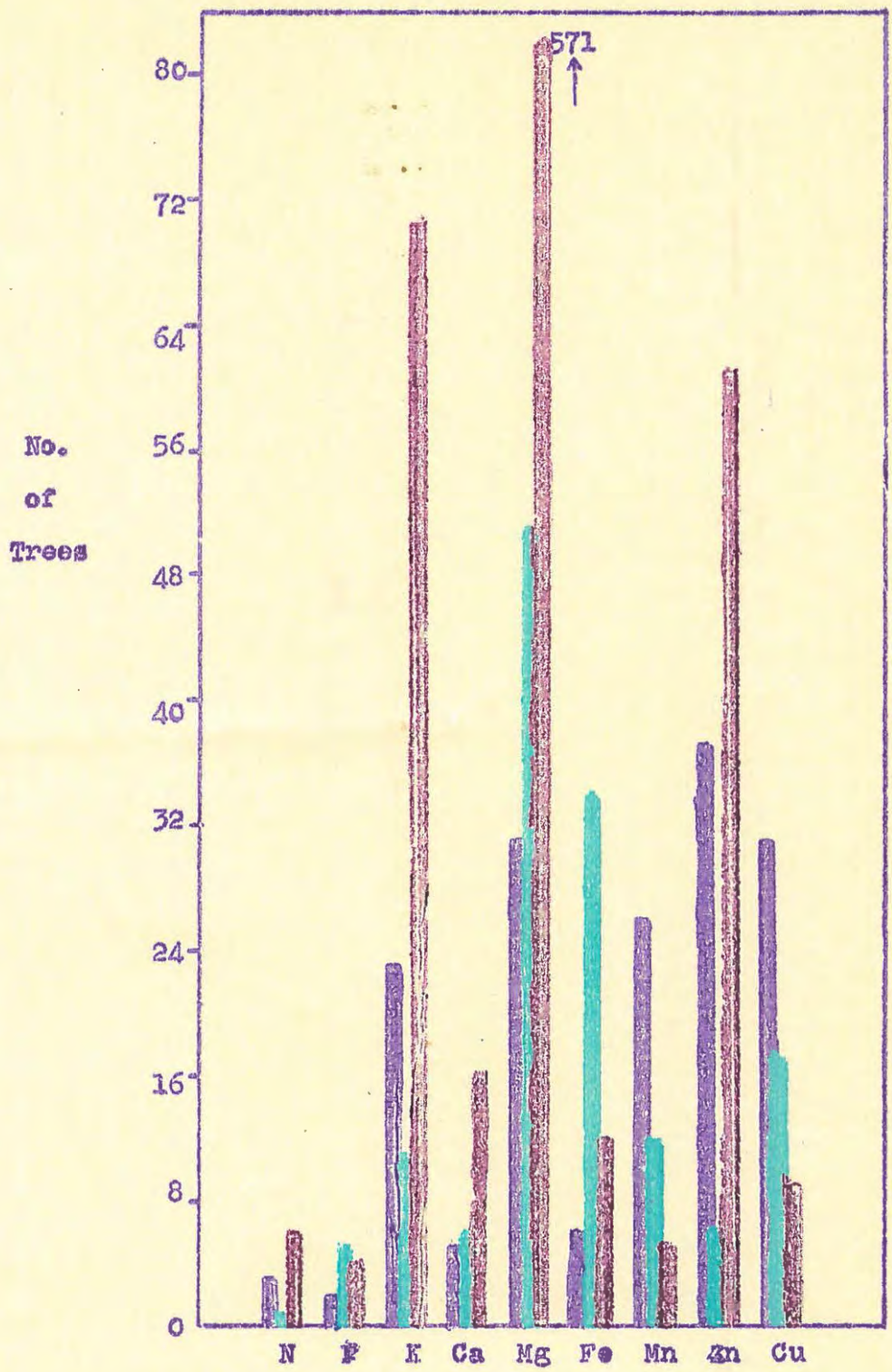


Fig. XVIII.

Minimum number of citrus trees to sample in uniform blocks in order to represent the nutrients to within 10 % of the mean values on a probability level of 19 to 1.

Block A Block B Block C

2.52: DISCUSSION.

The results recorded in the tables above speak for themselves and need not be discussed in detail. Only a few of the more important points will be dealt with.

As was to be expected, the poor orchard (Block C) showed the greatest degree of variation, despite the fact that particular care was taken to select a plot containing 8 fairly homogeneous trees as far as outward appearances were concerned. The vigorous young orchard (Block B) showed the least degree of variation. Nitrogen and phosphorus usually showed considerably less variation than the other elements studied, while potassium and magnesium usually showed the greatest degree of variation, followed by some of the trace elements like zinc, copper and manganese. When the concentration of an element was at a deficient level, as concluded from Critical Nutrient Tables (35), the variation was exceptionally large, for example magnesium in Block C, and zinc in Blocks C and A as compared to Block B, where zinc was apparently present in sufficient supply. It is interesting to note that where artificial phosphorus was applied (Block B), the leaf P apparently varied to a greater extent from tree to tree than in Block A where organic P (manure) was applied.

From Table 33, where the final statistics for the 3 orchards are grouped together, it is evident that if all the nutrient elements are to be determined in a sample, and this seems to be the most satisfactory procedure when interpreting leaf analysis data, even sampling 100% of the trees in a small block may fail to represent some of these nutrients to within 10% of the general mean values on a 19 to 1 probability level. In the poor orchard, Block C, potassium, magnesium and zinc would not even be represented to within 20% of the mean values if all the trees in the block are sampled. This, of course, is due to the large variations shown by these elements from tree to tree, as can be seen from Table 31. This orchard represents the type of problem to which leaf analysis would normally be applied in order to determine whether the trouble lies in nutrient unbalance. From Table 33 it is seen that an average of 7 trees in Block A, 5 in Block B, and all the trees in Block C, must be sampled to represent the majority of the elements to within 20% of the mean values at the 5% point.

The only conclusion that can be reached is that when a poor orchard is analysed, all the trees in a sampling locality must be sampled and that differences in most of the nutrient values of less than 20% should not be regarded as significant. This, of course, makes the interpretation of leaf analysis data rather difficult, even where the results are not "border-line". Take the case of zinc, for

example. If a value of 18 p.p.m. is obtained for a sample from a poor orchard, then, by the above reasoning, the correct value may as likely be 13 p.p.m. (acute deficiency (35)) as 24 p.p.m. (considered to be in the "optimum range" (35)). Such exhaustive sampling is never carried out for advisory purposes - it would not be practicable. If, then, doubt exists for zinc in a case where all the trees were sampled, how much more so if only 1-2% of the trees were sampled as is usually recommended?

In order to illustrate the ease with which faulty conclusions may be drawn if only a small percentage of the trees in a given orchard is sampled, Table 34 was drawn up. In this Table the probable error at the 5% point if only 1 tree in each block was sampled, is recorded. From these figures it is obvious that the odds are 1 to 19 against arriving at correct conclusions in all cases with the exception possibly of nitrogen in Blocks A and B and phosphorus in Blocks A and C. The chances are equally favourable of obtaining a very low value or a very high one. The probable range of the nutrients is exceptionally large in the case of the poor orchard, Block C. That the formula used in calculating the figures recorded in Table 34 is perfectly sound may be seen by comparing the highest and lowest values for each nutrient in Tables 27, 29 and 31. It must be concluded, therefore, that sampling only a small percentage of the trees in an orchard, as is usually recommended, may lead to entirely faulty interpretations as regards some of the nutrients, particularly if the orchard is a poor one.

The problem of sampling large blocks of trees must now be discussed. Many large orchards cover several tens of acres. If the trees in an orchard are perfectly uniform and if no large soil variations occur, then it is reasonable to assume that if, say, 10 trees must be sampled to represent a block of 16 trees to within 10% of the mean nutrient values, 10 randomised trees in a block of 1000 would also be sufficient. However, it is probably seldom that such a uniform distribution does in fact occur in a large orchard. Such orchards usually include large variations in the soil (35).

In order to arrive at an estimate of the variations likely to occur in a large block of trees, the results recorded in Table 35 may be studied. This Table gives the variations obtained from 5 randomised localities in a block of 1000 trees (orchard B). Eight trees (100 leaves per tree) were sampled in each locality, giving a total of 40 trees from the whole block. It must be emphasised that the statistics recorded in the last part of Table 35 are not strictly correct, since no attempt was made to determine the "true" coefficients of variation by sampling all 1000 trees. Nevertheless, it is evident that large variations occurred from locality to locality, particularly if these

are compared with the variations recorded for Block B in Table 29.

If it is assumed that the estimates of the coefficients of variation for the nutrients as recorded in Table 35 are reasonably representative, it is seen that an average of 31 localities, representing 248 trees, must be sampled in the block of 1000 trees to represent all the nutrients to within 10% of the mean values at the 5% probability level. Eight localities (64 trees) must be sampled in order to represent all the elements to within 20% of the mean values. In contrast, only 2 localities (16 trees) need be sampled in the block to represent nitrogen to within 10% of the mean value.

It must be remembered, however, that Block B was a particularly uniform one from outward appearances. Where the trees are not uniform, as for example in Block C, it may be taken that a still greater intensity of sampling would be necessary for adequate representation. However, the adoption of an intensive sampling programme for a large block of trees would appear to be out of the question, both from the point of the excessive amount of labour involved as well as the large number of leaves which would present a real problem during the analytical procedure. Moreover, there are serious arguments against the validity of taking a composite sample from a large orchard.

If a composite sample is taken from a large orchard by sampling a certain percentage of the trees, it is necessary to consider on what principle to select the individual trees. If the yield response to fertiliser treatment were linearly related to the nutrient content in the leaves, the matter would be relatively simple, for a random sample of the trees would give a correct indication of the response of the orchard as a whole. However, there appears to be doubt about this and most critical nutrient tables contain an optimum range, the so-called "luxury consumption" of plants (2); a range in which no apparent response is shown by plants with an increase in concentration of a particular nutrient. If this fact is true, then a random sample from an orchard in which some of the trees only are suffering from a nutrient deficiency will very probably contain enough material from trees in the "luxury consumption" range to push the mean nutrient content of the whole bulked sample into this range (93). Such observations have been made on apple trees, for instance, by Burrell et al (101). Some investigators recommend that samples should be taken from plants appearing typical of the area covered. Bathurst (34), too, in taking samples of citrus trees, advises that they should be taken from trees "representative of the block as a whole". This procedure, of course, involves a subjective estimate of what is typical or representative, and therefore may, to some extent, be distrusted; moreover, it hardly covers the possibility of the area having a minority of trees with a deficiency, among others with satisfactory nutrition.

A similar difficulty arises when selecting the leaves on individual trees. Some leaves on a tree may show typical deficiency symptoms while others appear to be perfectly healthy - which of these must be picked? There is no doubt that where the symptom is known to be caused by a particular element, the leaves showing these symptoms contain a very small concentration of that element, as was shown to be the case for zinc by Eve (102), for instance. However, leaves showing no pronounced symptoms appearing on the same tree as leaves depicting characteristic symptoms, are usually also low in that particular element - a fact established by the author. In order not to influence the concentration of the other nutrients, it appears best not to pick mottled leaves - the chlorotic spots may influence the nitrogen, magnesium and possibly other nutrient concentrations.

From the above discussion, then, it appears that taking a composite sample from a large orchard in order to obtain an idea of the nutrient status of the orchard as a whole, is not a reliable procedure. Rather should the orchard be divided into a number of smaller units which can be dealt with more satisfactorily from an intensive sampling point of view. Such a procedure is outlined in the suggestions for sampling below.

2.53: SUGGESTIONS FOR SAMPLING CITRUS TREES FOR LEAF ANALYSIS PURPOSES.

From the results recorded above it is clear that no standard sampling programme can be laid down to cover all cases. It is advisable to carry out a thorough preliminary survey of an orchard before deciding upon a final procedure. However, the following general suggestions may be given as a result of the sampling studies described above. These suggestions are made on the assumption that complete leaf analysis, covering all the nutrients, will be carried out:-

(1) Only a thoroughly trained and experienced operator should take the leaf samples.

(2) The samples for a particular study should be picked under the same climatic conditions, preferably between 8-10 a.m. on sunny days. Because of constancy of nutrient concentrations, the autumn and winter periods appear to be the best for sampling.

(3) Great care should be exercised when selecting the typical leaves to be picked (Section 2.1). When the trees are large and healthy, at least 50 average-sized spring-cycle leaves on fruit-bearing twigs should be picked, all around the tree in a band 1-7 feet from the ground. When the trees are medium sized and healthy, 25 leaves per tree will usually suffice. If the trees are in obvious poor condition, all the average-size typical leaves, or at least 70 (depending on the number on the tree), should be picked. Avoid picking leaves with obvious symptoms of deficiency, but note this condition for comparison with the analytical figures.

(4) Poor orchards need much more intensive sampling for adequate representation than healthy orchards.

(5) With the exception of nitrogen and phosphorus, and depending on the intensity of sampling, differences of less than 20% in the nutrient values at the 5% point should not be treated as significant.

(6) If a deficiency is suspected an intensive sampling procedure would be necessary to prove it conclusively.

(7) All the trees in experimental plots for research purposes should be sampled, when carrying out leaf analysis studies.

(8) When sampling large orchards, the following procedure is suggested:- Carry out a preliminary survey of the orchard, noting in particular the size and condition of the trees in various localities. If possible, dig a few scattered profile pits in order to establish large soil variations. Divide the orchard into a number of small blocks (50 - 100 trees), grouping together as far as possible all similar factors, such as tree size, healthy and poor trees, soil characteristics, such as type, slope, drainage, etc. Each small block must be sampled separately and as intensively as circumstances will allow. It is suggested that at least 20% of the trees in each block should be sampled. The results will indicate if a more intensive sampling is later necessary in certain localities. By analysing the samples obtained from each small block separately a fairly good estimate should be obtained of nutrient conditions in various parts of the orchard.

3: THE SAMPLING OF PINEAPPLE PLANTS FOR CHEMICAL ANALYSIS.

3.1: THE SAMPLING TECHNIQUE.

In this study the sampling technique as worked out for pineapple plants by Sideris et al at the Pineapple Research Institute of the University of Hawaii (36) and later also recommended by Nightingale (38), was followed. In this procedure the longest of the leaves produced after planting are detached from the stem of the plant and the basal white, non-chlorophyllous portion of the leaf is cut off and kept for analysis. With some experience it is easy to recognise and select these socalled "active" leaves (36).

In Fig. XIX a schematic representation is given of the leaf types usually found on a pineapple plant before reaching the fruiting stage.

A brief description of these leaves follows:-

A: Old leaves originally present on the planting material.

B: Also original leaves, but showing some new growth.

C: These are the most mature of the new leaves produced after planting. They have broad bases in relation to the rest of the leaf, with pronounced lateral points forming acute angles. They contain little or no succulent-brittle white tissue, the white tissue being mainly very fibrous.

D: The "active" or longest of the new leaves produced after planting. These are selected for sampling. The leaf-base consists of succulent-brittle white tissue, slightly broader than the green blade proper, and with non-expanded lateral points. They slant at an angle of more than 45° from the vertical.

E: The immature or shoulder leaves. The base is narrower than the green blade proper and it contains great amounts of succulent-brittle white tissue.

F: The youngest or apical leaves with large amounts of white tissue.

After blossom buds appear, all the leaves on the original stem become mature and no further analysis are carried out in Hawaii after this stage.

In sampling the "D" leaves, the following procedure was adopted:- The plant was held firmly with the left hand, while the proper leaf was grasped in the right hand as close to the stem as possible. The leaf was worked loose by gentle sideways pushing and pulling until it broke off close to the point of attachment to the stem. The white basal portion was cut off with a pair of stainless steel scissors and kept for washing and further treatment as described in Part II.

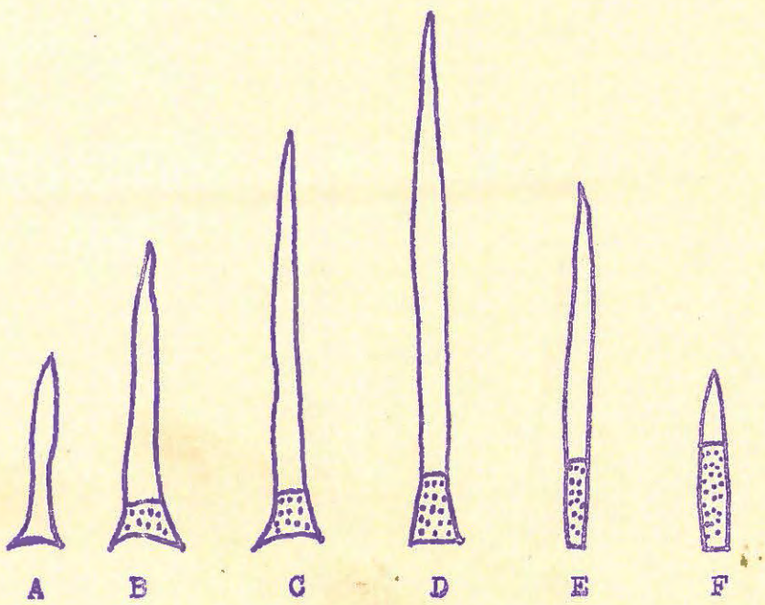


Fig. XIX.

Typical leaves of a non-fruit bearing pineapple plant.

A and B: Old leaves, originally present on planting material.

C: Fully mature leaf, produced after planting.

D: "Active" or longest leaves, correct for sampling.

E: Immature shoulder leaves.

F: Youngest or apical leaf.

▩ White non-chlorophyllous basal portion.

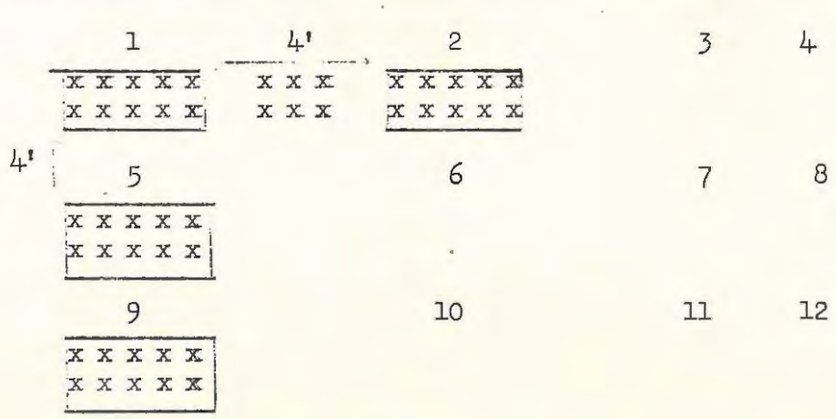
There appears to be no doubt from the results reported by the Hawaiian workers (36, 37, 38) that this leaf material is a good indicator of the nutrient status of the pineapple plant. A particular advantage, as in the case of citrus leaves, is the fact that these leaves are dated and it is a relatively simple matter to pick leaves of the same physiological age.

No reference could be found in the literature of any statistical work carried out in order to determine nutrient variations from plant to plant in a field. In work published later (103, 104, 105, 106, 107) the Hawaiian investigators still used the recommendations put forward by Nightingale (38) of selecting several key locations in a field and picking 10 leaves from 10 plants in a double-row bed (100 yds. long) in each location. As Cayenne pineapples are usually planted 1 foot apart in the rows, a double row, 100 yds. long, will contain about 600 plants. Sampling 10 plants out of 600 in a few "key" locations in a field would entail the sampling of only a fraction of 1% of the total number. This procedure appears to have been adopted arbitrarily. Leaves are usually picked at periodic intervals right through the initial stages of growth up to fruit bud initiation and the nutrient status determined at a particular stage of growth by reference to previously established graphs covering the whole period of growth.

3.2: EXPERIMENTAL PROCEDURE.

It was decided to study the variations in N, P, K, Ca, Mg, Fe, Mn, Zn and Cu in pineapple leaves by intensive sampling of blocks of plants according to the technique described above. All the sampling studies were carried out on smooth Cayenne pineapple plantations on a farm in the heart of the Bathurst pineapple producing area of the Eastern Cape Province.

The same principles were adopted as described in Section 2 for the citrus sampling studies. In order to keep soil variations and other factors as constant as possible, small plots were selected. In all cases, the sampling plots were of the following design:-



Each of the 12 plots consisted of 10 plants (5 x 2, double row) with 3 x 2 plants between plots in the rows. Three adjacent double rows were selected. This procedure gave 12 plots, each situated 4 feet from the adjacent plot, with a total number of 174 plants covering an area of about 36 square yards. Ten plants per plot were selected because this was the minimum number for supplying sufficient leaf material in order to carry out the analytical procedure accurately.

The following studies were carried out in the order given:-

(a) Internal variations within the "D" leaves.
(b) Diurnal variations in nutrient concentrations.
(c) Variations in 22-month old Cayenne slips.
(d) Variations in 14-month old Cayenne slips.
(e) Variations in 22-month old Cayenne tops.
(f) Variations from scattered samples taken over a large area.

3.3: INTERNAL VARIATIONS WITHIN THE "D" LEAVES.

3.31: VARIATIONS WITHIN THE WHITE BASAL TISSUE OF THE LEAF.

In order to determine whether any large variations occurred in the white basal portion of the leaf - the section used for chemical analysis (36, 37, 38) - the following experiment was carried out:- A large sample of 100 leaves was picked from adjacent plants (22-month old plantings from slips), the white basal portions were cut off close to the chlorophyllous part of the leaf, the portions were washed and then cut up into 3 sections as follows:-

(a) Base - the bottom third portion consisting entirely of succulent - brittle white tissue, about $\frac{1}{2}$" in width.
(b) Middle - the middle third portion consisting of some succulent - brittle tissue, but also containing some of the more fibrous white tissue found higher up, about $\frac{1}{2}$" in width.
(c) Top - the top third portion, still white with no chlorophyllous tissue, but much more fibrous in character than (a) or (b) $\frac{1}{2}$" in width.

After drying and grinding, the 3 samples so obtained were analysed in duplicate for the nutrients. The means of the results are recorded in Table 36.

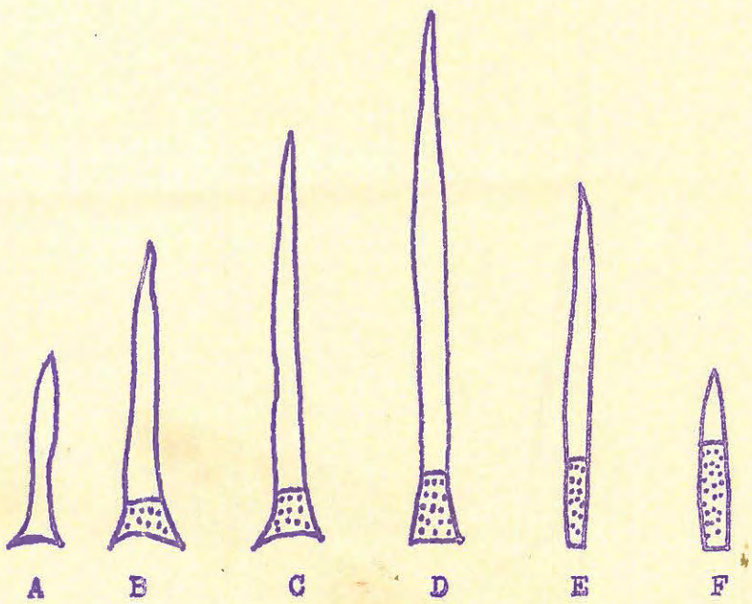


Fig. XIX.

Typical leaves of a non-fruit bearing pineapple plant.

A and B: Old leaves, originally present on planting material.

C: Fully mature leaf, produced after planting.

D: "Active" or longest leaves, correct for sampling.

E: Immature shoulder leaves.

F: Youngest or apical leaf.

▩ White non-chlorophyllous basal portion.

TABLE 36.

VARIATIONS WITHIN THE BASAL TISSUE.

ELEMENT	(a) BASAL THIRD	(b) MIDDLE THIRD	(c) TOP THIRD	PERCENTAGE DIFFERENCE (a)-(b)	(a)-(c)	(b)-(c)
N %	1.56	1.18	0.92	24%	41%	22%
P %	0.250	0.193	0.136	25	46	30
K%	3.86	3.41	2.89	12	25	12
Ca %	0.27	0.22	0.18	19	34	18
Mg %	0.33	0.24	0.19	27	42	21
Mn ppm	102	76	60	25	41	21
Zn ppm	24	17	14	29	42	18
Cu ppm	14	9	8	36	43	11
A V E R A G E				25%	39%	19%

It is evident that great variations in nutrient content occur in these 3 sections of the white basal tissue, the brittle-succulent bottom portion generally containing much higher values than the more fibrous top section. There appears to be a fairly systematic decrease in nutrient content in the direction of the chlorophyllous part of the leaf. The bottom portion is, of course, much higher in moisture than the upper portions of the leaf.

However, apart from the physiological interest of this phenomenon, it will have a profound effect on the reproducibility of the results obtained from analysing the whole of the white portion. The fact that variable portions of the highly nutrient concentrated basal part remain attached to the stem during the detachment of the leaf will cause large sampling errors. It was decided, therefore, in future to use only the middle third section of the white basal tissue for chemical analysis. The whole of the white portion is still sampled in the field but, after washing, the middle third is carefully cut out with stainless steel scissors and treated further as described in Part II. Since the width of the white basal portion usually varies with age, size and health of the plant, it is best to measure the width of this portion for an average sample in a series and then to cut out the middle third of this width, making the cuts as parallel as possible. After some practice, the procedure is simple and rapid.

It was felt that this procedure, besides minimising the possibility of leaching losses occurring during washing (Part II), would give a more reproducible sample than the previous method. From the figures in Table 36 it is clear that the average values given

by the middle portion will not differ greatly from the average of the whole white section, so that the values presented in this work may still be compared with nutrient values from elsewhere.

3.32: VARIATIONS IN "D" LEAVES ON THE SAME PLANT.

The Hawaiian workers apparently sample any 1 of the 4-10 "D" leaves usually occurring on a plant. In order to determine whether any large variations occurred amongst these leaves, the following experiment was carried out. A double row of Cayenne plants (slips), 20 per row, was selected so as to contain plants of more or less the same size. These plants contained about 6 "D" leaves each. Four composite samples were collected from each row by picking as follows:-

(a) Sample 1 contained 20 "D" leaves 1 picked on the North side of each plant.

(b) Sample 2 contained 20 "D" leaves 1 picked on the East side of each plant.

(c) Sample 3 contained 20 "D" leaves 1 picked on the South side of each plant.

(d) Sample 4 contained 20 "D" leaves 1 picked on the West side of each plant.

The 8 samples so obtained for the 2 rows were analysed in duplicate for the nutrient elements. The values for the 2 rows agreed closely and the means of the results are recorded in Table 37.

TABLE 37.

VARIATION IN "D" LEAVES.

ELEMENT	SAMPLE 1 NORTH	SAMPLE 2 EAST	SAMPLE 3 SOUTH	SAMPLE 4 WEST
N %	1.45	1.44	1.43	1.44
P %	0.185	0.186	0.183	0.184
K %	3.12	3.12	3.08	3.10
Ca %	0.31	0.30	0.30	0.30
Mg %	0.33	0.33	0.32	0.32
Fe ppm	45	42	43	43
Mn ppm	160	158	157	159
Zn ppm	24	23	23	24
Cu ppm	12	11	11	13

From these figures it appears that there are no significant differences in the nutrient content of the "D" leaves on the same plant, provided such leaves are truly "D" leaves and the middle third portion of the white basal tissue used for the analysis. It must be mentioned that the "D" leaves selected above were all more or less of the same length. It was perfectly obvious, though, that unless the sampler is experienced, he is bound to include some of the wrong

type of leaves in a sample.

In the subsequent studies, 2 "D" leaves were picked per plant, situated one on each side of the plant parallel to the rows. This represented about 25-50% of the total number of "D" leaves per plant.

3.4: DIURNAL VARIATIONS.

Nightingale (38) stated that pineapple leaf sampling may be carried out at any time between 8 a.m. - 4 p.m. He found no material differences in the nitrate content of leaves sampled from comparable plants during different times of the day. On the other hand, Sideris et al (108), in studying diurnal changes and growth rates as associated with ascorbic acid, titratable acidity, carbohydrate and nitrogenous fractions in the chlorophyllous region of pineapple leaves, reported great variations in these factors. Thus titratable acidity and nitrogenous fractions decreased by day and increased at night, while the reverse was true for total sugar content.

In view of these reports it was thought advisable to carry out a study of possible variations in nutrient concentrations in the white basal tissue during a day. Since no plants were available in Grahamstown, the experiment was only carried out between 7-11 a.m. on a bright, calm sunny day in July. Twenty adjacent plants (22-month old slips) of similar size were selected. One "D" leaf was picked from each plant at 7 a.m., 9 a.m. and 11 a.m. Care was exercised to pick leaves of similar length and size throughout. The 3 samples (20 leaves each) so obtained, were washed and further treated as described in Part II. The mean analytical results are recorded in Table 38.

TABLE 38.
DIURNAL VARIATIONS.

ELEMENT	7 a.m. (Before sunrise)	9 a.m.	11 a.m.	% Difference
N %	1.35	1.37	1.41	+ 4%
P %	0.186	0.188	0.197	+ 4
K %	2.94	2.90	3.25	+ 11
Ca %	0.28	0.27	0.28	0
Mg %	0.30	0.28	0.33	+ 10
Fe ppm	48	47	43	- 10
Mn ppm	176	161	172	- 2
Zn ppm	24	22	22	- 8
Cu ppm	11	10	11	0

As in the case of citrus leaves, there appeared to be an increase in the concentration of the major elements from sunrise towards noon. Again, if sampling errors are taken into account, these increases may

not be significant, except perhaps in the case of K and Mg. The small decreases in the case of the trace elements may or may not have any significance.

At any rate, it seems advisable that a sampling procedure should be carried out during a set period only. It was decided to sample all the plants during 8-10 a.m. in all the subsequent studies.

3.5: THE SAMPLING OF PINEAPPLE FIELDS.

3.51: EXPERIMENTAL:-

The sampling studies were carried out on 3 different plantations, adjacent to each other and more or less on the same general soil type. These were situated on the farm "Lyndhurst" of Mr. D.A.L. Dold in the Bathurst District. A brief description of the sampling blocks is given below:-

FACTOR	BLOCK 1	BLOCK 2	BLOCK 3
Variety	Smooth Cayenne	Smooth Cayenne	Smooth Cayenne
Planting material	Slips	Slips	Tops
Age of plants	22 months	14 months	22 months
Planting	Double rows, 4' apart, plants 1' apart in rows.	Same as 1	Same as 1
Description	Large, healthy	Medium, healthy	Uneven stand
Soil type	Brown sandy laom	Same as 1	Same as 1
Slope	Level	Slight slope	Level
Cultivation	Clean	Clean	Clean
Fertiliser	N twice/annum	Same as 1	Same as 1
Leaf symptoms	Mottled (Zn?)	Mottled	Mottled
No. "D" leaves per plant	6	4	5
Sampling date	July	July	July
Time of sampling	8 - 10 a.m.	8 - 10 a.m.	8 - 10 a.m.

The Blocks were laid out as described in Section 3.2. Particular care was taken to select an even stand of plants, as homogeneous as possible in outward appearance. This was particularly difficult in the case of Block 3 (tops), where the stand was rather uneven, probably due to this type of planting material. In selecting the plants in this Block, those that were much below average size were omitted. The utmost care was exercised during the picking of the samples as well as during the analytical procedure in order to keep interfering factors at a minimum. Each sample was analysed in duplicate. The means of the results are recorded in Tables 39, 41 and 43 with the statistical treatments (as for citrus) in Tables 40, 42 and 44. Table 45 records a summary of the statistics for the 3 Blocks while Fig. XX shows the number of plots to be sampled in order to represent the nutrients to within 10% of the mean values

at the 5% point.

Table 46 records the error to expect if 10% of the plants (i.e. 1 plot out of 12) are sampled from the 3 Blocks. These figures were calculated by using the formula, $D = \sqrt{\frac{2t^2d^2}{n}}$, as was done for citrus.

In order to obtain some idea of the variations likely to occur over a larger area the following experiment was included:- An area containing about 1500 plants (1/10 acre) was marked off in Plantation 1 (22-month old slips), i.e. 12 double rows of 60 plants per single row. Six random plots of 10 plants each were selected in every alternate double row. The 6 plots were sampled as above and the samples analysed for the nutrients. The results are recorded in Table 47.

TABLE 39.

BLOCK 1: 22-MONTH OLD SLIPS.

PLOT NUMBER	N %	P %	K %	Ca %	Mg %	Fe ppm	Mn ppm	Zn ppm	Cu ppm
1	1.16	0.193	2.89	0.24	0.26	25	105	16	9.0
2	1.28	0.184	2.25	0.36	0.33	31	123	16	8.6
3	1.38	0.221	3.08	0.30	0.31	35	105	17	10.4
4	1.25	0.212	2.33	0.35	0.34	25	127	17	10.0
5	1.18	0.195	2.67	0.22	0.26	19	104	16	11.5
6	1.28	0.211	2.62	0.32	0.28	24	117	18	9.6
7	1.39	0.225	2.97	0.33	0.30	22	121	17	9.3
8	1.46	0.236	2.92	0.39	0.34	28	100	18	11.3
9	1.23	0.205	2.90	0.21	0.26	18	109	20	10.9
10	1.17	0.183	2.76	0.24	0.27	19	106	15	10.8
11	1.35	0.194	2.94	0.24	0.29	25	110	17	10.5
12	1.25	0.215	2.73	0.31	0.32	24	97	18	10.4
Mean	1.28	0.206	2.75	0.29	0.30	25	110	17	10.2
Standard Deviation	0.09	0.017	0.25	0.06	0.03	5	9	1.4	0.91
Coeff. of Variation	7.4	8.2	9.3	20.0	10.7	20.0	8.7	8.2	8.9

TABLE 40

BLOCK 1: MINIMUM NUMBER OF PLOTS TO SAMPLE TO SHOW SIGNIFICANCE ON A 5% LEVEL IN DIFFERENCES EXCEEDING D% OF THE MEANS.

ELEMENT	COEFF. OF VARIATION (ADJUSTED)	D = 5%	D = 10%	D = 20%
Nitrogen	7.3	21	6	2
Phosphorus	8.2	27	7	2
Potassium	9.2	33	9	3
Calcium	20.0	160	40	10
Magnesium	10.3	42	11	3
Iron	19.6	149	38	10
Manganese	8.5	29	8	2
Zinc	6.5	17	5	2
Copper	8.4	28	7	2

TABLE 41.

BLOCK 2: 14-MONTH OLD SLIPS.

PLOT NUMBER	N %	P %	K %	Ca %	Mg %	Fe ppm	Mn ppm	Zn ppm	Cu ppm
1	1.36	0.235	2.48	0.32	0.31	49	144	12	9.3
2	1.42	0.203	2.12	0.37	0.38	67	174	12	10.3
3	1.38	0.224	2.58	0.39	0.33	64	161	12	8.8
4	1.41	0.215	2.78	0.31	0.30	61	144	12	9.4
5	1.36	0.212	2.50	0.30	0.34	47	164	13	9.7
6	1.37	0.202	2.63	0.35	0.33	55	189	12	9.6
7	1.35	0.235	2.50	0.32	0.31	50	145	12	9.3
8	1.41	0.205	2.10	0.36	0.37	65	172	13	10.1
9	1.38	0.224	2.60	0.39	0.33	65	161	12	8.8
10	1.41	0.211	2.78	0.31	0.30	62	145	12	9.4
11	1.36	0.212	2.50	0.30	0.34	45	164	13	9.7
12	1.37	0.206	2.60	0.34	0.32	55	185	12	9.6
Mean	1.38	0.215	2.52	0.34	0.33	57	162	12	9.5
Standard Deviation	0.02	0.012	0.21	0.03	0.02	8	16	0.5	0.45
Coeff. of Variation	1.8	5.4	8.5	9.7	7.3	13.8	9.7	4.5	4.7

TABLE 42.

BLOCK 2: MINIMUM NUMBER OF PLOTS TO SAMPLE TO SHOW SIGNIFICANCE ON A 5% LEVEL IN DIFFERENCES EXCEEDING D% OF THE MEANS.

ELEMENT	COEFF. OF VARIATION (ADJUSTED)	D = 5%	D = 10%	D = 20%
Nitrogen	1.7	2	1	1
Phosphorus	5.4	13	4	1
Potassium	8.4	28	7	2
Calcium	9.7	37	10	3
Magnesium	7.3	21	6	2
Iron	13.7	73	19	5
Manganese	9.7	37	10	3
Zinc	4.0	8	2	1
Copper	3.5	5	2	1

TABLE 43.

BLOCK 3: 22-MONTH OLD TOPS.

PLOT NUMBER	N %	P %	K %	Ca %	Mg %	Fe ppm	Mn ppm	Zn ppm	Cu ppm
1	1.30	0.192	2.38	0.28	0.31	51	103	14	9.1
2	1.27	0.188	2.03	0.37	0.34	48	96	13	7.8
3	1.27	0.214	2.99	0.27	0.30	60	112	16	8.7
4	1.29	0.165	2.04	0.33	0.32	46	122	16	10.0
5	1.23	0.152	1.73	0.37	0.34	50	145	13	8.2
6	1.35	0.224	2.96	0.27	0.30	48	107	17	8.9
7	1.30	0.194	2.39	0.29	0.31	50	103	14	9.0
8	1.30	0.188	2.03	0.37	0.34	48	96	13	7.8
9	1.27	0.209	3.00	0.27	0.30	49	116	16	8.7
10	1.30	0.165	2.03	0.33	0.32	46	122	16	9.5
11	1.25	0.151	1.76	0.37	0.34	49	115	13	8.1
12	1.33	0.222	2.95	0.27	0.30	47	106	17	8.8
Mean	1.29	0.189	2.36	0.32	0.32	49	112	15	8.7
Standard Deviation	0.03	0.26	0.50	0.05	0.02	4	14	1.6	0.66
Coeff. of Variation	2.6	13.7	21.0	14.4	5.3	7.5	12.2	10.7	7.6

TABLE 44.

BLOCK 3: MINIMUM NUMBER OF PLOTS TO SAMPLE TO SHOW SIGNIFICANCE ON A 5% LEVEL IN DIFFERENCES EXCEEDING D% OF THE MEANS.

ELEMENT	COEFF. OF VARIATION (ADJUSTED)	D = 5%	D = 10%	D = 20%
Nitrogen	2.5	3	1	1
Phosphorus	13.7	75	19	5
Potassium	21.0	170	43	11
Calcium	14.4	80	21	6
Magnesium	5.0	10	3	1
Iron	6.5	17	5	2
Manganese	12.0	57	15	4
Zinc	9.3	34	9	3
Copper	6.9	19	5	2

TABLE 45.

MINIMUM NUMBER OF PLOTS (10 PLANTS EACH) TO SAMPLE IN A BLOCK FOR A GIVEN DEGREE OF REPRESENTATION AT THE 5% POINT.

ELEMENT	BLOCK 1			BLOCK 2			BLOCK 3		
	Mean value	10%	20%	Mean value	10%	20%	Mean value	10%	20%
Nitrogen	1.28	6	2	1.38	1	1	1.29	1	1
Phosphorus	0.206	7	2	0.215	4	1	0.189	19	5
Potassium	2.75	9	3	2.52	7	2	2.36	43	11
Calcium	0.29	40	10	0.34	10	3	0.32	21	6
Magnesium	0.30	11	3	0.33	6	2	0.32	3	1
Iron	25	38	10	57	19	5	49	5	2
Manganese	110	8	2	162	10	3	112	15	4
Zinc	17	5	2	12	2	1	15	9	3
Copper	10	7	2	9.5	2	1	8.7	5	2
Average No. of Plots		15	4		7	2		14	4
Average No. of Plants		150	40		70	20		140	40

TABLE 46.

PERCENTAGE DIFFERENCE FROM THE MEAN VALUE AT THE 5% POINT BY SAMPLING 10% OF THE PLANTS (1 PLOT).

ELEMENT	BLOCK 1	BLOCK 2	BLOCK 3.
Nitrogen	23	6	8
Phosphorus	26	18	43
Potassium	29	27	66
Calcium	64	31	45
Magnesium	33	23	16
Iron	61	43	21
Manganese	27	31	38
Zinc	21	14	30
Copper	27	11	22

TABLE 47.

RANDOM SAMPLES TAKEN OVER 1/10 ACRE (1500 PLANTS)

(22-MONTH OLD SLIPS)

PLOT NUMBER	N %	P %	K %	Ca %	Mg %	Fe ppm	Mn ppm	Zn ppm	Cu ppm
1	1.24	0.217	3.37	0.23	0.26	27	100	18	12.0
2	1.12	0.152	2.52	0.18	0.23	17	124	14	9.0
3	1.23	0.210	3.21	0.23	0.25	30	149	16	9.8
4	1.21	0.213	2.60	0.23	0.28	20	140	16	9.5
5	1.57	0.282	4.16	0.35	0.29	45	111	21	13.0
6	1.25	0.202	2.65	0.25	0.27	34	128	17	10.0
Mean	1.27	0.212	3.09	0.24	0.26	29	125	17	11.0
Standard Deviation	0.15	0.041	0.63	0.06	0.02	10	18	2.3	1.7
Coeff. of Variation	12.2	19.6	20.4	23.7	8.5	34.8	14.4	13.5	15.5
Minimum No. of Plots for 10% repr.	20	51	55	75	10	161	28	25	32
Minimum No. of plots for 20% repr.	5	13	14	19	3	41	7	7	8

AVERAGE FOR 10% REPRESENTATION: 51 plots = 510 plants ($\pm$ 30%) .

AVERAGE FOR 20% REPRESENTATION: 13 plots = 130 plants ($\pm$ 9%).

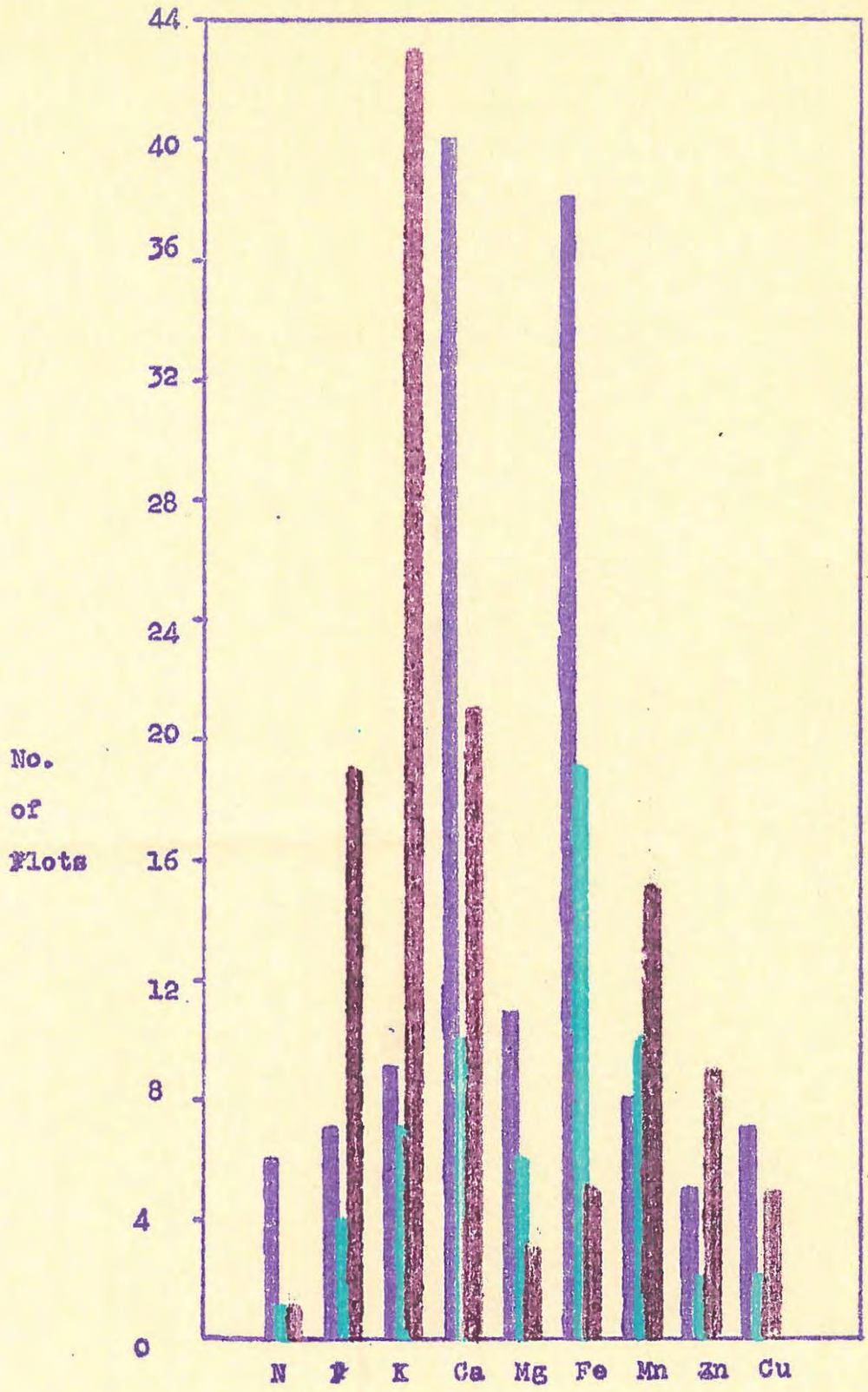


Fig. XX.

Minimum number of plots(10 plants each) to sample in uniform blocks of pineapple plants in order to represent the nutrients to within 10 % of the mean values on a probability level of 19 to 1.

3.52:- DISCUSSION.

From the Tables it is evident that the young plants (Block 2) showed the least degree of variation while the average variations for 22-month old slips and 22-month old tops were more or less the same. This may possibly be due to the fact that the younger plants contained considerably less white basal tissue on the leaves than the older plants with possibly a more even distribution of the nutrient concentration levels.

From Table 45, where the final statistics for the 3 blocks are grouped together, it is apparent that much more intensive sampling is necessary than hitherto practised in order adequately to represent the nutrient values of a group of pineapple plants. It is evident that even sampling all the plants in a small block of 120 plants will not represent all the nutrients determined to within 10% of the mean values at the 5% point, due to the large variations shown by some of the nutrients. By sampling an average of 33% of the plants in the 22-month old slips and tops blocks and 16% of the plants in the 14-month slips block, most of the nutrients could be represented to within 20% of the mean values at the 5% point.

To sample more than 20% of the plants in a block means intensive sampling, and if, even then, the results can only be trusted to be within 20% of the mean values, it is perfectly clear that the correct interpretation of such results is a difficult and often untrustworthy matter - faulty conclusions may easily be arrived at. This is apparent if the figures in Table 46 are studied, where the error in the nutrient values at the 5% point, if 10% of the plants in a block are sampled, is given. It is only in the case of the young plants (Block 2) that a certain degree of confidence in interpreting the results can be achieved. It is evident that when less than 1% of the plants in a field are sampled, which appears to be the usual practice, no real value can be attached to the results.

The figures in Table 47 record the variations likely to occur over a larger area (1500 plants, 1/10 acre). As in the case of citrus (Section2), it must be emphasised that the statistics recorded in the lower part of the Table are not strictly correct since no attempt was made to determine the "true" coefficients of variation by sampling all the plants - only about 4% of the plants were sampled. Nevertheless, it is clear from Table 47 that the nutrients varied to a greater extent over this larger area than over the smaller blocks. If it is assumed that the coefficients of variation recorded in this Table are a reasonable reflection of the " true" values over the whole area, it is seen that an average of 510 plants out of the total 1500 plants (about 30%) must be sampled in order to

represent most of the nutrients to within 10% of the mean values at the 5% point, while for 20% representation a minimum of 130 plants (about 9% of the total) must be sampled.

The particularly large variations shown by samples 2 and 5 in Table 47 pointed to possible variations in the soil. This was confirmed by soil analysis - the "available" soil nutrients in plot 2 being considerably lower than the average values over the whole area, while the values were higher than average in plot 5. Yet, when the leaf samples were taken the stand as a whole appeared to be fairly even, and it was only when the analytical figures were available and subsequent closer inspection of the plants, that it was noticed that the plants from Plot 2 and the plants in the vicinity were smaller than average, while those in Plot 5 and vicinity were larger and more healthy in appearance than the average plants over the whole plot. It must be mentioned that the plants in Plot 5 did not show the typical leaf-mottling symptoms so conspicuous in the other plants, particularly in those from Plot 2. In fact, judging from the appearance of the plants, the high nutrient values obtained for Plot 5 probably lie close to the optimum values for healthy 22-month old Cayenne plants. It is interesting to note that the figures recorded for P and K correspond closely to values laid down by Nightingale (103) for optimum yields from similarly aged plants in the Hawaiian Islands.

It seems imperative when sampling large pineapple fields that a preliminary sampling study should be carried out before deciding upon a definite programme. From Table 47 it is clear that the inclusion of Plots 2 and 5 was mainly responsible for the large coefficients of variation. It must be remembered that an area of 1/10 acre is really only a fraction of the size of large plantations, covering several acres. The fact that such large soil variations were found in a small area, coupled with individual differences in the size of the plants, even where apparently homogeneous planting material was used, makes the efficient sampling of large pineries for the purpose of ascertaining the general nutrient status of the plants, a hazardous and difficult problem.

This influence of the size of the plants on the nutrient concentrations has a very important bearing on efficient sampling. The homogeneity of a stand of plants is probably influenced to a large degree by efficient selection of the planting material - where this is not done the plants will acquire a very uneven stand. In many cases, however, it is a question of planting material that is available, without due regard to selection. Consequently, many commercial pineries, whether planted from slips, suckers, tops or stumps, contain a number of plants which are smaller or larger than

the average, and sometimes the stands are very uneven. Because the smaller plants usually differ greatly in nutrient concentrations from the average plants (Table 47), they should not be included in the samples.

The arguments against compositing when sampling large areas, as outlined in the case of citrus, are also applicable here. It seems essential that a large plantation should be divided up into a number of small blocks, each of which is to be sampled intensively and analysed separately. It is clear from Table 47 that if one composite sample is taken over a large area, the chances are very favourable of including material which will seriously upset the average nutrient values of the plants, resulting in incorrect interpretation.

3.53: SUGGESTIONS FOR SAMPLING PINEAPPLE PLANTS FOR LEAF ANALYSIS.

Because of the different types of planting material used and the factors discussed above, no standard sampling procedure to cover all cases can be laid down. It is advisable to carry out a thorough preliminary study of a field before deciding upon a final programme. The following suggestions are made as a result of the studies reported above:-

(1) Only a thoroughly trained and experienced operator should take the leaf samples.

(2) The samples should preferably be taken between 8-10 a.m. on calm, sunny days.

(3) The plants to be sampled should be selected with care, omitting those that vary considerably in size from the average.

(4) The correct leaves to be sampled should be selected with extreme care, as described in Section 3.1. One or two of the typical leaves may be sampled from any position on the plant.

(5) Only the middle third of the white basal portion of the leaf should be used for chemical analysis, as described in Section 3.1.

(6) Plants in an advanced stage of growth, containing relatively much white basal tissue on the typical leaves, should be sampled more intensively than young plants.

(7) Differences of less than 20% in the nutrient values at the 5% point should not be treated as significant, unless more than 30% of the plants in an area are sampled, or the sampling variations for that area have been established by experiment.

(8) In experimental plots for research purposes all the plants in the plots should be sampled and analysed separately.

(9) When sampling large plantations the following procedure is suggested:- The area should be divided up into a number of small blocks, grouping together all similar factors, such as plant size, poor and healthy plants,

soil characteristics such as type, slope, drainage, etc. This would involve a thorough preliminary survey of the area, including a few soil profile studies if possible. Each small block must be sampled separately and as intensively as circumstances will allow - at least 10% of the plants should be sampled if the plants are younger than 16 months while more than 20% of the plants should be sampled if they are at an advanced stage of growth. The analytical figures from the separate samples should give a fairly good estimate of the nutrient status of the plants over the whole are. The figure will also indicate "border line" spots where more intensive samplings should be carried out in order to confirm a deficiency or otherwise.

4. CONCLUSION.

It is realised that these sampling studies were by no means exhaustive and that there are many other factors which should be studied as well - factors such as the effect of fertiliser treatments irrigation and cultivation practises, rootstock, planting material, variety, soil type, climate, etc., on the variation in mineral composition of the leaves from plant to plant. However, such studies could only be carried out at an agricultural research station and it is hoped that this problem would be tackled in the future on lines as suggested in this thesis. Nevertheless, the pattern of the results recorded above would indicate that the sampling of citrus trees and pineapple plants should be carried out on a much more intensive scale than hitherto practised, in order to arrive at reliable conclusions.

The suggestions put forward in order to sample large citrus orchards or pineapple plantations for advisory purposes, may involve several days of sampling followed by several weeks of analytical work, a procedure which many institutions may be loth to accept. Yet, when the large variations as reported above are considered, with the realisation that they occurred in small plots sampled under the most exact conditions and with the utmost care that a sampler is capable of, that interfering factors which cannot normally be controlled when sampling large areas were kept at a minimum, and that the analytical error was largely eliminated by subtraction before these values were calculated, it will be evident that the author could not possibly arrive at any other conclusion but that of intensive sampling.

If leaf analysis is to be a useful tool in helping to assess the fertiliser needs of crop plants, it must be applied properly. As one of the essentials for proper application appears to be intensive sampling, as was shown to be the case for tree crops like citrus, and close-growing crops like pineapple plants, every effort should be made by investigators to meet this requirement, otherwise this valuable tool will suffer and may even become distrusted and fall into disfavour. The cost of artificial fertilisers is such that it justifies the spending of more than the usual care in determining the needs of specific crop plants.

By dividing the sampling area into a number of small units, depending on physical differences in the plants and the soil as determined by a preliminary survey, a much better idea of a fertiliser programme may be arrived at than by treating the field as one unit. It seems foolish and uneconomic to apply a specific fertiliser to an entire field when only some of the plants in a

particular spot may suffer from the deficiency. A case in point can be seen from the results in Table 47 where, in a small area, a group of perfectly healthy plants (Plot 5) appears with a group obviously suffering from some deficiencies (Plot 2).

In conclusion, it must be pointed out that the sampling of leaves is a far less laborious procedure than the sampling of soils. An expert operator can sample 10 citrus trees efficiently in $\frac{1}{4} - \frac{1}{2}$ hour, while 120 pineapple plants may be sampled in 1 hour. In view of the laborious and lengthy procedure of leaf analysis, it would be foolish and unforgivable if the sampler did not carry out his relatively simple part of the job properly by obtaining a truly representative sample. A truly representative sample appears to be, for all practical purposes, one obtained by exhaustive sampling. This appears to be particularly necessary when the nutrient falls in the "deficient range", a condition which leaf analysis is specifically expected to define. If such exhaustive sampling is not carried out when determining all the nutrients, the chances of arriving at a correct interpretation appear to be small indeed.

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THE ACCURATE DETERMINATION OF CALCIUM AND MAGNESIUM IN PLANT MATERIALS

by W. J. A. STEYN

THE ACCURATE DETERMINATION OF CALCIUM AND MAGNESIUM IN PLANT MATERIALS

by **W. J. A. STEYN**

OPSOMMING

Die belangrikste faktore wat die vlamfotometriese en volumetriese bepaling van kalsium en magnesium beinvloed, is ondersoek. Daar word gewys dat sodra fosfaat en sulfaat ione verwyder word uit die toetsoplossing, die nadelige invloed van ander elemente of geheel en al verdwyn, of baie verminder word. Snel metodes vir die noukeurige bepaling van kalsium, natrium, kalium (vlamfotometries) en kalsium en magnesium (volumetries), nadat nadelige ione verwyder is deur middel van ion-uitruiling, word beskrywe.

SUMMARY

A critical investigation of the interferences likely to occur in the flame-photometric and volumetric determination of calcium and magnesium is presented. It is shown that all interferences may either be eliminated or much suppressed by the removal of phosphate and sulphate ions from the test solution. Rapid methods are described for the accurate determination of calcium, sodium, potassium (flame-photometric) and calcium and magnesium (volumetric) after removal of interfering ions by ion-exchange.

Various workers[1, 2, 3, 4] have reported the interference of several ions, particularly of phosphates, in the versenate(EDTA) titration of calcium and magnesium. The interference of phosphate, sulphate and other ions has been reported in the flame-photometric determination of calcium[5, 6, 7, 8]. More or less satisfactory methods for the flame-photometric determination of calcium in air:acetylene mixtures have been worked out[5, 6, 8]. These methods, however, do not hold for the "cold" flame of the air : propane mixture used, for example, in the "Eel" flame-photometer. The removal of interfering phosphates has been satisfactorily accomplished by the use of anion-exchange resins for the EDTA determination of calcium[9, 10, 11].

The work described here was undertaken in order to investigate existing methods with a view to modifying them for rapid and accurate routine analysis of soils and plant materials.

EXPERIMENTAL

Apparatus and reagents. The flame-photometric work was carried out on the "Eel" of Evans Electroselenium, Limited. "Bottogas" was used with compressed air; the variation in the pressure of the town gas supply was such that accurate results could not be obtained with it. The low-heat flame of the "Eel" is admirable for the accurate determination of calcium, sodium and potassium since interference from emission by foreign elements is less likely than with hotter flames. All standard solutions and solutions containing interfering ions were prepared from Johnson Matthey "Specpure" chemicals. All other reagents were of "Analar" grade.

Interfering ions in the EDTA titration of Calcium and Magnesium. Controlled experiments were carried out on standard solutions containing varying amounts of the foreign ions likely to interfere in solutions of plant materials. The results are summarised below:

(i) *EDTA titration of Ca with screened murexide:* The indicator, murexide, was screened with naphthol green B to obtain a better end point in calcium titrations against EDTA. The end point was a clear dark blue without a trace of violet, was very definite, stable and much easier to recognise than when murexide alone was used.

Magnesium had no influence on the recovery of calcium between Ca/Mg ratios of 10:1 and 1:10. The only effect of phosphate between ratios of Ca/P from 15:1 to 1:4 was to delay the end point when the ratios became smaller than 3:1. Quantitative results for Ca were still obtained up to a ratio of 1:4 even though solid calcium phosphate was formed, provided the titration was continued with shaking to the stable end point.

When phosphate and heavy metals were present, however, the end point deteriorated rapidly with increasing concentration of heavy metals. The concentration of some heavy metals which could simultaneously be present with phosphate without interfering with the quantitative recovery of calcium were found to be:

Fe^{3+}, 10 *per cent.*; Mn^{2+}, 1 *per cent.*; Cu, $\frac{1}{2}$ *per cent.* and Zn, $\frac{1}{2}$ *per cent.* of the Ca. concentration.

The phosphorus in the solution was double the concentration of the Ca. When phosphorus was absent from the test solution, the amounts of heavy metals could be doubled without showing interference, a fact which seems to have been overlooked by previous workers.

(ii) *EDTA titration of Ca+Mg with Eriochrome Black T:* Standard solutions containing varying amounts of Ca, Mg, Na, K, Fe^{3+}, Mn^{2+}, Cu, Zn, and B were titrated against EDTA with Eriochrome Black T as indicator, both in the presence as well as in the absence of phosphate. Ferric iron interfered with the colour change of the indicator, apparently oxidising the indicator with the result that no change in colour was observed. Increased amounts of Mn^{2+} enhanced the interference of iron. In the presence of phosphorus, Fe^{3+} began to interfere when present in amounts greater than 1·5 *per cent.* of the Ca+Mg content. However, in the absence of phosphorus, Fe^{3+} could be present in amounts up to 25 *per cent.* of the Ca+Mg content without interfering.

A synthetic standard solution containing a mixture of different elements in the proportions listed below, was titrated against EDTA with Eriochrome Black T as indicator. The recovery of Mg was quantitative and no deterioration of the end point was observed (phosphate absent):

Ca	Mg	Na	K	Fe^{3}	Mn^{2}	Cu	Zn	B
20	1	1	40	5	0·1	0·02	0·02	0·02

Thus in the absence of phosphate the EDTA method for determining magnesium with Eriochrome Black T as indicator is very suitable for plant materials in which the concentrations of trace elements seldom exceed the figures given above. The titration was sensitive and accurate in the absence of phosphorus.

Interfering ions in the flame-photometric determination of Ca. The interference of phosphate, sulphate and some other ions in the flame-photometric determination of calcium has been reported by several workers[5, 6, 7, 8]. Some of these interferences were investigated using an "Eel" flame-photometer with an air : 'bottogas'" mixture.

Standard solutions containing varying concentrations of calcium, phosphate, sulphate and foreign cations were tested for the recovery of Ca. The suppression by sulphate and phosphate ions of the emission by calcium is shown diagramatically in Fig. 1, and is so serious that calcium cannot be determined accurately on the "Eel" unless these anions are removed from the test solution.

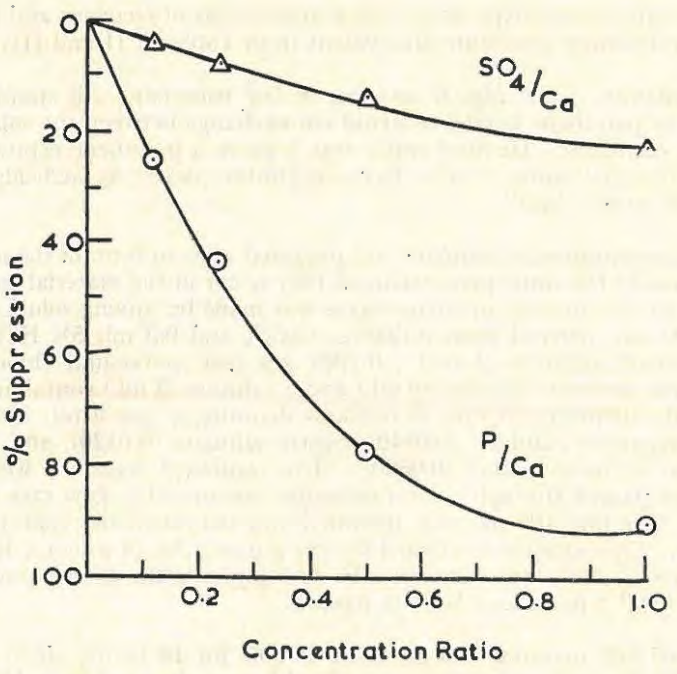

Fig. 1
Suppression of Ca by PO_4 and SO_4.

Magnesium had no effect on the emission of Ca for Ca/Mg ratios from 10:1 to 1:3. When the ratio was 1:4 and smaller, Mg suppressed the emission of Ca, the error being about 6 *per cent.* for a ratio of 1:8. This ratio, however, never occurs in plant materials and only very seldom in soils.

A synthetic standard solution containing a mixture of different cation chlorides in the proportions listed below, showed no interference with the recovery of Ca on the "Eel":

Ca	Mg	Na	K	Al	Fe^3	Mn^2	Cu	Zn	B
20	10	10	40	2	0·5	0·2	0·1	0·1	0·1

It is clear, therefore, that if phosphate and sulphate are absent, other elements normally occurring in plant materials will not interfere in the determination of calcium on a low-heat flame-photometer. It is of interest that the relatively high concentration of potassium showed no interference with calcium on the "Eel", whereas Strasheim and Nell[6] reported a suppression by K of the Ca emission when using an air:acetylene mixture. The author actually obtained quantitative recovery of Ca on the "Eel" from chloride solutions in which the K/Ca ratio was as much as 6:1.

Removal of phosphate and sulphate by ion-exchange. Exhaustive experiments were carried out with various anion exchange resins to find a suitable and rapid means for the qnantitative removal of polyvalent anions from the test solution prior to the determination of calcium and magnesium. This was satisfactorily accomplished with the resin, De-Acidite E, obtained from Hopkin and Williams, England. Similar results were obtained with the American equivalent, Amberlite IR-4B, which

is, however, much more expensive. The completeness of recovery and the reproducibility of the leaching procedure are evident from Tables I, II and III.

Determination of Ca, Mg, K and Na in leaf materials. All standard solutions were stored in polythene bottles to avoid ion-exchange between the solution and the walls of the container. Distilled water was kept in a polythene aspirator as it was found that distilled water from a Pyrex aspirator picked up sufficient calcium to be detectable on the "Eel".

The flame-photometer standard was prepared so as to contain the main elements in approximately the same proportions as they occur in the material to be analysed. A standard for the analysis of citrus leaves was made by mixing solutions of calcium chloride (100 ml., derived from 0·1248 g. $CaCO_3$ and 0·3 ml. 5N HCl), 0·2535 *per cent.* magnesium sulphate (3 ml.) , 0·1907 *per cent.* potassium chloride (10 ml.), 0·1271 *per cent.* sodium chloride (10 ml.) and a solution (3 ml.) containing the following hydrated substances (figures in brackets denoting g. per litre): ferrous sulphate (0·1659), manganous sulphate (0·0440), cupric sulphate (0·0120), and zinc sulphate (0·0500), and orthoboric acid (0·0500). The combined solutions were diluted to 1000 ml. and passed through an ion-exchange column at a flow rate of two drops per second. The first 100 ml. was discarded and the remainder collected in a polythene bottle. This solution contained Ca (50 p.p.m.), Na (5 p.p.m.), K (10 p.p.m.), Mg (0·75 p.p.m.), and, approximately, Fe (0·1 p.p.m.), Mn (0·03 p.p.m.), Cu (0·009 p.p.m.), Zn (0·03 p.p.m.) and B (0·03 p.p.m.).

Powdered leaf material (0·5 g.) dried at 105° for 16 hours, nitric acid (10 ml.) and about 65 *per cent.* perchloric acid (2 ml.) were digested in a 150 ml. conical flask fitted with a cover glass at a very low heat until the vigorous reaction subsided and then boiled until most of the solid particles had dissolved (usually about 1 hour). With the cover glass removed the solution was evaporated to dryness; any colouration in the residue was removed by adding a few drops of perchloric acid and evaporating again to dryness. The colourless residue was dissolved in water acidified with a few drops of 5N HCl, filtered and diluted to 100 ml.

The solution (10 ml.) thus prepared was adjusted to pH 3-4 by adding 10 *per cent.* ammonium carbonate (the required amount of which had previously been determined with the aid of methyl orange) and passed through a column (2 cms. diameter) of De-Acidite E resin in the chloride form (about 15 g., previously washed with 5 *per cent.* sodium carbonate, distilled water, 5 *per cent.* hydrochloric acid and finally distilled water until the washings were free of colour) at a rate of 1 drop per sec. The resin was washed with distilled water at the same flow rate until a total volume of 100 ml. had been collected.

Ca, Na and K were determined in the leachate without further dilution on the "Eel" flame-photometer. The instrument was calibrated by spraying the standard and adjusting the potentiometer control to give full-scale deflection on the lower linear scale for Ca (50 p.p.m.), Na (5 p.p.m.) and K (10 p.p.m.) respectively. The zero was set by spraying distilled water.

Ca+Mg was determined by titrating 50 ml. of the leachate, diluted to 100 ml. and containing 5 ml. of ammonium chloride buffer (67·4 g. and 570 ml. concentrated ammonia per litre) and 15 small drops of 0·4 *per cent.* Eriochrome Black T (in methanol containing 4 *per cent.* hydroxylamine hydrochloride) against 0·01 normal EDTA to a stable clear blue endpoint. Magnesium was obtained by subtracting the value due to the Ca (determined flame-photometrically) from the titer.

Perchlorate ions were found to be mainly responsible for exhausting the resin, hence the necessity of evaporating as much of the perchloric acid as possible during the digestion stage. 10 to 100 Samples, depending on the perchlorate content in plant residue, could be leached through the same column before regeneration of the resin was necessary. The resin was exhausted when phosphate could be detected in the leachate (molybdenum blue test). For routine analysis, a battery of 12 columns was prepared, the whole operation of leaching 12 samples and determining Ca, Na, K and Mg in the leachates taking about 1½ hours. It was better to regenerate the resin in an erlenmeyr flask rather than performing the operation in the column itself.

RESULTS

In Tables I, II and III experimental results showing the reproducibility and accuracy of the method are tabulated. In each case the solutions were passed through the ion-exchange column prior to analysis.

TABLE I

RECOVERY TESTS FROM SYNTHETIC STANDARDS

Foreign ions added			Calcium		Magnesium		Sodium		Potassium	
P p.p.m.	SO_4 p.p.m.	Fe:Mn:Cu:B:Zn p.p.m.	Theor. p.p.m.	Found p.p.m.	Theor. p.p.m.	Found p.p.m.	Theor. p.p.m.	Found p.p.m.	Theor. p.p.m.	Found p.p.m.
10	10	1:·05:·05:·05:·05	40	40·0	2	1·98	2	2·0	6	5·9
20	10	,,	40	39·5	2	2·01	2	2·1	6	6·0
50	10	,,	40	40·0	2	1·99	2	2·0	6	6·1
100	10	,,	40	40·0	2	2·02	2	1·9	6	5·8
200	10	,,	40	39·0	2	1·97	2	2·0	6	6·0

TABLE II

RECOVERY TESTS FROM PLANT SOLUTIONS

Calcium		Magnesium		Sodium		Potassium	
Added p.p.m.	Found p.p.m.	Added p.p.m.	Found p.p.m.	Added p.p.m.	Found p.p.m.	Added p.p.m.	Found p.p.m.
25	24	4·19	4·18	1·35	1·30	3·5	3·4
30	29	2·84	2·85	1·85	1·75	4·5	4·3
35	34	3·58	3·60	2·35	2·30	5·5	5·3
40	39	4·32	4·34	2·85	2·80	6·5	6·5
45	43	5·07	5·10	3·35	3·30	7·5	7·3
50	49	5·81	5·83	3·85	3·85	8·5	8·5

TABLE III

REPRODUCIBILITY OF LEACHING AND DIGESTION PROCEDURE
(Citrus Leaves)

No.	Wt. Leaves g.	Calcium %	Magnesium %	Sodium %	Potassium %
1	0·5238	3·74	0·46	0·16	0·35
2	0·4976	3·77	0·50	0·17	0·35
3	0·5012	3·76	0·48	0·16	0·34
4	0·5024	3·72	0·45	0·16	0·35
5	0·4864	3·77	0·50	0·15	0·34
6	0·5312	3·75	0·47	0·17	0·36
6	0·5167	3·77	0·50	0·17	0·35
8	0·5087	3·73	0·45	0·16	0·35
9	0·4952	3·75	0·47	0·16	0·34
10	0·5126	3·77	0·50	0·15	0·35
11	0·4856	3·76	0·49	0·16	0·33
12	0·5267	3·76	0·50	0·17	0·35
MEAN		3·75	0·48	0·16	0·35
Standard Deviation ..		0·018	0·02	0·008	0·01
% Standard Deviation ..		0·5	4·2	5	2·9

DISCUSSION

The digestion procedure was specially developed to provide a rapid means of dissolving plant material. The cover glass on the digestion flask (a funnel-shaped cone with a short tapered glass rod fused on the apex and extending to about ½ inch above the liquid in the flask) acted as a condenser which economised on the consumption of nitric acid. Twelve to twenty-four leaf samples could comfortably be brought into solution within 4 hours, depending on the size of hotplate available. The digestion proceeded smoothly and needed a minimum of attention.

From the tables it is clear that leaching was quantitative—all the phosphate and sulphate ions were removed from the test solution, while Ca, Mg, Na and K ions were not retained by the resin. All the anions in the test solution were exchanged for chloride ions. The leaching procedure was rapid when carried out on a routine basis.

There is no doubt that the removal of all anions except chloride from the test solution rendered both the versenate and the flame-photometric method accurate and reproducible. The reproducibility, as indicated in Table III, was well within the experimental error to be expected from normal chemical methods.

Since the development of the method, it has been used successfully and extensively by two assistants for the routine determination of Ca, Mg, Na and K in the leaves of various crops, particularly of citrus and of pineapples. It has also been successfully applied to the determination of exchangeable cations in soils.

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ZINC DETERMINATION

Determination of Zinc in Plants and Soils

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A critical investigation of the reliability of the dithizone and polarographic methods, for the determination of zinc, is presented. A satisfactory modification of the Cowling and Miller dithizone photometric method was devised by the close study of the influence of pH on zinc extraction, transmittance curves, and accuracy of the technique. With this modified procedure, 24 zinc determinations per day could be carried out. A modified polarographic method, aimed especially at the elimination of a preliminary separation step was found to lend itself also to the simultaneous determination of copper, nickel, manganese, and possibly cobalt.

Study of Dithizone Photometric Method

ZINC IN BIOLOGICAL MATERIALS has been determined most commonly by either photometric procedures, based on the red complex which zinc forms with diphenylthiocarbazone, usually referred to as "dithizone," or polarographic methods. Both of these methods were selected for careful study.

The most popular dithizone method is that of Cowling and Miller (*2*), published in 1941. Since then many workers have presented modifications of this method (*5–7*, *14–17*). The majority of these were aimed at a reduction in the time of the lengthy extraction technique employed by Cowling and Miller. However, in many cases, a loss of accuracy was involved by the modification.

The essential feature of the Cowling and Miller method consisted in a three-stage extraction technique. First, the zinc and other metals forming dithizone complexes were separated from iron, aluminum, calcium, and other substances by extracting the sample solution, buffered at a pH of 8.5, with a dithizone–carbon tetrachloride solution. The zinc was next separated from copper by extracting the organic phase with a dilute hydrochloric acid solution. Finally, the acid solution was neutralized, buffered at pH 8.5 with ammonium citrate–ammonia solution containing some sodium diethyl dithiocarbamate, and the zinc extracted with dithizone as before. Photometric measurements were carried out on a portion of the zinc dithizonate extract at a wave length of 530 to 540 mμ (*17*).

The critical points in this method which were selected for closer study were the effect of pH on the quantitative

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extraction of zinc, the most suitable wave length at which the transmittance measurements should be made, and possible sources of error in the general technique. Several important modifications have been introduced in the original method and the modified method is described below.

Apparatus

All photometric measurements, unless otherwise stated, were carried out by means of a Beckman Model DU spectrophotometer, using 1-cm. Corex glass cells. A Beckman Model G pH meter was applied for the determination of hydrogen ion concentrations.

All glassware, particularly the separatory funnels, were scrupulously cleaned before use, first with chromic acid solution, thereafter with distilled water, and finally with zinc-free water. Stopcocks were reground so as to eliminate the use of grease. The use of rubber was strictly avoided, as rubber contains appreciable amounts of zinc. A special rack was constructed to hold two sets of six separatory funnels, one set above the other. Borosilicate glass was used throughout.

Reagents

All reagent solutions were stored in polyethylene bottles to avoid the possibility of ion exchange from glass vessels. This was particularly essential for storing the zinc standards.

All acids, alkalies, and water were redistilled from all-borosilicate glass stills. Only high-grade chemicals were used and these were further purified where necessary, as described below.

Standard Zinc Solution. Analytical reagent grade zinc oxide powder was heated to constant weight in a platinum crucible and 0.2488 gram of this was dissolved in 10 ml. of hot 1*N* sulfuric acid, neutralized with redistilled ammonia, and made up to 1000 ml. (200 p.p.m. of zinc). A 5-p.p.m. solution was made up from this for use in preparing calibration curves.

Carbon Tetrachloride. Technical grade carbon tetrachloride was dried with calcium chloride and redistilled from a borosilicate glass still. Used carbon tetrachloride was reclaimed by distillation with dilute sodium hydroxide –sodium thiosulfate solution, dried, and redistilled as before.

1*N* Ammonium Hydroxide. Prepared from redistilled ammonia.

1*N* Hydrochloric Acid. Prepared from redistilled hydrochloric acid.

Dithizone Reagent. Analytical reagent grade diphenylthiocarbazone, 0.1 gram, was dissolved in 500 ml. of carbon tetrachloride by shaking in a 2-liter separatory funnel. It was then shaken with 20 ml. of 1*N* ammonium hydroxide in 1 liter of water to transfer the dithizone to the aqueous phase. The carbon tetrachloride was run off and the aqueous phase washed with several small portions of carbon tetrachloride. This was acidified with 50 ml. of 1*N* hydrochloric acid and shaken with 500 ml. of carbon tetrachloride to transfer the dithizone once more to the organic phase. The carbon tetrachloride phase was run off into an amber-glass bottle, diluted to 1 liter, and stored in a refrigerator.

Ammonium Citrate Buffer (0.5*M*). Dibasic ammonium citrate, 226 grams, was dissolved in 2 liters of water and the pH adjusted to 9 with redistilled ammonia. This solution was extracted with excess dithizone reagent and then washed with small portions of carbon tetrachloride until the washings were pure green.

Solution A. One liter of ammonium citrate buffer plus 150 ml. of 1*N* ammonium hydroxide were diluted to 4 liters.

Solution B. One liter of ammonium citrate buffer plus 320 ml. of 1*N* ammonium hydroxide were diluted to 4.5 liters.

Solution C. One volume of freshly prepared 0.2% sodium diethyl dithiocarbamate solution was mixed with 9 volumes of Solution B just before use.

0.02*N* Hydrochloric Acid. Prepared from 1*N* hydrochloric acid.

Procedure for Plant Material

Weigh out a portion of the powdered and dried plant material containing between 5 and 25 γ of zinc (usually 0.5 gram) into a wide-necked 150-ml. conical flask. Add a mixture containing 10 ml. of redistilled nitric acid plus 2.5 ml. of analytical reagent grade perchloric acid (60 to 70%) and cover with a reflux funnel. Digest on a hot plate at a very low heat until the vigorous reaction has subsided. Then boil gently until most of the material has dissolved. Remove the cover glass, rinse with water, and evaporate the contents of the flask to about 0.5 ml. At this stage, the contents should be colorless. If not, repeat the evaporation with a few drops of perchloric acid. Cool, dilute slightly, and filter through Whatman paper No. 40 which has been previously washed with hot, dilute perchloric acid. Wash with small portions of hot water and transfer to a 100-ml. separatory funnel. Add 1 ml. of 10% zinc-free ammonium citrate and 1 drop of 0.1% phenolphthalein, and titrate with redistilled ammonia until just pink. Then carry out the following extractions.

First Extraction (separation of zinc and other dithizone complex-forming metals from iron, aluminum, calcium, and other substance). Pipet 40 ml. of solution A and 10 ml. of dithizone reagent into the separatory funnel and shake vigorously for 2 minutes. The aqueous layer should now be yellow-orange, indicating excess of dithizone. Allow the carbon tetrachloride layer to separate and run this phase into a second separatory funnel, being careful not to let the aqueous layer enter the bore of the stopcock. Wash down with three 2-ml. portions of carbon tetrachloride, withdrawing each washing into the second funnel. Then add 5 ml. of carbon tetrachloride and shake for 0.5 minute. The carbon tetrachloride layer should now be pure green. Run this layer into the second funnel and flush with a small volume of carbon tetrachloride as before. Discard the contents of the first funnel.

Second Extraction (separation of zinc from other dithizone complex-forming metals). Pipet 40 ml. of 0.02*N* hydrochloric acid into the second funnel, shake vigorously for 2 minutes, allow layers to separate, and run off the carbon tetrachloride layer (keep for reclaiming). The drops on the surface and the remaining colored carbon tetrachloride are flushed out as before. Add 5 ml. of carbon tetrachloride, shake for about 15 seconds, and run out. This removes any impure carbon tetrachloride which may stick to the sides of the funnel.

Final Extraction (to recomplex the zinc for photometric measurement). Pipet 40 ml. of Solution C and 10 ml. of dithizone reagent into the separatory funnel. Shake vigorously for 2 minutes, allow to separate, and run the organic phase into a 50-ml. volumetric flask. Flush out with three 2-ml. portions of carbon tetrachloride to remove all of the colored phase. Make up to the mark with carbon tetrachloride and measure the transmittance of the solution at 520 mμ.

Calibration Curve. Treat standard solutions containing 0 to 25 γ of zinc exactly as above. Plot a calibration curve of micrograms of zinc *vs.* per cent transmittance.

Procedure for Soils

Soil solutions for total zinc were prepared from 100-mesh samples according to the hydrofluoric-perchloric evaporation technique of Sherman and McHargue (*15*). Soil extracts for acid-soluble zinc were prepared from 1-mm. samples according to the 0.1*N* hydrochloric acid-extraction procedure of Wear and Sommer (*23*).

An aliquot of the soil solution containing 5 to 25 γ of zinc is treated as above.

Experimental Results

Influence of pH on Extraction of Zinc. Cowling and Miller (*2*) reported that zinc could be quantitatively extracted as dithizonate from aqueous solutions at a pH of between 8 and 9,

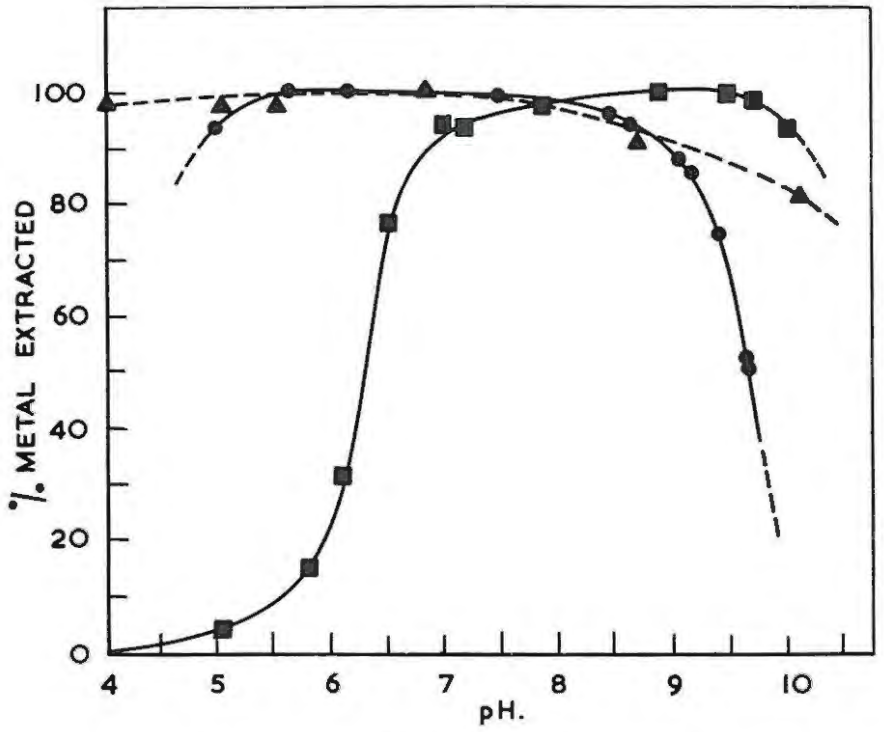

Figure 1. pH-Extraction relationships

■ Extraction of Zn with dithizone in CCl_4
● Extraction of Zn with carbamate and CCl_4
▲ Extraction of Cu with dithizone in CCl_4

and, also, that in the presence of carbamate, unless extreme care was taken to keep the conditions absolutely standard throughout, variable proportions of the zinc were extracted. Cholak, Hubbard, and Burkey (*7*) reported 100% extraction of zinc dithizonate from ammonium citrate solutions at pH 8.3, with a rapid decrease in extraction efficiency above this pH. On the other hand, Walkley (*22*) and Hibbard (*6*) reported complete extraction of zinc dithizonate from similar solutions between pH 7 to 10.

Because of these anomalies, a full investigation was carried out of the pH-extraction relationships of zinc-dithizone–carbon tetrachloride, of copper-dithizone–carbon tetrachloride, and of zinc carbamate. The results are shown in Figure 1.

These curves showed that the extraction of zinc dithizonate from ammonium citrate solution approached a maximum between pH 9 and 9.5, the amount of copper dithizonate extracted decreased appreciably above pH 9, and the extraction of zinc by carbamate fell off rapidly above pH 9.

On the basis of these results, all zinc dithizone–carbon tetrachloride extractions were carried out at a pH of between 9 to 9.5 rather than 8.5 to 9.0 as recommended by other workers. This modification ensured complete recovery of the zinc and minimized the danger of possible interference from copper. Moreover, Walkley (*22*) reported that the adsorption of zinc by suspended silica was appreciable at pH 8 but negligible at pH 9.5.

Transmittance Curves. Shirley and coworkers (*17*) published transmittance curves for dithizone and zinc dithizonate in carbon tetrachloride and, as a result of this work, recommended that photometric measurements of zinc dithizonate in carbon tetrachloride should be carried out at a wave length of between 530 and 540 mμ.

The results of a similar study carried out by the present authors are shown in Figure 2.

The curve for zinc dithizonate shows that maximum adsorption took place at about 535 mμ. For mixed-color methods, it is usually recommended that measurements be made at the wave length of maximum separation between the transmittance curves of pure reagent in solvent and metal complex in solvent. However, in the extraction procedure as described above, not all the excess of dithizone remains in the carbon tetrachloride phase—a proportion of it entering the ammoniacal aqueous phase. Thus this may not be regarded as a true mixed-color method in which the concentration of excess reagent is inversely proportional to the metal being determined. It is, therefore, more important to select a wave length at which absorption due to unchanged reagent is negligibly small.

In order to assist in the selection of the most suitable wave length at which measurements should be made, Table I was drawn up.

A wave length of either 515 or 520 mμ would be the most suitable (Table I). Although the separation between the curves was not as great at 520 as at 515 mμ, 520 mμ was finally selected because the difference is only slight while the sensitivity towards zinc dithizonate was greater.

All measurements were made at a wave length of 520 rather than at 535 mμ, as recommended by previous workers. An examination of the statistics in Table III showed that the authors were justified in making this modification.

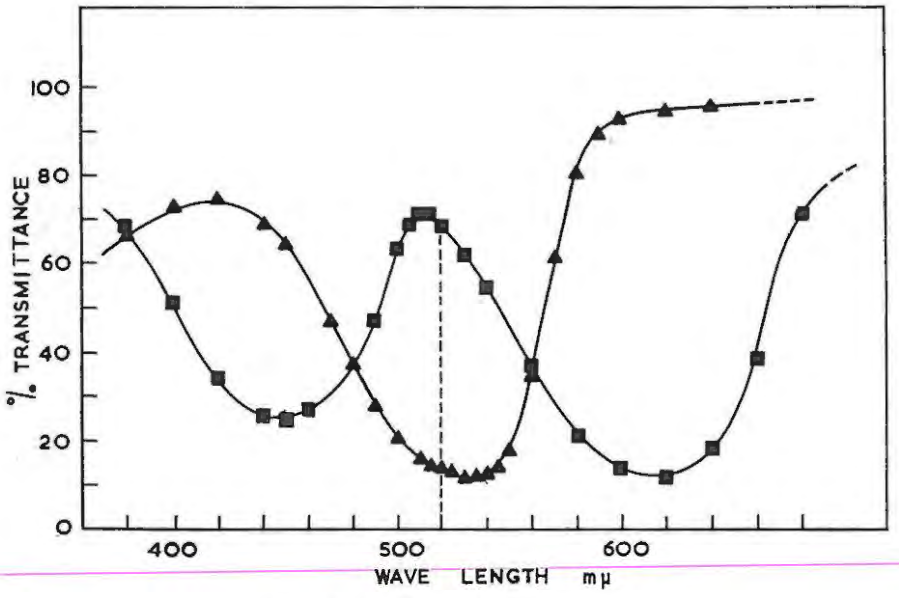

Figure 2. Transmittance curves

▲ Transmittance curve for zinc dithizonate in CCl_4
■ Transmittance curve for dithizone in CCl_4

Table I. Quantitative Examination of Transmittance Curves

Wave Length, mμ	Transmittance of Dithizone (T'), %	Transmittance of Zn Complex (T), %	Separation (T' − T), %	Loss in Sensitivity (T − T Min.[a]), %
505	68.5	17.5	51.0	5.7
510	71.0	15.9	55.1	4.1
515	70.8	14.2	56.6	2.4
520	68.2	13.3	54.9	1.5
525	65.0	12.6	52.4	0.8
530	61.9	12.0	49.9	0.2
535	57.5	11.8	45.7	0.0

[a] *T* min. equals transmittance of Zn complex at 535 mμ.

Technique. In the Cowling and Miller method, zinc was first extracted from the sample solution by shaking for 0.5 minute with 10 ml. of dithizone reagent, followed by repeated 0.5-minute extractions with 5-ml. portions of carbon tetrachloride until the organic phase had a pure green color. The present authors found, however, that by shaking for 2 minutes after addition of the dithizone, complete extraction of up to 25 γ of zinc could be obtained, and that the first extraction thereafter with carbon tetrachloride gave a pure green solution. Equilibrium had not been reached after shaking for only 0.5 minute, several additional extractions being necessary. For the sake of uniformity and to ensure complete equilibrium, the shaking time for all three extraction stages was increased to 2 minutes. By increasing the shaking time in this way, the total time for the analysis, as well as the total volume of carbon tetrachloride consumed, was actually reduced.

Instead of making a 5-ml. aliquot of the final extract to 25 ml. before photometric measurement, as described by Cowling and Miller, the authors decided that it would be safer and less time-consuming to transfer the complete carbon tetrachloride phase at the end of the final extraction to a 50-ml. volumetric flask. By the washing procedure as described, the complete colored phase, including the drop on the surface, could readily be transferred. This procedure was found to be more rapid and precise.

Previous workers (*17*) recommended the use of amber-glass separatory funnels and volumetric flasks in order that the colored extract should not deteriorate during extraction and before measurements can be carried out. The present authors carried out tests on the stability of the zinc dithizonate–carbon tetrachloride solutions. No deterioration could be detected in solutions which were left standing for 48 hours in the diffused light normally present in a laboratory. Direct sunlight, however, affected the stability of the colored solutions almost immediately. In view of these observations, no special precautions were taken to exclude light, though it is advisable to make the photometric measurements as soon as possible after completing the extraction. The dithizone reagent will keep satisfactorily for several months, if stored in a dark bottle in a refrigerator.

For the accurate delivery of 10 ml. of dithizone reagent, an all-glass automatic pipet was used. Besides obviating contact with the toxic solvent, the use of this pipet introduced a precise and rapid means of making exact additions to each separatory funnel.

Applicability of Modified Method. The photometric method was subjected to rigorous tests on a routine basis. Two laboratory assistants carried out total zinc determinations in triplicate on 30 different soil types and on 95 different leaf samples, taken mainly from citrus and pineapple plants from various regions. In all cases the results were accurate and precise. A feature of these tests was the ease and rapidity with which the assistants mastered the seemingly complex technique of the procedure.

The analyses were carried out most conveniently in batches of six. The most time-consuming factor in the procedure was the shaking period. Unsatisfactory results were obtained by shaking the separatory funnels mechanically, complete extraction of zinc being obtained only when the funnels were shaken vigorously by hand. By putting two assistants on the shaking operation, much of the tediousness was removed while the speed of the analysis was greatly increased. It was found that 24 determinations for zinc could be carried out comfortably in a normal working day. More than 100 leaf samples could be analyzed for zinc in a 5-day week by taking 24 samples into solution every day.

Study of Polarographic Method

Most of the methods described in the literature for the polarographic determination of zinc in plants and soils are based on the procedure originally developed by Stout, Levy, and Williams (*20*), which makes use of the separation of the zinc, and other minor constituents, from the major constituents of the sample by means of an extraction with dithizone in chloroform. This extraction is usually followed by a transfer to an acid solution and subsequent electrolysis in the presence of a suitable electrolyte. Of this type are the procedures of Walkley (*22*), Takazawa and Sherman (*21*), Menzel and Jackson (*11*), Jones (*9*), Martin (*10*), and Cholak, Hubbard, and Burkey (*7*), although authors of the last two references recommend the use of di-2-naphthylthiocarbazone instead of dithizone. As opposed to these, Reed and Cummings (*12*) remove the iron and aluminum by precipitation with ammonia at pH 4.5.

Although the supporting electrolyte generally used in all these procedures was a solution of potassium thiocyanate buffered to a pH of 4.6, other electrolytes such as ammonia (*11*) and biphthalate (*9*) have also been recommended.

These methods have a common disadvantage in that all necessitate the carrying out of a preliminary separation before the actual polarographic determination can be performed. This not only represents a considerable waste of time but is also a possible source of error A procedure was therefore sought which would eliminate the need for such a separation by making use of complexing agents.

A previous investigation by Eve and Verdier (*3*) showed that fluoride ions are efficient complexing agents for ferric ions, this being in accordance with the findings of West and Dean (*24*) and West, Dean, and Breda (*25*). As indicated above, thiocyanate solutions are particularly well suited to the determination of zinc in the presence of copper, lead, cadmium, nickel, and cobalt (*20*). A supporting electrolyte containing both these substances was therefore tried and has proved to be a most suitable medium for the direct determination of zinc in plants and soils.

Apparatus

The instrument used was a Leybold Nachfolger polarograph which had been slightly modified to ensure better moving contacts. As it was necessary to use high galvanometer sensitivities in this investigation, a compensator for the charging current was constructed and adapted to the instrument (*8*).

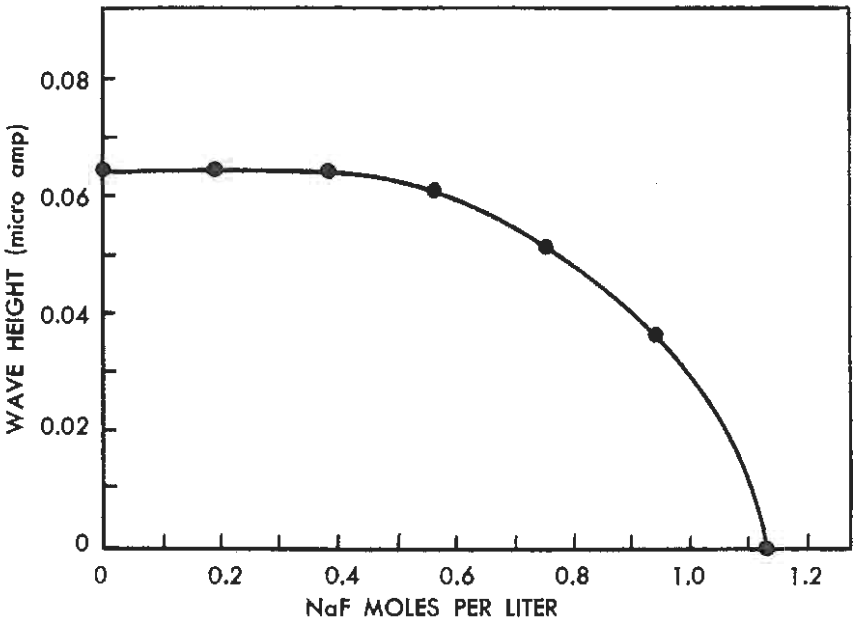

Figure 3. Influence of fluoride concentration on zinc waves

The cells were of internal-pool anode type. When determining half-wave potentials, the potential of the anode was measured against a saturated calomel electrode before and after recording a polarogram. The electrolysis cells were immersed in a thermostat and all readings were taken at 25° ± 0.1° C. A galvanometer with a sensitivity of 2 × 10^{-9} ampere per millimeter was used. The drop rate was 3.0 seconds and a mass of 3.0 mg. flowing per second into distilled water on an open circuit at a mercury height of 50.0 cm.

Reagents

Great care was taken in the preparation of solutions and only high-grade reagents were used. Polyethylene bottles were extensively used and proved to be satisfactory for storage. No effect of the type reported by Gatos (*4*), who found that storage in these bottles resulted in a depression of the polarographic maxima and waves, was noticed in this investigation. The following additional solutions were used:

Potassium thiocyanate, 15%. Prepared from analytical reagent grade potassium thiocyanate and zinc-free water.

Bromothymol blue, 0.1%. Dissolved in the minimum quantity of hot absolute alcohol and then diluted to volume with zinc-free water.

Agar-Agar solution, 0.2%. Dissolved in warm zinc-free water.

Standard zinc solution. As described above.

Procedure

Into a 25-ml. measuring flask, pipet out an aliquot of the plant or soil solution, prepared as described in Procedure for Plant Material, and containing 10 to 100 γ of zinc. If this solution is very acid, add ammonia to raise the pH to approximately 2. By means of a weighing funnel, add 0.40 gram of sodium fluoride and mix the contents of the flask well. At this stage, the yellow color due to the ferric ions disappears and a fine white precipitate separates out at the same time. Now add 1 ml. of 15% potassium thiocyanate solution, one drop of 0.1% bromothymol blue, and redistilled ammonia dropwise, with shaking, until the solution shows a faint, permanent, blue-green coloration (pH 6 to 7).

Finally add 0.2 ml. of 0.2% agar solution as a maximum suppressor, make up to the mark, and mix thoroughly. Transfer to an electrolysis vessel, remove the oxygen, and electrolyze the solution between 0.8 and 1.2 volts. Under these conditions, zinc gives a well defined wave at −1.03 volts *vs.* the saturated calomel electrode, which is suitable for quantitative determinations.

Experimental Results

Fluoride Concentration. In view of the conflicting statements concerning the formation of a ferric wave in fluoride medium (*3, 18, 19, 24, 25*) experiments were carried out to see under what conditions complexing was achieved. On the addition of a slightly acid ferric chloride solution to a solution 1*M* in sodium fluoride and 0.05*M* in potassium thiocyanate, no wave was obtained for the iron, though zinc, under the same conditions, gave a perfect wave. The formation of the white crystalline precipitate, mentioned earlier, did not appear to interfere in any way with the determination.

Because subsequent experiments showed that an increase in the fluoride concentration caused a suppression of the cobalt wave formed under the same conditions, its effect on the zinc wave was systematically studied. Figure 3 shows the variation of the height of the zinc wave with the fluoride concentration. These results show that the concentration of the fluoride should not be greater than 0.4*M* (0.4 gram in 25 ml.).

Influence of pH. In agreement with the results obtained by Shoemaker (*18*) it was found essential that the fluoride should not be added to ferric solutions which had a pH greater than 2. If the pH is greater, or very much lower than this value, complete complexing of the ferric ions does not take place and subsequent acidification or neutralization fails to bring about the desired effect. It was further found necessary to neutralize the resulting solution immediately after the addition of the fluoride for the following two reasons.

If the solution remains acid (pH less than 5), a rapidly growing wave is formed at about −0.5 volt *vs.* the saturated calomel electrode. This wave occurs even in the absence of iron. It disappears on shaking the electrolysis cell but reappears later.

If the pH is not brought to within a value of 6.0 to 7, another wave starting at about −1.3 volts is formed, which tends to interfere with the determination of zinc. It was assumed, in accordance with the findings of von Stackelberg and von Freyhold (*19*), that this wave was due to the iron in solution and no attempt was made to verify this. In a recent publication, Rulfs and Stoner (*13*), on the contrary, consider that the wave at −0.49 volt is due to the iron but the one at −1.36 volts is not. These apparently conflicting views reveal the need for a further complete investigation of the deposition of iron from fluoride solutions.

Thiocyanate Concentration. A study of the shape and separation of the nickel, zinc, cobalt, and manganese waves in thiocyanate-fluoride medium, showed that best results were obtained when the thiocyanate concentration was 0.062*M*. At this concentration, zinc, nickel, and manganese give well defined waves which are all suitable for the quantitative determination of these elements. The cobalt wave, however, is not well developed and tends to show a type of "prewave" with the consequent drawing out of the whole wave.

Maximum Suppressor and pH Indicator. The use of gelatin was found to shift the cobalt wave by approximately 0.05 volt towards more positive potentials, thus diminishing the separation

between the zinc and cobalt waves. Other ordinary suppressors, which are reduced at the dropping mercury cathode are not suitable because of the interference they caused at the high galvanometer sensitivities used. Eventually, agar-agar was found to be satisfactory. Bromothymol blue, which is added to the solution to serve as an indicator, was found to be reduced also at about −0.85 volt but at the concentrations required, even at the maximum galvanometer sensitivity, the wave produced was negligible. Measurements carried out with a pH meter showed that this indicator was suitable for use in these solutions as, in all cases, they had a pH between 6 and 7 when adjusted to the correct color.

Applicability of Procedure. Values of the half-wave potentials in sodium fluoride and in potassium thiocyanate are given for comparison (Table II). The above values for potassium thiocyanate alone refer to unbuffered solutions of this substance. Stout and associates (*20*) found that better separation is achieved in solutions buffered to a pH between 4 and 5, but gave no actual numerical values for the half waves. Moreover, these values show that with the above mixed indifferent electrolyte, good separation between the zinc and cobalt waves is achieved while the iron, which is usually present in much larger quantities than the other metals, will not interfere with the determination of any of the elements mentioned in Table II.

Other elements such as aluminum and molybdenum do not give waves in fluoride solutions, while calcium, magnesium, sodium, and potassium are reduced only at more negative values and therefore would not interfere.

In order to test the accuracy of the method for the determination of zinc under conditions somewhat similar to those likely to be encountered in plant and soil extracts, the following experiment was carried out.

A series of standards containing between 20 and 120 γ of zinc was analyzed polarographically: first, in the absence of any foreign ions, second, in the presence of 0.08 gram of iron, and third, in the presence of 0.08 gram of iron and 120 γ of copper, 60 γ of cobalt, 80 γ of nickel, and 2 mg. of manganese. This final solution was equivalent to a solution from 2 grams of a soil containing 1000 p.p.m. of manganese, 10 to 60 p.p.m. of zinc, 60 p.p.m. of copper, 30 p.p.m. of cobalt, and 40 p.p.m. of nickel, but corresponds to a larger excess of iron than would normally be met with in practice. The results obtained showed a straight-line relationship between the concentration of the zinc ions and their diffusion current, and further, that the above ions do not interfere with the determination of zinc even under conditions where their concentration is relatively larger than normally is to be expected.

Table II. Half-Wave Potentials of Trace Elements in Sodium Fluoride and Potassium Thiocyanate

Trace Elements	0.5M NaF (25)		0.4M NaF and 0.062M KCNS,	1M KCNS (26)
	V.	pH	V. at pH 6–7	V.
Cu	−0.003	5.1	−0.0	−0.0
	One wave only		−0.45	
Ni	−1.12	4.3–6.4	−0.68	−0.70
Zn	−1.14	4.2–6.4	−1.03	−1.06
Mn	−1.55	2.4–6.8	−1.55	−1.55
Co	−1.38	2.9–6.0	−1.3	−1.03

The method could further lend itself to the simultaneous determination of copper, nickel, manganese, and even possibly cobalt, though the presence of phosphate might interfere with the determination of manganese.

Statistical Comparison of the Two Methods

The accuracy and precision of the two methods were tested by comparing the results obtained on a soil solution, prepared by an exhaustive perchloric acid extraction technique. This ensured the presence of a large excess of foreign ions. The photometric measurements were carried out on three different instruments—a Hilger Spekker Absorptiometer fitted with a green filter (500 to 550 mμ), a Cenco-Sheard Spectrophotelometer set at first at 535 mμ and then at 520 mμ, and a Beckman Model DU spectrophotometer set at 520 mμ. The results are shown in Table III.

Discussion

Both the modified photometric method and the new polarographic method yielded accurate results, as there was no significant difference between the mean zinc values obtained by these two totally independent methods. The precision of the photometric procedure, with a standard deviation of only 0.52 p.p.m. in 30 p.p.m. of zinc, was very good. The difference in precision obtained on the Cenco when set at the two different wave lengths, justified the decision to carry out the measurements at 520 rather than at 535 mμ, as recommended by other workers. The increased precision obtained on the Beckman was partly due to the fact that it was possible to use an effective band width of only 1 mμ on this instrument. However, reasonably accurate and precise results can be obtained by the use of cheaper instruments like the Cenco and Spekker.

Although the accuracy of the results given by the polarographic method is much less than that of the photometric procedure, it has several advantages. The method is shorter and less tedious, fewer reagents are needed, and it could be used for the simultaneous determination of other metals present in the material analyzed.

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Table III. Statistical Comparison of Methods on a Standard Soil Solution

Factor	Photometer: Spekker Green filter 500–550 mμ	Cenco 535 mμ	Cenco 520 mμ P.p.M.	Beckman 520 mμ	Polarograph
Mean Zn (30 repl.)	30.7	30.8	30.9	31.0	31.5
Standard deviation	0.73	0.67	0.59	0.52	1.26
Stand. deviation, %	2.38	2.18	1.91	1.68	4.00
Standard error				0.095	0.222
Standard error of difference between means				0.70	
Difference between means (not significant)				0.5	

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