MINERAL COMPOSITION OF CHLOROTIC ORANGE LEAVES AND SOME OBSERVATIONS ON THE RELATION OF SAMPLE PREPARATION TECHNIQUE TO THE INTERPRETATION OF RESULTS

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Certain types of chlorosis of leaves of green plants are universally attributed to deficiencies or unbalanced conditions of one or more of the essential mineral elements. Agreement does not always exist, however, as to whether a specific type of leaf-symptom pattern is related to total content of the deficient element or only to a specific fraction of it. This is particularly true of the presumed deficiencies of iron and zinc where sample selection, contamination, and poor precision of analytical procedures are all complicating factors. An extensive review of all previous work is beyond the scope of this report. JACOBSON (**8**) and LINDNER and HARLEY (**10**) point out some of the more pertinent discrepancies with respect to iron content and iron chlorosis. More recently MCGEORGE (**11**) reports the lack of any apparent relationship between total Fe or Zn and the appearance of the specific deficiency symptoms for these elements in citrus, pear, and several other plants. Other studies have occasionally shown this same result in the case of Fe; but JACOBSON (**8**) found that surface contamination was a factor of major importance in producing discrepant results. When pear leaves from trees grown in calcareous soil were carefully washed in dilute HCl, a correlation was found between the low total Fe values found and the iron chlorosis shown. BENNETT (**1**) confirmed this observation with further studies, although he found a stronger relationship of chlorosis to an acid-soluble, or "active" Fe fraction than to the total. In Florida (**6**), New Zealand (**7**), and elsewhere citrus trees showing typical zinc deficiency symptoms have been found to contain less total zinc in their leaves than leaves from healthy trees.

In Florida when the acidity of the topsoil of the Lakeland and related series drops below pH 5.0 (between 4.5 and 5.0) for a considerable length of time, citrus trees may become unthrifty in appearance, sparsely foliated, and show typical iron-chlorosis leaf patterns. This interveinal chlorosis condition strongly resembles the lime-induced iron chlorosis pattern (**2**) and that associated with poor drainage. Exploratory tests in small containers showed that reducing the acidity by treatment with the carbonates of Ca, Na, or K (or extracting the soil with NH_4-acetate) all resulted in the disappearance of this symptom in young citrus seedlings. In connection with this general problem, some analyses were made to determine the mineral composition of leaves from trees affected with this type of chlorosis. This led to preliminary tests on the preparation of leaf samples for analysis. Several methods of washing were compared, and these were found to influence the apparent concentration of some of the elements present in small amounts. Total iron was found to be consistently lower in chlorotic leaves than in green leaves when both were carefully cleansed.

This report is concerned with the mineral composition of leaves from orange trees affected with this particular type of acid-soil chlorosis, and it gives certain information about sample preparation.

Materials and methods

Preliminary tests showed that, at least for the macro-nutrient elements, only minor variations in composition existed among the several leaves produced on a twig when a flush of growth occurred. This finding afforded an easy means of preparing several samples of whole leaves having almost identical composition. A number of twigs were harvested and samples were prepared in such a way that each twig contributed one leaf to each sample. By selecting twigs with leaves of uniform size, 6 to 8 reasonably identical samples were prepared for comparison of different washing treatments.

The washing treatments used were: (1) Wiped with moistened cheesecloth, (2) scrubbed with a fiber-bristled hand-brush in tap water, (3) scrubbed in 0.3N HCl, (4) scrubbed in a detergent[1] solution for different lengths of time, and (5) scrubbed in a detergent solution that was acidulated to 0.3N with HCl. All washing solutions were made with tap water. Each sample was rinsed in running tap water and in four separate distilled water baths. The drying, grinding (in a semi-micro Wiley mill equipped with a nickel-coated screen), storage, and analysis were all carried out as previously described (**13, 14**). Iron determinations made by standard chemical procedures on the 32 samples summarized in table II agreed closely with the spectrographic values listed.

In order to ascertain the amount of contamination contributed by the use of the Wiley mill, six samples were crushed manually with a porcelain mortar and pestle. These samples are compared with matched samples that were ground in the mill. One set of samples was collected from trees that had been sprayed with Cu and Zn to give some measure of the efficacy with which these elements could be removed. Four samples of picked leaves were sprayed with a ferrous sulphate solution containing hydroxylamine and Dreft and allowed to dry for 30 minutes before washing. This spray mixture showed some greening response when sprayed on chlorotic leaves on the trees.

[1] Dreft, a commercial "soapless" washing powder (sodium lauryl sulphate) was the only detergent tried. CHAPMAN *et al.* (4) suggest using Ivory soap, and BENNETT (1) states that a "detergent" is efficacious in removing dust residues from pear leaves.

Results and discussion

Iron was assumed to be a general surface contaminant and was used as an indicator of efficacy of washing in initial tests. Since nearly all citrus leaves have on them visible dust particles, no samples were analyzed without some washing treatment. One of the initial tests was to take twigs of growth from a mature seedling orange tree that appeared to be "normal" and form samples as described above. Duplicate samples were scrubbed with tap water, in 0.3N HCl, and in Dreft suds. The following total iron values were found respectively: 125, 155; 61.4, 62.8; and 47.5, 46.9 p.p.m. The detergent wash seemed somewhat superior to the acid wash and both were effective in greatly lowering the apparent iron concentration in the leaves. Since these values were lower than most of the published values for healthy leaves of tree crops, it was felt worthwhile to repeat the test on leaves that showed the iron-chlorosis pattern, taken from trees on very acid soil. In addition to the three treatments just described, one sample was wiped carefully with water-moistened cheesecloth, and two additional periods of detergent washing were included. The same strength of detergent wash was used for all three periods. One set of severely chlorotic, one set of mildly chlorotic, and two sets of green samples were taken from trees all in a small area of an orchard (Hamlin orange on Rough lemon roots). The leaves were mature when collected in December 1946. Table I shows the results, which are not entirely consistent. Scrubbing in water seems to have removed a larger proportion of the surface contamination from green leaves than from chlorotic ones. The acid wash was not superior to water alone on the green leaves, but appeared to be so on the chlorotic leaves. The detergent-washed leaves tended to become slightly lower in iron content with prolonged washing; but did not differ from each other significantly. This would indicate that the leaching out of internal leaf-iron was probably not a factor in these tests. The correspondence between the degree of greenness and total Fe content of the detergent-washed samples is striking.

Another collection of leaves from the same orchard was made during the rainy season of 1947. About 4-month-old leaves were collected on June 16. Two samples of mildly chlorotic leaves and two samples of green, each with 15 leaves, were given the washing treatments indicated in table I, with two added treatments, one in which it was desired to see if freshly applied iron could be removed and one in which the detergent was acidulated with HCl. The samples were analyzed for 11 mineral elements (table II). The similarity in composition of samples matched in the manner described is apparent. The only elements affected by the washing treatments were Fe, Al, Cu, and Na. There was apparently very little external matter on these leaves, which was probably due to the washing of the rains. The lowest Fe values were again found with the detergent-washed leaves. The behavior of Al, in respect to washing treatment, was identical to that of Fe. Copper likewise was lower in the leaves that were washed with the detergent, although some slight irregularities exist in the green-leaf series. Sodium and Fe apparently entered the leaves of the sprayed groups, as they averaged substantially higher for both of these elements. Likewise Na was found to increase with increase in time of detergent washing in the case of chlorotic leaves (the detergent contains Na). Green leaves were not significantly affected in this respect. The chlorotic leaves were significantly higher than the green leaves in N, P, K, and Na and lower in Ca, Mg, Fe, Mn, Cu, and B. Both types of leaves showed the same Al content.

A comparison of the proportion of each of these elements found in the leaves of a healthy Pineapple orange orchard on the same soil type, with the pH maintained above 5.0, can be seen in table III. The samples involved in this case were collected about three weeks later in the same year

TABLE I

THE EFFECT OF DIFFERENT METHODS OF WASHING ON THE APPARENT IRON CONTENT* OF HAMLIN ORANGE LEAVES

CONDITION OF LEAVES	WIPED WITH CHEESE-CLOTH	H_2O 3 MIN.**	0.3N HCl 3 MIN.	DREFT 1 MIN.	DREFT 3 MIN.	DREFT 6 MIN.	MEAN
Severely chlorotic	68.5	44.6	26.2	13.6	12.8	10.7	29.4
Mildly chlorotic	66.3	59.4	35.6	36.0	35.0	33.8	44.4†
Green A	125.1	51.6	51.2	44.9	42.1	40.2	59.2††
Green B	87.5	53.9	51.7	52.4	49.0	44.2	56.4††
Mean	86.8	51.9††	41.2††	36.5††	34.7††	32.2††	

* P.p.m. Fe on oven-dry basis (50° C).

** Entire sample in contact with washing medium for three minutes. During this time each leaf was scrubbed on both surfaces with a small wooden, fiber-bristled hand-brush.

† Difference from first mean is significant at odds greater than 19: 1.

†† Difference from first mean is significant at odds greater than 99: 1.

as those in table II. The most striking differences are the low Fe and Mn values found in the leaves from the orchard showing chlorosis. Again detergent washing resulted in lower Fe and Cu values than did the acid washing.

One washing test was made on leaves from old sweet seedling trees that were showing partial recovery from the acid-soil injury. Lime application had been made several months previous to sampling, and the chlorosis pattern was difficult to find. A few weeks before sampling the orchard was sprayed with a mixture containing $CuSO_4$, $ZnSO_4$, and hydrated lime. The leaves collected showed a very faint chlorotic pattern with the veins slightly greener than the interveinal areas. Triplicate samples were treated as shown in table IV. Zinc was determined in addition to the other trace elements already mentioned.

It is apparent that grinding these samples in the mechanical mill had

TABLE II

THE EFFECT OF SEVERAL METHODS OF WASHING HAMLIN ORANGE LEAVES ON THE MINERAL CONTENT FOUND BY ANALYSIS

WASHING TREATMENT	PERCENTAGE IN DRY LEAVES					P.P. M. IN DRY LEAVES					
	N	P	K	Ca	Mg	Fe	Mn	Cu	B	Na	Al
	Leaves showing moderate "iron" chlorosis pattern										
Wiped with moist cheesecloth	3.11	0.175	2.77	2.50	0.313	27.8	30.6	9.7	74.1	499	37
Scrubbed in tap water (3 min.)*	3.06	0.172	2.88	2.38	0.303	31.3	27.9	9.7	75.9	509	35
Scrubbed in 0.3N HCl (3 min.)	3.00	0.175	2.70	2.30	0.311	30.2	27.4	9.4	74.8	486	35
Scrubbed in Dreft (1 min.)	3.07	0.176	2.78	2.45	0.303	24.7	29.3	8.5	75.9	515	28
Scrubbed in Dreft (3 min.)	3.00	0.172	2.73	2.53	0.317	23.1	27.3	7.8	74.5	536	21
Scrubbed in Dreft (6 min.)	2.98	0.173	2.74	2.62	0.331	21.4	27.1	8.4	74.5	629	18
Scrubbed in Dreft + 0.3N HCl	3.00	0.171	2.67	2.53	0.311	24.1	28.4	8.0	74.3	561	21
Scrubbed in Dreft (3 min.)**	2.99	0.173	2.68	2.50	0.300	62.1	28.0	8.2	78.2	776	22
Mean for chlorotic-type leaves	3.026	0.1734	2.744	2.476	0.3111	30.59	28.25	8.71	75.28	563.8	27.12
	Green leaves from trees adjacent to those above										
Wiped with moist cheesecloth	2.80	0.147	2.16	3.03	0.358	39.3	37.0	10.6	83.6	433	34
Scrubbed in tap water (3 min.)	2.79	0.154	2.08	3.00	0.348	36.3	35.6	13.0	81.8	431	28
Scrubbed in 0.3N HCl (3 min.)	2.79	0.159	2.08	3.00	0.348	37.5	37.3	12.6	83.5	418	34
Scrubbed in Dreft (1 min.)	2.85	0.155	2.18	2.91	0.346	34.4	35.0	11.8	81.6	396	24
Scrubbed in Dreft (3 min.)	2.78	0.147	2.17	2.93	0.361	35.0	39.2	10.6	83.3	432	24
Scrubbed in Dreft (6 min.)	2.84	0.148	2.18	3.02	0.360	33.9	38.3	10.7	82.8	441	18
Scrubbed in Dreft + 0.3N HCl	2.94	0.155	2.16	2.90	0.348	33.7	38.4	10.0	78.5	434	21
Scrubbed in Dreft (3 min.)**	2.80	0.154	2.09	2.99	0.356	54.8	36.7	10.3	81.2	514	20
Mean for green-type leaves	2.824	0.1524	2.138	2.972	0.3531	38.11	37.19	11.20	82.04	437.4	25.38
L.S.D.† between leaf-type means 0.05	0.61	0.004	0.13	0.07	0.007	1.58	1.62	0.44	2.62	39	N.S.
L.S.D. between leaf-type means 0.01	0.83	0.006	0.18	0.09	0.009	2.17	2.21	0.59	3.56	53	N.S.
L.S.D. between any two values within leaf-type 0.05	N.S.	N.S.††	N.S.	N.S.	N.S.	2.24	N.S.	0.62	N.S.	55	4.65
L.S.D. between any two values within leaf-type 0.01	N.S.	N.S.	N.S.	N.S.	N.S.	3.07	N.S.	0.84	N.S.	74	6.22

* Length of time that the leaves were in contact with the washing solution.
** Leaves sprayed with 0.25% $FeSO_4 \cdot 7H_2O$ + 0.25% $NH_2OH \cdot HCl$ + 0.5% Dreft and allowed to dry 30 min. before washing.
† L.S.D.—Least significant difference at level indicated.
†† N.S.—Not significant.

TABLE III

EFFECT OF DREFT AND HCl AS WASHING AGENTS ON THE MICRO-NUTRIENT CONTENT OF 24 MATCHED PAIRS OF SAMPLES OF HEALTHY PINEAPPLE ORANGE LEAVES

SAMPLE TREATMENT	PERCENTAGE IN DRY LEAVES					P.P.M. IN DRY LEAVES				
	N	P	K	Ca	Mg	Fe	Mn	Cu	B	Na
Scrubbed in Dreft	2.65	0.141	2.11	3.01	0.426	90.69	86.72	6.92	77.34	527.2
Scrubbed in 0.3N HCl	....	....	....	....	....	93.53	88.05	7.17	78.02	530.4
Effect of treatment	....	....	....	....	....	**	N.S.	*	N.S.	N.S.

* Odds for significant difference between means exceeds 19: 1.
** Odds for significant difference between means exceeds 99: 1.

little or no influence on the concentration of these elements. The most efficacious washing treatment for the several elements involved is the combination of the detergent and acid. Iron was just as efficiently removed by the detergent alone. Copper and Zn were apparently partially removed by acid and partially by the detergent, since each of these removed substantial amounts of these elements and the lowest reproducible level was obtained with the acidulated-detergent washing. It is not possible to conclude, however, that the amounts of these elements remaining after washing are totally within the leaf. Although the proportion of Cu was greatly reduced by the acidulated-detergent wash, it seems unlikely that the remaining Cu was all internal. If so, it is from five to 10 times the concentration usually found in normal, unsprayed citrus leaves (**13**). Zinc, especially under sand culture conditions, frequently occurs in

TABLE IV

A COMPARISON OF THE APPARENT COMPOSITION OF SPRAYED* ORANGE LEAVES IN RESPECT TO CERTAIN MICRO-ELEMENTS AFTER VARIOUS WASHING AND GRINDING** TREATMENTS

SAMPLE TREATMENT		P.P.M. IN DRY LEAVES						
		Fe	Mn	Cu	B	Na	Zn	Al
Wiped with moist cheesecloth		57.7	27.3	209	169	513	208	133
Scrubbed in tap water		57.3	27.3	237	157	466	202	109
Scrubbed in 0.3N HCl		57.3	29.0	125	160	480	79	133
Scrubbed in Dreft		45.0	26.3	107	154	513	100	76
Scrubbed in Dreft (NG)		45.3	22.3	108	160	482	115	87
Scrubbed in Dreft + 0.3N HCl		45.0	22.7	67	165	517	62	65
Scrubbed in Dreft + 0.3N HCl (NG)		44.0	22.0	65	161	488	59	62
L.S.D. between any two values	0.05	4.9	2.4	46	N.S.	N.S.	44	15
	0.01	6.9	3.4	64	N.S.	N.S.	61	21

* Old sweet seedling trees sprayed with a nutritional spray containing copper and zinc a few weeks before sampling.

** All samples were ground in a semi-micro Wiley mill with a nickel-coated screen, except the two treatments marked (NG), which were pulverized in a porcelain mortar.

amounts from 50 to 100 p.p.m. It is possible, therefore, that the 60 p.p.m. found after the most drastic washing is largely absorbed Zn. Healthy leaves from commercial orchards, however, usually vary only from 20 to 40 p.p.m. (**12**). It appears doubtful that leaves previously sprayed with materials containing Fe, Cu, or Zn can be satisfactorily used for diagnostic purposes for these particular elements.

Manganese is inconsistent. Of several tests over the past few years, this is the only time that this element has been affected by washing treatment. It appears that some Mn contaminant was in the spray mixture, and this was affected by washing in much the same way as Fe.

Aluminum, under these conditions, did not behave in quite the same way as Fe. The spray appears to have influenced its removal in some way. The lowest values were obtained with the acidulated-detergent washing treatment.

The external contamination that we are most concerned with comes from colloidal particles, since sprayed leaves are usually avoided for trace-element analysis. Iron, Al, and Cu (perhaps Zn also) apparently are contributed by dust deposits on leaves. In the absence of spray residue, these elements appear to be removed as efficiently by the detergent alone.

Citrus leaves are glabrous and waxy and probably are capable of withstanding a more rigorous washing treatment than would more succulent leaves. It is possible that some mobile elements might be lost by leaching if the leaves become "waterlogged" with the detergent solution. With intact citrus leaves 8 to 10 minutes are required before internal wetting is evident. No evidence of leaching of even such a mobile ion as K has been found in the present studies, with six minutes as the maximum length of time that the leaves were in contact with the washing medium.

By carefully washing leaves of pear, corn, and tobacco in 0.3N HCl, JACOBSON (**8**) found a relationship between total iron and chlorophyll content. It is apparent from the present work that with citrus the action of a detergent is usually more efficient than acid when the leaves are thoroughly scrubbed on both surfaces.

MCGEORGE (**11**) recently found total Fe values ranging from 75 to 366 p.p.m. in Arizona citrus leaves of several varieties. Most values were greater than 150 p.p.m. No mention was made of a washing treatment for removal of external contamination. No difference existed between the green and chlorotic samples. The Mn values were all relatively low in comparison with those found with leaves from other citrus areas (**12**), and ranged from 10 to 32 p.p.m. The suggestion is made that the Mn deficiency pattern may be in reality an Fe toxicity condition.

In connection with a general survey of the composition of orange leaves from different localities, the present authors (**12**) collected 17 samples of newly matured leaves from different orchard locations in Arizona. These were rigidly selected for age, washed in Dreft solution, and handled in our regular procedure. The Mn values obtained were almost identical with those found by McGeorge. They ranged from 7 to 34 p.p.m. The total Fe values, however, were all quite low. One sample of leaves showing the iron chlorosis pattern showed 18 p.p.m. Fe. The over-all range of iron values for the 17 samples was only from 18 to 47 p.p.m. total Fe.

This wide discrepancy in Fe content along with others, such as the reported values for citrus in Florida (**5**) and California (**3**), leads to the suggestion that surface contamination has not received proper attention. Iron also increases with leaf age (**15**). In general, it seems possible that conclusions in regard to total Fe and iron-chlorosis and other Fe functions might become more harmonious if greater consideration were given to age of leaves sampled and removal of surface contamination.

From comparison with previous work (**3, 12**) it would appear that Fe and Mn are both present in inadequate amounts for proper leaf function in the chlorotic leaves grown under highly acid soil conditions. Observations indicate that extensive root damage is associated with this type of tree condition. This would seem to indicate that the availability of either of these elements is not the primary cause of the disorder. The real cause is not yet understood but may be some toxic reaction which damages or kills most of the small feeder roots. Under these conditions Fe and Mn absorption, or translocation, is apparently depressed to the point that the leaves are inadequately supplied.

In regard to the general mineral composition of leaves low in total Fe and showing the iron-chlorosis pattern, the present results show increased K and lowered Ca in comparison with green leaves. Since this has been quite generally found with other plants, CHAPMAN (**4**) recently predicted that the same relationship would prevail in citrus. He further points out that all other known mineral-nutrient deficiencies that have been examined (except K) induce this same result. Higher N percentage in Fe-chlorotic leaves apparently is not widely recognized with the tree crops, although it was noted in relation to mottle-leaf (zinc deficiency) in California (**9**) and in leaves with lime-induced chlorosis in Arizona (**11**). The increased P and lowered Mg observed in chlorotic leaves in the present study are variations that were not found in the lime-induced chlorotic citrus leaves in Arizona (**11**).

There seems to be an over-all similarity between the composition of citrus leaves showing the "iron-chlorosis" pattern whether it occurs in acid soil, calcareous soil, or poorly drained soil. Iron and manganese both appear to be unusually low in such leaves. For example, a sample of Valencia orange leaves obtained in Texas and showing "iron-chlorosis" symptoms contained 11.6 p.p.m. of Mn and 40 p.p.m. of Fe (**12**). Likewise, a leaf sample obtained in Arizona representative of Valencia oranges associated with overirrigation (**11**) contained 18 p.p.m. of Fe and 8.5 p.p.m. of Mn (**12**). The deficiency pattern was the same. Therefore, it appears that with the disorders of tree crops called "iron chlorosis" or "lime-induced chlorosis," consideration should be given to manganese as well as to iron nutrition.

Summary

Leaves from orange trees on highly acid soil that were in a chlorotic and unthrifty condition were used as test material for studies on preparation of samples of leaves for analysis. Eleven chemical elements were determined. The various treatments tried for removing surface contamination consisted in wiping both surfaces with moist cheesecloth, scrubbing in tap water, in HCl, in a neutral detergent (Dreft), and in an acidulated detergent solution. The surface contaminants consisted chiefly of natural clay and organic dust particles, although a few tests were made with leaves sprayed with Fe, Cu, and Zn.

With unsprayed leaves total Fe, Al, and Cu were reduced to minimal values by scrubbing in a neutral detergent solution, followed by adequate rinsing. Sodium was absorbed from the detergent solution by chlorotic leaves but not by healthy ones. No other element tested was influenced by washing treatment.

With leaves that were sprayed with Cu and Zn in the presence of lime, acidulated detergent solution consistently gave the lowest analytical values for these elements. There is some doubt, however, that such treatment completely removes surface contaminations of this type.

Grinding the leaf samples in a mechanical mill did not appreciably influence the composition in regard to the elements determined.

Total iron of the leaves cleansed with the detergent showed a strong inverse relationship to the degree of iron chlorosis. Severely chlorotic leaves showed from about 10 to 15 p.p.m. total iron; mildly chlorotic leaves from about 18 to 30 p.p.m. iron; and non-chlorotic leaves from about 35 to 100 p.p.m. Newly matured leaves from healthy orange trees usually show from 50 to 70 p.p.m. The chlorotic leaves also had an abnormally low Mn content.

The general composition of these chlorotic orange leaves and the need for greater emphasis on sampling methods and preparation of samples for analysis are discussed.

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