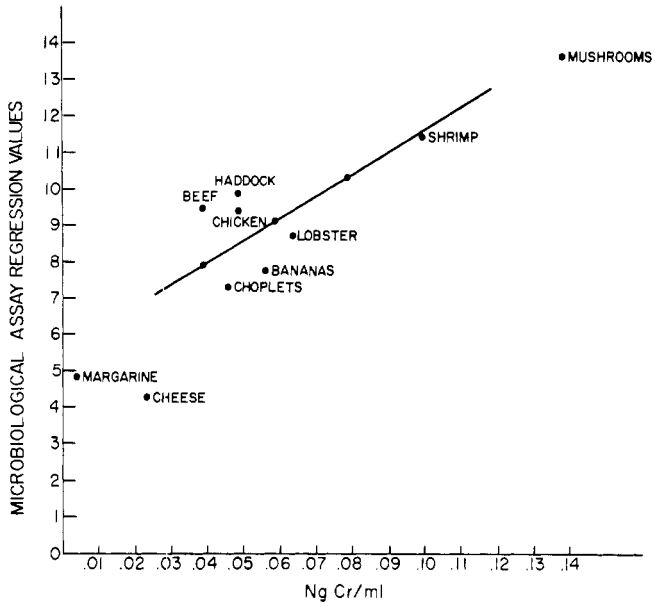

Figure 5. Biologically active glucose tolerance factor chromium distribution in different foods, with the relationship between microbiological assay regression values and the chromium in food samples. The slope of the line is given by the regression equation $y = a + bx$ or $y = 5.7273 + 54.7848x$.

the purified yeast fractions used to obtain the microbiological assay values. Using these fractions, a parallel relationship was established in this study between glucose tolerance factor bioassay values and the *Flavobacterium* microbiological assay. Thus, it appears that the growth response elicited in the bacteria by the yeast fraction is related to the biologically active chromium complex assayed by the rat epididymal fat pad assay (Mertz, 1969).

After the assay organism for the glucose tolerance factor was initially isolated, the microbiological assay was patterned after the vitamin B_{12} assay (Ford, 1953; Lichtenstein *et al.*, 1959) and the microbiological assay of vitamin B_6 (Toepfer and Polansky, 1970). The assay was used in a preliminary trial testing the content of the chromium factor in several foods, and good agreement was found between the results obtained with the rat bioassay and the microbiological assay. The microbiological assay can be applied directly to the testing of isolated yeast fractions to purify the active principle and to test for the content of glucose tolerance factor in natural sources.

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Preparation of Solutions for Atomic Absorption Analyses of Fe, Mn, Zn, and Cu in Plant Tissue

A. S. Baker* and R. L. Smith

Dry ashing results in decreases in the apparent levels of Cu, Zn, and Fe in many types of plant tissue. The high concentrations of extraneous ions often present in plant ash solutions interfere with the determination of Fe, Mn, Zn, and Cu by atomic absorption. A procedure is proposed involving wet ashing with HNO_3, $HClO_4$, and H_2SO_4 and complete extraction of the trace elements with pyrrolidine dithiocarbamic acid in $CHCl_3$. The $CHCl_3$ is evaporated and the residue containing the trace elements is solubilized by refluxing with HNO_3. Using the proposed procedure, good recovery of added trace elements was obtained and the relative standard deviation was below 2% for the four trace elements.

There are two difficult problem areas that affect the precision and accuracy of atomic absorption (AA) analysis of trace elements in plant tissue, elemental losses that occur during organic matter destruction and extraneous inorganic ion interferences with AA.

Dry ashing of tissues in open vessels at high temperatures in a muffle furnace is a common means of organic matter destruction primarily because of the economy in terms of equipment and technician time. Elemental losses due to volatilization and incorporation in some solid material, either in the ash residue or the ashing vessel, can be quite serious with dry ashing. Since the chlorides of many metals have relatively low melting points, Gorsuch (1970) warns of the danger of volatilization of metals from tissues that are high in Cl at the temperatures used for muffling. Kometani *et al.* (1972) found it helpful to treat filter papers on which air-borne particulate matter was collected with H_2SO_4 before muffling at 500° in order to drive off Cl. At 600° they found greater volatilization losses of Cu from the $CuSO_4$ than $CuCl_2$. This also can be predicted from the melting points of the two salts. Generally temperatures must exceed 500° in ordinary muffle

Western Washington Research and Extension Center, Puyallup, Washington 98371.

furnaces to completely ash most plant tissues and complete ashing is important for Cu recovery (Isaac and Jones, 1972).

Gorsuch (1970) states that losses of Fe, Mn, Zn, and Cu are minimal with proper wet oxidation procedures. We found this to be entirely true provided the ash solutions are not taken to dryness in the presence of dehydrated SiO_2 or $CaSO_4$.

Ash solutions of plant tissue of convenient concentrations for AA analyses of Fe, Mn, Zn, and Cu often have relatively high concentrations of extraneous matrix elements. This matrix may influence AA by trace elements due to atomization, chemical, ionization, molecular absorption, and light scattering interferences (Christian and Feldman, 1970; Slavin, 1964). The matrix also affects the physical characteristics of solutions which, in turn, influences the rate of aspiration (Trent and Slavin, 1964), droplet size, and proportion of the aspirated sample which enters the flame (Slavin, 1964).

This paper describes and discusses a procedure for obtaining a wet ash solution of plant tissue that is compatible with a solvent extraction of Fe, Mn, Cu, and Zn which minimizes losses during ashing and matrix interference in AA analysis.

EXPERIMENTAL SECTION

Reagents. *Ashing Catalyst.* Transfer 0.57 g of NH_4VO_3 to a 100-ml volumetric flask. Use heat to dissolve the salt in about 80 ml of water. Adjust to volume while hot and readjust to volume when cool.

Ashing Acids. Mix 1400 ml of concentrated HNO_3, 400 ml of 70% $HClO_4$, 200 ml of concentrated H_2SO_4, and 10 ml of the ashing catalyst.

Buffer. Transfer 460 g of trisodium citrate dihydrate to a 1-l. volumetric flask. Use heat to dissolve the salt in about 900 ml of water. Adjust to volume while hot and readjust to volume when cool.

Pyrrolidine Dithiocarbamic Acid (PDTCA). Add 18 ml of pyrrolidone to about 500 ml of $CHCl_3$ in a 1-l. volumetric flask. Cool the contents in an ice bath and add 15 ml of CS_2 slowly while swirling in the ice bath. Bring to volume with $CHCl_3$ and store in a refrigerator.

Diluting Solution. Dilute 80 ml of 1:1 (v/v) HNO_3 to 1 l. with water.

Standard Solution. Make four-element standards in 1.44 *M* H_2SO_4 so that each standard combines increments of the four trace elements up to 30, 15, 10, and 5 μg/ml of Fe, Mn, Cu, and Zn, respectively. The top standards are selected to produce between 60 and 70% absorption by AA.

Procedure. Transfer about 2.5 g of oven-dried plant tissue to a 100-ml volumetric or Kohlrausch flask. Transfer 20 ml of the ashing acids to the flasks, washing down the tissue adhering to the neck of the flask. Swirl the flasks to thoroughly wet the plant tissue. Allow the mixture to react without heat until foaming subsides. The action at this stage is quite varied with different plant tissues. With most, the oxidation is slow and easily controlled. With others, *e.g.*, Douglas fir needles, the reaction mixture rapidly heats up and foams over. Conflagration may occur if all the tissue is not wetted. The foaming and rate of oxidation can easily be controlled by placing the flasks in a shallow pan of cold water.

Place the flasks on a hot plate and bring the surface temperature slowly to 140 ± 20°. Reflux at this temperature for at least 4 hr. This can be accomplished in an ordinary forced draft hood. Place the flasks on a hot plate in a $HClO_4$ hood and bring the surface temperature up to 235 ± 20°. When the last of the HNO_3 is volatilized, the acid mixture will boil vigorously and may turn quite dark. Continue refluxing at this temperature until the solution turns clear yellow. Place a vented sheet Al reflector oven over the flasks and bring the surface temperature to 305 ± 15°. Continue heating at this temperature for at least 30 min after the copious fuming of $HClO_4$ ceases. The residual H_2SO_4 will reflux with no noticeable fumes if the surface temperature is kept below 320°.

Remove the flasks from the hot plate, cool, and add 5 ml of water and boil for about 30 min. Cool and filter the ash solution into a 60-ml cylindrical separatory funnel through a 5.5-cm Whatman no. 40 filter paper. Wash the flasks and filter papers with five 3-ml aliquots of water. Finally, wash the filter papers with two 2-ml aliquots of water, directing it along the edge of the filter. To other separatory funnels add 25 ml of the standard solutions. Treat the ash solution and standards as follows.

Add 4.5 ml of concentrated NH_4OH to the funnels, swirl, and allow to cool. Using a microelectrode pair or a combination electrode, adjust the pH to 2.00 ± 0.05 by dropwise additions of 1:1 NH_4OH and wash the electrodes with a small volume of water. Add 5 ml of PDTCA and shake for 5 min. Allow the phases to separate and run the $CHCl_3$ layer into a 30-ml beaker containing 2 ml of water. Add 3 ml of the buffer to funnels and swirl, bringing the pH up to 4.90 ± 0.15. Repeat the 5-min extraction using 4, 4, and 2 ml of PDTCA. If the $CHCl_3$ layer in the last extraction is still reddish, extract with another 2-ml aliquot of PDTCA.

Evaporate the $CHCl_3$ at a hot plate temperature of 70 ± 10° in a hood with a strong air current blowing over the beakers. The H_2O in the beaker and the air current prevent the organic phase from creeping on the sides of the beakers. The water phase need not be evaporated but it will do no harm if it drys at <80°.

Remove the beakers from the hot plate and add 2 ml of 1:1 HNO_3 under a watch glass. When the bubbling has subsided (in 2 to 3 min), place the covered beakers on a hot plate and bring the surface temperature to 120 ± 10° and reflux for about 30 min. Transfer the solutions quantitatively to 25-ml volumetric flasks and bring to volume with water.

The latter solutions are used to determine the trace element concentration by AA. The diluting solution is used to set the instrument on zero absorption. The four-element standards carried through the extraction procedure are used to obtain the standard curves. When any of the unknowns are higher than the top standard, the former can be diluted with the diluting solution. Sometimes a small amount of trace element contaminant can be detected in the blank standard. This error is cancelled when undiluted ash solutions are compared to the standards prepared in the same manner. A small negative error occurs when the ash solutions are diluted. This error is corrected by first estimating the amount of trace element in the blank standard from its absorption relative to that of the diluting solution and using the following formula.

$$y = x + AC/A + B$$

where A = ml of diluting solution, B = ml of unknown solution, C = μg/ml of estimated trace element contaminant in the blank standard, x = apparent μg/ml of trace element in the diluted solution, and y is the actual μg/ml of trace element in the diluted solution derived from the plant tissue, ashing acids, and filter paper.

A blank should be carried through the whole procedure although we have not found detectable amounts of the four trace elements in the reagent grade ashing acids or the filter paper.

General. Standards were made from primary standard metals or oxides. All other reagents were reagent grade except for the pyrrolidine, which was J. T. Baker practical grade. Serious Zn contamination occurs from contact with

natural or synthetic rubber. Thus, a completely plastic distilled water storage and dispensing system should be used, and plastic stoppers should be used for flasks.

In this laboratory, electric hot plates with a continuous heat control are used. An enclosed surface thermometer is used for calibrating the control knob with surface temperature.

It is important to note that if another type of oxidation vessel is used there is increased danger of obtaining an explosive mixture. After the initial refluxing at about 140°, much of the organic matter is oxidized or dissolved, but there is generally some undissolved, cotton-like, organic material remaining in the faintly yellow acid mixture. When the surface temperature is raised to about 235°, the HNO_3 is volatilized slowly from the volumetric flasks, resulting in a slow rise in boiling temperature and oxidation potential. By the time the last of the HNO_3 is volatilized, all but the most refractive organic material is oxidized. If a more open vessel is used, the HNO_3 may be volatilized so rapidly that an explosive mixture is obtained.

For shaking, it was found convenient to lay the separatory funnels in a horizontal shaker with a 1-in. thick piece of plastic-covered foam rubber inserted at each end to hold the funnels firmly in place.

To take advantage of the increased AA sensitivity that can be obtained with an organic solvent, the watch glass can be removed from the beaker containing the extracted trace element residue and the 1:1 HNO_3 and the contents dried at <80°. When the nitric acid odor cannot be detected, add exactly 1 ml of H_2O to each beaker and transfer the contents to a volumetric flask using a water miscible organic solvent, *e.g.*, methanol, to wash and bring to volume.

The AA analyses are performed in this laboratory with an 82-500 series Jarrell-Ash instrument using an air–acetylene flame and laminar flow burner.

DEVELOPMENT

Extraction. To provide ash solutions of plant tissue of convenient concentrations for AA analyses of Fe, Mn, Zn, and Cu, the extraneous matrix elements are often present in relatively high concentrations. Dilution to avoid matrix interference often results in the determination of one or more trace elements near the detection limit with an adverse effect on accuracy and precision. Approximate matching of standards with the extraneous ions of the sample is difficult or impossible when dealing with biological materials of variable matrix composition. After some unsatisfactory attempts to use high salt or acid concentrations to blank out matrix interference, it was decided that the most reliable procedure would be to separate the trace elements from the major constituents.

The method of Lakanen (1966) was selected for extraction because of the economy of the PDTCA reagent, the elimination of the need to determine the Mn shortly after extraction, and the completeness of the extraction. Lakanen recommended dry ashing at 450° of the residue remaining after evaporation of the $CHCl_3$. Ashing is incomplete at this temperature, and serious losses of Cu and Zn occur during ashing. When the residue was dissolved by refluxing with HNO_3, complete recovery of the four trace elements was obtained. In the presence of PO_4. high levels of Fe are difficult to extract completely at pH 4.9 and higher. Most of the Fe is extracted at pH 2.0. As Lakanen (1966) points out, the pH must be adjusted to between 4.6 to 10 for complete recovery of Mn.

The H_2SO_4 in the ash solutions and standards provides buffering near pH 2.0 due to the weak ionization of HSO_4^-. The citrate buffers well between pH 4 and 6. It is important that pH remain below about 6 to prevent precipitation of $CaHPO_4$. Particulate matter makes phase separation difficult because of the formation of stable bubbles.

Table I. Effect of Muffling and a Premuffling Phosphoric Acid Addition on the Recovery of Trace Elements, μg/ml

	Fe	Mn	Zn	Cu
Expected concentration[a]	8.00	8.00	4.00	4.00
Concentration found				
Not muffled	7.97	8.10	4.03	4.10
Muffled	7.97	7.99	3.69	0.38
Not muffled, H_3PO_4 added[b]	7.95	8.03	4.02	4.06
Muffled, H_3PO_4 added	3.95	6.35	3.42	3.24

[a] Calculated concentrations of trace elements on dilution to 25 ml. [b] 7 ml of dilute H_3PO_4 (2.91 g of 86% H_3PO_4/l. of 95% ethanol) added.

Ashing. Many dry ashing procedures were investigated but none proved entirely satisfactory for all four trace elements in all types of plant material. Variables included ashing temperatures from 420 to 650°, ashing periods from 2 to 16 hr, acidification of the tissue with ethanolic solutions of H_2SO_4 or HCl before ashing, and the use of $Mg(NO_3)_2$ as an ashing aid after predigestion of plant tissue with HNO_3 or preashing at 350°. Acidification of the plant tissue prevents etching of crucibles or borosilicate beakers used for ashing and consequent absorption of trace elements. The use of $Mg(NO_3)_2$ showed promise in terms of recovery tests of trace elements but it was not consistent, probably because of etching of the container surfaces. Puffing or rapid ignition with some predigested or preashed plant tissues during drying or muffling was difficult to control when $Mg(NO_3)_2$ was added.

It became obvious that wet ashing is a more reliable preparation. The H_2SO_4 was added for two reasons. Its high boiling point permits volatilization of all of the ClO_4 ion, preventing the formation of a precipitate (probably $KClO_4$) upon neutralization of the ash solution in the separatory funnels. Secondly, it is possible to finish the oxidation with a fairly consistent quantity of acid remaining, making neutralization more convenient. The addition of H_2SO_4 results in the formation of a $CaSO_4$ precipitate with most plant tissues. It is filtered off along with dehydrated SiO_2. Recovery tests indicated neither precipitate occludes detectable quantities of the four trace elements unless the ash solution is taken to dryness. Gorsuch (1970) points out that serious losses of Pb occur when $CaSO_4$ is precipitated, but $PbSO_4$ is much less soluble than the sulfates of Fe, Mn, Zn, or Cu.

Volumetric flasks are convenient vessels for wet ashing because the reflux action in the stem allows for stepwise oxidation with less technician attention and eliminates explosion hazards. Spray losses are negligible due to the conformation of the vessel. The VO_3 catalyst speeds oxidation of resistant organic materials (*e.g.*, oils and waxes) during the $HClO_4$ oxidation step and does not interfere in the AA determination of the four trace elements.

RESULTS AND DISCUSSION

Table I indicates the possible extent of dry ashing losses of trace elements. This experiment was conducted by transferring a solution of the Cl (Fe and Zn) and SO_4 (Mn and Cu) salts of the four trace elements to 30-ml borosilicate beakers and drying at 100°. An ethanol–H_3PO_4 solution was then added to some of the beakers, dried at about 80°, and baked at 200°. Some of the beakers were then muffled at 550° for 4 hr. All of the beakers were covered with a watch glass, and 5 ml of 2 *N* HCl was added and then refluxed for 1 hr at 120°. The solutions were transferred to 25-ml volumetric flasks and brought to volume with H_2O. The latter were analyzed for Fe, Mn, Zn, and Cu by AA. In the absence of H_3PO_4 a drastic loss of

Table II. Effects of Extraneous Inorganic Ions on the Apparent Trace Element Concentrations Determined by Atomic Absorption, μg/ml

Extraneous cation concentration[a]	P concentration, μg/ml 0	200	700	0	200	700
	Fe-6, μg/ml[b]			Mn-6, μg/ml		
Nil	6.00	6.00	6.00	6.00	6.00	6.00
Low	5.92	6.00	5.92	6.00	6.00	6.05
High	5.45	5.40	5.45	5.60	5.60	5.70
	Zn-5, μg/ml			Cu-3, μg/ml		
Nil	5.00	5.00	5.00	3.00	3.05	2.97
Low	4.98	4.98	4.90	2.99	2.94	2.94
High	4.87	4.80	4.80	2.67	2.81	2.78

[a] Nil, no extraneous cations added. Low, 200, 100, 50, and 10 μg/ml of Ca, Mg, K, and Na in solution. High, 2000, 500, 500, and 50 μg/ml of Ca, Mg, K, and Na in solution. [b] Indicates the actual concentration of the trace element that should be present in solution.

Cu occurred on muffling and a smaller but significant loss of Zn occurred. Under the same conditions there was no significant loss of Fe and Mn. The addition of H_3PO_4 improved Cu recovery but Fe, Mn, and Zn recovery was decreased with muffling. The latter probably resulted from the formation of a difficultly soluble dehydrated iron phosphate which occluded Mn, Zn, and Cu phosphates. Those beakers to which H_3PO_4 was added and which were subsequently muffled contained an obvious residue after refluxing with HCl. The amount of H_3PO_4 added was sufficient to result in 220 μg of P/ml in the final solution. This is equivalent to 0.22% P in 2.5 g of plant tissue.

The addition of the same H_3PO_4 solution to 2.5 g of a variety of plant tissue samples previous to muffling resulted in improved Cu recovery but definitely decreased Fe recovery. The Fe recovery was not decreased to the extent shown in Table I probably because other cations in the plant tissue compete with Fe for association with the PO_4·Mn and Zn recoveries were too variable to evaluate.

To evaluate the effects of some extraneous ions present in plant ash solutions on AA analyses, solutions were made up with constant amounts of Fe, Mn, Zn, and Cu and varying concentrations of common plant tissue cations and P. The Ca, Mg, K, and Na were added as Cl salts and the P source was H_3PO_4. The solutions were 0.4 *N* with respect to HCl.

The presence of low concentrations of extraneous cations and low or high levels of P did not markedly effect the AA analyses of the trace elements (Table II). Higher, but common, concentrations of alkali and alkaline earth metals resulted in depressions in the apparent concentration of each of the trace elements. When the hollow cathode beam was passed through the cooler luminous portion of the flame, negative P interference was serious with all four trace elements and the added cations tended to counter P interference. The same was true when a cooler air–hydrogen flame was used in conjunction with a total consumption burner.

Recovery tests were run on markedly different plant tissues using three methods of ash solution preparation. Levels of some extraneous ions in these tissues are shown in Table III. The trace elements for recovery tests were added by drying aliquots of standard trace element solutions in the ashing vessels prior to adding the plant tissues. Ash solutions were prepared by the proposed procedure. Another set was dry ashed and the trace elements extracted by the proposed procedure. Dry ashing was accomplished by transferring the tissue to 30-ml unetched Pyrex beakers and ashing at 550° for 4 hr. The ash was

Table III. Extraneous Ions in the Plant Tissue Used for Recovery Tests, ppm

	Ca	Mg	K	Na	P
Alder leaves	9800	2370	7900	280	1350
Douglas fir needles	2200	790	5500	290	1440
Corn cobs	300	260	5800	180	530
Corn grain	300	1330	6300	130	3640
Cabbage leaves	11,100	2640	36,700	2530	5650
Alfalfa	13,700	2530	22,200	2410	2800
Mixed forage	13,900	2530	20,000	1820	2600

dissolved by refluxing for 1 hr in 5 ml of 2 *N* HCl. The third procedure involved wet ashing with HNO_3 and $HClO_3$ without separating the trace elements from other matrix ions. The H_2SO_4 was left out because it is a poor medium for AA analysis. When ashing was completed, the residual $HClO_3$ was diluted, filtered into 50-ml beakers, and dried at 150 to 170°. It is necessary to filter off the dehydrated SiO_2 previous to drying to prevent losses of Fe and Mn. The residue was dissolved by refluxing in 5 ml of 2 *N* HCl. These were dried again at 100°, redissolved in 5 ml of 0.5 *N* HCl, and brought to a 25-ml volume with H_2O. The latter solutions were analyzed by AA using standards made up on 0.1 *N* HCl. Each analysis was run in triplicate.

The results of these tests (Table IV) indicate that recovery of added trace elements was generally better and more consistent by the proposed procedure. Individual elements in individual plant materials can be determined with little error by the other procedures, but considering all plant materials and all elements, the proposed procedure is the most reliable. The relative standard deviations were lowest for the proposed procedure. These were calculated from the pooled triplicate analyses. Bartlett's χ-square test for homogeneity of variances indicated that variances of individual tests were not significantly different within procedures except for dry ashing for Cu. For the latter, the variance was much greater on fir needles, corn cobs, and alfalfa than on the other tissues.

Mn analyses were not affected by the ashing procedure. Dry ashing losses of Cu can be quite marked, as indicated by the analyses on fir needles, corn cobs, and alfalfa. The losses on fir needles and corn cobs were probably due to volatilization of Cu, since both materials are low in other inorganic constituents (Table III) and were completely ashed. Although the corn grain is low in bases, it is quite high in P, which may account for the good recovery of Cu from this material. As pointed out above, the addition of H_3PO_4 improved Cu recovery. The low levels of native and recovered Cu on alfalfa were probably due to its incorporation in the carbonaceous residue of the ash. There was considerably more dark residue remaining with alfalfa than with the other materials after dry ashing at 550°. A subsequent dry ashing of the alfalfa at 650° resulted in an apparent tissue level of Cu of 13.9 ppm. Isaac and Jones (1972) found that of 13 elements analyzed, only Cu was significantly decreased due to incomplete ashing of plant tissue. Dry ashing resulted in only small decreases in Zn levels on most of the materials and the mean recovery was down about 5%. Dry ashing resulted in a 10% decrease in mean recovery of Fe.

Recovery of all four trace elements was reduced when they were not separated from the extraneous inorganic salts after wet ashing. Although the Fe showed the greatest decrease in recovery, the results are not strictly comparable due to dilution of some of the ash solutions for individual determinations. All of the Fe and Cu determinations were made on undiluted ash solutions. For the Zn determination, all but the fir needle ash solutions were diluted (1:2 = ash solution:0.1 *N* HCl). For Mn determinations, the fir needles and mixed forage ash solutions were

Table IV. The Mean Effects of Ash Solution Preparation on the Analysis of and Recovery of Added Trace Elements, ppm

Plant material		Fe			Mn			Zn			Cu		
		Wet E[a]	Dry E[b]	Wet NE[c]	Wet E	Dry E	Wet NE	Wet E	Dry E	Wet NE	Wet E	Dry E	Wet NE
Alder leaves	native	153.4	139.0	135.5	419.0	409.2	418.3	57.0	53.0	54.6	10.8	10.4	10.4
	recovered[d]	80.1	75.7	72.7	80.5	83.2	79.4	40.8	41.3	36.4	40.0	39.6	36.3
Douglas fir needles	native	99.7	98.7	96.4	239.5	230.2	233.6	11.1	9.0	10.8	3.6	2.9	3.8
	recovered	82.1	75.5	70.8	81.5	74.8	79.5	41.7	39.3	38.2	40.8	26.8	38.0
Corn cobs	native	149.7	145.3	146.4	6.5	6.5	6.6	25.5	21.9	24.5	59.8	37.1	56.5
	recovered	79.0	72.0	77.6	80.0	78.4	79.2	40.0	36.0	40.0	41.0	10.8	41.7
Corn grain	native	25.1	25.2	25.4	7.1	7.2	6.8	25.2	27.1	24.8	6.6	6.7	7.5
	recovered	79.2	80.0	76.0	79.1	80.3	77.4	40.6	37.3	40.1	42.1	41.8	38.2
Cabbage leaves	native	126.6	115.0	113.0	44.6	44.2	40.7	48.3	45.4	46.0	13.8	13.3	12.3
	recovered	78.9	66.2	67.7	80.0	78.7	73.8	39.4	39.9	36.7	40.0	39.5	36.3
Alfalfa	native	206.5	189.2	179.5	23.1	21.9	20.2	30.8	26.6	28.9	14.5	5.7	13.3
	recovered	82.5	64.6	68.7	79.2	80.1	73.3	39.8	37.6	37.8	40.2	33.3	37.4
Mixed forage	native	120.0	115.7	109.0	151.2	152.0	142.1	34.8	31.3	33.8	13.9	13.3	12.6
	recovered	79.7	71.5	68.4	79.3	81.5	79.1	39.8	35.7	37.8	40.8	36.8	37.4
Relative SD, %		1.56	2.44	1.66	0.84	1.05	1.50	1.16	2.37	2.68	1.90	6.32	2.50
Mean recovery, %		100.3	90.3	89.6	99.9	99.5	96.7	100.8	95.4	95.3	102.4	81.6	94.8

[a] Wet ashed and trace elements extracted with PDTCA. [b] Dry ashed and trace elements extracted with PDTCA. [c] Wet ashed and trace elements were not extracted. [d] Trace elements were added to the plant tissue in amounts calculated to make the materials 80, 80, 40, and 40 ppm higher in Fe, Mn, Zn, and Cu, respectively.

diluted 1:2 and the ash solutions of the alder leaves were diluted 1:4. Recovery of Mn was poorest on cabbage leaves and alfalfa, the two materials which were relatively high in inorganic constituents and on which the determinations were made on undiluted ash solutions.

Some workers may feel that the proposed procedure is too time consuming for the analyses of a large number of samples and for samples on which the sampling error may exceed the analytical error. The proposed procedure can serve as a reference procedure for assessing errors involved in other methods. With slight modifications, the extracting procedure can be used to increase sensitivity manyfold by concentrating trace elements from dilute aqueous solutions and by redissolving the extracted trace elements in suitable organic solvents.

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Vitamin B_6: Gas–Liquid Chromatography of Pyridoxol, Pyridoxal, and Pyridoxamine

Augustus K. Williams

The three major nonphosphorylated forms of vitamin B_6 have been separated by means of gas–liquid chromatography of their heptafluorobutyryl derivatives. The highly electronegative nature of the derivatives combined with the use of an electron capture detector provides a very sensitive and somewhat specific assay for these compounds. Data are presented which suggest that quantitative determination of pyridoxol is possible at least within the range of 1.0–10.0 ng and for pyridoxal and pyridoxamine within the range of 2.0–20.0 ng.

At present a number of methods are available for both qualitative and quantitative determination of vitamin B_6. The most extensively utilized method, microbiological growth stimulation, is reliable and extremely sensitive. This method, however, suffers from the disadvantage of requiring considerable physical manipulation and uncertain specificity.

Recent advances in the technique of gas–liquid chromatography present a potential means of assaying the major vitamin B_6 components with satisfactory sensitivity and specificity, in addition to a significant reduction of analysis time.

Due to the low volatility of vitamin B_6 it has been nec-

Horticultural Crops Laboratory, Richard B. Russell Agricultural Research Center, U. S. Department of Agriculture, Agricultural Research Service, Athens, Georgia 30604.