METHODS OF ANALYSIS SOILS—BIOCHEMISTRY LABORATORY SERVICE

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INTRODUCTION

This report details the standard methods of analysis currently used by the Soils - Biochemistry Laboratory Service of the B. C. regional forest research laboratory. Routine determinations include total nitrogen, phosphorus and potassium in foliage; total nitrogen, nitrate, ammonia, and available phosphorus and potassium in soils; soil pH and conductance; and amino acids and soluble proteins from plant sources. Instruction sheets for the use of the equipment mentioned are available from the Laboratory Service.

As the need arises, more methods will be added and existing ones will be modified.

SAMPLE PREPARATION

Theory

Sample preparation varies with the analysis to be performed and the characteristics of the particular sample, which should be considered before sampling.

There is less likelihood of change in both plant material and soil if stored dry rather than wet. However, changes in ammonium, nitrate and nitrite occur on drying, and these should be analysed immediately after sampling.

Air-drying soil is less likely to cause changes than oven drying.

If it is necessary to express results on an oven dry basis, a separate sample should be oven dried to determine moisture content. Freeze-drying plant material is less likely to cause changes than other methods of drying.

With exchangeable potassium and extractable phosphorus, changes occur with some soils on air drying (see appropriate sections) and a preliminary assessment of the significance of such changes may be necessary. Soil pH and electrical conductance may be measured with field moist or air-dry samples, subject to precautions discussed in sections 6 and 7. Total nitrogen, phosphorus, and potassium and amino acids and proteins may be analyzed in freeze-dried plant material. Amino acids and proteins may also be analyzed in fresh material after collecting samples.

Procedure

Plant material

- Freeze-dry samples immediately after collection. To transport samples, place them in a dry-ice chest.
- When dry, grind samples with a ball-mill or Wiley-mill to pass a 32mesh screen and freeze-dry again.
- 3. Store in glass jars at 0°C.

Soil

- Bring samples to laboratory as soon as possible and pass them through a 10 mm sieve, discarding stones and pieces of root but not soil clods.
- Crumble soil clods as they dry: this is essential with very clayey soils, as dried clay clods may be difficult to break.
- 3. Work air-dry soil through a 2 mm sieve using a large rubber stopper. Discard stones and pieces of root but not soil aggregates. Crush aggregates with a mortar and pestle by rolling the pestle over them without applying pressure, until they are small enough to pass a 2 mm screen.

- 4. Mix thoroughly the sieved material by rolling it on paper.
- 5. Store one quart of soil per sample; use paper bags if samples are to be analyzed within 2 months, or glass jars if they are to be stored for longer periods.

TOTAL NITROGEN

Theory - Kjeldahl method

Nitrogen in nitrogenous compounds is converted to ammonia by boiling with excess sulphuric acid, which fixes ammonia as ammonium sulphate. The digest is then made basic with strong alkali to release ammonia which is captured by distillation into boric acid solution and determined by titration with standard hydrochloric acid.

This method does not include all nitrate and nitrite. The amount included varies with the organic content of the soil. However, as nitrate and nitrite usually constitute a very small fraction of the total nitrogen of soils, and as organic nitrogen can be converted to available forms, Kjeldahl nitrogen provides an index of the nitrogen fertility of a soil. Nitrate and nitrite can be determined by the Kjeldahl method if they are reduced to ammonia before analysis (Brenner & Shaw 1958).

Digestion of nitrogenous compounds is accelerated by adding potassium sulphate to the sulphuric acid so that the mixture refluxes at a higher temperature (345°C with K₂SO₄ vs 329°C without K₂SO₄). Avoid loss of sulphuric acid during refluxing. If the salt:acid ratio increases to about 1.3:1, the temperature of the digest will exceed 400°C and loss of nitrogen by pyrolytic decomposition may occur.

The digestion is further accelerated by the addition of a catalyst, mercuric oxide. This greatly decreases the digestion time (e.g. 12 hr with—out HgO vs $1\frac{1}{2}$ hr with HgO) but has the disadvantage that when the digest is treated with alkali some of the ammonium in the digest reacts with the precipitated mercuric oxide to form an ammonium complex. However, this mercury—

ammonium complex can be decomposed by adding thiosulphate to the alkali, according to the reaction

$$_{\rm NH_3}$$
 $_{\rm NH_3}$ $_{\rm$

The ratio of $\mathrm{Na_2S_2O_3.5H_2O}$ to HgO must be at least 5.

Material undigested at the end of the refluxing time can be treated with hydrogen peroxide to complete its digestion. Since the decomposition temperature of hydrogen peroxide in sulphuric acid is about 140°C, the digestion flasks must be cooled before peroxide is added.

Determination of ammonia by distillation into boric acid is a direct method requiring only one standard reagent. Neither the volume nor the strength of the boric acid solution used to collect the distillate needs to be known accurately because the ammonium borate formed is titrated back to boric acid.

$$NH_3 + H_3BO_3 \rightarrow NH_4^+ + H_2BO_3^-$$

 $H^+ + H_2BO_3^- \rightarrow H_3BO_3$

Bumping during distillation is avoided by using steam to liberate the NH_3 .

The distillate is titrated to an end point of 4.8, which is the pH of 0.12 M H₃BO₃. This is the approximate concentration of boric acid when about 25 ml of distillate is added to 5 ml of saturated boric acid. Since the pH of a solution of a weak acid depends on the concentration of the acid, about 25 ml of distillate should come over. If it is less, distilled water may be added; if considerably more, an end-point correction may be necessary.

Reagents and Materials

1. Alkali reagent

- 670 g low carbonate NaOH
- + 67 g Na₂S₂O₃ · 5H₂O
- + 1350 ml double distilled ${\rm H_20}$ (to volume of 1.5 L) stir, cover, and allow to cool.

2. Standard HCl, approximately 0.3 N

HCl can be standardized against a $\mathrm{Na_2CO_3}$ or a borax solution of accurately known normality.

(a) Prepare approximately 0.3 N HCl by adding 26 ml of conc. (36%) HCl to distilled H₂O in a l L. volumetric flask, make to volume with distilled water.

For standardization against Na₂CO₃

- (b) Weigh accurately about 1.06 g dry Na₂CO₃ (i.e., weigh to 5 decimal places e.g. 1.05993 g). Carefully transfer it to a 1 L volumetric flask and make up to volume with boiled, cooled, double distilled water (CO₂ free). This is about 0.02 N; calculate exact normality on basis that 1 N weight of Na₂CO₃ is 52.9965 g.
- (c) Pipette four 10 ml aliquots of Na2CO3 solution into 4 vials with a 10 ml volumetric pipette. Titrate with HCl solution to an end point of 4.8. Calculate normality of HCl on basis Normality1 x Volume1 = Normality2 x Volume2.

(For standardization against borax see A. J. Vogel, 1961, page 238).

3. Saturated H₃BO₃

- 250 g/4L; set on shaker 1 hr.
- use double distilled water.

- 1. Kjeldahl digestion apparatus to hold 100 ml and 30 ml Kjeldahl tubes.
- 2. Kjeldahl distillation apparatus to fit 100 ml and 30 ml Kjeldahl tubes
- 3. Titrator with motorized burette accurate to 1 ul.
- 4. Ball mill with capsules large enough to hold 10 g of soil.

Procedure

Mineral Soil

- Take 2 sub-samples of dried 2 mm soil with a 250 ml beaker and spread them separately on brown paper.
- Divide both sub-samples by quartering them and then quartering a quarter
 to get a sample of about 10 g from both sub-samples.
- 3. Ball mill both 10 g sub-samples until they pass a 35 mesh screen.
- 4. Weight accurately about 500 mg of these duplicate sub-samples.
- 5. Place each in a clean, dry 100 ml Kjeldahl flask. Tap the sides to ensure that all the sample goes to the bottom of the flask. Errors from weighing and loss of sample on the sides of the flask are the most important sources of variation.
- 6. To each flask add 0.13 g HgO, 2.6 g K2SO4 and 4 ml concentrated H2SO4.
- 7. Digest for 30 min at low heat (set voltage regulator at 60), then 1 hr at high heat (set voltage regulator at 110). The digestor will hold 5 samples in duplicate and two blanks.
- 8. Cool flasks, add 4 drops of 30% H202.
- 9. Re-heat at high heat for 15 min.
- 10. Cool, add 15 ml of double distilled water to each flask. Cool flasks.
- 11. Dispense 5 ml of saturated boric acid into a 40 ml vial and place it under the downspout of the distillation apparatus.

- 12. Swirl a flask until all undissolved material is suspended; connect to distillation apparatus.
- Beware of alkali reagent bumping. Wear safety goggles. If there is excessive bumping when adding the alkali, acid fumes will distill into the boric acid and titration will be invalid. Bumping is avoided by cooling flasks and suspending undissolved material before adding alkali. If these precautions are observed and bumping still occurs, add 20 ml of distilled water at step 5.
- 14. Steam distill for 4 min with the downspout under the surface of the boric acid, then distill for 1 min with the downspout above the level of the boric acid.
- 15. With the titrator, titrate the distillate with standard HCl to an end point of pH 4.8.
- 16. Calculate % N in sample by:

% N = (Equivalents of acid added to sample - equivalents of acid added to blank) (14.01)(100)

Sample weight (g)

Programmed calculator cards for this calculation are available from the Laboratory Service.

Plant material or Litter

- Subdivide main sample as described for soil, steps 1 and 2, obtain two 1 g sub-samples
- 2. Ball mill each sub-sample to pass a 35 mesh screen.
- 3. Weigh accurately about 100 mg of each duplicate sub-sample into weighing cups made from N free paper. Wrap the paper around the sample using forceps and drop it into the bottom of a 30 ml Kjeldahl tube.

- 4. To each flask add 0.13 g HgO, 1.3 g K2SO,, and 1.5 ml H2SO,.
- Digest as described for soil, steps 7-9. Include two blanks in each digestion.
- 6. Add 5 ml of double distilled water to each flask.
- Distill samples as described for soil, steps 11-14, but add 10 ml instead of 35 ml of alkali reagent to flasks.
- Titrate distillates and calculate % N as described for soil, steps
 15-16.

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PHOSPHORUS

Theory

Total P in Plant Material

Total phosphorus in foliage is determined by ashing samples in a muffle furnace to remove organic material. Phosphorus in the ash is dissolved in dilute acid and determined colorimetrically. Before ashing, the sample is mixed with magnesium acetate and dried, making loss of phosphorus unlikely at temperatures up to 650°C. At temperatures lower than 550°C, a black ash is obtained with magnesium acetate-sample mixtures, indicating some carbon residue which retains phosphorus. The ash is wetted with dilute hydrochloric acid and boiled gently to convert any pyrophosphate to orthophosphate. The sample is heated to dryness and then baked to dehydrate silica, which becomes less soluble and less likely to interfere with P determinations. Phosphorus is then dissolved in dilute acid before analysis. Available P in Soils, bicarbonate method

Available phosphorus may be determined in soil extracts made with 0.5 M sodium bicarbonate at pH 8.5. This is one of many methods of extracting "available phosphorus" from soils, and different methods may give different results. The decision as to which method of extracting available phosphorus provides the most accurate determination for a certain species requires knowledge of which method gives results most closely correlated with phosphorus uptake by that species. A high correlation has been found between sodium bicarbonate extractable phosphorus and uptake of phosphorus by sorghum (Moser et al. 1959). Because CO₂ production by plants raises bicarbonate levels in the vicinity of roots, this method may approximate phosphorus extraction by plants. The relation between phosphorus determined

by this method and expected yield response is as follows: 5 ppm, a response; between 5 and 10 ppm, a probable response; greater than 10 ppm, a response unlikely. The crops used in field experiments to establish these levels were wheat, oats, alfalfa and cotton (Olsen et al. 1954). If an estimate of phosphorus fertility for some years after the time of analysis is desired, the technique can be modified to include organic phosphorus (Legg and Black 1955).

Available P in Soils, acid fluoride method

Another index of available phosphorus in soils is the amount extracted with a solution of hydrochloric acid and ammonium fluoride (Bray) and Kurtz 1945). Though this method has been found to be poorly correlated with phosphorus yield in sorghum plants (Moser et al. 1959), it is frequently used and is therefore included here. Test values obtained are interpreted as: less than 3 ppm, very low; 3-7 ppm, low; 7-20 ppm, medium; greater than 20 ppm, high.

An increase in acid extractable phosphorus has been shown to occur in some soils as a result of air-drying (Allen & Grimshaw 1962). Therefore, if it is necessary to compare data to results obtained with freshly collected soil, the sample should be analyzed immediately after collection and compared to an air-dry sample to determine whether a change occurs on drying.

Colorimetric determination of P

The colorimetric determination of phosphorus is based on the measurement of the absorption of light passing through a solution of a colored phosphorus-containing compound. The latter is obtained by converting phosphorus to orthophosphate, reacting this with sodium molybdate to produce phosphomolybdic acid, and then reducing the phosphomolybdic acid to molybdenum

blue which is determined colorimetrically. Two forms of phosphomolybdic acid occur in aqueous solution, a yellow and a colorless form. The amount of the yellow form depends on the acidity of the solution. Since molybdenum blue is produced mainly by the yellow form, the acidity of the solution affects the amount of color produced. Therefore, samples must be neutralized before adding molybdate so they will be of the same acidity as the standards. Ions other than phosphate will form heteropoly acids with molybdate, e.g. silicate, arsenate, germanate and tungstate, and these interfere with phosphate determination. Silicate is removed by baking the acid wetted ash to dehydrate it and render it insoluble. The other ions mentioned are unlikely to be present in significant amounts; however, if interference occurs they may be removed by volatilization from HBr/H2SOL solution. Ferric ion in high concentrations will interfere, giving the solution a greenish color. This can be eliminated by reducing ferric to ferrous ion, which interferes less, by extracting the phosphomolybdic acid with isobutyl or octyl alcohol before reduction; or by running the test solution through a cation exchange column. This last treatment will also remove lead, antimony and copper which also interfere if they are present in high enough concentrations. If soil extracts contain sufficient organic material to interfere with colorimetry, the phosphomolybdic acid may be extracted with octyl or iso-butyl alcohol before it is reduced (Watanabe and Olsen 1962; Schaffer et al. 1953).

Reagents and Materials

1. Magnesium acetate

- 500 g $Mg(C_2H_3O_2)_2$. $4H_2O$ in double distilled water to make 1 L.

- set on shaker until dissolved, make to 1 L, if necessary.

2. 2N HCl

- 172 ml of conc (36%) HCl diluted to 1 L with double distilled water.

Caution: when diluting add acid to water

3. .OLN HCl

- 0.86 ml of conc (36%) HCl diluted to 1 L with double distilled water.

4. Extracting solution, bicarbonate

- 42 g of NaHCO3 dissolved to make 1 L with double distilled water.
- adjust to pH 8.5 with 1 M NaOH(4 g of NaOH dissolved to make 100 ml).
- store in a tightly stoppered, polyethylene bottle;
 make up fresh every month.

 (Check pH of solution before use; if it is not 8.5, make fresh solution.)

5. Extracting solution, acid fluoride

- dissolve 18.5 g of NH₄F in double distilled water, make to 500 ml with distilled water, store in polyethylene bottle.
- dilute 20.2 ml of conc (36%) HCl to 500 ml with double distilled water.
- for extracting solvent, measure 15 ml of NH₄F solution and 25 ml of HCl solution into a 500 ml volumetric flask, make to volume with double distilled water.

6. Molybdate reagent

- dissolve 25 g of Na₂Mo O₄ · 2H₂O in 10 N H₂SO₄,

make to 1 L with 10 N H₂SO₄ (280 ml of conc (98%) H₂SO₄ diluted to

1 L. <u>Caution</u>: add H₂SO₄ <u>slowly</u> to water. <u>Wipe up spills immediately</u>.

<u>Strong acid</u>.)

7. Hydrazine sulphate

- 1.5 g hydrazine sulphate dissolved in double distilled water to make 1 L.

8. P standards

- weigh 4.3942 g dry KH_2PO_4 into a weighing boat.
- carefully rinse into a 1 L volumetric flask, make to 1 L with double distilled water. This contains 1000 ppm P.
- with a volumetric pipette, transfer 10 ml of 1000 ppm stock to a 100 ml volumetric flask, make to volume.

This contains 100 ppm P.

For	use ml	of 100 ppm stock.	make to 1 L with double distilled water
0.1 ppm	1		
0.2	2		
0.3	3		
0.4	4		
0.5	5		
0.7	7		
1.0	10		

Store tightly stoppered.

- 1. Muffle furnace
- 2. Sand bath
- 3. Wrist-action shaker
- 4. Steam bath
- 5. Colorimeter or spectrophotometer capable of operation at 825 nm.

Procedure

Plant Material

- Accurately weigh duplicate samples of ground, dried foliage of about 1 g into crucibles.
- Add 3 ml of Mg(OAc)₂ solution, mix, and evaporate to dryness on the sand bath.
- 3. Cover and ash at 550°C for 32 hr in the muffle furnace.
- 4. Remove and allow crucibles to cool.
- 5. Add sufficient 2N HCl to wet thoroughly the ash.
- 6. Slowly evaporate to dryness on the sand bath.
- Transfer to a small hot-plate and bake 5-10 min after the ash turns white.
- 8. Cool crucibles.
- 9. Add 5 ml of 2N HCl, warm crucible to dissolve ash.
- 10. Filter through dampened Whatman #40 filter paper into a 100 ml volumetric flask.
- 11. Rinse twice the crucible and filter paper with .OlN HCl.
- 12. Make flasks to volume with double distilled water.
- 13. Place a 10 ml aliquot of this solution in a 50 ml beaker, add about 25 ml double distilled water, and neutralize with NH₄OH with the pH meter.
- 14. Pour neutral solution into a 100 ml volumetric flask, rinse the beaker into the volumetric flask with double distilled water, and make to volume with double distilled water.
- 15. Pipette duplicate 10 ml aliquots of the neutral diluted solution into 50 ml volumetric flasks.

- 16. Pipette duplicate 10 ml aliquots of standards into 50 ml volumetric flasks.
- 17. To each flask, add 5 ml of molybdate reagent and swirl flask.
- 18. To each flask, add 2 ml of hydrazine sulphate solution and make to volume with double distilled water.
- 19. Stopper and shake well.
- 20. Unstopper and place in steam bath 10 min.
- 21. Remove to ice bath, stopper and shake well.
- 22. Read at 825 nm within half an hour. (If samples have an absorbance greater than .400, they are too concentrated. Redetermine P from step 16 with a 5 ml aliquot of the neutral diluted solution.)
- 23. Calculate concentration of P in dilute extract from standard curve. (Once a standard curve has been established further runs may be made with only a blank and two other concentrations of P as checks.)
- 24. Determine the value of P in ppm in foliage by:

$P = \underbrace{\text{(ppm in dilute extract) (dilution factor)}}_{\text{weight of sample (g)}}$

The dilution factor using a 10 ml sample aliquot is x1000; for a 5 ml sample aliquot it is x2000.

Available P in Soils, bicarbonate method

- Weigh duplicate 5 g samples of soil sieved through a 2 mm screen into 250 ml erlenmeyer flasks.
- 2. Add 1 tsp of carbon black and 100 ml of NaHCO3 extracting solution.
- Shake for 30 min on the Burrel wrist-action shaker at a setting of 2.
 Higher results will be obtained if the shaking speed increases greatly.
- 4. Filter the suspension through Whatman #40 filter paper.

Add more carbon black or filter through membrane filters, if necessary, to obtain a clear filtrate.

- Pipette duplicate 10 ml aliquots of the filtrate into 50 ml volumetric flasks.
- Pipette duplicate 10 ml aliquots of standards into 50 ml volumetric flasks.
- 7. To flasks with standards, add 10 ml of bicarbonate extracting solution.
- 8. Determine P as described for plant material, steps 17-23.
- 9. Determine the value of P in ppm in soil by:

P = (ppm in extract) (dilution factor)

The dilution factor here is x20.

Available P in Soil, Acid fluoride method

- Weigh duplicate 1 g samples of soil sieved to pass a 2 mm screen into 50 ml screw top test tubes.
- 2. Add 7 ml of extracting solution.
- 3. Stopper and shake tube lengthwise for 1 min, filter immediately through Whatman #42 filter paper into 30 ml test tubes. If the filtrate is not clear, pour it again through the filter into a clean tube.
- 4. Pipette duplicate 2 ml aliquots into 30 ml test tubes.
- Pipette duplicate 2 ml aliquots of standards into 30 ml test tubes.
 Add 2 ml of acid fluoride extracting solution.
- 6. To all tubes, add 1 ml of molybdate reagent and shake well.
- 7. To all tubes, add 0.4 ml of hydrazine sulphate solution and shake.
- 8. To sample tubes, add 6.6 ml of distilled water.
- 9. To standard tubes, add 4.6 ml of distilled water.
- 10. Stopper and shake well.

- 11. Unstopper, place a clean glass marble on each tube, and heat on steam bath 10 min.
- 12. Remove to ice bath, stopper and shake well.
- 13. Read at 825 nm within half an hour.
- 14. Calculate concentration of P in extract from standard curve.
- 15. Calculate concentration of P in soil by:
 P = (ppm in extract) (dilution factor)
 The dilution factor here is X 7.

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POTASSIUM

Theory

Total Potassium in Plant Material

Total potassium in foliage is determined on ashed samples. Potassium in the ash is dissolved in dilute acid and determined by atomic absorption spectroscopy. If total phosphorus is to be determined in the same sample, it is ashed with Mg(OAc)₂ as described. If loss of P is not important, the sample may be dry ashed at 480°C for about 2 hr. The exact time required for complete ashing should be determined for particular samples.

Available Potassium in Soils

Available potassium in soils is determined in extracts made with neutral 1.0 N NH₄OAc. This represents the sum of the water-soluble and exchangeable potassium in soils, and is probably the most universally employed index of potassium availability in soils. Additional potassium may be released from non-exchangeable forms over a period of time, and since the cropping period for trees is long, a measure which includes release rates as well as exchangeable potassium may be a more complete index of potassium availability in forest soils. Mycorrhizal associations may also enable trees to obtain potassium from non-exchangeable forms. A common method for determining water soluble, exchangeable and non-exchangeable potassium is to extract it with boiling nitric acid.

In some soils exchangeable potassium changes with drying. If exchangeable potassium is very high, potassium may be fixed during drying, whereas if it is low, there may be release of potassium from non-exchangeable forms during drying (Attoe 1948). Therefore, a check should be made and if

changes in potassium occur with drying, analysis should be performed on field moist soil. A separate sample should be dried to determine moisture content so results can be expressed on a dry-weight basis.

Determination of Potassium by Atomic Absorption Spectroscopy

The determination of potassium (and other metal ions) by atomic absorption is based on the measurement of the absorption of resonance radiation by free atoms in the gaseous state. This absorption follows the Beer-Lambert law. In the atomic absorption unit, the resonant radiation is provided by hollow cathode tubes and the free atoms are produced in the burner. Since potassium has a low ionization potential, sensitivity is lost by ionization of potassium free atoms in the burner. The addition of excess sodium to samples and standards suppresses potassium ionization by providing an excess of free electrons in the flame. If a sample containing much more sodium than potassium is compared to standards without sodium, erroneously high values for potassium will be obtained. The ease with which a metal salt dissociates to produce free atoms varies with the anion with which it is associated; therefore, the same reagents present in samples are added to standards. Some aqueous soil extracts contain substances that interfere with potassium determination; their effect can be eliminated by the addition of tetraphenylboron to the sample (Temperli and Misteli 1969). Interfering substances in a sample can be detected by the use of an internal potassium standard. If duplication of the sample matrix in standards or elimination of interference is not practical, the concentration of potassium in samples may be determined by the standard additions technique (Rubeska and Moldan 1969). If calcium and magnesium are to be determined in the sample, 1000 ppm strontium may be added to suppress interference by phosphate, aluminium, silicate and sulphate (David 1960).

Reagents and Materials

Reagents for ashing foliage are the same as those described for phosphorus (third section).

1. Neutral 1.0 N $\mathrm{NH}_{\mathrm{L}}\mathrm{OAc}$ extracting solution

- 77.08 g dry ammonium acetate dissolved to make 1 L with distilled water. Adjust to pH 7.0 with acetic acid or ammonium hydroxide.

2. 1.0 N Nitric acid extracting solution

- 65 ml of conc (69%) HNO3, diluted to 1 L. Caution: add acid to water.

3. 0.1 N Nitric acid rinse solution

- 6.5 ml conc (69%) HNO_3 diluted to 1 L.

4. 1000 ppm NaCl (for diluting foliage ash extracts)

- 2.5 g NaCl dissolved to make 1 L with distilled water.

5. K standards

- weigh accurately 1.90691 g KCl into a weighing boat, carefully rinse into a 1 L volumetric flask, dissolve and make to volume with distilled water. This contains 1000 ppm K.
- with a volumetric pipette transfer 10 ml to a 100 ml volumetric flask, make to volume with distilled water. This contains 100 ppm K.
- to make standards:

For	use: stock	<u>ml</u>
0.2 ppm	100 ppm	1
0.6	u ·	3
1	m .	5
2	"	10

For	use: stock	<u>ml</u>
3	100 ppm	15
4	1000 ppm	2
6	m .	3

make to 500 ml with the appropriate diluting solution.

6. Diluting solutions:

for foliage standards

- 2.54 g NaCl + 5 cc 2N HCl diluted to 1 L with distilled $\rm H_2O$. for $\rm NH_LOAc$ soil extract standards

-2.54 g NaCl diluted to 1 L with neutral 1.0 N NH, OAc.

for nitric acid soil extract standards

-2.54 g NaCl diluted to 1 L with 0.31 N HNO $_3$ (=20.15 ml of conc HNO $_3$ diluted to 1 L with distilled water).

- Centrifuge capable of 1800 x g, when holding 50 ml screw top centrifuge tubes.
- 2. Shaker
- 3. Muffle furnace
- 4. Sand bath
- 5. Oil bath useful for determination of non-exchangeable K.
- Atomic absorption spectrophotometer.

Procedure

Plant Material

- 1. If P is to be determined in the same sample, ash as described for
 - P. If loss of P is not critical, accurately weigh duplicate samples of about 1 g of dried, ground foliage into crucibles, cover and ash

- at 480°C to a grey ash which does not contain large lumps of carbon (about 2 hr).
- Treat the ash as described in third section for plant material, steps
 4 to 12.
- Pipette 10 ml or 5 ml aliquots of the concentrated ash solution into a 100 ml volumetric flask, depending on K concentration (see 5).
- 4. Make to volume with 1,000 ppm NaCl.
- 5. Determine K in this solution by atomic absorption using standards made with HCl and NaCl. Solutions must have an absorbance less than 0.400 or readings are not accurate. A preliminary check can be made to determine which dilution is most useful.
- 6. Determine value of K in ppm in foliage by:

The dilution factor is x1000 if a 10 ml aliquot is taken in 3, x2000 if a 5 ml aliquot is taken.

Available K - exchangeable and water soluble K

- 1. Place 10 g of soil in a screw-top 50 ml centrifuge tube.
- 2. Add 25 ml of NH, OAc solution and shake lengthwise for 10 min.
- 3. Centrifuge to obtain a clear supernatant.
- 4. Decant supernatant into a 100 ml volumetric flask.
- 5. Repeat steps 2-4 three times.
- 6. Make flasks to volume with NH₄OAc solution, stopper and shake well. If solution is cloudy, filter through 1.2 u membrane filters into 125 ml erlenmeyer flasks.
- 7. Add 0.25 g NaCl to each flask and swirl to dissolve.

- 8. Determine K in this solution by atomic absorption using standards made with NH₄OAc and NaCl. If extracts have absorbances greater than 0.400, dilute with NH₄OAc extracting solution containing 1000 ppm NaCl and change the dilution factor accordingly.
- 9. Determine value of available K in ppm in soil by:

K = (ppm in extract) (dilution factor)

The dilution factor here is x10.

Long Term Available K - exchangeable, non-exchangeable, and water soluble K

- 1. Place 10 g of soil in a 100 ml beaker.
- 2. Add 25 ml of 1.0 N nitric acid.
- 3. Place on sand bath or, preferably, oil bath at 113°C for 25 min.
- 4. Remove the beaker and filter into a 100 ml volumetric flask.
- 5. Rinse the soil with four 15 ml portions of O.1 N nitric acid.
- 6. Cool the flask and contents, add 0.25 g NaCl to each flask.
- 7. Dilute to volume with distilled water.
- 8. Determine K in this solution by atomic absorption using standards made with HNO3 and NaCl. If extracts have absorbances greater than 0.400, dilute with 0.31 N nitric acid containing 1000 ppm NaCl and change the dilution factor accordingly.
- 9. Determine K in samples by:

K = (ppm in extract)(dilution factor)

The dilution factor here is x10.

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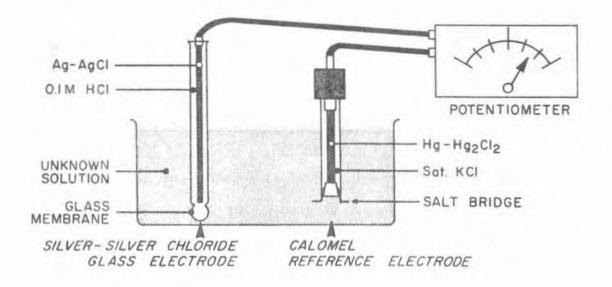
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SOIL pH

Theory

Hydrogen ion activity or pH is measured potentiometrically, hydrogen ion activity and concentration being related by the equation $pH = -log \ cH^{+} \cdot fH^{+} \ where \ cH^{+} = hydrogen \ ion \ concentration$ $fH^{+} = hydrogen \ ion \ activity \ coefficient$

Thin membranes of the proper glass are selectively permeable to H⁺ ions and if a cell is set up as follows:



the potential that develops across the glass membrane can be directly related to the pH of the unknown solution. The potentiometer is calibrated to read pH directly by setting the meter against standard pH buffers. The glass electrode-calomel reference electrode system is applicable to practically all solutions and may be used satisfactorily in semi-solid materials as long as there is adequate electrical contact. Glass electrodes do not function well in solutions of very high pH when appreciable quantities of certain metal ions (particularly Na⁺) are present. Under these conditions,

they begin to act as Na⁺ ion electrodes because of the solubility of Na salts in the glass at high pH. The titrator in the service lab has a special high pH combination electrode made with glass that does not act as a Na⁺ membrane. This glass is extremely fragile.

The titrator functions as a pH meter at the "read" setting. It can be used for small samples (1 to 2 g of soil) since it has a combination glass-reference electrode and only one electrode need be immersed in the sample. As the glass membrane on this electrode is delicate, it cannot be used for a saturated soil paste nor for very gravelly suspensions. If a glass membrane is scratched, the internal standard may leak and the electrode must be discarded. To measure the pH of very thick pastes or very gravelly suspensions, a field pH meter should be used as it has sturdier electrodes. A larger sample is required for this as two electrodes must be immersed in the sample.

The proper soil-water ratio for pH measurements is debatable. The pH of a soil water mix increases with dilution. (This increase in pH is directly related to the dilution of soluble salts in the mix. It parallels the periodic changes in pH measurements of soils in nature which tend to go down in dry seasons and up in rainy seasons as a result of salt leaching.) With the advent of stronger glass electrodes, pH measurements have been taken on thicker soil suspensions on the premise that these values more closely approximate conditions around roots. However, at very low moisture contents, readings may be inaccurate because of poor electrical contact. Furthermore, in clay soil pastes or soils saturated with bivalent cations there is a negative liquid junction at the KCl salt bridge (calomel electrode) sufficient to give erroneously low pH values. The conventions of

soil-water ratios of 1:2.5 and 1:1 have been adopted by various workers. These ratios result in suspensions in which the clay settles on standing so readings may be taken with the glass electrode in the suspension, but the calomel electrode in the clear supernatant. Liquid junction errors can be reduced or eliminated by increasing the salt concentration in the solution so the ionic strength is greater than .005; measuring pH with the calomel electrode in the clear supernatant of a suspension may not eliminate this error (Clark 1966). The cause of the liquid junction potential is controversial (Coleman et al. 1950, Peech et al. 1953, Clark 1966).

The effect of dilution on soil pH may be overcome by taking measurements in .01 M CaCl₂ solution. This results in measurements which are lower than measurements in water, but are independent of dilution. In non-saline soils such measurements are also independent of the periodic changes in measured pH as a result of changes in salt concentration mentioned previously.

The decision on what soil-water ratio to use and whether to make measurements in salt solution depends on what comparisons are to be made.

If comparison to a value in the literature is desired, the same measurement conditions are required.

Reagents and Materials

- 1. .Ol M CaCl₂
 - dissolve 1.11 g CaCl₂ in distilled H₂O, make to 1 L with distilled water.
- 1. pH meter

Procedure

- Prepare a soil suspension by adding the appropriate amount of water or .Ol M CaCl₂ to soil in a beaker. Let it stand for 30 min, stirring several times.
- 2. To prepare a saturated soil paste, add distilled water in small amounts to soil while stirring and occasionally tamping the beaker on the bench. At saturation the paste glistens, flows slightly and slides cleanly off a spatula unless the soil has a high clay content; these conditions do not change after the paste stands for at least one hour.
- 3. Read pH of suspensions with the titrator electrode or a field pH meter. Add a little more water to saturated pastes if they are to be measured with the combination electrode.

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SOLUBLE SALTS BY ELECTRICAL CONDUCTANCE

Theory

The electrical conductance of a soil extract is directly related to the concentration of ionized salts in the extract and can be used to measure the salt concentration. Obviously, the salt concentration in an extract will depend on the soil-water ratio used to make the extract. Also, the absolute amount of the salt removed from a soil will vary somewhat with the amount of water used to make the extract. Generally more salt is extracted from a soil if a larger volume of water is used. Thus the concentration of salt in a 1:2 soil-water extract may be more than half the concentration of salt in a 1:1 soil-water extract. The ratio used depends on the purpose of the measurement. To follow changes in salt content with time or treatment a 1:2.5 soil-water ratio may be used; to obtain a measure as close as possible to field salt content a saturated soil paste may be used (a saturated paste contains about 4 times the water of soil at the permanent wilting point and about 2 times the water of soil at field capacity). With a 1:2.5 soil-water extract, a specific conductance approaching 1.0 milli-mho indicates abnormally high soluble salt concentration (Wilde et al. 1964). With a saturated paste, specific conductance about 8 millimho indicates soluble salt content which may restrict yield of crops other than salt tolerant crops (Bower & Wilcox 1965).

Electrical conductivity is the reciprocal of the electrical resistivity of the conductor and is measured in mho per cm (1 mho = $\frac{1}{\text{ohm}}$), in milli-mho per cm (1 milli-mho = 1 mho x 10^{-3}), or micro-mho per cm (1 u mho = 1 mho x 10^{-6}).

The conductivity meter in the laboratory service has a built-in standard resistor for calibration, so it is not necessary to calibrate it against standard KCl. The conductivity of electrolyte solutions varies with temperature, increasing about 2% per C degree. Measurements are made at 25°C and a correction is necessary if it is required to measure a solution at a very different temperature. The distance between electrodes in the conductivity cell is not necessarily 1 cm but the cell is engraved with a correction factor to convert readings to mho per cm.

Reagents and Materials

- 1. 0.1% Na hexametaphosphate
 - 100 mg of Na hexametaphosphate dissolved to make 100 ml with 2X distilled water.
- 1. Conductivity meter
- 2. Wrist-action shaker

Procedure

Samples should not be oven dried before taking conductance measurements since drying at $105\,^{\circ}\text{C}$ increases the solubility of CaSO_4 • $2\text{H}_2\text{O}$.

- 1. For 1:2.5 soil-water suspensions, weigh 10 g of air-dry soil into a 50 ml erlenmeyer flask, add 25 ml of double distilled water, stopper and set on shaker for 1 hr. Filter the suspension with Whatman #40 paper (if the first drops of filtrate are turbid, discard them).
- 2. For saturated pastes, prepare as described under pH, filter with suction through Whatman #40 paper using a buchner funnel and collect the filtrate in a test tube. If this filtrate is turbid, filter through

membrane filters.

3. Using the conductivity meter, read extracts immediately or add 1 drop of 0.1% sodium hexametaphosphate if the extract is to stand for more than an hour. (This prevents precipitation of CaCO₃).

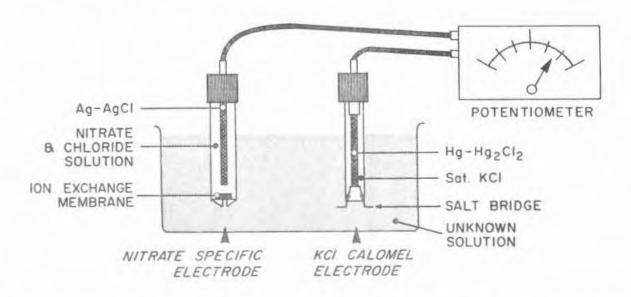
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NITRATE

Theory

Nitrate may be determined potentiometrically with a nitrate specific electrode operating on the same principle as a pH meter except the cell is set up as follows:



The potential that develops across the ion exchange membrane is measured in millivolts and related to nitrate ion concentration using a calibration curve. The ion exchange membrane was a high (but not absolute) specificity for nitrate, and nitrate and chloride in the internal solution provide a stable potential between the inside of the membrane and the Ag-AgCl electrode, so changes in potential across the membrane are due mainly to changes in the nitrate activity of the unknown solution. Interfering ions for the nitrate electrode include ClO_4 and I, which should not be present, and ClO_3 , Br and S which should be less than 1/100 of the nitrate concentration. Nitrite at concentrations equal to nitrate concentration will interfere by +2%, and other interfering ions, at concentrations equal to nitrate, interfere by less than 1%. These include Cl_4 , OAc_4 , CO_3 , S_2O_3 ,

SO₃, F, SO₄, H₂PO₄, PO₄ and HPO₄, and are important if present in large amounts. Organic ions rapidly contaminate the ion exchange membrane and should not be present. Their presence is indicated by extreme drift of readings. Readings obtained when drifting is minimal are not related to nitrate concentration if the membrane is contaminated with organic ions. The ion exchange membrane is slowly contaminated in normal use, and the ion exchange liquid should be changed once a month or more often if in constant use. The reference electrode solution is drained after use to prevent KCl crystals plugging the liquid junction.

The electrode responds to nitrate ion activity, which is directly related to nitrate concentration. The relation between activity and concentration is different in solutions of very different total ionic strength. Therefore, samples and standards should have about the same total ionic strength or the readings will have to be corrected. Total ionic strength of samples can be estimated from conductance measurements.

Nitrate is not considered to be strongly held by soil colloids and can be extracted by short periods of shaking with water. However, aqueous extracts frequently contain organic and colloidal matter and chloride which interfere with conventional colorimetric determination, so extracts have been prepared with salts that flocculate colloidal matter (e.g. saturated Ca(OH)₂), and precipitate chloride (e.g. .02 M CuSO₄ with .007 M AgSO₄). These solutions may be used for determination with the ion specific electrode rather than colorimetric determination, but correction must be made for the increased total ionic strength of the extract and for any interfering ions. Similarly, if nitrate is to be determined in 2N KCl extracts used for determination of ammonium, correction must be made for the

KCl. Mahendrappa (1969) measured nitrate in saturated Ca(OH)₂ extracts using a calibration curve made with solutions of similar ionic strength. He found close agreement between this method and nitrate determination by steam distillation, with less variation using the nitrate electrode. Aqueous extracts can be prepared with distilled water containing a drop of wetting solution per litre of water. If extracts are to stand for more than an hour, the water should contain 10 ppm phenylmercuric acetate as a preservative. If turbid solutions are obtained they may be filtered through 1.2 u membrane filters.

Determination of nitrate in soils may be invalidated by rapid changes of nitrate due to ammonification, nitrification and other chemical and biological changes during transportation and storage of the soil. Therefore, samples should be analyzed for nitrate immediately after sampling. If this is impossible, they should be stored deep-frozen or rapidly air-dried, though changes in nitrate contents as a result of these procedures have been demonstrated (Allen and Grimshaw 1962).

Reagents and Materials

1. Extracting solution.

- 1 drop wetting agent (polyoxyethylene ether mixtures, e.g. Triton X-100, Tween 80, Span-80, etc.) and 1 ml 0.1% phenylmercuric acetate in 1 L double distilled water.

2. 0.1% phenylmercuric acetate. Caution: TOXIC

- 100 mg phenylmercuric acetate dissolved to 100 ml in double distilled water.
- 1. Nitrate specific electrode, for use with calomel electrode and

potentiometer with expanded millivolt scale.

Procedure

- Add 100 ml of extracting solution to 50 g of soil in a 125 ml erlenmeyer flask.
- 2. Shake 10 min.
- Filter through Whatman #40 paper. If filtrate is turbid filter through 1.2 u membrane filter.
- 4. Take reading with nitrate specific electrode.
- 5. Calculate nitrate concentration from standard curve.

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AMMONIUM

Theory

Ammonium may be determined colorimetrically with Nessler's reagent. When this reagent is added to a dilute ammonium salt solution, the liberated NH3 reacts to form an orange-brown product which remains in colloidal solution. The reaction may be represented as:

$$2K_2(HgI_4) + 2NH_3 \rightarrow NH_2Hg_2I_3 + 4KI + NH_4I$$

Since the colored product flocculates on long standing, the colorimetric reading must be taken before flocculation occurs. Arsenate, chromate, cyanide, dichromate, permanganate and high concentrations of metal ions interfere. Compounds readily hydrolyzed by alkali to produce NH₃ (e.g. amides) interfere and must be absent. Soil extracts of exchangeable ammonium made with 2N KCl cannot be analyzed directly by this method as the KCl interferes. Extracts containing interfering substances are analyzed by rapid steam distillation with MgO into boric acid or dilute H₂SO₄ before determination of NH₃ with Nessler's reagent. If 1 mg or more N is collected in the distillate, NH₃ may be determined by titration as in the Kjeldahl method. Nessler's reagent is sensitive in the range of 0.1 to 1.5 ppm NH₃ (i.e., 2 to 20 ug in 20 ml of distillate).

Reagents and Materials

- 1. NH3 standards.
- —Weigh 3.140 g dry $\mathrm{NH_4Cl}$ into a weighing boat, carefully rinse it into a 1 L volumetric flask and dilute to the mark with double distilled water. This contains 1000 ppm $\mathrm{NH_4}$.
 - -With a volumetric pipette, transfer 1 ml to a 100 ml volumetric

flask and make to volume with double distilled H_2O . This contains 10 ppm NH_3 .

To make	Use ml of 10 ppm stock	Make to 100 ml with double
0.1 ppm	1	distilled H ₂ O
0.2	2	
0.3	3	
0.5	5	
0.7	7	
1.0	10	
1.5	15	

2. Nessler's reagent

Use double distilled $\rm H_2O$ throughout, since normal distilled $\rm H_2O$ may contain detectable $\rm NH_3$

- dissolve 3.5 g KI in 10 ml H₂0, add 4% HgCl₂ solution, with stirring, until a slight red precipitate remains. (About 33 ml are required add the last few ml dropwise.)
- add, with stirring, 12 g of NaOH dissolved in 25 ml H2O, make to 100 ml with H2O.
- add a few more drops HgCl₂ to permanent turbidity, let it stand for at least one day, decant the pale yellow supernatant.
- store in a tightly stoppered dark bottle.
- prior to analysis, dilute 10 ml to 100 ml.

<u>CAUTION</u>: Mercuric chloride is highly toxic and mercury tends to accumulate in certain tissues. Wash immediately glassware used to prepare reagent. Wash hands before eating or smoking. Wipe up spills immediately with warm water, soap and gloves. If spilled on clothing, do not wear again until laundered;

take a shower as soon as possible.

1. Colorimeter or spectrophotometer capable of operation at 366 nm.

Procedure

Observe precautions described above.

- Pipette duplicate 5 ml aliquots of samples or standards into a test tube.
- Add 1.0 ml of diluted Nessler's reagent, mix and allow to stand
 min. Caution: Do not pipette Nessler's reagent by mouth.
- 3. Read at 366 nm within 50 minutes.
- 4. Calculate concentration of NH_3 from standard curve.

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AMINO ACIDS

Theory

Amino acids are extracted from plant tissue in a mixture of chloroform, methanol and water. If necessary, the residue is further extracted with hot 80% ethanol. Chlorophyll and fatty materials are removed from the chloroform mixture by saturating it with chloroform to produce two phases. The amino acids remain in the aqueous layer while chlorophyll and fatty materials are retained in the chloroform layer. The aqueous layer is concentrated, placed on a cation exchange column and rinsed with water to remove sugars and organic acids. The amino acids are then eluted from the column with 2N NH_LOH. The eluate containing the amino acids is dried under vacuum and then dissolved in a known volume of 30% propanol. If desired, phenolics in the extract may be removed by rinsing the column with acetone after rinsing with water. Removing phenolics does not appear to improve the separation of amino acids in extracts of Douglas fir.

Known amounts of the 30% propanol containing extracted amino acids and standard amino acid mixtures at two concentrations are spotted on mixed silica gel/cellulose thin layer plates. Amino acids are separated first by high voltage electrophoresis in formic/acetic acid buffer, then by chromatography in n-propanol/water/propyl acetate/acetic acid/pyridine. This combination does not separate lysine from ornithine nor glutamine from citrulline; however, it does separate the amino acids found in Douglas-fir foliage. With other tissues or other species, one-directional chromatograms in other solvents should be run to check that only one of each pair of these overlapping aminoacids is present. After chromatography, the plates are dipped in ninhydrin

reagent which reacts with amino acids to produce colored spots. The reaction is carried out in a CO₂ atmosphere with 0% humidity, either at room temperature for 16 hr or at 50°C for 1 hr. More background color develops on heated plates. Gamma-aminobutyric and aspartic acid react only slightly at room temperatures and heating improves the sensitivity for these amino acids. The plates are coated with cellulose acetate to facilitate removal of the spots. The color is eluted from the spots and absorbance is measured at 510 nm. The amount of amino acid present in a spot is calculated from standard curves prepared from plates containing amino acid standards. Standard plates are run with each set of sample plates since the position of amino acids and intensity of their reaction with ninhydrin may vary slightly from run to run.

Reagents and Materials

1. M.C.W. solvent

- methanol/chloroform/water, 120:50:30 volume/volume.

2. 2N NH₄OH

-133 ml conc(28% NH3) NHLOH diluted to 1 L with distilled water.

3. Formic/Acetic buffer

- 17 ml formic acid (91%) + 57 ml glacial acetic acid (99%) made to 1 L with distilled water.

4. n-Propanol solvent

- n-propanol/distilled water/n-propyl acetate/acetic acid/pyridine, 120:60:20:4:1 volume/volume.

5. Ninhydrin reagent

- 100 mg cadmium acetate + 10 ml H_2 0 + 5 ml glacial acetic acid.
- 1 g ninhydrin + 100 ml acetone.
- mix aqueous and acetone solutions immediately before use.

6. Cellulose acetate solution

- 6 g cellulose acetate + 2 g camphor + 2.7 ml diethylene glycol.
- + 33 ml n-propanol + 67 ml acetone.
- set on shaker for 4 hr.

7. Color eluting solvent

- 100 ml methanol + 100 ml ethyl acetate + 100 ml distilled H₂0 + 3 ml. acetic acid (glacial) + 3 g cadmium acetate.

8. Standard amino acid mixture

- purchased standards, .OlM.
- mix equal amounts of appropriate amino acids.

1. Shaker

- 2. Centrifuge capable of 1,800 x g when holding 15 ml centrifuge tubes.
- 3. Flash evaporator designed for small amounts of materials.
- 4. Ion exchange columns.
- 5. Thin layer plates, 500 u thick, prepared using 12.5 g cellulose powder MN300 G, 5 g silica gel G, and 100 ml water for five plates.
- 6. D-C power supply capable of delivering 1000 V at 40 mamp.
- Sealed chromatography oven with gas inlet and exit ports (large dessicator will do).
- 8. Colorimeter or spectrophotometer capable of operation at 510 nm.

Procedure

The technique for extraction of 1 g of tissue is described; this can be scaled down to 100 mg, using smaller chromatography columns.

 Place one g of ground freeze-dried tissue in a 50 ml centrifuge tube, add 20 ml of M.C.W. solvent.

- 2. Shake tube 5 min at room temperature.
- 3. Centrifuge to obtain a clear supernatant, decant supernatant.
- 4. Repeat extraction 3 times.
- 5. Combine M.C.W. supernatants.
- 6. On a trial sample, check, as follows, whether further extraction is necessary. Reflux with 20 ml 80% EtOH for 3 min; filter extract and concentrate the filtrate to about 25 ul; spot the concentrate on filter paper; spray with ninhydrin (0.5% in acetone) and heat at 100°C for 5 min. If a purple spot appears, further extraction is necessary. In this case, extract residue with 20 ml 80% EtOH under reflux for 3 min, filter, repeat extraction twice. Combine filtrates.
- 7. To the combined M.C.W. supernatants, add 20 ml CHCl3, then 30 ml H20.
- 8. Mix, centrifuge to separate layers.
- Pipette off upper layer with a bulb. Discard bottom layer. Add EtOH filtrates, if further extraction was necessary (step 6).
- 10. Concentrate to about 2 ml under vacuum at temperature less than 40°C.
- 11. Place concentrate on a column of Dowex 50W-X8 cation exchange resin 7 cm tall, wet bed volume 5 ml.
- 12. Elute column with 20 ml H₂0 at a flow rate of 1.5 to 1 ml/min. Discard eluate.
- 13. Elute column with 60 ml 2N NH, OH at a flow rate of 1.5 to 1 ml/min.
- 14. Dry eluate under vacuum at less than 40°C.
- 15. Dissolve residue in 500 ul of 30% propanol.
- 16. Spot duplicate 5 ul aliquots on thin layer plates.
- 17. Spot aliquots of mixtures of standard amino acids, at two concentrations on two other thin layer plates (amounts equivalent to 1 ul and 3 ul of

- each amino acid/standard).
- 18. Separate the amino acids in one direction by electrophoresis in formic/acetic-acid buffer, at 1000 to 600 V, 40-60 mamp, for 20 min.
- 19. Dry the plate.
- 20. Separate amino acids at right angles to the first separation by chromatography in n-propanol solvent at room temperature until the solvent front comes to within 1 cm of the top of the plate (about 3½ hr).
- 21. Dry the plate.
- 22. Flood with ninhydrin reagent, dry quickly with hair-dryer.
- 23. Place in chromatography oven over separate containers of conc H2SO4 and NaOH pellets.
- 24. Purge oven with CO, at 4 lb. pressure for 30 min.
- 25. Turn off CO2, close exit port, turn on fan and heater for 1 hr.
- 26. Remove plates, coat them with cellulose acetate.
- 27. When dry, remove spots to 15 ml conical test tubes (as a blank, include a background spot).
- 28. Add 1.5 ml of color eluting solvent, stopper and shake gently for 10 min.
- 29. Centrifuge at 1000 xg for 10 min, decant.
- 30. Read absorbance at 510 nm within 4 hr.
- 31. Calculate amount of each amino acid present from standard curves.

 If possible, spots should be eluted and read the same day as the colors are developed to keep the background reading low.

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PROTEINS

Theory

Soluble proteins are extracted from plant tissue and separated by electrophoresis on polyacrylamide gels. Samples are mixed with 8 M urea which breaks phenolic-protein H-bonds. The urea slurry is placed on a discontinuous polyacrylamide gel and extraction and separation are carried out in one step by electrophoresis. Protein bands are stained with Amido black and can be quantified by densitometry. Active enzymes can be demonstrated directly in the gels or in solution after elution of proteins.

Reagents and Materials

1. pH 3.35 Buffer

- 3.37 g KOH dissolved to 1 L with distilled H₂O; adjust to pH 3.35 with citric acid using the pH meter.

2. pH 4.35 Buffer

- 3.37 g KOH dissolved to 1 L with distilled H20; adjust to pH 4.35 with citric acid using the pH meter.

3. 3M Urea

- 180 mg urea per ml of appropriate buffer solution.

4. Catalysts

- 1 mg ascorbic acid + 25 ug $FeSO_4$ + 1 ul of 30% H_2O_2 per ml of gel /buffer solution.
- dissolve ascorbic acid and FeSO $_4$ in gel/buffer solution first, add ${\rm H}_2{\rm O}_2$, with stirring, just before pouring gel into chamber.

5. Electrode Buffer

- 5.56 g glycine dissolved in distilled $\mathrm{H}_{2}\mathrm{O}$ to make 2 L; adjust to

pH 4.35 with citric acid using the pH meter.

6. 8 M Urea

- 48 g urea dissolved to make 100 ml with distilled water.

7. Saturated Urea containing ascorbate

- 60 g urea + distilled H20 to 100 ml, set on shaker 3 hr.
- filter, add 15 mg Na ascorbate per ml of filtrate.

8. Staining Solution

- 0.7 g Amido Black 10 B + 10 ml acetic acid (glacial) + 90 ml H20.
- filter and use filtrate.

9. Destaining Solution

- methanol/H20/acetic acid 5:5:1 volume/volume
- 1. Polyacrylamide gel electrophoresis chamber.
- 2. D-C power supply capable of delivering 500 V, 150 mamp.
- Electrophoretic destaining apparatus.

Procedure

- 1. Prepare 14 cm wide, 3 mm thick gels in a vertical gel electrophoresis chamber as follows: plug of 40 ml 16% Cyanogum in pH 3.35 buffer; running gel of 70 ml. 16% Cyanogum in pH 3.35 buffer containing 3 M urea; spacer gel of 4% Cyanogum in pH 4.35 buffer containing 3 M urea. Have the cooling water running while the gels polymerize.
- 2. Fill chamber with electrode buffer.
- 3. Mix 50 mg of freeze-dried, ground tissue with 500 ul of 8 M urea, or pulverize 100 mg of fresh tissue with 500 ul of 8 M urea. For bark samples, use saturated urea containing 1.5% Na ascorbate. Using a Drummond micro-pipette, transfer 50-200 ul of slurry to 1 cm wide

sample slots.

- 4. Cover chamber, turn on current to 250 V (45 mA) for 10 min.
- 5. Increase current to 450 V (80 mA) for 1-2 hr.
- 6. Turn off current, drain chamber, remove gel slab.
- 7. Stain for 1 hr.
- Destain for 30-90 min in destaining apparatus, checking slab every
 min to insure even destaining.

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- McMullan, E.E. and Ebell, L.F. 1970. Disc electrophoresis of soluble proteins of conifer foliage. Phytochemistry 9: 2281-2285.