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Analysis of Detritus and Organic Horizons for Mineral and Organic Constituents

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The analysis of the chemical composition of plant matter (leaves, stems, roots, and detritus) is critical for studies of nutrient turnover in ecosystems and of the biotic pools of important biogeochemical elements, as well as for understanding nutrient and chemical limits to plant growth. Understanding the organic constituents of plant litter and their transformation into those forming soil organic matter is also a critical need given their link to the biogeochemistry of other elements and the storage of carbon in ecosystems.

The chemical analysis of plant materials for mineral constituents is fairly straightforward, although different materials may need either different pretreatment or slightly different digestion methods. In contrast, the analysis of organic constituents is not straightforward because of both the underlying complexity of the compounds themselves and the variety of methods that can be used, none of which are perfect. Our intent in this chapter is to review these methods and recommend standard protocols. In the case of organic constituents, we recognize that these recommendations may become dated rapidly if the most modern instrumentation becomes generally available.

Sample Pretreatment

Aboveground plant materials, including plant litter, organic layers, and woody detritus, should be dried at 65 °C in an oven before homogenizing. For plant litter this may take 48 hours, whereas woody debris may take up to a week. Complete drying should be monitored by measuring sample weight loss; when weights have stabilized for 24 hours, drying is complete. Once dry, materials should be homogenized

by grinding in a Wiley mill or similar grinder for macroelement analysis. Hand grinding with a mortar and pestle may be warranted for trace element analysis. Materials should be ground to pass a 60-mesh (0.246 mm) screen for carbon-hydrogen-nitrogen (CHN) or carbon-nitrogen-sulfur (CNS) combustion analyses, or a 20-mesh (0.833 mm) to 40-mesh (0.417 mm) screen for wet digestion. Samples should be stored either in a warm oven or in a desiccator to prevent rehydration prior to elemental analysis.

Analysis of root materials is slightly more complex. Roots removed from soils need to be cleaned of soil without unduly fragmenting the roots or leaching water-soluble compounds, and thus should not be dried before processing. In addition, Fe and various other metals may form insoluble coatings on root surfaces, particularly in soils with fluctuating water tables. Such coatings are virtually impossible to remove, and thus interpretation of the chemical analysis of these elements from roots must include this pool of surface-bound, nonorganic material. Field- or greenhouse-moist roots can be placed in a beaker or other acid-washed, cleaned glass or polyethylene container and gently swirled in a phosphate-free, dilute detergent solution. Only a small amount of detergent is needed, and plant roots do not need to be mechanically rubbed, since the purpose of this step is to break the surface tension. After rinsing, roots are swirled in a 0.01 mol/L NaEDTA solution for 5 minutes, which complexes cations bound at the surfaces, including metals. After rinsing in deionized water, roots should be placed immediately in paper bags or small envelopes and dried for 24 hours at 65 °C. Once dry, roots can be ground and stored as discussed previously.

Available Methods

Organic Matter Digestion for Mineral Analysis

There are several published methods for organic matter digestion and chemical analysis, including both dry ashing and various wet digestion/ashing techniques. We will not discuss dry ashing techniques because although these are quite fast and simple to perform, they may cause loss of elements due to volatilization, sorption on crucible surfaces, or particulate loss. Dry ashing can never be used for the analysis of volatile elements such as nitrogen or sulfur. Nitrogen and sulfur are most easily analyzed on a CHN or CNS analyzer, although wet ashing and analysis are still possible if access to such analyzers is limited. Wet digestion techniques vary in the oxidants and acids used to oxidize organics and dissolve chemical constituents. In this chapter we avoid procedures that use perchloric acid because it is extremely dangerous to use, it requires specialized hoods, and much safer alternatives exist. We recommend two fairly straightforward wet digestion methods, one for major element analysis and one for trace element analysis.

Organic Constituents

Numerous methods exist for the analysis of organic constituents in detritus and organic horizons. Although only two are recommended as standard procedures, the or-

ganic chemistry of detritus and organic horizons is extremely complex. Depending on the question being asked, additional analysis methods may be needed. Three classes of methods are used to determine organic constituents: (1) mass loss methods that determine composition by sequential extraction, hydrolysis, and oxidation; (2) end-product methods that analyze the chemical constituents resulting from oxidation or thermal decomposition; and (3) nondestructive methods based on the spectral properties of samples.

Mass Loss Methods

Gravimetric determinations of organic fractions after various extraction, hydrolysis, and oxidation steps are collectively called proximate analysis. Given the complex nature of organic compounds to be found in undecomposed and decomposed plant litter, these methods define constituents operationally based on their resistance to various chemical treatments. This classic method has routinely been applied to determine organic constituents of forest products (Ryan et al. 1990). In this method the first step is to extract polar and nonpolar compounds, followed by hydrolysis of the remaining material in heated 72% H_2SO_4 (approx. 12 M). The remaining mass, minus ash, is the so-called Klason, or acid-resistant, lignin.

Proximate analysis may not produce well-defined chemical constituents. That is, lignin as measured by proximate analysis may contain other chemical constituents such as condensed tannins. This mismatch is less problematic for undecomposed gymnosperm wood, the substrate for which proximate analysis was originally developed, but the mismatch increases as one applies the analysis to angiosperm or decomposing wood because the lignin in these substrates can be partially hydrolyzed by acid (Effland 1977). The mismatch between chemical composition and operational definitions becomes an especially significant issue for leaves and fine roots, which contain aliphatic compounds such as cutin and suberin, as well as condensed tannins that may be acid-resistant but not extracted by the solvents usually used in proximate analysis (Trofymow et al. 1995).

An alternative proximate analysis method was developed by Van Soest (1963a

method to give a more complete analysis of constituents (Ryan et al. 1990; Gallardo and Merino 1993).

End-Product Methods

The end products of classic proximate analysis can be further analyzed, but this is rarely done in ecological studies. An alternative method for decomposing organic samples into constituent fractions is alkaline cupric-oxide oxidation (Hedges and Parker 1976). The oxidation products are then analyzed by capillary gas chromatography (Hedges and Parker 1976; Hedges and Mann 1979), gas chromatography and mass spectroscopy in series (Goñi and Hedges 1990a), or high-performance liquid chromatography (Kögel-Knabner and Ziegler 1993). The technique has been applied mainly to lignin and less often to cutin (Goñi and Hedges 1990a,b).

The main goal of the studies that employ end-product methods has been to identify the source of the organic portion of marine sediments. This is possible because the ratios of oxidation products (e.g., syringyl:vanillyl phenols in the case of lignin, or C_{14}:C_{16} fatty acids in the case of cutin) can indicate derivation from angiosperm versus gymnosperm taxa and/or woody versus nonwoody tissues. Similar applications are possible for plant detritus (Hedges et al. 1988) and organic horizons (Kögel-Knabner 1986; Kögel-Knabner and Ziegler 1993). The main drawback of the technique is that the oxidation itself is time-consuming and the efficiency of the oxidation process in producing detectable end products is difficult to determine (Goni and Hedges 1990a). A faster thermochemolysis method uses tetramethylammonium hydroxide (TMAH) (Hatcher et al. 1995). This method appears more suited to large numbers of samples and may become standard with time.

In addition to chemical treatments, heat can be used to decompose the organic fractions in a sample. One such method that has recently shown great promise is analytical pyrolysis (Kögel-Knabner et al. 1992; Preston et al. 1994), in which samples are heated gradually to an upper limit of 320–750 °C. The pyrolysis products are then analyzed with a mass spectrometer or a gas chromatograph–mass spectrometer linked in series. Of all the methods we reviewed, this system gives the most detail on the chemical structure of constituents such as carbohydrates, lignin, fatty acids, and aromatic and aliphatic esters and how they are transformed by decomposition. A second method involving thermal decomposition of organic fractions is differential scanning calorimetry combined with differential thermogravimetry (Reh et al. 1990). In this system, extremely small samples (3–5 mg) are heated at a constant rate from 100 to 800 °C to burn the organic compounds at their characteristic combustion temperatures. The amount of heat released at each temperature (calorimetry) and rate of mass loss (thermogravity) are used to identify the amount of each compound present in the sample. This method holds great promise, because even the form of constituents (e.g., amorphous versus crystalline cellulose) can be determined.

Nondestructive Methods

Near-infrared reflectance (NIR) spectroscopy is a nondestructive technique that uses the reflectance of dried, ground organic material to indirectly determine concentra-

tions of ash, nitrogen, and organic constituent contents of dried, ground samples. Although this method has gained widespread use for qualitative analysis of oilseed, grains, and forages, its acceptance as an ecological analysis tool has been slow (Wessman et al. 1988a). Numerous tests, however, have shown its applicability to fresh as well as decomposed plant litter (Wessman et al. 1988a; McClellan et al. 1991a,b; Joffre et al. 1992; Gillon et al. 1993). The technique is based on the fact that individual plant constituents have characteristic absorbance properties in the near-infrared spectral region (i.e., 1100–2500 nm). While these peaks are clearly defined in pure compounds, they are not as well defined in plant material. Reflectance spectra of plant litter and organic horizons exhibit overlapping absorption peaks that correspond to overtones and combinations of C-H, O-H, or N-H chemical bonds. Material-specific calibration of absorption spectra against wet chemistry data enables one to determine the abundance of a wide range of constituents. Once this calibration is completed, however, an extremely large number of samples can be assayed in a short time.

Nuclear magnetic resonance (NMR) spectroscopy is another nondestructive means to determine organic constituents of plant litter, soil organic horizons, and mineral soil (Preston 1993; Baldock and Preston 1995; Preston 1996). The technique, which was originally developed for use in organic chemistry and biochemistry, is based on the fact that atomic nuclei have a characteristic magnetic moment or spin that can be altered by radio frequency waves in a strong magnetic field (Jardetzky and Roberts 1981). The frequency of radio waves required to alter the spin is affected by the chemical bonding associated with the nuclei, resulting in the so-called chemical shift. Phenolic bonds, for example, modify the energy required to alter the spin differently than alkyl bonds.

With the advent of Fourier transform techniques and application of the cross-polarization magic angle spinning (CPMAS) method, NMR has become a powerful tool for the analysis of the complex nature of organic matter in soil and litter as it can be applied to dilute solutions, complex solids, extracts, and gels. For organic compounds the nuclei examined are ^{13}C, but other nuclei such as ^{15}N and ^{31}P can also be examined. Although NMR is an excellent analysis tool, and should be applied more widely, the instrumentation and training needed to use it are generally not available to ecologists. This is indeed unfortunate because the method has already been used to examine many long-held hypotheses concerning the chemical nature of soil organic matter (Kögel-Knabner et al. 1992; deMontigny et al. 1993; Preston et al. 1994; Preston 1996).

Suggested Standard Methods

Recommending a single method for the analysis of organic constituents is difficult given that none of the currently available methods is without problems of either chemical precision (e.g., gravimetric determinations) or availability (e.g., NMR). Indeed, recommending a single method is counter to the recent, healthy trend to examine organic constituents from several approaches (Kögel-Knabner et al. 1992; de Montigny et al. 1993; Preston et al. 1994). Nonetheless, we have selected two complementary methods for general application based on their availability and training requirements. These are the forest products–based proximate analysis (Ryan et al.

1990) and NIR spectroscopy (Wessman et al. 1988a). Proximate analysis has been widely used in the past, and despite problems of interpretation (e.g., is the acid-resistant fraction really lignin?) the method can be applied to a wide range of materials with a minimum of expensive equipment and training. Results of proximate analysis for litter have also been the basis for many existing litter decomposition models (Aber et al. 1990; Parton et al. 1994), a trend that is likely to continue for some time. NIR spectroscopy has been selected because it is extremely fast and can be applied to more samples than would be possible with other methods. Given its calibration to proximate analysis, it is subject to the same limitations of interpretation; however, this also makes it completely consistent with current litter decomposition models. Finally, NIR spectroscopy has the advantage that it can be linked to remote sensing (Wessman et al. 1988b), allowing one to potentially examine large-scale patterns of aboveground litter quality.

H_2SO_4–H_2O_2 Digestion for Plant Major Element Analysis

Perhaps the most common technique for plant material analysis is wet digestion using sulfuric acid and hydrogen peroxide in a block digester. As with any sulfuric acid digest, $CaSO_4$ may precipitate, and thus this digest should not be used for calcium analysis. In addition, $CaSO_4$ may precipitate in the glass tubes after repeated use, and thus tubes used for H_2SO_4 digests may never be used for calcium analysis.

Materials

1. 20- or 40-position block digester with tube rack
2. 50, 70, or 75 mL calibrated block-digestion tubes
3. Concentrated reagent-grade H_2SO_4
4. H_2O_2 (30%), reagent-grade, low P

Procedure

1. Weigh 200–300 mg of dried, ground material into acid-washed block-digestion tubes. This weight may need to be doubled in the case of woody material. Care should be taken to ensure that powders are placed near the bottom of the tube and do not adhere near the top.
2. Add 5 mL concentrated H_2SO_4 to each tube, swirling to wet the material and to wash down any powder from the sides.
3. Add 2 mL H_2O_2 very slowly and carefully to each tube, swirling constantly to reduce the vigorous boiling that will ensue.
4. When all tubes have finished boiling (1–5 minutes), place the rack in the block that has been preheated to no more than 170 °C, manually turn the heater on, and digest for 1 hour. The temperature must reach 230 °C before the heater is turned off, but if this temperature is reached before the hour, turn off the heater and allow tubes to sit until the end of the hour.
5. Remove the rack from the block, turn off the heater, and allow the tubes to cool. When tubes are cool to the touch, add another 2 mL H_2O_2 to each tube.

6. When the block has cooled to 175 °C or below, turn on the heater, place the rack with tubes in the block, and digest for an additional 2 hours, taking care that the final temperature does not exceed 350 °C. At least 1 hour of this final digest should be at 330–350 °C to ensure complete removal of the H_2O_2 because H_2O_2 interferes with Murphy and Riley (1962) phosphorus analysis.
7. After tubes are cool, solutions are saved in acid-washed polyethylene or glass vials. Analysis of nitrogen, phosphorus, potassium, magnesium, sulfur, and various trace elements can be performed from this digest.

Calculations

The concentration in mg element/g tissue (C_{tissue}) of an element (C) is calculated as

$$C_{tissue} = C_{digest} \times V_{digest}/M_{dry}$$

where

C_{digest} = the concentration of the digest (mg/L)
M_{dry} = the dry mass of the sample digested (g)
V_{digest} = the volume of the calibrated digestion tube, typically 0.05, 0.07, or 0.075 L

For roots or organic materials that might have high ash contents, results are often expressed on an ash-free dry-mass basis. Ash is determined using a muffle furnace (see later), and fractional ash-free dry mass ($F_{ash\text{-}free}$) is calculated as:

$$F_{ash\text{-}free} = (M_{dry} - M_{ash})/M_{dry}$$

where

M_{dry} = the dry mass of sample
M_{ash} = the ash mass of sample

Ash-free concentration of elements of samples ($C_{ash\text{-}free}$) in units of mg element/g ash-free tissue is calculated as

$$C_{ash\text{-}free} = (C_{digest} \times V_{digest})/(M_{dry} \times F_{ash\text{-}free})$$

where C_{digest}, V_{digest}, M_{dry}, and $F_{ash\text{-}free}$ are defined above.

Special Considerations

A standard plant sample (e.g., from National Institute of Standards and Technology [NIST]) and at least two sample replicates should be brought through the digestion procedure with every batch of 40 samples. Wood standards are not available from NIST; however, three wood standards ranging in amount of decay are available from Phillip Sollins or Mark Harmon at Oregon State University (see Contributor List, this volume, for addresses). Finally, blank matrix material for the automated analy-

sis should be made by following the preceding procedure without adding tissue material.

Digestion of woody material may be incomplete with the procedure described earlier, leading to an underestimate of mineral element concentrations. Addition of either K_2SO_4 or Na_2SO_4 can raise the boiling temperature sufficiently to increase the recovery of nitrogen, phosphorus, and other elements from woody material (Dan Binkley, personal communication).

Digestion for Trace Element Analysis

Microwave or hot plate digestion of plant materials is the most commonly used procedure for trace element or micronutrient analysis. Nitric acid is a more powerful oxidant than sulfuric acid, and thus peroxide is rarely needed. Obviously, nitrogen cannot be determined in these digests. This technique is particularly useful if automated microwave digestion equipment is available. If not, commercially available microwave ovens may be used with microwave Parr bombs that are available from Cole-Parmer. The microwave procedure is the easiest digestion procedure, although the resulting digestate is strongly acid, which may cause problems for analysis if dilution is not possible. Hot-plate digestion is the most low-tech procedure, but it requires more operator time. In all cases, samples can be evaporated to dryness and redissolved in weak HNO_3 to avoid the excess acid problem.

Here we describe digestion using a hot plate. If there is access to an automated microwave digestion system, follow the instructions included with the machine, since models vary. If microwave digestion is done manually, the technique is equally simple, but microwave digestion should only be attempted in ovens made for this purpose, because the power increments on standard microwave ovens are generally not fine enough and they lack safety features. Specific methods for Parr bomb digestion depend on the size of bomb purchased; directions are included with the specific bombs. Although microwave digestion is easy and convenient, more samples may be processed at the same time using a hot plate.

Materials

1. 20 mL closed Teflon vials (available from Cole-Parmer)
2. Large, adjustable hot plate
3. Concentrated HNO_3, ultrapure or trace metal grade
4. 25 mL calibrated volumetric flasks (polypropylene or Teflon for trace metal analysis)

Procedure

1. With large hot plates, up to 20 samples may be digested at one time. Place 200 mg of dried and ground plant material in the vials and add 2 mL concentrated HNO_3.
2. Close the lids and digest the mixture on a hot plate for 1 hour at ~120 °C. This

is generally the lowest heat setting on commercial hot plates and is the point at which a light reflux, or condensation, is first observed on lids.

3. If colorimetric analysis is to be employed, the digestion can stop here. Samples are then quantitatively transferred to 25 mL volumetric flasks and brought to volume with deionized (DI) water.
4. For analysis in which acid concentrations are a problem (e.g., graphite-furnace atomic-absorption spectrometry, inductively coupled plasma (ICP) spectrometry, or ICP-Mass Spectrometry (ICP-MS)), evaporate digestates to dryness at moderate heat (75–105 °C) taking care not to char the residue. Bring residues up to volume (usually 20 mL, although this will vary with the sensitivity needed for analysis) with 3% HNO_3. Note that if samples are evaporated to dryness, then the residue may be redissolved by adding a known mass of HNO_3 rather than bringing samples to volume in a small volumetric flask. Adding acid by mass is significantly more accurate than using a small volumetric flask. Because variable amounts of HNO_3 are reduced and thus lost during the digestion step, digest solutions cannot be brought to volume using known weights of acid unless samples are first brought to dryness.

Calculations

To determine the concentration of a trace element in a material, use the same calculations as for macroelement analysis, described earlier. The only difference is that the digest volume for trace element analysis is typically 0.02 L. For roots or materials that might have high ash contents because of adhering soil, results should be expressed on an ash-free dry-weight basis as described under the preceding procedure.

Special Considerations

Although some researchers have added several drops of H_2O_2 to the NO_3 digestion, this is generally more useful for animal tissues with high lipid contents than for plant materials.

Teflon vials used for trace metal analysis should be cleaned by boiling in 25% aqua regia (1:3 HNO_3:HCl). Solutions should be stored in polyethylene bottles that have been heated for 48 hours in 5–10% HCl.

A plant material standard (e.g., from NIST) should be processed through the entire digestion procedure with every batch of 20 samples, along with one blind replicate and at least one blank. The analysis of standards is crucial to identify problems with the procedure such as incomplete digestion or contaminated reagents.

Forest Products–Based Proximate Analysis

The forest products–based proximate analysis recommended is a series of extraction, hydrolysis, and oxidation steps (Fig. 8.1). These are used to determine gravimetrically the proportions of general classes of organic constituents.

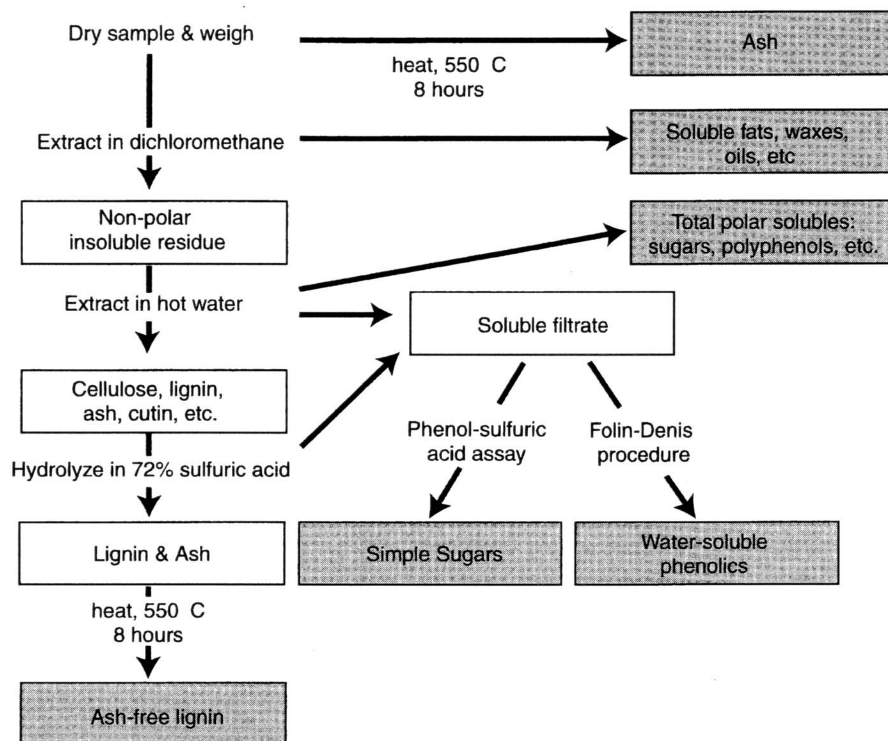

Figure 8.1. Flow diagram of steps used in proximate analysis. The open boxes indicate intermediate components separated by chemical and/or heat treatments. The shaded boxes indicate components resulting from final calculations. Modified from Ryan et al. (1990).

Materials

The materials for the various extractions and hydrolysis steps overlap; therefore, materials specific to each are indicated by the abbreviations to the right of the materials list (NPE = nonpolar extractives; PE = polar extractives; SA = sugar analysis; TA = tannin analysis; AH = acid hydrolysis; ASH = ash).

1. Pre-ashed fritted glass filtering crucibles (TA, AH, ASH)
2. Pre-ashed Gooch filtering crucible (NPE, PE, AH)
3. Wire hangers to suspend extraction thimbles (NPE)
4. BD-20 or BD-40 block-digestion tubes; straight tubes without volume markings are acceptable (NPE, PE)
5. Rubber stoppers with cold finger setup (see Figure 8.2) (PE, SA, TA, AH, ASH)
6. Walter crucible holders (NPE)
7. Buchner flask (NPE)
8. 100 mL beakers (NPE)
9. 15 mL round-bottomed Pyrex test tubes (AH)
10. 125 mL Erlenmeyer flask (AH)

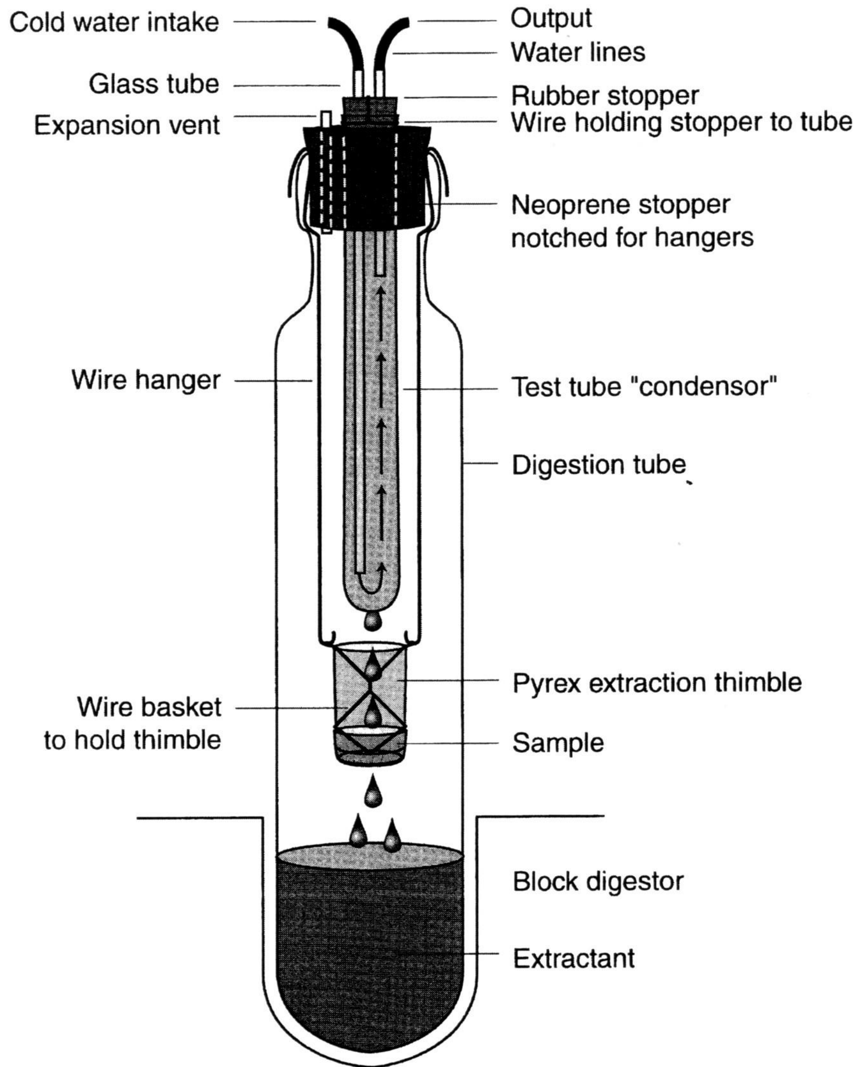

Figure 8.2. Cold finger extraction system used for nonpolar extraction. Based on the system developed by C. A. McClaugherty (personal communication).

11. 250 mL Erlenmeyer flask (PE, AH, SA, SA)
12. Glass funnels, small (AH)
13. 50 mL volumetric flasks (TA)
14. 500 mL volumetric flask (TA)
15. 1000 mL volumetric flasks (SA, TA)
16. Eppendorf pipettes, calibrated to known volume (SA, TA)
17. Block digester or sonicating bath (NPE, PE)
18. Autoclave or hot plate (AH)

19. Heating water bath (TA)
20. Desiccator (NPE, PE, AH, ASH)
21. Drying oven (NPE, PE, AH, ASH)
22. Muffle furnace (ASH)
23. Analytical balance (NPE, PE, AH)
24. Spectrophotometer (SA, TA)
25. Dichloromethane (CH_2Cl_2) (NPE)
26. Deionized water (PE, SA, TA, AH)
27. H_2SO_4 concentrated and 72% (AH)
28. Dextrose (SA). Dextrose standards used in the sugar analysis procedure are based on a primary standard of 200 μg of dextrose/mL. To prepare this, take 200 mg of dextrose that has been oven-dried at 105 °C and cooled in a desiccator for 30 minutes and add it to 1000 mL of deionized water in a 1 L volumetric flask. This stock solution is then diluted to form seven standards that systematically increase from 0 to 50 μg/mL.
29. Phenol, redistilled reagent grade or phenol solution of known density (SA). If phenol solution of known density (mg/mL) is not available, then phenol solution can be made by adding 20 g of deionized water to 80 g of redistilled reagent-grade phenol that has been placed in a beaker. Phenol is very toxic and must be handled with rubber gloves under a hood.
30. 1 M Na_2CO_3 (TA)
31. Folin-Denis reagent or Folin-Ciocalteau's reagent. Folin-Denis reagent is made by adding, in the following order, 50 g of sodium tungstate, 10 g of phosphomolybdic acid, and 25 mL of orthophosphoric acid to 375 mL of deionized water in a 1 L flat-bottomed flask fitted with a reflux condenser. The flask with several glass beads inside is refluxed on a hot plate for 2 hours, cooled, and emptied into a 500 mL volumetric flask, which is brought to volume with deionized water.
32. Phenol reagent, available through Sigma (TA)
33. Sodium tungstate for the Folin-Denis reagent (TA)
34. Phosphomolybdic acid for the Folin-Denis reagent (TA)
35. Orthophosphoric acid for the Folin-Denis reagent (TA)
36. Tannic acid standards (TA). Prepare standards based on a stock solution of 100 mg of tannic acid in 1000 mL of deionized water. This results in a concentration of 0.1 mg tannic acid/mL. Standards are made in the range of 0–0.6 mg tannic acid /mL by pipetting 0–6 mL of the stock solution into a 50 mL volumetric flask and adding deionized water to up to the final volume. These tannin standards are not stable and must be made fresh each day an analysis is conducted.

Procedure

Nonpolar Extractives

The nonpolar extraction, an adaptation of the method described by TAPPI (1976), removes oils, waxes, and fats from samples. Cutin and suberin are not effectively

removed by this extraction. Note that CH_2Cl_2 used in the extraction is volatile, moderately flammable, and toxic. It should only be used under a fume hood and handled with gloves. Any CH_2Cl_2 remaining should be saved and disposed of properly (i.e., do not pour down the sink!).

The TAPPI method employs Soxhlet extractors, an expensive system that is difficult to use with many samples. To increase the number of samples that can be processed, one can use either of the following two methods:

1. Place approximately 1 g of sample in a pre-ashed, preweighed Gooch filtering crucible and record the weight. Use a number 2 pencil to mark the number of the sample (other markings will be removed by the solvent). Place 75 mL of CH_2Cl_2 in a block-digester tube that contains the extraction thimble held up by wire hangers. Under a fume hood place the block-digester tube in a cold block-digester and seal it with a rubber stopper containing a cold finger connected to a cold water circulating system (Fig. 8.2). The block-digester temperature is set to 56 °C, and once the CH_2Cl_2 begins to boil (at 40 °C), condense on the cold finger, and drip onto the sample, the extraction is continued for 5 hours. Allow the block digester to cool before removing the extraction thimbles.
2. Place approximately 1 g of sample in a pre-ashed, preweighed Gooch filtering crucible and record the weight. Use a number 2 pencil to mark the number of the sample (other markings will be removed by the solvent). Set the Gooch filtering crucible inside a 100 mL beaker. Under a fume hood add 30–40 mL of CH_2Cl_2 into the Gooch crucible, pouring slowly so that the solvent moves through the crucible without overflowing. Set the 100 mL beakers with crucibles into a sonication bath, making sure they are packed tightly to avoid tipping during the sonication. One may need to use water-filled beakers to properly pack the beakers with samples. Sonicate for 30 minutes at 60–70 watts of power. Remove the Gooch crucible and suction off the CH_2Cl_2 using a Buchner flask fitted with a Walter crucible holder. Pour off the CH_2Cl_2 remaining in the 100 mL beakers into a waste container and repeat the extraction twice more, using fresh CH_2Cl_2 for each extraction. Once the extraction is completed, place the extraction thimbles in a drying oven placed in a fume hood and dry at 50 °C for 12 hours. Cool in a desiccator and weigh the extraction thimble and sample to determine the mass lost during extraction.

Polar Extractives

The polar extractives procedure, an adaptation of TAPPI (1981), removes water-soluble polyphenols and simple sugars but not condensed tannins. The residue remaining after the nonpolar extraction is removed from the extraction thimble with a spatula.

1. Carefully weigh approximately 500 mg of the residue and place in a clean block-digestion tube. Depending on the size of the block-digester tube, add 25 or 75 mL of deionized water to the tube, being careful to wash all adhering fibers from the sides of the tube.

2. Place the tube in a block digester preheated to 104 °C, adjusting the temperature to allow gentle boiling for 3 hours.
3. Once the extraction is completed, filter the water and residue into pre-ashed, preweighed Gooch filtering crucibles.
4. Wash the fiber residues with deionized water several times. The filtrate should be captured in a 50–250 mL volumetric flask if sugar and tannin contents are to be determined, brought up to final volume with deionized water, and saved.
5. Dry the fiber residues and filtering crucible at 50 °C for 12 hours.
6. Cool in a desiccator and determine the total weight remaining minus the weight of the crucible. The mass lost is the polar extractives.

Acid Hydrolysis

The H_2SO_4 hydrolysis process follows that described by Effland (1977) and removes cellulose and hemicellulose.

1. Weigh out 200 mg of the oven-dried, extracted fiber and place it in a 15 mL round-bottomed Pyrex test tube.
2. Add 2 mL of 72% H_2SO_4 to the tube and heat in a water bath set at 30 °C for 1 hour, mixing occasionally to assure complete dissolution.
3. Add 6 mL of deionized water to the test tube and transfer the solution and remaining fiber to a 125 mL Erlenmeyer flask.
4. Use another 50 mL of deionized water to rinse the test tube thoroughly, transferring all the water and fiber to the Erlenmeyer flask. The flask is covered by a small glass funnel to reduce water loss.
5. A secondary hydrolysis is then carried out in an autoclave set at 120 °C for 1 hour. If an autoclave is not available, the secondary hydrolysis can be carried out on a hot plate, boiling the solution for 4 hours. If the hot plate system is used, add deionized water periodically to maintain volume.
6. The resulting solution is filtered through a pre-ashed, preweighed Gooch filtering crucible, saving the filtrate if sugar content is to be determined.
7. Wash the fibers caught in the crucible with deionized water to remove the acid. As with polar extractions, capture the filtrate in 100 or 250 mL volumetric flasks and bring up to final volume before storing.
8. Dry the crucible and fibers at 50 °C for 12 hours.
9. Cool in a desiccator and weigh, subtracting the weight of the crucible.

Permanganate Oxidation

The mass removed by acid hydrolysis is considered to be hemicellulose and cellulose, while the remaining fraction is acid-resistant lignin, cutin, suberin, and remaining ash. To determine the fraction of acid-resistant material that is either cutin or suberin, the acid-resistant fraction is oxidized with potassium permanganate ($KMnO_4$) because lignin is soluble in this substance. The remaining material should be cutin (for leaves) or suberin (for roots) and ash that has not dissolved in the preceding extraction, hydrolysis, and oxidation steps.

Total Sugars

To determine the sugar content of either the polar extractives or the H_2SO_4 hydrolyzed fiber, we recommend the phenol–sulfuric acid colorimetric method described by DuBois et al. (1956). This method is based on the fact that sugars and their derivatives produce a yellow color with an absorbance peak at 490 nm in the presence of phenol and strong sulfuric acid. The filtrate resulting from either the polar extraction or acid hydrolysis is diluted to fall within the range of standards. A 20 to 1 dilution is a good starting point when filtrates were prepared in a 250 mL volumetric flask, and a 50 to 1 dilution is a good starting point when a 100 mL volumetric flask is used.

1. 2 mL of the diluted filtrate is pipetted into a Pyrex test tube, with duplicates of each filtrate and a standard prepared simultaneously.
2. 80 mg of phenol is added into each tube using a precalibrated Eppendorf pipette and the solution mixed with a touch mixer (e.g., Vortex). The volume of phenol to be added will depend on the exact density of the stock solution used. Phenol is very toxic; it must be handled under a hood and by individuals wearing gloves.
3. 5 mL of concentrated H_2SO_4 is rapidly added to each tube with a large-bore pipette to ensure good mixing.
4. Place the tubes under a hood for 10 minutes, then mix them with a Vortex and place in a 25–30 °C water bath for 20 minutes. Absorbance is measured at 490 nm using a spectrophotometer.

Total Polyphenols

The Folin-Denis method (Allen et al. 1974) is used to determine the quantity of water-soluble polyphenols in the polar extracts.

1. Add 0.5–3 mL of the polar extract with an Eppendorf pipette into a 50 mL volumetric flask. The amount used will range from 0.25 to 3 mL depending on the type of material being analyzed and the size of the volumetric flask used to prepare polar extracts; the key point is to have concentrations within the range of the standards.
2. Record the amount added to determine the dilution factor. For standards add 1 mL to the 50 mL volumetric flasks.
3. Add deionized water to each flask containing extracts of standards so that it is two-thirds full, and add 2.5 mL of Folin-Denis or Folin-Ciocalteau's phenol reagent to the flask, allowing it to sit 3 minutes.
4. Add 10 mL of 1 mol/L Na_2CO_3 solution to the volumetric flask and bring up to full volume with deionized water.
5. Shake 10 times and place in a 25 °C water bath for 25 minutes.
6. Examine the flasks carefully for precipitate. Those with precipitate will have to be either remade or spun down with a centrifuge.
7. Read at 760 nm with a spectrophotometer.

Ash

The amount of ash in samples is determined by the standard muffle-furnace method. One gram of sample is oven dried, cooled in a desiccator, and then added to a ceramic crucible that has been preweighed. The sample is heated in a muffle furnace at 450–550 °C for 4 hours, allowed to cool, then placed in a desiccator. The fraction of ash is determined as the ratio of mass remaining after ashing to the initial sample. At a minimum, the fraction of ash should be determined for the entire sample. For some types of material, such as wood, ash content is minimal (<1%) and might be ignored. Leaf and root material, however, can have very high ash contents, and adjustments must be made to correctly calculate the organic fractions. It is also highly recommended that the ash content of the acid-resistant fraction (ARF) be determined because some of the ash may dissolve during the extraction and hydrolysis processes.

Calculations

The following calculations are based on the assumption that the ash fraction is not removed by the extraction or hydrolysis steps. We feel this assumption is preferred over the assumption that ash is removed equally by each treatment. More precise estimates of the effect of ash content can be made by comparing the initial ash content to that remaining after the extraction and hydrolysis steps are completed.

The fraction of the sample consisting of ash (A) is

$$A = M_{final}/M_{initial}$$

where M_{final} and $M_{initial}$ are the sample mass corrected for the mass of the crucible after and before heating in the muffle furnace, respectively.

The fraction in NPEs is calculated as

$$\text{NPE} = M_{anpe}/[M_{initial} \times (1 - A)]$$

where

M_{anpe} = the mass after nonpolar extraction
$M_{initial}$ = the initial mass of the sample
A = the proportion of ash of the initial sample

The fraction in PEs is calculated as

$$\text{PE} = M_{ape}/[M_{anpe} \times (1 - A)]$$

where

M_{ape} = the mass after polar extraction
M_{anpe} = the mass after nonpolar extraction
A = the ash content of the initial sample

M_{ape} is calculated as

$$M_{ape} = M_{anpe} \times M_{act\text{-}ape}/M_{act\text{-}anpe}$$

Where $M_{act\text{-}ape}$ and $M_{act\text{-}anpe}$ are the actual mass remaining after polar extraction and the actual mass of polar extract free fiber used in the analysis (approximately 500 mg), respectively. This corrects for the fact that less than the full amount of non-polar extracted fiber was used in the analysis.

The proportion in ARF is calculated as

$$\text{ARF} = M_{aah}/[M_{ape} \times (1 - A)]$$

where

M_{aah} is the mass after acid hydrolysis
M_{ape} is the mass after polar extraction
A is the initial ash content

M_{aah} is calculated as

$$M_{aah} = M_{ape} \times M_{act\text{-}aah}/M_{act\text{-}ape}$$

Where $M_{act\text{-}aah}$ and $M_{act\text{-}ape}$ are the actual mass of fiber remaining after acid hydrolysis and the actual mass of polar extract free fiber used in the analysis (approximately 200 mg), respectively. This corrects for the fact that less than the full amount of polar extracted fiber was used in the analysis.

The proportion in polar extract sugar is calculated after developing a linear regression between the absorbance of the dextrose standards and their concentration. This regression is used to determine the concentration of dextrose equivalents (CDE) in the samples in units of μg/mL based on the absorbance value of the sample. The total mass in grams of the sugar (M_{sugar}) in the sample is calculated as

$$M_{sugar} = CDE \times V_{extract} \times DF \times 10^6$$

where

CDE = concentration of dextrose equivalents as mg/mL
$V_{extract}$ = the total volume of extract or filtrate resulting from the polar extraction or acid hydrolysis (mL)
DF = the dilution factor used to prepare the samples

The proportion of the total sample in polar extractive sugars (PES) or acid hydrolyzed sugars (AHS) is computed as

$$\text{PES} = M_{sugar}/[M_{initial} \times (1 - A)]$$

$$\text{AHS} = M_{sugar}/[M_{initial} \times (1 - A)]$$

where

M_{sugar} = the mass in sugar of either the polar extracts or acid hydrolysates
$M_{initial}$ = the initial mass of the sample
A = the initial ash content of the sample

The fraction in water-soluble polyphenols (WSP) is calculated by first developing a regression between the absorbance of the tannic acid standards (mg/mL). This means that polyphenol concentration is given in tannic acid equivalents and not the concentration of the polyphenol compounds actually present. The concentration of "tannin" (*CT*) of each sample is then calculated from the absorbance value. The total mass (in grams) of tannin (M_{tannin}) in the polar extracts is calculated as

$$M_{tannin} = CT \times V_{extract} \times DF \times 10^3$$

where

CT = tannin concentration in mg/mL
$V_{extract}$ = the total volume of extract or filtrate resulting from the polar extraction or acid hydrolysis (mL)
DF = the dilution factor used to prepare the samples

The proportion of polar extractive tannin (PET) is computed as

$$\text{PET} = M_{tannin}/[M_{initial} \times (1 - A)]$$

where

M_{tannin} = the mass in tannin of the polar extracts
$M_{initial}$ = the initial mass of the sample
A = the initial ash content of the sample

Special Considerations

We strongly recommend duplicate analysis of all samples. Given the number of samples being analyzed, it is extremely easy for a small amount of fiber to be overlooked and thus influence results. Duplicate samples that differ markedly should be rerun to determine which value is correct.

As with any proximate analysis of organic constituents, one must be aware that the operational definition (e.g., acid resistance) may not exactly match the chemical definition (lignin). Therefore, one has to be extremely careful about the interpretation of data generated by proximate analysis. This may not be a major concern if the general aspects of decomposition are to be modeled, where emphasis is on general classes of organic constituents of litter (labile versus resistant). For models considering the biochemical nature of the organic constituents and how they are altered by decomposition, the level of resolution offered by proximate analysis is probably inadequate. Therefore, CuO oxidation, NMR, or other methods will be required.

NIR Analysis of Organic Fractions

NIR spectroscopy is a rapid, nondestructive method for determining the major organic constituents of fresh and decomposing plant litter. It may also be used to determine nitrogen concentrations. In the most recent instruments, reflectance is determined from 400 to 2500 nm at intervals of 2 nm. Although this range includes some of the visible spectrum (400–700 nm), it has provided some useful information in terms of correlations (Gillon et al. 1993).

Prior to analysis, samples need to be dried and ground to pass a 20- or 40-mesh sieve. The determination of NIR spectral properties takes 2–3 minutes per sample. Before the proportion of organic constituents can be determined by NIR spectroscopy, a calibration to wet chemistry methods (e.g., proximate analysis) must be made. This is the most time-consuming step in the NIR method. To be most useful, the calibration samples must be representative of the overall population of interest, these samples must be accurately analyzed in terms of wet chemistry, and the correct mathematical processing of the spectral data must be determined.

Materials

1. NIR spectrometer with spinning sample module
2. Personal computer
3. Software for spectral analysis and calibration
4. 10–20 sample cups with quartz glass windows
5. Sample cup covers (paperboard)
6. Black iodized aluminum washers to reduce effective size of cell for small samples
7. Marking pens
8. Tweezers

Procedure

In most cases those interested in the NIR method will have to consult laboratories that possess the required equipment and expertise. While ecological laboratories generally do not possess this equipment, NIR spectrometers have been used extensively in crop and food sciences. Therefore, the equipment may be available locally. The procedure can be divided into two steps: development of routine regression equations from calibration samples, and subsequent scanning of samples for routine analysis.

1. To scan samples with large quantities, take approximately 2 g of dried, powdered, and well-mixed sample and place it in the sample cup with quartz window face down. To ensure the quartz window is not scratched, fill the sample cup on a soft surface. Place a paperboard cover on top of the filled sample cup and push firmly into place. Mark the sample number on the paperboard and turn the sample cup over and inspect to see that the sample material fills the entire window and the paperboard is not visible. Although it is possible to fill

a single sample cup, determine its reflectance, empty the cup, and then repeat the process sample by sample, it is most efficient to fill 10–20 cups, then determine their reflectance.

2. In the case of samples that are less than 2 g in weight, one can use a simple system to reduce effective size of the quartz window. This involves placing an aluminum washer that has a black finish similar in color to the walls in the sample cups. This "micro-cup" is then filled with the smaller sample and the procedure continues as usual.

   Follow the instructions that come with the spectrometer and software to determine the NIR reflectance spectra. The usual procedure consists of opening the door of the spinning sample module, placing the sample cup in two clamps, and closing the door to analyze the sample. A ceramic surface, which acts as a standard, is presented to the detectors when the door is opened. When the door is closed, the sample is presented to the detector. Once the sample is analyzed, open the door, take the sample out, and remove the paperboard cover with the tweezers to empty the sample cup. Because this is a nondestructive method, it is recommended that the sample be saved for reanalysis. One should be able to scan 80–100 samples per day.

3. Samples to be used for developing the calibration equations can be analyzed using the proximate analysis described earlier or for nitrogen using the method described in Chapter 7, this volume. A minimum of 50 samples are recommended to develop correlations between NIR reflectance and the wet chemical analysis. If the entire population of samples is scanned before the wet chemistry is performed, the newer analysis software that is available can be used to select representative samples (Infrasoft International 1993).

Calculations

To report actual spectral data reflectance (R) is usually converted to absorbance (A) using the following equation:

$$A = \log(1/R)$$

Because NIR spectrometers usually come with analysis software (e.g., Infrasoft International 1993), we will not describe all the combinations of mathematical procedures or treatments that can be used to create calibration equations. In most cases those wishing to develop their own prediction equations will initially need to seek outside expertise or become trained in the procedures. Here we will review the basic options involved in creating prediction equations so that novices have some foundation on which to base their initial decisions.

The overall approach is to try a number of mathematical procedures to determine which has the best fit to the wet chemistry calibration data. The first consideration is whether to use the original spectral data or to use the first or second derivative. Using the derivatives of the spectra is usually preferred because it eliminates effects caused by particle arrangement and moisture (Wessman et al. 1988a). In determining the derivatives, one must decide the segment length, expressed as the number

of data points, over which the derivative is to be determined. The second consideration is choosing the algorithm to be used to determine the calibration equation: either stepwise regression (SR) or partial least squares (PLS). In SR, the wavelengths most highly correlated to the chemical constituent are added to the calibration equation, and this process continues until the addition of a wavelength does not increase the variation explained by the equation. The PLS algorithm is a combination of principal components analysis and multiple linear regression. This is advantageous because all the spectral data are included in the principal components analysis, whereas in the SR algorithm only a few wavelengths may be used (Bolster et al. 1996). When presenting the calibration equations, it is important to specify the derivative used, its segment length, and the calibration algorithm used.

Special Considerations

The quality of the calibration data is a major limitation in using NIR reflectance spectra to determine organic constituents of fresh plant and decomposing litter. The interpretation of results from NIR analysis has all the limitations of the original wet chemical methods (e.g., acid-resistant material may not be entirely lignin). The NIR spectrometer is extremely delicate and should be kept in a vibration- and dust-free environment. We therefore recommend that sample cups be filled and emptied under a hood or in a room separate from the instrument.

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STANDARD SOIL METHODS

FOR

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