Fast, safe, and reliable methods for extraction of major inorganic cations from small quantities of woody plant tissues

RAKESH MINOCHA[1] AND WALTER C. SHORTLE

USDA Forest Service, Northeastern Forest Experiment Station, P.O. Box 640, Durham, NH 03824, U.S.A.

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Two simple and fast methods for the extraction of major inorganic cations (Ca, Mg, Mn, K) from small quantities of stemwood and needles of woody plants were developed. A 3.2- or 6.4-mm cobalt drill bit was used to shave samples from disks and increment cores of stemwood. For ion extraction, wood (ground or shavings) or needles were either homogenzied using a Tekmar Tissumizer or frozen and thawed (three times) in 0.01 M HCl. After filtration through a 0.45-μm nylon filter, the extract was analyzed for ion content using direct current plasma atomic emission spectrometry. Quality control samples of pine needles obtained from the National Institute of Standards and Technology, and individually pooled wood samples of red spruce (*Picea rubens* Sarg.) and red oak (*Quercus rubra* L.), were used to compare these two methods of extraction with the most commonly used method of wet ash digestion. The results of either method of extraction (freezing–thawing or homogenization) were higher than or similar to those obtained by wet digestion. Direct use of drill shavings eliminates the need for making wood chips by hand and grinding in a Wiley mill. Moreover, both approaches are relatively safe, since they do not require the use of hot concentrated acids and strong oxidizing agents. These methods may be particularly useful for the analysis of major inorganic cations from extremely small size samples (25 mg) such as individual annual growth rings of mature trees.

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Deux méthodes simples et rapides ont été mises au point pour extraire les principaux cations inorganiques (Ca, Mg, Mn, K) à partir de petites quantités de bois et d'aiguilles chez les plantes ligneuses. De la cisaille de bois a été produite à partir de disques et de carottes à l'aide d'un foret au cobalt de 3,2 ou 6,4 mm. Pour l'extraction des cations, la mouture ou la cisaille de bois et les aiguilles ont été soit homogénéisées à l'aide d'un «Tekmar Tissumizer» ou congelées et dégelées trois fois dans du HCl 0,01 M. Après filtration avec une filtre de nylon de 0,45 μm, le contenu en ions des extraits a été déterminé directement par spectrométrie d'émission atomique à plasma à courant direct. Des échantillons d'aiguilles de pin, pour le contrôle de qualité, obtenus du «National Institute of Standards and Technology» ainsi que des échantillons de bois d'épinette rouge (*Picea rubens* (Raf.) Sarg.) et de chêne rouge (*Quercus rubra* L.) regroupés individuellement ont été utilisés pour comparer les deux méthodes d'extraction avec la méthode de digestion humide qui est la plus communément utilisée. Les résultats de l'une ou l'autre des méthodes d'extraction (gel–dégel ou homogénéisation) étaient plus élevés ou semblables à ceux obtenus par digestion humide. L'utilisation directe de la cisaille de bois élimine la nécessité de produire des copeaux de bois à la main et de les broyer dans un broyeur Wiley. De plus, les deux approches sont relativement sécuritaires étant donné qu'elles ne nécessitent pas l'utilisation d'acides chauds et concentrés et de puissants agents d'oxydation. Ces méthodes peuvent être particulièrement utiles pour l'analyse des principaux cations inorganiques dans des échantillons de très petite dimension (25 mg) tels que les cernes annuels individuels chez les arbres matures.

[Traduit par la rédaction]

Introduction

Changes in the external environment have been shown to influence the physiological processes and composition of living trees. Pillay (1976) and Bondietti et al. (1989) suggested that year to year variations in the ion content of a tree ring or increment core are indicative of the composition of the nutrients taken up by the tree during that growth period, and as such may be used to develop environmental models. Patterns of ion mobilization have also been shown to be reliable markers of wood decay processes in living trees (Safford et al. 1974). Similar patterns of mobilization of one or more inorganic cations (Ca, K, Mg, and Mn) or changes in Al:Ca ratios may also indicate environmental stress and injury (Blanchard et al. 1978; Shortle and Smith 1988; Bondietti et al. 1989, 1990). Therefore, the changes in the ion content of wood may be used to evaluate the current growth potential as well as to predict vulnerability of trees to environmental stress, injury, and infection.

Most of the current methods for determination of the concentration of inorganic ions in wood require the formation of wood chips from stem disks followed by grinding in a Wiley mill. The most widely used methods of dry or wet ash digestion for analysis of woody as well as herbaceous plant tissues require a rather large sample size (100–1000 mg) and are also time-consuming, laborious, costly, and above all, hazardous because of the use of concentrated acids (Wolf 1982; Matusiewicz and Barnes 1985; Wikoff and Moraghan 1986; Kingston and Jassie 1986; Zarcinas et al. 1987; Goyal and Hafez 1990; Momoshima and Bondietti 1990).

Direct current plasma (DCP) and inductively coupled plasma atomic emission spectroscopy (AES) are very useful for multielement analysis from a variety of samples. However, their use is currently limited by the requirement of extensive pretreatment of samples including preparation, digestion, and concentration steps. Attempts have been made to minimize or eliminate the pretreatment steps with varying degrees of success (Kuennen et al. 1982).

[1]Author to whom all correspondence should be addressed.

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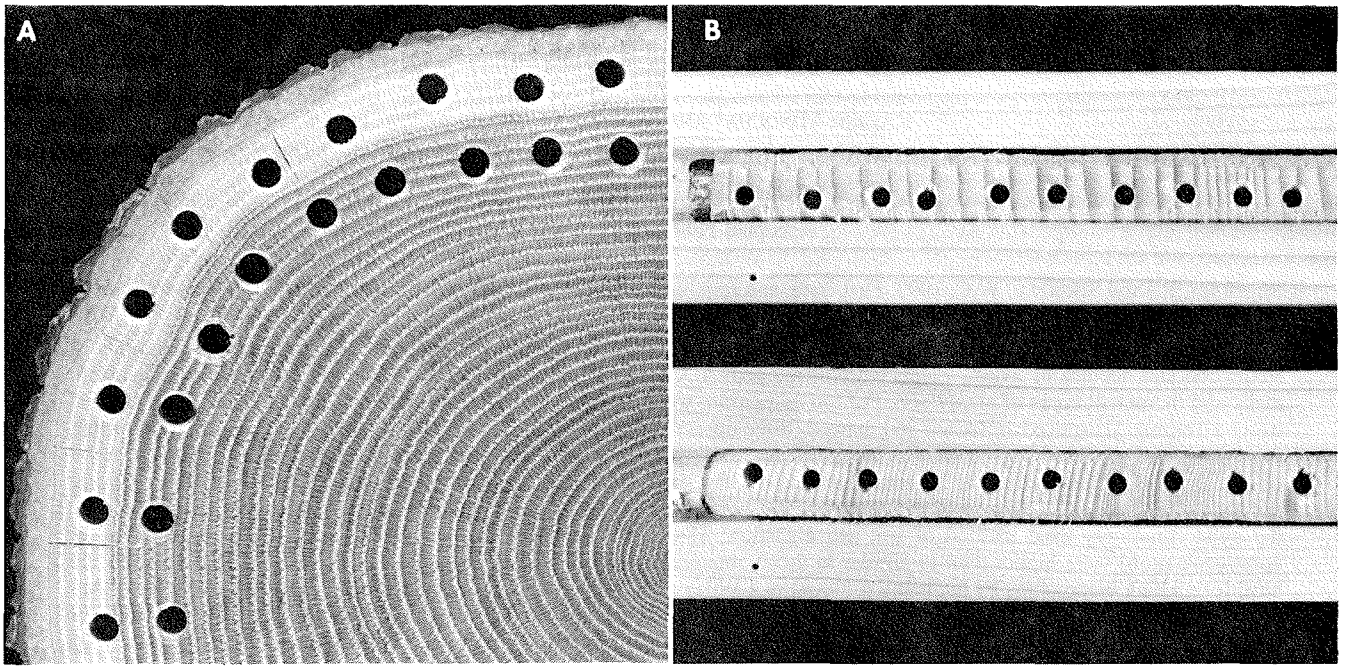

FIG. 1. (A) An oak wood disk showing the arrangement for taking sapwood and heartwood samples using a 6.4 mm diameter drill bit. (B) Increment cores of 12 mm diameter from spruce glued to grooves in wood blocks. A 3.2 mm diameter drill bit was used to drill holes within single annual rings of a fast-growing tree (top) or at a set distance regardless of number of rings per hole (bottom).

In this paper we report two simple, fast, and reliable methods for inorganic cation extraction from small samples of wood and conifer needles. Direct use of drill shavings eliminates the need for chipping and grinding. Extraction of ions with 0.01 M HCl replaces the hot concentrated acids and strong oxidizing agents used in wet ash digestion methods.

Materials and methods

Tissue samples

No standard reference material for nutrient content of wood is available from the National Institute of Standards and Technology, NIST (formerly the National Bureau of Standards, NBS) for use in methods development and quality control. Samples of pine needle tissue are available as a dry powder (NBS 1575). This reference material was used for comparison of our digestion and extraction methods for major inorganic cations. A comparison of wet ash digestion and freezing–thawing was also done on fresh needles from 1-year-old red spruce (*Picea rubens* Sarg.) seedlings grown in the greenhouse and on ovendried, 2- to 3-year-old needles of mature red spruce trees.

Sapwood of an air-dried disk (5 cm thick) taken from each of eight mature red spruce trees was chipped and ground in a Wiley mill to pass through a 420-μm sieve. The ground wood from these trees was thoroughly mixed and stored for use as an in-house reference material and to compare the new methods of extraction with a standard wet ash digestion procedure (Isaac and Johnson 1976).

As an alternative to grinding, wood shavings drilled from the transverse surface of air-dried disks (5 cm thick) of two different tree species from northern New England, red spruce and red oak (*Quercus rubra* L.), were used for comparing extraction methods to wet ash digestion. One tree of each species was felled in the winter, and 5-cm disks were cut from the stem 1–2 m above ground. The sapwood–heartwood boundary in spruce was marked with soft pencil while the disks were still fresh because the color distinction between these tissues disappears as the wood dries. In the case of oak, however, a visible sapwood–heartwood boundary remains even in air-dried wood. The disks were split in half and air dried. The radius was marked every 10° around the disks (Fig. 1A). The midpoint between cambium and the sapwood–heartwood boundary was chosen along each radius to take samples for sapwood. For heartwood samples, a point was selected along the radius that was at the same distance inward from the sapwood–heartwood boundary as the point for sapwood collection was outward from the boundary (Fig. 1A). The wood surface was cleaned by drilling a 2–3 mm deep hole with a 7.9-mm titanium twist bit in an electric drill. The shavings generated were dusted off the surface and discarded. A fine drill shavings sample was then taken with a 3.2-mm cobalt twist bit in an electric drill. A coarse drill shavings sample was also collected from the same hole with a 6.4-mm cobalt twist bit. Fine as well as coarse shavings from 17 different holes were separately collected in two clean Pyrex dishes and transferred to polyethylene vials for storage until the time of analysis. The sample yield per hole ranged from 80 to 120 mg for fine shavings and 290 to 420 mg for coarse shavings.

The drill method of sample collection was also tested on air-dried 12 mm diameter increment cores from mature red spruce trees. Holes were drilled either at 1-cm intervals or within individual annual rings beginning at 5 mm inside the cambium (Fig. 1B). The surface of the core was cleaned with a 3.2-mm bit to a depth of 1-2-mm, and the shavings were discarded. The shavings from individual holes were then collected on weighing paper using a 3.2-mm cobalt twist bit. The drill bit was cleaned with a brush between samples. The yield per hole ranged from 20 to 30 mg in dated tree-ring tissue. Based on this trial, 25-mg samples of ground or drilled wood were used to compare new extraction methods with the standard wet digestion method that used 100–200 mg tissue per sample. All samples, except fresh red spruce needles, were oven-dried at 80°C for 16–24 h and cooled in desiccators over silica gel for 1–2 h before weighing. Data sets consisted of five replicates of each tissue for each extraction method.

Wet digestion

The procedure of Isaac and Johnson (1976) as modified by Michaelson and Ping (1990) was used for wet ash digestion (WD). Briefly, 200 ± 0.5 mg of well-mixed sample was transferred to a 75-mL block digest tube followed by the addition of 7 mL of digestion mixture (97 g of selenous acid dissolved in 100 mL ultrapure

water and added slowly to a 4-kg bottle of concentrated sulfuric acid). Two Teflon boiling stones and 0.5 mL of 50% hydrogen peroxide were added to the digestion mixture in each tube. The tube was vortexed and placed in a preheated (400°C) block for 30 s and then removed. The step of adding 0.5 mL hydrogen peroxide and heating for 30 s was repeated until the solution became clear (about 4 mL). Before adding hydrogen peroxide, the tubes were removed from the heating block to allow them to cool for 1 min in order to avoid loss of volume due to effervescence. Once the solutions were clear the digestion was allowed to continue for another 55 min (total digestion time 60 min). After cooling the tubes to room temperature, ultrapure water was added to bring the volume to 75 mL and the solution was transferred to acid-cleaned storage bottles until ready for analysis. Two blanks were digested per batch of samples (G.J. Michaelson, University of Alaska Fairbanks, personal communications).

Extraction by homogenization

For homogenization (HO), 1 mL of 0.01 M HCl was added to approximately 25 mg of ovendried sample contained in a 12- or 15-mL glass test tube. The samples were homogenized at 24 000 rpm using a Tekmar Tissumizer for 90 s. The probe was rinsed with 1 mL of 0.01 M HCl into the test tube. Another 3 mL of 0.01 M HCl was added to this tube to bring the total volume to 5 mL. The extract was filtered using a 45-μm nylon syringe filter, and the filtrate was stored in acid-washed bottles at room temprature until the time of analysis.

For whole dry or fresh needles, 50 mg material in 10 mL of acid was used to produce a representative sample of needles and still keep the sample acid ratio the same.

Extraction by freezing–thawing

For freezing–thawing (FT), the samples were frozen at –20°C and thawed at room temperature, repeating the process two more times. The duration of the freezing step varied from 4 h to a few days. Samples were allowed to thaw completely (not to exceed 4–5 h) before refreezing. The samples were filtered without any HO. Other details of sample weight and extraction volume were identical with the homogenization method.

Cation analysis

The concentrations of major inorganic cations, Ca, K, Mg, and Mn, were determined by DCP–AES using the method AES0029 1986, as described in the Environmental Protection Agency regulation 40 CFR part 136.

Statistical analysis

Cochran's test for homogeneity of variance was performed on data sets involving comparison of more than two treatments (Cochran 1941). In cases where the null hypothesis of homogeneity was accepted, analysis of variance was carried out using SAS version 6.7 (SAS Institute Inc. 1989). If the treatment differences were significant, Duncan's multiple range test was performed to separate the multiple treatment means (Duncan 1955). In cases where the null hypothesis of homogeneity was rejected, Student's *t*-test for unequal variances was performed comparing each treatment separately with the standard treatment. For data sets involving only two treatments, Satterthwaite's *t*-test for equal or unequal variances was performed using the same version of SAS. All tests were performed for $P \leq 0.01$.

Results

Wood

A sample of 25 mg of wood shavings extracted, in 5 mL of 0.01 M HCl, provided the desired precision (≤5% variation between replicates) for extraction of ions. Initially both the HO and FT methods were tested on the in-house reference material (ground red spruce sapwood). The results shown in Fig. 2 indicate no significant difference between the amounts of Ca, Mg, and Mn extracted by any of the three methods. However, the amount of K extracted by FT was significantly higher than the K extracted by WD and HO.

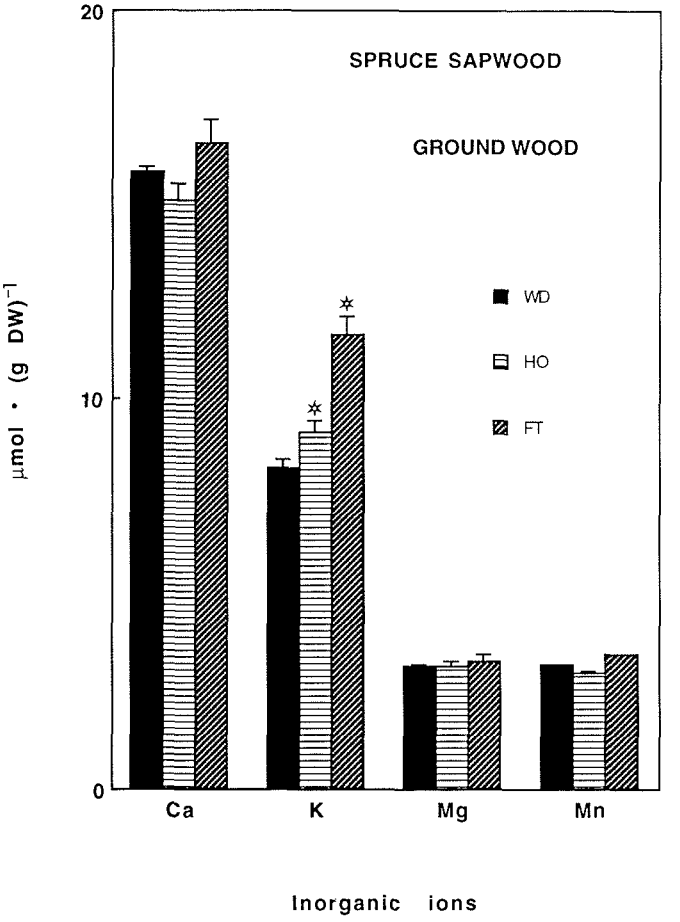

FIG. 2. Comparison of wet digestion (WD), homogenization (HO), and freezing–thawing (FT) for extraction of inorganic cations from ground spruce sapwood used as a reference. Data are mean + SE of five replicates. *, significant difference from wet digestion at $P \leq 0.01$. DW, dry weight.

To avoid the step of making wood chips by hand and grinding in a Wiley mill, wood was pooled by collecting shavings generated by either a 3.2- or 6.4-mm drill bit as described under Materials and methods. Both fine and coarse drill shavings were used to compare HO and FT with WD. The results presented in Figs. 3 and 4 for red spruce sapwood and heartwood show that the amounts of Ca, Mg, and Mn extracted by HO or FT were similar to the amounts obtained by WD except in the case of Mn, where FT yielded significantly higher amounts than WD or HO (Figs. 3B and 4B). The K yield, as in the case of ground spruce wood, was also significantly higher with HO and FT than with WD. Both WD and HO produced identical results regardless of the size of shavings.

In oak sapwood, there was complete extraction of Mg by all three methods but Mn was present in barely detectable quantities (Figs. 5A and 5B). In oak heartwood, both Mg and Mn were present in very low quantities (Figs. 6A and 6B). However, whereas K levels were higher for HO and FT than for WD for both oak sapwood and heartwood as in the case of red spruce, Ca extraction was consistent but not complete by either HO or FT (Figs. 5 and 6). Heating the tubes after extraction resulted in total extraction of Ca (Fig. 6B). Here

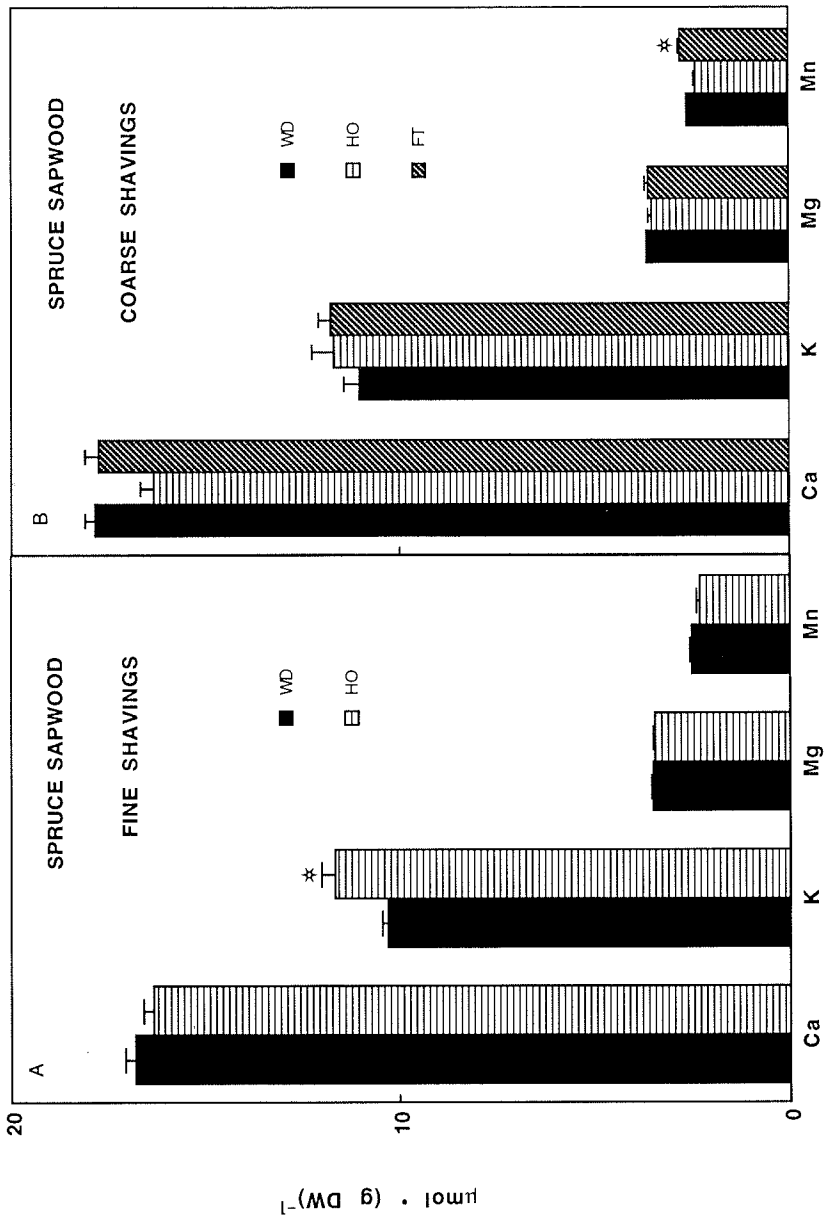

FIG. 3. Comparison of wet digestion (WD), homogenization (HO), and freezing–thawing (FT) for extraction of inorganic cations from red spruce sapwood fine shavings and coarse shavings. Only WD and HO were compared for fine shavings. Data are mean + SE of five replicates. *, significant difference from wet digestion at $P \leq 0.01$. DW, dry weight.

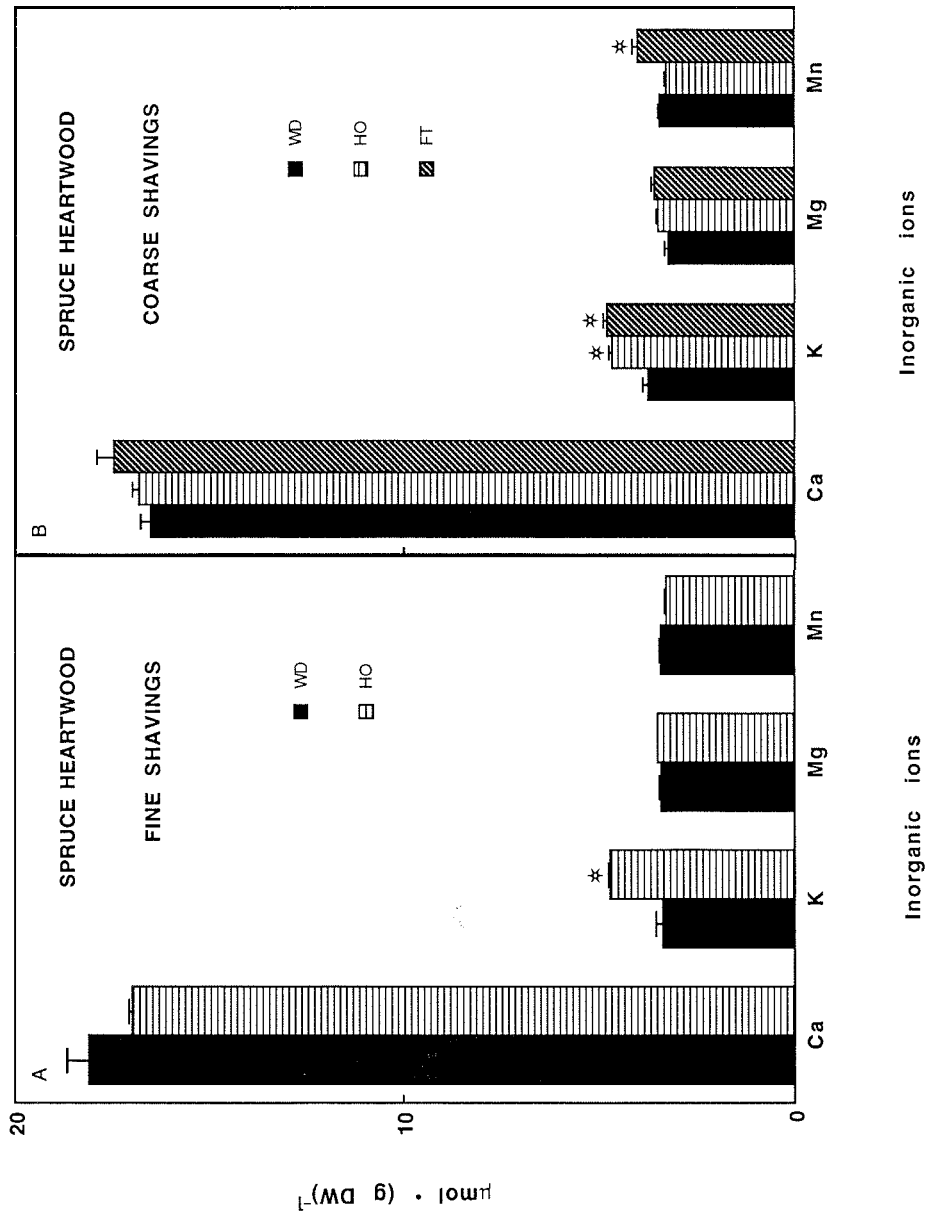

FIG. 4. Comparison of wet digestion (WD), homogenization (HO), and freezing–thawing (FT) for extraction of inorganic cations from red spruce heartwood fine shavings and coarse shavings. Only WD and HO were compared for fine shavings. Data are mean + SE of five replicates. *, significant difference from wet digestion at $P \leq 0.01$. DW, dry weight.

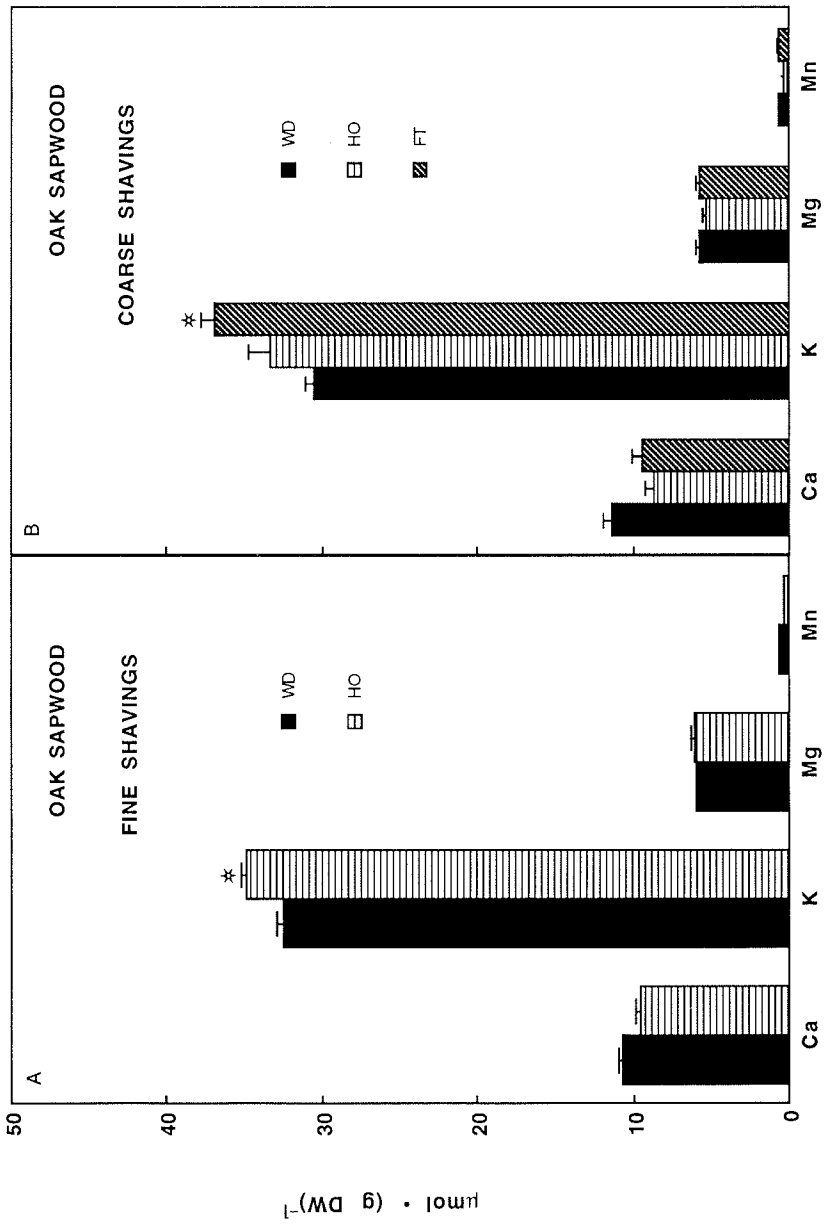

FIG. 5. Comparison of wet digestion (WD), homogenization (HO), and freezing–thawing (FT) for extraction of inorganic cations from oak sapwood fine shavings and coarse shavings. Only WD and HO were compared for fine shavings. Data are mean + SE of five replicates. *, significant difference from wet digestion at $P \leq 0.01$. DW, dry weight.

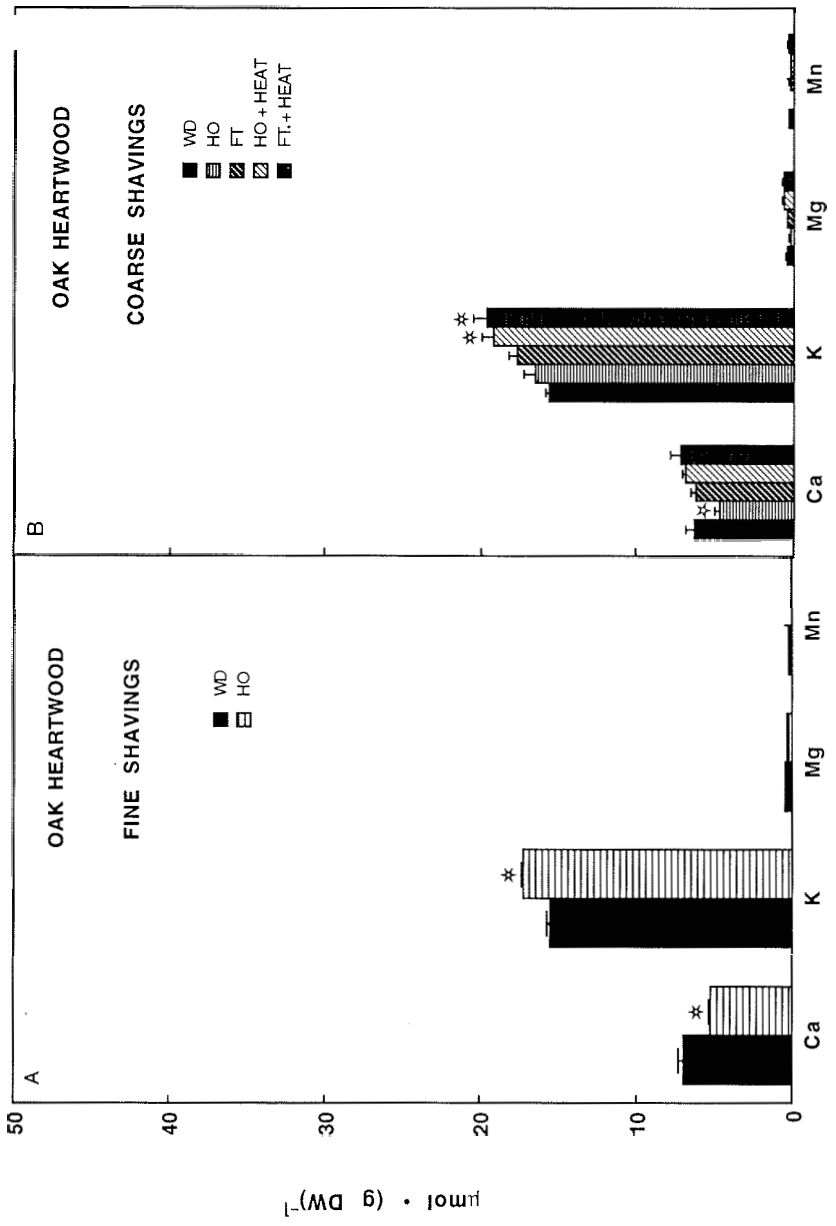

FIG. 6. Comparison of wet digestion (WD), homogenization (HO), HO + heat, freezing–thawing (FT), and FT + heat for extraction of inorganic cations from oak heartwood fine shavings and coarse shavings. Only WD and HO were compared for fine shavings. Data are mean + SE of five replicates. *, significant difference from wet digestion at $P \leq 0.01$. DW, dry weight.

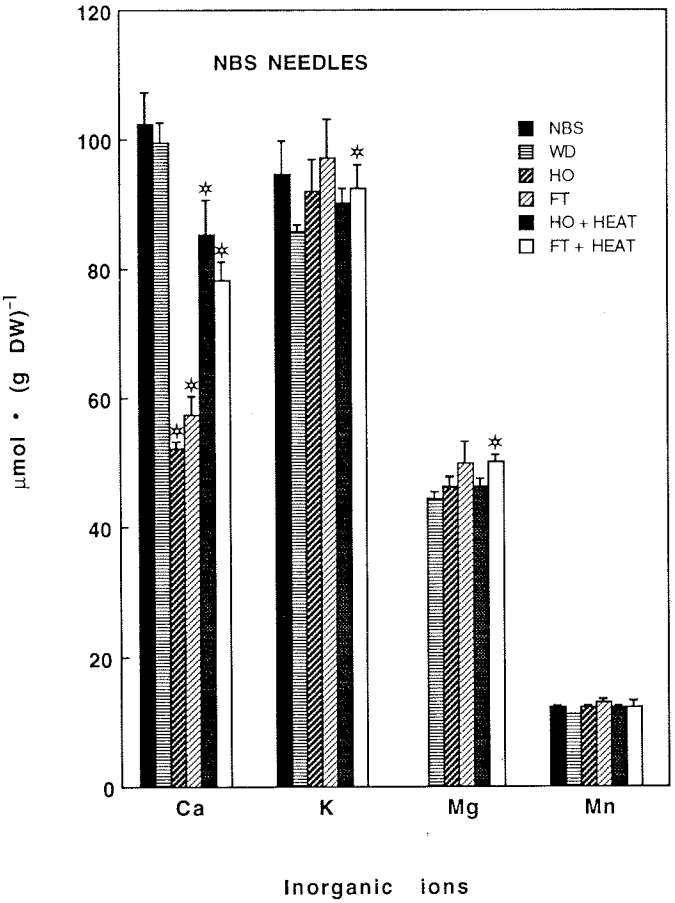

FIG. 7. Comparison of reported results with wet digestion (WD), homogenization (HO), HO + heat, freezing–thawing (FT), and FT + heat for extraction of inorganic cations from ground pine needles obtained from NIST. Data are mean + SD of five replicates. *, significant difference from wet digestion at $P \leq 0.01$. DW, dry weight.

again the size of drill bit used had no effect on the results obtained by either WD or HO.

Needles

A quality control sample of powdered dry needles of pine (NBS 1575) was used for comparison of these methods for needles. With the exception of Ca, the extraction of the other three ions was complete by all three methods. There was general agreement between the values given by NIST and the values obtained by our digestion and extraction methods for these three ions (Fig. 7). The yield of Ca could be improved from 50% to about 80% by heating the test tubes at 95°C for 1 h after FT or HO. However, when fresh needles from 1-year-old seedlings of red spruce were used, FT resulted in significantly higher extraction of all four inorganic ions, including Ca (Fig. 8A). FT was not suitable for extraction of these ions from 2- to 3-year-old dried needles of mature red spruce trees. The yields were consistently lower for each ion (Fig. 8B).

Discussion

The use of drill-bit shavings directly for digestion of samples or for other extraction procedures has many advantages besides eliminating the need for chipping and milling: (*i*) direct sampling of small quantities of specific tissue can be easily recorded by saving the drilled sample source (Fig. 1); (*ii*) in case more sample is needed from contiguous tissue, successively larger drill bits can be used to collect the additional sample needed; (*iii*) drill bits are easier to clean than Wiley mills; (*iv*) electric drills are relatively inexpensive compared with mills; (*v*) drilling is fast; and finally (*vi*) nonferrous drill bits are readily available, if iron determination is required.

Drilling within individual tree rings of a increment core yielded enough sample to be used with either one of these two extraction procedures. Thus it provides a faster approach to study the historical record of ions within a tree.

Acid digestion of the sample is generally the slowest step in the process of complete extraction and quantification of inorganic ions from woody plant tissues. Some of the disadvantages of the currently used digestion procedures are as follows: (*i*) difficulty in digesting a large number of samples; (*ii*) need for special equipment, e.g., digest block or pressure bomb; (*iii*) large sample dilutions limiting analysis of micronutrients; (*iv*) possible volatilization; (*v*) chemical explosion hazard; (*vi*) poor solution clarity; and (*vii*) coprecipitation (Anderson and Henderson 1986). In contrast, the main advantages of HO and FT are as follows: (*i*) a large number of samples can be extracted at one time; (*ii*) simplicity; (*iii*) no heat is required for most extractions and thus there is no worry about evaporation of samples to dryness; (*iv*) there is no need for a fume hood; (*v*) it is safe since extraction is done with a small amount of dilute nonoxidizing acid; (*vi*) there is no coprecipitation; and finally (*vii*) a relatively small amount of sample is needed (20–25 mg) for extraction. However, these new methods can only be used reliably for four major inorganic ions for the tissues tested so far. Higher levels of K were consistently obtained by HO and FT over WD. One possible explanation for this could be that the use of high temperature during extraction in the case of wet digestion and dry ashing causes volatilization of K present in the tissue (Jackson 1962).

The extraction of Ca from dry pine needles, though incomplete, can yield very useful information. The reproducibility of the replicates indicates that a consistent defined fraction of Ca such as a soluble or noncovalently bound fraction is being extracted by this method. Thus, if our goal is to study changes in a particular fraction of Ca (and not total Ca) in relation to a stress or change in physiological state of the tissue, HO and FT may be successfully applied. Recently, Fink (1991) showed that in Norway spruce (*Picea abies* (L.) Karst.) needles, the major fraction of Ca is bound extracellularly on the outside walls of mesophyll cells in the form of insoluble Ca oxalate crystals and could be extracted with 2 M HCl. Calcium pectate is bound in the middle lamella of cell walls and could be extracted with 2 N acetic acid. Therefore, it is likely that a pretreatment with a nonpolar solvent before applying HO or FT may extract Ca completely out of needles.

The difference in the effectiveness of FT for extraction of ions from fresh versus dry red spruce needles is hard to explain. One possible reason for this could be the age difference between the needles tested. It is known that most of the Ca in the young spruce needles is present as soluble fraction, and as the needles grow older the excess Ca is stored in the form of insoluble Ca oxalate crystals (Fink 1991). The use of powdered dry spruce needles along with heating may improve the recovery as was seen in the case of pine needles.

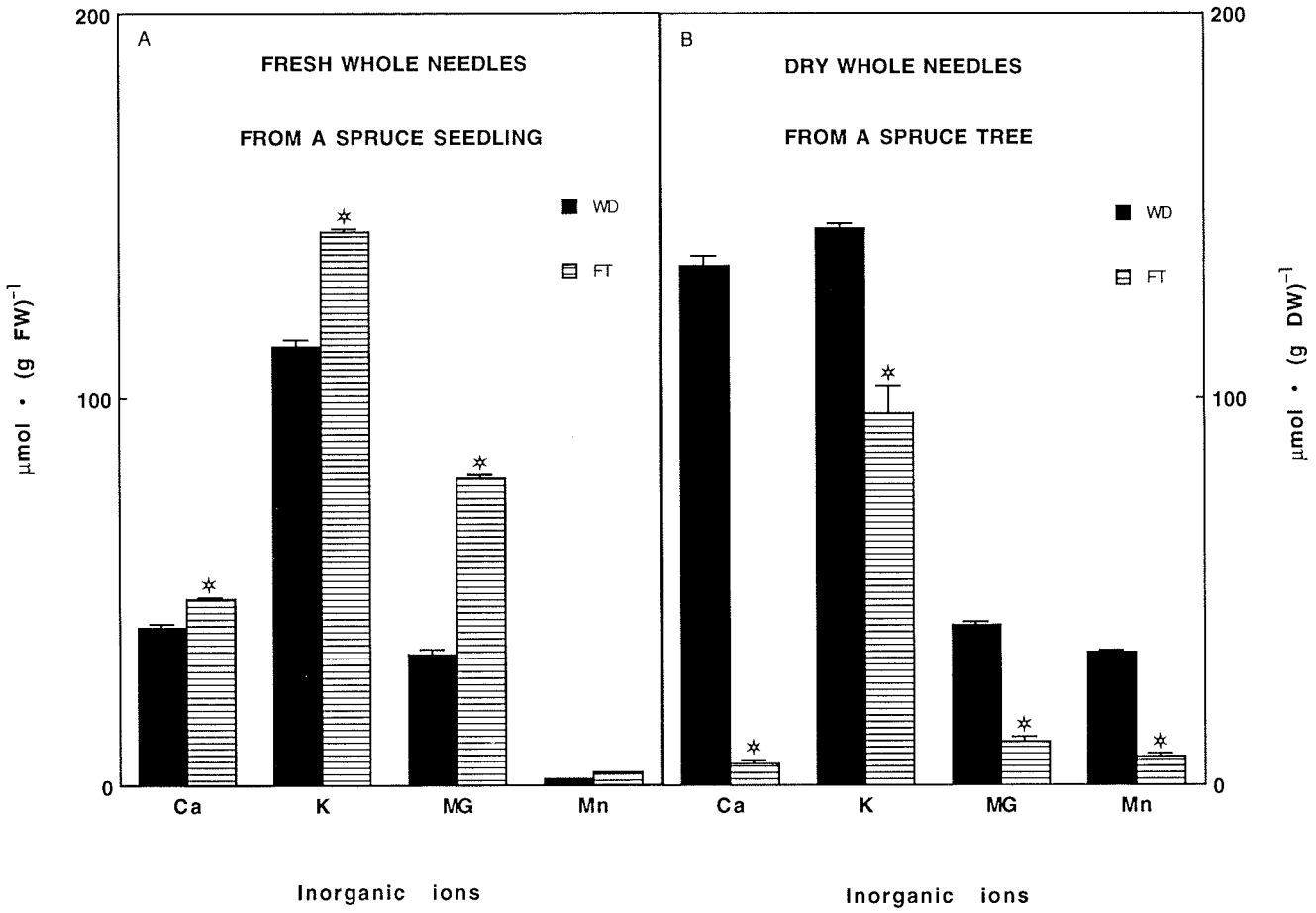

FIG. 8. Comparison of wet digestion (WD) and freezing–thawing (FT) for extraction of inorganic cations from fresh 1-year-old needles of red spruce seedlings and ovendried 2- to 3-year-old whole needles of mature red spruce trees. Data are mean + SE of five replicates. *, significant difference from wet digestion at $P \leq 0.01$. DW, dry weight.

Work is in progress to test the use of HO and FT for multielement analysis of herbaceous plants. Attempts are also being made to use real sample matrix calibration for multielement analysis by DCP-AES in order to verify that higher values of K are not caused by some organic material in the sample (Kuennen et al. 1982).

Conclusions

Both HO and FT produced identical results independent of the size of the drill bit used. There was excellent agreement between HO, FT, and the standard WD method for the analysis of Ca, Mg, and Mn for spruce and oak wood. Higher levels of K were consistently obtained by HO and FT over WD. For mature pine needles, neither HO nor FT extracted all of Ca. Heating of needles at 95°C for 1 h enhanced extraction by 50%. Whereas FT completely extracted all four ions from fresh spruce needles, it did not extract any of the ions from whole dry needles from mature red spruce trees.

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