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## Technical note

# Extraction and estimation of the quantity of calcium oxalate crystals in the foliage of conifer and hardwood trees

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The main goal of this study was to develop a method for the extraction and indirect estimation of the quantity of calcium oxalate (CaOx) in the foliage of trees. Foliar tissue was collected from a single tree of each species (five conifers and five hardwoods) for comparison of extractions in different solvents using 10 replicates per species from the same pool of tissue. For each species, calcium (Ca) and oxalate were extracted sequentially in double deionized water and 2N acetic acid, and finally, five replicate samples were extracted in 5% (0.83N) perchloric acid (PCA) and the other five in 2N hydrochloric acid (HCl); three cycles of freezing and thawing were used for each solvent. Total ions were extracted by microwave digestion. Calcium was quantified with an inductively coupled plasma emission spectrophotometer method and oxalate was eluted and quantified using a high performance liquid chromatography method. This experiment was repeated again with two conifer and two hardwood species using four trees per species, and two analytical replicates for each tree. We report here that, regardless of age of individual trees within a species, time of collection or species type, the third extraction in PCA or HCl resulted in near equimolar quantities of Ca and oxalate ( $r^2 \geq 0.99$ ). This method provides an easy estimate of the quantity of CaOx crystals using a small sample of foliar tissue. An additional benefit of PCA is that it precipitates the nucleic acids and proteins, allowing the quantification of several free/soluble metabolites such as amino acids, polyamines, organic acids and inorganic elements all from a single sample extract.

**Keywords:** aspen, beech, birch, conifers, fir, hardwoods, hemlock, HPLC, maple, oak, organic acids, pine, spruce.

## Introduction

Calcium (Ca) is often available in less-than-adequate quantities needed for growth in various forested ecosystems in the USA (Huntington 2005, Johnson et al. 2008, Warby et al. 2009). Calcium is a necessary macronutrient for all plants due to its involvement in critical cellular processes and the structural stability of the cell wall (Eklund and Eliasson 1990, Hepler 1994, White and Broadley 2003, Boudsocq and Sheen 2010). Because of the multiple functions of Ca in plants it is vital to forests, and its depletion in the soil can potentially threaten the long-term health of trees and overall forest productivity (Huntington et al. 2000). Low levels of soluble Ca decrease a

plant's ability to defend against abiotic stressors (Monroy et al. 1993, Trewavas and Knight 1994, Christie and Jenkins 1996, Knight et al. 1997, Gong et al. 1998).

Calcium oxalate (CaOx) crystals are known to provide mechanical support, mineral balance and waste sequestration (Cote 2009 and references therein). Unlike Ca, whose cellular contents depend upon bioavailability in the soil, oxalate is synthesized within plants. Since both Ca and oxalate are cytotoxic in excess amounts, the synthesis of oxalic acid, and the subsequent formation of CaOx, must be regulated within cells in order to maintain both Ca and oxalic acid levels within a species-specific homeostatic range (Franceschi and Nakata 2005).

Calcium oxalate crystal formation not only depends on Ca levels, but is also regulated by external pressures such as herbivory, which initiates its production as a defense mechanism (Molano-Flores 2001).

Calcium can also be found within cells in forms other than CaOx, such as calcium pectate and calcium acetate. Similarly, plants also accumulate other salts of oxalate besides CaOx (Libert and Franceschi 1987, Kinzel 1989). Under Ca-limiting conditions cells have specific mechanisms to release sequestered Ca. Volk et al. (2002) showed that under low Ca conditions, newly formed CaOx crystals partially dissolve releasing both Ca and oxalate in the leaves of water cabbage (*Pistia stratiotes* L.). There was a concomitant increase in oxalate oxidase, the enzyme responsible for catabolism of free oxalate to carbon dioxide and hydrogen peroxide, indicating that this process of crystal dissolution is enzymatic. Therefore under Ca deficit conditions, this pool of CaOx could serve as a reservoir for Ca. Understanding basic cellular and molecular strategies of how these crystals are dissolved may provide insight into their possible use as bioavailable sources of Ca and oxalate. However, to get to that point we first need to establish a reliable method for extraction and quantitation of CaOx crystals. Recently da Costa et al. (2009) reported a qualitative method for the extraction and determination of CaOx crystals from croton (*Codiaeum variegatum*) that required large quantities of leaf tissue. Our study was aimed at developing a method for extraction and indirect estimation of CaOx in tree leaves. In order to demonstrate the broad applicability of this method, we used both conifer and hardwood (total 10) species and tested multiple trees per species. Sequential extractions were done in three steps starting with double deionized water (ddH<sub>2</sub>O), followed by 2N acetic acid (CH<sub>3</sub>COOH), and for the final step, one half of the samples were extracted in 5% (0.83N) perchloric acid (PCA, HClO<sub>4</sub>) and the other half in 2N hydrochloric acid (HCl). The extraction in PCA is of special interest because it precipitates nucleic acids and proteins, allowing the quantification of several metabolites such as amino acids, polyamines, organic acids and soluble inorganic elements from the same extract.

## Materials and methods

### Site description

For the first experiment, with the exception of red spruce (*Picea rubens* Sarg.) all foliar samples were collected from trees growing on level terrain in the vicinity of the USDA Forest Service laboratory in Durham, NH, USA (N 43 08.535 W 70 56.950). Soils in this area are moderately deep to deep Inceptions, formed in coarse glacial till, but in the mesic temperature regime. Red spruce foliage was collected from Hedgehog Mountain, Albany, NH, USA (N 43 58.453 W 71 22.030). The Hedgehog Mountain site consists of moderately deep Spodosols formed in coarse glacial till in the frigid temperature regime.

For the second experiment, foliage was collected from two conifer and two hardwood species (four trees per species and two analytical replicates for each). Sugar maple (*Acer saccharum* Marshall) and black oak (*Quercus velutina* Lam.) were collected from East Foss Farm (N 43 07.135 W 70 56.209) and white pine (*Pinus strobus* L.) from the Woodman horticultural Farm (N 43 08.862 W 70 56.439) in Durham, NH, USA. Red spruce foliage was collected from a plantation established at the Kingman Farm in Madbury, NH (N 43 10.353 W 70 55.762). Soils at these sites are also moderately deep to deep Inceptions, formed in coarse glacial till, but in the mesic temperature regime.

Total foliar Ca concentrations of the trees sampled from these sites indicated that soil Ca varied among sites (foliar Ca has a strong relationship with soil Ca). In addition, intra-site variation in total Ca between four replicate trees of the same species were observed possibly because of microsite variability in soil chemistry.

### Foliar sampling

In the first experiment, visually healthy foliage was collected from a tree of each of 10 different species that varied in age and diameter at breast height (dbh) in the summer/early fall of 2010 using a pole pruner. In order to achieve homogeneity of samples for different methods of extraction of 10 replicates, foliage from several branches of a single tree of each species was pooled. The species tested were five conifers [red spruce, Norway spruce (*Picea abies* (L.) Karst.), white pine, eastern hemlock (*Tsuga canadensis* (L.) Carrière) and balsam fir (*Abies balsamea* (L.) Mill)] and five hardwoods [sugar maple, yellow birch (*Betula alleghaniensis* Britton), red oak (*Quercus rubra* L.), American beech (*Fagus grandifolia* Ehrh.) and trembling aspen (*Populus tremuloides* Michx.)]. For first experiment, only current year foliage was used for the conifers.

The second set of samples was collected in mid to late June of 2014 from four trees each of: red spruce, white pine, sugar maple and black oak. Two analytical samples were taken from each tree. For this experiment both current and past year foliage was analyzed for the conifer species. Trees sampled for this experiment also varied in age and dbh, but were within the same range of age and dbh as those collected in the first experiment.

### Sample processing

For hardwood species 10 g of punched leaf disks (6.4 mm in diameter), excluding main veins, were collected using a paper punch; for conifers, needles were cut to 1–2 mm in length with scissors. In the second experiment, 4 g of pooled tissue was collected for each species.

### Sequential extractions for Ca and oxalate salts

In order to evaluate the amount of Ca and oxalate extractable in solvents of increasing strength, 10 replicates per species were used for the first experiment. Fresh foliage tissue (300 mg)

was placed in 10 ml of ddH<sub>2</sub>O and subjected to three freeze–thaw cycles. Previous work from our laboratory has shown that repeated freezing and thawing of samples is a fast and reliable procedure for extracting soluble nutrients and metabolites (Minocha et al. 1994). Freeze–thawing not only eliminates the need for various tissue homogenizers but its simplicity allows for a large number of samples to be processed simultaneously. After the third thaw, samples were vortexed for 30 s at top speed (2700 rpm) on a Vortex Genie 2 mixer (Fisher Scientific, Pittsburg, PA, USA). The foliage was allowed to settle and the supernatant was carefully transferred to a clean scintillation vial using a pipette. The pellets were then washed with 40 ml ddH<sub>2</sub>O and dried overnight at 50 °C. The dried tissue was reconstituted with 10 ml of 2N acetic acid, and the freeze–thawing, washing and drying steps described above were repeated. Following this, the 10 replicates were split into two groups of five; one group was reconstituted in 10 ml of 5% PCA and the other in 10 ml of 2N HCl. Samples were again frozen and thawed three times and kept frozen at –20 °C until analyses.

#### Extraction of total foliar Ca

For total foliar Ca analysis, a sub-sample was taken from each pool of tissue and dried at 70 °C for 7 days. The samples were then ground using a shatterbox (SPEX SamplePrep, Metuchen, NJ, USA) for 1 min to produce a fine homogeneous powder. Approximately 0.2 g of each tissue sample was digested in 9 ml of concentrated nitric acid in Teflon vessels using a microwave (MARS Xpress, CEM Corporation, Matthews, NC, USA) following EPA method #3052 (Microwave assisted acid digestion of siliceous and organically based metrics, 1996).

#### Quantification of Ca ions

Total Ca extracts (digested in concentrated acid) were diluted to 50 ml volume with ddH<sub>2</sub>O before analyzing for total Ca. All previously frozen samples from sequential extractions of Ca were also diluted 2.5 times for the conifers and 5 times for the hardwoods with the appropriate matrix before Ca analysis using a simultaneous axial inductively coupled plasma emission spectrophotometer (ICP-AES, Vista CCD, Varian, Palo Alto, CA, USA) and Vista Pro software (Version 4.0). National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) standards for Eastern white pine needles (SRM 1575A) and apple leaves (SRM 1515) were used since no standards are available for fresh foliar tissues. We did use an in-house ground wood reference sample for quality control and assurance. For all samples, a standard curve was repeated after every 20 samples, and check standards were run after every recalibration and after every 10 samples.

#### Extraction of total foliar oxalate

Total oxalate was extracted according to Hönow and Hesse (2002) in 2N HCl.

#### Quantification of foliar oxalate

Oxalic acid content was determined using a high performance liquid chromatographic method (HPLC) optimized in our laboratory for this report based on published methods (Castellari et al. 2000, Bai et al. 2006, Pereira et al. 2010). Frozen samples were thawed, vortexed and filtered through a 0.45- $\mu$ m nylon syringe filter (Pall Corporation, Port Washington, NY, USA) before placing into 2 ml autosampler vials. The samples were quantified using a Series 200 Perkin-Elmer HPLC system using 20  $\mu$ l injections, and UV/VIS detector (Perkin-Elmer Corporation, Norwalk, CT, USA); absorbance was measured at 215 nm. A Phenomenex Synergi-Hydro-RP 4  $\mu$ m C18 column, 100  $\times$  4.6 mm id with a Security Guard, 5  $\mu$ m, 4  $\times$  3 mm id C18 guard column at 25 °C (Phenomenex, Torrance, CA, USA) was used with 25 mM potassium phosphate buffer containing 0.5 mM Tetra-Butyl ammonium Hydrogen Sulfate (THS) and 1% MeOH (pH 2.5) at a flow rate of 1.0 ml min<sup>-1</sup>; oxalate typically had a retention time of <1.5 min. Perkin-Elmer TotalChrom software (Version 6.2.1) was used to integrate data. A calibration curve based on the elution profiles of the oxalic acid standard was used to estimate sample concentration.

#### Statistical analysis

For each species, the effect of solvent on foliar Ca and oxalate concentrations was tested using a one-way ANOVA (Systat 10.2, Systat Inc., Chicago, IL, USA). Separate ANOVA were conducted for Ca and oxalate concentrations as dependent variables; solvent type was used as the treatment factor. Means from significant ANOVA were compared using Tukey's test ( $P \leq 0.05$ ).

## Results

#### Sequential extractions

For all species except aspen, the amount of Ca extracted from foliar tissue with ddH<sub>2</sub>O was lower than the amount extracted in the second step with acetic acid (see Figures S1a, b and S2 available as Supplementary Data at *Tree Physiology* Online). Comparison of the third extraction in PCA or HCl revealed that, for all species, PCA extracted either slightly higher or similar amounts of Ca than HCl (see Figure S1a and b available as Supplementary Data at *Tree Physiology* Online); both of these solvents extracted more Ca than the previous two steps with ddH<sub>2</sub>O and acetic acid.

In contrast to the extractions of Ca, the amount of oxalate extracted from foliar tissue in the first step with ddH<sub>2</sub>O was higher than the amount extracted in the second step with acetic acid for 8 of the 10 species (see Figures S1c, d and S2 available as Supplementary Data at *Tree Physiology* Online).

Both Ca and oxalate quantities extracted in water varied within a relatively small range for all species (see Figure S2 available as Supplementary Data at *Tree Physiology* Online). However, for extractions with acetic acid, there was more variation for Ca

compared with oxalate among the 10 species. Although the quantities of Ca and oxalate extracted by HCl or PCA were similar within a species, there was variation observed among different species (Figure 1, Figure S2 available as Supplementary Data at *Tree Physiology* Online). The combined sum of the amount of Ca or oxalate extracted from all three sequential steps also varied with tree species (see Figure S2 available as Supplementary Data at *Tree Physiology* Online).

In general, no correlation was observed between Ca and oxalate for the water or acetic acid extractable fractions (Figure 1a and b). Regardless of species, the third fraction extracted by either dilute PCA or HCl resulted in a nearly perfect correlation between the amounts of Ca and oxalate extracted (Figure 1c and d).

In the second part of the experiment where the sequential extractions were done with replicate trees of four selected species, the ratio of Ca to oxalate for the third extract in PCA were again close to one (Figure 2). The pooled data (mean of two analytical replicates for four trees of each species), as expected, followed a nearly 1 : 1 relationship for Ca and oxalate (Figure 3a). In addition the sum of Ca extracted by the three fractions was directly proportional to the amount of total foliar Ca (quantified

by hot acid digest) for each species (see Figure S3 available as Supplementary Data at *Tree Physiology* Online) and across species (Figure 3b). Sequential extractions in HCl were not repeated in the second experiment because: (i) in the first experiment HCl was shown to extract amounts similar to those extracted by PCA; and (ii) one of the objectives of this study was to use one extract for all analyses including elements and cellular metabolites of interest.

## Discussion

The method described here is a significant advancement over those published earlier to estimate the quantity of CaOx in plants in two ways: first, it uses a small quantity of foliar tissue, and second, the same sample following CaOx extraction can be used to analyze other small metabolites (amino acids, polyamines, inorganic ions, etc.), if desired. Ilarslan et al. (1997) indirectly calculated the quantity of CaOx crystals in developing soybean seeds by quantifying total and soluble oxalate and assuming that the difference between the two fractions was insoluble oxalate present in the form of CaOx. The authors confirmed this information by the use of X-ray diffraction and polarizing microscopy

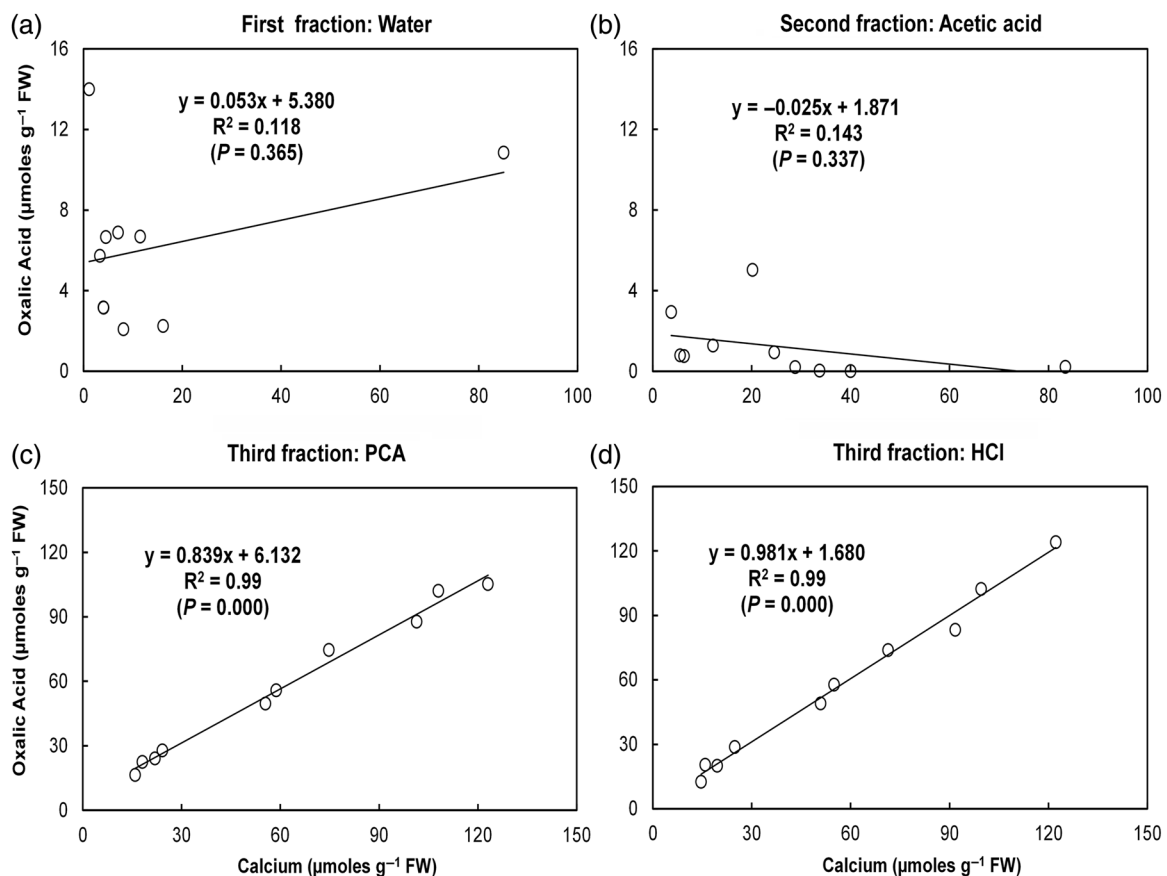


Figure 1. Relationship between the amount of calcium and oxalic acid extracted sequentially in: water (a), then acetic acid (b) and finally in PCA (c) or HCl (d) from pooled foliage from a single tree from each of five conifer and five hardwood species. Data are means of 10 analytical replicates per species for water and acetic acid extractions, and five for the third step of sequential extraction in PCA or HCl.

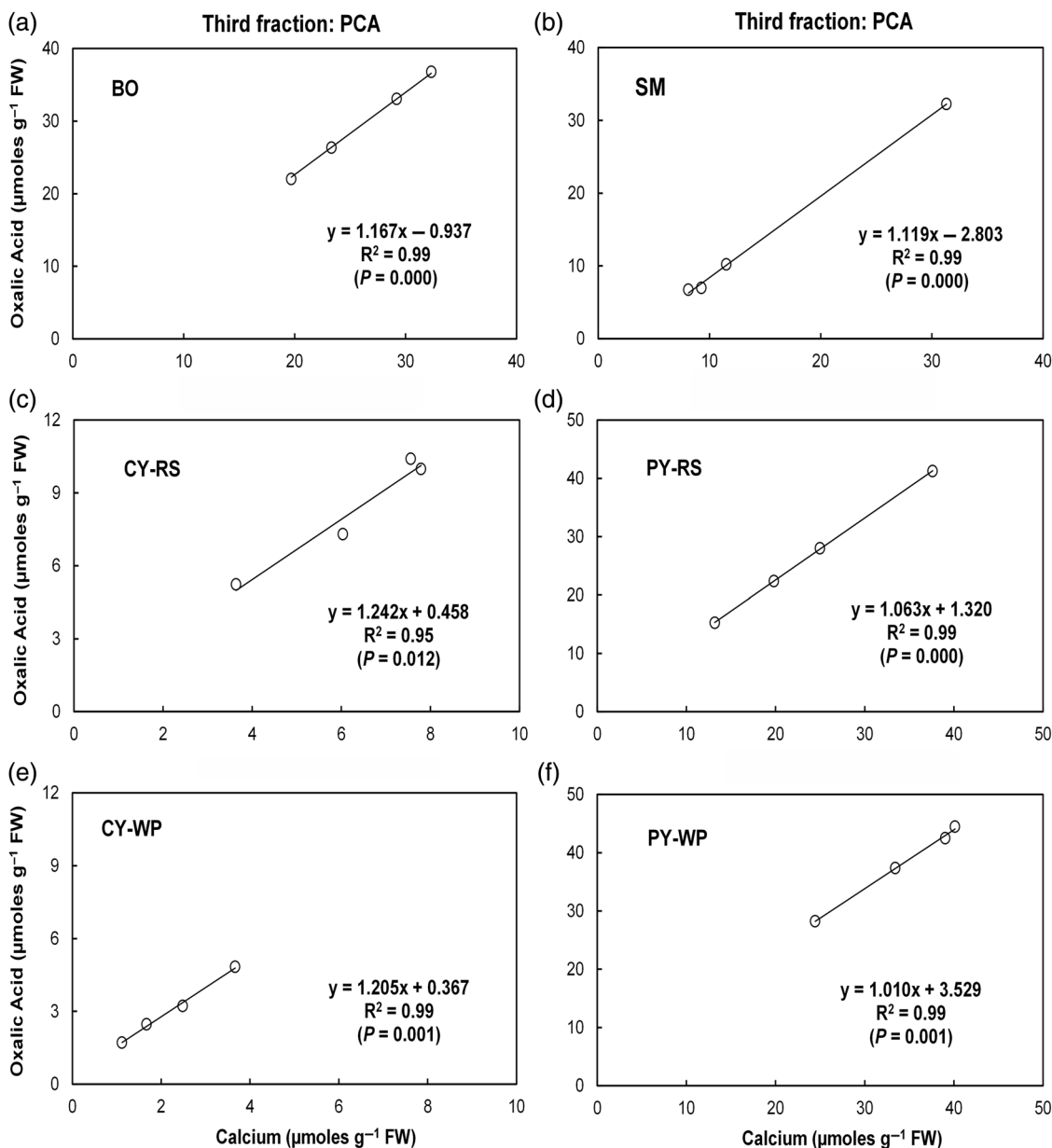


Figure 2. The relationship between foliar calcium and oxalic acid extracted sequentially in the third extraction with PCA (after extractions in water and then in acetic acid) in: black oak (a); sugar maple (b); current year (CY) and past year (PY, one-year-old) red spruce (c and d) and white pine (e and f). Data are means of four trees per species (two analytical replicates were averaged for each tree, eight separate samples were individually analyzed for each species).

techniques. da Costa et al. (2009) extracted and concentrated CaOx crystals from croton (*C. variegatum*). However, this method requires large quantities of leaf tissue and does not provide quantitative estimations.

Most forest ecological studies quantify total Ca concentration in the foliage for estimating Ca sufficiency levels for a species. However, total Ca may not be the most relevant measure to evaluate Ca sufficiency for physiological functions because a part of the total Ca is irreversibly committed to structural components of cells or tied up as CaOx and is not pertinent to a plant's present metabolic needs. Different species require different but

specific amounts of Ca for optimum growth. This could explain the observed variation among species for the amounts of Ca extracted by weak acid. Total foliar Ca (quantified by hot acid digest) was directly proportional to the sum of Ca extracted by the three sequential fractions. In the present study, this relationship was true for individual trees of the same species as well as across species (Figure 3b) in line with the earlier report from the findings of Borer et al. (2004).

The small amount of water-soluble oxalate possibly included sodium hydrogen oxalate, potassium hydrogen oxalate and some free oxalate (Hönow and Hesse 2002). This fraction



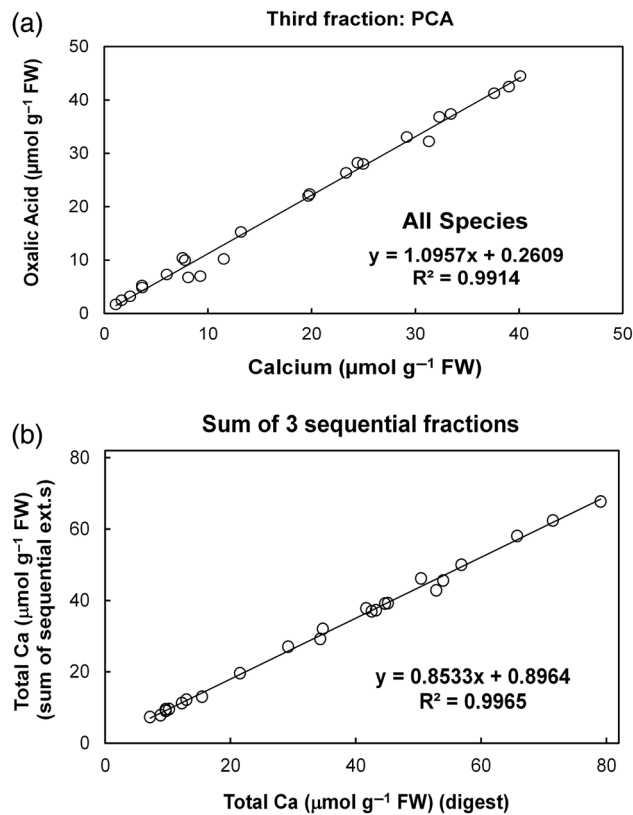


Figure 3. (a) The relationship between foliar calcium and oxalic acid of black oak, sugar maple, red spruce and white pine extracted sequentially in the third fraction with PCA (after extractions in water and then acetic acid). The data used here are combined from black oak, sugar maple, red spruce and white pine and are the mean of four trees per species (two analytical replicates were averaged for each tree, eight separate samples were analyzed for each species). (b) Relationship between the sum of Ca extracted by the three sequential fractions (water, acetic acid and PCA) and total foliar Ca concentration (digest). The data used here are combined from black oak, sugar maple, red spruce and white pine and are mean of four trees per species (two analytical replicates were averaged for each tree, eight separate samples were analyzed for each species).

should be easily available for physiological processes in the cell (Fink 1991, Borer et al. 2004). These authors reported that water-soluble Ca reaches a homeostatic level inside cells when Ca sufficiency status is attained by the plant. Any remaining Ca is then stored as other salts that are extractable with acetic acid, HCl or PCA. As CaOx is relatively insoluble in water ( $K_{sp} = 1.3 \times 10^{-9}$ ) (Kinzel 1989), the Ca and oxalate in the water-soluble fraction was most likely not present as CaOx crystals. Acetic acid mostly extracts Ca as Ca pectate (Fink 1991); CaOx crystals are insoluble in acetic acid, which is a weaker proton donor than oxalic acid. Solubility and Ca yields increased in the order of ddH<sub>2</sub>O < acetic acid < PCA, HCl (see Figure S1 available as Supplementary Data at *Tree Physiology* Online).

Regardless of individual trees of the same species or tree across species, near equimolar concentrations of Ca and oxalate ( $r^2 \geq 0.99$ ) were extracted in the third step of sequential extractions in 5% PCA or 2N HCl following water and 2N acetic

acid (Figures 1c, d and 2) These data provide indirect evidence that CaOx crystals are being extracted by both PCA and HCl. The amount of Ca and oxalate extracted was species-specific. The variability observed among trees within a species or different species with regard to the concentration of Ca, oxalate and CaOx crystals may be due to the phenology of growth, site chemistry and innate differences among species. Exploring and understanding the mechanisms that control the formation of CaOx and release of Ca from these crystals in plants is vital to discern the relationship between Ca bioavailability and forest health (White and Broadley 2003).

The fact that direct extraction in PCA yields all three fractions of soluble Ca (see Figures S2 and S4 available as Supplementary Data at *Tree Physiology* Online), along with other nutrients and soluble metabolites, supports integrated or streamlined sample collection and processing. Future research will help delineate which of the soluble fractions of Ca changes more rapidly in these species in response to an increase in total Ca during the growing season, thus providing valuable information for advancing our understanding of plant available Ca.

## Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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## Conflict of interest

None declared.

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