Rapid Quantification of Proanthocyanidins (Condensed Tannins) with a Continuous Flow Analyzer

James K. Nitao,[†,‡,§] Bruce A. Birr,[#] Muraleedharan G. Nair,[*,‡] Daniel A. Herms,[⊥,⊗] and William J. Mattson[#]

Department of Entomology, Michigan State University, East Lansing, Michigan 48824; Bioactive Natural Products Laboratory, Department of Horticulture, Michigan State University, East Lansing, Michigan 48824; North Central Experiment Station, Forestry Sciences Laboratory, 5985 Highway K, Rhinelander, Wisconsin 54501; and The Dow Gardens, 1018 West Main Street, Midland, Michigan 48640

Proanthocyanidins (condensed tannins) frequently need to be quantified in large numbers of samples in food, plant, and environmental studies. An automated colorimetric method to quantify proanthocyanidins with sulfuric acid (H_2SO_4) was therefore developed for use in a continuous flow analyzer. Assay conditions were optimized using 50% methanol extracts of paper birch, sugar maple, and quaking aspen leaves. Short extraction times and centrifugation of samples prevented proanthocyanidin degradation that otherwise occurred in 50% methanol extracts of aspen leaves. Extraction of birch and maple proanthocyanidins with 50% methanol was comparable to or better than that with 70% acetone. Proanthocyanidin levels in aspen were lower when extracted with aqueous methanol, but relative differences among samples were consistent with those found in aqueous acetone extracts. Results from the automated sulfuric acid assay were highly correlated with those of the conventional BuOH−HCl method for proanthocyanidins and, except for birch, with the Folin−Denis assay for total phenolics. This new technique significantly improves assay processing rate and repeatability compared to conventional colorimetric proanthocyanidin assays.

Keywords: *Proanthocyanidins; condensed tannins; colorimetric assay; Betula; Acer; Populus*

INTRODUCTION

Proanthocyanidins (condensed tannins) consisting of oligomers and polymers of flavan-3-ol units are the most widely distributed type of tannins in the plant kingdom (*1*). In addition to these widespread 3-hydroxy forms, proanthocyanidins comprising 3-deoxy subunits also exist but are very rare, having been identified only in sorghum and maize (*2*).

Humans consume significant quantities of proanthocyanidins in foods, such as fruits, vegetables, cereals, legumes, and grains, as well as in beverages, including tea, cocoa, and red wine (*3*). In addition to influencing the flavor and appearance of foods (*4*), dietary proanthocyanidins are hypothesized to be beneficial, possibly due to their antioxidant properties and their ability to complex with macromolecules and metal ions (*5*). Proanthocyanidins can also have deleterious effects. When tannin-rich plants are used in feeds, nutritional values of forage and growth rates of livestock and poultry are often reduced (*6*). In crop plants, proanthocyanidins have been implicated as resistance traits against pathogens and pests (*4*, *7*). In nature, these compounds can influence the behavior of plant-feeding insects and mammals (*1*, *8*). Many proanthocyanidins also possess antimicrobial activity (*9*, *10*). In addition to having a multitude of effects on biotic interactions, proanthocyanidins affect nutrient cycling in ecosystems (*11*). Proanthocyanidins leached from leaf litter can increase nitrogen availability for trees growing in infertile, acidic soils (*12*), and the microbial decomposition and nutrient dynamics of the litter itself can be affected by proanthocyanidin content (*13*, *14*).

Methods to quantify proanthocyanidins have been studied and compared extensively (*1*, *3*, *15*). The most commonly used colorimetric methods are the vanillin assay and BuOH−HCl assay (*16*, *17*). Each technique has its advantages and drawbacks, but results from the two assays tend to be correlated (*15*, *18*). The BuOH−HCl assay is more specific to proanthocyanidins, relying on the oxidative cleavage of proanthocyanidin molecules to produce anthocyanidin chromophores, whereas the vanillin assay can react positively with non-proanthocyanidin flavanols and fail to react with certain proanthocyanidin flavanols (*19*). Both techniques are affected by the presence of water, but the vanillin assay is especially sensitive to solvent composition, as well as temperature conditions and experimental variation (*1*, *19*−*21*).

A third, less commonly used colorimetric method employs sulfuric acid and is thought to work on the same principal as the BuOH−HCl assay. Sulfuric acid

* Author to whom correspondence should be addressed [telephone (517) 353-2915; fax (517) 432-2310; e-mail nairm@pilot.msu.edu].

† Department of Entomology, Michigan State University.

‡ Bioactive Natural Products Laboratory, Michigan State University.

§ Present address: USDA-ARS, Nematology Laboratory, Building 011A, Room 165B, BARC-West, Beltsville, MD 20705-2350.

North Central Experiment Station.

⊥ The Dow Gardens.

⊗ Present address: Department of Entomology, Ohio State University−OARDC, Wooster, OH 44691.

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was originally used for phenolics by Bate-Smith and Rašper (*22*) to quantify what they believed to be a leucoanthocyanidin (monomeric flavanol) in sorghum; methanolic sulfuric acid solutions were mixed either with extracts of sorghum grain or with the grain itself, and a chromophore with an absorbance maximum at 550 nm was produced. Subsequently, this assay was used to measure what were also presumed to be monomeric leucoanthocyanidins in corn pericarps and sorghum (*23*, *24*). Bae and co-workers (*21*) modified this procedure to quantify proanthocyanidins in a bacterial culture medium by using concentrated sulfuric acid; peak absorbance after reaction with proanthocyanidins was at 580 nm rather than at 550 nm. Chromophore development was rapid, highly stable, and unaffected by the presence of water, methanol, or culture medium. Schofield (*25*) applied this method to quantify proanthocyanidins in willow leaf samples and obtained results similar to those from the BuOH−HCl technique (*26*). The characteristic color reaction of sulfuric acid with proanthocyanidins has also been used for histochemical staining to identify proanthocyanidins vacuoles in plant tissues (*27*).

Despite the widespread need to routinely quantify proanthocyanidins in food, plant, and soil samples, no automated technique is available for this important class of natural products. Automated methods to measure total phenolics based on the Folin−Denis assay (*28*) and the reduction of Fe(III) (*29*) have been developed; however, redox assays such as these measure non-proanthocyanidin phenolic compounds together with proanthocyanidins and are subject to interference from non-proanthocyanidin reductants such as ascorbic acid (*1*, *19*). In standard proanthocyanidin assays, the sample processing rate is low, procedures are time-consuming, and chromophore development is subject to experimental variation in temperature and timing. Optimization of sample extraction and automation of proanthocyanidin analysis would expand sampling capability and improve repeatability.

In the process of investigating stress-induced physiological changes in plants, we developed an automated technique based on the sulfuric acid method to quantify proanthocyanidins in plant extracts. The apparatus required proanthocyanidin samples to be prepared in aqueous methanol; therefore, studies were first conducted to improve proanthocyanidin stability during sample extraction and handling in 50% methanol. Once these procedures were identified, extraction characteristics were compared between aqueous methanol and aqueous acetone, a solvent known to be the highly effective for proanthocyanidin extraction. Leaf samples from three tree species were then extracted and analyzed with the automated sulfuric acid method, the widely used BuOH−HCl assay for proanthocyanidins, and the Folin−Denis assay for total phenolics to determine the correlation between these different techniques.

MATERIALS AND METHODS

Proanthocyanidin Standards. Paper birch (*Betula papyrifera*) proanthocyanidin standard was provided by Tom Clausen (University of Alaska, Fairbanks, AK), and quaking aspen (*Populus tremuloides*) proanthocyanidin standard was supplied by Karl Kleiner (York College of Pennsylvania, York, PA). Crude quebracho proanthocyanidin was provided by Ann Hagerman (University of Miami, Oxford, OH) and purified using Sephadex LH-20 (*30*, *31*). Sugar maple (*Acer saccharum*) proanthocyanidins were isolated according to the procedures of Hagerman and Butler (*30)* and Bae et al. (*21*). Sugar maple leaves were collected fresh, then freeze-dried (see Plant Material), and extracted in 70% aqueous acetone (0.1% ascorbic acid). The acetone was evaporated in vacuo, and the aqueous portion was partitioned with ethyl acetate. The aqueous layer was concentrated, and ethanol was added to produce a concentration of 80%. Proanthocyanidins were adsorbed onto Sephadex LH-20, washed with 95% aqueous ethanol, and eluted with 50% aqueous acetone.

Plant Material. Fully expanded sugar maple and paper birch leaves were collected from trees grown in experimental field plots, 4 weeks after bud break (The Dow Gardens, Midland, MI). Birch and maple trees were 6 and 9 years old, respectively. Quaking aspen leaves were collected from 15-week-old rooted cuttings grown in an environmentally controlled greenhouse under various atmospheric ozone and CO_2 conditions (U.S. Forest Service North Central Experiment Station, Forestry Sciences Laboratory, Rhinelander, WI); both immature and mature leaves were collected. Fully expanded, mature quaking aspen leaves were also sampled from field-grown, reproductively mature trees at least 10 years old at the Michigan State University Tree Research Center (East Lansing, MI). Samples from individual maple, birch, and greenhouse aspen trees were kept separate during collection and storage, whereas leaves from field aspen trees were pooled. Leaves were frozen and transported in dry ice and stored at −40° C. Samples were freeze-dried in a tray freeze-drier at 5° C initially prechilled to −40° C. Dried leaves were milled and stored at −40° C until extraction.

Aqueous acetone, which is often used for extracting proanthocyanidins (*32*−*35*), caused unstable baseline absorbance measurements when used in the continuous flow analyzer. Therefore, 50% aqueous methanol, a recommended alternative to aqueous acetone (*1*), was chosen as the extraction solvent for use in the analyzer. Although ascorbic acid stabilizes proanthocyanidins in aqueous methanol (*36*), this antioxidant was not used for aqueous methanol extractions because it interferes with the Folin−Denis assay, which was conducted in parallel in the automated analyzer.

Leaf samples were extracted in 15 mL polypropylene screw-top centrifuge tubes. Between 0.070 and 0.080 g of leaf powder was weighed into each tube. Solvent was added (100:1 mL/g), and tubes were tightly capped. Unless stated otherwise, extractions were conducted at 25 °C on an orbital shaker with the tubes lying horizontally to maximize agitation. Extractions were conducted in the dark to minimize potential effects from light. Extracts were light green in color. Immediately after extraction, samples were centrifuged at 2500 rpm for 10 min to firmly pellet the crude leaf powder, and the supernatants were used for analysis.

Butanol—HCl Assay. The butanol−HCl method of Porter et al. (*17*) was used as the standard method to measure proanthocyanidins in experiments optimizing and comparing extraction techniques. Reaction mixtures consisted of 0.5 mL of proanthocyanidin standard or plant extract in either 50% methanol or 70% acetone (5.5 mM ascorbic acid), 3.0 mL of 5% concentrated HCl in 1-BuOH, and 0.1 mL of 2% $NH_4(SO_4)_2 \cdot 12H_2O$ in 2 N HCl. Plant extracts were diluted when necessary to ensure proanthocyanidin concentrations fell within the range of the standard curve. The mixtures were heated at 95 °C for 50 min in covered test tubes and then cooled with ice water. Absorbance at 550 nm was recorded (Shimadzu UV-260 spectrophotometer). Absorbances of unheated reaction mixtures from representative plant samples and of blank controls were compared to verify that plant pigments did not interfere with the assay.

Stability of Proanthocyanidins in Aqueous Methanol. Studies were conducted to optimize extraction and increase proanthocyanidin stability in aqueous methanol. In preliminary studies, proanthocyanidin measurements were observed to be highly variable during the extraction of aspen leaves from greenhouse trees; because of their sensitivity to extraction and handling conditions, greenhouse aspen leaves were chosen for optimization studies. Relative treatment differences in aspen proanthocyanidin concentrations were of interest and not

absolute concentrations. Therefore, to conserve purified aspen proanthocyanidin, quebracho proanthocyanidin was used to construct standard curves in stability experiments only.

The effect of extraction time on proanthocyanidin quantification was tested. Portions of leaf samples from different aspen trees were pooled and extracted in 50% methanol at 25 °C for 0.5, 1, and 2 h (three replications perctime period). In a second study, samples were extracted for 1.5, 3, 6, and 24 h (five replications per time period). Relative proanthocyanidin concentrations were determined with the BuOH−HCl assay relative to the quebracho proanthocyanidin standard curve.

Once extracted into aqueous methanol, proanthocyanidins appeared to be interacting with the aspen leaf powder, resulting in the binding or breakdown of the proanthocyanidins. To test this hypothesis, a subset of leaf samples was pooled and extracted in 50% methanol at 25 °C under four treatment regimes: (1) samples were extracted for 0.5 h; (2) samples were extracted for 24 h; (3) samples were extracted for 0.5 h and centrifuged, and only the supernatants were returned to the shaker for an additional 24 h; and (4) samples were first heated to 100 °C for 2 min in 50% methanol, rapidly cooled in ice water, and then extracted for 24 h. All samples (five replications perctreatment) were centrifuged after extraction and analyzed for proanthocyanidins with the BuOH−HCl assay relative to quebracho standard.

Because of potential interactions between proanthocyanidins and leaf powder, the efficacy of centrifuging was assessed as a means to reduce postextraction proanthocyanidin loss by minimizing surface area between extract and leaf residue during handling of samples. A subset of greenhouse aspen leaf samples was pooled and extracted for 0.5 h and immediately pelleted by centrifugation. Samples were then allowed to sit in centrifuge tubes at 25 °C without shaking for 3, 6, and 24 h and at 5 °C for 24 h. Results were compared to proanthocyanidin concentrations of samples analyzed immediately after extraction and centrifugation (0 h). Extracts were analyzed with the BuOH−HCl assay relative to quebracho proanthocyanidin standard (five replications per treatment).

Statistical analyses for these and subsequent experiments were conducted with SigmaStat software (SPSS, Chicago, IL).

Comparison of Aqueous Methanol and Acetone for Extraction. Efficacy of proanthocyanidin extraction was compared between 50% methanol and 70% acetone supplemented with ascorbic acid, a solvent frequently used to extract plant proanthocyanidins. Leaf powders from four to five trees each of paper birch, sugar maple, field-grown aspen, and greenhouse-grown aspen were pooled, keeping tree species and growing conditions separate. Each of the four pooled samples was weighed into two groups. One group was extracted in 50% methanol, and the second group was extracted in 70% acetone (5.5 mM ascorbic acid). After extraction for 0.5 h, the extracts were centrifuged, and proanthocyanidins in the supernatants were quantified with the BuOH−HCl assay using corresponding birch, maple, and aspen proanthocyanidin standard curves in 50% methanol and 70% acetone (5.5 mM ascorbic acid). Each solvent and tree combination had five replicates.

The relationship between proanthocyanidin estimates obtained through extraction by 50% methanol and 70% acetone was determined. Leaves were sampled from 16 greenhouse-grown aspen trees and milled, keeping samples from individual trees separate. Each initial sample was in turn divided and weighed into paired groups. One sample from each pair was extracted in 50% methanol, and the other was extracted in 70% acetone (5.5 mM ascorbic acid). Samples were extracted for 0.5 h, centrifuged, and assayed with the BuOH−HCl method. Proanthocyanidins were quantified using quaking aspen proanthocyanidin standard curves in 50% methanol and 70% acetone (5.5 mM ascorbic acid).

Automated H_2SO_4 Assay. The procedures of Bate-Smith and Rašper (*22*) and Bae et al. (*21*) were modified for the continuous flow analyzer by using concentrated H_2SO_4 diluted to 43% with methanol (v/v) as a reagent and by heating reaction mixtures. Dilution of acid was required to prevent damage to the apparatus and must be conducted with appropriate care due to the vigorous boiling of methanol during mixing of the stock reagent. The short reaction time of 7 min within the apparatus in combination with the diluted acid necessitated that reaction mixtures be heated to accelerate the reaction; otherwise, no measurable chromophore was produced.

Leaf samples were extracted in 50% methanol (100:1 mL/g) for 0.5 h on a shaker (25 °C, dark) and centrifuged. Extracts were decanted through disposable filter columns (200−300 μm pore size; Fisher Scientific) into 3.5 mL polystyrene sample cups (Sarstedt, Inc., Newton, NC), which served as autosampler vials. An air-segmented, continuous flow analyzer (RFA-300 Rapid Flow Analyzer and 301 autosampler; Astoria-Pacific International, Clackamas, OR) was equipped with nonmetal connections, flexible acid-resistant tubing, and glass tubing.

The mixing ratio of sample and acid reagent flow rates were adjusted according to proanthocyanidin concentration ranges in extracts. For greenhouse aspen leaf samples (proanthocyanidin concentrations in extracts ∼5−160 μg/mL), the sample and acid reagent flow rates were set to 118 and 745 μL/min, respectively, to yield a mixing ratio of 1:6.3 (v/v sample/43% H_2SO_4 reagent). For birch, maple, and field aspen leaves (proanthocyanidin concentrations in extracts ∼60−1300 μg/mL), the sample flow was reduced to 74 μL/min, resulting in a 1:10.1 mixing ratio. The flow rate for the air injection line was 226 μL/mL. Fifty percent methanol was used as wash solution. Autosampler durations for uptake of sample and wash solutions were set to 75 and 25 s, respectively.

The analyzer was configured to mix sample and acid reagent for 1 min at room temperature using a 10-turn mixing coil. The mixture was then heated to 50 °C for 3.7 min with a heating "bath" (4 mL glass tubing coiled against a thermostatically controlled heating element). The reaction was allowed to continue for an additional 2.5 min through 15- and 25-turn mixing coils exposed to room temperature. Absorbance was measured at 580 nm. Standard curves were constructed for each sample set with proanthocyanidins from appropriate tree species in 50% methanol. Autosampler, standard curve construction (polynomial fit), quantification, and data capture were controlled through RFA-PC Softpac software. Mixing coils, heating bath, and software were obtained from Astoria-Pacific International.

The production of the 580 nm anthocyanidin chromophore (*21*) in the modified H_2SO_4 technique was verified by examining absorbance spectra (400−650 nm) of reaction solutions. To obtain sufficient quantities for analysis, volumes were scaled up in culture tubes under conditions simulating the continuous flow analyzer process. Proanthocyanidin standards were dissolved in 50% MeOH (250 μg/mL). Birch, maple, and aspen leaves were extracted for 0.5 h in 50% methanol and centrifuged. Proanthocyanidin standard solution or leaf extract (118 μL) was diluted with 356 μL of 50% methanol and mixed with 43% H_2SO_4 to achieve a ratio of 1:6.3 (v/v sample/H_2SO_4). Mixtures were gently shaken for 1 min at room temperature and then shaken for 3.7 min in a 50 °C water bath and finally shaken for 2.5 min at room temperature.

Comparison of Automated H_2SO_4, BuOH—HCl, and Folin—Denis Methods. The modified H_2SO_4 assay was compared with the BuOH−HCl method for proanthocyanidins and the Folin−Denis assay for total phenolics by analyzing identical leaf extracts with each method. Leaves from 25 trees each of birch, sugar maple, and greenhouse aspen were freeze-dried and milled, keeping samples from individual trees separate. Samples were extracted with 50% methanol (100:1 mL/g) for 0.5 h (25 °C, dark) on a shaker. Extracts were centrifuged, and the supernatants were analyzed for proanthocyanidin and total phenolics. Proanthocyanidin standards from each of the appropriate tree species were used for standard curves.

A second channel of the continuous flow analyzer processed each sample with the Folin−Denis assay (*37*) in parallel with the H_2SO_4 assay. The analyzer was configured to dilute samples with 1.5% sodium lauryl sulfate surfactant in water to 1:81 of the original sample concentration by mixing the sample stream (118 μL/mL) sequentially with two diluent streams (947 μL/min each). The diluted sample was mixed with

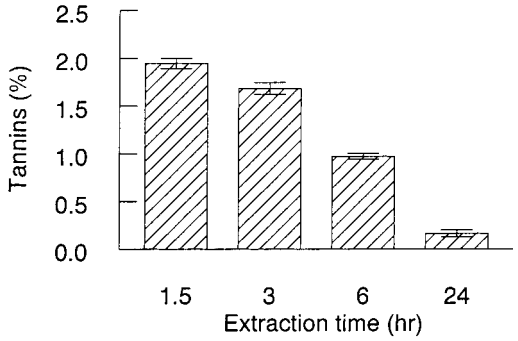

Figure 1. Effect of extraction time on tannin yield from leaves of quaking aspen. Tannins are expressed as percent of leaf dry weight. Values are mean ± SE. Time effect was significant (analysis of variance, $p < 0.001$), and all means were significantly different from each other (Tukey test, $p < 0.05$).

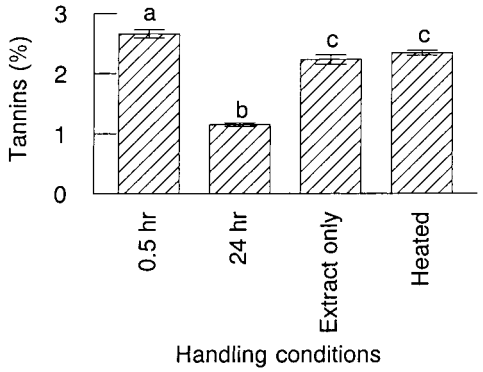

Figure 2. Effect of extraction and handling conditions on tannin yield from leaves of quaking aspen: "0.5 hr", extracted for 0.5 h; "24 h", extracted for 24 h; "extract only", extract incubated for 24 h without leaf residue after 0.5 h of extraction; "heated", sample first heated for 2 min at 100° C and extracted for 24 h. Tannins are expressed as percent of leaf dry weight. Values are mean ± SE. Means with different letters are significantly different from each other (Tukey–Kramer test, $p < 0.05$).

Folin–Denis reagent (74 μL/min) and saturated sodium carbonate solutions (74 μL/min). Reagents were prepared according to the method of Rosenblatt and Peluso (*36*). Air line flow rate was 166 μL/min. Absorbance was recorded at 750 nm.

RESULTS AND DISCUSSION

Stability of Proanthocyanidins in Aqueous Methanol. Increasing the extraction time of greenhouse aspen leaves reduced proanthocyanidin yields when 50% methanol was used. Compared to the 0.5 h extraction, the proanthocyanidin yield was slightly lower for extractions carried out for 1 and 2 h. Proanthocyanidin concentrations expressed as percentages of leaf dry weight after 0.5, 1, and 2 h extraction durations were calculated to be 2.5 ± 0.14, 2.3 ± 0.04, and 2.3 ± 0.09%, respectively (mean ± SE) (significant time effect, ANOVA, $p < 0.05$). A second experiment in which leaves were extracted for longer time periods (1.5, 3, 6, and 24 h) showed more dramatic reductions in proanthocyanidin yield (Figure 1). The decline was especially severe when extraction duration was >3 h. On the basis of these results, we adopted 0.5 h as a standard extraction time because extractions beyond 0.5 h had no benefit and potentially reduced proanthocyanidin yield.

The disappearance of proanthocyanidins during longer extraction of greenhouse aspen leaves was caused in part by an interaction between the extract and the leaf powder. This was evident when proanthocyanidin yields were compared from leaves that were (1) extracted for 0.5 h, (2) extracted for 24 h, (3) extracted for 0.5 h followed by the extracts without the leaf residue placed being placed on a shaker for 24 h, and (4) heated briefly at 100 °C and extracted for 24 h. As with the previous experiment, proanthocyanidin yield was lower after 24 h of extraction relative to 0.5 h of extraction (Figure 2); 24 h of extraction resulted in 57% lower proanthocyanidin yield than the 0.5 h extraction. Proanthocyanidin loss was only 16% lower than the 0.5 h extraction if the extracts were incubated for 24 h without the leaf residue. Briefly, heating the leaf powder in aqueous methanol at 100 °C helped to stabilize the proanthocyanidins, as proanthocyanidin yield was only 12% lower than that resulting from the 0.5 h extraction. Differences in proanthocyanidin levels after 24 h between experiments (Figures 1 and 2) are probably due to variation introduced from the pooling of different leaf ages and trees for each experiment.

These results suggest that the time-dependent disappearance of proanthocyanidins in 50% methanol may be due to enzymatic oxidation or interactions with other heat-sensitive components in the leaves. Polyphenol oxidase has been suspected to be active in 50% methanol, but details of proanthocyanidin oxidation in this solvent are poorly understood (*35*). Once proanthocyanidins are extracted out of cellular vacuoles into solution, they may also begin to bind to proteins in the leaf residue, progressively removing them from the solvent.

Consistent with the results of the previous experiment, minimizing the surface area between the extract and the leaf residue by centrifugation reduced proanthocyanidin loss during postextraction handling. Proanthocyanidin concentration (percent leaf dry weight) was 2.2 ± 0.04% (mean ± SE) when extracts were analyzed immediately after 0.5 h of extraction (0 h of treatment). In comparison, proanthocyanidin yields in extracts stored with pelleted residues at 25 °C for 3, 6, and 24 h were 2.1 ± 0.05, 2.1 ± 0.04, and 1.8 ± 0.02%, respectively. Of these, only the treatment group stored for 24 h at 25 °C was significantly lower than the 0 h treatment ($p < 0.05$, Dunnett's test, pairwise comparison against 0 h treatment). Storing the extracts and pelleted residues in the cold further reduced proanthocyanidin loss. Proanthocyanidin concentration of extracts stored with centrifuged residues at 5 °C was 2.0 ± 0.02% (not significantly different from that of 0 h treatment, $p > 0.05$, Dunnett's test). Centrifuging samples immediately after extraction can be not only a preliminary step for removing leaf particles before analysis but also a means of preserving proanthocyanidins during handling of sensitive samples such as those of the greenhouse-raised aspen leaves in this study.

Unlike leaves from greenhouse aspen trees, leaves from paper birch, sugar maple, and quaking aspen grown in the field did not exhibit a pronounced sensitivity to extraction time. Tests comparing 0.5 and 24 h extractions of field-grown aspen, sugar maple, and paper birch leaves in 50% methanol detected no significant decline ($p > 0.05$, t tests, $n = 5$); proanthocyanidin values (mean ± SE) for 0.5 and 24 h extractions, respectively, were 6.4 ± 0.3 and 6.4 ± 0.3 for field-grown aspen, 10.0 ± 0.4 and 10.5 ± 0.7 for maple, and 10.7 ± 0.4 and 9.7 ± 0.5% for birch. It is possible that experimentally elevated atmospheric ozone and CO_2 conditions in the greenhouse affected aspen leaf proper-

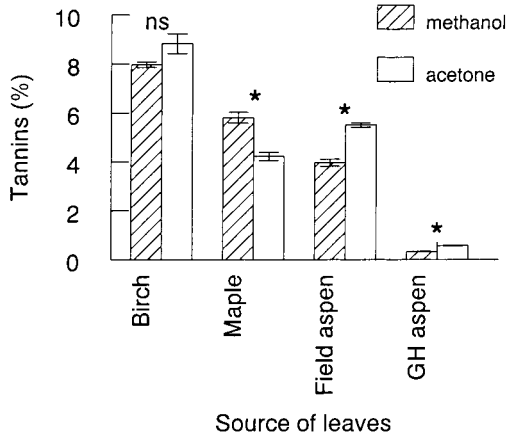

Figure 3. Effect of extraction solvent on tannin yield from leaves of paper birch, sugar maple, and quaking aspen grown in the field and quaking aspen grown in the greenhouse ("GH aspen"): "Methanol", 50% aqueous methanol; "acetone", 70% aqueous acetone. Tannins are expressed as percent of leaf dry weight. Values are mean ± SE. Asterisks indicate significant differences in tannin yield between solvents for a particular tree species or type (*t* test, $p < 0.05$; ns = no significant difference between solvents).

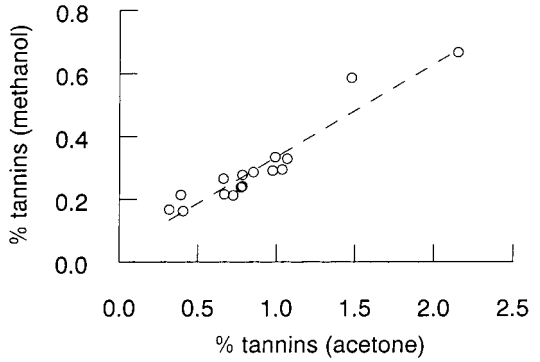

Figure 4. Relationship between tannin estimates of 16 quaking aspen leaf samples extracted with 70% acetone and 50% methanol. Tannins are expressed as percent of leaf dry weight.

ties and induced higher levels of polyphenol oxidase activity. Alternatively, proanthocyanidin losses during extraction may be proportionally higher in greenhouse aspen leaves because their proanthocyanidin concentrations are inherently low and changes could be more easily detected.

Comparison of Aqueous Methanol and Acetone for Extraction. Differences in extraction efficiencies between 50% methanol and 70% acetone varied with tree species (Figure 3). There was no significant difference in extraction efficiency between the two solvents when paper birch leaves were extracted (*t* test, $p > 0.05$). Extraction with aqueous methanol gave higher proanthocyanidin estimates than with 70% acetone for sugar maple leaves, whereas 70% acetone gave higher proanthocyanidin concentrations when extracting field- and greenhouse-grown quaking aspen leaves (*t* tests, $p < 0.05$). Species-dependent differences in solvent extraction efficiencies are commonly observed when plant leaves are examined, although usually 70% acetone is found to be superior over aqueous methanol (*31*−*34*).

Typically when extraction solvents are compared, the effect of extraction efficiency on the relative ranking of proanthocyanidin estimates among samples is not considered. To address this issue, a more detailed comparison between 50% methanol and 70% acetone was undertaken for greenhouse-grown aspen leaves. As was observed in the previous experiment, proanthocyanidin concentrations were higher overall when 70% acetone was used. However, proanthocyanidin measurements between 50% methanol and 70% acetone were linearly correlated when the 16 pairs of samples were analyzed (Figure 4). Thus, despite the difference in extraction efficiencies, both solvents resulted in similar rankings of proanthocyanidin levels for the samples ($R^2 = 0.91$, $p < 0.001$, $y = 0.296x + 0.039$).

Automated H_2SO_4 Assay. The use of diluted instead of concentrated H_2SO_4 and the short reaction time of the continuous flow analyzer necessitated heating reaction mixtures to accelerate chromophore development. Empirical tests determined that the combination of 43% H_2SO_4 and heating at 50 °C resulted in satisfactory chromophore production, whereas higher acid concentrations and temperatures caused charring of plant extracts. The low reaction temperature had the added benefit of preventing the formation of vapor bubbles, which would have disrupted sample flow and absorbance measurements. The standard BuOH−HCl assay requires heating to 95° C for chromophore development, and our attempts to adapt this method to the continuous flow analyzer were unsuccessful due to solvent boiling in the analyzer lines.

The anthocyanidin chromophore in the modified H_2SO_4 method was confirmed by examining the absorbance spectra of proanthocyanidin standards and leaf extracts reacted with 43% H_2SO_4 under conditions similar to those of the automated assay. Birch, maple, and aspen proanthocyanidin standards reacted with H_2SO_4 diluted in methanol and heated at 50 °C produced absorbance maxima in the 580−590 nm region (Figure 5A) consistent with the spectra observed by Bae and co-workers (*21*). Similar results were obtained when 50% methanol extracts of birch, maple, and aspen leaves were tested (Figure 5B). The 580 nm absorbance differs slightly from the 550 nm maximum recorded by Bate-Smith and Rašper (*22*), who were dealing with leucoanthocyanidins or possibly 3-deoxy proanthocyanidins (*38*) as opposed to 3-hydroxy proanthocyanidins. The potential contribution of conversion or addition of other phenolic products to this shift must also be considered. Leaf extracts in the modified H_2SO_4 reaction also exhibited strong absorbance at 450 nm, but this absorbance appeared not to interfere with the detection of the anthocyanidin chromophore.

Comparison of Automated H_2SO_4, BuOH—HCl, and Folin—Denis Methods. The automated H_2SO_4 and the conventional BuOH−HCl methods resulted in very similar relative measurements of proanthocyanidin concentrations. Proanthocyanidin estimates were highly correlated between techniques when extracts from birch, sugar maple, and aspen leaves were analyzed (Figure 6). Regression analyses for all species were significant and yielded large coefficients of determination (R^2), indicating that proanthocyanidin estimates from the two assay techniques were linearly related. For paper birch, an $R^2 = 0.85$ ($p < 0.0001$, $y = 0.562x + 2.951$, $n = 25$) was observed. Higher R^2 values, which indicate closer fits to straight-line relationships, were found when the proanthocyanidin assays were compared for sugar maple ($R^2 = 0.93$, $p < 0.0001$, $y = 1.061x + 1.215$, $n = 25$) and quaking aspen ($R^2 = 0.95$, $p < 0.0001$, $y = 0.681x + 0.035$, $n = 25$). Although the proanthocyanidin assays gave closely parallel results with respect to relative

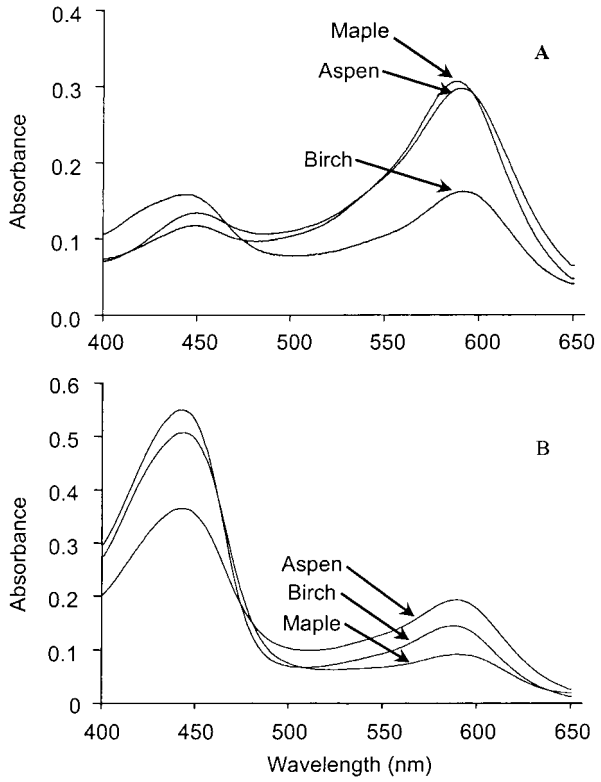

Figure 5. Absorbance spectra of (A) tannin standards and (B) 50% methanol extracts of paper birch, sugar maple, and quaking aspen leaves. Samples were reacted with 43% H_2SO_4 in methanol under conditions simulating the automated assay.

proanthocyanidin concentrations, estimates of absolute proanthocyanidin concentrations differed between techniques, as indicated by the regression slopes departing from one in the birch and aspen comparisons. The lack of correspondence in absolute proanthocyanidin concentrations is typical when proanthocyanidin assays are compared because the vanillin−HCl, BuOH−HCl, and concentrated H_2SO_4 methods are differentially sensitive to structural variation in proanthocyanidin molecules (*15*, *21*).

Differences among tree species were striking when the automated H_2SO_4 and the Folin−Denis assays were compared (Figure 7). The H_2SO_4 and Folin−Denis assays were linearly related for sugar maple and quaking aspen ($p < 0.001$, $n = 25$). The association between these assay results was more diffuse than that observed between the H_2SO_4 and BuOH−HCl assays, as reflected by the lower R^2 values; for sugar maple, the R^2 was 0.64 ($y = 1.016x + 14.713$), and for quaking aspen, R^2 was 0.78 ($y = 1.482x + 2.581$). No relationship could be detected between the two assays when paper birch was examined ($p = 0.43$, $n = 25$). Because the Folin−Denis assay is not specific for proanthocyanidins (*19*), the lower correlation between it and the H_2SO_4 assay compared to between the two proanthocyanidin analyses is not unexpected. The extent to which the results from the total phenolics and H_2SO_4 methods in birch are unrelated is particularly dramatic and underscores the fact that redox assays such as the Folin−Denis technique do not always produce results similar to those of proanthocyanidin assays. In addition, phenolic compounds other than proanthocyanidins are known to occur in birch species, such as hydrolyzable tannins and flavonoids (*39*, *40*), and may have affected the Folin−Denis more than the proanthocyanidin assay.

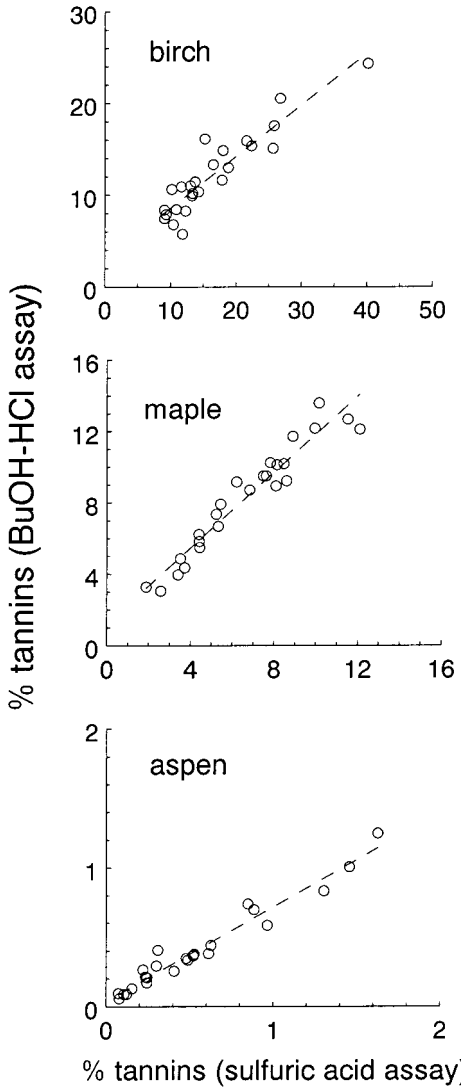

Figure 6. Relationship between condensed tannin measurements obtained from the automated H_2SO_4 assay and the BuOH−HCl assay on 25 leaf samples each from paper birch, sugar maple, and quaking aspen trees. Tannins are expressed as percent of leaf dry weight.

Conclusions. The modified H_2SO_4 assay is the first automated colorimetric method for quantification of 3-hydroxy proanthocyanidins (condensed tannins). The assay benefits from the robustness and rapidity of the H_2SO_4 reaction introduced by Bate-Smith and Rašper (*22*) and refined by Bae et al. (*21*). Total reaction time per sample in the automated assay is slightly more than 7 min, as opposed to 15 and 50 min for the conventional vanillin−HCl and BuOH−HCl methods, respectively. The actual sample processing rate is ~30 samples/h. In addition to the usual benefits of improved repeatability and sample processing rate, automation alleviates a significant inconvenience of the original H_2SO_4 technique, which is the repeated handling of concentrated H_2SO_4 when large numbers of samples are analyzed manually.

Our studies demonstrated that the H_2SO_4 reaction yields proanthocyanidin measurements that are highly correlated with the standard BuOH−HCl assay. Further testing with other types of proanthocyanidin samples will be necessary, but these results suggest that the automated H_2SO_4 assay can be used to measure proanthocyanidin concentrations whenever the BuOH−HCl

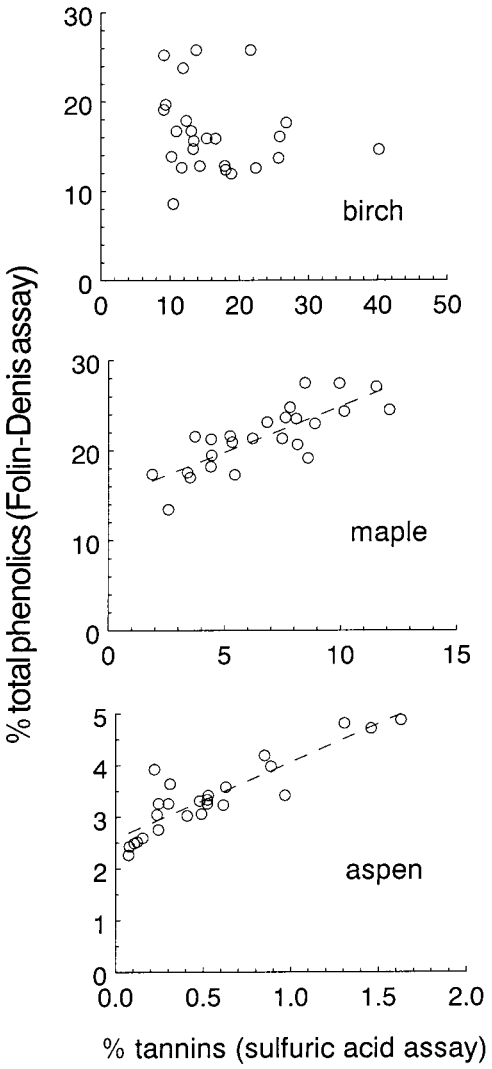

Figure 7. Relationship between condensed tannin measurements obtained from the automated H_2SO_4 assay and total phenolics measurements obtained from the Folin−Denis assay on 25 leaf samples each from paper birch, sugar maple, and quaking aspen trees. Tannins and total phenolics are expressed as percent of leaf dry weight.

assay would be considered. The linear relationship between the proanthocyanidin estimates from the two assays validates the new technique and also indicates that results from the H_2SO_4 method can be easily equated to the BuOH−HCl assay, if desired. Because the linear regression equation between assays varies with proanthocyanidin source, preliminary testing would be needed for each type of proanthocyanidin analyzed. Because only extracts can be processed with the automated H_2SO_4 assay, bound proanthocyanidins must still be quantified by direct analysis of the leaf residue.

Leucoanthocyanidins and 3-deoxy proanthocyanidins may potentially interfere with quantification of 3-hydroxy proanthocyanidins during analysis of certain sorghum and corn varieties using the automated H_2SO_4 assay. In cold or unheated acids, these compounds can produce pigments that absorb at 550 nm and that are unstable, especially at high temperatures (*22*−*24*, *38*). Unlike anthocyanidins formed from the cleavage of 3-hydroxy proanthocyanidins (*21*), the products of sorghum leucoanthocyanidins and 3-deoxy proanthocyanidins break down during the heating step of the BuOH−HCl assay (*23*). Further testing will be necessary to determine if these pigments form during the automated H_2SO_4 procedure, and, if so, whether they break down during the 50 °C reaction step of the assay. However, in general, the potential complications from 3-deoxy proanthocyanidins are limited because they are rare, having been identified only in sorghum and possibly in corn (*2*).

The automated H_2SO_4 technique is presently limited to aqueous methanol as the sample solvent. This precludes the use of aqueous acetone for extraction unless an extra step to dry and redissolve the extracts in aqueous methanol is imposed, a process that would likely offset the benefits of automation. This limitation is not serious if the purpose of the assay is to measure relative differences in proanthocyanidin concentration among experimental samples. Extractions with aqueous methanol and aqueous acetone give results that are linearly correlated with each other, indicating that the two solvents provide very similar representations of relative proanthocyanidin concentrations among plant samples even when extraction efficiencies differ. The simple linear relationship also implies that results from 50% methanol can easily be related to measurements obtained with 70% acetone when more accurate estimates of absolute proanthocyanidin concentrations are needed. Moreover, our studies determined that degradation of plant proanthocyanidin samples in aqueous methanol can be reduced by controlling extraction time and postextraction sample handling. Further increases in proanthocyanidin stability can probably be achieved through the addition of ascorbic acid during extraction, an antioxidant that we chose not to use in our studies to avoid interference with the Folin−Denis assay.

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