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# Plant tissue extraction method for complexed and free cyanide

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## Abstract

A method for free cyanide and strongly-complexed cyanide measurement within plant tissue was developed to study uptake and movement of cyanide species separately from cyanide metabolism and metabolite movement by a willow plant (*Salix eriocephala* var. Michaux). Spike recoveries from solutions with and without plant tissue, using various solvent combinations, and background control tissue contributions were investigated to obtain an accurate and precise extraction method for measurement of complexed and free cyanide concentrations within plant tissue. The optimum extraction technique involved the freezing of plant tissue with liquid nitrogen to facilitate homogenization prior to extraction. Homogenized willow tissue samples, 1 to 1.5 g-fresh weight, were ground a second time under liquid nitrogen followed by grinding in slurry with 2.5 M NaOH. The slurry was brought to 100 mL volume, sonicated for five minutes, extracted in the dark for sixteen hours, and analyzed without filtration for total and free cyanide by acid distillation and microdiffusion respectively. Sample tissue extraction controls found recoveries of 89% and 100% for 100 $\mu g\ L^{-1}$ $CN_T$ as KCN and $K_4Fe(CN)_6$ spiked in willow tissue slurries. Methanol, hexane, and 2-octanol inclusion in the solvent matrix with 2.5 M NaOH interfered with the cyanide analytical technique while chloroform reacted with NaOH and free cyanide in solution. Filtration was not included due to increased cyanide loss, and analysis of control tissue showed minimal release of cyanide or interference of plant tissue with the cyanide analytical method. Tissue cyanide concentrations from hydroponically-exposed tissue using the optimal extraction method agreed with tissue cyanide stable isotope ($^{15}N$) results.



**Keywords:** cyanide; ferrocyanide; extraction; plant analysis; plant concentration; *Salix eriocephala* var. Michaux; speciation; willow

# 1. Introduction

Measurement of cyanide within plant tissue is important for evaluation of phytoremediation of cyanide in soil and groundwater (Ebbs *et al.*, 2003) and also for assessing routes of cyanide toxicity to both plant and animals. For a phytoremediation system, cyanide must be taken up from solution and assimilated within plant tissue as plant tissue containing cyanide, particularly in the free form, can be toxic if consumed (ATSDR, 1997; Köster, 2001). The fate of the cyanide and the potential risks associated with the presence of cyanide compounds within the plant tissue must be considered. Therefore, the removal of solution cyanide together with evidence of assimilation within plant tissue determines remediation effectiveness.

Cyanide occurs naturally in plant tissue due to the breakdown of cyanogenic glycosides (Selmar *et al.*, 1990) as well as cyanide release during ethylene synthesis (Yip and Yang, 1988), but can also occur due to uptake from contaminated water and soil. Previous methods for cyanide determination in plant tissue have utilized various solvent extraction techniques with an emphasis on the determination of cyanide release potential, primarily from the breakdown of cyanogenic glycosides, rather than the concentration of the individual chemical forms (e.g. free cyanide, strongly-complexed cyanide) present.

Some studies of plant tissue analysis for cyanide have employed extraction methods similar to the conventional distillation (APHA/AWWA/WEF, 1998) and microdiffusion (ASTM, 1998) analytical techniques with colorimetric determination. Howe and Noble (1985) digested plant tissue samples directly in a distillation apparatus with $MgCl_2$ and $NaH_2PO_4$ prior to color development using chloramine-T and pyridine barbituric acid reagent. Tittle *et al.* (1990) performed an acid-digest on tissue directly within a cyanide distillation unit followed by analysis

of the liberated free cyanide colorimetrically and by gas chromatography after bromination. Mizutani *et al.* (1987) also utilized bromination for analysis of cyanide in apple samples ground under distilled water. Forensics analysis for chemical poisoning has provided additional examples for cyanide analysis of biological samples. The analytical methods for total and free cyanide content of animal tissue are modified distillation (Nolte and Dasgupta, 1996) and microdiffusion (Swanson and Krasselt, 1994) techniques. Analytical concerns for animal tissues are similar to those for plants in that the samples must be preserved to prevent cyanide release prior to analysis and the samples contain high amounts of various organic compounds that may interfere with cyanide detection.

None of the reported techniques for cyanide analysis in plant tissue have explored the issues of cyanide recovery during the extraction process or the extent to which plant tissues or plant compounds interfere with the analysis. Many of the plant studies have been concerned only with free cyanide concentration and release potential, primarily from cyanogenic glycosides. As such, preparation of samples is directed towards purifying the cyanogenic glycoside rather than removing cyanide analytical interferences. The only study to address total cyanide concentration, rather than simply free cyanide, did not investigate cyanide recovery or potential analytical interferences (Howe and Noble, 1985).

Breakdown of the plant tissue in a plant sample and the analytical interference issues that can result are of concern for cyanide analysis. Yet, disruption of the tissue is necessary to bring about complete release of plant cyanide content to solution. Typical methods for disrupting the membranes separating the cellular compartments within plant tissues involve a three day methanol/chloroform (2:1 v/v) extraction (Cohen *et al.*, 1998; Hart *et al.*, 1998). However, a wide variety of compounds liberated from disrupted plant tissue, particularly organics or sulfides,

can interfere with the cyanide detection technique (APHA/AWWA/WEF, 1998). Also, the release of naturally-occurring cyanide or cyanide-reactive compounds into solution can artificially elevate cyanide concentration measurements for plant tissue exposed to external cyanide sources. Cyanogenic glycosides release cyanide upon enzymatic hydrolysis, at neutral pH, to form the highly volatile $HCN_{(g)}$, for the purpose of protection versus herbivory or as a nitrogen source in seedling development. Hydrolysis of cyanogenic glycosides can also occur during plant tissue extraction in solvents such as methanol or ethanol (Forslund and Jonsson, 1997; Kobaisy *et al.*, 1996; Selmar *et al.*, 1990). Highly alkaline pH conditions, such as those used for preserving and analyzing aqueous cyanide samples, denature the hydrolytic enzymes, preventing the release of cyanide and minimizing interference (Halkier and Møller, 1990; Lechtenberg *et al.*, 1994).

Analytical methods that have been used to determine the cyanide content of tissue extracts have involved the use of picrate paper (Jacobs *et al.*, 1996) according to the Feigl-Anger method (Aikman *et al.*, 1996), the use of NaOH-soaked paper (Grossman and Kwiatkowski, 1995), or the bromination of cyanide trapped in sodium hydroxide solution (Mizutani *et al.*, 1987; Tittle *et al.*, 1990). Picrate paper is calibrated based upon the color change of the paper while cyanide trapped on NaOH-soaked paper is extracted and analyzed via gas chromatography. Analysis is performed in a closed flask to capture released cyanide gas. Recovery was only 16% for the NaOH-soaked paper (Grossman and Kwiatkowski, 1995) and the analytical precision of picrate paper is limited by the discernment of the color range relative to controls, particularly compared to detection ability with aqueous cyanide analyses (APHA/AWWA/WEF, 1998). The bromination technique involves additional sample handling, increasing the potential for free

cyanide loss. Another limitation of all three analyses is that only free cyanide is targeted unless the sample is acid-distilled prior to analysis.

Development of an extraction method yielding a solution that can be analyzed with distillation for total cyanide and microdiffusion for free cyanide was the focus of this study. Detection limitations and sample preservation were a concern for developing a methodology to extract and analyze cyanide within willow plant tissue. The analysis of cyanide in an aqueous sample by distillation (APHA/AWWA/WEF, 1998) and microdiffusion (ASTM, 1998) offers precision, a lower detection limit, and the ability to measure complexed cyanide. To preserve the natural distribution of chemical cyanide species within the tissues, it is necessary to preserve the extracts with the addition of a strong base solution and to limit additional cleanup steps to minimize the potential for free cyanide losses. The investigation and method development for the analysis of cyanide in plant tissues was structured with these limitations in mind. The objective of the investigation was to develop a method that minimized cyanide losses from the system while providing an accurate and precise measure of cyanide content and speciation within plant tissue. Experiments involved spiking control (without tissue) extract solutions and simulated tissue extracts with cyanide to assess recovery, comparing solvent extract mixtures, and assessment of background tissue cyanide concentrations to obtain the optimal extraction method.

## 2. Methods

Solvent combinations were examined to assess the potential interference of solvent with the cyanide analytical technique using solutions spiked with cyanide but without the presence of plant tissue. Willow tissue from plants grown in the absence of cyanide was utilized to assess background cyanide concentrations in willow and to determine cyanide recovery from slurries prepared by adding ground plant tissue to cyanide-spiked solutions. Willow growth and treatment is described in Ebbs *et al.* (2003), as is the cyanide concentration and speciation of exposed willow tissue obtained in support of hydroponic experiments. Exposed pea tissue was used for some preliminary solvent testing as a surrogate for willow tissue to assess the effect of solvent combinations on extraction. Pea tissue samples were grown for preliminary testing due to their common use and rapid growth (Bushey, 2003). Willow tissues were also used to supplement research examining prospects for cyanide phytoremediation by this species (Ebbs *et al.* 2003).

Willow and pea tissue from plants exposed to cyanide were used to examine the effects of tissue homogenization prior to extraction. Preparation of plant tissue prior to extraction is important for obtaining uniform, consistent results as determined by the standard deviation of the sample replicates. Extraction tests were performed on plant tissue samples with (willow) and without (pea) grinding under liquid nitrogen (i.e., sample homogenization) prior to extraction in 2.5 M sodium hydroxide (NaOH). Grinding of plant tissue under liquid nitrogen increases recovery of tissue content by rupturing cells (Halkier and Møller, 1990; Lechtenberg *et al.*, 1994) and improves measurement precision while simultaneously minimizing cyanide losses to volatilization. Willow and pea root tissue taken from plants exposed to 2 ppm total cyanide

($CN_T$) as $K_4Fe(CN)_6$ for 20 and 7 days, respectively, were extracted in 2.5 M NaOH with and without homogenization. The results demonstrated that the inclusion of liquid nitrogen grinding significantly reduced the standard deviation of specific tissue total cyanide content replicates from 27 to 10% ($p \leq 0.0185$) (Bushey, 2003). Statistical analysis was assessed using a t-test to determine p, the probability of the results being the same.

Extract solutions were analyzed for total cyanide by distillation (APHA/AWWA/WEF, 1998) and free cyanide by microdiffusion (ASTM, 1998), with cyanide content determined colorimetrically. The pH was reduced in each method by additional $H_2SO_4$/water (1:1 v/v) to overcome the additional buffering capacity of the 2.5 M NaOH extract solution relative to the conventional 1.6 g $L^{-1}$ NaOH concentration used in the analytical methods cited.

## 2.1. SOLVENT SELECTION

Solvent choice is important for maximizing tissue breakdown and cyanide recovery without distorting the chemical species of cyanide present or interfering with analysis. Analysis of solids for cyanide content involves a 16-hour extraction in 2.5 M NaOH (APHA/AWWA/WEF, 1998). The combination of both strong alkalinity during extraction and high acid concentration during the distillation, as employed in solid extraction, provides a wide pH range to break apart plant tissue while preventing enzymatic action during extraction and distillation. Previous literature on cyanide extraction from plants discusses the use of basic solution for tissue extraction, and also the use of methanol ($CH_3OH$) and chloroform ($CHCl_3$) to disrupt biological membranes (Cohen *et al.*, 1998; Hart *et al.*, 1998). Each of these solvents was examined in this study. Hexane ($C_6H_{14}$) and 2-octanol ($2\text{-}C_8H_{17}OH$) were also examined to broaden the range of solvent polarity

based upon a recommended method for preventing fatty acid interference in cyanide analysis (APHA/AWWA/WEF, 1998). Results obtained with 2.5 M NaOH were used as the baseline for comparison. Alternative solvents were investigated against the baseline recovery using cyanide-spiked solutions without tissue. Some preliminary investigations were performed with pea tissues grown in hydroponics and exposed to cyanide. Sodium hydroxide was always included in the solvent matrix to maintain a high pH in the extract and minimize volatilization losses during extraction. The various solvent matrices were assessed based upon cyanide recovery and the extent of interference with the cyanide analytical technique relative to the baseline levels.

The recommended conditions for the analysis of cyanide on solids, 2.5 M NaOH extraction for 16 hours, were chosen based on their use in pretreatment of solid waste samples containing "insoluble cyanide" (APHA/AWWA/WEF, 1998). The high pH (>12.5) obtained with 2.5 M NaOH reduces cyanide interactions with both inorganic and organic solids (Bushey and Dzombak, 2004; Chank, 1997). The potential for reducing the extraction time to less than 16 hours was not investigated. Sonication, which is often used in solid extractions, was included but only for 5 minutes of mixing prior to longer-duration extraction because of concern about loss of free cyanide due to sonication-induced heating.

Methanol in combination with 2.5 M NaOH was the first solvent matrix examined. Concerns about methanol interference with cyanide colorimetric analysis suggested evaporation of the methanol prior to distillation. To examine the effect of evaporation in addition to potential methanol spectrophotometric interference, eight 50 $\mu g\ L^{-1}$ $CN_T$ as KCN sample spikes were prepared: two NaOH controls and six volumetric flasks containing 30 mL 2.5 M NaOH brought to 100 mL volume with methanol (MeOH). Three of the MeOH: NaOH samples were evaporated overnight in an oven at 60°C while the others were sealed and mixed for 16 hours.

Aliquots were drawn from the sodium hydroxide portion of each sample and analyzed for total cyanide before and after distillation.

Testing of chloroform as an extraction enhancer was performed using extraction of cyanide-spiked solvent mixtures. As sodium hydroxide-chloroform mixtures can be explosive (DeForest, 1976), flasks for samples containing NaOH-chloroform mixtures were left open during mixing but kept closed during sonication due to possible volatilization that could occur during heating. Spike samples containing 70 $\mu g\ L^{-1}$ $CN_T$ as KCN without tissue were prepared for 2.5 M NaOH and for 2.5 M NaOH/chloroform (35:15 v/v). After sonication and a 16-hour extraction in the dark, the sodium hydroxide solution was withdrawn and analyzed for total cyanide.

Hexane and 2-octanol were examined using 2.5 M NaOH/Hexane and 2.5 M NaOH/2-Octanol (4:1 v/v) mixtures in 50 mL centrifuge tubes. The ratio was chosen based on the recommended value for preventing fatty acid interference during distillation (APHA/AWWA/WEF, 1998). Samples spiked with 37 $\mu g\ L^{-1}$ $CN_T$ as KCN were prepared for each solvent combination, including a 2.5 M NaOH control. The tubes were sealed, covered to prevent light intrusion, sonicated for 20 minutes, and rotated for 16 hours prior to total cyanide analysis without filtration of the sodium hydroxide phase.

## 2.2. SAMPLE SPIKE RECOVERY

Once solvent selection studies concluded, solutions spiked with known concentrations of cyanide were used to determine the extent of cyanide recovery with the optimal solvent, to investigate the impact of filtration, and to examine possible interferences caused by the presence of plant tissue. Sets of 200 mL volumetric flasks spiked with 100 $\mu g\ L^{-1}$ $CN_T$ as KCN and $K_4Fe(CN)_6$ were

prepared with and without homogenized willow root tissue [~800 mg-fresh weight (FW)] from plants that had not been treated with cyanide. Two samples were included for each possible treatment, with each sample analyzed for total cyanide before and after filtration with a 0.2 μm Millipore Type HN filter. Therefore, four conditions existed for each cyanide species with duplicates run for each condition. All samples were subjected to a five-minute sonication followed by a 16-hour extraction in the dark under constant mixing. Variation in the extraction pH and time were not investigated. Control flasks containing 100 $\mu g\ L^{-1}$ KCN (undistilled) and $K_4Fe(CN)_6$ (distilled) as cyanide (CN) and not subjected to the extraction procedure were used for recovery comparison. Cyanide-spiked solutions containing plant root tissue that had not been treated with cyanide were utilized for recovery determination, as certified cyanide-containing plant tissues are not available.

### 2.3. CONTROL TISSUE

The cyanide concentration and speciation in willow root, stem, and leaf tissue unexposed to KCN and $K_4Fe(CN)_6$ hydroponic solutions were measured as plant tissue could contribute to the background cyanide level as well as release organic compounds that interfere with the detection methods. The root, stem, and leaf unexposed tissue samples for each of four replicates within a hydroponic uptake study (Ebbs *et al.*, 2003) were homogenized and ~1700 mg-FW of tissue was subjected to the optimal extraction method and analyzed for total cyanide and free cyanide. Two tissue extractions were performed for each tissue within each replicate.

# 3. Results

The results obtained in the solvent selection, spike recovery, and control tissue tests are summarized here. All original, unreduced data are available in Bushey (2003).

## 3.1. SOLVENT SELECTION

The results from inclusion of methanol in the solvent matrix are given in Table I for 52 μg $L^{-1}$ $CN_T$ as free cyanide (KCN) in solution standards. Elevated cyanide levels for MeOH: NaOH relative to NaOH were measured in both undistilled and distilled samples without evaporation, although the increase was not significant as determined by a t-test ($p \leq 0.312$). MeOH interference with spectrophotometric cyanide detection was of greater concern than the slightly elevated cyanide sample results for each trial. The presence of methanol in the NaOH trap for methanol-containing samples, evident by foaming, substantiated concerns over interference. MeOH interference with the spectrophotometric cyanide determination is magnified during subsequent analyses as MeOH accumulates within the distillation unit. The differences between control and MeOH-containing samples increases with repeated use of the distillation unit. Attempts to evaporate the methanol prior to distillation reduced free cyanide recovery significantly ($p \leq 0.0001$) from 47 to <13 μg $L^{-1}$ while not removing the methanol completely. As expected, cyanide concentrations were consistently higher for undistilled samples compared to distilled samples for all three treatments.

In the presence of chloroform, KCN solutions show a disappearance of free cyanide from the solvent matrix (initial total cyanide, $CN_T$, ~60 μg $L^{-1}$) to non-detectable levels (Table I). Chloroform reacts with the free cyanide. Chloroform also reacts violently with NaOH, releasing

gas, pressurizing the extraction vessel to dangerous levels, and increasing the potential for free cyanide loss.

Results from tests of 2.5 M NaOH/Hexane and 2.5 M NaOH/2-Octanol (4:1 v/v) solvent mixtures for tissue extraction are given in Table I. The control 2.5 M NaOH solution served as the basis for comparison with a 37 $\mu g\ L^{-1}$ $CN_T$ solution as KCN. Total cyanide for NaOH/Hexane and NaOH/2-Octanol solutions without tissue increased to 45 and 40 $\mu g\ L^{-1}$. As only one sample was collected and analyzed per solvent mixture, the concentration changes within the solution cannot be evaluated for significance. However, foaming in the distillation unit for all samples containing hexane and 2-octanol was more excessive than that with methanol and indicated potential interference of the organic solvent with the cyanide analytical method.

## 3.2. SAMPLE SPIKE RECOVERY

Cyanide spike sample results are given in Table II for 2.5 M NaOH extractions from simulated tissue extracts containing willow root tissues spiked with 100 $\mu g\ L^{-1}$ $CN_T$ as KCN or $K_4Fe(CN)_6$, as well as from control samples containing no root tissue. As expected due to the relative volatility, KCN samples exhibited a lower recovery than $K_4Fe(CN)_6$ samples. Filtration decreased sample cyanide recovery, particularly for KCN spike samples. Recovery in filtered, KCN-spike samples decreased 4% ($p \leq 0.31$) for samples with plant tissue and 5% ($p \leq 0.13$) for those without tissue. For $K_4Fe(CN)_6$ samples, recovery decreased 5% ($p \leq 0.12$) for filtration of solution check samples without tissue. The presence of plant tissue also decreased recovery for unfiltered KCN-spike samples from 96 to 84% ($p \leq 0.0007$). All free cyanide recoveries are based

upon results for undistilled spike samples without tissue while those for ferrocyanide are based upon distilled samples.

### 3.3. CONTROL TISSUE

Results for analysis of 2.5 M NaOH extracts of homogenized (i.e., ground under liquid nitrogen), unexposed willow root, stem, and leaf tissue are given in Table III for samples grown for a hydroponic willow uptake study (Ebbs *et al.*, 2003). Tissue extractions and analysis yielded low background cyanide concentrations for all three tissues. Stem and leaf background total and free cyanide concentrations were near non-detect and not significant ($p \leq 0.112$ and 0.161 for stem and leaf) at 0.04 mg kg-fresh weight$^{-1}$ (mg kg-FW$^{-1}$) as CN. Values were higher for root tissue, measuring 0.46 and 0.22 mg kg-FW$^{-1}$ as CN for total and free cyanide, respectively, but still were not significant ($p \leq 0.180$) as the deviation was also higher.

## 4. Discussion

The objective was to develop a more accurate and precise technique for the measurement of plant tissue cyanide content and speciation. Tissue extracts from plants were analyzed for total cyanide by the digestion-distillation method (APHA/AWWA/WEF, 1998) and free cyanide by the microdiffusion method (ASTM, 1998). Analysis of both total and free (diffusible) cyanide species provides information on tissue sample cyanide speciation. Solvent choice is important for maximizing tissue breakdown and cyanide recovery during extraction without adversely affecting cyanide analysis. The baseline for comparison was extraction in 2.5 M NaOH after homogenization with grinding under liquid nitrogen, as a high pH in the extract solution was required to prevent free cyanide loss and prevent changes in the chemical species of cyanide present. Methanol, chloroform, hexane, and 2-octanol were examined as potential additions to 2.5 M NaOH in the extraction matrix. Slight changes in cyanide recovery were found for methanol, hexane, and 2-octanol (Table I). Chloroform extraction without tissue resulted in complete loss of free cyanide (Table I). All of the organic solvents interfered with the spectrophotometric analysis of cyanide after distillation when added in addition to NaOH. The 1.6 g $L^{-1}$ NaOH cyanide traps used in the distillation method foamed for samples containing the alternative solvents with increasing interference with each subsequent distillation. Hexane and 2-octanol interfered with the total cyanide analysis to a greater extent than methanol due to higher volatility and lower solubility. Attempts to remove methanol by evaporation prior to distillation significantly reduced cyanide recovery (Table I).

Previous studies on plant extraction for cyanide suggested that adding organic solvents to 2.5 M NaOH could increase recovery. However, organic solvents interfered with the total cyanide

analytical technique without significantly increasing recovery from plant tissue. The violent reaction between chloroform and sodium hydroxide (DeForest, 1976) justifies the exclusion of the former from the solvent matrix. Also, the inclusion of chloroform results in unaccountable losses of free cyanide from solution. Finally, a comparison of cyanide recovered from 2.5 M NaOH extracts of willow plants exposed to $^{15}N$-labeled cyanide in a hydroponic uptake study to the values calculated from the stable isotope results (Ebbs *et al.*, 2003) are in agreement. Therefore, 2.5 M NaOH was determined to be the optimal solvent for extraction of plant tissue for analysis of free cyanide and total cyanide.

Recoveries of cyanide from simulated tissue solutions spiked with KCN or $K_4Fe(CN)_6$ and extracted with 2.5 M NaOH were used to examine whether to filter tissue samples prior to distillation and also to determine cyanide recovery during the extraction procedure (Table II). Although ferrocyanide has been shown to bind to the cell walls of plant tissue at pH 6 (Ebbs *et al.*, 2003), this sorption of ferrocyanide did not change recovery from tissue-containing samples. For free cyanide, recovery significantly decreased when plant tissues were present in the extracts. While partitioning of free cyanide to plant tissue was possible, a more likely cause of the losses was volatilization during the extraction process. Filtration only increased the potential for additional cyanide loss and was not included in the final extraction procedure. Recoveries from unfiltered samples containing plant tissue were 84% for KCN and 100% for $K_4Fe(CN)_6$ (Table II). These values do not account for recovery of cyanide in the standard distillation technique, which is approximately 95% for free cyanide and 98-100% for ferrocyanide samples (results not given here). Therefore, the adjusted extraction recovery was 89% for free-cyanide-exposed plant tissue. These recoveries are significantly higher than those reported for some alternative cyanide

analytical methods using treated papers (Grossman and Kwiatkowski, 1995) or a modified microdiffusion technique (Yip and Yang, 1988).

Analysis of control tissue is important for assessing the background contribution to total and free cyanide tissue concentrations. Disruption of plant tissue could release compounds that interfere with cyanide analysis. The release of endogenous cyanide, possibly from cyanogenic glycosides or ethylene production, could also influence the results of cyanide analyses. The background levels were consistent with those previously reported for plant tissue free (Tittle *et al.*, 1990; Yip and Yang, 1988) and total (Howe and Noble, 1985) cyanide. The low cyanide concentrations in the root, stem, and leaf control tissue (Table III) for both total and free cyanide relative to exposed tissue (Ebbs *et al.*, 2003) negate concern over tissue breakdown and endogenous cyanide levels producing significant interferences.

The optimal tissue extraction method, 2.5 M NaOH extraction of samples homogenized by grinding under liquid nitrogen, provided reproducible results and high total and free cyanide spike recoveries for willow plant tissue. Results for hydroponically-exposed tissue agreed with tissue stable isotope tracer ($^{15}N$) uptake data (Ebbs *et al.*, 2003). Preserved (frozen) plant tissue samples were homogenized by grinding under liquid nitrogen in a ceramic crucible. A weighed amount of homogenized sample (approximately 1 – 1.5 g-FW) was ground a second time under liquid nitrogen prior to grinding under 2.5 M NaOH to a fine paste. The sample was rinsed from the crucible and brought to volume with 2.5 M NaOH in a 100 mL volumetric flask. Following a five-minute sonication, the sealed flask was covered with aluminum foil to prevent light intrusion and subjected to a 16-hour extraction with constant mixing. After extraction, volumes of the tissue slurry were pipetted off and analyzed, without filtration, for total cyanide by

distillation (APHA/AWWA/WEF, 1998) and free cyanide by microdiffusion (ASTM, 1998) with the pH adjusted to the desired value by adding additional $H_2SO_4$/water (1:1 v/v).

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**Table I** Results from the solvent selection experiments (s.e. = standard error of the mean). Extractions with NaOH in combination with hexane or 2-octanol were not replicated. The total cyanide concentration as KCN in the initial standard solution for the methanol, chloroform, and organic solvent extractions were 52, 60, and 37 μg $L^{-1}$, respectively... Total cyanide analysis after extraction was determined by distillation according to *Standard Methods* 4500-CN-C/E (APHA/AWWA/WEF, 1998). Details concerning each specific solvent experiment appear in the text. NE = no evaporation; EV = evaporation at 60°C for 18 hours.

| **Solvent combination** | **μg $L^{-1}$ as $CN_T$** | |
|---|---|---|
| *Methanol* | Undistilled | Distilled |
| NaOH | 51.9 (0.0) | 46.3 (1.3) |
| NaOH/MeOH (NE) | 54.4 (0.5) | 47.6 (1.4) |
| NaOH/MeOH (EV) | 13.8 (0.2) | 12.2 (0.1) |
| | | |
| *Chloroform* | Filtered | Unfiltered |
| NaOH | 57.3 (3.1) | 65.7 (0.5) |
| NaOH/chloroform | 1.0 (0.0) | 1.0 (0.0) |
| | | |
| *Organic solvents* | | Distilled |
| NaOH | | 36.8 |
| NaOH/hexane | | 45.2 |
| NaOH/2-octanol | | 40.1 |

**TABLE II** Free cyanide and ferrocyanide recovery with optimal extraction method. Recovery evaluated using 100 μg $L^{-1}$ $CN_T$ aqueous spike samples (KCN and $K_4Fe(CN)_6$) with and without control root tissue added. Filtration performed with 0.2 μm Millipore Type HN filter. Approximately 800 mg-FW of homogenized willow control tissue added to selected flasks. Total cyanide concentrations measured by distillation according to *Standard Methods* 4500-CN-C/E (APHA/AWWA/WEF, 1998). Recovery determined relative to unextracted, distilled ($K_4Fe(CN)_6$) and undistilled (KCN) check standards (s.e. = standard error of the mean). Boldface values represent recovery using the optimum extraction method for unfiltered, tissue-containing samples.

| | KCN | | | | $K_4Fe(CN)_6$ | | | |
|---|---|---|---|---|---|---|---|---|
| | No Tissue | | Tissue | | No Tissue | | Tissue | |
| Filter | N | Y | N | Y | N | Y | N | Y |
| % Recovery | 95.5 | 90.7 | **83.5** | 80.0 | 100.0 | 95.0 | **100.5** | 101.0 |
| (s.e.) | (1.5) | (3.3) | (1.5) | (6.0) | (0.0) | (3.5) | (1.5) | (1.0) |

**TABLE III** Control willow tissue total and free cyanide concentrations (s.e. = standard error of the mean). Detection limits for total and free cyanide in the extract solution are 1 and 2.5 μg $L^{-1}$ as CN. Approximately 1700 mg-FW of homogenized tissue extracted in 100 mL 2.5 M NaOH (*n* = 4). Based upon total and free cyanide measured by distillation (APHA/AWWA/WEF, 1998) and microdiffusion (ASTM, 1998).

| | | Total Cyanide (mg CN kg-$FW^{-1}$) | Free Cyanide (mg CN kg-$FW^{-1}$) |
|---|---|---|---|
| Root | Average | 0.46 | 0.22 |
| | (s.e.) | (0.4) | (0.1) |
| Stem | Average | 0.04 | 0.08 |
| | (s.e.) | 0.03) | (0.03) |
| Leaf | Average | 0.04 | 0.03 |
| | (s.e.) | 0.04) | (<0.01) |