

PRESERVING LEAVES FOR TANNIN AND PHENOLIC GLYCOSIDE ANALYSES: A COMPARISON OF METHODS USING THREE WILLOW TAXA

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Abstract—Members of the Salicaceae often produce phenolic glycosides and condensed tannins. There is much debate on the best method for the preservation of leaf material prior to chemical analysis. Published results indicate freeze-drying, a method commonly used for tannin analysis, may be inappropriate for phenolic glycosides, unless done in a manner to prevent thawing during the drying process. Another commonly employed method, air-drying, is appropriate for phenolic glycosides but inappropriate for condensed tannins. I present evidence using willow leaves that demonstrates that: (1) leaves freeze-dried in external flasks without temperature control contain lower concentrations of phenolic glycosides (salicortin and 2'-cinnamoylsalicortin), (2) air-dried leaves have reduced concentrations of condensed tannins, while (3) vacuum-dried fresh leaves have high concentrations of both phenolic glycosides and condensed tannins. Freeze-drying caused salicortin and 2'-cinnamoyl salicortin concentrations to drop by 20 mg/g and 4 mg/g, respectively. Salicin, a product of salicortin and 2'-cinnamoyl salicortin degradation, is absent in vacuum-dried leaves, present in air-dried leaves and very high in freeze-dried leaves. Thus, the presence of salicin in this system is an artifact of the preservation technique. Condensed tannin concentrations dropped nearly 20 mg/g when leaves were air-dried. Thus, vacuum-drying fresh leaves allows researchers to quantify phenolic glycosides and condensed tannins from the same leaf material.

Key Words—Salicaceae, willows, phenolic glycosides, salicortin, 2'-cinnamoyl salicortin, salicin, condensed tannins, leaf preservation.

INTRODUCTION

Willows and other Salicaceous plant species produce two main secondary chemicals: phenolic glycosides and/or condensed tannins (Julkunen-Tiitto, 1986,

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1989; Lindroth et al., 1987). The concentrations of the phenolic glycosides may vary among individuals (Nichols-Orians et al., 1993) and in some cases influence the susceptibility of plants to both insect and mammalian herbivores (Rowell-Rahier, 1984; Tahvanainen et al., 1985; Basey et al., 1988; Lindroth and Peterson, 1988; Lindroth et al., 1988; Clausen et al., 1989; Lindroth, 1992). Condensed tannins are bioactive as well, especially against microbes (Schultz, 1989; Nichols-Orians, 1991a).

Salix sericea Marshall and *Salix eriocephala* Michx. produce different secondary chemicals. *S. sericea* produces two main phenolic glycosides, salicortin and 2'-cinnamoylsalicortin (Nichols-Orians et al., 1992) and very low concentrations of condensed tannins. *S. eriocephala* produces high concentrations of condensed tannins and no phenolic glycosides. Interestingly, these species hybridize and *S. sericea* × *S. eriocephala* hybrids produce both tannins and phenolic glycosides at intermediate concentrations (Orians and Fritz, 1995). When studying the bioactivity of these secondary chemicals, it is critical that leaves be preserved in a manner that prevents degradation.

Salicortin is quite labile and when improperly preserved it degrades to salicin (Lindroth and Pajutee, 1987; Julkunen-Tiitto and Meier, 1992). Even relatively nonlabile chemicals like tannins are lost when improperly preserved (Hagerman, 1988). Given the bioactivity of both these chemical types, it is of interest to determine the method of preservation that will prevent their degradation (Lindroth and Pajutee, 1987; Hagerman, 1988; Julkunen-Tiitto and Gebhardt, 1992; Julkunen-Tiitto and Meier, 1992).

Julkunen-Tiitto and Meier (1992) have suggested that air-drying at low temperatures is sufficient to prevent the degradation of phenolic glycosides. However, air-drying leaves is known to result in the degradation of tannins (Hagerman, 1988). Van Sumere et al. (1983) suggest that lyophilization (freeze-drying) is inappropriate for low- and intermediate-molecular weight phenolics. When drying leaves for tannin analysis, Hagerman (1988) recommends fresh extraction or freeze-drying prior to extraction. The exact method depends upon the species and time of year. Thus, the method appropriate for one set of chemicals is often inappropriate for another. The lack of a standard technique has made it difficult to compare studies and has hindered researchers in their efforts to determine the bioactivity of foliar chemicals. This study was initiated after preliminary results indicated that vacuum-drying was appropriate for both phenolic glycosides and tannins.

Here I compare three methods of preservation (air-drying, freeze-drying, and vacuum-drying). Based on studies by Hagerman (1988), Van Sumere et al. (1983), and Julkunen-Tiitto and Meier (1992), I expected concentrations of phenolic glycosides to be lowest in freeze-dried leaves and concentrations of condensed tannins to be lowest in air-dried leaves. The results indicate that air-drying results in a loss of tannins, freeze-drying causes extensive conversion of

salicortin and 2'-cinnamoylsalicortin to salicin, while vacuum-drying preserved both the phenolic glycosides and the tannins. Thus, of the three methods, vacuum-drying is superior for both chemicals.

METHODS AND MATERIALS

Plants. *Salix sericea* Marshall and *Salix eriocephala* Michx. are abundant in the northeastern United States and eastern Canada (Argus, 1986). They produce leaves continuously from May until September at our field site near Milford, New York, and adult plants reach a height of 3–4 m. *S. sericea* × *S. eriocephala* hybrids are abundant at the field site.

Cuttings from field plants at the study site were brought back to Williams College. Cuttings were rooted, potted, and then placed in a common garden on Williams College campus. I used a total of 36 one-year old cuttings for this study (12 of each plant species). Four plants of each species were randomly assigned to each treatment. Thus all the leaves removed from one plant were processed the same way.

Leaf Material. Two adjacent leaves (the first two fully mature leaves) from eight different shoots were collected from each plant. The methods of collection were as follows. For freeze-drying, leaves were immediately frozen in liquid nitrogen, transported to the lab, placed in flasks and attached to a Labconco (model 77500) freeze-drier. Care was taken to limit thawing once frozen. This freeze-drier has external flasks (that cannot be temperature regulated) attached to a central vacuum. Therefore it was impossible to prevent thawing completely prior to sublimation.

For air-drying, leaves were put into a cooler containing ice, transported back to the lab, and air-dried at 60°C for 24 hr.

For vacuum-drying, leaves were put into the ice cooler, transported back to the lab, and immediately vacuum dried (a freeze-dryer was used but the leaves were never frozen). Once dry, leaves were ground in a Wiley mill (size 30 mesh), weighed, and stored in the freezer until chemical analyses were performed.

Chemical Analysis. The phenolic glycosides were assayed using standard techniques (Nichols-Orians et al., 1992, 1993). Briefly, leaf powder (30 ± 3 mg) was extracted in cold MeOH (10 mg leaf powder/1 ml MeOH) with sonication for 10 min. Cold water was constantly flushed through the sonicator to prevent the heating of samples. Extracts were centrifuged and filtered ($0.45 \mu\text{m}$) then placed in crimp-top vials. Extracts were kept in the freezer until analysis (48 hr or less). The concentration of glycosides was quantified with an HPLC (Hewlett-Packard) and a UV detector set at 274 nm. A reverse-phase Nova-Pak C_{18} ($4 \mu\text{m}$) column (Waters) and a gradient system of distilled water and MeOH

was used. The samples were injected through an LS-3200 automated sampler (SGE). 1,3-Dimethoxybenzene was used as the internal standard. Purified standards of salicin, salicortin, and 2'-cinnamoylsalicortin were used to determine concentrations.

The condensed tannins also were extracted using standard techniques (see Nichols-Orians, 1991a,b). Briefly, approximately 300 mg leaf powder was washed with ether and extracted with 70% acetone for 3 hr in a 40°C water bath. Acetone was removed under reduced pressure and all extracts were diluted to 8 ml with distilled water. Two techniques were used to quantify condensed tannin concentrations: the butanol/HCl method and the vanillin method (Bate-Smith, 1977; Butler et al., 1982; Hagerman and Butler, 1989). The concentration of the condensed tannin (milligrams per gram dry leaf) in each species was determined using purified tannins collected from each of the species.

RESULTS AND DISCUSSION

The method of preservation had a statistically significant effect on the concentrations of the phenolic glycosides (see treatment effect in Table 1). As expected, freeze-dried *S. sericea* or hybrid leaves had the lowest concentrations of salicortin and 2'-cinnamoylsalicortin (Figure 1A and B) (*S. eriocephala* does not produce phenolic glycosides). The difference in phenolic glycoside concentration between air-dried and vacuum-dried leaves was not significant. However, the presence of a breakdown product, salicin, is the best indicator of salicortin or 2'-cinnamoylsalicortin degradation. No salicin was present in the vacuum-dried leaves but was present in both the air-dried and freeze-dried *S. sericea* leaves (Figure 1C).

TABLE 1. SIGNIFICANCE OF EFFECTS OF LEAF PRESERVATION TECHNIQUE ON CONCENTRATIONS OF PHENOLIC GLYCOSIDES IN *S. sericea* AND HYBRID LEAVES USING TWO-WAY ANALYSIS OF VARIANCE WITH PLANT SPECIES AND PRESERVATION TECHNIQUE AS MAIN EFFECTS^a

Source of variation	Phenolic glycoside					
	Salicortin		2-Csalicortin ^b		Salicin	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Taxon	34.95	<0.001	5.95	0.025	165.98	<0.001
Treatment	13.33	<0.001	4.02	0.036	432.62	<0.001
Taxon × Trtmt	1.34	NS	0.01	NS	71.35	<0.001

^aConcentrations were LN transformed prior to analyses.

^b2-Csalicortin = 2'-cinnamoylsalicortin.

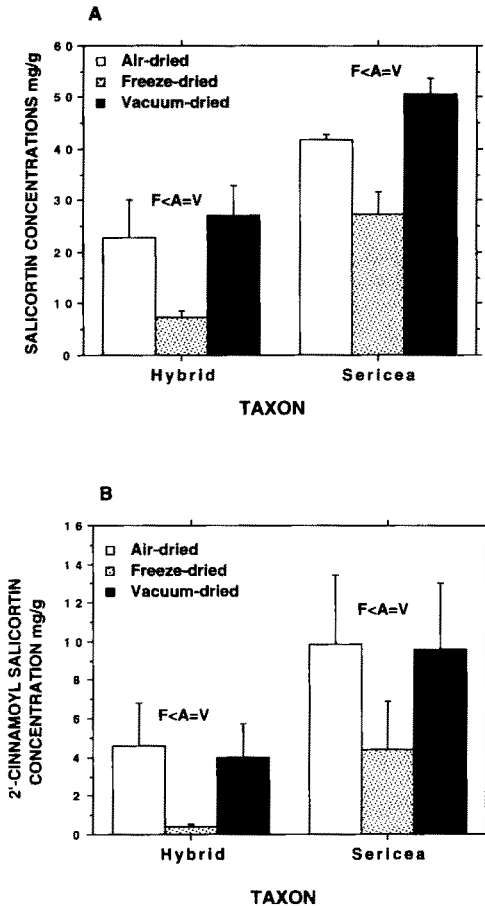


FIG. 1. The concentration (milligrams per gram dry leaf) of (A) salicortin, (B) 2'-cinnamoylsalicortin, and (C) salicin as a function of plant taxon and preservation treatment. Data were ln transformed prior to analyses and the Bonferroni method was used to determine which treatments were significantly different. A = air-dried, F = freeze-dried, V = vacuum-dried.

When vacuum-dried, the mean concentration of salicortin was 50 mg/g in *S. sericea* leaves and 27 mg/g in hybrid leaves. When freeze-dried, the mean concentration dropped to approximately 27.5 mg/g in *S. sericea* and 7.4 mg/g in hybrid leaves. Salicin concentrations increased from zero in vacuum-dried leaves to approximately 21 mg/g and 10 mg/g in *S. sericea* and hybrid freeze-dried leaves, respectively.

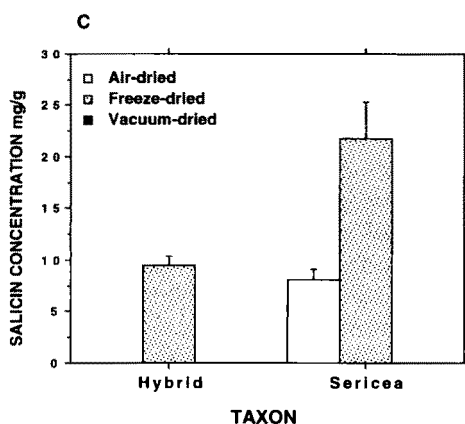


FIG. 1. Continued.

If samples are placed in a freeze-dryer with a temperature-regulated drying chamber (set below freezing), degradation can be prevented (Lindroth, personal communication). It is critical, therefore, not to use freeze-driers with external flasks when analyzing phenolic glycosides. Many researchers have reported the presence of salicin in their study species, yet my results show that its presence may be merely an artifact of the preservation technique.

Condensed tannin concentrations also varied with preservation technique (Table 2). As expected, both the vanillin and the butanol/HCl method show that concentrations are lowest in air-dried leaves and highest in the freeze- and

TABLE 2. SIGNIFICANCE OF EFFECTS OF LEAF PRESERVATION TECHNIQUE ON CONCENTRATIONS OF CONDENSED TANNINS IN LEAVES USING TWO-WAY ANALYSIS OF VARIANCE WITH PLANT TAXON AND PRESERVATION TECHNIQUE AS MAIN EFFECTS^a

Source of variation	Condensed tannin method			
	Vanillin method		Butanol/HCl method	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Taxon	260.49	<0.001	149.64	<0.001
Treatment	4.35	0.023	8.55	0.001
Taxon × Trtmt	0.86	NS	1.42	NS

^aConcentrations were LN transformed prior to analyses.

vacuum-dried leaves (Figure 2A and B). The loss of tannin with air-drying is consistent with the results of Hagerman (1988). It is standard methodology to freeze-dry leaves prior to tannin analysis. I found no difference in concentration between freeze-dried and vacuum-dried leaves. Clearly vacuum-drying is an excellent method when quantifying the concentrations of condensed tannin.

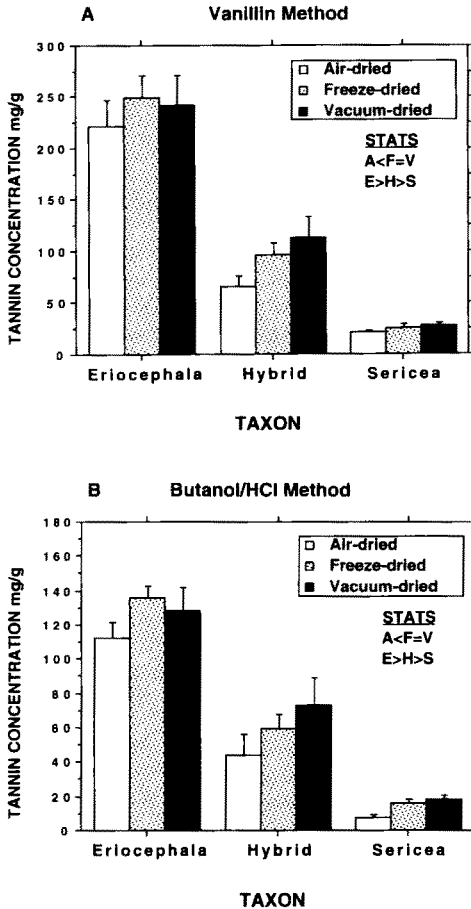


FIG. 2. The effects of plant taxon and preservation treatment on the concentration of condensed tannin (milligrams per gram dry leaf) as determined with the (A) vanillin method and (B) butanol/HCl method. Data were transformed prior to analyses and the Bonferroni method was used to determine which taxa and treatments were significantly different. A = air-dried, F = freeze-dried, V = vacuum-dried, E = *S. eriocephala*, H = hybrid and S = *S. sericea*.

Overall, this technique allows researchers to quantify both phenolic glycosides and condensed tannins from the same leaf material. For plants that contain both chemical types, such as *S. sericea*, *S.s.* × *S.e.* hybrids, and most *Populus* sp., this technique would be ideal. Further study will be required to determine if vacuum-drying will be the method of choice for other chemicals, such as hydrolyzable tannins and alkaloids. Van Sumere et al. (1983) suggest that some phenolics might be lost during freeze-drying under high-vacuum conditions because many of the volatile phenolics are concentrated on the ice trap. Whether these same chemicals would be lost in the absence of freezing is unknown. If vacuum-drying proves to be generally appropriate, it would alleviate the need for liquid nitrogen and facilitate research in remote sites. I conclude that researchers studying salicaceous species should adopt this preservation technique as it prevents the degradation of both phenolic glycosides and condensed tannins and avoids the need for liquid nitrogen—a cooler of ice and a vacuum pump will do.

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