Effect of Sample Preparation Method on Birch (*Betula pendula* Roth) Leaf Phenolics

Markku Keinänen* and Riitta Julkunen-Tiitto

Department of Biology, University of Joensuu, P.O. Box 111, FIN-80101 Joensuu, Finland

Sample preparation methods for high-performance liquid chromatographic analysis of birch leaf phenolics were compared. The methods tested were: (1) air-drying at ambient temperature, (2) oven-drying at 40 °C, (3) oven-drying at 80 °C, (4) freeze-drying, prefreezing with liquid N_2, (5) freeze-drying, prefreezing at −18 °C, (6) freeze-drying without prefreezing, (7) storing frozen for 12 days without drying, and (8) immediate extraction of fresh samples. Although there were significant differences among methods in absolute concentrations of phenolics, none of the methods altered considerably the phenolic profile of birch leaves. For quantitative analysis, the samples should be analyzed immediately. Alternatively, the samples may be stored fresh frozen, at least for a short time. Among the drying methods, the highest concentrations of phenolics were obtained by freeze-drying of slowly frozen leaves (method 5). Freeze-drying without prefreezing resulted in significantly lower concentrations of flavonoid glycosides than method 5. Oven-drying did not prove to be a good alternative to conventional freeze-drying, nor did it show any advantage over simple air-drying.

Keywords: *Betula pendula; sample preparation; drying methods; flavonoid glycosides; phenolic compounds*

INTRODUCTION

The chemical composition of dried plant material is likely to be different from that of fresh tissue. Fresh plant tissues should therefore be preferred in phytochemical studies (Harborne, 1984). If this is not possible, the collected tissues must be preserved for later extraction and analysis. Although the plant material may be stored frozen, most often it is dried.

After collection of plant material, enzymatic activity may quickly lead to irreversible changes in phenolic compounds, such as oxidation and subsequent polymerization or decomposition (Harborne, 1984; Waterman and Mole, 1994). Rapid drying shortens the time the compounds are subject to enzyme action, because the reactions require some water. However, the concentration of enzymes and substrates increases during the drying process, which is likely to facilitate the reactions (Waterman and Mole, 1994). On the other hand, fast drying at high temperatures may induce polymerization or degradation of thermolabile phenolics (Walker, 1975; Karchesy, 1989). In addition, during the first stages of warming, even the enzymes may be transiently stimulated.

Quick freezing and subsequent freeze-drying combine efficient enzyme inactivation with low drying temperature, and thus could be considered as an ideal drying method for phytochemical studies (Waterman and Mole, 1994). However, freezing, as well as conventional freeze-drying of prefrozen plant tissue, has been reported to induce dramatic changes in total and relative concentrations of *Salix* and *Populus* phenolic glycosides (Lindroth et al., 1987; Lindroth and Pajutee, 1987; Julkunen-Tiitto and Tahvanainen, 1989). Furthermore, compounds of low or intermediate molecular weight may be partly removed from plant material during freeze-drying by high vacuum (Van Sumere et al., 1983).

The purpose of this study was to investigate the validity of several commonly used sample preparation methods for the analysis of birch leaf phenolics. Flavonoid glycosides and small molecular phenolic compounds were analyzed by high-performance liquid chromatography (HPLC). For plant phenolics, comparisons of sample preparation methods are often carried out by measuring tannins or total phenolics with the aid of some color test. It is obvious that different types of phenolics or individual compounds may not respond similarly, and detailed HPLC studies are therefore preferable. Preliminary testing is especially important before studies utilizing quantitative data of individual compounds, such as chemotaxonomic surveys and cultivar fingerprinting, research on chemical ecology, or quality assessment of food products. This study forms a part of methodological testing for a quantitative survey of flavonoids and other phenolics in birch species (Keinänen, 1993).

MATERIALS AND METHODS

Sample Preparation. Composite samples of birch leaves (*Betula pendula* Roth) were collected from the lower branches (from ca. 60 to 180 cm) of a single tree growing in an open field near the University of Joensuu. Samples were taken between 18.30 and 19.30 p.m. on 7 rainless days during a 10-day period in September. On each day, 20−25 flowerless branches with short shoot leaves were cut from different sides of the tree. The branches were brought to the laboratory within 5 min and treated immediately.

Preliminary studies indicated that quantitative variation of phenolics is smaller among short-shoot leaves than among long-shoot leaves. Therefore, only short-shoot leaves were included in the study. Seriously damaged or diseased leaves and leaves differing greatly in size were not included. The petioles were cut from the leaves, and only the leaf blades were used.

On each day, 90−126 leaves were pooled and randomly divided among eight treatments and a separate sample for dry-weight determination. The treatments were as follows: (1) air-drying at ambient temperature

* Author to whom correspondence should be addressed (e-mail keinanen@joyl.joensuu.fi).

S0021-8561(96)00168-9 CCC: $12.00

Table 1. Effect of Sample Preparation Method on the Concentration[a] of (+)-Catechin and Flavonoid Glycosides[b]

sample preparation method	(+)-catechin	myricetin 3-*O*-galactoside	quercetin 3-*O*-				flavonoid glycosides[c]
			galactoside	glucuronide	arabinoside	rhamnoside	
1, air-drying	1.79 ± 0.16	0.59 ± 0.06	5.66 ± 0.21	1.83 ± 0.05	1.11 ± 0.05	0.47 ± 0.03	9.7 ± 0.35ab
2, oven-drying at 40 °C	1.72 ± 0.12	0.54 ± 0.04	5.41 ± 0.25	1.77 ± 0.06	1.05 ± 0.05	0.49 ± 0.03	9.3 ± 0.37a
3, oven-drying at 80 °C	1.60 ± 0.12	0.52 ± 0.03	6.13 ± 0.26	1.98 ± 0.08	1.22 ± 0.07	0.55 ± 0.04	10.4 ± 0.42bc
4, freeze-drying (N_2)[d]	1.74 ± 0.11	0.55 ± 0.04	6.25 ± 0.27	2.02 ± 0.07	1.21 ± 0.05	0.56 ± 0.03	10.6 ± 0.41bc
5, freeze-drying (−18 °C)[e]	1.90 ± 0.17	0.54 ± 0.01	6.64 ± 0.07	2.20 ± 0.03	1.30 ± 0.01	0.59 ± 0.04	11.3 ± 0.10cd
6, freeze-drying (unfrozen)[f]	2.03 ± 0.14	0.58 ± 0.06	5.88 ± 0.35	1.85 ± 0.08	1.13 ± 0.07	0.50 ± 0.04	9.9 ± 0.55ab
7, fresh frozen	1.91 ± 0.15	0.60 ± 0.05	6.93 ± 0.37	2.37 ± 0.13	1.34 ± 0.09	0.61 ± 0.03	11.8 ± 0.64de
8, fresh	1.96 ± 0.14	0.62 ± 0.04	7.21 ± 0.27	2.46 ± 0.09	1.42 ± 0.07	0.62 ± 0.03	12.3 ± 0.44e
p	0.016	0.501	0.000	0.000	0.000	0.002	0.000
lsd[g]	0.24		0.54	0.18	0.11	0.08	0.92

[a] Expressed as mg/g of dry weight; flavonoid glycosides quantified as quercetin 3-galactoside. [b] Data are means ± SE, $n = 7$. [c] The sum of flavonoid glycosides; means not sharing the same letter differ at $p < 0.05$. [d] Prefreezing with liquid N_2. [e] Prefreezing at −18 °C. [f] Freeze-dried without prefreezing. [g] Least significance difference.

(ca. 22 °C), (2) drying in a vented oven at 40 °C, (3) drying in a vented oven at 80 °C, (4) freeze-drying of leaves frozen by immersion in liquid N_2, (5) freeze-drying of frozen leaves kept at −18 °C for 24 h, (6) freeze-drying by placing the samples directly into the freeze-dryer without being frozen first, (7) storing at −18 °C for 12 days without drying, and (8) extracting the fresh samples immediately. The freeze- and oven-drying lasted for 12 h and the air-drying for 7 days. The drying process or extraction of fresh leaves was started within 30 min from sampling. To avoid systematic errors, the treatments were started in a different order each day. Two ovens were used alternately at 40 or 80 °C. The number of leaves per treatment varied among days (10−14 leaves/treatment); on each day the treatments had an equal number of leaves. The samples were stored in paper bags for 7−14 days at room temperature until extracted and analyzed. The dry weights obtained from separate dry-weight determination for each day were used for all treatments. The dry weights were calculated from samples of 10−14 leaves oven-dried at 80 °C for 12 h.

Extraction. The fresh leaves (1.3−1.8 g) were cut into 10−20 mm^2 pieces and left to steep for 15 min in 80% ethanol. The samples were extracted twice with 50 mL of 80% ethanol using an Ultra-Turrax homogenizer for 3 min. The extract was filtered, and the residue was washed with 20 mL of 96% ethanol. The combined extract was evaporated to dryness at 40 °C.

The dry leaves were cut into 10−20 mm^2 pieces. A 120 mg sample was allowed to steep for 15 min in 80% ethanol and then extracted twice with 7 mL of 80% ethanol in a similar manner as the fresh leaves. Before analysis, the extracts were dissolved in water−methanol (1:1 v/v).

Apparatus. The HPLC apparatus was a Hewlett-Packard system consisting of a quaternary pump (HP 1050), an autosampler (HP 1050), and a photodiode array detector (HP 1040A) coupled with an analytical workstation (HP 79994A). The compounds were separated on a 60 mm × 4.6 mm i.d. HP Hypersil ODS II (3 μm) column. The elution solvents were aqueous 2.4% tetrahydrofuran plus 0.25% orthophosphoric acid (A) and methanol (B). The samples were eluted according to the following gradient: 0−4 min, 2−12% B in A; 4−30 min, 12−35% B in A; 30−45 min, 35−60% B in A. The flow rate was 2 mL/min and the injection volume 20 μL. The analyzed compounds were detected at 280 nm [Z_1 and (+)-catechin], 320 nm [hydroxycinnamic acid derivatives (HCA)], and 360 nm (flavonoid glycosides).

Materials. Quercetin 3-galactoside, quercetin 3-arabinopyranoside, and quercetin 3-rhamnoside were obtained from Roth, (+)-catechin and chlorogenic acid were from Aldrich-Chemie, and picein was from Extrasynthese. HPLC grade methanol and tetrahydrofuran (Lab-Scan) were used.

Identification and Quantification of Birch Leaf Phenolics. The identification of (+)-catechin, chlorogenic acid, and flavonoid glycosides was based on comparison of retention and spectral characteristics of the corresponding peaks with standard compounds. Myricetin 3-galactoside, quercetin 3-glucuronide, and hydroxycinnamic acids were tentatively identified by their UV spectra and relative retention order (Harborne and Boardley, 1984; Dallenbach-Tölke et al., 1987). A major peak (Z_1) with almost identical spectrum with picein (glucoside of 4-hydroxyacetophenone), but eluting later than picein, is probably 3,4′-dihydroxypropiophenone 3-β-D-glucopyranoside, which has been reported from leaves of *Betula alba* (Tschesche et al., 1974), *B. platyphylla* var. Japonica (Mori et al., 1992), *B. pendula*, and *B. pubescens* (Ossipov et al., 1996). Flavonoid glycosides were quantified as quercetin 3-galactoside, hydroxycinnamic acid derivatives as chlorogenic acid, and Z_1 as picein.

Statistical Analysis. The statistical model was a randomized complete blocks design, each day representing a block. Multiple comparisons of individual means were tested by Fisher's protected least significant difference method (Snedecor and Cochran, 1980).

RESULTS AND DISCUSSION

The highest concentrations of phenolics were obtained by immediate extraction of fresh leaves, which may be considered as a control treatment (Tables 1 and 2). Storing the leaves frozen at −18 °C for 12 days resulted in consistently lower concentrations of phenolics than immediate extraction, although the difference was significant only for chlorogenic acid (Table 2). With few exceptions, the concentrations of phenolics in drying treatments were lower than in extractions of fresh leaves (methods 7 and 8).

Except for Z_1 and myricetin 3-galactoside, analysis of variance showed significant differences among the methods for all of the compounds analyzed. The results for individual quercetin glycosides were almost identical. Within a group of phenolics, i.e. flavonoid glycosides or hydroxycinnamic acid derivatives, individual compounds responded somewhat similarly to the tested methods.

In oven-drying, the concentration of flavonoid glycosides was significantly higher at 80 °C than at 40 °C (Table 1). In contrast, the yields of other phenolic

Table 2. Effect of Sample Preparation Method on Concentration[a] of Nonflavonoid Phenolics[b]

sample preparation method	Z_1	HCA_1[c]	HCA_2[c]	HCA_3[c]	chlorogenic acid	total HCA[d]
1, air-drying	7.84 ± 0.33	0.194 ± 0.014	0.432 ± 0.016	0.185 ± 0.011	0.226 ± 0.007	1.038 ± 0.027ab
2, oven-drying at 40 °C	7.40 ± 0.29	0.200 ± 0.010	0.433 ± 0.010	0.181 ± 0.010	0.227 ± 0.006	1.040 ± 0.018ab
3, oven-drying at 80 °C	7.35 ± 0.24	0.182 ± 0.009	0.415 ± 0.012	0.184 ± 0.008	0.214 ± 0.006	0.995 ± 0.015a
4, freeze-drying (N_2)[e]	7.52 ± 0.27	0.177 ± 0.006	0.414 ± 0.013	0.180 ± 0.006	0.222 ± 0.009	0.992 ± 0.021a
5, freeze-drying (−18 °C)[f]	7.76 ± 0.29	0.206 ± 0.009	0.438 ± 0.013	0.190 ± 0.009	0.221 ± 0.006	1.054 ± 0.033ab
6, freeze-drying (unfrozen)[g]	7.79 ± 0.38	0.195 ± 0.010	0.457 ± 0.020	0.192 ± 0.010	0.234 ± 0.015	1.079 ± 0.041b
7, fresh frozen	7.94 ± 0.35	0.227 ± 0.010	0.466 ± 0.018	0.212 ± 0.011	0.240 ± 0.010	1.146 ± 0.036bc
8, fresh	8.23 ± 0.28	0.234 ± 0.015	0.477 ± 0.017	0.220 ± 0.010	0.262 ± 0.006	1.193 ± 0.029c
p	0.125	0.000	0.028	0.001	0.002	0.000
lsd[h]		0.019	0.042	0.021	0.022	0.084

[a] Expressed as mg/g of dry weight; hydroxycinnamic acids quantified as chlorogenic acid, Z_1 as picein. [b] Data are means ± SE, $n = 7$. [c] Hydroxycinnamic acid. [d] The sum of chlorogenic acid and unidentified hydroxycinnamic acids; means not sharing the same letter differ at $p < 0.05$. [e] Prefreezing with liquid N_2. [f] Prefreezing at −18 °C. [g] Freeze-dried without prefreezing. [h] Least-significance difference.

compounds were consistently lower by drying at 80 °C than at 40 °C, although the differences were not significant. Drying at temperatures above 60 °C is usually considered to be too extreme for phytochemical studies (Julkunen-Tiitto, 1985; Waterman and Mole, 1994). Drying at 80 °C may thus have induced reactions leading, for example, to oxidative condensation or decomposition of thermolabile compounds, such as (+)-catechin. However, common flavonoid glycosides are thought to be relatively thermostable, and rapid drying even at about 100 °C has been recommended (Markham, 1982). On the other hand, thermal denaturation of enzymes usually begins at about 40−50 °C (Taiz and Zeigler, 1991). The differences between the oven-drying methods could thus be explained by the combination of two factors: slower rate of enzyme inactivation at 40 °C and differences in thermostability of the phenolic compounds present in birch leaves.

In freeze-drying, the material being dried is usually prefrozen (Flink and Knudsen, 1983). In this study, three methods of freeze-drying were compared, two of which involved prefreezing of plant material: quick freezing in liquid N_2 (method 4), and slow freezing at −18 °C (method 5). In addition, leaves were placed in the freeze-dryer without being frozen first (method 6), as suggested by Waterman and Mole (1994).

Among freeze-drying methods, the highest concentrations of phenolics were obtained by freeze-drying of slowly frozen leaves (method 5), although the difference between methods 5 (slow freezing) and 4 (quick freezing) was significant only for HCA_1 (Table 2). Slow freezing gives rise to larger ice crystals than rapid freezing (Flink and Knudsen, 1983), which may cause a more complete rupture of cell structure. Thus, better solvent access during the short extraction could partly explain differences between the methods.

Freeze-drying without prefreezing the leaves (method 6) resulted in significantly lower concentrations of flavonoid glycosides than method 5 (Table 1). This could be partly due to enzymatic activity, because in leaves placed directly into the freeze-dryer it is allowed to continue, albeit only for a limited time. However, for phenolics other than flavonoid glycosides, there were no significant differences between methods 5 and 6. On the other hand, concentrations of (+)-catechin and HCA_2 were significantly higher by freeze-drying without prefreezing (method 6) than by freeze-drying of leaves frozen in liquid N_2 (method 4).

Because of its simplicity, air-drying is often applied when large numbers of samples need to be analyzed. Because the drying process is slow at ambient temperature, metabolic processes may continue longer than in other methods tested. Thus, it was of interest to examine whether simple air-drying is inferior to more complicated methods of drying. In this study, oven-drying at 40 or 80 °C did not show any advantage over air-drying at ambient temperature. The only drying method significantly better than air-drying was freeze-drying of slowly frozen leaves (method 5), which resulted in highest phenolic concentrations of all the drying methods tested.

Our results suggest that for quantitative analysis of birch leaf phenolics, the samples should be analyzed immediately. Alternatively, samples may be stored fresh frozen, at least for a short time. Freeze-drying of leaves frozen at −18 °C is preferred as a drying method. Oven-drying at either of the tested temperatures (40 and 80 °C) did not prove a better alternative to freeze-drying. Higher concentrations of phenolics might be achieved by drying at some intermediate oven temperature with faster enzyme inactivation than at 40 °C. If the samples must be dried at low temperature to avoid thermal damage, a method of enzyme inactivation, such as a short initial drying period at higher temperature or microwaving, should be tested.

Although there were significant differences among methods in absolute concentrations of phenolics, none of the methods altered the phenolic profile of birch leaves dramatically. The choice of the method depends thus on the aims of the research and the equipment available. For comparative purposes, all of the methods tested may be considered acceptable, if used consistently. Nevertheless, to examine changes associated with drying, a few samples of undried tissue should also be analyzed.

ACKNOWLEDGMENT

We thank Sinikka Sorsa for skillful assistance in laboratory work.

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Received for review March 13, 1996. Revised manuscript received June 12, 1996. Accepted June 13, 1996.[⊗] The study was partly supported by the Finnish Cultural Foundation and Emil Aaltonen Foundation.

JF960168X

[⊗] Abstract published in *Advance ACS Abstracts*, August 1, 1996.