Comparison of Methods for the Extraction of Flavonoids from Birch Leaves (*Betula pendula* Roth.) Carried Out Using High-Performance Liquid Chromatography

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The efficiency of commonly used methods and solvents for the extraction of flavonoids from dried birch leaves was compared, and the responses of individual compounds were determined. The three methods compared were (1) a short extraction with a clipping homogenizer in an unheated solvent, (2) refluxing, and (3) Soxhlet extraction. The solvents tested (pure and 80% aqueous solvent) were acetone, ethanol, and methanol. In general, the aqueous solvents were superior to the nonaqueous solvents. Methanol was the most efficient nonaqueous solvent, and in refluxing and Soxhlet extraction, the yield of flavonoid glycosides was comparable to that in aqueous methanol. Soxhlet extraction and refluxing were found to be efficient methods with several solvents. Refluxing was more efficient than homogenizing, but neither method revealed any significant differences among the aqueous solvents. The responses of individual compounds were fairly similar, except in procedures resulting in the lowest yields. Refluxing in nonaqueous methanol is recommended as the most convenient exhaustive extraction method for flavonoid glycosides. If the use of a heated solvent is undesirable, homogenizing in aqueous ethanol is recommended as the best compromise between safety and efficiency.

INTRODUCTION

Silver birch (*Betula pendula* Roth.) supplies a valuable raw material to the Finnish plywood and cellulose industry. However, birches have been cultivated on a small scale because of their susceptibility to damage by mammalian herbivory (Rousi, 1990). In recent years, the chemical defense of birch species against herbivory has been the subject of several studies (Bryant, 1981; Reichardt et al., 1984; Palo, 1987; Bryant et al., 1989; Iason and Palo, 1991; Vainiotalo et al., 1991; Tahvanainen et al, 1991; Rousi et al., 1993). The growing interest in the secondary chemistry of birches and the possibility of resistance breeding (Rousi, 1990) has emphasized the need for a better understanding of birch phytochemical variability and taxonomy.

Foliar and bud flavonoids have proved to be good taxonomic indicators of birch species (Hänsel and Hörhammer, 1954; Wollenweber, 1975; Pawlowska, 1983). The leaves of *B. pendula* contain the following flavonoid glycosides: quercetin 3-rutinoside (rutin), quercetin 3-galactoside (hyperin), quercetin 3-glucuronide, quercetin 3-arabinopyranoside, quercetin 3-arabinofuranoside, quercetin 3-rhamnoside (quercitrin), and myricetin 3-galactoside (Dallenbach-Tölke et al., 1986). The presence of myricetin 3-digalactoside, several minor flavonoid glycosides, methylated flavones (acacetin and apigenin 7,4′-dimethyl ether), and various phenolic acids has also been reported (Hänsel and Hörhammer, 1954; Pawlowska, 1980).

The study of phytochemical variation in birches at different levels of taxonomic hierarchy requires a large number of samples. However, earlier surveys have been based on only a few individuals of each taxon, and differences between geographical origins have not been investigated. In addition, recent studies using high-performance liquid chromatography (HPLC) have revealed the presence of several new flavonoid glycosides in birch leaves (*B. pendula*, *B. pubescens*) (Dallenbach-Tölke et al., 1986, 1987). Consequently, the results of earlier studies need to be confirmed by modern chromatographic methods.

The use of HPLC has made it possible to quantify flavonoid glycosides accurately and routinely. Accordingly, Harborne and Williams (1988) have suggested that in phytochemical investigations, more effort should be made to determine (a) the total concentration of flavonoid glycosides and (b) the percentage of the different glycosides relative to that total concentration. Hence, it may be possible to distinguish between taxa with qualitatively identical flavonoid profiles if there are quantitative differences. Such measurements have provided useful information in chemotaxonomic studies (Harborne et al., 1985) and cultivar "fingerprinting" (Van Sumere et al., 1985). Preliminary studies of the flavonoid profiles of various birch species (M. Keinänen, unpublished results) indicate that it may not be possible to distinguish certain taxa qualitatively. However, the determination of the relative composition of flavonoid glycosides might still prove useful.

The utilization of quantitative flavonoid data in taxonomic studies underlines the importance of appropriate methodologies because inconsistent sampling and extraction practices can easily affect the phenolic composition of the material studied. Quantitative extraction of phenolic compounds is difficult, and chemical changes may occur during extraction (Ribéreau-Gayon, 1972). In addition, because of the variation in the structure and the solubility of flavonoid glycosides, different extraction procedures may selectively favor the yield of one glycoside over another.

Several methods and solvents have been used in the extraction of flavonoid glycosides (e.g., Markham, 1989), but no single procedure has proved superior to others. The purpose of this study was to compare the efficiency of commonly used extraction methods and solvents in the extraction of flavonoids from dried birch leaves and to determine the responses of individual compounds to different procedures. The three extraction methods compared were (1) a short extraction with a clipping homogenizer in an unheated solvent, (2) refluxing, and (3) Soxhlet extraction. The tested solvents were acetone,

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ethanol, and methanol. The effect of both pure and 80% aqueous solvent on flavonoid extractability was tested.

EXPERIMENTAL PROCEDURES

Apparatus. A Hewlett-Packard HPLC system consisting of a quaternary solvent delivery system (HP 1050), an autosampler (HP 1050), and a photodiode array detector (HP 1040A) coupled with an analytical workstation (HP 79994A) was used. The absorption spectra of the compounds were recorded between 210 and 400 nm. The compounds were separated on a 60-mm × 4.6-mm i.d. HP Hypersil ODS II (3-μm) column. The elution solvents were A (aqueous 1.8% tetrahydrofuran + 0.25% *o*-phosphoric acid) and B (100% methanol). The samples were eluted according to the following gradient: 0–5 min, 100% A; 5–10 min, 0–15% of B in A; 10–20 min, 15–30% of B in A; 20–30 min, 30–35% of B in A; 30–40 min, 35–50% of B in A; and 40–45 min 50% B in A. The gradient system has been used for phenolic glycosides and flavonoids of willows (Meier et al., 1988). The flow rate was 2 mL/min and the injection volume 20 μL. The analysis was simultaneously monitored at 220, 230, and 270 nm.

Materials. Rutin, hyperin, quercetin 3-arabinopyranoside, quercitrin, myricitrin (myricetin 3-rhamnoside), and acacetin (apigenin 4′-methyl ether) standards were obtained from C. Roth (Karlsruhe, Germany); (+)-catechin and chlorogenic acid were obtained from Aldrich-Chemie (Steinheim, Germany). HPLC-grade methanol (Lab-Scan, Dublin, Ireland) was used. The acetone (Merck, Darmstadt, Germany) was of analytical reagent quality. The absolute ethanol was purchased from Oy Alko Ab (Finland).

Sample Preparation. A composite sample of *B. pendula* leaves was collected in August 1991 from stands growing on a clear felling near Joensuu, Finland. Current-year-growth twigs (20) were collected from the upper half of 30 individuals, 1.5–3.0 m in height. The twigs were air-dried at room temperature on a laboratory table for 3 weeks. After drying, the leaves were separated from the shoots and milled. The powdered material was stored in an air-tight glass container at −20 °C until used.

Extraction. A sample (250 mg) of dried material was extracted with three different methods (homogenizing, refluxing, and Soxhlet extraction) using pure and 80% (v/v) aqueous methanol, ethanol, and acetone as solvents.

Method I. The sample was extracted twice with 25 mL of solvent using an Ultra-Turrax homogenizer for 3 min. The extract was filtered, and the residue was washed with 20 mL of solvent. The combined extract was evaporated to dryness.

Method II. The sample was refluxed with 25 mL of solvent for 30 min. The extract was then treated as in method I.

Method III. The sample was extracted in a Soxhlet apparatus for 18 h. The solvent (3 × 70 mL) was changed after 3 and 6 h. The extracts were evaporated to dryness.

Five replicate extractions were processed with each method–solvent combination. All evaporations were performed on a rotary evaporator under vacuum at 40 °C. Before analysis, the extract was dissolved in water–methanol (1:1 v/v).

Identification of Birch Leaf Phenolics. A chromatogram of *B. pendula* leaf extract is shown in Figure 1. The identification of (+)-catechin, chlorogenic acid, hyperin, quercetin 3-arabinopyranoside, and quercitrin was based on comparisons of the retention and the spectral characteristics of the corresponding peaks with those of the standards. Quercetin 3-glucuronide and quercetin 3-arabinofuranoside were tentatively identified by their characteristic UV–vis spectra and relative elution order (Harborne and Boardley, 1984; Dallenbach-Tölke et al., 1987). The spectra of peaks 4 and 5 were characteristic of a myricetin 3-glycoside. Peak 4 (M_1) consisted of at least two overlapping compounds with similar spectral characteristics, one of which could be myricetin 3-galactoside, previously reported from *B. pendula* (Dallenbach-Tölke et al., 1987). The glycoside corresponding to peak 5 (M_2) coeluted with myricitrin, but its spectrum did not exhibit the characteristic hypsochromic shift of a flavonol 3-rhamnoside (Harborne, 1964). The compound corresponding to peak 11 was tentatively identified as a methylated apigenin derivative. It coeluted with acacetin, but its spectrum resembled that of apigenin 7,4′-dimethyl ether (Wollenweber, 1974; Voirin, 1983). The spectrum of peak 1 (Z_1) resembled that of 4-hydroxyacetophenone or its glucoside (picein). A few minor peaks with characteristics of cinnamic acid and flavonoid spectra were also detected. The analyzed compounds were relatively quantified using salicin as the reference compound.

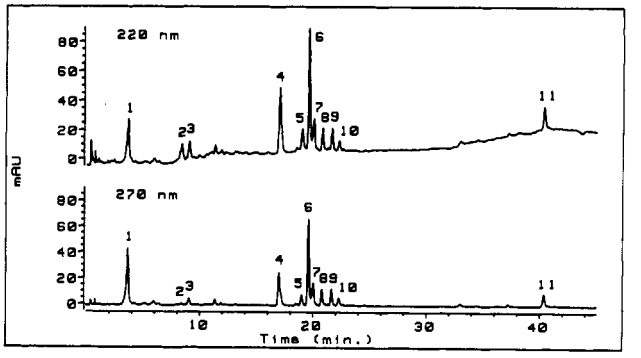

Figure 1. HPLC chromatogram of *B. pendula* leaf sample extracted in Soxhlet apparatus with 100% methanol. Peaks: (1) Z_1 (unknown), (2) (+)-catechin, (3) chlorogenic acid, (4) myricetin 3-glycoside (M_1), (5) myricetin 3-glycoside (M_2), (6) hyperin, (7) quercetin 3-glucuronide, (8) quercetin 3-arabinopyranoside, (9) quercetin 3-arabinofuranoside, (10) quercitrin, and (11) apigenin derivative.

Statistical Analysis. The results were analyzed by a three-way analysis of variance. The statistical model was a 3 × 3 × 2 factorial of the extraction method, the solvent, and the use of aqueous or nonaqueous solvent. Multiple comparisons of individual means and levels of factors were tested by Tukey's honestly-significant-difference method (Sokal and Rohlf, 1981).

RESULTS

The yield of analyzed compounds (milligrams of salicin per gram of dry weight) using each method–solvent combination is shown in Table I. The differences between methods and solvents in the yield of flavonoid glycosides were more pronounced with nonaqueous solvents than with aqueous solvents. In general, aqueous solvents were superior to nonaqueous solvents. Water improved especially the efficiency of the weakest solvent, pure acetone. Methanol was the most efficient nonaqueous solvent, and in refluxing and Soxhlet extraction, the yield of flavonoid glycosides was comparable to that in aqueous methanol.

Soxhlet extraction was the most efficient extraction method with almost all the tested solvents. In extractions using alcoholic solvents and pure acetone, Soxhlet extraction and refluxing were superior to homogenizing. In contrast, when aqueous acetone was used, the yield of flavonoid glycosides was smaller in Soxhlet extraction than in refluxing ($P = 0.001$) or homogenizing ($P = 0.050$). Refluxing was more efficient than homogenizing, but there were no significant differences among aqueous solvents with either extraction method.

With few exceptions, individual compounds responded similarly to different factors. The choice of a specific procedure was less critical in extracting the apigenin derivative (peak 11, Figure 1) than in extracting flavonoid glycosides or other phenolics (Table I). The yield of the apigenin derivative differed significantly only between extraction methods (three-way analysis of variance, $P = 0.006$); refluxing and Soxhlet extraction were more efficient than homogenizing. The yield of (+)-catechin was smaller in Soxhlet extraction than in refluxing, although in extractions using nonaqueous solvent, the difference was not significant ($P > 0.05$; aqueous solvents, $P = 0.007$). The response of chlorogenic acid and Z_1 (peak 1, Figure 1) to extraction procedures resembled that of flavonoid glycosides.

Table II shows the responses of individual flavonoid glycosides to extraction methods and solvents. The

Table I. Influence of Extraction Procedures on the Yield[a] of Phenolic Compounds[b]

extraction procedure	flavonoid glycosides[c]	apigenin derivative[d]	(+)-catechin	Z_1[e]	chlorogenic acid	total phenolics[f]
acetone						
homogenizer	19.2 ± 1.11	6.44 ± 0.39	0.49 ± 0.33	1.30 ± 0.03	0.39 ± 0.12	27.8 ± 0.40
reflux	40.4 ± 4.34	7.12 ± 1.01	2.96 ± 0.25	6.30 ± 0.34	2.05 ± 0.24	58.9 ± 2.76
Soxhlet	52.2 ± 1.35	7.13 ± 0.68	2.93 ± 0.48	9.33 ± 0.28	2.95 ± 0.10	74.5 ± 0.70
ethanol						
homogenizer	41.4 ± 1.63	5.74 ± 0.43	1.41 ± 0.46	4.78 ± 0.33	1.75 ± 0.12	55.0 ± 1.00
reflux	64.6 ± 1.69	6.69 ± 0.83	3.96 ± 0.24	13.65 ± 0.15	4.26 ± 0.14	93.1 ± 1.24
Soxhlet	72.0 ± 1.36	6.35 ± 0.40	3.53 ± 0.21	14.12 ± 0.45	4.35 ± 0.14	100.4 ± 0.67
methanol						
homogenizer	57.8 ± 2.24	6.87 ± 0.96	3.65 ± 0.34	12.24 ± 0.10	3.69 ± 0.07	84.3 ± 1.57
reflux	77.3 ± 2.74	6.70 ± 1.20	4.86 ± 0.19	14.25 ± 0.07	5.84 ± 0.03	109.0 ± 0.93
Soxhlet	80.4 ± 2.53	6.84 ± 0.82	4.59 ± 0.52	14.50 ± 0.73	5.02 ± 0.12	111.4 ± 1.64
80% acetone						
homogenizer	73.3 ± 4.15	6.09 ± 0.74	3.66 ± 0.32	13.53 ± 0.09	5.84 ± 0.13	102.4 ± 1.84
reflux	79.2 ± 3.09	5.94 ± 0.66	4.99 ± 0.68	14.18 ± 0.49	5.60 ± 0.26	109.9 ± 2.01
Soxhlet	67.8 ± 2.24	7.21 ± 0.55	3.98 ± 0.29	14.17 ± 0.20	4.87 ± 0.09	98.1 ± 1.43
80% ethanol						
homogenizer	71.1 ± 2.33	6.08 ± 0.63	3.57 ± 0.33	13.54 ± 0.22	5.62 ± 0.06	99.9 ± 1.12
reflux	79.2 ± 1.11	6.73 ± 0.26	4.41 ± 0.92	13.73 ± 0.33	5.62 ± 0.24	109.7 ± 0.78
Soxhlet	82.5 ± 2.17	6.56 ± 0.32	4.19 ± 0.45	13.36 ± 0.18	6.37 ± 0.08	114.0 ± 1.27
80% methanol						
homogenizer	71.1 ± 3.50	5.83 ± 0.79	4.26 ± 0.26	14.75 ± 0.22	4.93 ± 0.03	100.9 ± 2.15
reflux	77.8 ± 2.46	6.65 ± 0.54	4.62 ± 0.92	13.39 ± 0.31	5.47 ± 0.11	107.9 ± 1.35
Soxhlet	83.3 ± 2.60	6.25 ± 0.22	3.83 ± 0.39	13.50 ± 0.11	6.25 ± 0.16	113.1 ± 0.91

[a] Relatively quantified as mg of salicin/g of dry wt. [b] Data are means ± SE, $n = 5$. [c] The sum of flavonoid glycosides. [d] Peak 11, Figure 1. [e] See Figure 1. [f] Total phenolics = the sum of analyzed compounds.

Table II. Influence of Extraction Procedures on the Relative Amount (Percent)[a] of Flavonoid Glycosides[b]

extraction procedure	myricetin 3-*O*-		quercetin 3-*O*-				
	gly (M_1)[c]	gly (M_2)[c]	gal[c]	glur[c]	ara(p)[c]	ara(f)[c]	rha[c]
acetone							
homogenizer	17.8 ± 0.5	8.2 ± 0.3	44.6 ± 0.2	7.5 ± 0.3	7.4 ± 0.4	9.9 ± 0.2	4.7 ± 0.3
reflux	23.3 ± 0.8	8.2 ± 0.3	40.4 ± 0.9	8.3 ± 0.6	7.8 ± 0.4	8.0 ± 0.2	4.0 ± 0.2
Soxhlet	26.5 ± 0.3	7.4 ± 0.1	37.3 ± 0.4	10.4 ± 0.3	7.5 ± 0.2	7.2 ± 0.1	3.6 ± 0.1
ethanol							
homogenizer	26.8 ± 0.6	7.4 ± 0.2	38.4 ± 0.4	10.7 ± 0.3	6.7 ± 0.4	7.1 ± 0.2	2.9 ± 0.1
reflux	27.4 ± 0.2	7.4 ± 0.2	38.3 ± 0.2	9.6 ± 0.3	6.7 ± 0.1	7.1 ± 0.1	3.6 ± 0.1
Soxhlet	28.3 ± 0.3	7.5 ± 0.2	36.6 ± 0.3	10.6 ± 0.1	6.8 ± 0.3	6.8 ± 0.3	3.4 ± 0.3
methanol							
homogenizer	30.8 ± 0.8	7.3 ± 0.1	35.5 ± 0.2	11.3 ± 0.3	6.2 ± 0.2	6.2 ± 0.2	2.7 ± 0.1
reflux	27.8 ± 0.8	7.2 ± 0.2	37.2 ± 0.2	11.7 ± 0.2	6.5 ± 0.2	6.5 ± 0.2	3.2 ± 0.1
Soxhlet	29.2 ± 0.4	6.9 ± 0.1	35.3 ± 0.7	12.6 ± 0.3	6.9 ± 0.3	6.1 ± 0.1	2.9 ± 0.2
80% acetone							
homogenizer	29.5 ± 0.6	5.8 ± 0.1	37.4 ± 0.4	12.7 ± 0.6	6.1 ± 0.2	6.0 ± 0.2	2.6 ± 0.2
reflux	29.8 ± 0.4	6.7 ± 0.2	34.8 ± 0.5	13.1 ± 0.3	6.3 ± 0.2	6.4 ± 0.1	2.9 ± 0.1
Soxhlet	28.7 ± 0.3	7.2 ± 0.1	36.3 ± 0.3	11.3 ± 0.2	6.9 ± 0.2	6.7 ± 0.1	2.9 ± 0.1
80% ethanol							
homogenizer	30.4 ± 0.5	6.9 ± 0.3	34.8 ± 0.5	12.1 ± 0.6	6.2 ± 0.3	6.4 ± 0.1	3.3 ± 0.3
reflux	29.6 ± 0.1	6.9 ± 0.1	34.5 ± 0.4	12.9 ± 0.4	6.6 ± 0.1	6.4 ± 0.1	3.1 ± 0.1
Soxhlet	29.6 ± 0.5	6.8 ± 0.0	35.5 ± 0.2	13.3 ± 0.2	6.3 ± 0.2	5.9 ± 0.1	2.7 ± 0.1
80% methanol							
homogenizer	30.7 ± 0.3	6.6 ± 0.1	35.6 ± 0.3	12.0 ± 0.3	6.2 ± 0.2	6.2 ± 0.1	2.6 ± 0.1
reflux	29.4 ± 0.1	6.8 ± 0.1	34.6 ± 0.5	13.5 ± 0.2	6.7 ± 0.1	6.2 ± 0.2	2.8 ± 0.2
Soxhlet	29.2 ± 0.2	6.6 ± 0.1	35.5 ± 0.1	13.6 ± 0.1	6.3 ± 0.2	5.9 ± 0.1	2.8 ± 0.2

[a] Percent of total flavonoid glycosides. [b] Data are means ± SE, $n = 5$. [c] gly = glycoside, gal = galactoside, glur = glucuronide, ara(p) = arabinopyranoside, ara(f) = arabinofuranoside, rha = rhamnoside; for M_1 and M_2, see Figure 1.

profiles of different procedures are fairly similar, except in procedures resulting in the lowest yields (i.e., mainly in extractions using pure acetone). However, the profiles of nonaqueous and aqueous solvents are slightly different. The proportions of quercetin 3-glucuronide and M_1 are higher in extractions with aqueous solvents. With most solvents, the proportion of quercetin 3-glucuronide is also higher in Soxhlet extractions and refluxing than in homogenization. In successive Soxhlet extractions, the proportion of quercetin 3-glucuronide and M_1 increased with most solvents (Table III). These changes in the quantitative flavonoid profile are most apparent in complete extractions (i.e., by using aqueous alcohols or pure methanol).

DISCUSSION

The choice of the extraction procedure for flavonoid glycosides depends on whether the purpose is to extract the glycosides quantitatively or merely to determine the glycoside composition. The glycosidic link is relatively labile, and glycosides inevitably undergo a certain amount of degradation during their isolation (Harborne and Williams, 1975). Consequently, quantitative extraction enabled by high temperatures or extended extraction periods is possibly accomplished at the cost of the degradation of the more labile compounds. For this reason, Soxhlet extraction is not recommended for flavonoid glycosides by Ribéreau-Gayon (1972) and Swain (1976).

Table III. Relative Amount (Percent)[a] of Flavonoid Glycosides in Successive Soxhlet Extractions[b]

	myricetin 3-*O*-		quercetin 3-*O*-					
solvent[c]	gly (M_1)[d]	gly (M_2)[d]	gal[d]	glur[d]	ara(p)[d]	ara(f)[d]	rha[d]	total flavonoid glycosides[e]
acetone								
I	24.2 ± 0.5	7.8 ± 0.1	39.7 ± 0.4	8.7 ± 0.3	8.1 ± 0.2	7.7 ± 0.1	3.9 ± 0.1	41.2 ± 1.00
II	36.0 ± 0.4	6.2 ± 0.2	28.2 ± 0.6	17.4 ± 0.4	5.3 ± 0.3	4.9 ± 0.3	2.0 ± 0.2	4.4 ± 0.43
III	34.5 ± 0.8	6.2 ± 0.4	28.2 ± 0.7	17.1 ± 0.4	5.6 ± 0.2	5.8 ± 0.4	2.6 ± 0.3	6.5 ± 0.37
ethanol								
I	28.2 ± 0.3	7.9 ± 0.2	37.2 ± 0.3	9.1 ± 0.1	7.0 ± 0.3	7.0 ± 0.3	3.5 ± 0.3	62.1 ± 0.37
II	30.2 ± 0.2	5.6 ± 0.4	30.6 ± 0.5	20.1 ± 0.5	5.5 ± 0.1	5.5 ± 0.1	2.5 ± 0.1	4.7 ± 0.16
III	28.4 ± 0.3	4.9 ± 0.3	34.4 ± 0.7	18.8 ± 0.3	5.7 ± 0.2	5.2 ± 0.1	2.6 ± 0.2	5.2 ± 0.23
methanol								
I	29.0 ± 0.4	7.1 ± 0.1	36.0 ± 0.7	11.5 ± 0.3	7.1 ± 0.3	6.3 ± 0.1	3.0 ± 0.2	78.3 ± 1.15
II	36.2 ± 1.2	0.9 ± 0.6	11.2 ± 0.7	50.5 ± 1.7	1.2 ± 0.5	0	0	1.2 ± 0.04
III	38.2 ± 1.4	0	6.4 ± 0.7	55.4 ± 0.8	0	0	0	0.9 ± 0.05
80% acetone								
I	28.7 ± 0.3	7.3 ± 0.1	36.7 ± 0.2	10.8 ± 0.2	7.0 ± 0.2	6.6 ± 0.1	2.9 ± 0.1	57.9 ± 0.97
II	29.1 ± 0.4	6.2 ± 0.7	31.6 ± 1.3	17.0 ± 1.4	6.5 ± 0.5	7.2 ± 0.5	2.5 ± 0.4	3.6 ± 0.12
III	28.5 ± 0.3	7.2 ± 0.2	35.2 ± 0.7	11.7 ± 0.4	6.6 ± 0.2	7.5 ± 0.4	3.3 ± 0.3	6.4 ± 0.08
80% ethanol								
I	29.6 ± 0.5	6.8 ± 0.0	35.6 ± 0.2	13.2 ± 0.2	6.3 ± 0.2	5.9 ± 0.1	2.7 ± 0.1	82.3 ± 0.99
II	44 ± 2.1	0	13 ± 4.9	42 ± 3.9	0	0	0	0.1 ± 0.03
III	+	0	0	+	0	0	0	+
80% methanol								
I	29.1 ± 0.2	6.6 ± 0.1	35.6 ± 0.1	13.5 ± 0.1	6.4 ± 0.2	5.9 ± 0.1	2.8 ± 0.2	83.0 ± 1.20
II	54 ± 6.4	0	10 ± 4.2	34 ± 3.6	0	0	0	0.1 ± 0.02
III	+	0	0	+	0	0	0	+

[a] Percent of total flavonoid glycosides. [b] Data are means ± SE, $n = 5$; +, trace amounts. [c] I, II, III = extractions for 3, 3, and 12 h, respectively. [d] gly = glycoside, gal = galactoside, glur = glucuronide, ara(p) = arabinopyranoside, ara(f) = arabinofuranoside, rha = rhamnoside; for M_1 and M_2, see Figure 1. [e] Relatively quantified as mg of salicin/g of dry wt.

Selective decomposition could be partly responsible for the larger proportion of quercetin 3-glucuronide obtained by more effective extraction methods (refluxing and Soxhlet extraction, Table II) and its increase in successive Soxhlet extractions (Table III). Quercetin 3-glucuronide is known to be resistant to chemical hydrolysis in acid–alcohol mixtures, whereas 3-galactosides and especially 3-rhamnosides and 3-arabinosides are more easily degraded (Harborne, 1965). However, the degradation of flavonoid glycosides to the corresponding aglycones was not detected. In addition, there were no significant differences in the extractability of the two quercetin 3-arabinosides, although degradation of the less stable furanose form may have occurred during Soxhlet extraction (Table II). Therefore, it seems likely that the differences observed in the flavonoid profiles between the extraction procedures are mostly due to the varying solubility of the glycosides.

Aqueous solvents have proved to be effective in the extraction of phenolic compounds, especially from dried plant material (e.g., Harborne, 1984). However, the presence of water may have certain disadvantages. Refluxing in water is deleterious for small molecular weight phenolic glycosides (Steele et al., 1969), and the use of a nonaqueous solvent is thought to reduce their chemical hydrolysis (Lindroth and Pajutee, 1987). Flavonoid glycosides are commonly extracted with aqueous solvents, and the extract is reduced to an aqueous residue or to dryness by heat-assisted vacuum rotary evaporation. Most flavonoids and their glycosides are usually considered to be stable under these conditions (Swain, 1976; Markham, 1982).

In this study, detrimental effects due to the presence of water were not detected, except in the Soxhlet extraction of (+)-catechin. Condensed tannins, which consist of catechins, are thermally labile and susceptible to oxidation (Walker, 1975; Karchesy et al., 1989; Porter, 1989). Oxidative reactions induced by high temperatures, such as nonenzymic condensation and polymerization, could thus account for the low yield of (+)-catechin in the Soxhlet extraction (Table I). The differences between aqueous and nonaqueous solvents may be explained by the enhancement of these processes due to the presence of water.

The use of aqueous solvents in the extraction of flavonoid glycosides from dried birch leaves was clearly advantageous, although good results can also be obtained with nonaqueous methanol. Nonaqueous ethanol and acetone proved to be poor solvents for the phenolic compounds from dried birch leaves. In the extraction of phenolic compounds, methanol or mixtures of methanol and ethanol (1:1, v/v) are often preferred to ethanol (Van Sumere et al., 1985). However, there were no significant differences between the aqueous alcohols, as was found also by Sauvesty et al. (1992) in the extraction of phenolic compounds from maple leaves. Aqueous acetone was as good as aqueous alcohols in refluxing and homogenizing but was a less suitable solvent for Soxhlet extraction.

Soxhlet extraction and refluxing were found to be efficient methods with several solvents. The highest yields were obtained by Soxhlet extraction with aqueous alcohols. On the other hand, although Soxhlet extraction is effective, it is tedious and time-consuming and may be harmful to the more labile compounds, such as (+)-catechin or quercetin 3-arabinofuranoside. When aqueous alcohols and nonaqueous methanol were used, refluxing was only slightly less effective than Soxhlet extraction. Because water is difficult to remove rapidly and completely, refluxing in nonaqueous methanol is recommended as the most convenient exhaustive method for flavonoid glycosides.

If the use of a heated solvent is undesirable, homogenizing in an aqueous solvent or nonaqueous methanol is a relatively rapid and gentle alternative to refluxing. Although nonaqueous methanol was not as efficient as aqueous solvents in homogenization, the relative composition of flavonoid glycosides can be determined reliably. However, the use of an aqueous solvent may increase the solubility of certain glycosides, such as quercetin 3-glucuronide. Because ethanol is a relatively inexpensive and nontoxic solvent, homogenizing in aqueous ethanol is recommended as the best compromise between safety and

efficiency, in accordance with Graham (1991) and Sunnerheim-Sjöberg et al. (1992).

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