mineral nutrition.

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Registry No. LAL, 18810-04-3; CPA, 11075-17-5; EDTA, 60-00-4; Zn, 7440-66-6.

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Phenolic Constituents in the Leaves of Northern Willows: Methods for the Analysis of Certain Phenolics

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Several phenolic components were extracted from willow samples by using the same extraction procedure, purified, and analyzed by spectrophotometry and gas chromatography. The components analyzed were total phenolics, condensed tannins, and phenolic glycosides. Methods for drying the material, extraction, and analysis are tested and discussed.

Higher plants may produce a great variety of secondary phenolic compounds, the role of which in the metabolism of the plant itself has not been adequately explained. Many of these compounds are bound with sugars in living plants. Sugar conjugates of phenolic aglycons may decrease the toxicity or reactivity and increase the solubility of the compounds to make it easier for them to be transported or stored without harm to the plant producing them (Vickery and Vickery, 1981).

In Finland, the genus *Salix* contains about 20 native species and numerous hybridized forms. The secondary chemistry of Salicaceae has been studied for taxonomic purposes because morphological identification is often very difficult. Because certain phenolics are bitter tasting and/or have the ability to precipitate plant and animal proteins, they have been considered as defense compounds against animal predators and microbes [e.g., Markham (1971) and Feeny (1976)].

The present work was carried out as part of an investigation on the relationship between willow feeding herbivores and the secondary chemistry of Salicaceae. The object of this study was to determine the distribution and seasonal variation of certain phenolics and their dependence on fertilization in laboratory and field conditions. This paper describes the methods developed and tested for isolation and characterization of certain phenolic components in *Salix* extracts.

EXPERIMENTAL SECTION

Equipment. For colorimetric determinations a Hewlett-Packard dual-wave spectrophotometer was used and for column chromatography an LKB fraction collector equipped with a UV-detector. The gas chromatograph used to analyze the trimethylsilyl derivatives of willow samples was a Packard 433 equipped with a flame ionization detector. A fused silica SE-52 capillary column 25

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m in length with an inner diameter of 0.32 mm and a phase layer of 0.25 μm was used. The temperature program was started at 190 °C followed immediately by a rise of 8 °C/min to 295 °C. The detector and injector temperatures were 300 and 270 °C, respectively. Nitrogen was used as the carrier gas, a split ratio was 1:20, and the injected volume was 1 μL.

Materials. For testing the methods 3-year-old willow leaves collected from the cultivated stands of *Salix* cv. *aquatica* and *Salix viminalis* and from the native stands of *Salix phylicifolia* and *Salix myrsinifolia* were used. Composite leaf samples were immediately dried after collection in a circulating air oven at 48 °C. For testing, the drying temperature the leaves were dried at room temperature (at 20 °C in the laboratory) and in the oven at 48, 60, and 90 °C. After drying, the samples were milled to dust and stored in plastic containers in a desiccator in a cold room at 4 °C until used. The moisture content of the oven-dried leaves was about 4–6%. For every different extraction three replicate runs were used.

Extraction. A sample of 0.5–3 g of oven-dried and milled leaves was extracted by a Soxhlet extractor with 80% aqueous acetone (300 mL) for 20 h. The solvent was changed 3 times (3 × 100 mL) during the extraction. The combined acetone extracts were concentrated to 30–40 mL in a vacuum circulating evaporator. The residue was refluxed for a few seconds, filtrated hot, and diluted to 100 mL with water. The total phenolics and leucoanthocyanins were determined from the cooled filtrate.

Phenolic glycosides and catechins were fractionally extracted from the filtrate by liquid–liquid extraction with 340 mL of ethyl acetate. The extraction time was 60 h, with several successive solvent changes (with 100 and 3 × 80 mL). The ethyl acetate extract was reduced with the evaporator to dryness and dissolved in a small volume (2–3 mL) of distilled water. The aqueous sample was purified by polyamide column chromatography (Thieme, 1964). The column used was 40 cm high and its inner diameter was 16 mm. Before application of the sample, the column was stabilized with distilled water for 2 h. The sample was eluted through the column at first with water (300 mL) and then with 50% aqueous ethanol (400 mL), monitoring by the UV detector at 280 nm. Water and alcohol eluates were freeze-dried and redissolved in 10 mL of methanol.

Phenolic Glycoside and Catechin Analysis. A gas chromatographic method was used. A 0.2–1 mL aliquot of methanol solution was taken to a Teflon-stoppered vial and methanol concentrated to dryness. The residue was then treated with 0.4 mL of *N,N*-formamide and 0.2 mL of Tri-Sil Z (Pierce Chemical Co.). The vial was shaken vigorously for about a minute and allowed to react for 10–12 h at 4 °C. Silylated samples were found to be stable for 2–3 weeks, after that, numerous extra small peaks could be detected. The standard compounds used were salicin, fragilin, salidrosid, picein, tremuloidin, (+)-catechin, triandrin, salicortin, salireposide, vimalin, populin, and grandidentatin. Arbutin were used as an internal standard, because it has not been found in willows. The identification of phenolic glycosides and (+)-catechin was based on the retention times of the authentic compounds. An electronic integrator was used to quantify the compounds.

Total Phenolic Determination. A certain amount (50–100 μL) of crude extract was diluted with water to 2 mL in a 10-mL measuring flask. One milliliter of Folin–Ciocalteu phenol reagent was added and the flask vigorously shaken. Immediately, 5 mL of 20% sodium carbonate solution was pipetted and the mixture made up to 10 mL, shaking thoroughly again. After 20 min the absorptivity of the mixture was read at 700 and 735 nm without background measurements. The spectrophotometer was set to zero against air. Before the samples were measured, they were centrifuged if necessary because of a precipitate formation.

Leucoanthocyanins Determination. Depending on the species, 0.1–0.5 mL of crude extract was put into homogeneous volumetric flasks, butanol–concentrated HCl (95:5) (Bate-Smith, 1981) was added up to 4 mL, and the flaskware thoroughly shaken. The samples were hydrolyzed for 2 h at 95–98 °C, cooled at room temperature in the dark, and adjusted to 4 mL with butanol–HCl. The absorptivity of red pigments was measured at 550 nm. The background determinations were made in the same way without hydrolyzing the samples. Standard curves were prepared by using commercially available anthocyanidin.

Condensed Tannin Determination with Vanillin–HCl. For these determinations 0.1–0.5 mL of crude extracts were taken and put into tubes covered with aluminum foil. Three milliliters of 4% vanillin (w/v) in methanol (Broadhurst and Jones, 1978) was added, and the tubes were shaken vigorously with a mixer. Immediately after that 1.5 mL of concentrated HCl was pipetted and the tubes were shaken again. The absorbances were read at 500 nm after being allowed to stand for 20 min at room temperature. The results were plotted after a (+)-catechin standard made in the same manner. The interference background of the crude extract was corrected by preparing the test without vanillin.

RESULTS AND DISCUSSION

Sample Preparation and Crude Extraction. Phenolics are usually susceptible to different factors (e.g., acidic solutions and high temperatures) during the extraction process. Thus, every precaution should be taken, as it is essential that the processing scheme used does not cause chemical breakdown of the compounds and gives comparable and reproducible results.

The classical Soxhlet apparatus has used to obtain crude extracts. Powdered materials were continuously extracted, the solvent being changed a few times. The solvent temperature never exceeded 60 °C. After the organic solvent from the combined crude extract was vaporized, it was refluxed for a few seconds. In this process some of the lipid-soluble components was deposited on the walls of the boiling flask. The deposit did not contain any phenolics when screened with the Folin–Ciocalteu test. Soxhlet extraction is a rather effective but quite slow procedure, so the extraction time is very important. Six hours was a long enough extraction time for all of the species screened (Figure 1). During this time over 95% of the phenolics were been extracted from the leaves of three different willow species and an additional 14 h gave only about 5% of total extractable phenolics. The results did not seem to be dependent on the concentration of phenolics in the leaves.

Preferably, fresh plant tissues should be used for analysis (Harborne, 1973). Often this is not possible, and therefore dried material was used. Care should be taken in drying the samples: it should be done quickly by using low enough temperatures to prevent possible chemical changes in the plant material (Harborne, 1973). Drying at room temperature may enhance the enzymic degradation and thus lower the amounts of phenolics in the samples. Drying the willow leaves at 20 °C took 2–4 days, while, in an oven with good ventilation at 48 °C, 5–10 h was required to reach over 95% dry weight content.

I tested the effect of drying temperature on total phenolics and leucoanthocyanins (Figures 2 and 3). Drying

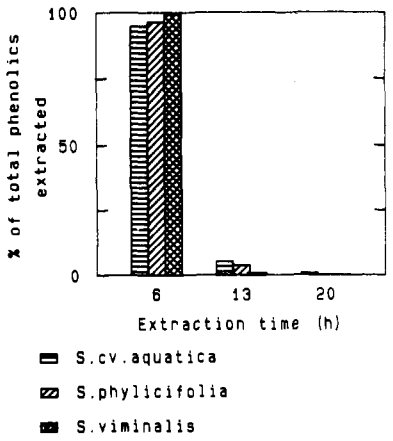

Figure 1. Dependence of total phenolics in the leaves of *Salix* sp. on extraction time. The amounts are mg equiv of phenol determined by the Folin–Ciocalteu phenol reagent.

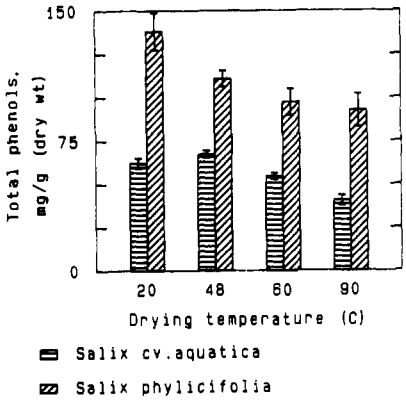

Figure 2. Effect of the drying temperature on total phenolics in leaves of willow twigs (determined as in Figure 1). Vertical bars represent the standard errors.

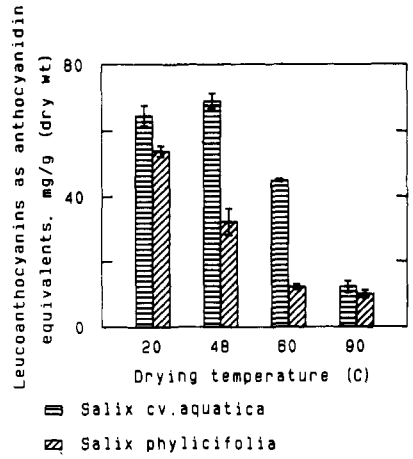

Figure 3. Changes in the level of leucoanthocyanin after drying willow leaves at different temperatures (determined by the BuOH–HCl test; contents plotted against anthocyanidin equivalents). Vertical bars represent the standard errors.

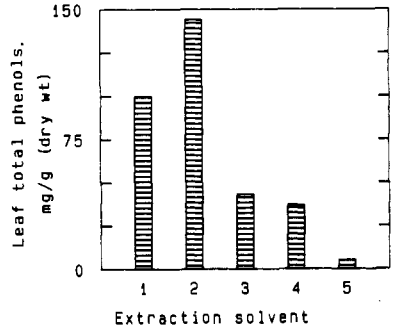

Figure 4. Effect of extraction solvent on total phenolics in the leaves of *Salix* cv. *aquatica* (1 = 80% acetone; 2 = 80% methanol; 3 = distilled water; 4 = 0.2 M NaCl; 5 = diethyl ether). The amounts were determined as in Figure 1.

the willow leaves below 50 °C yielded the highest amounts of total phenolics. Increasing the temperature above 60 °C lowered the phenolic amounts considerably, and using 90 °C for drying gave only about 60% of the amount in *Salix cv. aquatica* leaves obtained below 50 °C. The differences of total phenolics were not as high in *S. phylicifolia*, even though the reproducibility was lower, especially at higher temperatures (SE = 8–10%). The effect of drying on leucoanthocyanins was even stronger than on total phenolics (Figure 3). It is possible that at high temperatures certain phenolics (e.g., polyphenolic condensed tannis) may simply decompose or combine with the other plant component so fast that it is difficult to get free again during the extraction. Also, at higher temperatures some easily volatile phenolics may be lost by volatilizing.

Many phenolic compounds are soluble in polar solvents. The choice of solvents depends on the number of hydroxyl groups and sugars in the molecules. For crude total phenolic extracts, aqueous alcohols (Thieme, 1964; Binns et al., 1968; Pearl and Darling, 1970) and acetone (Steele et al., 1973) have often been used as solvents.

The result of testing different solvents for the phenolics in *Salix cv. aquatica* leaves is shown in Figure 4. Aqueous methanol (50%) (v/v) is rather effective, but it is known to be a rough solvent for certain phenolic compounds, e.g., for glycosides (Steele et al., 1969). Aqueous 80% acetone gives a quite acceptable extraction of the total phenolics. In this research it has been used as the solvent for the first extraction because, unlike other solvents, it does not decompose pure glycosides under the test conditions (Steele et al., 1969) and it may inhibit enzymatic degradation during the extraction. Pure water itself is not a very good solvent. It yields quite a low amount of phenolics and its effect is known to be very deleterious for glycosides. According to Steele et al. (1969), tremuloidin when refluxed in water is almost completely converted to salicin and populin. In water fragilin yielded again salicin and other unknown compounds (Steele et al., 1969). Recovery with diethyl ether is also very low. It has been found to be a usable solvent for low molecular weight phenolic acids (Harborne, 1973). It can be used for the purification of lipid soluble pigments (e.g., chlorophylls) from plant extracts.

When 50% and 80% acetone, and 50% methanol are compared as extracting solvents and their effect on certain phenolic groups in *S. myrsinifolia* leaves is compared (Figure 5), the differences between total phenolics and leucoanthocyanins are the same as in the test shown in Figure 4, except that 50% acetone gives the highest leucoanthocyanin amounts. But if we want to compare single glycosides (Figure 6), we can see the effect of methanol in the proportions of salicin and salicortin. The explanation for this may be either that the solvent systems (acetone or methanol) have different abilities to extract compartmentalized phenols or that salicortin simply decomposes into salicin during extraction with methanol. Salicortin is reported to be a very labile glycoside and is probably the precursor of salicin (Thieme, 1965). The effect of methanol may be the same on other glucosides in willows, especially in the extraction of salicin derivatives (e.g., fragilin). Steele et al. (1969) have shown that tremuloidin refluxed in methanol produces small amounts of salicin and an unknown compound. They have shown that nei-

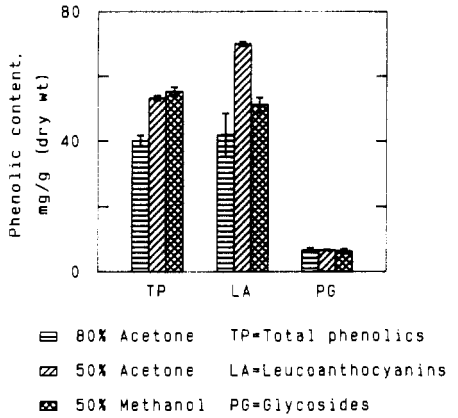

Figure 5. Effect of extraction solvent on the phenolics in the leaves of *S. myrsinifolia* (determined as in Figure 1 and Figure 3). Vertical bars represent the standard errors.

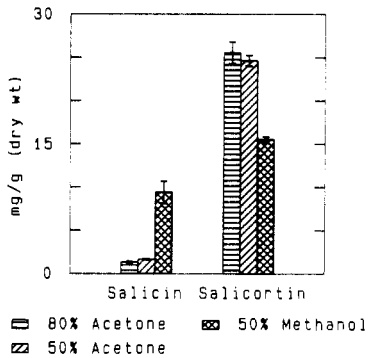

Figure 6. Effect of methanol and acetone as extraction solvents on the glycosides in the leaves of *S. myrsinifolia* (determined by GLC). Vertical bars represent the standard errors.

ther acetone nor ethyl acetate has any detectable effect on tremuloidin.

Total Phenolics and Tannin Determination. There are several methods for total phenolic determinations in plant material [e.g., Singleton and Rossi (1965) and Folin and Denis (1912)]. The most often used methods are based on the ability of phenolics to react with oxidizing agents. I have used the commercially available Folin–Ciocalteu phenol reagent, which contains sodium molybdate and sodium tungstate, 2.5% and 10%, respectively. This reagent, like others, is nonspecific for any phenolics and the color yielded depends on hydroxyl groups and their place in the molecules. But in spite of the fact that phenolic reagents are unspecific, in certain circumstances (e.g., absence of interfering substances) we may get only relative results for phenolics.

When testing Folin–Denis and Folin–Ciocalteu reagents with certain phenolic standards, I found, as Singleton and Rossi (1965) have also reported earlier, that Folin–Ciolalteu reagent produces more intense blue coloration than the very commonly used Folin–Denis reagent. The difference between these reagents was 0.1–0.15 absorbance unit, depending on the standards. It has been shown, too, that Folin–Ciocalteu reagent does not react so readily with interfering reducing substances such as Folin–Denis reagent (Singleton and Rossi, 1965).

It is obviously very difficult to choose suitable standards for total phenolic determinations in plant extracts due to the chemical heterogeneity of plant products and the unspecificity of phenolic reagents. Thus, it is only possible to get relative equivalents with the standards used.

The reactivity of different phenolic standards to Folin–Ciocalteu reagents is shown in Table I. All the standards tested showed an almost linear relationship between absorptivity and standard concentration with the amounts of phenol reagent (1 mL) and sodium carbonate (1 g) used in the 10-mL final volume. Gallic acid and 3,4-dihydroxylbenzoic acid produced the most intense reaction. The substitution of a hydroxyl group in the position two or three in addition to position four considerably increases the sensitivity of the reaction.

Table I. Reactivity of Different Phenolics with Folin–Ciocalteu Phenol Reagent[a]

phenolics	coefficient A	coefficient B	rel intensity compared with gallic acid (=100%)
gallic acid	+0.0568	0.01233	100
3,4-dihydroxybenzoic acid	+0.1520	0.01187	96
ellagic acid	+0.0303	0.01148	93
tannic acid	+0.0455	0.00940	76
2,4-dihydroxybenzoic acid	+0.0171	0.00924	75
3,4-dihydroxycinnamic acid	−0.0628	0.00899	73
(+)-catechin	+0.0710	0.00887	72
phenol	+0.1112	0.00863	70
4-hydroxycinnamic acid	+0.0510	0.00654	53
2,6-dihydroxybenzoic acid	+0.0630	0.00653	53
2,5-dihydroxybenzoic acid	−0.0396	0.00448	36
4-hydroxybenzoic acid	+0.1480	0.00430	35

[a] Results are expressed as coefficients of the linear equation $y = A + Bx$, which are calculated by using the least-squares method.

I have found the vanillin–concentrated HCl test for leucoanthocyanins and catechins quite reproducible and sensitive. It yielded nearly the same amounts for catechins purified from plant extracts as did GLC using (+)-catechin as the standard. The butanol–HCl color test used for leucoanthocyanins is quite specific but in some samples reproducible problems might occur.

Purification of the Extracts and GLC Analysis. The crude water extract was exhaustively extracted by using ethyl acetate. In this method the recovery value is relative to the time of liquid–liquid extraction. After 20 h 30% of salicin was extracted, but in 60 h over 80% recovery was reached for the whole method.

Ethyl acetate soluble fractions were purified with polyamid columns. Polyamide absorbents are effective for the separation even of macro amounts of different kind of compounds. A polyamide column has a capacity hundreds of times greater than one based on simple adsorption, because it functions by hydrogen bonding with phenolic groups and the amide groups in the interior of the particles as well as on the surface (Geissman, 1962). Glycosides are weakly adsorbed on polyamide and are readily separated from the more strongly adsorbed compounds (e.g., aglycon).

Eluates were immediately freeze-dried after collections. During this process no loss of salicin could be detected due to sublimation or decomposition. The water eluate contained mostly the glucosides found in willows, and the ethanol eluate consisted mostly of catechins with only traces of glycosides depending on the samples.

Glycosides and catechins were analyzed by high-sensitivity gas–liquid capillary chromatography. Due to their chemical and physical nature the GLC analyses must be carried out on a more volatile and less polar derivative (Bolan and Steele, 1968). Commercially available Tri-Sil Z reagent was used for derivatizing the compounds. This reagent, a premixed trimethylsilylation reagent containing *N*-(trimethylsilyl)imidazole in silylating-grade pyridine, gave consistently reproducible results. The reagent components and reaction byproducts were eluted through the column a very short time after injection (Figure 7).

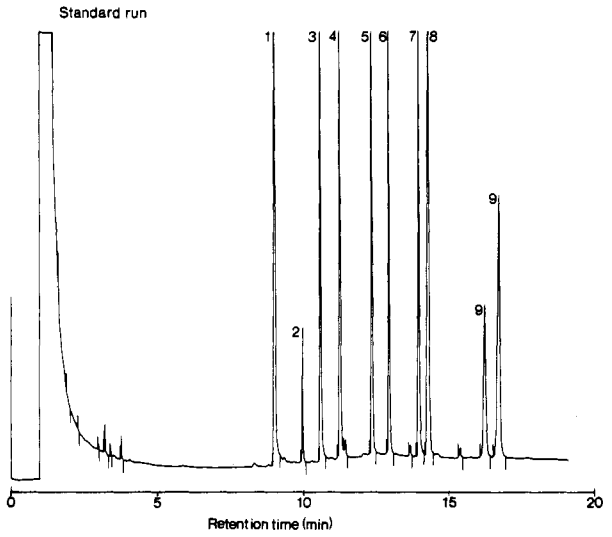

Figure 7. Gas chromatogram of a mixture of the trimethylsilyl derivatives of reference glycosides (1 = salicin; 2 = fragilin; 3 = picein; 4 = salidroside; 5 = vimalin; 6 = triandrin; 7 = tremuloidin; 8 = populin; 9 = salicortin). Grandidentatin, salireposide, and (+)-catechin have been eluted separately. The retention time for (+)-catechin was between triandrin and tremuloidin and for grandidentatin two 2 min after salicortin.

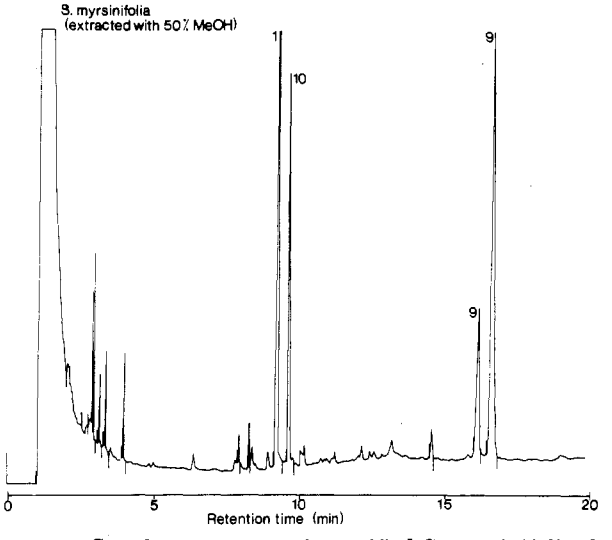

Figure 8. Gas chromatogram of a purified *S. myrsinifolia* glycoside extract. The number of the peak refers to the reference glycosides shown in Figure 7. The internal standard, arbutin (10), elutes shortly after salicin.

The sample mixture was allowed to react for about 12 h after adding the Tri-Sil Z reagent. Preliminary work showed that this time is adequate for the completion of silylation. In silylation I used larger volumes of silylation reagent than recommended by the producers in order to obtain better derivative formation; otherwise small extra peaks might occur.

With the column used, all standard compounds gave quite distinct and symmetrical peaks (Figure 7). All standards except salicortin gave one peak. Salicortin gave two separate peaks that were very close to each other. The required separation was reached with retention times to all other standards except salicortin and salireposide. Salireposide overlapped with the second peak of salicortin. The analyzing time for standards was quite short, never exceeding 30 min.

The GLC spectrum of the purified glycosidic composition of *S. myrsinifolia* leaves extracted with 50% methanol is shown in Figure 8. *S. myrsinifolia* contains two phenolic glycosides, salicin, and salicortin. Besides these peaks the chromatogram shows some minor peaks, which do not respond with any of the known reference components. The ethanol fraction of *S. myrsinifolia* leaves was also screened quantitatively for catechins with vanillin–HCl and with gas chromatography. The results indicated that this species does not yield detectable amounts of catechins.

In every extraction process there exists a possibility of decomposition, although every effort is made to minimize decomposable phase. However, I believe that the majority of the isolated and identified components was present in these growing plants as such and not as artifacts.

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Registry No. Gallic acid, 149-91-7; 3,4-dihydroxybenzoic acid, 99-50-3; ellagic acid, 476-66-4; 2,4-dihydroxybenzoic acid, 89-86-1; 3,4-dihydroxycinnamic acid, 331-39-5; (+)-catechin, 154-23-4; phenol, 108-95-2; 4-hydroxycinnamic acid, 7400-08-0; 2,6-dihydroxybenzoic acid, 303-07-1; 2,5-dihydroxybenzoic acid, 490-79-9; 4-hydroxybenzoic acid, 99-96-7; methanol, 67-56-1; water, 7732-18-5; acetone, 67-64-1; diethyl ether, 60-29-7; sodium chloride, 7647-14-5; salicin, 138-52-3; salicortin, 29836-41-7; fragilin, 19764-02-4; picein, 530-14-3; salidroside, 10338-51-9; vimalin, 19764-36-4; triandrin, 19764-35-3; tremuloidin, 529-66-8; populin, 99-17-2; grandidentatin, 15732-48-6; salireposide, 16955-55-8.

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