Analytical approaches to the determination of simple biophenols in forest trees such as *Acer* (maple), *Betula* (birch), *Coniferus*, *Eucalyptus*, *Juniperus* (cedar), *Picea* (spruce) and *Quercus* (oak)

Danny R. Bedgood, Jr., Andrea G. Bishop, Paul D. Prenzler and Kevin Robards*

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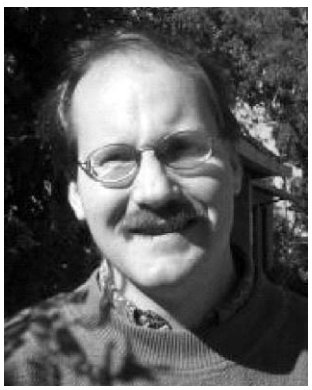
Analytical methods are reviewed for the determination of simple biophenols in forest trees such as *Acer* (maple), *Betula* (birch), *Coniferus*, *Eucalyptus*, *Juniperus* (cedar), *Picea* (spruce) and *Quercus* (oak). Data are limited but nevertheless clearly establish the critical importance of sample preparation and pre-treatment in the analysis. For example, drying methods invariably reduce the recovery of biophenols and this is illustrated by data for birch leaves where flavonoid glycosides were determined as 12.3 $\pm$ 0.44 mg g^{-1} in fresh leaves but 9.7 $\pm$ 0.35 mg g^{-1} in air-dried samples (data expressed as dry weight). Diverse sample handling procedures have been employed for recovery of biophenols. The range of biophenols and diversity of sample types precludes general procedural recommendations. Caution is necessary in selecting appropriate procedures as the high reactivity of these compounds complicates their analysis. Moreover, our experience suggests that their reactivity is very dependent on the matrix. The actual measurement is less contentious and high performance separation methods particularly liquid chromatography dominate analyses whilst coupled techniques involving electrospray ionization are becoming routine particularly for qualitative applications. Quantitative data are still the exception and are summarized for representative species that dominate the forest canopy of various habitats. Reported concentrations for simple phenols range from trace level (<0.1 μg g^{-1}) to in excess of 500 μg g^{-1} depending on a range of factors. Plant tissue is one of these variables but various biotic and abiotic processes such as stress are also important considerations.

Introduction

Phenolic compounds are ubiquitous in the plant kingdom being the most abundant secondary metabolites.[1] Although biophenols are found in all plants their quantitative distributions vary between different tissues of the plant and within different populations of the same plant species. There has been intense interest in biophenols of fruits, vegetables, herbs and spices, and cereals as evidenced by the many reviews[2–19] on various aspects of their chemistry. Papers on biophenols in these application areas fall into various categories as those concerned with isolation and identification

*krobards@csu.edu.au



Dan Bedgood

Dan Bedgood is a Senior Lecturer in Chemistry at Charles Sturt University, having earned his PhD in Inorganic Chemistry at Montana State University. After a visiting position at the University of Illinois, Urbana-Champagne, he did his post-doctoral studies at the University of Queensland as an ARC Research Associate, followed by teaching positions at North Carolina State University, Lenoir Community College, and Arizona State University. Since joining CSU in 2002 Dan has expanded his research interests beyond platinum chemistry to remote sensing of olive groves and the examination of phenolic and volatile compounds important in the production of high quality olive oils.

Andrea Bishop

Andrea Bishop obtained her PhD in Physical Chemistry from Monash University in 1999 for work examining fundamental properties of polymer and aprotic electrolytes. After a two year Postdoctoral Fellowship at Uppsala University, Sweden, she returned to Australia to take up a position at Charles Sturt University. Now a Senior Lecturer in Chemistry at CSU, Andrea applies her physical and analytical chemistry experience in projects with regional significance and which involve a central theme of phenolic chemistry. These currently include monitoring rice during storage and olive oils during processing, and the sources and fates of dissolved carbon in aquatic systems.

of novel compounds, investigations of ontogeny, chemotaxonomy (which extends to adulteration and authenticity testing), bioactivity, herbal medications, defense functions and allelopathy.[21] It has been argued that allelopathic potential can only be appreciated when we have established a good understanding of methods for extraction and isolation of phenolic compounds.[22] This consideration applies equally to other application areas.

In marked contrast to the above reviews, those devoted to the analysis and occurrence of biophenols in forest trees are limited[23] although interest in the high molecular mass phenols of forest trees has been considerable.[24–28] These phenols comprise the bulk of the structural matrix of plants, lignin being the second most abundant compound in nature. The interest can be attributed to various factors. Hydrolysis products of lignins may provide unique bioactive substances. Oaks have been one of the classic model systems in elucidating the role of biophenols in plant-herbivore interactions.[26] Interest in lignin can also be attributed to the geosciences because the composition of its phenols is characteristic for the types of vascular plant tissues from which they derive.

Interest in the biophenols of forest trees is also related to their contribution to dissolved organic carbon (DOC).[29,30] Plants are the ultimate source of the allochthonous† phenolic fraction of DOC and forest trees constitute a significant component of this input.[31] Indeed, lignin has been used as a stable tracer for terrestrial organic input in aquatic environments.[32] Various routes from plant matter to DOC can be identified. Direct leaching from intact bark and leaves is one option. Leachates from different plant species can differ qualitatively and quantitatively in their profile of water-soluble phenols.[33] Formation of leaf litter followed by leaching and/or degradation and release into the soil provides a second route. Phenols have been identified in varying quantities in soils derived from many natural and cultivated ecosystems.[34]

In contrast to the interest in complex biophenols of forest trees, simple phenols have attracted somewhat less attention. This can be ascribed to the assumption that simple phenols are labile with relatively short residence time in the aquatic environment. However, this has not been established unequivocally and there is current debate regarding their resistance.[35] Moreover, photochemical reactivity of dissolved lignin suggests that a significant portion of the high molecular mass material is converted to smaller molecules in relatively short time periods.[36] Regardless of such considerations, simple phenols (if indeed labile) are still useful as short term markers giving valuable information about carbon that results from a flow or flood event and for developing ecological models of carbon flow.

In this paper, analytical methods used for the determination of low molecular mass biophenols in forest trees are examined. Basic terminology used to describe these and related compounds is presented in Table 1. The review brings together a broad body of literature that is currently diverse and found in ecological, nutritional, and other journals reflecting individual interests. It covers a broad range of plant species, many being important riparian species not previously reviewed and that are significant to carbon flows in riverine systems. Sample types are also diverse and include leaves/needles, roots, wood, bark and phloem. It is hoped to stimulate interest in quantitative investigations of these species in comparison with much current literature that examines unique phenols for the sake of discovery of new compounds. In contrast, known compounds have received little attention on analytical methodology, quantification and seasonal variation.

Analytical approaches

This section presents an overview of techniques for the determination of biophenols in forest trees. Analytical techniques for these sample types parallel methods used for phenolic compounds in fruits and vegetables. However, there are differences between the two areas in the relative number of papers devoted to different aspects of their chemistry. Analyses of fruits and vegetables for phenolic content are driven by diverse purposes[2]—more recently related to the potential health benefits of these compounds. The efficient and targeted isolation, identification and quantification of compounds,

† Allochthonous input refers to any organic matter that originates from outside the river, such as the fall of leaf litter from riparian zones and matter leached from humus during floods or rainfalls.

Paul Prenzler

Paul Prenzler is a Senior Lecturer in Chemistry at Charles Sturt University. He obtained his PhD in 1992 in Inorganic Chemistry from the University of Queensland. This was followed by postdoctoral appointments at the Advanced Research Laboratory of Hitachi, Japan, the Australian National University and the University of Melbourne. Since joining CSU in 1997 he has diversified his research interests to include studies in wine, olives and ecology. A central theme is the role phenolic compounds have in these systems. This has led to further research initiatives aimed at a more complete understanding of their antioxidant activity.

Kevin Robards

Kevin Robards is Professor of Chemistry and Head, School of Science and Technology. His research interest is the application of analytical science to food and environmental systems to ensure sustainable development of natural resources. The study of biophenolic chemistry is an important component of this strategy.

Table 1 Terminology of biophenols

Class of phenol	Sub-class	Common examples
Benzoic acids		Salicylic acid
Cinnamic acids		Ferulic acid, caffeic acid
Coumarins		Aesculetin
Flavonoids	Flavones	Luteolin, apigenin
	Isoflavones	Daidzein, genistein
	Flavonols	Quercetin, kaempferol
	Flavanonols	Dihydroquercetin
	Flavanones	Hesperitin, naringenin
	Flavan-3-ols	Catechins
	Chalcones	Phloridzin, arbutin
	Dihydrochalcones	
	Anthocyanidins	Cyanidin, delphinidin
	Anthocyanins	Anthocyanidin glycosides
Tannins	Condensed tannins	Proanthocyanidins: oligomers of anthocyanidins
	Hydrolysable tannins—these are esters of a polyol with either:	
	gallic acid, or	Gallotannins
	hexahydroxydiphenic acid	Ellagitannins
Lignins		High molecular mass biopolymer based on phenylpropanoid units, unique to vascular land plants

often with the intention of discovery of novel compounds is a common theme. Similar themes are seen in analysis of phenols in forest trees but a new dimension relating to environmental issues gains prominence. Regardless of the purpose of such studies, the methodology typically follows the traditional approach of extraction, separation and detection as demonstrated by the isolation of biophenols from a methanolic extract of the leaves of silky oak (*Grevillea robusta*).[37] Representative data summarized in Table 2 demonstrate the diversity of approaches.

Sample collection

Sample handling covers both sample collection/storage and sample pre-treatment/clean-up. The importance of the first step is often ignored although the use of fresh *versus* dried material remains as a controversial issue. Comparisons of sample preparation methods have often been based on measurement of tannins or total phenols using a colorimetric method. However, individual compounds or classes of phenols may respond differently and high performance procedures are essential for meaningful comparisons. A drying method has been established to effect phenol concentration[56] although Julkunen-Tiitto and Sorsa[20] state "most of the flavonoids are considered to be fairly stable under different pre-handling and extraction conditions..." Indeed, there were no qualitative or quantitative differences between the phenolic glucoside content of fresh and oven-dried samples of willow leaves and bark[46] but these data contrast sharply with other studies in which oven-drying caused changes in glucoside content.[45] For instance, the effect of various drying treatments (air-, oven- and freeze-drying) relative to extraction from fresh leaves has been investigated for birch[41] (Table 3). There were significant concentration differences among the methods for the majority of phenols. With the exception of a few phenols, the concentrations after drying treatments were lower than in extractions of fresh leaves. Maximum recovery of both flavonoids and hydroxycinnamic acids was obtained by immediate extraction of fresh leaves. Differences between various oven drying methods were attributed to two processes: competing effects of drying temperature on rate of enzyme inactivation and differences in the thermostability of the phenols. Other relevant considerations are the enhanced concentrations of enzymes and substrates encountered during drying processes that may facilitate degradation processes. The use of intact *versus* homogenized leaves, in which destruction of leaf compartmentalization during the crushing of fresh leaves releases hydrolytic enzymes into the cytoplasm, may also account for observed differences.

Differential effects of various drying treatments on phenolic content have also been reported for flavonoids in willow.[20,46,57] Purple willow was selected for this study because of its particular phenolic content containing representative biophenols from three different flavonoid sub-groups. In contrast with leaf and bark tissue, where oven or room temperature drying provided satisfactory agreement with results for fresh samples, oven-drying of whole twigs produced dramatic changes in glucoside composition. The magnitude of the changes was different in different willow species. Freezing or freeze drying produced substantial changes in the glucoside composition of leaves. Pre-handling methods generally impacted the concentrations of individual biophenols and analysis of fresh material is desirable. However, where practical constraints necessitate sample preservation, then dessication at room temperature and freeze-drying at low temperature, preferably without pre-freezing, are the best methods (at least in the case of mature willow leaves). Alternatively, vacuum-drying fresh leaves permits quantification of both phenolic glycosides and condensed tannins from the same leaf material.[57]

A comprehensive study of sample treatment[58] investigated the effects of different methods of sample drying and storage, and the choice of extraction solvent and analysis method on the concentrations of 14 individual hydrolysable tannins and insoluble ellagitannins in birch leaves. More reliable results were achieved with freeze- and vacuum-drying of the leaves than air- or oven-drying. Leaf storage at −20 °C was generally preferable to room temperature or 4 °C storage. However,

Table 2 Analytical methods used for the determination of various biophenols in different samples

Sample	Analyte	Sample handling	Extraction	Clean-up	Measurement[a]	Ref.
Beech, birch and ash dusts	Phenolic acids and flavonoids	Ground samples	Aqueous methanol	Diethyl ether partitioning	RPLC, ESI	38
Birch—inner bark	Phenolic glycosides	Fresh homogenised samples	Aqueous methanol	Column chromatography	Structural elucidation by MS and NMR	39
Birch leaves	Flavonoids		Comparison of extraction methods		RPLC, 220, 230 and 270 nm	40
Birch leaves	Flavonoids	Vacuum dried leaves	Aqueous acetone		Folin Ciocalteau reagent and RPLC	1
Birch leaves	Phenolic acids and flavonoids	Comparison of sample preparation methods			RPLC, 280, 320 and 360 nm	41
Birch leaves	Hydroxycinnamic acids and flavonoids	Fresh leaves fixed in acetone, air dried, ground	Aqueous acetone		RPLC, ESI	42
Silk oak leaves	Novel phenols		Methanol	Column chromatography	Structural elucidation by MS and NMR	37
Oak—cork	Phenolic acids and aldehydes, ellagitannins and proanthocyanidins	Ground samples	Aqueous methanol	Diethyl ether partitioning	RPLC, 325 nm	43
Chestnut wood	Phenolic acids and aldehydes	Ground wood chips	Aqueous ethanol		RPLC, 280 and 320 nm plus fluorescence	44
Willow leaves	Phenolics	Comparison of drying methods	Comparison of extraction solvents		Folin Ciocalteau reagent and GC	45
Willow leaves, bark and twigs	Phenolic glucosides	Comparison of sample handling methods			GC	46
Norway spruce needles	Flavonoids	Fresh leaves frozen in liquid nitrogen, ground	Methanol–chloroform–formic acid or aqueous methanol. Extraction of cell wall bound phenolics using aqueous alkali		RPLC, 280 and 320 nm	47
Norway spruce	Phenolic acids and aldehydes	Acid hydrolysis	SFE		RPLC, ESI and diode array	48
Pine needles	Phenolic acids	Powder	Acidified aqueous methanol	Diethyl ether partitioning and SPE	RPLC, 270 nm	49
Pine needles	Flavonoids	Freeze dried material	Aqueous methanol, reflux	SPE	RPLC, 350 nm	50
Eucalyptus leaf litter	Phenolic acids and aldehydes		Distilled water, room temperature, 24 h, dark	Diethyl ether partitioning	RPLC	51
Eucalyptus leaves	Phenolic acids and flavonoids	Fresh leaves frozen in liquid nitrogen, ground	Aqueous methanol	SPE	Comparison of RPLC (280 and 320 nm) and MECC (214 nm)	52
Eucalyptus wood, bark and leaves	Flavonoids, phenolic acids and aldehydes	Ground samples	Aqueous methanol, room temperature, 24 h	Diethyl ether partitioning	RPLC, 325 nm	53
Eucalyptus leaves	Flavonoids, phenolic acids	Ground samples	Aqueous methanol, room temperature, 24 h	Diethyl ether partitioning	RPLC, 325 nm	54
Eucalyptus wood	Ellagitannins	Fresh samples	Cold aqueous acetone, 24 h, dark		RPLC, ESI	55

[a] RPLC, reversed phase liquid chromatography; ESI, electrospray ionization; SFE, supercritical fluid extraction; SPE, solid phase extraction; MECC, micellar electrokinetic capillary chromatography.

Table 3 Effect of sample treatment on recovery of (+)-catechin, flavonoid glycosides and hydroxycinnamic acids from birch leaves

Sample preparation method	Recovered concentration/mg g^{-1} dry weight		
	(+)-catechin	Flavonoid glycosides	Hydroxycinnamic acids
Air-drying	1.79 ± 0.16	9.7 ± 0.35	1.038 ± 0.027
Oven-drying at 40 °C	1.72 ± 0.12	9.3 ± 0.37	1.040 ± 0.018
Oven-drying at 80 °C	1.60 ± 0.12	10.4 ± 0.42	0.995 ± 0.015
Freeze-drying (prefrozen at liquid N_2)	1.74 ± 0.11	10.6 ± 0.41	0.992 ± 0.021
Freeze-drying (prefrozen at −18 °C)	1.90 ± 0.17	11.3 ± 0.10	1.054 ± 0.033
Freeze-drying (unfrozen)	2.03 ± 0.14	9.9 ± 0.55	1.079 ± 0.041
Fresh frozen	1.91 ± 0.15	11.8 ± 0.64	1.146 ± 0.036
Fresh	1.96 ± 0.14	12.3 ± 0.44	1.193 ± 0.029

[a] Selected data reproduced from ref. 41.

as noted by the author "each plant species, with its presumably unique" hydrolysable tannin "composition, is likely to have a unique combination of ideal conditions for tissue preservation and extraction." Thus, generalizations are dangerous and this makes the challenge of metabolomics a daunting prospect.

Sample pre-treatment

Sample pre-treatment and quantification steps are closely related due to the limited selectivity of the latter. There is however considerable variation in methodology and some procedures (*e.g.* spectrophotometric measurement of total phenols) require more rigorous sample pre-treatment than others. Fundamental aspects of sample preparation have recently been reviewed.[59] The importance of mass transfer in multiphasic systems on method choice, optimization and design was noted. Further consideration of sample pre-treatment needs to be given to the various sources of biophenols. For example, some plant tissues will yield simple phenols directly from solvent extraction, whereas other materials such as lignin may give simple phenols through breakdown reactions of polymeric material. These may occur naturally or as part of sample preparation. For instance, flavones (apigenin and luteolin), flavonols (quercetin, myricetin and kaempferol) and proanthocyanidins were investigated[60] in acid hydrolyzed extracts of laurel. Significant differences in the hydrolysis times were required to optimize recovery of free phenols from the different classes of phenols. Regardless of origin of the simple phenols, similar techniques may be applied for identification and quantification.

Lignin as a high molecular mass phenolic biopolymer is outside the scope of this review. Nevertheless, some mention of lignin methods is justified because its degradation products are simple phenols. The typical procedure involves copper(II) oxide oxidation of lignin in a bomb to monomeric phenols[32] that are usually analysed by HPLC. Alternatively, woods have been delignified at 170 °C with aqueous sodium hydroxide or tetramethylammonium hydroxide and the resulting liquors acidified and extracted with dichloromethane.[61] Fifteen simple phenols and related compounds were examined in the extracts using capillary electrophoresis.

Less severe conditions have been used to release ester bound (released after alkaline hydrolysis) and glycoside bound (released after acid hydrolysis) phenolic acids from a methanolic extract of spruce needles ground in liquid nitrogen.[62] Free phenolic acids were obtained by simple methanolic extraction and a fourth fraction, cell wall bound phenolic acids was obtained by alkaline hydrolysis of the residual material after methanolic extraction. The extracts were analysed by HPLC using gradient elution. The paper is also notable for the histochemical studies of the phenolics which were found in most of the vacuoles and cell walls of the needle tissue. Soluble (or free) phenols were recovered from spruce needles with a mixture of methanol, chloroform and formic acid.[47] This solvent produced higher yield and a more complex pattern of extractable phenols than extraction with aqueous methanol. The residue from this extraction was sequentially extracted with methanol, water, methanol, acetone and diethyl ether and the final residue was hydrolysed with aqueous sodium hydroxide (1 M, 17 h, 80 °C) for the isolation of insoluble cell-wall bound phenols. Mild acidic hydrolysis (2 M HCl; 100 °C; 40 min) was used to liberate aglycones from flavonoid glycosides.[33,47] Biophenols occurred mainly as the latter in leaves and roots but the aglycones prevailed in senescent or dead organs.

Solvent extraction—choice of method. For plant tissues, solvent extraction of either fresh or dried samples remains as a popular approach. Several extraction procedures and solvents have been used to recover phenols from the tissue of trees (see Table 2) but no single procedure has proved superior in all cases.[45] A wide variation is seen in all aspects of the process. For example, extraction times have varied widely between *ca.* 3 min (*i.e.* methods employing simple shaking)[52] and 24 h.[51,54,63,64] In some cases, a short-time homogenization (*e.g.* 1.5 min) at elevated temperature has been followed by standing, with[65] or without shaking, at room or sub-ambient temperature for various times up to 24 h.[66] In the case of dry leaves, steeping is a sensible precaution prior to extraction to ensure effective solvation.[41,67]

Flavones and flavonols were recovered from laurel leaves by methanolic extraction using an ultrasonic bath.[60] However, comparison of a conventional sonicator with a less traditional homogenizer showed improved efficiency and consistency in recovery of biophenols from both tender and tough leaves using the latter system.[68] The use of an Ultra Turrax homogenizer has become popular[39,41,67,69–74] and this typically involves a short (30–180 s) homogenization. For example, leaves and buds of white birch were extracted with methanol in an Ultra Turrax homogenizer for 30 s and then the extract was

left on ice for 30 min.[75] The sample was centrifuged and vacuum evaporated to dryness. The dry extract was stored at −20 °C prior to LC-MS. In buds, the major biophenols were hydrolysable tannins and flavonoid aglycones, whereas, later during leaf development, the flavonoid glycosides accounted for most of the total low molecular mass phenols. The sharp increase in flavonol glucosides during ontogeny might be due to a change in growth phase from exponential to stationary (less competition with protein synthesis) and/or the exposition of unfolding leaves to UV-radiation.

Reflux[33] and Soxhlet extraction have also been employed. Simple extraction with homogenization, solvent reflux and Soxhlet extraction using aqueous acetone, methanol or ethanol was compared for recovery of flavonoids from birch leaves.[76] Refluxing in aqueous methanol was recommended for recovery of flavonoid glycosides although it was noted that the use of a heated solvent is frequently undesirable. In another study,[50] flavonoids were recovered from freeze-dried pine needles by refluxing with aqueous methanol followed by reduction in extract volume and clean-up using solid phase extraction (SPE). This procedure contrasted with the preparative scale isolation in which the pine needles were defatted and depigmented by Soxhlet extraction with carbon tetrachloride prior to extraction with methanol and clean-up using a short column packed with an RP18 phase.

The simple expedient of sample immersion in the appropriate solvent was chosen to recover birch leaf surface flavonoids.[77] Leaves were frozen and stored at −80 °C prior to extraction with aqueous ethanol for 20 s. The ethanolic extracts were filtered and analysed by HPLC. Extraction efficiency was quoted as 97% but details of the calculation were not provided. Flavonoid data were reported as both mass of a particular compound per unit of leaf mass and mass of compound per leaf. The use of dry mass is important to compensate for dilution effects during seasonal growth. Data were also reported for the flavonoid concentration per unit of leaf surface area.

Solvent extraction—choice of solvent. In each of these procedures (simple extraction, Soxhlet extractor, sonicator, homogenizer/blender), the choice of a suitable extraction solvent remains a critical factor. A comparison of the magnitude of phenolic losses during extraction with methanol, water, acetone, diethyl ether and chloroform showed that methanol and acetone were most suitable as solvents whilst water may be the source of considerable losses.[78] Nevertheless, in some circumstances, water is seen as the most realistic solvent as is the case in allelopathic studies[51] despite incomplete extraction of biophenols. Aqueous extraction is also most appropriate for duplicating environmental leaching of phenols from leaves and bark.

The pH of the extractant is manipulated in some cases to alter partitioning behaviour as in the extraction of phenolic acids and flavonoids from pine needles with acidified (HCl) aqueous methanol.[49,79] The extraction solvent was added to the powdered pine needles and shaken for 2 h at room temperature. In other cases, notably the recovery of anthocyanins, pH control is essential.[69] Thus, homogenization with acidified (pH 1) methanol was the most effective solvent for recovery of anthocyanins, flavonols and tannins from leaves of Eucalyptus.[66]

Diethyl ether has been used to extract biophenols from the phloem of pine shoots[80] but this solvent choice is unusual and in the more typical case, a solvent of higher polarity is employed. For example, methanol is commonly used as in the extraction of phenols from crushed spruce needles.[81] An unusual feature of the extraction procedure was the homogenization of the needles with diatomaceous earth at liquid nitrogen temperature prior to addition of methanol. The use of methanol in aqueous mixtures has been advantageous for extracting many phenols and particularly phenolic glycosides from dried plant material.[38,43,53,54,82–84]

Aqueous methanol was used for recovery of proanthocyanidins, ellagitannins and flavonol glycosides from Eucalyptus and cork (*Quercus suber*).[43,64] Tannins were extracted from dried (procedure not specified!) ground leaves with aqueous methanol for 24 h at room temperature in the dark.[64] Methanol was removed under vacuum "and the aqueous solution was extracted with (di)ethyl ether and freeze dried. The aqueous solution was used for quantitative analysis of total phenols, proanthocyanidins and ellagitannins." Total phenols and proanthocyanidins were determined colorimetrically whilst ellagitannins were estimated by HPLC evaluation of the ellagic acid obtained following acid hydrolysis. Individual phenols including proanthocyanidins, ellagitannins and flavonoids were determined by HPLC on the lyophilized material. It appears that the lyophilized material is in fact the ether extract. This interpretation is supported by a related paper from the same group[54] although a chromatogram is shown in the paper[64] for "aqueous leaf extract".

Methanol has been reported as a suitable solvent for short-time extraction procedures but longer extraction times induced changes in some phenolic glycosides.[46] Aqueous acetone extraction yielded slightly higher concentrations of all glycosides in willow bark. The effect of aqueous acetone *versus* aqueous methanol as extractant was demonstrated for the extraction of the glycosides, salicin and salicortin from willow leaves.[45] The concentration of salicin was greater in aqueous methanol extracts whereas the reverse applied for salicortin. Moreover, the relative proportions of the two glycosides were reversed with the two solvents. These differences may reflect the relative extraction efficiencies of the two solvents for compartmentalized phenols. Alternatively, salicortin may decompose to salicin during methanolic extraction.

Aqueous acetone has been used in a number of situations. Birch has been studied extensively and has a well established ecology and phytochemistry.[85,86] Vacuum dried birch leaves were homogenized into a powder and stored at −20 °C.[65,85] The powder was suspended in aqueous acetone for 1 h at room temperature with continuous stirring and then centrifuged. The extract was reduced to the aqueous phase by evaporation at room temperature and the resulting aqueous phase was frozen and lyophilized. This extract was used for the determination of soluble phenols (total phenols by Folin Ciocalteau and soluble proanthocyanidins) whilst the acetone insoluble residue was used to determine cell wall bound proanthocyanidins and lignin. In a related paper, significant seasonal changes were observed in the concentrations (mg g^{-1}) and

amounts (mg leaf^{-1}) of phenols in the leaves of mountain birch.[1] The seasonal trends of phenolic classes differed drastically. Concentrations of soluble proanthocyanidins increased through the season, whereas cell wall-bound proanthocyanidins, gallotannins and flavonoid glycosides declined after an initial increase in young leaves. There was no evidence of a trade-off between leaf growth and production of phenols which is the basic assumption of the growth/differentiation balance and protein competition model hypotheses. Annual variations in concentrations of major leaf phenols as high as 50% have been reported for birch populations.[73]

Aqueous acetone was also used for extraction of proanthocyanidins from pine bark.[87] The crude extract was fractionated on Sephadex LH-20 to produce three procyanidin fractions, one with shorter oligomers, one with longer oligomers and one with polymers. Aqueous acetone was also used for the extraction of hydrolysable tannins from sapwood of Eucalyptus.[55] Extraction was performed at low temperature in the dark for 24 h. The extracts were centrifuged to remove solids prior to HPLC analysis.

The choice of extraction solvent for recovery of hydrolysable tannins and insoluble ellagitannins from birch leaves was investigated.[58] Of the extraction solvents tested, aqueous acetone was superior to pure acetone, or aqueous methanol. In contrast, pure methanol was superior to pure acetone. The percentage of water in the acetone also had an effect on the yield of hydrolysable tannin sub-groups. The addition of 0.1% ascorbic acid into 70% acetone significantly increased the yield of ellagitannins, presumably by preventing their oxidation.

Solvent extraction—precautions. Various precautions taken to protect the phenols during extraction include manipulation in the dark[51,55,64,66,81] or with "minimum exposure to light"[52] and the addition of various stabilizers.[88,89] The dynamic nature of the sample must be considered in the design of any extraction procedure. Relevant questions are whether the sample tissues are intact, preserving compartmentalization; is the determination to include free or total phenols? The latter is important as some of the phenols are attached to cell walls while others are in cytoplasmic vacuoles. Methanol combines good solvent action for phenols with the ability to disrupt cell walls and inhibit enzyme action. Ethanol is more lipophilic than methanol so it exhibits superior extraction of polymeric and hydrophobic phenols.[89]

Sample clean-up and newer developments. The extract is often analysed directly although a preliminary clean-up involving liquid liquid partitioning or SPE has been used in some situations. Thus, biophenols recovered from Eucalyptus tissues by aqueous methanolic extraction were partitioned into diethyl ether prior to HPLC.[53] Gradient elution with photodiode array detection at 325 nm was used and the resulting chromatograms (Fig. 1) show the phenolic profiles of wood, bark and leaves of a single species of Eucalypt. This variation in phenolic composition between different plant parts is well documented. The wood extract contained several phenolic acids and aldehydes, some ellagitannins and no flavonoids. Ellagic acid was abundant in wood and this is readily explained by the hydrolysis of numerous wood ellagitannins.[44] Some flavonoids were detected in the bark extract although phenolic acids and aldehydes and ellagitannins were also major components. The most abundant compounds in the leaf extracts were flavonol glycosides.

In an unusual approach, powdered bark from Norway spruce was treated with pentane to remove hydrophobic substances such as terpenes prior to methanolic extraction[88] rather than the more conventional approach of post extraction clean-up. Conventional column chromatography has been used for sample clean-up although this is more common in preparative scale than analytical separations.[50] For example, hydrolysable tannins were extracted from fresh oak leaves with aqueous acetone at room temperature.[90] Chlorophylls and waxes were precipitated by concentration of the extract under vacuum and removed by filtration. The resulting filtrate was fractionated by Sephadex chromatography and various reversed phase gels to yield five new tannins together with 26 structurally known tannins and related compounds including gallotannins, ellagitannins, proanthocyanidins and simple phenol glucoside gallates.

The increasing demand for new extraction techniques, amenable to automation with reduced solvent consumption and analysis times has seen the introduction of a diverse range of techniques. Of these, accelerated solvent extraction,[91] SPE[50] and supercritical fluid extraction (SFE) are relevant. Knotwood was sampled and stored at −18 °C[92,93] and extracts were obtained by sequential extraction with hexane and aqueous acetone in an accelerated solvent extractor. Lipophilic compounds were removed first with the hexane and then the hydrophilic phenols were recovered with aqueous acetone.

Extraction with supercritical carbon dioxide using countercurrent flow has been applied to recovery of low molecular mass phenols from aqueous standards plus a dilute acid hydrolysate of spruce.[48] Extraction efficiencies ranged from 2 to 100% in the standard solutions and 11 to 98% in the hydrolysate depending on the various phenolic classes. Major differences in extraction efficiencies were noted for some phenolic acids (*e.g.* ferulic acid, 24.5% was extracted in the hydrolysate *versus* 51.5% in the standard solution). These differences were attributed to the effect of matrix components. No correlation was observed between polarity indices (log K_{ow}) and extraction efficiencies for the investigated compounds, however, a relationship between retention in reversed phase HPLC and extractability could be established.

SPE has been the most attractive of the newer technologies for biophenols.[89] Although applications to forest trees are limited, work on other plant materials is notable[94] for its sample clean-up procedure and optimization of the mobile phase gradient. A combination of microwave-assisted extraction (MAE) and SPE was applied prior to liquid chromatographic identification and quantification of phenolic acids in plant materials.

Lipophilic materials were extracted from dried and ground pine needles with hexane.[78] The residue was mixed with acetone for 20 min at room temperature and then filtered. The resulting solution was mixed with Florisil and evaporated to dryness allowing adsorption of the phenols. The Florisil

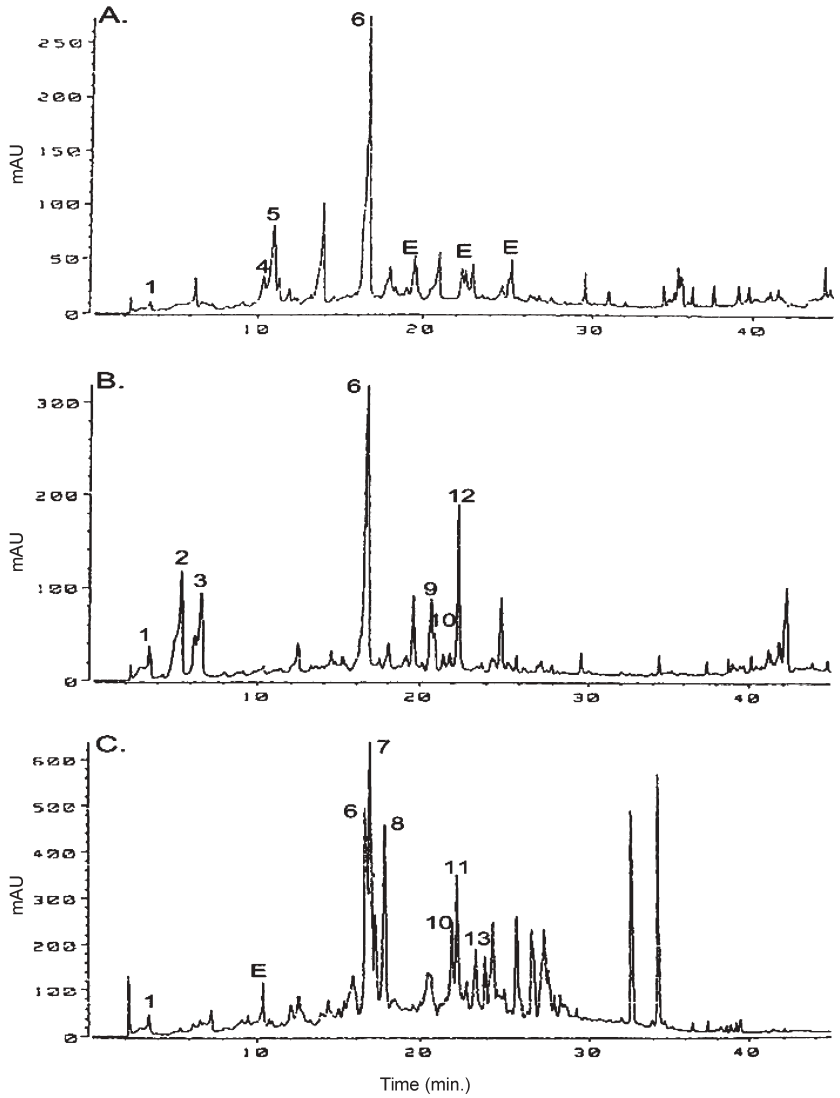


Fig. 1 HPLC chromatograms of aqueous methanolic extracts (room temperature, 24 h) of *Eucalyptus camaldulensis* tissues: A. wood; B. bark; and C. leaves. Compounds are identified as: 1. gallic acid; 2. protocatechuic acid; 3. protocatechuic aldehyde; 4. vanillin; 5. syringic aldehyde; 6. ellagic acid; 7. unidentified flavonal; 8. eriodictyol; 9. quercetin; 10. leuteolin; 11 and 12. unidentified flavonals; 13. kaempferol, E = ellagitannins. Reproduced (and adapted) with permission from ref. 53.

adsorbent was then added to a C18 SPE cartridge which was eluted with methanol. Although acetone had good elution properties it exhibited poor selectivity. The eluted phenols were silylated with BSTFA and analysed by gas chromatography (GC) using a flame ionization detector. Recovery from the florisil/C18 sorbent ranged from 32% for ferulic acid to 88% for gentisic acid. The high-precision quantification of 9 phenolic acids in the pine needles was achieved.

In an alternative procedure for phenolic acids in powdered pine needles,[49] the phenols were partitioned from aqueous methanol into diethyl ether and subjected to clean up by reversed phase SPE prior to HPLC. Similarly, phenols extracted from Eucalyptus with aqueous methanol[52] were defatted by passing through a reversed phase C18 cartridge prior to HPLC or micellar electrokinetic capillary chromatography (MECC). The electropherogram of the Eucalyptus extract contained a broad hump that is typical of the interference due to humic substances.[95] Although a reversed phase chromatogram was not shown for the Eucalyptus extract[52] a humic hump is also typically seen in these separations. Based on the electropherogram data, reversed phase SPE does not appear to eliminate this source of interference which has not been specifically addressed in relation to forest trees. However, data from other sources are useful. For instance, various methods based on the use of chemical reagents have been compared in the analysis of environmental water[96] and the optimum procedure was addition of sodium sulfite to the humic-containing water.

Quantification

Methods of separation and quantification are identical with those used for phenols extracted from foods and "edible" plants.[2] Traditional methods for the determination of the

phenolic component relied on colorimetric measurement of total phenols using one of a number of reagents of varying selectivity. The diversity of phenolic compounds means that selection of a reagent and/or absorbing wavelength will be a compromise although this is less of a problem where a single class of phenols predominates. The Folin–Ciocalteau reagent is the classic reagent recommended for total phenols.[49,51,60,64,82,97] The blue colour formed after 15–60 min is measured at 725–735 nm and results are expressed in terms of mass equivalents of a commonly occurring phenol, for example, gallic acid.[98] Reaction time and temperature are important variables that must be optimized. Indeed, Skerget *et al.*[60] attributed the reduction in total phenols after 5 min reaction at 50 °C to the decomposition of biophenols. The limitations of this method have been recognized[33] but it still seems to be considered adequate for relative comparisons.[98]

Other approaches to measurement of total phenols include reaction with iron(III) chloride and measurement at 510 nm.[99] Measurement in the ultraviolet and infrared regions of the spectrum are also suitable as demonstrated by the application of infrared spectroscopy for the measurement of extracts derived from hardwood (including oak and maple) leaves. The extracted DOC was separated into fractions of different polarities by sequential adsorption on XAD macroreticular resins.[31] Infrared spectra of the fractions were measured in perdeuterated pyridine to suppress inter- and intra-molecular hydrogen bonding. Integration of the hydroxyl stretching band (3500–2375 cm^{-1}) in spectra that had been corrected for non-phenolic contributions in this region (from alcohols and carboxylic acids) provided a measure of phenolic content. Alternative approaches to the measurement of total phenols may include the integrated response from chromatographic separations. Despite continued use[60,100] and its obvious attractions in terms of simplicity, the measurement of total phenols is not a preferred approach. The poor correlation between individual and total phenols led Salminen *et al.*[26] to caution against the uncritical use of summary quantifications of composite phenolic fractions in ecological studies. The same caution should be applied to studies in other areas.

Separation methods. The need for profiling and identifying individual phenolic compounds has seen traditional methods replaced by high performance chromatographic analyses. The limited volatility of many phenols has restricted the application of GC to their separation. However, with suitable derivatization (*e.g.* trimethylsilylation) some phenols are amenable to GC and GC-MS.[101,102] For instance, hydrophilic extracts of knotwood comprising lignans, stilbenes and flavonoids were analysed by GC and GC-MS after silylation with bis(trimethylsilyl)trifluoroacetamide (BSTFA).[92,93] Heneicosanoic acid and betulinol were used as internal standards and a correction factor of 1.2 was used for lignans that were calculated against betulinol. There were no substantial amounts of polymeric compounds in the extracts although a number of oligomeric species were identified. Silylation with the less expensive and less toxic bis(trimethylsilyl)acetamide has been recommended[103] following a comparison of its use with that of BSTFA for derivatization of phenolic acids. In this procedure, conventional heating of the reaction mixture was replaced by microwave irradiation to produce much more rapid reaction without loss of phenols. A procedure that has not been applied to forest trees deserves consideration. In this method, methylated derivatives of phenolic acids and flavonoids were prepared by phase transfer catalysis and analysed by GC-MS.[104]

Simple phenols derived from copper(II) oxide oxidation of lignin were analysed by GC-MS[105] using selected ion monitoring. Data were expressed relative to organic carbon as the sum of the six syringyl and vanillyl lignin-phenols. Glycosides are generally considered unsuited to GC because of relatively high molecular masses and low volatility but silylation has been applied to the effective GC separation of phenolic glycosides from willow leaves.[45]

Of the various capillary electrophoretic techniques, simple capillary zone electrophoresis was not suited for the separation of low molecular mass phenolic and neutral lignin degradation compounds.[61] Switching to MECC enhanced the separation performance considerably.[52] Alternatively, microemulsion electrokinetic chromatography provided separations of lignin degradation products.[61] A comparison of MECC on fused silica and HPLC on a C18 phase emphasized the complementarity of the two techniques.[52] Elution order differed between the two techniques confirming the different separation mechanisms operating for the two systems. Separation efficiencies were much higher in MECC (theoretical plates 10^4–10^5) than in HPLC (10^3–10^4). Nevertheless, HPLC currently represents the most popular and reliable technique for analysis of phenols.[41,43] This usually involves separation of the underivatized phenols although pre-column derivatization has been used to improve recovery of phenols from aqueous samples.[106]

The typical system involves reversed phase HPLC comprising a C18 stationary phase.[38,41,49,51,54,55,64,84] Columns are commonly 100 to 300 mm in length[49,55] with 10 μm or increasingly 5 μm packings[52,66,81] thus favouring the shorter columns. Shorter columns (60–75 mm) with even smaller particles (3 or 4 μm) are also becoming popular[41,58,70,73] although a 250 mm column with 2.5 μm particles has been used.[88]

In some instances, isocratic elution has provided adequate resolution due to the restricted range of analytes or selectivity effects of one or more components of the mobile phase.[51,107] However, gradient elution has usually been mandatory[38,41,55,58,66,84] in recognition of the complexity of the phenolic profile of most samples. Numerous mobile phases have been employed but binary systems comprising an aqueous component and a less polar organic solvent such as acetonitrile or methanol remain common.[41,58,77,108] Acid (acetic, formic or phosphoric acid) is usually added to both components[33,38,49,54,58,63,64,66] to maintain constant acid concentration during gradient runs. Ternary phases have had little use[79] but offer greater flexibility in control of selectivity and will likely increase in popularity. The optimization of mobile phase composition and gradient is challenging given the complexity and variability of biophenolic extracts. Optimization has been facilitated by the commercial availability of modern computer-based optimization methods. However, even these modern systems have their limitations and experience difficulty coping with the complex chromatograms and manual intervention becomes mandatory.[109]

Detection. Routine detection in HPLC is typically based on measurement of UV absorption.[49] The most commonly used wavelength for routine detection has been 280 nm[41,51] although no single wavelength is ideal for all classes of phenols since they display absorbance maxima at distinctly different wavelengths.[48] Alternatively, photodiode array detection has been common[41] and Conde *et al.*[54] used this technique with a bandwidth of 150 nm. The use of a broad bandwidth is a simple expedient but represents a significant development. Newer instruments facilitate full spectral collection with display as a composite integrated wavelength response and this approach is certainly recommended for any new or unusual samples. In other cases, selected wavelengths have been employed as in the determination of flavanones (quantified at 280 nm as naringenin), flavones (349 nm as apigenin, luteolin or acacetin) and flavonols (349 nm as kaempferol or quercetin) using the corresponding aglycones as external standards for both the flavonoid aglycones and their derivatives.[77,108] Cinnamates, flavonoids and anthocyanins were identified at 325 nm, 370 nm and 520 nm, respectively in tissues of horseradish trees.[110] In this work, the photodiode array system was used to advantage as the composite response was monitored between 200 and 600 nm.

Although not applied to forest trees, an interesting development is the application of a microbore column with electrochemical detection to the separation of 15 flavonoids.[111] Detection limits were improved 600-fold relative to conventional detection with UV. The 15 flavonoids were divided into two groups based on their hydrophobicity and were resolved by isocratic elution with aqueous methanol containing 0.5% phosphoric acid. A linear correlation was obtained between the retention factor of each flavonoid and the logarithmic partition coefficients between 1-octanol and water.

Identification and measurement. Quantification has been most commonly performed by external calibration[50,54,64,67,74,88,112] although internal standardization has been used in limited cases as in quantification of phenols in *Eucalyptus marginate* employing gallic acid as internal standard.[52] In a further example, rutin was used as an internal standard[20,63] for the determination of hydrolysable tannins. Quantification was performed by LC-MS using negative ion ESI. Peak areas were converted from 'rutin units' to appropriate units by using response factors to authentic catechin plus non-commercial hydrolysable tannin standards. The lack of authentic standards is a common problem. This has been addressed by preparative scale recovery and purification of the relevant compounds[20] and the use of a single compound to quantify a given compound class.[67,112] For instance, flavonoid glycosides were quantified as quercetin 3-galactoside, hydroxycinnamic acid derivatives as chlorogenic acid and an unknown compound as picein.[41] Quantification of glycosides as the corresponding aglycones assuming similar molar absorptivity for the pair is common.[42,54,64,72,74,112] Correction factors have been applied in some instances as in the correction of anthocyanin data for chlorophyll absorbance.[66]

Compound identification is based on comparison of retention and spectral data with those of authentic standards[43,49,51,54,64,77,108] and literature data.[51] Given the number and diversity of biophenols, identifications based on the latter must be considered as tentative at best. Co-chromatography[33] and TLC have also been used to aid identification[54] whilst preparative HPLC and GC-MS have also been employed.[64] For instance, flavonoid glycosides isolated by preparative HPLC were hydrolysed with trifluoroacetic acid and the resultant aglycones characterized by retention and UV spectral data in HPLC. The corresponding sugars were trimethylsilylated and identified by GC-MS. This is a useful approach but does not permit full characterization as it does not necessarily indicate the order in which aglycone and sugar are assembled.

Mass spectrometry (MS) now excels in providing unsurpassed opportunities for compound "identification". LC-MS interfacing has been achieved in a number of ways but it was with the advent of atmospheric pressure ionization (API) techniques that LC-MS came of age. API is a soft ionization source suitable for the analysis of polar, non-volatile, thermolabile and high molecular mass molecules such as plant phenols. API-based interfacing systems which are liquid-based include ESI. Such systems have revolutionized the application of LC-MS to biophenols[38,42,48,58,113–115] and represent a superior analytical technique compared to conventional UV detection because of enhanced selectivity and the ability to resolve co-eluting peaks.[66] Choice of elution conditions is not usually a problem although in some instances the need to accommodate the electrospray system may require a compromise. For instance, eluants containing phosphate are not preferred for ESI[27] and the concentration of formic acid in the mobile phase was reduced for the ESI analysis of leaf phenolics in birch.[115]

Of the two modes, positive and negative ionization, the latter has been favoured[42,58,63] although positive ion has been used in specific instances as for detection of anthocyanins.[66] It also provided useful data in the analysis of hydrolysable tannins but was not as easily interpretable as negative ion data.[27] In negative ion ESI of flavanones, flavonols and flavones of birch leaves, compound identification was based on the *m/z* value of the deprotonated molecular ion and the deprotonated dimer.[77,108] Methoxylated flavonoids exhibited an additional ion corresponding to loss of a methyl group from the deprotonated molecular ion.

In a typical application, low-molecular mass phenolic compounds in mountain birch were quantified by HPLC with UV detection at 280 nm[42] but identification was achieved by LC-MS using negative ion ESI. Analysis of healthy sapwood and the reaction zones (tissue of antimicrobial defence) from the sapwood of Eucalyptus trees by HPLC with ESI-MS revealed a diverse range of hydrolysable tannins in both healthy sapwood and in reaction zone extracts.[55] The hydrolysable tannins comprised over 30 gallotannins, ellagitannins and phenols. The majority of these compounds are beyond the range of GC analysis. HPLC was more suitable yet even here problems of co-elution precluded peak identification based on retention and UV spectral data alone. Unequivocal identification was based on mass chromatograms generated for specific deprotonated molecular ions, on mass spectra of the deprotonated molecular ion at specific retention times and on MS-MS of selected daughter ions. Characteristic fragmentations were observed in the spectra. For instance, loss of 44 mass

units from the deprotonated molecular ion was characteristic of a free carboxyl and loss of 18 mass units was characteristic of C-glucosidic ellagitannins.

Procyanidins extracted from pine bark were analyzed using reversed-phase and normal-phase LC-MS in the negative ion mode.[87] Although diode array provides sensitive detection of procyanidins, the identification of individual compounds was impossible. In contrast, ESI produced simple spectra generally comprising a molecular ion peak plus multiply charged ions. Positive ion spectra were suitable for oligomers up to pentamers but the decreased ionization efficiency of higher oligomers favoured negative ion mode. Using the latter, pine bark was found to contain procyanidins from monomers through decamers and higher polymers.

Some problems remain in ESI, the major limitation being the strong dependency of the response on the nature of the analyte and mobile phase. Thus, generation of ESI-based mass spectral libraries is difficult. Moreover, it is difficult to optimize conditions for a typical extract containing a broad range of analytes although instruments now incorporate provision for programmed operation of spectral conditions.

Applications

This section presents a brief overview of some relevant applications. Data are presented (Table 4) on the phenolic profile of representative species that dominate the forest canopy of various habitats. Deciduous trees such as oak, elm, alder, beech and birch are characteristic of Northern Hemisphere temperate regions whilst coniferous forests of pines, firs, spruces and related species are the dominant vegetation in sub-Arctic regions. Eucalyptus and casuarina characterize the floodplain and upper reaches of river systems in Australia. Of all the genera that populate the world's forests, none dominate regionally to the extent that Eucalyptus does. The systematic treatment of phenolic profiles based on forest type was precluded by the patchy and incomplete data compounded by procedural differences. Classifications that distinguish hardwood (*e.g.* oak, ash) *versus* softwood (*e.g.* pine and spruce) are similarly restricted.

Although there is significant variation in the qualitative and quantitative distribution of biophenols in forest trees,[38] a number of phenols are common to many species as shown by the data of Table 4. Eucalyptus species are typical in that they contain many phenols common to other species (Table 4) but a number of novel phenols have also been isolated from eucalyptus.[53,123–128] For instance, three novel phenol glycosides acylated with (+)-oleuropeic acid, called cypellocarpins A, B and C, were isolated from the dried leaves of *Eucalyptus cypellocarpa* along with seven known compounds.[125] The cypellocarpins are rare examples of phenol glycosides in which a monoterpenoid acid is esterified with glucose. Moreover, different eucalyptus species were differentiated by the number and concentrations of phenols observed in their leaves.[54,64] For example, *E. camaldulensis* and *E. rudis* showed the highest concentrations and variety of flavonol glycosides, whilst *E. globulus* was characterized by high concentrations of ellagitannins. The influence of genetic factors on biophenol content has been examined in many papers and results have repeatedly shown that species differences were more important in determining phenolic profile than region or any other single characteristic.[89] It is these features that facilitate the use of biophenols as markers for chemotaxonomy and for DOC sources. Indeed, phenolic composition is used in chemotaxonomy[67] more than any other secondary metabolite.[129]

It is important to recognize that biophenol content is affected both qualitatively and quantitatively by a range of factors apart from genetic and procedural considerations. Apart from species specificity, the occurrence of some biophenols is plant-part specific and their relative proportions in various tissues may change substantially as the plant matures. This is illustrated by the data for eucalyptus in Fig. 1. Quantitative differences in phenol distribution within different plant parts has also been demonstrated for the concentrations of catechin, *cis*-coniferin, *cis*-isoconiferin, and *cis*-syringin between the inner and outer bark in beech trees.[130] In the case of beech, birch and ash, phenolic contents varied considerably between the wood dust and the corresponding leaves.[38] Plant-part specificity of particular phenols has also been demonstrated, for example, in the leaves and stems of bilberry which were characterized by distinctive phenols.[83]

Maturation-dependence is seen in the rapid decrease in the content of eleven flavonols[131] during the early phases of needle outgrowth of Norway spruce. Soluble phenols exhibited a differential accumulation pattern in spruce needles with skimmin (a glycoside of umbelliferone), 4-coumaroyl esters and 4-hydroxybenzoic acid 4-*O*-glucoside accumulating slowly as needle development progressed whilst most phenols showed an increase in concentration during the later phases of needle differentiation.[47] Kaempferol 3-*O*-glucoside was unusual in that it showed a rapid turnover and/or translocation from a soluble to an insoluble (cell wall bound) pool. In the case of oak leaves, old leaves were much richer in proanthocyanidins than young leaves, whereas the opposite was observed for total hydrolysable tannins and flavonoid glycosides.[26] However, when quantified as individual compounds, hydrolyzable tannins and flavonoid glycosides showed highly variable seasonal patterns.

Geographical influences are evidenced by the increase in flavonol content of needles associated with a decrease in latitude of the growing region.[131] Furthermore, the southern region produced more highly oxygenated flavonols than the other regions.

The role of biophenols in plants is at least partly stress-related. Thus, we expect to see a change in phenolic content in plants exposed to biotic or abiotic stresses[132] such as that observed in extracts of inner and outer bark of *Fagus sylvatica* following infection with *Cryptococcus fagisuga* (beech scale) feeding in the parenchyma tissue.[130] Highest concentrations of (2*R*,3*R*)-(+)-glucodistylin, (2*S*,3*S*)-(−)-glucodistylin and 3-*O*-(β-D-xylopyranosyl)taxifolin occurred in European beeches strongly infested with beech scale. The concentration of other phenols was lowered by attack whilst still others were unaffected by infestation. The effect of pathogen presence on the concentration of phenolic acids was investigated in phloem of shoots from Scots pine trees (*Pinus sylvestris L.*) growing with annosum root.[80] The combined concentrations of ferulic and salicylic acids represented the largest fraction of acids

Table 4 Representative data for the biophenolic content of plant parts from various sources

	Beech		Birch	Oak						Chestnut	Pine		Spruce			Eucalypt			
Phenol	Wood dust	Leaf primordia	Leaves/ mg g^{-1}	Wood/ mg g^{-1}	Heartwood/ μg g^{-1}	Dust/ μg g^{-1}	Bark	Leaves	Cork/ μg g^{-1}	Wood/ μg g^{-1}	Shoot phloem/ μg g^{-1}	Needles/ μg g^{-1}	Bark	Needles/ μg g^{-1}	Roots	Wood	Bark	Leaves/ μg g^{-1}	Leaf litter
Gallic acid		Present		14.8	99.8–1867	63–176			12	3.3	0–191	3				Present	Present	0.4–77	Major
(+)-Catechin	Present		0.4–5				Minor	Present					2	Present					
Vanillic acid		Present		1.5	4.2–30.3	1.8–2.0			28	0.3	1.9–197	8–25.0		1.0–1.3	0.06–0.14	Present			Trace
Chlorogenic acid		Present	2.1–9.7								0–437								Minor
Ellagic acid				0.7		183–213			192	1.9						Present	Present	80–157	Major
Ferulic acid		Present			44.2–110.3	0.5–1.3			20	0.8	Present			0.8–1.1				Present	Minor
Protocatechuic acid					0.3–5.7				44			40–70		300–340	0.008–0.1	Present	Present	Trace-22	Major
Quercetin glycosides	Present		0.6–74.1																
Vanillin				0.7	13.7–23.8	1.5–2.9			18			3.7–6.9				Present			Trace
4-Hydroxy benzoic acid					16.2–29.2						1.9–17.3	4.5–11.7		0.8–1.1; 330–600	0.12–0.15				Minor
Caffeic acid					2.3–18.2				8		0–9.3								Minor
Ellagitannins																Present	Present	0.1–4.9	
Kaempferol glycosides			0.7–5.6											Present					Major
p-Coumaric acid					0.2–3.8							5–18		0.5–90; 170–190					
Syringic acid				1.4	4.8–33.0	1.6–2.7				1.2		5							
Syringaldehyde				1.6	4.3–14.7	1.8–3.8				Trace						Present			
Hydroxycinnamic acids			1.2								0–101.9								
Quercetin												0–700					Present	0–61	Present
Scopoletin				0.1		1.1–2.3			8										
Coniferaldehyde				0.9		3.3–4.3			18										
Proanthocyanidins			107 ± 18 soluble; 42 ± 9 bound					Present											
Sinapaldehyde				1.6					1										
Taxifolin	Present											10–600	0.0–0.23						
Aesculetin						0.8–2.5			6										
Epicatechin			Present					Present											
Cinnamic acid											0–80.3			90–110					
Protocatechuic aldehyde									9								Present		
Rutin								Present										0–127	
Quercitrin							Major												
Total phenols			17.2–40.4; 127.4 ± 19.8		8700–38100						274.5–2749	23–41; 1200–3800		67,000–73,000	54–66				
Reference	38	116	1,40–42,65, 67,73,117	118	89	84	119	120,121	43,54	122	80	49,50	88	33,62,81	33	53	53	52,54,64	51

isolated, benzoic and chlorogenic acids were also present as major constituents. Variations in phenolic content were found to be dependent on factors such as genotype, stage of disease development and tree growth conditions, such as insolation exposure and moisture access within root zones. Phenolic metabolite production increased in the needle crowns of Norway spruce which had been inoculated with *Sirococcus conigenis*.[81] Compared to controls, the concentrations of all but one, picein, of five detected phenolic compounds increased significantly in the needle crowns of inoculated specimens.

Abiotic stresses such as pollution also impact phenolic content, which has been used as a biomarker of atmospheric pollution.[49] Most studies on the impact of environmental pollution on biophenol content have involved controlled experimental conditions and presented data on total phenols only. Many studies examine only ozone impact.[133] However, recent studies have involved natural conditions and reported data for total phenols plus individual simple phenols.[49,62] These studies showed that the response was dependent on the pollutant and varied with the particular biophenol. Histochemical methods did not reveal any differences in localization of biophenols among the needles of Norway spruce collected from four sites which were characterized by different levels of environmental pollution damage.[62] Moreover, there were no significant differences in total Folin–Ciocalteau phenol contents of the needles but HPLC revealed marked alterations in the forms of seven phenolic acids. Thus, concentrations of conjugated forms of phenolic acids were higher in damaged needles (255.9 μg g^{-1}) than in healthy needles (189.8 μg g^{-1}) while content of esterified phenolic acids incorporated into cell walls was higher in needles from healthy trees (101.1 μg g^{-1}) than in damaged needles (78.3 μg g^{-1}). Similar observations have been reported[134] in relation to total and specific phenols in pine needles while contents of (+)-catechin and various gallic acid derivatives in birch leaves decreased significantly along a pollution gradient from a smelter.[42] Contents of flavonol glycosides slightly increased with the distance from the smelter whereas hydroxycinnamic acid derivatives remained unaffected.

Foliage and litter quality in *E. globulus* plantations in Gippsland, south-eastern Australia were characterized on three sites covering a range of soil types, inherent soil fertility and fertiliser treatments.[135] Phenol concentrations in foliage and litter varied significantly between sites, with the least fertile site showing significantly higher concentrations of phenols in recent litter. Phenols extracted in cold trichloroacetic acid decreased from old foliage to litter at all sites. Temporal variations in the levels of phenols in leaves of *E. nitens* seedlings[66] have also been observed. However, longer term variations, if any, are unknown.

The isolation of novel phenols from forest trees has been reported[39,47,71,90,112,120,125] in a number of publications. Such studies typically involve larger-scale isolation of the biophenols often involving preparative scale HPLC and with off-line characterization. In many cases, nuclear magnetic resonance spectrometery (NMR) is needed for full characterization and until recently it has been difficult to obtain adequate sensitivity for on-line studies of this type. For example, air dried leaves of silky oak were extracted with boiling methanol for 3 h[37] and evaporated to dryness *in vacuo*. The extract was suspended in water and extracted sequentially with hexane, chloroform, ethyl acetate and butanol. Column chromatography of the chloroform- and butanol-soluble fractions on silica gel and Sephadex LH-20, respectively yielded several phenolic fractions. Further purification was achieved with preparative scale HPLC. Seven new phenols apart from known compounds were identified by 2D NMR and MS.

Methanolic extraction, liquid–liquid partitioning and column chromatography on polyamide, silica gel and Sephadex LH-20 were used to recover novel phenols from bark of infected beech.[130] Characterization was achieved by TLC, circular dichroism, IR, UV and MS plus several two-dimensional (COSY, HMQC, HMBC) NMR techniques. A similar extraction and partitioning procedure was used[136] to isolate phenols from Myrtaceae. However, no new compounds were isolated illustrating one of the frustrating features of such work. The analysis of phenolic acids in Eucalyptus,[51] spruce and bilberry[33] also demonstrates an important point. Current wisdom suggests that the phenolic acids occur naturally as the all-*trans* isomers and thus, where measurable amounts of *cis* isomer are observed it is assumed that this is a feature of the analytical methodology. Peak areas for the isomers are summed and data are reported as the single isomer.[33,51] Future developments may depend on closer attention to such seemingly anomalous data that should not be dismissed as artefactual. Xenobiotic phenols are ubiquitous in the environment and their presence in bio-extracts is not unexpected. The authors are not aware of any work that addresses this possibility.

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Danny R. Bedgood, Jr., Andrea G. Bishop, Paul D. Prenzler and Kevin Robards*
Charles Sturt University, School of Science and Technology, Locked Bag 588, Wagga Wagga 2678, Australia. E-mail: krobards@csu.edu.au

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