Available online at www.sciencedirect.com
SCIENCE DIRECT®
Pergamon
Biochemical Systematics and Ecology 31 (2003) 115–127
biochemical systematics and ecology
www.elsevier.com/locate/biochemsyseco

Plant-part specific and temporal variation in phenolic compounds of boreal bilberry (*Vaccinium myrtillus*) plants

Johanna Witzell [a,*], Rolf Gref [b], Torgny Näsholm [a]

[a] *Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, SE-901 83 Umeå, Sweden*
[b] *Department of Silviculture, Swedish University of Agricultural Sciences, SE-901 83 Umeå, Sweden*

Received 22 October 2001; accepted 5 April 2002

Abstract

Leaves and stems of bilberry were analysed for phenolic metabolites using a simple method of extraction and HPLC analysis. Hydroxycinnamic acid derivatives (HCAs) and flavonoids were identified on the basis of their UV-spectra. Temporal fluctuations in the levels of selected phenolic compounds were followed during the growth season of 2000. Qualitative and quantitative differences were detected in phenolic profiles between leaf and stem tissues. The most abundant peak in leaf samples was tentatively identified as chlorogenic acid (5-*O*-caffeoylquinic acid). In stems, this compound was found at much lower levels than in leaves and an unidentified p-coumaric acid derivative dominated the phenolic profile. The most abundant flavonols in leaves and stems were quercetin derivatives. The total sum of methanol-soluble phenolics was generally higher in leaves than in stems. The concentrations of some alkaline hydrolysis products were higher in stems than in leaves, indicating that a larger part of the phenolic pool was incorporated into lignified cell walls in the stems. Individual phenolic compounds differed in their seasonal fluctuation patterns. It is suggested that the observed plant-part specific and within-seasonal variation may influence the ecological interactions between bilberry and its natural enemies.

Keywords: HPLC; Phenolic acids; Flavonoids; *Vaccinium myrtillus*; Chemical defence

* Corresponding author. Tel.: +46-90-786-9392; fax: +46-90-786-5901.
E-mail address: johanna.witzell@genfys.slu.se (J. Witzell).

0305-1978/03/$ - see front matter © 2002 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0305-1978(02)00141-2

1. Introduction

Bilberry (*Vaccinium myrtillus* L.) is an ericaceous dwarf shrub that is commonly found in the herbaceous layer of boreal forests (cf. Jäderlund et al., 1998 and refs. within). Modern food chemists are interested in bilberry due to its potential as a source of biologically active non-nutrient compounds (so-called functional food). These include phenolic compounds, e.g., phenolic acids and flavonoids, which may act as natural antioxidants, anticarcinogens and antimicrobial agents (Häkkinen, 2000; Häkkinen and Auriola, 1998; Häkkinen et al., 1998, 1999a; Kähkönen et al., 1999; Madhavi et al., 1998; Prior et al., 1998; Rauha et al., 2000). In recent years, the phenolic compounds of bilberry litter have also received increasing interest in ecological studies on allelopathic relationships in forest soils (Pellissier, 1993; Gallet, 1994; Gallet and Lebreton, 1995; Jäderlund et al., 1998).

The phenolic compounds in the nutritionally important bilberry fruits have been extensively investigated using modern analytical techniques (e.g. HPLC and GC-MS) in various studies, including Häkkinen (2000); Häkkinen and Törrönen (2000) and Häkkinen et al. (1999b). However, recent reports on the variation in phenolic concentrations and composition in the vegetative tissues of growing bilberry plants are scarce. In early studies quercetin-3-rhamnoside (quercitrin), quercetin-3-glucoside (isoquercitrin) and quercetin-3-arabinoside (avicularin) were identified among the flavonol glycosides in the leaves of Central European bilberry plants by Ice and Wender (1953) using absorption chromatography and, later, von Friedrich and Schönert (1973) using paper chromatography and thin layer chromatography. The latter authors also reported the presence of caffeic and chlorogenic acids in the ratio 1:5 in dried bilberry leaves. However, they did not find arbutin or hydroquinone, which had previously been reported to be present in bilberry leaves (von Friedrich and Schöenert, 1973). Using liquid chromatography (HPLC), Sticher et al. (1979) analysed water extracts of freeze-dried bilberry leaves for the presence of arbutin, methylarbutin, hydroquinone and hydroquinonemonomethylether, but found no evidence of these compounds. They did not, however, report data concerning phenolic acids or flavonoids. Pellissier (1993) analysed phenolics in water extracts of bilberry leaves after alkaline hydrolysis and identified a number of phenolics, including caffeic acid, vanillic acid, quercetin and kaempferol. Gallet (1994) and Gallet and Lebreton (1995) studied phenolic constituents in bilberry leaves and litter, and name caffeic acid and catechol as the major phenolic monomers in bilberry leaves. Fraisse et al. (1996) identified chlorogenic acid, quercetin-3-D-galactoside (hyperoside), isoquercitrin, quercetin-3-glucuronide, avicularin and quercitrin in bilberry, but did not mention caffeic acid.

Plant phenolics show marked qualitative and quantitative variation at different genetic levels (between and within species and clones) (e.g., Hakulinen et al., 1995; Nichols-Orians et al., 1993) and between different physiological and developmental stages (Kause et al., 1999). They also vary in response to environmental factors, such as light intensity and nutrient availability (see Herms and Mattson, 1992 and refs. therein). Thus, the existing information on the chemistry of Central European bilberry populations may not be directly comparable to data on Scandinavian popu-

lations. In the present study, we report a simple extraction and HPLC-analysis method for the quantification and partial identification of phenolic acids and non-anthocyanidin flavonoids in the vegetative tissues of boreal bilberry plants. We also investigated the plant-part specific variation in phenolic profiles in bilberry plants grown in the herbaceous layer of a boreal spruce forest (in northern Sweden), and followed the fluctuations in the concentrations of selected phenolics in bilberry leaves during one growth season. The results of the study provide new information on the chemical variation of bilberry plants in the boreal ecosystem and can be used as background information for chemoecological studies, where the role of phenolics in biotic interactions between boreal bilberry plants and their natural enemies is investigated.

2. Material and methods

2.1. Standards and reagents

The standard compounds gentisic acid, chlorogenic acid, p-coumaric acid, syringic acid, luteolin, kaempherol, myricetin and quercitrin (quercetin-3-L-rhamnoside) were purchased from Sigma (St. Louis, MO), caffeic acid, ferulic acid and rutin (quercetin-3-rutinoside) from Aldrich (Steinheim, Germany), apigenin and hyperoside (quercetin-3-D-galactoside) from Fluka (Buchs, Switzerland). Methanol and acetic acid were HPLC grade (J.T. Baker, Deventer, Holland). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA).

2.2. Sample preparation and extraction of phenolics

Plant material was collected from an experimental forest at Svartberget research station in Vindeln, northern Sweden (64° 14′N, 19° 46′E). The forest is a late successional Norway spruce (*Picea abies*) forest and bilberry is the dominating ericaceous shrub in the herbaceous layer (see Nordin et al., 1998 and Strengbom et al., 2002 for further details on the site). The experimental plots from which the plant material was collected were unfertilized. To study the within-seasonal variation, material was collected from five plots in late June, mid-July, and early and late August 2000. At each time point, 8–10 current annual shoots were collected at each plot. The shoots originated from ramets, which were located at least 5 m apart from each other. In the testing of the plant-part specific variation with different extraction and hydrolysis methods, material from three plots, collected in early August, was used. The plant material was air-dried at room temperature for about two weeks and stored at −20 °C until analysed (within six months of harvest).

The leaves were detached from the shoots and all the healthy, full-grown leaves were crushed manually inside a paper bag until a powdery consistency was obtained. The stems were cut into small pieces with scissors until the material was fine-grained and homogenous. The bulk samples of leaf and stem material obtained in this way were both divided into five sub-samples, each of which was randomly assigned to

different extraction or hydrolysis procedures (see below). Each sub-sample was extracted or hydrolysed and analysed with HPLC in triplicate. About 15 mg of plant material was used in each replicate analysis. The material was weighed in eppendorf vials and three tungsten carbide beads, (Qiagen GmbH, Hilden, Germany), 3 mm in diameter, were added to each vial together with 1000 μL of an extraction solvent (cf. Weiss et al., 1999). We tested the following extraction solvents: methanol, 80% aqueous MeOH, and 50% aqueous MeOH. All solvents were cooled to +4 °C before they were added and the vials were kept on ice. The samples were shaken in a Retsch mixer mill (Model MM2 at speed setting 80; Retsch GmbH & CO. KG, Haan, Germany) for 2 min, after which they were centrifuged at +4 °C (12000 rpm, 5 min in a Hettich EBA 12 centrifuge) and the supernatant was transferred to a vial. The residue was extracted as before with 500 μL of solvent, and the supernatants were combined. The solvent was evaporated to dryness in a vacuum concentrator (Savant, Speed Vac Concentrator SC 1000 with a Savant condensation trap and a vacuum pump). The dried samples were dissolved in 400 μL water:methanol (1:1) and filtered through a disposable syringe filter (pore size 0.2 μm; Agilent Technologies, Palo Alto, CA) before the injection of 20 μL into the HPLC system.

Alkaline hydrolysis was carried out using a method modified from Kader et al. (1996). Briefly, the samples were hydrolysed in 2 M NaOH under nitrogen at room temperature for 3 h. The samples were acidified with concentrated HCl and extracted with ethyl acetate (3×1000 μL). The upper phase was separated and evaporated to dryness under nitrogen. The samples were dissolved in water:methanol (1:1) and filtered as described above. Acid hydrolysis was carried out in 1 M HCl-methanol at 90 °C for 30 min. After quick cooling in a cold water bath the samples were extracted with ethyl acetate and HPLC-samples were prepared as described above. The hydrolysis procedures were applied solely as a means to obtain qualitative information and were not specifically optimised for bilberry tissues.

HPLC analyses were performed using a Waters 717 HPLC system (plus Autosampler, 600 Controller, 996 photodiode array detector and Millenium 32 chromatography manager software). A reversed-phase C-18 column (Thermo Quest HyPURITY™ C18, 100×4.6 mm i.d., 3 μm particle size) was used as the solid phase and in each analysis the mobile phase consisted of a gradient of solvents A (aqueous 1% acetic acid) and B (solvent A in methanol, 4:6, v/v). After testing different gradients, flow velocities and column temperatures, the gradient was set as follows: 15% B in A (0–5 min); 15–20% B in A (5–15 min), 20–35% B in A (15–25 min), 35–60% B in A (25–40 min) and 60–15% B in A (40–55 min) followed by washing and re-equilibration (10 min) of the column to initial conditions. The flow rate was 1.2 mL/min and the column temperature 40 °C. UV-spectra were recorded between 200 and 400 nm. Eluted compounds with a UV-spectrum matching that of either chlorogenic acid or p-coumaric acid standards were classified as hydroxycinnamic acids (HCAs), those with a spectrum resembling quercetin or its derivatives (rutin, hyperoside) were classified as flavonoids. The compounds of interest were quantified as chlorogenic acid (HCAs) or quercitrin (flavonoids) equivalents, according to their respective peak areas as monitored at 320 nm. Due to low number of replicate analyses, a Kruskall-Wallis test was applied on data to test the effects of different extrac-

tion solvents on phenolic yield from leaf and stem material. One-way ANOVA was used to the effect of sampling time on phenolic levels. When necessary, the data were log (x + 1) transformed to improve the normality and homoscedasticity.

3. Results and discussion

3.1. Evaluation of the methodology

Various procedures can be used to extract phenolics from plant tissues (Harborne, 1998; Waterman and Mole, 1994). Methanol is commonly used as an extraction solvent for this purpose, and its use in aqueous mixtures has been found to be advantageous for extracting phenolic glycosides from dried plant material (Harborne, 1998). We compared pure methanol with methanol containing 20 or 50% water as extraction solvents for dried bilberry tissues. The mean concentrations of two peaks representing HCAs and two peaks representing flavonoid compounds, obtained from the extracts using the three solvents, are shown in Table 1. There were no statistically significant differences in the concentrations of these phenolics extracted with the different solvents (Kruskall–Wallis test, χ^2 values 0.103–0.230, df=2, P values 0.891–0.930, Table 1). However, in leaf samples the phenolic yields tended to be higher when samples were extracted with pure methanol or 80% methanol, rather than 50% methanol (Table 1). In contrast, in stem samples the mean concentrations of the analysed compounds tended to be higher in 50% methanol extracts than in methanol and 80% methanol extracts. This indicates a more pronounced glycosylation of phenolics in stems. Since the variation between replicate extractions was lowest when 100% methanol was used (Table 1), and the presence of water increases the time required for drying the samples in the evacuated centrifuge, pure methanol is recommended as the extraction solvent in routine analysis of phenolics from dried bilberry tissues.

The extraction procedure we used requires only a small amount of plant material and a small volume of extraction solvent. The simultaneous homogenisation of the plant material and extraction of phenolics using tungsten carbide beads and a mixer mill allowed up to 50 samples to be processed within a normal working day. Thus, the time required per extraction and the need for manual work was reduced as compared to extraction using mortar and pestle or motorized homogenisers. The HPLC conditions described above provided a relatively good separation of phenolic acids and flavonoids (Figs. 1 and 2a,b) and allowed accurate integration of peaks at wavelengths in the range 240–360 nm. The vegetative tissues of bilberry also contained some phenolic compounds with absorption maxima near 220 nm. For the accurate quantification of these compounds acidification of the eluent water with phosphoric acid (pH 3.0–3.3) instead of acetic acid is recommended, to reduce background absorption at low wavelengths.

Table 1
Mean concentrations (μmol/g DW) and standard deviation (n=3) of representative HCAs (peaks 2 and 5) and flavonoids (peaks 10 and 12) in bilberry (*Vaccinium myrtillus* L.) leaves and stems extracted with different solvents (100% methanol, aq. 80% methanol and aq. 50% methanol). For an explanation of the numbering of the peaks, see Fig. 2a

Extraction solvent:		Peak 2		Peak 5		Peak 10		Peak 12	
	Leaves:	mean	*SD*	mean	*SD*	mean	*SD*	mean	*SD*
MeOH		118.8	*7.3*	0.60	*0.09*	18.98	*3.8*	1.11	*0.2*
aq. 80% MeOH		124.5	*6.8*	0.56	*0.09*	19.76	*2.4*	1.14	*0.1*
aq. 50% MeOH		108.2	*18.1*	0.42	*0.10*	17.97	*5.4*	0.89	*0.3*
	Stems:	mean	*SD*	mean	*SD*	mean	*SD*	mean	*SD*
MeOH		9.54	*1.7*	0.08	*0.01*	8.33	*1.4*	0.99	*0.2*
aq. 80% MeOH		9.62	*3.9*	0.11	*0.03*	8.31	*4.8*	0.91	*0.4*
aq. 50% MeOH		11.56	*3.9*	0.13	*0.05*	8.51	*4.0*	1.13	*0.6*

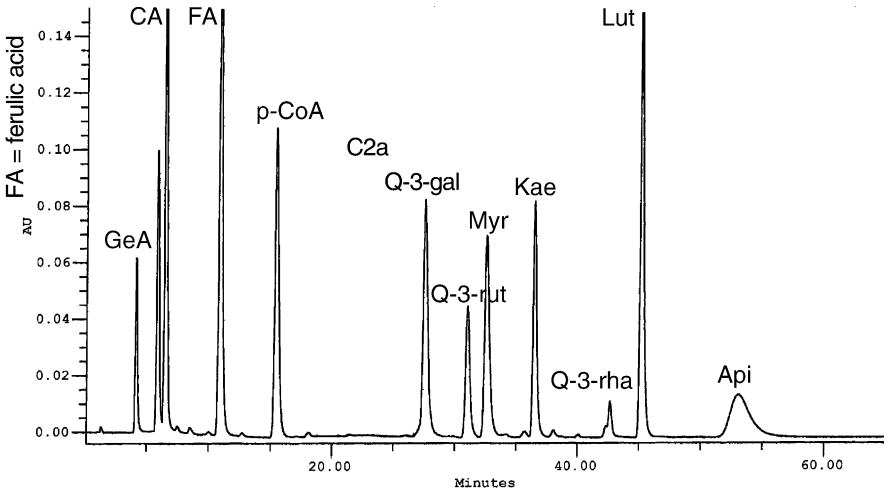

Fig. 1. HPLC-separation of selected phenolic standards at 320 nm. GeA=gentisic acid, CQA=chlorogenic acid, CA=caffeic acid, FA=ferulic acid, p-CoA=p-coumaric acic, Q-3-gal=quercetin-3-D-galactoside (hyperoside), Q-3-rut=quercetin-3-rutinoside (rutin), Myr=myricetin, Kae=kaempherol, Q-3-rha=quercetin-3-rhamnoside (quercitrin), Lut=luteolin, Api=apigenin.

3.2. Phenolic profiles in leaves and stems of bilberry

The UV-spectra of the eluted compounds revealed that the most abundant phenolics in methanol extracts of blueberry tissues were hydroxycinnamic acid derivatives (HCAs). Flavonoids in bilberry tissues were mainly quercetin derivatives, as indicated by their spectral similarity with quercetin and its glycoside standards (rutin and hyperoside). The leaves and stems also contained some compounds with UV-spectra resembling that of the (+)-catechin standard. The histological location of the analysed compounds still remains to be determined. Preliminary tests showed that a brief (30 sec) flushing of fresh bilberry shoots with methanol yielded several phenolic metabolites in considerable amounts (J. Witzell, unpublished). This suggests that in bilberry leaves some phenolics are epicuticular and may thus readily act as positive or negative signals to parasites and herbivores attacking bilberry plants.

There were quantitative differences in the levels of phenolic compounds between stem and leaf samples (Table 2). Kruskall–Wallis test on the data presented in Table 1 indicated that only one of the tested compounds (a flavonoid, peak 12) did not differ between leaves and stems (χ^2=0.593, *df*=1, *P*=0.441; for the other compounds *P*=0.001). In stems, a hydroxycinnamic acid derivative (peak 21) with a UV-spectrum similar to that of the p-coumaric acid standard dominated the chromatographic profile. In leaf samples, on the other hand, the most abundant peak (peak 2 in Fig. 2 a) had a UV-spectrum matching that of the chlorogenic acid standard. Hydrolysis seemed to degrade the compound(s) corresponding to peak 2 and increase the area of peak 3 (see Table 2). The latter co-eluted with, and had a UV-spectrum similar to that of the caffeic acid standard and could therefore represent the caffeoyl moiety

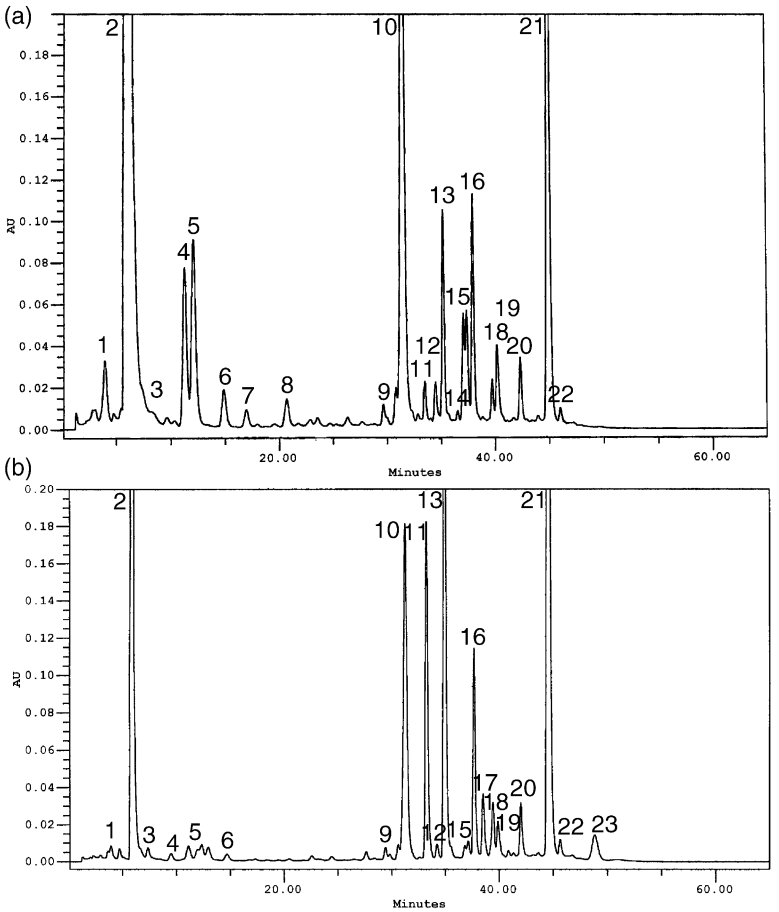

Fig. 2. HPLC-separation of phenolic compounds extracted with methanol from (a) the leaves and (b) stems of bilberry (*Vaccinium myrtillus*) and detected by UV absorbance at 320 nm.

of chlorogenic acid. In the methanol extracts of leaf samples, peak 3 (assigned as caffeic acid) was detected only as a small shoulder on peak 2 (Table 2). Therefore, it appears that the major phenolic compound in the analysed bilberry leaves was chlorogenic acid. This finding is consistent with the earlier report of Fraisse and co-workers (1996). In plants, at least two isomers of chlorogenic acid have been reported: neochlorogenic acid (3-*O*-caffeoylquinic acid) and cryptochlorogenic acid (4-*O*-caffeoylquinic acid) (Nakatani et al., 2000). To our knowledge, the presence of these isomers in bilberry has not been confirmed, but it is possible that the large peak (peak 2) contained at least two isomers, which were not separated under the analytical conditions we used.

In stems, the concentration of HCA(s), detected as peak 2, and the concentration of the main quercetin derivative (peak 10), was lower than in leaves. Compared to leaves, stems contained higher levels of some flavonoids (e.g., peak 11) (Table 2).

Table 2
Concentrations (μmol/g DW) of selected hydroxycinnamic acid derivatives and flavonoids in leaves stems of bilberry (*Vaccinium myrtillus* L.). measured in methanol extracts and after hydrolyses (mean and standard deviation. *n*=3). For an explanation of the numbering of the peaks, see Fig. 2a. For the peak numbering, see Fig. 2. (*n.d.*=not detected)

Group of phenolics	**Peak nr**	**Leaves: *MeOH***		***Acid hydrolysis***		***Aalkaline hydrolysis***		**Stems: *MeOH***		***Acid hydrolysis***		***Alkaline hydrolysis***	
		mean	*(sd)*	mean	*(sd)*	mean	*(sd)*	mean	*(sd)*	mean	*(sd)*	mean	*(sd)*
HCAs:													
	2	118.89	*(7.32)*	28.13	*(1.84)*	*n.d.*		9.45	*(1.72)*	1.51	*(0.59)*	*n.d.*	
	3	3.09	*(0.00)*	18.90	*(1.68)*	8.28	*(3.00)*	*n.d.*		0.98	*(0.33)*	86.14	*(9.83)*
	5	3.57	*(0.45)*	3.97	*(0.89)*	32.13	*(8.17)*	0.13	*(0.02)*	3.11	*(0.98)*	0.25	*(0.03)*
	6	0.60	*(0.09)*	0.32	*(0.07)*	3.42	*(0.93)*	0.09	*(0.01)*	0.16	*(0.03)*	2.51	*(0.94)*
	7	0.29	*(0.04)*	0.22	*(0.20)*	0.29	*(0.02)*	*n.d.*		*n.d.*		0.42	*(0.13)*
	8	0.79	*(0.10)*	*n.d.*		*n.d.*		*n.d.*		*n.d.*		*n.d.*	
	13	3.18	*(0.68)*	*n.d.*		0.10	*(0.02)*	7.21	*(2.45)*	*n.d.*		0.36	*(0.01)*
	17	*n.d.*		*n.d*		0.11	*(0.03)*	0.68	*(0.16)*	*n.d.*		*n.d.*	
	19	0.40	*(0.07)*	0.38	*(0.03*	0.50	*(0.17)*	0.50	*(0.04)*	*n.d.*		0.77	*(0.14)*
	20	1.05	*(0.11)*	2.24	*(0.04)*	*n.d.*		0.66	*(0.11)*	0.96	*(0.12)*	*n.d.*	
	21	9.90	*(1.48)*	1.34	*(0.23)*	*n.d.*		17.65	*(2.01)*	1.20	*(0.34)*	*n.d.*	
	23	*n.d.*		*n.d.*		*n.d.*		0.87	*(0.41)*	*n.d.*		*n.d.*	
	Sum:	***141.76***		***55.50***		***44.83***		***37.24***		***7.92***		***90.45***	
Flavonoids:													
	9	0.59	*(0.03)*	*n.d.*		0.27	*(0.07)*	0.19	*(0.03)*	0.03	*(0.01)*	0.47	*(0.02)*
	10	18.98	*(3.82)*	2.44	*(0.24)*	5.03	*(1.39)*	8.33	*(1.41)*	1.03	*(0.34)*	9.91	*(0.23)*
	12	0.47	*(0.12)*	*n.d.*		*n.d.*		0.19	*(0.02)*	*n.d.*		*n.d.*	
	11	0.94	*(0.20)*	*n.d.*		*n.d.*		7.02	*(0.78)*	*n.d.*		0.15	*(0.03)*
	14	1.48	*(0.38)*	0.31	*(0.14)*	1.39		0.18	*(0.05)*	*n.d.*		0.67	*(0.25)*
	15	1.90	*(0.28)*	0.42	*(0.15)*	0.23	*(0.06)*	0.20	*(0.05)*	*n.d.*		0.66	*(0.51)*
	16	2.20	*(0.27)*	*n.d.*		0.15	*(0.04)*	3.55	*(0.75)*	*n.d.*		*n.d.*	
	Sum:	***26.56***		***3.17***		***7.07***		***19.66***		***1.06***		***11.86***	

The high level of peak 3 (assigned as caffeic acid) after alkaline hydrolysis of stem samples (Table 2) indicates that a large part of the total pool of this compound may have been bound to cell walls of the lignified cells in stem tissues.

In addition to quantitative differences, there were also qualitative differences in the phenolic profiles of leaves and stems. Peaks 7 and 8 were characteristic of leaf tissue, and were not present in detectable amounts in methanol extracts of stem samples (Figs. 2a, b; Table 2), (Fig. 3). On the other hand, two unknown compounds with UV-spectra matching the UV-spectrum of p-coumaric acid (peaks 17 and 23) appeared to be characteristic of stems.

3.3. Temporal fluctuation in the concentrations of phenolic acids and flavonoids—ecological implications

In the present study we wanted to obtain information on possible temporal fluctuations in leaf phenolics during a growth season. Five compounds representing three HCAs and two flavonoids were chosen for analysis with one-way ANOVA on the

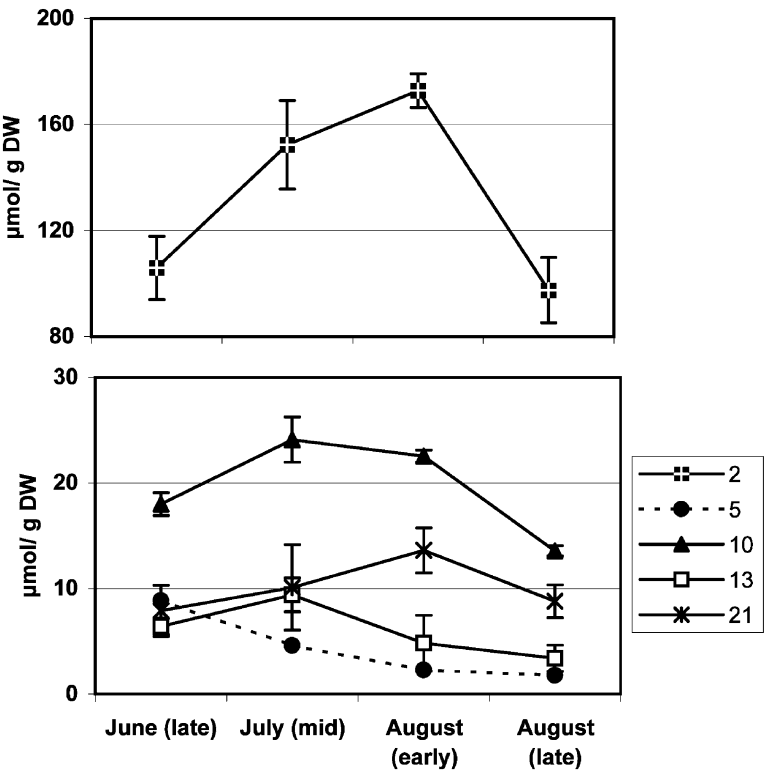

Fig. 3. Fluctuation in concentrations of selected phenolic compounds in bilberry (*Vaccinium myrtillus*) leaves during one growth season. For an explanation of the numbering of the peaks, see Fig. 2a. The mean concentrations of five replicates are shown. Vertical bars represent standard errors of the means.

effect of sampling date. There was a statistically significant decrease in concentration of a ferulic acid derivative (peak 5) during the growth season (log (x + 1) transformed data: *MS*=1313, *df*=3, *F*=6642, *p*=0,004) Within the study period, the main HCA (chlorogenic acid isomer(s)) showed an increasing trend from July to early August, but by the end of August it had decreased again, to a level similar to the initial concentration found in June. One-way ANOVA detected no significant sampling date effect on this compound, but multiple comparisons indicated that the concentration of this compounds was significantly higher in early August than in late August (LSD test, *P*=0,047). The main flavonol (peak 10), and a p-coumaric acid derivative (peak 13) appeared to reach their highest levels in mid-July, but significant differences were not detected by one-way ANOVA. The level of the peak 21 was very stable throughout the growth season. These findings indicate that individual compounds vary in their within-seasonal dynamics, and that temporal fluctuations may be found in the levels of some phenolics in bilberry during the growth period. In nature, temporal variation in the prevailing concentration of phenolics in bilberry leaves is to be expected, since the synthesis and accumulation of phenolics is known to be regulated by a complex interaction between intrinsic plant factors (ontogeny and phenology) and external factors, both abiotic (e.g. light and nutrient availability) and biotic (e.g. natural enemies) (Beckman, 2000; Booij-James et al., 2000; Herms and Mattson, 1992). Seasonal trends in concentrations of individual phenolics have also been reported earlier, for instance in birch (*Betula*) species (Kause et al., 1999).

In boreal forests, bilberry plants are continuously and simultaneously consumed by insect herbivores and infected by several fungal species. If phenolic compounds have defensive functions in bilberry (cf. Dixon and Paiva, 1995; Nicholson and Hammerschmidt, 1992), any plant-part specific or temporal variation in the quality and quantity of phenolics has the potential to affect their resistance to herbivores and parasites. We therefore suggest that the observed plant-part specific variation, together with the seasonal fluctuation in biologically active phenolic metabolites, may affect the ecological interactions between bilberry and its natural enemies.

Acknowledgements

We thank Margareta Zetheström for her invaluable help and advice during the method development and John Blackwell for revising the English language. The study was financially supported by Academy of Finland and the Jenny and Antti Wihuri Foundation.

References

Beckman, C.H., 2000. Phenolic storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defense responses in plants? Physiol. Mol. Plant Path. 57, 101–110.

Booij-James, I.S., Dube, S.K., Jansen, M.A.K., Edelman, M., Mattoo, A.K., 2000. Ultraviolet-B radiation

impacts light-mediated turnover of the photosystem III reaction center heterodimer in *Arabidopsis* mutants altered in phenolic metabolism. Plant Physiol. 124, 1275–1283.
Dixon, R.A., Paiva, N.L., 1995. Stress-induced phenylpropanoid metabolism. Plant Cell 7, 1085–1097.
Fraisse, D., Carnat, A., Lamaison, J.-L., 1996. Composition polyphénolique de la feuille de myrtille. Ann. Pharm. Franc. 54, 280–283.
von Friedrich, H., Schönert, J., 1973. Untersuchungen über einige Inhaltsstoffe der Blätter und Früchte von *Vaccinium myrtillus*. Planta Med. 24, 90–100.
Gallet, C., 1994. Allelopathic potential in bilberry-spruce forests: influence of phenolic compounds on spruce seedlings. J. Chem. Ecol. 20 (5), 1009–1024.
Gallet, C., Lebreton, P., 1995. Evolution of phenolic patterns in plants and associated litters and humus of a mountain forest ecosystem. Soil Biol. Biochem. 27 (2), 157–165.
Hakulinen, J., Julkunen-Tiitto, R., Tahvanainen, J., 1995. Does nitrogen fertilization have an impact on the trade-off between willow growth and defensive secondary metabolism? Trees–Structure and Function 9, 235–240.
Harborne, J.B., 1998. Phytochemical Methods, third ed. Chapman & Hall, London.
Herms, D.A., Mattson, W.J., 1992. The dilemma of plants: to grow or defend. Quart. Rev. Biol. 67, 283–335.
Häkkinen, S., 2000. Flavonols and phenolic acids in berries and berry products. Dissertation. Kuopio University Publications D. Medical Sciences 221.
Häkkinen, S., Auriola, S., 1998. High-performance liquid chromatography with electrospray ionisation mass spectrometry and diode array ultraviolet detection in the identification of flavonol aglycones and glycosides in berries. J. Chrom. A. 829, 91–100.
Häkkinen, S.H., Törrönen, A.R., 2000. Content of flavonols and selected phenolic acids in strawberries and *Vaccinium* species: influence of cultivar, cultivation site and technique. Food Res. Int. 33, 517–524.
Häkkinen, S.H., Kärenlampi, S.O., Heinonen, I.M., Mykkänen, H.M., Törrönen, R., 1998. HPLC method for screening of flavonoids and phenolic acids in berries. J. Sci. Food Agric. 77, 543–551.
Häkkinen, S., Heinonen, M., Kärenlampi, S., Mykkänen, H., Ruuskanen, J., Törrönen, R., 1999a. Screening of selected flavonoids and phenolic acids in 19 berries. Food Res. Int. 32, 345–353.
Häkkinen, S.H., Kärenlampi, S.O., Heinonen, I.M., Mykkänen, H.M., Törrönen, A.R., 1999b. Content of flavonols quercetin, myricetin and kaempferol in 22 edible berries. J Agric. Food Chem. 47, 2274–2279.
Ice, C.H., Wender, S.H., 1953. Quercetin and its glycosides in leaves of *Vaccinium myrtillus*. J. Am. Chem. Soc. 75, 50–52.
Jäderlund, A., Norberg, G., Zackrisson, O., Dahlberg, A., Teketay, D., Dolling, A., Nilsson, M.-C., 1998. Control of bilberry vegetation by steam treatment — effects on seeded Scots pine and associated mycorrhizal fungi. For. Ecol. Man. 108, 275–285.
Kader, F., Rovel, B., Girardin, M., Metche, M., 1996. Fractionation and identification of the phenolic compounds of highbush blueberries (*Vaccinium corymbosum* L.). Food Chem. 55 (1), 35–40.
Kause, A., Ossipov, V., Haukioja, E., Lempa, K., Hanhimäki, S., Ossipova, S., 1999. Multiplicity of biochemical factors determining quality of growing leaves. Oecologia 120, 102–112.
Kähkönen, M.P., Hopia, A.T., Vuorela, H.J., Rauha, J.-P., Pihlaja, K., Kujala, T.S., Heinonen, M., 1999. Antioxidant activity of plant extracts containing phenolic compounds. J. Agric. Food Chem. 47, 3954–3962.
Madhavi, D.L., Bomser, J., Smith, M.A.L., Singletary, K., 1998. Isolation of bioactive constituents from *Vaccinium myrtillus* (bilberry) fruits and cell cultures. Plant Science 131, 95–103.
Nakatani, N., Kayano, S., Kikuzaki, H., Sumino, K., Katagiri, K., Mitani, T., 2000. Identification, quantitative determination, and antioxidative activities of chlorogenic acid isomers in prune (*Prunus domestica* L.). J. Agric. Food Chem. 48, 5512–5516.
Nichols-Orians, C.M., Fritz, R.S., Clausen, T.P., 1993. The genetic basis for variation in the concentration of phenolic glycosides in Salix sericea: clonal variation and sex-biased differences. Biochem. Syst. Ecol. 21, 535–542.
Nicholson, R.L., Hammerschmidt, R., 1992. Phenolic compounds and their role in disease resistance. Annu. Rev. Phytopathol. 30, 369–389.

Nordin, A., Näsholm, T., Ericson, L., 1998. Effects of simulated N deposition on understorey vegetation of a boreal coniferous forest. Funct. Ecol. 12, 691–699.

Pellissier, F., 1993. Allelopathic effect of phenolic acids from humic solutions on two spruce mycorrhizal fungi: *Genococcum graniforme* and *Laccaria laccata*. J. Chem. Ecol. 19, 2105–2114.

Prior, R.L., Cao, G., Martin, A., Sofic, E., McEven, J., O'Brien, C., Lischner, N., Ehlenfelt, M., Kalt, W., Krewer, G., Mainland, M., 1998. Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of *Vaccinium* species. J. Agric. Food Chem. 46, 2686–2693.

Rauha, J.-P., Remes, S., Heinonen, M., Hopia, A., Kähkönen, M., Kujala, T., Pihlaja, K., Vuorela, H., Vuorela, P., 2000. Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolics compounds. Int. J. Food Microbiol. 56, 3–12.

Sticher, O., Soldati, F., Lehman, D., 1979. Hochleistungsflüssigchromatographische Trennung und quantitative Bestimmung von Arbutin. Methylarbutin, Hydrochinon und Hydrochinonmonomethylaether in Arctostaphylos-, Bergenia-, Calluna- und Vaccinium Arten. Planta Med. 35, 235–261.

Strengbom, J., Nordin, A., Näsholm, T., Ericson, L., 2002. Parasitic fungus mediates changes in nitrogen exposed boreal forest vegetation. J. Ecol. 90, 61–67.

Waterman, P.G., Mole, S., 1994. Analysis of Phenolic Plant Metabolites. Blackwell Scientific Publications, Oxford.

Weiss, M., Schmidt, J., Neuman, D., Wray, V., Christ, R., Strack, D., 1999. Phenylpropanoids in mycorrhizas of the Pinaceae. Planta 208, 491–502.