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Influence of Altitudinal Variation on the Content of Phenolic Compounds in Wild Populations of *Calluna vulgaris*, *Sambucus nigra*, and *Vaccinium myrtillus*

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This study deals with the effect of altitudinal variation on the content of phenolic compounds in three traditional herbal plants, which are also consumed as food in Central Europe. Herbs of *Calluna vulgaris* (L.) HULL, flowers and fruits of *Sambucus nigra* L., and berries of *Vaccinium myrtillus* L. collected in the Naturpark Sölktäler (Austria) were extracted using accelerated solvent extraction (ASE). Identification and quantification of the constituents in the polar extracts (methanol 80%, v/v) were achieved by means of RP-HPLC-PDA and/or LC-PDA-MS analysis with external standards. 3,5-*O*-Dicaffeoylquinic acid was identified in flowers of *S. nigra* for the first time. Rising concentrations of flavonoids and especially flavonol-3-*O*-glycosides with adjacent hydroxyl groups in ring B in *C. vulgaris* and *S. nigra* with increasing altitude were observed. Anthocyanins from the berries of both *S. nigra* and *V. myrtillus* occurred in decreasing amounts with rising altitude. *C. vulgaris* showed the best radical scavenging capacity based on the DPPH assay.

KEYWORDS: ***Calluna vulgaris*; *Sambucus nigra*; *Vaccinium myrtillus*; flavonoids; hydroxycinnamic acids; HPLC; LC-MS; DPPH assay; altitudinal variation**

INTRODUCTION

In recent years, there has been an increased interest in edible plants rich in phenolic compounds including flavonols, flavones, anthocyanins, and different hydroxycinnamic acids due to their possible beneficial effects on human health (*1, 2*). In addition to genetic or age-related factors, numerous exogenous factors such as environmental parameters including UV radiation, time of harvest, and damage caused by pests as well as competition with other individuals/species have been been proven to affect the amounts of these polyphenol constituents in the plant tissue (*3, 4*). The availability of data describing the variation of beneficial compounds is crucial and contributes to the knowledge about the quality of the plant material used. Two representatives of the Ericaceae family, *Calluna vulgaris* (L.) HULL (common heather) and *Vaccinium myrtillus* L. (bilberry), as well as *Sambucus nigra* L. (common elder), a member of the Caprifoliaceae, are examples for plants that are almost always collected from natural resources in Europe (*5, 6*). *V. myrtillus* and *S. nigra* are utilized to manufacture jams, juices, wines, and, due to their high content of anthocyanins, natural dyes (*7, 8*). The plant material is also part of traditional folk medicine for treating urinary tract disturbances (*C. vulgaris*) (*9*) and the common cold (*S. nigra*) (*6*) and has even shown anticarcinogenic effects in mice (extracts of *V. myrtillus*) (*10*). These three plants comprise a high content of flavonoids and/or hydroxycinnamic acids (*11*−*13*), the antioxidative capacity of which (*14*) serves as the basis for their beneficial effects as part of the human diet. Documented radical scavenger properties of *C. vulgaris*, as well as of *V. myrtillus* and *S. nigra* berries, indicate a strong in vitro antioxidative potential (*15*−*19*).

We know from conifers and various angiosperms (*Picea abies* Karst., *Soldanella alpina* L., *Ranunculus glacialis* L., *Arnica montana* cv. ARBO) that both the content and the activity of compounds that are part of their antioxidant defense system vary in relation to altitude (*20*−*22*). A correlation to the exposure to UV radiation was indicated (*23*). However, there is only limited information about the altitudinal relationship of secondary metabolite profiles in the plants studied. The aim was to investigate the profiles of the main antioxidative active phenolic compounds (flavonoids, anthocyanins, and hydroxycinnamic acids) with respect to their altitudinal variation. By collecting plant material during two consecutive growing periods the consistency of the metabolite profiles should be examined. No such studies have been performed previously. We selected a limited area—the Naturpark Sölktäler in Upper Styria (Austria)—in order to have uniform climatic conditions.

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Table 1. Extraction Parameters Used during Accelerated Solvent Extraction

parameter	*C. vulgaris*	*S. nigra* flowers	*S. nigra* fruits	*V. myrtillus*
preheat (min)	0	0	0	0
static (min)	5	5	10	7
flush (%)	100	100	100	100
purge (s)	60	60	60	100
cycles	3	3	3	3
pressure (bar)	60	68.9	60	60
temperature (°C)	60	60	100	80

MATERIALS AND METHODS

Chemicals. Chlorogenic acid (5-*O*-caffeoylquinic acid) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO), kuromanin chloride (cyanidin-3-*O*-glucoside) and keracyanin chloride (cyanidin-3-*O*-rutinoside) were obtained from Extrasynthese (Genay, France), cynarin (1,3-*O*-dicaffeoylquinic acid) was bought from Sequoia Research Products (Oxford, U.K.), hyperoside (quercetin-3-*O*-galactoside), isoquercitrin (quercetin-3-*O*-glucoside), quercetin dihydrate, and rutin (quercetin-3-*O*-rutinoside) were obtained from Carl Roth (Karlsruhe, Germany), and *p*-coumaric acid and taxifolin (dihydroquercetin) originated from Fluka Chemie AG (Buchs, Switzerland). All solvents were of HPLC grade, bought either from Roth or Sigma Aldrich (Steinheim, Germany). Formic acid 98%, acetic acid 100%, and trifluoroacetic acid reagent grade originated from Roth or Merck (Darmstadt, Germany). Red onions (*Allium cepa* L.) serving as a natural source of taxifolin-3-*O*-glucoside (*24*) were purchased in a local supermarket. Powdered elderberry extract in capsules (Rubini, Iprona AG, Italy, lot 04517) was analyzed as a comparative sample.

Sample Collection. Samples were collected at two (*S. nigra*, 670 and 1000 m) and three (*C. vulgaris*, 800, 1000, and 1500 m; *V. myrtillus*, 800, 1200, and 1500 m) different altitudes during the growing periods of 2004 and 2005 (years 1 and 2, respectively) in the Naturpark Sölktäler in Upper Styria (Austria; coordinates N 47° 20′−N 47° 27′, E 13° 56′−E 14° 02′) (**Tables 2** and **3**). The minimum distance between the single sampling sites of *C. vulgaris* and *V. myrtillus* at one altitude was 10 m. The plant material was picked in the time periods when it is typically harvested for commercial purposes. It was transported in paper bags (*C. vulgaris*) or cool bags (*S. nigra*, *V. myrtillus*) and was air-dried immediately prior to storage in brown glass bottles with screw caps at room temperature except for fresh elderberries, which were kept at −30 °C until analysis. In the case of heather and elderflowers supplementary plant material was collected in order to draw comparisons between different habitats. The numbers of samples collected per altitude per year are specified in **Tables 2** (*C. vulgaris*, *V. myrtillus*) and **3** (*S. nigra*).

Herbs of C. vulgaris. In addition, five samples of heather originating from Carinthia (Austria; 400, 450, 1500, 1800, and 1900 m; coordinates N 46° 34′, E 14° 31′−E 14°34′ and N 46° 40′−N 46° 41′, E 13° 53′−E 13° 54′) were harvested in 2006. A total of 73 samples was analyzed.

Flowers and berries of S. nigra. In eastern Styria 10 samples of elderflowers were collected supplementarily in 2004 and 2005 (5 specimens each), at 790 m above sea level (coordinates N 47° 17′, E 15° 32′−E 15° 33′). In total, 56 specimens of elderflowers were investigated.

Elderberries were collected when they showed a dark violet color; reddish green colored fruits were excluded from the harvest. Whenever possible, elderflower and elderberry samples from years 1 and 2 were collected from the same individual plants. The sampling sites of elderflowers and elderberries were the same in several instances.

All plant samples were collected in cooperation with the Naturpark Sölktäler or by kind permission of the owners (Carinthia, eastern Styria). Identification was carried out by using specialist literature (*25*).

Extraction of Flavonoids, Anthocyanins, and Hydroxycinnamic Acids. All samples were ground by means of an IKA A10 analysis mill. Extraction was conducted in 11 mL steel cells using accelerated solvent extraction (ASE) with a Dionex ASE 200 system, using methanol 80% (MeOH; v/v) as solvent, which was degassed for 15 min by sonication before application. The parameters of previously published methods (*26, 27*) concerning temperature, solvent composition, and pressure were modified for the analysis of our samples. Extraction parameters for all samples investigated are given in **Table 1**.

Prior to extraction, frozen elderberries were freeze-dried for 48 h (Vitris Sentry freeze-dryer). One gram (*C. vulgaris*, *S. nigra* berries), 0.5 g (*S. nigra* flowers), and 2 g (*V. myrtillus*), respectively, of exactly

Anthocyanins | Hydroxycinnamic acids | Flavonols

Comp. No.	Anthocyanins	R_1	R_2	R_3	R_4
1	Delphinidin-3-*O*-galactoside	galactose	-H	-OH	-H
2	Delphinidin-3-*O*-glucoside	glucose	-H	-OH	-H
3	Cyanidin-3-*O*-galactoside	galactose	-H	-H	-H
4	Delphinidin-3-*O*-arabinoside	arabinose	-H	-OH	-H
5	Cyanidin-3-*O*-glucoside	glucose	-H	-H	-H
6	Petunidin-3-*O*-galactoside	galactose	-H	-OH	$-CH_3$
7	Cyanidin-3-*O*-arabinoside	arabinose	-H	-H	-H
8	Petunidin-3-*O*-glucoside	glucose	-H	-OH	$-CH_3$
9	Peonidin-3-*O*-galactoside	galactose	-H	-H	$-CH_3$
10	Petunidin-3-*O*-arabinoside	arabinose	-H	-OH	$-CH_3$
11	Peonidin-3-*O*-glucoside	glucose	-H	-H	$-CH_3$
12	Malvidin-3-*O*-galactoside	galactose	-H	$-OCH_3$	$-CH_3$
13	Peonidin-3-*O*-arabinoside	arabinose	-H	-H	$-CH_3$
14	Malvidin-3-*O*-glucoside	glucose	-H	$-OCH_3$	$-CH_3$
15	Malvidin-3-*O*-arabinoside	arabinose	-H	$-OCH_3$	$-CH_3$
16	Cyanidin-3-*O*-sambubioside-5-*O*-glucoside	sambubiose	glucose	-H	-H
17	Cyanidin-3,5-*O*-diglucoside	glucose	glucose	-H	-H
18	Cyanidin-3-*O*-sambubioside	sambubiose	-H	-H	-H
	Flavonol glycosides	R_1	**Position 2-3**		
19	Rutin	rutinose	double bond		
20	Isoquercitrin	glucose	double bond		
21	Hyperoside	galactose	double bond		
22	Taxifolin-3-*O*-glucoside	glucose	single bond		
	Hydroxycinnamic acids	R_1	R_2		
23	5-*O*-caffeoylquinic acid	caffeoyl	-H		
24	3,5-*O*-dicaffeoylquinic acid	caffeoyl	caffeoyl		

Figure 1. Compounds detected and quantified in *Calluna vulgaris*, *Sambucus nigra*, and *Vaccinium myrtillus*.

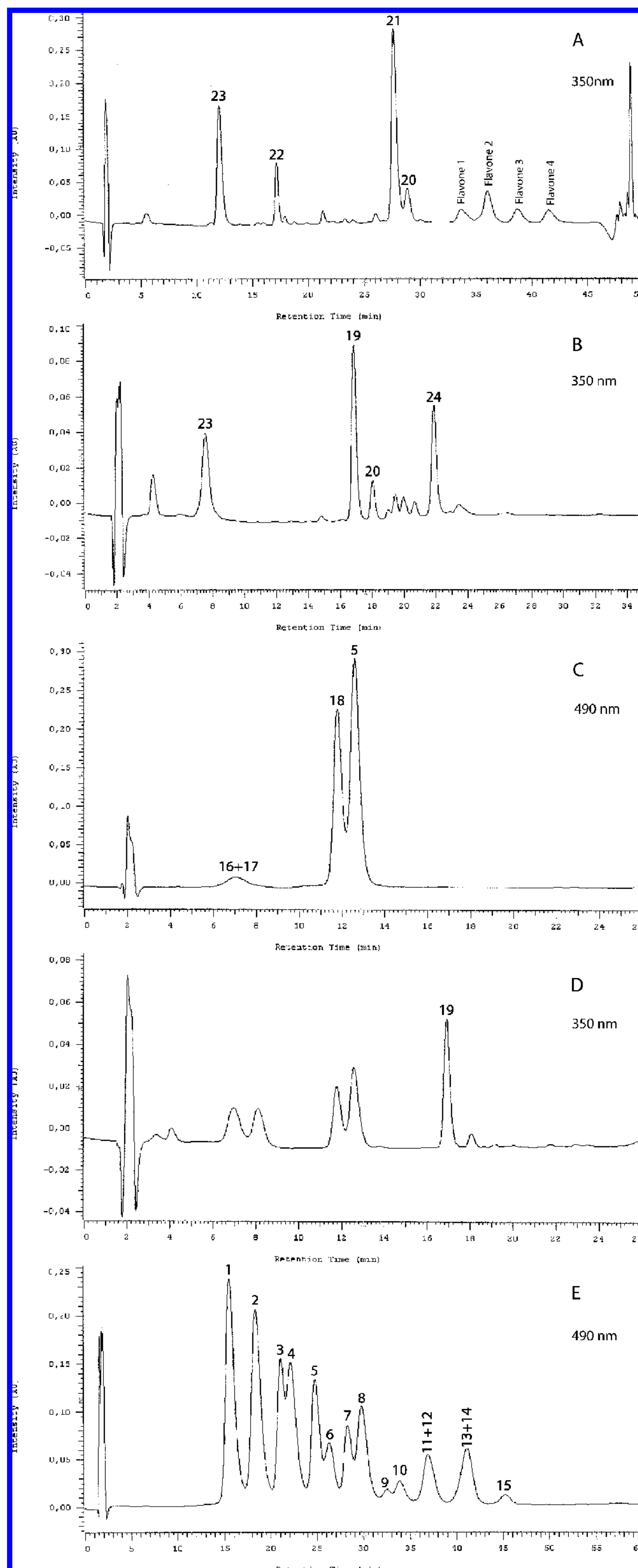

Figure 2. HPLC-PDA chromatograms of *Calluna vulgaris* (**A**), *Sambucus nigra* flowers (**B**) and berries (**C**, **D**), and *Vaccinium myrtillus* (**E**). For peak identification see **Figure 1**; for chromatographic conditions see Materials and Methods.

weighed powder was mixed 1:1 with diatomaceous earth (DE) or sea sand (*V. myrtillus*) and was then subjected to ASE. After centrifugation (Eppendorf centrifuge 5810 R; 10 min, 2500 rpm) of the extracts, the supernatants were quantitatively pipetted into a 25 mL (*C. vulgaris*, *V. myrtillus*) or 50 mL (*S. nigra* flowers and fruits) volumetric flask and brought to volume with MeOH 80% (v/v).

HPLC Analysis. All samples were analyzed in triplicate by HPLC-PDA immediately after the extraction process. The chromatographic system consisted of a LaChrom Merck Hitachi system. A Phenomenex Synergi Hydro-RP, 150 × 2 mm column (*C. vulgaris* and *V. myrtillus*) and a Phenomenex Synergi Polar-RP, 150 × 2 mm column (flowers and fruits of *S. nigra*), with a guard column (Opti-Guard C18, 1 mm), were used. The injection volumes were 5 μL for *C. vulgaris* and *S. nigra* and 10 μL for *V. myrtillus*.

C. vulgaris. Extracts of *C. vulgaris* were analyzed using a modification of the method described by Schieber et al. (*28*): gradient, 2% acetic acid in water (v/v) (A) and 0.5% acetic acid in acetonitrile (AcCN)/water (50:50) (v/v) (B); 10−30% B (0−13 min), 30% B (13−43 min), 100% B (43−51 min), 10% B (51−60 min); column temperature, 25 °C; detection wavelengths, 288 nm (taxifolin-3-*O*-glucoside), 320 nm (hydroxycinnamic acids), and 350 nm (flavonol-3-*O*-glycosides).

S. nigra Flowers. For elderflowers the method described by Dawidowicz et al. (*27*) was modified: gradient, AcCN (A) and 0.1% trifluoroacetic acid in water (v/v) (B); 9−22% A (0−13.5 min), 22% A (13.5−35 min), 22−9% A (35−35.1 min), 9% A (35.1−44 min); column temperature, 30 °C; detection wavelengths, 320 nm (hydroxycinnamic acids) and 350 nm (flavonol-3-*O*-glycosides).

Fruits of *S. nigra* and *V. myrtillus* were studied on the basis of the HPLC method described by Kaack et al. (*7*): gradient, water/AcCN/acetic acid/trifluoroacetic acid (50.4:48.5:1:0.1) (v/v) (A) and 0.1% trifluoroacetic acid in water (v/v) (B).

Conditions for *S. nigra* berries: 20−60% A (0−20 min), 60−20% A (20−21 min), 20% A (21−30 min); column temperature, 30 °C; detection wavelengths, 490 nm (anthocyanins) and 350 nm (rutin).

V. myrtillus. Conditions: 17−35% A (0−60 min), 35−17% A (60−60.1 min), 17% A (60.1−70 min); column temperature, 35 °C; detection wavelength, 490 nm.

The flow rate in all separations was 0.3 mL/min.

Identification of Phenolic Compounds (HPLC-PDA). Identification was based on cochromatography of authentic standard substances and LC-PDA-MS analysis when required. In these cases the same columns as in the RP-HPLC-PDA analyses were employed. Taxifolin-3-*O*-glucoside (**22**) was identified by HPLC comparison of a methanolic extract of the dry skin of red onions according to refs *29* and *30*.

LC-MS Analysis of Hydroxycinnamic Acids. The identity of the caffeoylquinic acids in elderflower extracts was elucidated by comparison with LC-MS data from cynarin and a hierarchical scheme for the LC-MS^n identification of chlorogenic and dicaffeoylquinic acids (*31, 32*). Gradient elution program: mobile phase, AcCN/acetic acid (1000:5) (v/v) (A) and water/AcCN/acetic acid (980:20:5) (v/v) (B), 4−33% A (0−90 min), 33−100% A (90−95 min), 100% A (95−100 min), 100−4% A (100−101 min), 4% A (101−110 min); flow rate, 0.300 mL/min; column temperature, 25 °C. Electrospray ionization (positive mode): capillary voltage, 15.00 V; source voltage, 5.00 kV; normalized collision energy, 35%; capillary temperature, 320 °C; sheath gas flow, 80 arbitrary units; auxiliary gas flow, 10 arbitrary units; mass range, 150−1000 amu.

LC-MS Analysis of Anthocyanins. Anthocyanins in the bilberry extracts were identified by comparison with (*33, 34*) and analysis of cyanidin-3-*O*-glucoside (**5**). Extracts (10 μL) were subjected to a gradient elution program. Gradient: 0.5% formic acid in water (v/v) (A) and water/AcCN/acetic acid/formic acid (50:48.5:1:0.5) (v/v) (B), 17−35% B (0−60 min), 35% B (60−70 min), 35−17% B (70−70.1 min), 17% B (70.1−80 min); flow rate, 0.2 mL/min; column temperature, 30 °C. For electrospray ionization (positive mode) the conditions were as described above except for a lower capillary temperature (220 °C).

Quantification (HPLC-PDA). All components were quantified by external standard method and molecular weight correction factors when necessary. Chlorogenic acid (**23**) was used for the quantification of hydroxycinnamic acids, taxifolin in the case of **22**, rutin (**19**) for flavonol glycosides (**19**−**21**), and **5** in the case of anthocyanins. Correlation coefficients of the calibration curves ranged from 0.9996 to 0.9999. Coeluting anthocyanins were calculated as **5**.

The repeatabilities of retention times and of the peak area were studied (within-day precision) by analyzing **19** (100 μg/mL) 10 times within 1 day. The coefficients of variation (CV) were 0.12% (retention times) and 1.18% (peak area). The between-day precisions of three

Table 2. Average Amount of Phenolic Constituents in Percent (± Standard Deviation; w/w, Dry Weight) in Herbs of *Calluna vulgaris* and Fruits of *Vaccinium myrtillus* Collected in the Naturpark Sölktäler at Three Different Altitudes[a]

Fruits of *Vaccinium myrtillus*

	year 1			year 2		
compound	800 m	1200 m	1500 m	800 m	1200 m	1500 m
1	0.39 ± 0.11 a***	0.28 ± 0.05 b	0.28 ± 0.11 b	0.36 ± 0.08 a	0.26 ± 0.08 b	0.22 ± 0.06 b
2	0.37 ± 0.10 a***	0.27 ± 0.04 b	0.26 ± 0.08 b	0.35 ± 0.07 a	0.24 ± 0.07 b	0.21 ± 0.04 b
3	0.28 ± 0.12 a***	0.18 ± 0.03 b	0.16 ± 0.05 b	0.22 ± 0.05 a	0.15 ± 0.05 b	0.13 ± 0.03 b
4	0.24 ± 0.06 a***	0.20 ± 0.03 b	0.20 ± 0.07 b	0.28 ± 0.06 a	0.17 ± 0.06 b	0.16 ± 0.03 b
5[b]	0.26 ± 0.12 a*	0.18 ± 0.02 ab	0.16 ± 0.04 b	0.24 ± 0.05 a**,***	0.15 ± 0.05 b	0.13 ± 0.02 b
6	0.15 ± 0.04 a***	0.09 ± 0.01 b	0.09 ± 0.02 b	0.12 ± 0.02 a	0.09 ± 0.02 b	0.08 ± 0.01 b
7	0.15 ± 0.06 a***	0.11 ± 0.02 b	0.10 ± 0.03 b	0.16 ± 0.04 a	0.09 ± 0.03 b	0.08 ± 0.02 b
8	0.25 ± 0.07 a***	0.16 ± 0.02 b	0.15 ± 0.04 b	0.21 ± 0.04 a	0.15 ± 0.04 b	0.13 ± 0.02 b
9[b]	0.06 ± 0.02 a***	0.03 ± 0.004 b*	0.03 ± 0.005 c	0.04 ± 0.01 a**,***	0.03 ± 0.01 b	0.03 ± 0.003 b
10	0.07 ± 0.02 a***	0.05 ± 0.01 b	0.05 ± 0.01 b	0.07 ± 0.01 a	0.04 ± 0.01 b	0.04 ± 0.004 b
11/12[b]	0.22 ± 0.08 a***	0.10 ± 0.02 b*	0.08 ± 0.02 c	0.14 ± 0.03 a**,***	0.09 ± 0.03 b	0.07 ± 0.01 b
13/14	0.21 ± 0.07 a***	0.10 ± 0.02 b	0.09 ± 0.02 b	0.15 ± 0.03 a	0.09 ± 0.03 b	0.07 ± 0.01 b
15	0.05 ± 0.01 a***	0.03 ± 0.005 b	0.02 ± 0.004 b	0.04 ± 0.01 a	0.03 ± 0.01 b	0.02 ± 0.002 b
	$n = 10$	$n = 10$	$n = 10$	$n = 10$	$n = 10$	$n = 10$

Herbs of *Calluna vulgaris*

	year 1			year 2		
compound	800 m	1000 m	1500 m	800 m	1000 m	1500 m
20[b]	0.09 ± 0.01 a***	0.15 ± 0.03 b	0.28 ± 0.11 c	0.13 ± 0.03 a***	0.14 ± 0.03 a**,***	0.29 ± 0.09 b
21[b]	0.38 ± 0.06 a***	0.63 ± 0.14 b	1.31 ± 0.43 c	0.44 ± 0.11 a*,***	0.57 ± 0.15 b***	1.33 ± 0.32 c
22	1.23 ± 0.14 a	1.23 ± 0.19 a	1.10 ± 0.27 a	1.23 ± 0.24 a	1.18 ± 0.30 a	1.24 ± 0.24 a
23	0.95 ± 0.22 a	1.00 ± 0.15 a	0.92 ± 0.25 a	0.94 ± 0.20 a	1.05 ± 0.14 a	1.11 ± 0.16 a
	$n = 11$	$n = 11$	$n = 11$	$n = 13$	$n = 11$	$n = 11$

[a] Statistically significant differences are marked with different letters. Level of significance is indicated by asterisks: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. Results of compounds **5**, **9**, **11**/**12**, **20**, and **21** at 800 m: asterisks are correlated to the comparison of the values from 800/1200 (1000) m before the comma and to the values from 800/1500 m after the comma. Results at 1200 (1000) m: asterisks are correlated to the comparison of the values from 1200 (1000)/1500 m. n = number of samples/altitude/year. [b] Kruskal−Wallis test, broken down by vegetation period. In all other cases samples of one altitude were pooled and analyzed by two-way analysis in a 2 × 2 design.

Table 3. Average Amount of Phenolic Constituents in Percent (± Standard Deviation; w/w, Dry Weight) in *Sambucus nigra* Flowers and Berries Harvested at Two Altitudes in the Naturpark Sölktäler[a]

	year 1		year 2	
compound	670 m	1000 m	670 m	1000 m
	Flowers of *S. nigra*			
19	2.63 ± 0.50 a***	1.97 ± 0.69 b	2.28 ± 0.56 a	1.67 ± 0.66 b
20[b]	0.05 ± 0.01 a*	0.20 ± 0.23 b	0.04 ± 0.01 a**	0.17 ± 0.20 b
23	1.67 ± 0.36 a	1.78 ± 0.30 a	1.26 ± 0.16 a	1.43 ± 0.32 a
24	1.56 ± 0.24 a	1.57 ± 0.39 a	1.70 ± 0.32 a	1.38 ± 0.29 a
	$n = 10$	$n = 12$	$n = 12$	$n = 12$
	Fruits of *S. nigra*			
5	0.86 ± 0.35 a*	0.69 ± 0.25 b	0.95 ± 0.27 a	0.68 ± 0.27 b
16/17	0.12 ± 0.05 a	0.11 ± 0.04 a	0.16 ± 0.06 a	0.13 ± 0.08 a
18	0.98 ± 0.33 a	0.92 ± 0.22 a	1.23 ± 0.42 a	1.05 ± 0.35 a
19	0.33 ± 0.10 a*	0.39 ± 0.12 b	0.25 ± 0.10 a	0.34 ± 0.11 b
	$n = 12$	$n = 10$	$n = 10$	$n = 9$

[a] Statistically significant differences are marked with different letters. Level of significance is indicated by asterisks: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. n = number of samples/altitude/year. [b] Kruskal−Wallis test, broken down by vegetation period. In all other cases samples of one altitude were pooled and analyzed by two-way analysis in a 2 × 2 design.

standard substances (**19** and **23**, 100 μg/mL; **5**, 250 μg/mL; six injections each) were also measured during 4 weeks in 18 runs, and the CVs were 1.3% (**19**), 2.4% (**23**), and 4.0% (**5**). All determined values were considered to be acceptable.

Radical Scavenging Capacity. In vitro nonspecific radical scavenging capacity of the plant extracts was determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) according to the method of Schneider (*35*). Extracts were diluted with MeOH 80% (v/v), and all measurements were conducted in triplicate. The inhibition ratio was determined spectrophotometrically at 535 nm after 30 (*C. vulgaris*, *S. nigra* flowers) or 60 min of incubation (berries of *S. nigra* and *V. myrtillus*), subtracting the corresponding background values (diluted extract without reagent). Inhibition percentage was calculated using the following formulas:

$$\text{abs}_{\text{control}} = \text{abs}_{\text{MeOH}} + \text{DPPH} - \text{abs}_{\text{blank}}$$

$$\text{inhibition percentage} = [(\text{abs}_{\text{control}} - \text{abs}_{\text{sample}})/\text{abs}_{\text{control}}] \times 100$$

(abs = absorption)

Results were expressed as IC_{50} values (concentration of inhibitor to cause a half-maximum inhibition of the reaction) in relation to control using Microsoft Excel for calculation. Five concentrations were used for investigating *C. vulgaris* (82.5−2.5 μg/mL), *S. nigra* flowers (250−8.25 μg/mL), and *V. myrtillus* (250−16.5 μg/mL) and four concentrations in the case of *S. nigra* berry extracts (250−25 μg/mL). The mean CV (percent) of the absorption of **5**, **20**, **21**, **23**, taxifolin, quercetin (1−0.033 mg/mL, four concentrations each) and **19** (1−0.1 mg/mL, four concentrations) amounted to 1.8%, which was considered to be acceptable.

Statistics. Statistical methods used were the Kolmogorov−Smirnov test (examining normal distribution), a two-way-analysis of variance in a 2 × 2 design, and the Kruskal−Wallis test (conducted in the case of values that were not normally distributed, broken down by vegetation period) and were all performed with SPSS for Windows 14.0 statistical software.

RESULTS AND DISCUSSION

Herbs of C. vulgaris. *Identity and Content of Phenolic Compounds.* In addition to compounds **20**−**23** (**Figure 1**) four substances with UV spectra typical for flavones were named flavones **1**−**4**, quantified and calculated as **19** equivalent. The compounds identified have all been described as constituents

Table 4. Average IC_{50} Values (Micrograms per Milliliter ± Standard Deviation) in the DPPH Assay of Extracts from *Calluna vulgaris*, *Sambucus nigra*, and *Vaccinium myrtillus*[a]

herb	670 m	800 m	1000 m	1200 m	1500 m
C. vulgaris (year 1)		9.4 ± 1.0 a **,***	8.4 ± 0.8 b*		8.2 ± 1.3 c
C. vulgaris (year 2)		9.4 ± 1.1 a	8.4 ± 0.6 b		7.5 ± 0.7 c
S. nigra flowers (year 1)	13.2 ± 1.7 a*		15.4 ± 3.6 b		
S. nigra flowers (year 2)	15.0 ± 2.3 a		17.4 ± 3.5 b		
S. nigra fruits (year 1)	57.9 ± 14.2 a		64.9 ± 13.4 a		
S. nigra fruits (year 2)	65.5 ± 11.1 a		67.3 ± 14.9 a		
V. myrtillus fruits (year 1)		37.6 ± 4.6 a ***,*		56.4 ± 4.7 b***	48.8 ± 10.4 c
V. myrtillus fruits (year 2)		48.1 ± 11.7 a		64.6 ± 10.4 b	49.1 ± 6.9 c

[a] Samples of one altitude were pooled and analyzed by two-way analysis in a 2 × 2 design. Statistically significant differences are marked with different letters. Level of significance is indicated by *, $p \leq 0.05$; **, $p \leq 0.01$; and ***, $p \leq 0.001$. Results of *C. vulgaris* and *V. myrtillus* at 800 m altitude: asterisks are correlated to the comparison of the values from 800/1200 (1000) m before the comma and the values from 800/1500 m after the comma. Results at 1200 (1000) m: asterisks are correlated to the comparison of the values from 1200 (1000)/1500 m.

in herbs and flowers of *C. vulgaris* in earlier work (*36, 37*). A characteristic HPLC chromatogram is presented in **Figure 2**.

C. vulgaris yielded greater amounts of **20** and **21** with increasing altitude in the first year and also in year 2 with the exception of no difference found between the altitudes of 800 and 1000 m in the case of **20** (**Table 2**). Extracts comprised 0.13−0.26% of flavone **1**, 0.25−0.45% of flavone **2**, and 0.10−0.21% of flavone **3** on average. All three constituents occurred in significantly greater amounts with increasing altitude in both years. However, the highest amounts of flavone **4**, ranging from 0.10 to 0.19% on average, were found in *C. vulgaris* collected at 1000 m above sea level in both vegetation periods. In addition, the concentration of flavone **4** was also higher in the second year ($p = 0.000$). The variation of the flavonoid fraction turned out to be closely related to the altitude, because we found the percentages of five of six flavonoid compounds rising significantly with increasing altitude. Samples collected in 2006 in Carinthia confirmed our findings from the Naturpark Sölktäler (data not shown). It can be concluded that environmental factors at higher altitude lead to elevated levels of flavonols, above all, **20** and **21**, in dry herbs of common heather.

Radical Scavenging Capacity. C. vulgaris extracts showed decreasing IC_{50} values in the DPPH assay with rising altitude in both growing periods (**Table 4**). This is probably linked to the flavonoid concentrations, which were up to 3-fold higher in the samples from 1500 m in comparison to the valley (800 m). The extracts of *C. vulgaris* turned out to be the most potent radical scavenging samples in our study, suggesting this plant deserves further investigation.

Flowers and Fruits of *S. nigra*. *Identity and Content of Phenolic Compounds in Elderflowers.* Compounds **19**, **20**, **23**, and **24** were quantified; for a typical HPLC profile, see **Figure 2**. **24** was identified in *S. nigra* for the first time (**Table 3**). LC-MS-analysis also led to the identification of 3-*O*-caffeoylquinic, 4,5-*O*-dicaffeoylquinic acid, and *p*-coumaric acid, which were not quantified in our study. Caffeic acid and kaempferol glycosides (*6*) were not detected.

Higher amounts of **23** as well as a distinct tendency toward greater percentages of **24** in year 1 were revealed. Specimens growing at 670 m above sea level had higher concentrations of **19** in both years. However, **20** was found in increasing concentrations with rising altitude. Elderflowers collected in eastern Styria at 790 m contained a profile similar to the individuals collected at 1000 m in the Naturpark Sölktäler (data not shown).

Radical Scavenging Capacity of Elderflowers. Samples collected at 670 m reached lower IC_{50}values in the DPPH assay than the extracts from 1000 m above sea level (**Table 4**). Also, IC_{50} values were significantly lower in specimens from year 1, which correlates well with the differences in the amounts of flavonols and hydroxycinnamic acids.

Identity and Content of Phenolic Compounds in Elderberries. Four anthocyanins (**5**, **16−18**) and **19** were quantified. Identification was achieved by comparison of RP-HPLC-PDA analysis with literature data (*34*). Our findings match the results from earlier work on wild-grown (*34*) as well as cultivated elderberries (*19*). For a characteristic HPLC chromatogram see **Figure 2**. It seems that cultivated and wild-grown elderberries show similar anthocyanin profiles. However, neither **20** and quercetin-3-*O*-arabinoside as described by Määttä-Riihinen et al. (*34*) nor the three minor anthocyanins cyanidin-3-rutinoside, pelargonidin-3-glucoside, and -sambubioside (*19*) and **21** (*6*) were detected either in our wild-grown berries or in powdered elderberry extract from cultivation.

The anthocyanin **5** occurred in decreasing amounts, whereas the quercetin-glycoside **19** showed rising quantities in samples collected at higher altitude (**Table 3**).

Radical Scavenging Capacity. In general, elderberry extracts were the least potent radical scavenging samples in our study (**Table 4**). Results indicate that in fruits of *S. nigra* growing at locations with enhanced UV radiation (e.g., 1000 m) biosynthesis is shifted to the more potent radical scavenging compound, which was **19** [IC_{50} (**19**) = 9.5 μM; IC_{50} (**5**) = 10.9 μM]. This happens possibly due to the differing positions in the steps of biosynthesis (*30*) and formation at different points in time. However, the precise correlation between these parameters remains to be discovered.

Berries of *V. myrtillus*. *Identity and Content of Anthocyanins.* Fifteen anthocyanins (compounds **1−15**, **Figure 1**) were quantified, although some coeluting pairs were calculated together. For a representative HPLC chromatogram see **Figure 2**.

In general, we observed decreasing amounts of all anthocyanins with rising altitude (**Table 2**). *V. myrtillus* had higher concentrations of compounds **3**, **6**, **8**, **13/14**, and **15** in year 1 ($p = 0.044−0.002$). Our findings concerning the identity and number of anthocyanins in bilberries match the literature data (*8, 33, 38, 39*). Acylated anthocyanin compounds were present only in trace quantities, if any. In most cases frozen fruit powder, fruit juice, or fresh fruits were analyzed (*8, 33, 34*). To our knowledge this is the first study investigating the variation of the amounts of anthocyanins in wild-grown bilberries in correlation to the altitude. The anthocyanin profile with higher amounts of delphinidin glycosides than of cyanidin glycosides on average matched well with the results of a previous comparison of eastern and southern European plants with those from Scandinavia (*34*). Jaakola et al. (*38*) reported on elevated gene expression and corresponding flavonoid

biosynthesis including cyanidin glycosides under enhanced UV radiation in bilberry leaves. Our results with air-dried bilberries are contradictory to that, revealing lower amounts of anthocyanins with increasing sea level, where rising solar radiation can be assumed (*4*).

Radical Scavenging Capacity. Bilberries from year 1 showed lower IC_{50} values ($p = 0.006$) in the DPPH assay (**Table 4**), which correlates with our findings above. Surprisingly, samples collected at 1200 m altitude showed the least radical scavenging capacity among the *V. myrtillus* extracts, although fruits collected at 1500 m above sea level contained the smallest concentrations of anthocyanins. Possibly other compounds in the extract, which were not investigated here, contributed to this effect more than the anthocyanins. Further investigation is needed to elucidate this question.

Recent papers have suggested that increasing solar radiation at higher altitude causes elevated biosynthesis of ortho-dihydroxylated flavonoids in contrast to the flavonoids lacking this feature (*21, 23*), in order to take advantage of the better radical scavenger properties of the former (*14*). Higher concentrations of these constituents were found at higher altitude in the case of *C. vulgaris* (both flavonols) and *S. nigra* flowers (**20**) and fruits (**19**). In addition, results from elderflowers suggest that within the subclass of flavonols with adjacent hydroxyl groups in ring B, the ones with elevated radical scavenging properties seem to be biosynthetically preferred at higher altitude. This corroborates the theory of Petitjean-Freytet et al. (*40*), who suggested a possible influence of increasing altitude on higher amounts of flavonols in elderflowers and the findings in refs *22*, *23*, and *38*, which associated enhanced UV radiation with activation of flavonoid biosynthesis. Variation of flavonoid biosynthesis in correlation to the altitude together with the presence of hydroxycinnamic acids functioning as important UV shields in the cell seem to be part of the plant's protective mechanism. However, the reason for decreasing amounts of all anthocyanins in bilberries and **5** in elderberries with rising altitude is still unclear.

In general, plant material with leaves, that is, herbs of *C. vulgaris*, showed the best radical scavenging capacity in our study, followed by flowers of *S. nigra*. Fruits of *V. myrtillus* reached better results in the DPPH assay in comparison to berries of *S. nigra*, which is in agreement with the findings of Nakajima et al. (*41*). One of the reasons for that might be that parts of the plant organism containing chloroplasts (green leaves, pedicel, calyx) face an enhanced production of reactive oxygen species (ROS) caused by the photosynthesis process. Test results from pure substances in the DPPH assay partly corroborate this supposition by showing a decrease in radical scavenging capacity from flavonols to anthocyanins (**20** > **19**, **21** > **5**, $IC_{50} = 8.6-10.9\ \mu M$). Thus, the hypothesis that plants from higher altitudes contain higher amounts of radical scavenging compounds as a result of their exposure to more severe climatic conditions including enhanced solar radiation cannot be affirmed in general. On the basis of our results, distinct differences between the amounts of phenolic compounds due to altitudinal impact can be expected at least in the case of the flavonols, flavones, and anthocyanins investigated in these species.

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