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Altitudinal variation of secondary metabolite profiles in flowering heads of *Arnica montana* cv. ARBO

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Abstract

The altitudinal variation on the contents of secondary metabolites in flowering heads of *Arnica montana* was assessed. Plants of *A. montana* cultivar ARBO were grown in nine experimental plots at altitudes between 590 and 2230 m at Mount Patscherkofel near Innsbruck/Austria. The total contents of sesquiterpene lactones and flavonoids were not positively correlated with the altitude of the growing site. However, the proportion of flavonoids with vicinal free hydroxy groups in ring B to flavonoids lacking this feature significantly increased with elevation. Additionally, the level of caffeic acid derivatives also positively correlated with the altitude of the growing site. In particular amounts of 1-methoxyoxaloyl-3,5-dicaffeoylquinic acid significantly increased in higher sites and samples from the summit region contained 85% more of this compound than samples from valley sites. These results are discussed with regards to chemosystematic studies comparing samples collected in different altitudes as well as in the light of a UV-B protective and radical scavenging function of phenolics and their significance for plant life in environments with elevated UV-B radiation.

Keywords: *Arnica montana*; Altitudinal effects; Chemical ecology; Phenolics; Caffeic acid esters; Sesquiterpene lactones; Natural products; UV radiation

1. Introduction

Various factors, such as age of the plant, season, microbial attack, grazing, radiation, competition, and nutritional status, have been proven to have an impact on the secondary metabolite profile in higher plants (Harborne, 1982). A factor rarely assessed is the altitude of the growing site. Many environmental factors like precipitation, mean temperature, soil, wind speed, low and high temperature extremes, duration of snow-cover, length of the vegetation period, and the intensity of radiation under clear sky conditions have been reported to differ between low and high altitude sites in temperate zones (Körner, 1999). From these variables, the higher solar radiation at higher altitudes has been most intensely discussed to have an impact on secondary metabolite profiles in higher plants (Körner, 1999). An increase of irradiance under clear sky conditions in the summer has been demonstrated for the European Alps. Blumthaler et al. (1997) found an increase of 8% for total irradiance, an increase of 9% for UV-A radiation, and an increase of 18% for erythemal effective radiation per 1000 m of altitude. A negative impact of the enhanced UV-B radiation on plant life at higher altitudes is generally assumed and an increase of the contents of phenolic

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compounds and carotenoids with rising altitude has been postulated as a response to increasing UV radiation (Körner, 1999). In particular, phenolics are considered to possess an UV-B protective function for the plants producing them, because flavonoids as well as caffeic acid derivatives are potent UV-B-absorbing compounds. The induction of enzymes involved in the biosynthesis of flavonoids under experimentally enhanced UV radiation is well established (Wellmann, 1975; Jaakola and Määttä-Riihinen, 2004). The role of phenolics in protecting plants from peak UV-B exposure might not be limited to UV-B screening (Markham et al., 1998b). Indeed observed changes in ratios between different types of flavonoids (*ortho*-dihydroxylated versus other flavonoids) indicate that the radical scavenging potential of *ortho*-dihydroxylated flavonoids like luteolin is also contributing to the UV-B protective activity (Markham et al., 1998a). For leaves of different plant species a positive correlation of the total flavonoid content with enhanced UV-B radiation is well documented under greenhouse conditions (Tevini et al., 1981; Cuadra et al., 1997; Wilson et al., 1998; Wulff et al., 1999), and even for plants grown in cell suspension culture (Min-Soo et al., 1998).

However, it remains unclear whether elevated peak UV-radiation under clear sky conditions at higher altitude sites has an effect on plant secondary metabolism under natural conditions. Moreover, all studies performed up to now investigating plants at different altitudes were performed on wild populations and are not conclusive whether the observed variations are a response of individual plants to environmental factors related to altitude or a genetic adaptation of the populations growing at different altitudes to their specific environment (McDougal and Parks, 1984; Polle et al., 1992; Veit et al., 1996; Bachereau et al., 1998; Ruhland and Day, 2000, 2001; Zidorn and Stuppner, 2001a; Zidorn et al., 2005b).

The present study addresses these questions by a phytochemical investigation of flowering heads from genetically homogenous populations of *Arnica montana* cv. ARBO, which have been grown in nine different sites at Mount Patscherkofel near Innsbruck/Tyrol/Austria. Previous chemosystematic investigations in different genera of the Asteraceae (Zidorn and Stuppner, 2001a,b; Zidorn et al., 2002, 2005b) showed that flowering heads are best suited for quantitative comparative phytochemical investigations, because flowering heads are least affected by seasonal variations of secondary metabolite contents (Zidorn and Stuppner, 2001a).

The HPLC/DAD and HPLC/MS results from flowering heads of *A. montana* cv. ARBO reported in the following communication are demonstrating a significant increase of the concentration of phenolic acids (caffeic acid derivatives) and of the ratio of *ortho*-dihydroxylated versus other flavonoids with elevated altitude. This indicates that biosyntheses of certain phenolics are indeed induced by elevated peak UV-B radiation occurring at higher altitude sites under natural conditions.

2. Results

2.1. Compound identification

Plants of *A. montana* were successfully cultivated in nine different altitudes in Innsbruck, Götzens, and at Mount Patscherkofel near Innsbruck (Table S1). Flowering heads were collected from all experimental sites in the second year of the trial and investigated for their contents of sesquiterpenoids and phenolics (Table 1).

Peaks were characterized by retention times, UV, and MS spectra. Sesquiterpene lactones were numbered from **S1** to **S14** with increasing HPLC retention times; full names of these and all other compounds investigated are given in Table 2. The identity of the sesquiterpene lactones was assigned according to Seeber (1996) and Seeber et al. (1997).

Phenolics were grouped into flavonoids (**F**) and caffeic acid derivatives (phenolic acids, **P**) based on their characteristic UV spectra (**F**: a broad maximum at 350 nm, **P**: a maximum at 330 nm with a shoulder at 295 nm). Peaks assignable to flavonoids (**F1**–**F6**) and phenolic acids (**P1**–**P9**) were also numbered consecutively with increasing HPLC retention times. Compounds **F1**–**F6** as well as minor compounds (<1.00 mg/g in all investigated samples) chrysoeriol (**CHR**) and pectolinarigenin (**PCT**) were identified with authentic reference compounds isolated from *A. montana* in previous studies (Merfort, 1984, 1985, 1992; Merfort and Wendisch, 1987, 1988, 1992; Ebert et al., 1988). Caffeic acid derivatives **P1** (chlorogenic acid, Roth, Karlsruhe, Germany) and **P4** [3,5-dicaffeoylquinic acid, isolated from *Leontodon hispidus* L. (Zidorn and Stuppner, 2001b)] were also identified using reference compounds. **P5** was isolated and identified as 1-methoxyoxaloyl-3,5-dicaffeoylquinic acid as described below. The other caffeic acid derivatives (**P2**–**P3**, **P6**–**P9**) were characterized by their retention times and MS data only (see Section 4).

Table 1
Overview about total amounts (mg/g) of flavonoids (**F**), caffeic acid derivatives (**P**), and sesquiterpene lactones (**S**) in flowering heads of *A. montana* cv. ARBO grown in different altitudes[a]

Site	$\Sigma_{\mathbf{F1–F6}}$		$\Sigma_{\mathbf{P1–P9}}$		$\Sigma_{\mathbf{S1–S14}}$	
	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x
1	19.88	3.51	28.10	1.52	9.81	0.39
2	22.42	0.82	33.55	2.38	7.96	0.91
3	17.80	3.23	31.92	0.32	11.04	0.13
4	21.30	2.34	33.74	4.78	8.81	2.19
5	23.45	1.21	30.41	0.79	12.95	1.21
6	20.70	0.27	38.01	5.01	11.37	1.22
7	21.53	1.58	32.81	4.76	11.75	1.75
8	18.26	1.48	31.33	2.73	10.53	0.59
9	17.39	1.40	38.03	3.77	9.81	0.28

[a] A detailed table with quantification results for each particular compound is available in the Supplementary material (Tables S2–S5).

Table 2
Correlations of the contents of *A. montana* secondary metabolites with the altitude of the growing site

Common name	Abbreviation	*r*	*p*
Quercetin 3-*O*-β-D-glucosid	**F1**	0.247	0.224
Patuletin 3-*O*-β-D-glucosid	**F2**	0.162	0.430
Kaempferol 3-*O*-β-D-glucosid	**F3**	−0.534	0.005
Kaempferol 3-*O*-β-D-glucuronid	**F4**	−0.219	0.282
6-Methoxykaempferol 3-*O*-β-D-glucosid	**F5**	−0.157	0.444
Hispidulin	**F6**	0.113	0.581
Sum of flavonoids **F1**–**F6**	$\Sigma_{\mathbf{F1–F6}}$	−0.067	0.746
Chlorogenic acid	**P1**	−0.209	0.307
Unknown hydroxycinnamate ester	**P2**	−0.017	0.935
Unknown hydroxycinnamate ester	**P3**	−0.218	0.284
3,5-Dicaffeoylquinic acid	**P4**	0.102	0.621
1-Methoxyoxaloyl-3,5-dicaffeoylquinic acid	**P5**	0.705	0.000
4,5-Dicaffeoylquinic acid	**P6**	0.338	0.091
Unknown hydroxycinnamate ester	**P7**	0.544	0.004
Unknown hydroxycinnamate ester	**P8**	0.449	0.021
Unknown hydroxycinnamate ester	**P9**	0.628	0.001
Sum of caffeic acid derivatives **P1**–**P9**	$\Sigma_{\mathbf{P1–P9}}$	0.422	0.032
Dihydrohelenalin	**S1**	−0.738	0.000
Helenalin	**S2**	0.074	0.721
Acetyl-dihydrohelenalin	**S3**	0.067	0.746
Acetyl-helenalin	**S4**	0.225	0.268
Methacryloyl-dihydrohelenalin	**S5**	0.255	0.210
Methacryloyl-helenalin/isobutyryl-dihydrohelenalin	**S6/S7**[a]	0.295	0.143
Isobutyryl-helenalin	**S8**	0.082	0.691
Tigloyl-dihydrohelenalin	**S9**	0.143	0.485
Tigloyl-helenalin	**S10**	0.379	0.056
2-Methylbutyryl-dihydrohelenalin	**S11**	0.110	0.593
Isovaleryl-dihydrohelenalin	**S12**	0.150	0.463
2-Methylbutyryl-helenalin	**S13**	0.105	0.611
Isovaleryl-helenalin	**S14**	0.240	0.237
Sum of sesquiterpene lactones **S1**–**S14**	$\Sigma_{\mathbf{S1–S14}}$	0.298	0.139

[a] These two compounds were not separable by HPLC using the employed analytical system.

2.2. Structure elucidation of 1-methoxyoxaloyl-3,5-dicaffeoylquinic acid (*P5*)

Compound **P5** was isolated from the methanolic extract of commercially available plant material of *A. montana* by partitioning of the methanolic extract, which was re-dissolved in a mixture of MeOH and H_2O (1/1, v/v) with CH_2Cl_2 and BuOH and successive Sephadex LH-20 column chromatography (CC) of the BuOH layer. HRFABMS spectra of compound **P5** in the negative mode yielded a signal at $m/z = 601.1245$ $[M - H]^-$ (m/z calculated for $C_{28}H_{25}O_{15} = 601.1188$) indicative for a molecular formula of $C_{28}H_{26}O_{15}$. An ESIMS experiment in the negative mode showed besides a $[M - H]^-$ signal at $m/z = 601$ the following major fragments: $m/z = 557$ $[M - COOH]^-$, $m/z = 515$ $[M - COCOOCH_3]^-$, $m/z = 439$ $[M - \text{caffeoyl}]^-$, $m/z = 395$ $[M - \text{caffeoyl} - COOH]^-$, and $m/z = 353$ $[M - \text{caffeoyl} - COCOOCH_3]^-$.

^{1}H NMR, ^{13}C NMR in combination with HHCOSY, HSQC, and HMBC experiments measured in MeOH-d_4 showed that compound **P5** was a quinic acid derivative, which had two caffeoyl moieties and an additional acyl moiety as substituents. Acylation of the hydroxy group in position 1 was proven by the pronounced downfield shift of C-1 of the quinic acid moiety in comparison with the literature data for 1,3,5-triacylquinic acid derivatives (Agata et al., 1993; Zidorn et al., 2005a) and quinic acid derivatives lacking an acyl moiety in position 1 (Cheminat et al., 1988). Acylations of positions 3 and 5 of the quinic acid moiety were evidenced by the pronounced downfield shift of the signals assignable to the protons geminal to the respective hydroxy groups ($\delta_{H\text{-}3} = 5.52$ ppm, $\delta_{H\text{-}5} = 5.44$ ppm) and by the fact that the signal assignable the proton geminal to the unsubstituted hydroxy group (H-4, $\delta_{H\text{-}4} = 3.96$ ppm) showed HHCOSY correlations to both the signal of H-3 and the signal of H-5. These two acyl moieties in positions 3 and 5 were identified as caffeoyl moieties by HMBC crosspeaks from the signals assignable to H-3 and H-5 to the carbonyl groups of the caffeic acid moieties as well as by HMBC crosspeaks from signals assignable to protons H-7″/H-7‴ and H-8″/H-8‴ of the caffeic acid moieties to these carbonyl signals.

However, the substituent in position 1, which according to MS data had a molecular mass of 87 units was only represented by two further carbonyl signals ($\delta_{C\text{-}1'} = 168.2$ ppm, $\delta_{C\text{-}2'} = 168.0$ ppm). These data were interpretable only if an exchange of ^{1}H to ^{2}H from the solvent deuteromethanol or an exchange of an OCH_3 moiety against an OCD_3 moiety were supposed. Experiments to reverse the exchange failed. Therefore, a small amount of **P5** was re-isolated and analyzed by NMR using the aprotic solvent DMSO-d_6. Results obtained from ^{1}H NMR and phase sensitive HSQC experiments indicated the presence of a methoxy group ($\delta_H = 3.73$ ppm, $\delta_C = 55.8$ ppm), which was missing in the spectra recorded in MeOH-d_4. Conclusively, compound **P5** is 1-methoxyoxaloyl-3,5-dicaffeoylquinic acid (Fig. 1). This compound has formerly been reported only as a natural product from *Achyrocline satureioides* DC. (Asteraceae, Gnaphalieae) (Robinson et al., 1996). Interestingly, these authors, who also recorded their NMR spectra in MeOH-d_4 obviously also failed to obtain signals for the proposed methoxy moiety (Robinson et al., 1996). Besides that NMR data for **P5** in MeOH-d_4 are reported only partially in Robinson et al. (1996). Therefore, NMR data measured in MeOH-d_4 and DMSO-d_6 are summarized in the experimental part (Section 4.6).

2.3. HPLC quantification results

HPLC investigations (Fig. S5) of the total contents of sesquiterpene lactones showed no significant correlation with the altitude of the growing site (Fig. 2). Amounts of particular sesquiterpene lactones also showed no correlation with the altitude of the growing site (Table 2) except for dihydrohelenalin (**S1**), which showed a highly signifi-

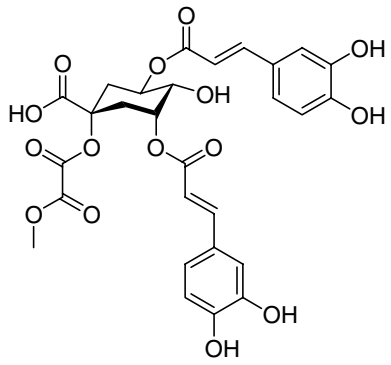

Fig. 1. Structure of 1-methoxyoxaloyl-3,5-dicaffeoylquinic acid from *A. montana*.

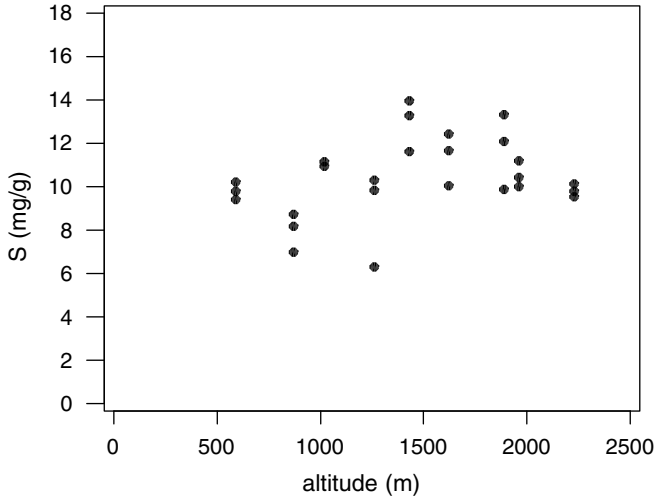

Fig. 2. Correlation of the altitude of the growing site and the total content of sesquiterpenoids (mg/g) in flowering heads of *A. montana*. Each dot represents one analyzed batch. Correlation $r = 0.298$ (0.139).

cant decrease with altitude [$r = -0.738$ (0.000)]. This result is hard to interpret and might be of limited relevance as dihydrohelenalin is one of the minor sesquiterpene lactones in flowering heads of *A. montana* occurring in negligible amounts only (0.03–0.08 mg/g, Table S4).

HPLC analyses (Fig. S4) of the total contents of flavonoids also revealed no positive correlation with the altitude of the growing site (Fig. 3, Table 2). A closer inspection of the HPLC data revealed that the ratio of flavonoids with a 3′,4′-dihydroxylation pattern in ring B to flavonoids lacking this feature [(**F1** + **F2**)/(**F3** + **F4** + **F5** + **F6**)] showed a highly significant increase with the altitude of the growing site (Fig. 4; Table 2).

Finally, the correlation of the contents of caffeic acid derivatives was investigated. The total contents of compounds **P1**–**P9** showed a significantly positive correlation with the altitude of the growing site (Fig. 5; Table 2). The regression equation obtained for the content of caffeic acid derivatives in dependence of the altitude of the experimental plot was $\Sigma_{(P1-P9)}$ (mg/g) = 28.2 + 0.00342 * altitude (m). Thus, an increase of approximately 10% of the

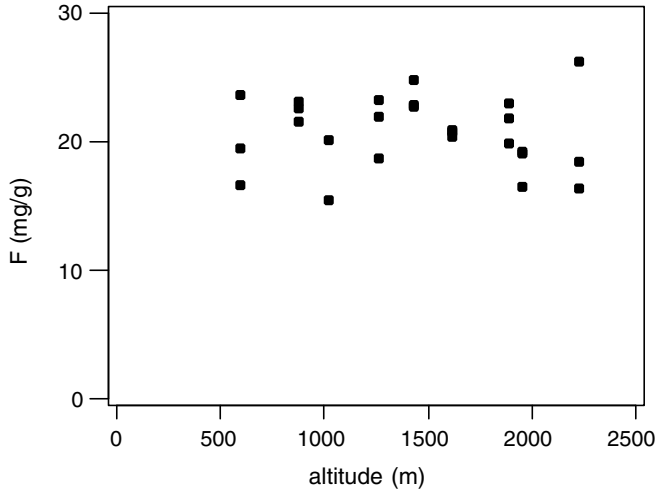

Fig. 3. Correlation of the altitude of the growing site and the total content of flavonoids (mg/g) in flowering heads of *A. montana*. Each dot represents one analyzed batch. Correlation $r = -0.067$ (0.746).

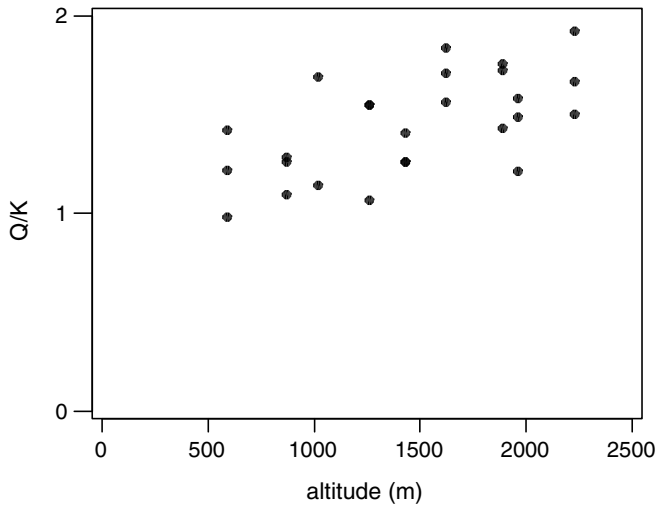

Fig. 4. Correlation of the altitude of the growing site and the quotient of ortho-dihydroxy-substituted flavonoids to other flavonoids {Q/K = [(**F1** + **F2**)/(**F3** + **F4** + **F5** + **F6**)]} in flowering heads of *A. montana*. Each dot represents one analyzed batch. Correlation $r = 0.607$ (0.001). Regression equation (3′,4′-dihydroxyflavonoids)/(other flavonoids) = 1.03 + 0.000291 * altitude (m).

total of caffeic acid derivatives is to be expected at an altitude of 2000 m above mean sea level (a.m.s.l.) as compared to 1000 m a.m.s.l. In contrast to the total of compounds **P1**–**P9**, the contents of compounds **P1**, **P2**, **P3**, and **P4** showed no significant increase with altitude ($p > 0.100$). On the other hand, the content of compound **P6** showed a weak ($r < 0.4$) weakly significant ($r < 0.100$) positive correlation and contents of compounds **P5**, **P7**, **P8**, and **P9** showed medium ($0.4 < r < 0.6$) to pronounced ($0.6 < r < 0.8$) significant ($p < 0.050$) to highly significant ($p < 0.010$) positive correlations to the altitude of the growing site. The content of 1-methoxyoxaloyl-3,5-dicaffeoylquinic acid (**P5**) was most pronouncedly correlated to the altitude of

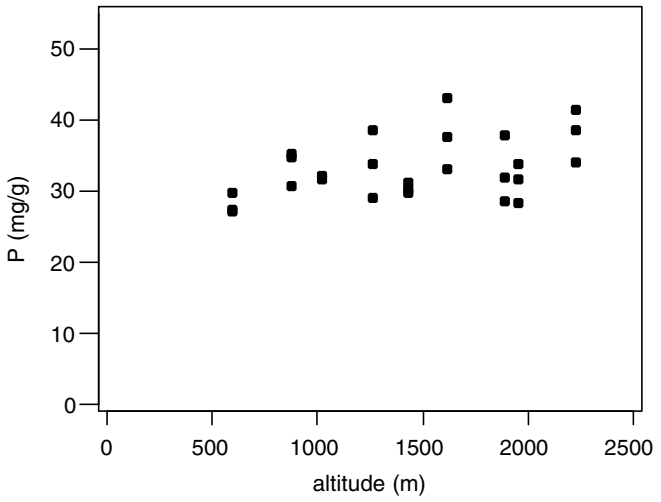

Fig. 5. Correlation of the altitude of the growing site and the total content of phenolic acids (mg/g) in flowering heads of *A. montana*. Each dot represents one analyzed batch. Correlation $r = 0.422$ (0.032). Regression equation, total of phenolic acids (mg/g) = 28.2 + 0.00342 * altitude (m).

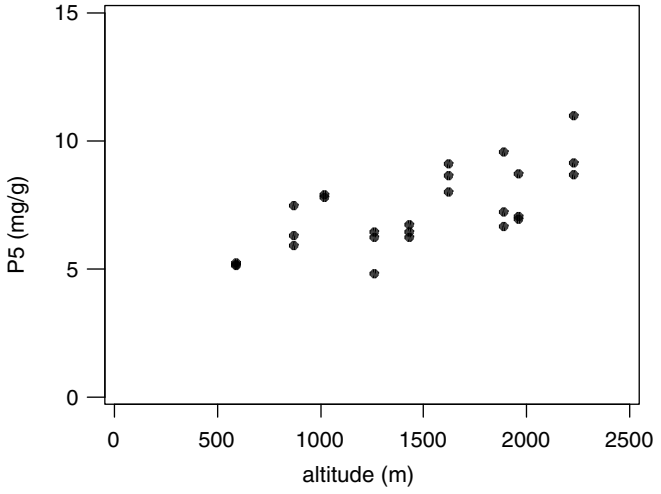

Fig. 6. Correlation of the altitude of the growing site and the content of 1-methoxyoxaloyl-3,5-dicaffeoylquinic acid (**P5**) (mg/g) in flowering heads of *A. montana*. Each dot represents one analyzed batch. Correlation $r = 0.705$ (0.000). Regression equation, content of methoxyoxaloyl-3,5-dicaffeoylquinic acid (**P5**) (mg/g) = 4.29 + 0.00205 * altitude (m).

the growing site (Fig. 6). For the content of 1-methoxyoxaloyl-3,5-dicaffeoylquinic acid the following regression equation was calculated: Content **P5** (mg/g) = 4.29 + 0.00205 * altitude (m). Thus, flowering heads collected in 2000 m a.m.s.l. are expected to contain more than 30% more of **P5** than flowering heads collected at 1000 m a.m.s.l.

3. Discussion

The fact that the level of sesquiterpene lactones in flowering heads of *A. montana* is not correlated to altitude was not unexpected, because sesquiterpene lactones are commonly considered to be compounds, which have an antifeedant activity. None of the known biological activities of sesquiterpenoids is in any way related to factors, which are changing with the altitude of the growing site, e.g., sesquiterpenoids neither absorb radiation in the UV-B range nor do they have pronounced radical scavenging activity.

The lack of correlation of altitude and the total content of flavonoids was rather unexpected, because of earlier observations on wild populations of *Leontodon helveticus* Mérat emend. Widder, which indicated a highly significant positive correlation of the altitude of the growing site and the total amount of flavonoids (Zidorn and Stuppner, 2001a). However, the pronounced increase of the ratio of 3′,4′-dihydroxylated flavonoids to flavonoids without that substitution pattern (quercetin versus kaempferol derivatives) corroborates the hypothesis of Markham et al. (1998a). These authors postulated that the role of phenolics in prohibiting damage by UV-B radiation is not restricted to direct absorption of UV-B radiation but is also effective through the radical scavenging of phenolics. As *ortho*-dihydroxylated flavonoids have a far greater radical scavenging potential (by a factor of 3–4 times) than flavonoids without *ortho*-dihydroxy groups (Rice-Evans et al., 1996), the increase in the relative amount of 3′,4′-dihydroxylated *Arnica*-flavonoids will be of UV-B damage protective value for the plants growing at higher altitude sites.

The main result of the current study is the pronounced statistically significant increase of caffeic acid derivatives with the altitude of the growing site. The total content of the detected phenolic acids, which are all caffeoylquinic acid derivatives and therefore all possess one or more *ortho*-dihydroxylated aromatic rings, increased approximately in the same order (10%) per 1000 m of altitude as the total irradiance according to Blumthaler et al. (1997). The presented data also indicate that some dicaffeoylquinic acid derivatives are particularly linked to the altitude of the growing site. 1-Methoxyoxaloyl-3,5-dicaffeoylquinic acid, which has not been reported from *A. montana* previously, accounts for approximately 60% of the total increase of caffeic acid derivatives in flowering heads of *A. montana* in response to environmental factors related to the altitude of the growing site. It is safe to assume that the stress factor with the greatest impact on the induction of the biosynthesis of caffeic acid derivatives is the enhanced peak UV-B radiation at higher altitude sites (Blumthaler et al., 1997). It is well established that enhanced UV-B radiation indirectly causes damage to plants by inducing the formation of free radicals, which subsequently target lipids, proteins, carbohydrates, and nucleic acids (Blokhina et al., 2003). It was also demonstrated that phenolic compounds play a vital role in the hydrogen peroxide scavenging system of plants, which besides phenolics comprises peroxidase, ascorbic acid, and glutathione (Takahama and Oniki, 1997). The present report demonstrates for the first time an induction of this system by factors related to the altitude of the growing site (presumably enhanced UV-B radiation) in genetically homogenous populations grown along an altitudinal gradient.

An influence of other factors than UV on the changes in the secondary metabolite profile cannot be ruled out on the basis of the present data. However, some of the factors usually changing with altitude in mountain ranges of Central Europe are particularly unaffected by altitude in the Tyrolean Central Alps. The cloud cover, which is decreasing the amount of solar radiation in mountain ranges of the peripheral chains of the Alps as compared to lowland sites, is nearly identical between the weather stations of Innsbruck University (N 47°15′, E 11°23′, altitude: 578 m a.m.s.l.) and Mount Patscherkofel (N 47°13′, E 11°28′, altitude: 2247 m a.m.s.l.) with Innsbruck University receiving 47.4% of the theoretically possible sunshine hours and Mount Patscherkofel receiving 46.4% (ZAMG, 2002). Another related factor is also virtually constant between the two adjacent stations: the yearly amount of precipitation is 883.1 mm for Innsbruck University and 878.8 mm for Mount Patscherkofel. More important, the seasonal distribution of the precipitation is also similar between the two stations. Other factors like yearly mean temperature (8.9 °C versus 0.0 °C), mean temperature in July (18.3 °C versus 7.9 °C), and days with frost (96.7 versus 222.5) differ of course considerably between the valley and the mountain summit (ZAMG, 2002). However, it is hard to see any adaptive advantage of the described altitudinal changes in secondary metabolite profiles against factors related to the temperature regime of the growing site. One potential indirect influence of the temperature during the growing season is the delay of the flowering period at higher altitudes as compared to low altitude sites (see harvesting dates in Section 4), which results in higher altitude populations to flower around the solstice, whereas the flowering period starts at the end of May in lowland sites. Therefore, irradiation during and immediately before the flowering period of *A. montana* is not only enhanced in higher altitudes because of lessened atmospheric filtering but also because of higher solar irradiation during the peak flowering season.

In applied botany, the possibility to cultivate *A. montana* in a wide range of altitudes in the Alps is of interest to farmers in alpine regions looking for alternative crops in high mountain farming, especially in regions like Bavaria, Austria, and the Southern Tyrol, where family owned small farms still account for most of the farming activities (Bätzing, 2003). The demonstrated differences between drug material from high and low altitude are also in-line with traditional popular believe that medicinal plants collected at higher altitudes contain more active ingredients than plants from lower altitude sites. The fact that similar and high levels of sesquiterpene lactones were found in samples from all altitudes investigated are also of importance for the potential of *A. montana* cv. ARBO as a crop for the production of medicinal products (either the flowering heads themselves or extracts and preparations derived from them), because these helenalin derivatives (Fig. S3) are the main active constituents of *A. montana* extracts (Klaas et al., 2002).

The impact of the presented findings for comparative chemosystematic studies is hard to estimate. The results indicate that the altitude of the collection site has an impact on the (quantitative) secondary metabolite profile. However, as recently demonstrated for related New Zealand neophytes from the Lactuceae tribe of the Asteraceae family (Zidorn et al., 2005b), phytochemical differences between different taxa are usually more pronounced than intraspecific variations of populations from a particular taxon growing at different altitudes. For intraspecific chemosystematic studies, which generally will deal only with a limited degree of phytochemical variation, the altitude of the growing site has to be taken into account. In cases subtle phytochemical differences between high and low altitude populations of plant species growing in the wild are observed the ultimate proof for a genetic basis of the observed variation will require cultivation experiments under identical growing conditions.

On the basis of our previous experiments with other taxa from the Asteraceae family (Zidorn and Stuppner, 2001a; Zidorn et al., 2005b) it might be speculated that the plasticity of *A. montana*, which ultimately results in quantitative differences between high and low altitude populations is only one factor contributing to differences in secondary metabolite profiles of natural populations growing in different altitudes in the wild. Genetic adaptation to the specific environment of the growing site is the other, probably more important, factor to be taken into account. Studies assessing differences between wild populations of *A. montana* are underway and might give further insights into the importance of genetic adaption versus plasticity for the observed phytochemical differences between plants collected in different altitudes.

4. Experimental

4.1. Plant material

Plantlets from *A. montana* cultivar ARBO (von Raison et al., 2000) were purchased from Saatzucht Steinach GmbH (Steinach/Germany) in May 2002. The cultivar *A. montana* cv. ARBO was derived from numerous accessions collected in the wild in Austria, Germany, and Switzerland as well as from accessions from various botanical gardens from these three countries. All in all 55 accessions contributed to the breeding program of the cultivar. As free pollination between different accessions was possible during the breeding, it is not possible to assign the relative contribution of the particular accessions to the genome of *A. montana* cv. ARBO (Bomme and Daniel, 1994; U. Bomme/Freising/Germany, personal communication).

Plantlets were pre-adapted to outdoor conditions in 5 l pots. Then plants were successively bedded out to the experimental sites (July–August 2002) in the botanical garden of Innsbruck (site 1), in Götzens (site 2), and on seven sites at the western slopes of Mount Patscherkofel (sites 3–7). Per site 98 plantlets ($2 \times 7 \times 7$) were planted out at to two plots a 1 m^2. The experimental plots were protected from grazing animals by wire-cages (1 m × 1 m × 1 m).

The exact locations of the nine experimental sites are given in Table S1. Flowering heads were collected during the peak of the flowering season 2003. In detail, flowering heads were collected on May 26th (sites 1 and 2), June 2nd (site 3), June 12th (sites 3 and 4), June 17th (sites 4, 6, and 7), June 25th (sites 5, 6, and 8), July 1st (sites 6 and 8), July 8th (sites 8 and 9), July 15th (site 9), and July 22nd (site 9). To exclude ontogenetic differences, only flowering heads with at least two rows of flowering ray florets were collected (Douglas et al., 2004). Terminal flowering heads of each stem were collected separately from lateral capitula. For the investigations reported in this communication only terminal flowering heads were analyzed, though analyses of three batches of terminal and lateral flowering heads of site 7 showed no significant differences (data not shown). Flowering heads were collected in batches of twelve, air-dried and afterwards kept at −20 °C until analysis. For each site three batches of flowering heads were collected and analyzed separately.

4.2. Extract preparation

Each batch of twelve air-dried flowering heads was ground and divided into two parts. One was used for the quantification of the sesquiterpene lactones, the other for the analysis of flavonoids and phenolic acids. Extraction for the analysis of sesquiterpene lactones was performed using a modification of the system described by Douglas et al. (2004) but using CH_2Cl_2 instead of $CHCl_3$ for extraction. In detail, to 500.0 mg ground plant material 5 ml of a stock solution containing 0.200 mg/ml of the internal standard santonin and 15 ml CH_2Cl_2 were added and the mixture was sonicated for 10 min. The sample was then filtered, rinsed (3×2 ml CH_2Cl_2), evaporated to dryness, re-suspended in MeOH (1 ml), and placed on a C18 solid phase extraction column (Chromabond, Merck, Darmstadt, Germany) pre-wet with 3/2 MeOH/H_2O (3 ml). The sample flask and column were then rinsed (3/2 MeOH/H_2O, 1 ml). The column was allowed to drain dry and the combined eluants were cooled at −20 °C (30 min), filtered ready for analytical HPLC. Comparisons with extracts made with $CHCl_3$ and experiments using a higher number of ultra sonication cycles and longer times of sonication showed that the chosen approach resulted in an exhaustive extraction (extraction rate of >98%).

Extraction for the analysis of phenolics was performed using the procedure described by Zidorn et al. (2005b). After adding 2.5 mg of the internal standard compound cynarin as a stock solution in MeOH, $(CH_3)_2CO$, and H_2O (3/1/1, v/v/v), ground flowering heads (500.0 mg) were sonicated twice for 30 min with a mixture of MeOH, $(CH_3)_2CO$, and H_2O (3/1/1, v/v/v) and once for 30 min with a mixture of MeOH and H_2O (1/1, v/v) (total extraction volume 25 ml for each cycle). The extract were filtered, the remaining plant material rinsed with 20 ml of a mixture of MeOH, $(CH_3)_2CO$, and H_2O (3/1/1, v/v/v) and the combined extracts were filled up to 100.0 ml with a mixture of MeOH, $(CH_3)_2CO$, and H_2O (3/1/1, v/v/v); 10.0 ml of this solution were brought to dryness in vacuo and re-dissolved in 2.00 ml of a mixture of MeOH, $(CH_3)_2CO$, and H_2O (3/1/1, v/v/v). After filtration, this solution was used for HPLC analysis. Comparative investigations using different extraction media and longer times of sonication, and a larger number of sonication cycles proved that the chosen procedure led to an exhaustive extraction. All quantitative analyses were run in triplicate.

4.3. HPLC analyses

HPLC analyses of sesquiterpene lactones were performed using HP-1090 and HP-1100 ChemStations (Agilent, Waldbronn, Germany) equipped with DAD detectors and by applying the following parameters: column, Zorbax SB-C18 4.6×150 mm (3.5 μm material); guard column, Merck LiChrospher 100 RP-18 (5 μm material); mobile Phase A, H_2O; phase B, MeOH; flow rate, 1.00 ml/min; injection volume, 5 μl; detection wavelength, 225 nm; oven temperature, 40 °C; linear gradient, 0 min 45% B, 15 min 50% B, 30 min 57% B, 35 min 95% B, 40 min stop; post time, 12 min.

Phenolics were analyzed using HP-1090 and HP-1100 ChemStations equipped with DAD detectors and by applying the following parameters: column, Phenomenex Synergi Hydro-Rp 80A 150×4.6 mm (4 μm material); guard column, Phenomenex Security Guard C18 (ODS, Octadecyl) 4.0 mm × 3.0 mm; mobile phase A, H_2O/HCOOH/CH_3COOH (99/0.9/0.1, v/v/v); phase B, MeCN/MeOH/HCOOH/CH_3COOH (89/10/0.9/0.1, v/v/v/v); flow rate, 1.00 ml/min; injection volume, 10 μl; detection wavelength, 350 nm; oven temperature, ambient; linear gradient, 0 min 5% B, 5 min 15% B, 20 min 16% B, 35 min 18% B, 45 min 19% B, 55 min 27.5% B, 60 min 65% B, 65 min 98% B, 70 min stop; post time, 12 min.

The amounts of sesquiterpene lactones were estimated by comparing the peak areas obtained for the particular sesquiterpene lactones **S1**–**S14** with the peak area obtained for the internal standard santonin (**SAN**). The amounts of phenolics were estimated by comparing the peak areas obtained for the particular flavonoids **F1**–**F6** and caffeic acid derivatives **P1**–**P9** with the peak area obtained for the internal standard cynarin (**CYN**). For quantitative analyses only phenolics reaching at least the threshold of 1.00 mg/g dried plant material in at least one of the investigated samples were taken into account.

4.4. HPLC–MS analyses

Analyses were performed using the HPLC system described above. The HPLC was coupled to a Bruker Esquire 3000^{plus} iontrap (Bremen, Germany). The following parameters were employed: Ionization, negative ESI; scanning range m/z 50–1200; nebulizer 40 psi; dry gas 10 l/min; dry temperature 300 °C. Flavonoids and caffeic acid derivatives were detected as $[M - H]^-$ signals using these

parameters. Unidentified caffeic acid derivatives (**P2**–**P3**, **P6**–**P9**) were characterized as follows: **P2** (Rt = 28.7 min, $m/z = 516$), **P3** (Rt = 33.5 min, $m/z = 516$), **P6** (Rt = 43.7 min, $m/z = 516$), **P7** (Rt = 48.5 min, $m/z = 602$), **P8** (Rt = 52.5 min, $m/z = 688$), and **P9** (Rt = 59.5 min, $m/z = 764$). Thus, compounds **P2**, **P3**, and **P6** were identified as isomeric dicaffeoylquinic acids, compound **P7** as an isomer of compound **P5**, and compound **P8** as a tricaffeoylquinic acid derivative. According to the molecular mass compound **P9** might represent a hitherto unknown methoxyoxaloyl-tricaffeoylquinic acid derivative.

4.5. Isolation of 1-methoxyoxaloyl-3,5-dicaffeoylquinic acid (*P5*)

P5 was isolated from commercially available flowering heads of *A. montana* [Fl. Arnicae CS., PhE, Mag. Kottas Heilkräuter (Vienna, Austria), batch A318268-00]. Fine ground flowering heads (984 g) were exhaustively extracted with MeOH to yield 245 g of crude extract after evaporation of the solvent in vacuo. The extract was re-suspended in a mixture of H_2O and MeOH (2/1, v/v) and then successively partitioned with CH_2Cl_2 and BuOH. The BuOH layer (57 g) was dissolved in MeOH (100 ml), centrifuged, filtered and separated by Sephadex LH-20 column chromatography (CC) using MeOH as an eluant. Fractions rich in **P5** were successively re-chromatographed over Sephadex LH-20 using a mixture of MeOH, $(CH_3)_2CO$, and water (3/1/1, v/v/v) as an eluant to yield 27 mg of **P5**. Re-isolation of **P5** (0.5 mg) for NMR spectra recorded in DMSO-d_6 was achieved by Sephadex LH-20 CC from other fractions containing **P5**.

4.6. NMR data of 1-methoxyoxaloyl-3,5-dicaffeoylquinic acid (*P5*)

^{1}H NMR (300 MHz, CD_3OD): quinic acid moiety, δ 2.75 (1H, br *d*, $J = 15.0$ Hz, H-2a), 2.48 (1H, *dd*, $J = 15.0$; 3.5 Hz, H-2b), 5.52 (1H, *ddd*, $J = 3.5$, 3.5, 3.5 Hz, H-3), 3.96 (1H, *dd*, $J = 9.5$; 3.5 Hz, H-4), 5.44 (1H, *ddd*, $J = 9.5$; 9.5; 4.0 Hz, H-5), 2.63 (1H, br *d*, $J = 13.5$ Hz, H-6a), 2.02 (1H, *dd*, $J = 13.5$; 9.5 Hz, H-6b); methoxyoxaloyl moiety, not detectable; caffeoyl moiety I, δ 7.11 (1H, *d*, $J = 2.0$, H-2″), 6.80 (1H, *d*, $J = 8.0$ Hz, H-5″), 7.01 (1H, *dd*, $J = 8.0$; 2.0 Hz, H-6″), 7.61 (1H, *d*, $J = 16.0$ Hz, H-7″), 6.30 (1H, *d*, $J = 16.0$ Hz, H-8″); caffeoyl moiety II, δ 7.07 (1H, *d*, $J = 2.0$, H-2‴), 6.79 (1H, *d*, $J = 8.0$ Hz, H-5‴), 6.97 (1H, *dd*, $J = 8.0$; 2.0 Hz, H-6‴), 7.62 (1H, *d*, $J = 16.0$ Hz, H-7‴), 6.35 (1H, *d*, $J = 16.0$ Hz, H-8‴). ^{13}C NMR (CD_3OD, 300 MHz): quinic acid moiety, δ 80.7 (C-1), 33.0 (C-2), 73.6 (C-3), 71.8 (C-4), 71.2 (C-5), 37.8 (C-6), 174.2 (C-7); methoxyoxaloyl moiety, δ 168.2* (C-1′), 168.0* (C-2′), not detectable (C-3′); caffeoyl moiety I, δ 127.8 (C-1″), 115.4 (C-2″), 146.8 (C-3″), 149.6 (C-4″), 116.5 (C-5″), 123.2 (C-6″), 147.4 (C-7″), 115.1 (C-8″), 168.7 (C-9″); caffeoyl moiety II, δ 127.8 (C-1‴), 115.3 (C-2‴), 146.8 (C-3‴), 149.8 (C-4‴), 116.5 (C-5‴), 123.0 (C-6‴), 147.9 (C-7‴), 115.2 (C-8‴), 167.7 (C-9‴), * signals might be exchangeable. Referenced to solvent residual and solvent signals at 3.31 ppm (^{1}H NMR) and 49.0 ppm (^{13}C NMR), respectively.

^{1}H NMR (500 MHz, $(CD_3)_2SO$): quinic acid moiety, δ 2.41 (2H, *m*, H-2a,b), 5.32 (1H, *ddd*, $J = 3.5$, 3.5, 3.5 Hz, H-3), 3.79 (1H, *dd*, $J = 9.5$; 3.5 Hz, H-4), 5.19 (1H, *ddd*, $J = 9.5$; 9.5; 4.0 Hz, H-5), 2.44 (1H, *m*, H-6a), 1.92 (1H, *m*, H-6b); methoxyoxaloyl moiety, δ 2.41 (3H, *s*, H-3′); caffeoyl moiety I, δ 7.09 (1H, *d*, $J = 2.0$, H-2″), 6.76 (1H, *d*, $J = 8.0$ Hz, H-5″), 7.00 (1H, *dd*, $J = 8.0$; 2.0 Hz, H-6″), 7.47 (1H, *d*, $J = 16.0$ Hz, H-7″), 6.25 (1H, *d*, $J = 16.0$ Hz, H-8″); caffeoyl moiety II, δ 7.06 (1H, *d*, $J = 2.0$, H-2‴), 6.78 (1H, *d*, $J = 8.0$ Hz, H-5‴), 7.00 (1H, *dd*, $J = 8.0$; 2.0 Hz, H-6‴), 7.50 (1H, *d*, $J = 16.0$ Hz, H-7‴), 6.25 (1H, *d*, $J = 16.0$ Hz, H-8‴). ^{13}C NMR (CD_3OD, 300 MHz, only signals detectable by HSQC): quinic acid moiety, δ 31.5 (C-2), 71.1 (C-3), 69.9 (C-4), 70.3 (C-5), 35.6 (C-6); methoxyoxaloyl moiety, 55.8 (C-3′); caffeoyl moiety I, δ 114.7 (C-2″), 115.7 (C-5″), 120.9 (C-6″), 145.0 (C-7″), 114.7 (C-8″); caffeoyl moiety II, δ 115.3 (C-2‴), 115.7 (C-5‴), 120.9 (C-6‴), 144.9 (C-7‴), 114.5 (C-8‴). Referenced to solvent residual and solvent signals at 2.50 ppm (^{1}H NMR) and 39.5 ppm (^{13}C NMR), respectively.

4.7. Data analysis

Correlation coefficients and linear regression equations were calculated using the MINITAB 13.31 software package.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2005.11.018.

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