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# Chemotaxonomic significance of flavonoids and phenolic acids in the *Hieracium rohacsense* group (*Hieracium* sect. *Alpina*; Lactuceae, Compositae)

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## Abstract

Five apomictic taxa from the *Hieracium rohacsense* group were studied for their phenolic constituent composition. The following substances represent dominant compounds in the leaves: chlorogenic acid, 3,5-dicaffeoylquinic acid, luteolin 7-*O*- $\beta$ -D-glucopyranoside, luteolin 4'-*O*- $\beta$ -D-glucuronopyranoside and apigenin 4'-*O*- $\beta$ -D-glucuronopyranoside. Within the group only quantitative differences were found, luteolin 7-*O*-glucoside being the most important chemotaxonomic marker. Each taxon has its own specific quantitative pattern, invariable within the taxon. Based on these characteristic profiles, *H. rohacsense* can be distinguished from a closely related and still undescribed taxon from Mt. Pip Ivan. The proportion of luteolin 7-*O*-glucoside to apigenin 4'-*O*-glucuronoside also clearly separates the individuals of two morphologically close species—*H. ratezaticum* and *H. pseudocaesium*, which corresponds to a few slight but recognisable morphological and phenological characteristics. The ontogenetic stage of leaf development and seasonal variation are also important factors, which must be taken into consideration, as the quantity of the substances changes during leaf ontogeny and with season.

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**Keywords:** *Hieracium*; Asteraceae; Apomixis; Chemotaxonomy; Flavonoids; Phenolic acids; HPLC

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## 1. Introduction

The genus *Hieracium* L. (hawkweed) is one of the largest genera in the World. There are more than 11,000 species names included in the Index Kewensis (Royal Botanic Gardens Kew, 1993). Such a rich nomenclature has resulted from several objective and partially subjective reasons: (i) the taxa of the subgenus *Hieracium* are mostly apomictic tri- and tetraploids with diplosporic formation of the seeds, only a small fraction of the populations are sexual diploids (cf. Schuhwerk, 1996), while in the subgenus *Pilosella* (Hill) Gray, besides aposporic apomixis and sexuality, the mode of reproduction is known to be amphimictic (Krahulcová et al., 2000); the American representatives of the subgenus *Chionoracium* Sch. Bip. are diploid, sexual (Beaman, 1990; Schuhwerk, 1996); (ii) the genus has a world-wide distribution; (iii) *Hieracium* taxa occur in a large variety of habitats; (iv) there are different taxonomic approaches to the classification of the apomictic taxa in this genus.

The flavonoids and related phenolic compounds are the most widely used of all secondary constituents in taxonomic studies, mainly due to their ubiquitous occurrence in vascular plants, their structural variability and chemical stability (Harborne and Turner, 1984). These secondary compounds have been successfully used as taxonomic markers in different agamic genera such as *Crataegus* (Sinnott and Phipps, 1983), *Rubus* (Bammi and Olmo, 1966), *Potentilla* (Asker and Fröst, 1970) and *Hierochloa* (Weimarck, 1970).

However, little attention has been paid to the phenolic composition of the “true” hawkweeds (subgenus *Hieracium*), compared with the subgenus *Pilosella* (Hill) Gray, which has been analysed by many authors (Duquénois and Greib, 1956; Duquénois and Haag-Berrurier, 1962; Haag-Berrurier and Duquénois, 1962, 1963; Mihele, 1970, 1971; Constantinescu et al., 1971; Shelyuto et al., 1977; Dombrowicz et al., 1992). The major constituents of the representatives of *Hieracium* s. str., which have been studied, were identified as mono- and diglycosides of luteolin and apigenin, together with smaller amounts of the corresponding free aglycone; phenolic acids, predominantly chlorogenic, 1,5-dicaffeoylquinic and 3,5-dicaffeoylquinic acids, have also been reported (Haag-Berrurier and Duquénois, 1969; Guppy and Bohm, 1976; Giner et al., 1992; Petrović et al., 1996, 1999, Zidorn et al., 2002). Bate-Smith et al. (1968) studied the presence of the coumarin umbelliferone in different taxa of the subgenera *Hieracium* and *Pilosella*. Species-specific patterns of flavonoid glycosides were reported from five taxa belonging to the subgenera *Stenotheca* (Monnier) Fr. and *Hieracium* (Guppy and Bohm, 1976). Flavonoid aglycones deposited on the plant surfaces of *Hieracium intybaceum* All. and *H. amplexicaule* L. were identified by Wollenweber (1984) and Wollenweber et al. (1997).

The *Hieracium rohacsense* group (*Hieracium* sect. *Alpina* Fries) includes species in the morphological position *H. alpinum* < *H. bifidum* (in the sense of Flora Europaea: Sell and West (1976), having more or less stellate trichomes on peduncles and involucre. However, this character might be a result of a convergent evolution rather than a close phylogenetic evolution (Mráz, unpublished). The following taxa of the

*H. rohacsense* group were included in the present chemotaxonomic study: *Hieracium rohacsense* Kit; a still undescribed *Hieracium* “population from Mt. Pip Ivan”; *Hieracium borsanum* Zahn ex Mráz; *Hieracium ratezaticum* (Nyár. et Zahn) Mráz; *Hieracium pseudocaesium* Degen et Zahn. All taxa studied are apomicts, which has been proven by isolation experiments. Except one triploid ( $2n=27$ )—*H. borsanum*, the other taxa examined in this study are tetraploids ( $2n=36$ ) (Mráz, 2001; Mráz, in prep.).

The aims of the present study were to examine flavonoid and phenolic acid patterns of the studied taxa (the substances of the taxa from the sect. *Alpina* have not yet been analysed) and to investigate whether these secondary compounds could be used as discriminatory taxonomic markers in this group, especially between closely related taxa such as *H. rohacsense* s. str. and the “population from Mt. Pip Ivan”.

## 2. Materials and methods

### 2.1. Plant material

Living plants were collected during botanical expeditions in 1996–1999 by the corresponding author, then transferred into the experimental field in the Botanical Garden of the P.J. Šafárik University in Košice, Slovakia (48°44'03" N, 21°14'15" E; 240 m a.s.l.) and grown under identical field conditions. The origin of the collections is given below. Data on localities are accompanied by the collection numbers of individual analysed plants in brackets. The same numbers can be found on the voucher specimens of the analysed plants in the herbarium of SAV. Additionally, there are also unnumbered voucher specimens originating from the same populations as the analysed plants deposited in the herbarium SAV. (Abbreviations used: Po-Poland, Ro-Romania, Sk-Slovakia, Uk-Ukraine).

#### 2.1.1. *Hieracium rohacsense*

- Po, Tatry Zachodnie Mts., Mt. Grzes (= Mt. Lúčna), 1640 m a.s.l. (No. 611, 612, 614, 615)
- Sk, Západné Tatry Mts., Roháčska dolina valley, 1350 m a.s.l. (No. 101, 102, 104, 114, 115)
- Sk, Západné Tatry Mts., below the Zábľa saddle above the Látaná dolina valley, 1550–1600 m a.s.l. (No. 122, 127)
- Sk, Západné Tatry Mts., Račkova dolina valley, 1500 m a.s.l. (No. 190, 191, 195, 207, 208, 210)
- Sk, Západné Tatry Mts., Zadná Tichá dolina valley, 1550 m a.s.l. (No. 488, 490, 491, 492)
- Sk, Západné Tatry, Mt. Roh, 1540 m a. s. l. (No. 861, 862)
- Sk, Vysoké Tatry Mts., Kobylika dolina valley, 1650 m a.s.l. (No. 481)
- Sk, Nízke Tatry Mts., saddle between Mt. Kráľička and Mt. Lajštroch, 1500–1600 m a.s.l. (No. 38, 40, 42)

### 2.1.2. *Hieracium* “population from Mt. Pip Ivan”

- Uk, Marmarosh Mts., Mt. Pip Ivan, 1850–1900 m a.s.l. (No. 49, 50, 52, 53, 57, 58, 59, 60, 61, 65, 66, 68)

### 2.1.3. *Hieracium borsanum*

- Ro, Munții Rodnei Mts., by the path Borșa–Stația Meteo, 1560 m a.s.l. (No. 380, 383)
- Ro, Munții Rodnei Mts., by the path Borșa–Stația Meteo, 1780–1800 m a.s.l. (No. 386, 390)

### 2.1.4. *Hieracium ratezaticum*

- Ro, Munții Retezatului Mts., Zănoaga lake, 1850–1980 m a.s.l. (No. 532, 533, 534, 535, 536, 537, 538, 540, 542, 543, 544)
- Ro, Munții Retezatului Mts., Bucura lake, 1800–1900 m a.s.l. (No. 558, 566, 567, 568, 569, 570)

### 2.1.5. *Hieracium pseudocaesium*

- Ro, Munții Retezatului Mts., between Tăul Negru lake and Tăul Gemenele lake, 1800 m a.s.l. (No. 545, 546, 547, 548, 549)
- Ro, Munții Retezatului Mts., Bucura lake, 1800–1900 m a.s.l. (No. 554, 555, 556, 557, 560, 561, 562, 563, 564, 565)

## 2.2. *Sampling of the plant material for HPLC analysis*

The content of secondary metabolites can vary during organ ontogeny (Harborne and Turner, 1984). For this reason, six–eight individual leaves of different ontogenetic stages, from the oldest (outer) leaves to the youngest (inner) ones (for example see Fig. 1), were taken in June 2000 from the rosette of one or two individual plants per taxon. Subsequently the individual leaves were separately analysed for phenolic acid and flavonoid content.

Based on the analysis of different ontogenetic stages, mature, fully developed, so-called “aestival” leaves were collected from individual plants and chosen for quantitative analyses of the phenolic constituents to compare differences among the taxa. The mature leaves are the large leaves in the rosette (40–60 mg of dry mass), which develop during cultivation at the end of spring and early summer. For the present study they were collected in June 2000.

Additionally, so-called “autumnal” leaves were included into the study. They could be defined as small rosette leaves (weighing 10–25 mg after drying), which appear at the end of vegetation period (after flowering) and persist from the end of August until winter. They were collected at the beginning of September 2000.

All leaves were dried at room temperature.

## 2.3. *Quantitative determination of phenolic compounds*

For the quantification of phenolic substances, methanol extracts of dry homogenised leaves from individual plants were analysed by HPLC using an HPLC system

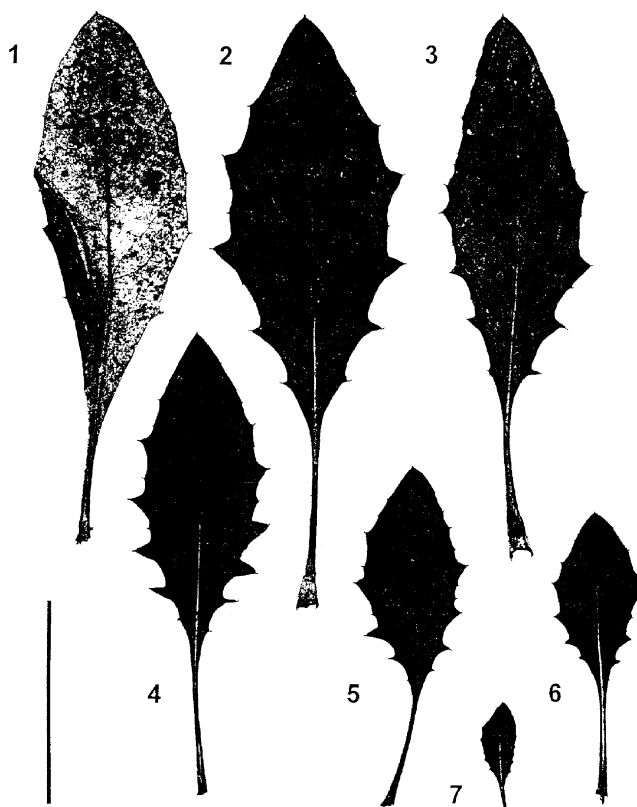


Fig. 1. Leaf rosette spectrum of a *H. rohacsense* plant analysed by HPLC, from the oldest outer leaf (ontogenetic stage 1) to the youngest inner leaf (ontogenetic stage 7).

by Ecom (Prague, Czech Republic) (pump, UV detector, integrator, injector). Analyses were carried out with a Phenomenex Selectosil C18 column 5  $\mu\text{m}$ , 4.6 $\times$ 250 mm, with a flow rate of 0.7 ml min<sup>-1</sup> at 30 °C. The gradient consisted of mobile phase A: H<sub>2</sub>O : acetonitrile : H<sub>3</sub>PO<sub>4</sub> (80:19:1) and B: 80% acetonitrile, gradient program: 0 min—5% B; 5 min—12% B; 25 min—20% B; 30 min—5% B. Detection was performed at 340 nm. Standard compounds used were luteolin 7-*O*- $\beta$ -D-glucopyranoside (Roth company) and chlorogenic acid (Fluka company).

#### 2.4. Isolation and identification of compounds

The compounds were extracted from homogenised leaves with methanol at room temperature. The extract was separated by column chromatography on Sephadex LH 20 column with methanol as eluent. The substances were further purified by means of preparative HPLC with the following conditions: column Separon SGX C18 7  $\mu\text{m}$ , 8 $\times$ 250 mm (Tessec company), flow rate 4 ml min<sup>-1</sup>, mobile phase: acetonitrile : H<sub>2</sub>O (1:9), wavelength 340 nm. Identity of the compounds was confirmed on the

basis of NMR spectra (1D TOCSY, 2D DQF-COSY, HSQC, HMBC experiments) using a Bruker DRX-600 spectrometer.

### 2.5. Statistical analysis

A primary matrix of the data on the amount of secondary compounds (in g) per 100 g of leaf dry mass for each individual plant was analysed using principal component analysis based on a correlation matrix of variables and canonical discriminant analysis (Krzanowski, 1990; Legendre and Legendre, 1998). While principal component analysis does not include any weighting of variables, canonical discriminant analysis weights variables in order to get the best possible separation of objects into the predefined groups. The groups in the discriminant analyses correspond in the present study to the taxa recognised on the basis of morphological characteristics. All statistical analyses were performed using the SAS statistical program package (SAS Institute, 1989). The *t*-test was used to evaluate the statistical significance of differences of secondary compound content between “autumnal” and “aestival” leaves and Scheffé’s test for statistical difference of secondary compound content among taxa.

## 3. Results and discussion

All *Hieracium* taxa included in the present study accumulated the following five compounds as the major components of the leaf extract: (1) chlorogenic acid; (2) luteolin 7-*O*- $\beta$ -D-glucopyranoside; (3) 3, 5-dicaffeoylquinic acid, (4) luteolin 4'-*O*- $\beta$ -D-glucuronopyranoside and (5) apigenin 4'-*O*- $\beta$ -D-glucuronopyranoside. The first three substances were previously found as the dominant leaf constituents in seven *Hieracium* species from the Balkan Peninsula analysed by Petrović et al. (1999). On the other hand, compounds (4) and (5) have been reported previously only from some North American species of *Hieracium* (Guppy and Bohm, 1976). In our study no qualitative interspecific differences in the phenolic acid and flavonoid patterns in the *Hieracium rohacsense* group were found. However, the taxa exhibited a statistically significant quantitative variability of the compound contents. This is in accordance with previous studies (Giner et al., 1992; Petrović et al., 1999), in which only quantitative differences were reported.

### 3.1. Variation during leaf ontogeny

The amount of the studied flavonoids and phenolic acids was found to change during leaf rosette ontogeny. The outside, oldest leaves had a generally lower content of flavonoid compounds and phenolic acids than the mature, biggest leaves from the middle part of the rosette. At this stage of fully developed “aestival” leaves the amount of compounds reached maximal values. The inner, smaller and younger leaves of the rosette again contained a lower quantity of phenolic substances (Fig.

2). Phenolic acid content was more variable than the flavonoid composition, therefore correlations with ontogenetic stage were less clear and obvious, but still recognisable.

### 3.2. Seasonal variation

Comparison of two seasonal types of leaves—mature “aestival” vs. “autumnal” leaves showed significant differences in the content of flavonoids. The former contained a higher amount of all flavonoid substances than the latter. Even in the youngest “aestival” leaves from the rosette with the lowest flavonoid quantity the content of the substances was still significantly higher than in the “autumnal” ones (Tables 1 and 2). Because of the strong absorbance in the UV wavelength region, flavonoids and hydroxycinnamic acid conjugates play an important role in the protection of plant tissues against excessive UV irradiation and in scavenging of reactive oxygen radicals induced by harmful UV-B irradiation (Burchard et al., 2000; Markham et al., 1998). The high amount of the phenolic substances may therefore present an adaptation of plants growing in the alpine and sub alpine habitats to higher intensity of UV irradiation. Lowland or forest plants were reported to contain a lower amount of the protective phenolics, mainly flavonoid compounds (e. g. Petrović et al. (1999); Zidorn and Stuppner (2001). This may be also an explanation of the significantly decreased quantity of flavonoids in the “autumnal” leaves, which appear at the end of vegetation period and persist till winter with lower levels and length of irradiation. Due to higher variability of the content values, with two exceptions (Table 1), the differences in phenolic acid quantity between the two leaf types were not statistically significant.

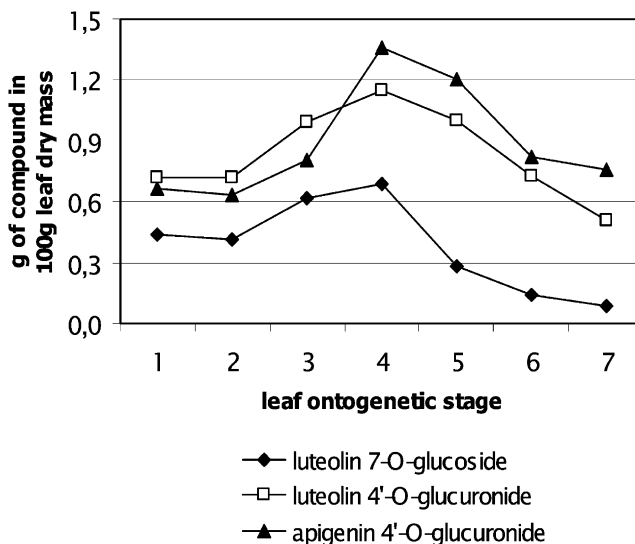


Fig. 2. Changes of flavonoid content in the leaves of a *H. rohacsense* plant during leaf rosette ontogeny from the oldest outer leaf (ontogenetic stage 1) to the youngest inner leaf (ontogenetic stage 7).

Table 1

Content of phenolic acids and flavonoid compounds (g of substance/100 g leaf dry mass) in the “aestival” leaves in the taxa of the *Hieracium rohacsense* group

| Average content of compounds (g/100g dry mass) in the “aestival” leaves <sup>a</sup> |              |                                     |                                    |                                    |                         |                           |
|--|--------------|-------------------------------------|------------------------------------|------------------------------------|-------------------------|---------------------------|
| Taxon  | No Ind./Pop. | luteolin 7- <i>O</i> -glucoside     | luteolin 4'- <i>O</i> -glucuronide | apigenin 4'- <i>O</i> -glucuronide | Chlorogenic acid        | 3,5-dicaffeoylquinic acid |
| <i>H. rohacsense</i>   | 12/4         | 0.632* <sup>b</sup><br>(0.245)<br>a | 1.366*<br>(0.415)<br>a             | 4.228*<br>(0.868)<br>a             | 2.869*<br>(0.770)<br>a  | 1.007<br>(0.269)<br>a     |
| <i>H.</i> “population from Mt. Pip Ivan”   | 9/1          | 0.988*<br>(0.200)<br>b              | 0.830*<br>(0.183)<br>b             | 2.588*<br>(0.530)<br>b             | 2.180<br>(0.634)<br>b,c | 1.384<br>(0.282)<br>a,b   |
| <i>H. borsanum</i>   | 4/2          | 0.148<br>(0.062)<br>c               | 0.870*<br>(0.222)<br>a             | 2.098*<br>(0.897)<br>b             | 2.775<br>(0.836)<br>b,c | 0.948<br>(0.306)<br>a     |
| <i>H. ratezaticum</i>  | 9/2          | 0.745*<br>(0.187)<br>a              | 1.153*<br>(0.284)<br>a             | 3.138*<br>(1.806)<br>b             | 1.899<br>(1.074)<br>c   | 0.984<br>(0.110)<br>b     |
| <i>H. pseudocaesium</i>  | 6/2          | 0.270*<br>(0.072)<br>c              | 1.432*<br>(0.354)<br>a             | 1.565*<br>(0.964)<br>a             | 1.696*<br>(0.739)<br>b  | 1.003<br>(0.309)<br>b     |

<sup>a</sup> ind: number of individuals analysed; pop: number of populations analysed (SD) given in parenthesis.

<sup>b</sup> \*denotes statistically significant differences by *t*-test between “autumnal” and “aestival” leaves ( $P < 0.05$ ). Means in columns sharing the same letter are not significantly different (Scheffé’s test,  $P < 0.05$ ).

### 3.3. Interspecific variation

As stated above, only quantitative differences were found among the analysed taxa (Tables 1 and 2). The related taxa *H. rohacsense* and the plants from “population from Mt. Pip Ivan” can be distinguished based on quantity of luteolin 7-*O*-glucoside, luteolin 4'-*O*-glucuronide and apigenin 4'-*O*-glucuronide in both “autumnal” and “aestival” leaves (Fig. 3). There are slight morphological differences between the taxa and also in their isozyme pattern (Mráz et al., 2001).

Two groups of plants are visible on the PCA ordination diagram of “autumnal” leaves (Fig. 4) only slightly overlapping along the first component axis. The first one is formed by *H. ratezaticum* and *H. rohacsense*, while the second one includes “population from Mt. Pip Ivan” and *H. pseudocaesium*, leaving three out of four individuals of *H. borsanum* in an intermediate position between these two groupings. However, no visible groupings were visible on the PCA ordination diagram of “aestival” leaves (diagram not shown). The ordination diagram of canonical discriminant analysis of “aestival” leaves (Fig. 5) clearly separates plants of *H. rohacsense* from the rest of the material, whereas “population from Mt. Pip Ivan” forms a rather compact grouping. The second diagram of canonical discriminant analysis, that of “autumnal” leaves (Fig. 6), shows a similar separation as corresponding PCA. The

Table 2

Content of phenolic acids and flavonoids (g of substance/100 g leaf dry mass) in the “autumnal” leaves in the taxa of the *Hieracium rohacsense* group

| Average content of phenolic compounds (g/100 g dry mass) in the “autumnal” leaves <sup>a</sup> |              |                                    |                                    |                                    |                       |                           |
|--|--------------|------------------------------------|------------------------------------|------------------------------------|-----------------------|---------------------------|
| Taxon  | No Ind./Pop. | luteolin 7- <i>O</i> -glucoside    | luteolin 4'- <i>O</i> -glucuronide | apigenin 4'- <i>O</i> -glucuronide | chlorogenic acid      | 3,5-dicaffeoylquinic acid |
| <i>H. rohacsense</i>   | 17/5         | 0.138<br>(0.067)<br>a <sup>b</sup> | 0.783<br>(0.315)<br>a              | 1.869<br>(1.071)<br>a,c            | 1.905<br>(0.600)<br>a | 0.375<br>(0.104)<br>a     |
| <i>H.</i> “population from Mt. Pip Ivan”   | 12/1         | 0.437<br>(0.116)<br>b              | 0.344<br>(0.089)<br>b              | 2.870<br>(0.555)<br>b              | 2.976<br>(0.676)<br>b | 0.639<br>(0.112)<br>b     |
| <i>H. borsanum</i>   | 4/2          | 0.136<br>(0.025)<br>a              | 0.644<br>(0.080)<br>b              | 2.813<br>(0.684)<br>a              | 2.177<br>(0.635)<br>b | 0.635<br>(0.047)<br>a,c   |
| <i>H. ratezaticum</i> s. str.  | 17/2         | 0.305<br>(0.095)<br>c              | 0.405<br>(0.168)<br>a,c            | 3.929<br>(0.761)<br>b              | 2.623<br>(0.540)<br>c | 0.463<br>(0.183)<br>b,c   |
| <i>H. pseudocaesium</i>  | 15/2         | 0.081<br>(0.031)<br>a              | 0.920<br>(0.238)<br>b,c            | 2.775<br>(0.867)<br>c              | 2.447<br>(0.726)<br>b | 0.546<br>(0.173)<br>c     |

<sup>a</sup> ind: number of individuals analysed; pop: number of populations analysed, (SD) given in parentheses.

<sup>b</sup> Means in columns sharing the same letter are not significantly different (Scheffé's test,  $P < 0.05$ ).

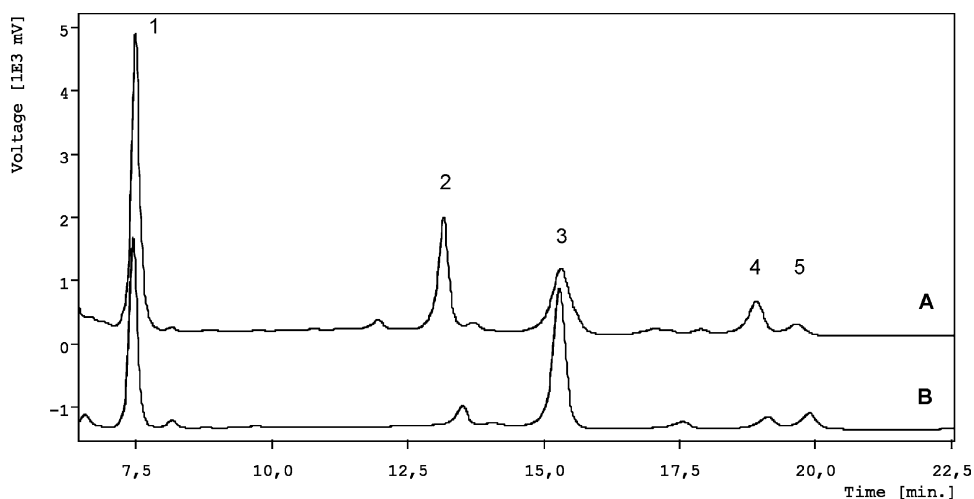


Fig. 3. HPLC chromatograms of the leaf methanol extract of *Hieracium* “population from Mt. Pip Ivan” (A) and *H. rohacsense* (B). 1 chlorogenic acid, 2 luteolin 7-*O*- $\beta$ -D-glucopyranoside, 3 3,5-dicaffeoylquinic acid, 4 luteolin 4'-*O*- $\beta$ -D-glucuronopyranoside, 5 apigenin 4'-*O*- $\beta$ -D-glucuronopyranoside.

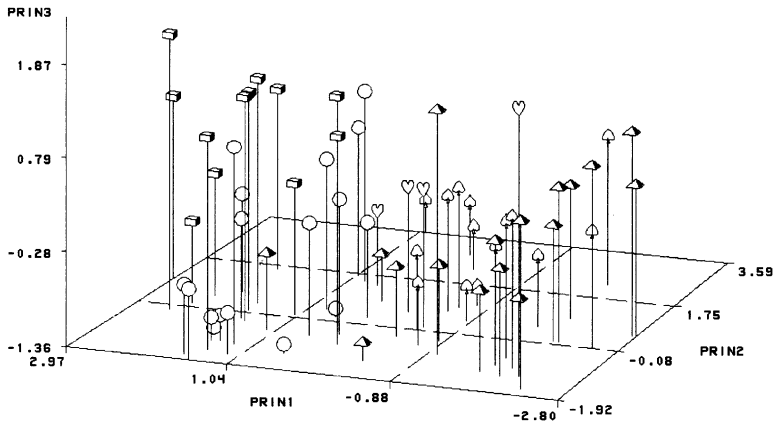


Fig. 4. Ordination diagram of the principal component analysis of “autumnal” leaves. The first three component axes account for 45.8, 26.2 and 12.4% of variation among plants respectively. Cube, *H. rohacsense*; pyramid, *H.* “population from Mt. Pip Ivan”; heart, *H. borsanum*; ball, *H. ratezaticum* and spade, *H. pseudocaesium*.

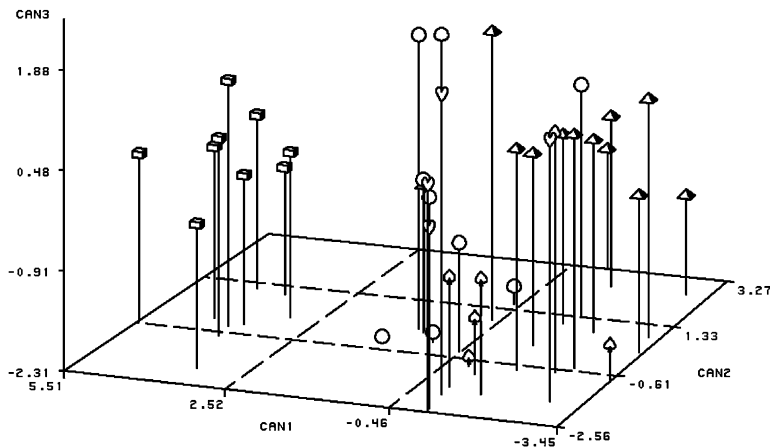


Fig. 5. Ordination diagram of the canonical discriminant analysis of “aestival” leaves. The first three canonical axes account for 78.5, 15.5 and 5.0% of variation among groups of plants, respectively. Symbols as in Fig. 4.

most important information resulting from these analyses is the clear separation of *H. rohacsense* from the plants from “Mt. Pip Ivan”, justifying the taxonomic separation of the latter, on one hand, and *H. ratezaticum* from the closely related taxon—*H. pseudocaesium*, on the other hand. The compounds which contribute most to the separation of “autumnal” leaves along the first canonical axis are (2) and (5). In the case of “aestival” leaves, (2), (4) and (5) are the compounds most strongly correlated with the first canonical axis.

*H. ratezaticum* was collected in nature as one taxon. However, during 3 years

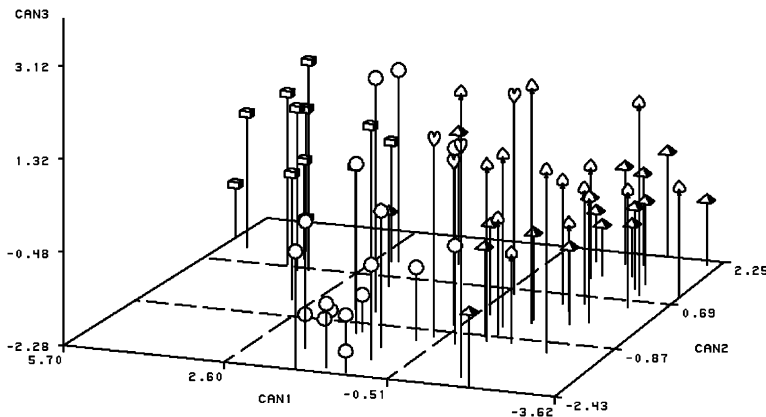


Fig. 6. Ordination diagram of the canonical discriminant analysis of “autumnal” leaves. The first three canonical axes account for 78.7, 12.8 and 6.8% of variation among groups of plants, respectively. Symbols as in Fig. 4.

(1998–2000) of the experimental cultivation the cultivated plants were found to belong to the two morphologically distinguishable groups. One group of plants, for which we found later the correct name *H. pseudocaeisium*, has darker green leaves with purple spots (this feature is most obvious during spring; there are no spots in the second group) and denser and simpler trichomes on the involucral bracts (the involucrem is apparently darker). The flowering time of this group is approximately 10–15 days earlier compared to the second group of plants—*H. ratezaticum*. The morphological and phenological differentiation is supported also by the flavonoid content. *H. ratezaticum* is a close relative of *H. pseudocaeisium* and it appears to differ from the latter taxon by a significantly higher quantity of luteolin 7-*O*-glucoside. On the other hand, it contains a decreased content of apigenin 4'-*O*-glucuronide. The interspecific differences in the substance content remain stable if we compare the “autumnal” and “aestival” leaves. Because the plants used for HPLC analyses were cultivated under the same climatic and mainly light conditions (experimental field), this interspecific variability should be based genetically.

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