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INTERSPECIFIC AND INTRASPECIFIC VARIATION OF PHENOLICS IN THE GENUS *EQUISETUM* SUBGENUS *EQUISETUM**

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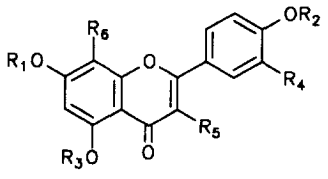
Key Word Index—*Equisetum arvense*; Equisetaceae; medicinal plants; flavonoids; phenolics; styrylpyrones; chemotaxonomy; variation study; HPLC.

Abstract—The patterns of phenolics in methanolic extracts from the overground sporophytes of all species of the subgenus *Equisetum* are given. From the interspecific variation in the accumulated flavonoid glycosides and other phenolics, all taxa of the subgenus can be distinguished. The retention characteristics in a standard HPLC method and maxima of the on-line UV spectra of all compounds detected are given. The HPLC method allows the quantification of phenolics in herbal remedies containing *Equisetum*, as well as in the crude drug material and therefore could be useful for both quality control and chemotaxonomic studies. All species show quantitative and qualitative variations during plant development. As an example, we present chromatograms of different developmental stages of *E. arvense* and accumulation dynamics of some phenols in that species. Phenolics accumulating in different organs of the plant are distinct. Apart from the spores, only diploid tissues are able to accumulate flavonoids. The haploid vegetative gametophytes accumulate caffeic acid esters and styrylpyrones in high contents. The rhizomes are also free of flavonoids, but contain various styrylpyrones, besides hydroxycinnamoyl esters. Styrylpyrones seem to replace flavonoids in gametophytes and sporophytic rhizomes of *Equisetum* species.

INTRODUCTION

Equisetum arvense L. (Equisetaceae, subgenus *Equisetum*) is a well-known and widespread pteridophyte distributed in the northern hemisphere. Its sterile stems are used as medicines in various countries, constituting the 'Equiseti herba' of European Pharmacopeias (DAB 10, Ph. Helv. VII, ÖAB 90, Ph. Pol. III, Ph. Ross 9 and Ph. Hung.). The indications of *Equisetum* preparations are related to the reputed aquaretic and antihaemorrhagic properties of the plant.

The other taxa of the subgenus are known as potential adulterations of Equiseti herba. While *E. palustre* has been shown to appear quite frequently as an adulterant, the role of the other taxa of the subgenus as possible adulterants is as yet uncertain. All *Equisetum* species, especially the hybrids, are extremely variable in their morphology and their identification by morphological characters can be particularly difficult. We published earlier an HPLC method to prove the identity of Equiseti herba and for the detection of a possible adulteration with *E. palustre* [1]. Apart from some minor compounds, all



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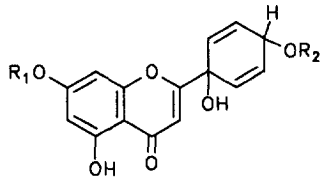

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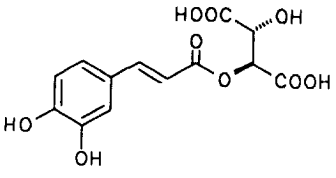

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*Dedicated to Prof. Dr Franz-C. Czygan, Wüzrburg, on the occasion of his sixtieth birthday.

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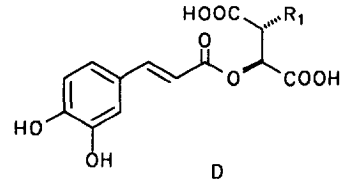

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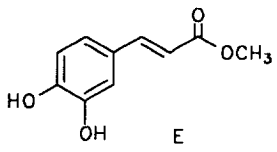

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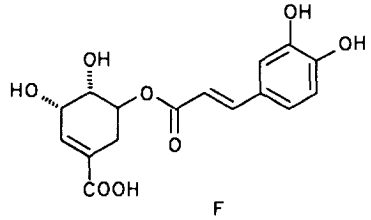

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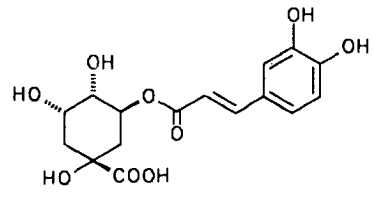

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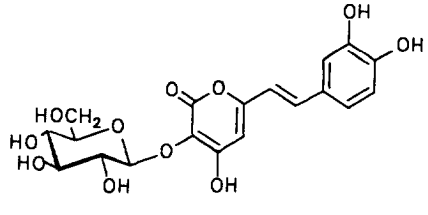

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phenolics in sprouts of *E. arvense* and the other species of the subgenus (*E. palustre* L., *E. telmateia* Erhr., *E. pratense* Erhr., *E. sylvaticum* L., *E. fluviatile* L. and *E. bogotense* H.B.K.) are now elucidated [2–14]. These cryptic characters have proven to be very useful in chemotaxonomy. For all species, we analysed samples from various regions of their distribution range. We found two discrete chemotypes of *E. arvense* [5, 6], while all other species of the subgenus show no significant intraspecific variation with geography. The aim of the present study was to examine inter- and intraspecific variation within the subgenus *Equisetum*. These data could provide evidence for plant classification and contribute to achieving better quality control of crude drug materials.

RESULTS AND DISCUSSION

Chromatographic identification and quantification

For the quantification of plant flavonoids, most pharmacopeias require a standard method, based on spectrophotometric determination of flavonoids as aluminium chelate complexes of the aglycones after hydrolysis of the glycosides [15]. As there is a calculation with the same given specific absorption for different aglycones, this method is only approximate. The method fails with extracts containing *C*-glycosides and is not reproducible if the extracts contain flavonols as well as flavones in various ratios [16–21]. The latter case is true for *E. arvense*, so RP-HPLC allows a lot more selective determination, not only of the flavonoids, but also of all other phenolics in the water–methanol extract [14].

To obtain fingerprint chromatograms of the genuine compounds, HPLC analysis must be done with the unhydrolysed extracts, though HPLC methods involving prior hydrolysis of glycosides have the advantage of much shorter run times and easier peak identification. To report interspecific variation, in all cases of co-elution the accumulation of the co-eluted compounds in the analysed plants has been proved by means of DC analysis. For distinguishing the different taxa, DC analysis might be helpful also in cases of mixed or adulterated samples, with plant material of two or more species [1]. In all such cases the extract (85% methanol) of the unknown sample should be compared with the extracts of determined species; while looking for hybrids, with extracts of the sympatric parents. Due to the poor reproducibility of R_f values, such data are not given for the isolated compounds. Also in HPLC analysis variations in R_ts may be caused for example by differences in solvent composition, temperature and stationary phase material. Therefore, methods for checking peak identity, e.g. ratio plots and on-line spectra with PDA detectors are necessary for exact peak identification. For this reason (Table 1) the maxima of the on-line spectra of all compounds are listed. It might also be helpful to calculate relative R_ts correlated to the R_t of the internal standard. Such quotients appear to be less sensitive to variation.

The compounds identified in various taxa differ in some cases from those reported in the literature [19–22]. Such results were obtained only by comparaison of R_f values using PC or TLC and could be incorrect. We found this to be true in particular for kaempferol 7-*O*-glycosides and free caffeic acid. In all species aglycones could be detected only in very old samples (> 10 years) or samples that had been stored under humid conditions and/or dried improperly. They are always accompanied by lowered contents of flavonoid glycosides and may be artefacts formed during improper drying and storage or extraction. Caffeic acid methyl ester is most likely formed during extraction with methanol by trans-esterification; it can be detected mainly in samples with high amounts of caffeic acid conjugates and only after extraction with methanol.

Table 1. Retention times and maxima (sh = shoulder, the most intense band in each spectrum is underlined) of the on-line UV spectra of phenolics detected regularly in sporophytic sprouts of taxa of *Equisetum* subgenus *Equisetum*

No.	R_t	Compound
1	7.1	Chlorogenic acid (219sh, 228sh, 323)
2	7.6	Kaempferol glycoside 'p1' (264 344)
3	8.0	Kaempferol 3-*O*-sophoroside-7-*O*-glucoside (264, 344)
4	8.8	Hydroxycinnamic acid 'C1' (216, 233sh, 325)
5	10.2	Phenolic 'p7' (286, 326)
6	10.9	Monocaffeoyl-*meso*-tartaric acid (216, 233sh, 327)
7	13.4	Quercetin 3,7-*O*-diglucoside (254, 348)
8	15.0	Protoapigenin-4'glucoside (204, 229, 249, 299)
9	15.4	Hydroxycinnamic acid (274, 327)
10	16.2	Phenolic 'P3' (276)
11	16.6	5-*O*-Caffeoylshikimic acid
12	16.8	Kaempferolglycoside 'P2' (264, 338)
13	17.5	Kaempferol 3-*O*-7-*O*-diglucoside (264, 346)
14	18.0	Hydroxycinnamic acid 'C2' (215, 327)
15	18.7	Kaempferol 3-*O*-rutinoside-7-*O*-sophoroside (264, 346)
16	19.3	Kaempferol 3-*O*-rutinoside-7-*O*-glucoside (264, 346)
17	19.8	Equisetumpyrone (218, 252, 371)
18	21.2	Kaempferol 3-*O*-(6''-*O*-malonylglucoside)-7-*O*-glucoside (264, 346)
19	22.5	Phenolic 'P5' (308)
20	23.4	Phenolic 'P6' (226, 313)
21	23.8	Quercetin 3-*O*-sophoroside (255, 352)
22	25.0	Caffeic acid methylester (231sh, 372)
23	25.4	Kaempferol 3-*O*-(6''-*O*-acetylglucoside)-7-*O*-glucoside (264, 346)
24	25.8	Protogenkwanin-4'-*O*-glucoside (231, 250, 296)
25	26.0	Gossypetin 7-*O*-glucoside (258, 344sh, 381)
26	26.1	Quercetin 3-*O*-glucoside-7-*O*-rhamnoside (256, 354)
27	26.5	Dihydrokaempferol
28	27.2	Quercetin 3-*O*-rutinoside-7-*O*-rhamnoside (256, 354)
29	28.0	Kaempferol 3-*O*-sophoroside (264, 345)
30	28.3	Quercetin 3-*O*-(6''-*O*-malonylglucoside)-7-*O*-rhamnoside (255, 353)
31	28.6	Luteolin 5-*O*-glucoside (242sh, 342)
32	30.6	Kaempferol 3-*O*-glucoside-7-*O*-rhamnoside (264, 346)
33	31.2	Kaempferol 3-*O*-rutinoside-7-*O*-rhamnoside (264, 346)
34	32.8	Quercetin 3-*O*-glucoside (256, 354)
35	33.5	Quercetin 3-*O*-rutinoside (256, 354)
36	33.5	Kaempferol 7-*O*-glucoside (264, 365)
37	35.6	Apigenin 5-*O*-glucoside (259, 333)
38	35.8	Kaempferol 3-*O*-(6''-*O*-malonylglucoside)-7-*O*-rhamnoside (265, 348)
39	36.3	Quercetin 3-*O*-(6''-*O*-malonylglucoside) (256, 354)
40	37.6	Apigenin 4'-*O*-glucoside (266, 326)
41	38.6	Luteolin 5-*O*-(6''-*O*-malonylglucoside) (242sh, 342)
42	39.5	Dicaffeoyl-*meso*-tartaric acid (214, 242, 328)
43	39.5	Kaempferol 3-*O*-(6''-*O*-acetylglucoside)-7-*O*-rhamnoside (264, 346)
44	39.8	Quercetin 3-*O*-rhamnoside (256, 349)
45	40.2	Kaempferol 3-*O*-glucoside (264, 346)
46	41.2	Kaempferol 3-*O*-rutinoside (264, 346)
47	42.5	Genkwanin 5-*O*-glucoside (260, 333)
48	44.0	Kaempferol 3-*O*-(6''-*O*-malonylglucoside (264, 346)
49	44.7	Apigenin 5-*O*-(6''-*O*-malonylglucoside) (259, 333)
IS	46.0	Naringenin (internal standard)
50	46.5	Kaempferol 3-*O*-rhamnoside (264, 346)
51	52.3	Genkwanin 5-*O*-(6''-*O*-malonylglucoside) (260, 333)
52	53.6	Kaempferol 7-*O*-rhamnoside (264, 365)
53	54.7	Genkwanin 4'-*O*-glucoside (266, 326)

Crude drug material of *E. arvense* is sometimes adulterated with material of other *Equisetum* species, because the plants are harvested mainly from wild stands. Therefore, in quality control, there is also a need for proving the identity of the crude drug material besides standardization and quantification of the extract.

Interspecific variation

The flavonoid biochemistry of the barren sprouts of the species in the subgenus *Equisetum*, can be divided into three groups. These correlate with a classification concept based on morphological and micromorphological characters [23].

Species accumulating only kaempferol glycosides

Equisetum bogotense *H.B.K.* This South American species is the only one in the subgenus whose distribution area is outside the holarctic. It is unlikely that crude drug material of Equiseti herba is adulterated with *E. bogotense*, although in South America the plants are used in herbal medicine with similar indications [24]. The plants showed very simple patterns of phenolics. Only two flavonoids, kaempferol 3-*O*-sophoroside (**29**) and kaempferol 3-*O*-sophoroside-7-*O*-glucoside (**3**) could be detected. The plants contained also 5-*O*-caffeoyl-shikimic acid (**11**) and equisetumpyrone (**17**). The latter is only in fertile stems.

Equisetum palustre *L.* Due to its reputed toxicity [25], crude drug material of Equiseti herba should always be checked for an adulteration with *E. palustre*. The main flavonoid glycoside, accumulated over the entire vegetation period in this species was kaempferol 3-*O*-rutinoside-7-*O*-glucoside (**16**). Only in some plants could we detect kaempferol 3-*O*-rutinoside-7-*O*-sophoroside (**15**). The accumulation of this compound was obviously unrelated to geographic origin or developmental variation. Apart from that, all samples of *E. palustre* examined show very similar patterns of accumulated phenolics.

As non-flavonoid compounds, monocaffeoyl-*meso*-tartaric acid (**6**) and a few unidentified compounds could be detected. The compound 'P5' (**19**) has proved to be useful for the detection of *E. palustre* as adulteration in Equiseti herba [1].

Equisetum telmateia *Ehrh.* This species was distinct from the two others in this group by the accumulation of acetylated glycosides: kaempferol 3-*O*-(6″-*O*-acetylglucoside)-7-*O*-glucoside (**23**) and kaempferol 3-*O*-(6″-*O*-acetylglucoside)-7-*O*-rhamnoside (**43**). Acetylated compounds could not be detected in any other species in the subgenus. The main compounds in all analysed samples were **23** and the corresponding non-acylated kaempferol 3,7-*O*-diglucoside (**13**). In the plants a variety of further kaempferol glycosides could be detected (Table 2). Of these, kaempferol 3-*O*-rutinoside-7-*O*-rhamnoside (**33**) was characteristic for *E. telmateia* and could not be detected in any other species of the subgenus.

Due to their disjunct distribution, North American plants have been regarded as a different taxon, ssp. *braunii* [26]. We could not detect the differences between plants of North America (British Columbia) and Europe which have been reported by others [4, 20].

Species accumulating kaempferol glycosides and quercetin glycosides

Equisetum pratense *Ehrh.* Kaempferol 3-*O*-rutinoside-7-*O*-glucoside (**16**) could be detected as the main compound in all samples. Apart from the minor compound quercetin 3,7-*O*-diglucoside (**7**), all flavonol glycosides accumulated in *E. pratense* could also be detected in *E. sylvaticum* (Table 2).

Equisetum sylvaticum *L.* The species was distinct from *E. pratense* in accumulating additionally the 7-*O*-rhamnosides **30** and **32**, as well as several malonylated flavonol glycosides (Table 2). The major compounds in all examined samples were kaempferol 3-*O*-(6″-*O*-malonylglucoside)-7-*O*-rhamnoside (**38**) and quercetin 3-*O*-(6″-*O*-malonylglucoside) (**39**). In earlier investigations [2] these compounds may have been overlooked because of their strong bonding to polyamide. From RP materials they are eluted easily, usually 2–5 min after the corresponding unmalonylated glycosides.

Species accumulating quercetin and kaempferol glycosides, as well as flavone glycosides

Equisetum fluviatile *L.* The species showed a constant and simple, but very complex pattern of accumulated phenolics. The main compounds were kaempferol-3,7-*O*-diglucoside (**13**), kaempferol-3-*O*-(6″-*O*-malonylglucoside)-7-*O*-glucoside (**18**) and apigenin-4′-*O*-glucoside (**40**) (Table 2). All compounds were accumulated over the entire growing season and plants from different origins showed very similar patterns of phenolics.

Equisetum arvense *L.* In this species morphological plasticity appears to be combined with the most diverse phenolic biochemistry. Thus, the plants showed notable intraspecific variation. Plants with only slight morphological differences showed distinct patterns of phenolics. Morphologically similar plants from different origins revealed the occurrence of two chemotypes (or chemodemes, see ref. [27]). The chemotypes were distinct in their accumulation of luteolin 5-*O*-glucoside (**31**) and luteolin 5-(6″-*O*-malonylglucoside) (**41**). While plants from North America and Asia (Japan, China, Siberia) are able to accumulate these flavone glucosides. European plants do not contain luteolin glycosides. In Scandinavia and Scotland the area of distribution of both types overlap and we found plants with intermediate patterns between both types, obviously produced by cross-breeding. In all cases the plants were almost identical in their morphological characters [5, 6, 14]. Quercetin 3-*O*-(6″-*O*-malonylglucoside) (**39**) was found to be abundant in all *E. arvense* samples examined. In European plants it was always the major flavonoid and comprised between 28 and 50% of the total flavonoid content. In plants from Asia and North America luteolin 5-(6″-*O*-malonylglucoside) (**41**), which was absent in plants from Europe,

Table 2. Hydroxycinnamic acid derivatives, flavonoids and styrylpyrones accumulated in *E. pratense* (PR), *E. bogotense* (B), *E. sylvaticum* (S), *E. telmateia* (T), *E. palustre* (P), *E. arvense*, Europe (A_{Eur}), *E. arvense*, Asia (A_A) and *E. fluviatile* (F).

	PR	S	B	T	P	A_{Eur}	A_A	F
Apigenin 4′-*O* glucoside (**40**)	—	—	—	—	—	+	+	++
Apigenin 5-*O*-(6″-*O*-malonylglucoside) (**49**)	—	—	—	—	—	+	+	—
Apigenin 5-*O*-glucoside (**37**)	—	—	—	—	—	++	++	—
5-*O*-Caffeoylshikimic acid (**11**)	+	+	+	—	—	+	+	—
Chlorogenic acid (**1**)	—	—	—	—	—	+	+	+
Dicaffeoyl-*meso*-tartaric acid (**42**)	F	F	—	—	—	++	++	—
Equisetumpyrone (**17**)	—	—	+	—	+	+	+	F
Genkwanin 4′-*O*-glucoside (**53**)	—	—	—	—	—	+	+	—
Genkwanin 5-*O*-glucoside (**47**)	—	—	—	—	—	+	+	—
Genkwanin 5-*O*-(6″-*O*-malonylglucoside) (**51**)	—	—	—	—	—	+	+	—
Gossypetin 7-*O*-glucoside (**25**)	F	F	F	F	F	F	F	F
Kaempferol 3-*O*-glucoside (**45**)	+	+	—	++	+	+	+	+
Kaempferol 3-*O*-glucoside-7-*O*-rhamnoside (**32**)	—	+	—	+	—	—	—	—
Kaempferol 3-*O*-rhamnoside (**50**)	—	—	—	+	—	—	—	—
Kaempferol 3-*O*-rutinoside (**46**)	+	+	—	+	+	—	—	—
Kaempferol 3-*O*-rutinoside-7-*O*-glucoside (**16**)	++	+	—	++	++	+	+	—
Kaempferol 3-*O*-rutinoside-7-*O*-rhamnoside (**33**)	—	+	—	+	—	—	—	—
Kaempferol 3-*O*-rutinoside-7-*O*-sophoroside (**15**)	—	—	—	—	++	—	—	—
Kaempferol 3-*O*-sophoroside (**29**)	+	+	+	+	+	+	+	+
Kaempferol 3-*O*-sophoroside-7-*O*-glucoside (**3**)	+	—	+	—	++	—	—	—
Kaempferol 3-*O*-(6″-*O*-acetylglucoside)-7-*O*-glucoside (**23**)	—	—	—	++	—	—	—	—
Kaempferol 3-*O*-(6″-*O*-acetylglucoside)-7-*O*-rhamnoside (**43**)	—	—	—	++	—	—	—	—
Kaempferol 3-*O*-(6″-*O*-malonylglucoside) (**48**)	—	+	—	—	—	+	+	+
Kaempferol 3-*O*-(6″-*O*-malonylglucoside)-7-*O*-glucoside (**18**)	—	+	—	—	—	+	+	++
Kaempferol 3-*O*-(6″-*O*-malonylglucoside)-7-*O*-rhamnoside (**38**)	—	++	—	—	—	—	—	—
Kaempferol 3,7-*O*-diglucoside (**13**)	+	+	—	++	++	+	+	++
Kaempferol 7-*O*-glucoside (**36**)	—	—	—	—	+	—	—	—
Kaempferol 7-*O*-rhamnoside (**52**)	—	—	—	+	+	—	—	—
Luteolin 5-*O*-glucoside (**31**)	—	—	—	—	—	—	++	—
Luteolin 5-*O*-(6″-*O*-malonylglucoside) (**41**)	—	—	—	—	—	—	+	—
Monocaffeoyl-*meso*-tartaric acid (**6**)	+	+	—	—	+	+	+	—
Protoapigenin 4-*O*-glucoside (**8**)	—	—	—	—	—	+	+	F
Protogenkwanin 4′-*O*-glucoside (**24**)	—	—	—	—	—	+	+	F
Quercetin 3-*O*-glucoside (**34**)	+	+	—	—	—	++	+	—
Quercetin 3-*O*-glucoside-7-*O*-rhamnoside (**26**)	—	+	—	—	—	—	—	—
Quercetin 3-*O*-rutinoside (**35**)	++	+	—	—	—	—	—	—
Quercetin 3-*O*-rutinoside-7-*O*-rhamnoside (**28**)	—	+	—	—	—	—	—	—
Quercetin 3-*O*-sophoroside (**21**)	—	+	—	—	—	++	+	—
Quercetin 3-*O*-(6″-*O*-malonylglucoside) (**39**)	—	++	—	—	—	+	+	—
Quercetin 3-*O*-(6″-*O*-malonylglucoside)-7-*O*-rhamnoside (**30**)	—	+	—	—	—	—	—	—
Quercetin 3,7-*O*-diglucoside (**7**)	+	—	—	—	—	+	+	—

++ Main compound (> 500 μg g^{-1} dry weight), + = detected, only the compounds in bold were accumulated over the entire growing season. F = detected in fertile sprouts only, — = not detected (> 15 μg g^{-1} dry weight).

Numbers in brackets refer to Table 1 and Figs 5 and 6.

was the major flavonoid comprising some 50–60% of the total flavonoid. Some samples of North American origin containing, besides **41**, also remarkably large amounts of quercetin 3-*O*-(6″-*O*-malonylglucoside) (**39**). In some plants from the whole distribution range, apigenin 5-*O*-glucoside (**37**) accumulated in larger amounts [14]. In all samples examined a wide variety of other flavone and flavonol glycosides could be detected (Table 2). *Equisetum arvense* accumulates also large amounts of dicaffeoyl-*meso*-tartaric acid (**42**), a depside which is typical for that taxon and could only be detected, in green sprouts, of *E. arvense* and its hybrids [7, 14], whereas the monoacylated derivative (**6**) could be detected in other taxa of the subgenus as well (Table 2).

Hybrids

Hybrids are believed to play a role in the adulteration of crude drug material of *E. arvense*, Equiseti herba. Morphological characters of *Equisetum* hybrids are quite variable and identification based on morphology is often difficult. The specific flavonoid patterns, shown by HPLC, can be used additionally to distinguish hybrids

Table 3.

No.	Formula	R_1	R_2	R_3	R_4	R_5	R_6
1	G						
3	A	glc	H	H	H	Osoph	H
6	C						
7	A	glc	H	H	OH	Oglc	H
8	B	H	glc				
11	F						
13	A	glc	H	H	H	Oglc	H
15	A	soph	H	H	H	Orut	H
16	A	glc	H	H	H	Orut	H
17	H						
18	A	glc	H	H	H	Omaglc	H
21	A	H	H	H	OH	Osoph	H
22	E						
23	A	glc	H	H	H	acglc	H
24	B	Me	glc				H
25	A	glc	H	H	—	OH	OH
26	A	rha	H	H	OH	Oglc	H
28	A	rha	H	H	OH	Orut	H
29	A	H	H	H	H	Osoph	H
30	A	rha	H	H	OH	Omaglc	H
31	A	H	H	glc	OH	H	H
32	A	rha	H	H	H	Oglc	H
33	A	rha	H	H	H	Orut	H
34	A	H	H	H	OH	Oglc	H
35	A	H	H	H	OH	Orut	H
36	A	glc	H	H	H	OH	H
37	A	H	H	glc	H	H	H
38	A	rha	H	H	H	Omaglc	H
39	A	H	H	H	OH	Omaglc	H
40	A	H	glc	H	H	H	H
41	A	H	H	maglc	OH	H	H
42	D						
43	A	rha	H	H	H	acglc	H
44	A	H	H	H	OH	Orha	H
45	A	H	H	H	H	Oglc	H
46	A	H	H	H	H	Orut	H
47	A	Me	H	glc	H	H	H
48	A	H	H	H	H	Omaglc	H
49	A	H	H	maglc	H	H	H
50	A	H	H	H	H	Orha	H
51	A	Me	H	maglc	H	H	H
52	A	rha	H	H	H	OH	H
53	A	Me	glc	H	OH	H	H

acglu = — (6″-*O*-acetyl)-*β*-D-Glucopyranoside, glc = — *β*-D-glucopyranoside, maglc-(6″-*O*-malonyl)-*β*-D-glucopyranoside, rha = — α-L-rhamnoside, rut = — 2-*O*-(α-L-rhamnoside)-*β*-D-glucopyranoside, soph = (2-*O*-*β*-D-glucopyranoside)-*β*-D-glucopyranoside.

from their sympatric parents. Patterns of phenolics from hybrids have been published elsewhere [30].

Mild oceanic climates seem to favour the establishment and development of gametophytic stands. Therefore, hybrids predominately develop in these regions. High humidity without drastic changes in temperature and little competition are necessary requirements for hybrids to establish small colonies [28, 29]. Therefore, crude drug material of Equiseti herba collected from Atlantic regions is more likely to contain higher percentages of hybrids. In contrast to other reports [31, 32], we could not detect hybrids in commerical samples of Equiseti herba, which originated mainly from Eastern Europe and China.

Intraspecific variation. Little attention has been paid to such variations in the past. For all taxa of *Equisetum*, we found developmental changes in flavonoids over the entire growing season, as well as variation due to certain environmental factors. Factors, e.g. UV irradiation and water supply, cause only quantitative differences, while the patterns of the accumulated phenolics always remained stable. In contrast, phenolics accumulated in the sterile sprouts of the plants differed depending on develo-

pmental stage and geographical origin. In *E. arvense*, for example, a decrease in all flavonols was observed during the growing season. At the same time, the proportion (per cent of total flavonoid content) of the individual compounds also varied. While the development of the total amount (per cent dry weight) of the main flavonoids quercetin 3-*O*-glucoside (**34**) and quercetin 3-*O*-(6″-*O*-malonylglucoside) (**39**) was different over several years of observation (Fig. 1), we found few differences in the proportional content (per cent of total flavonoid content) of these compounds in several years of observation (Fig. 2). Interesting was the decrease of the initially major compound, quercetin 3-*O*-(6″-*O*-malonylglucoside) (**39**) and a simultaneous increase of the non-malonylated derivative towards the end of the growing period (Fig. 2). Regarding the flavones accumulating in *E. arvense*, genkwanin 5-*O*-(6″-*O*-malonylglucoside) (**51**) was the only flavone glycoside to accumulate over the entire growing season. Most other flavones could only be detected in the Spring (Fig. 3), apart from luteolin 5-*O*-glucoside (**31**) and its 6″-*O*-malonyl derivative (**41**) which appear as major flavonoids only in the North-American/Asian chemotype.

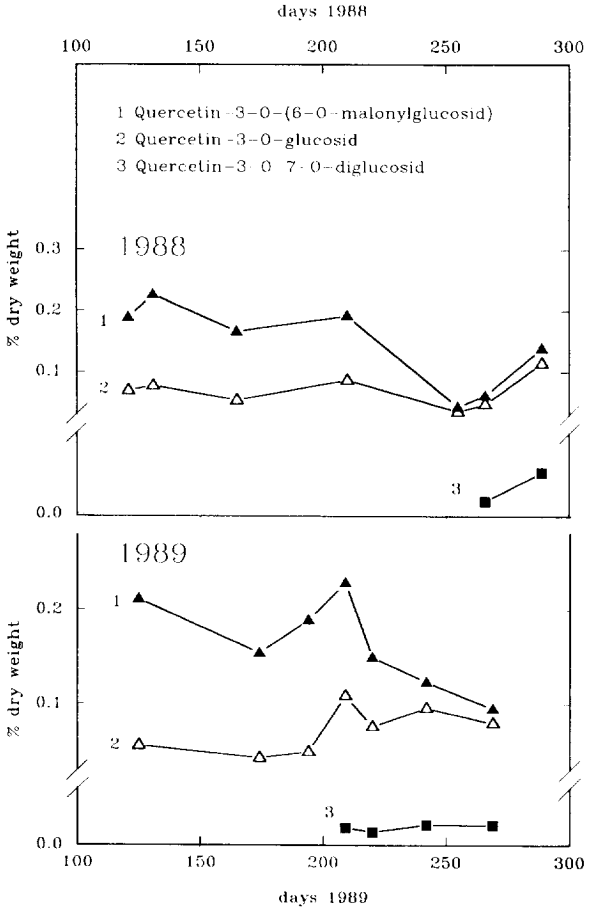

Fig. 1. Accumulation profile of quercetin glucosides (absolute content, per cent dry weight) in *Equisetum arvense* during two growing seasons.

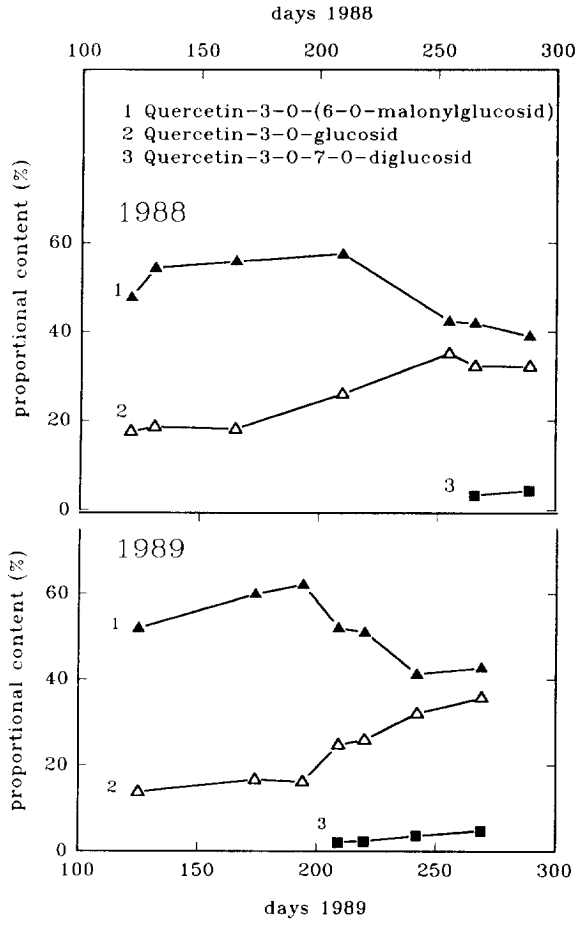

Fig. 2. Accumulataion profile of quercetin glucosides (proportional content, per cent total flavonoids) in *Equisetum arvense* during two growing seasons.

The decrease in content is even more pronounced in the accumulation profile of the caffeic acid conjugates in *E. arvense*, which could be detected in plants with very large amounts only in the early stages of plant development (Fig. 4). As these compounds are known to be active against pathogenic fungi and bacteria, there might be a function for these depsides in the plant defence system during early spring. With increasing accumulation of silica in the epidermal cells during plant development, the plants become better protected and the content of the depsides decrease.

To demonstrate the distinct patterns and amounts of accumulated phenolics, Fig. 5 shows an HPLC chromatogram of very young sprouts of *E. arvense* in contrast to the Autumn pattern shown in Fig. 6 (same plant). We found similar variations in some of the other taxa described here. For example, all species accumulating 3,7-*O*-diglycosides in the course of their development always accumulated the 3-*O*-glucosides prior to the 3,7-*O*-diglycosides (compare Fig. 1). All observed variations were found to be similar in three years of observation, while cultivating the plants together under identical conditions. Thus, the seasonal changes are predictable

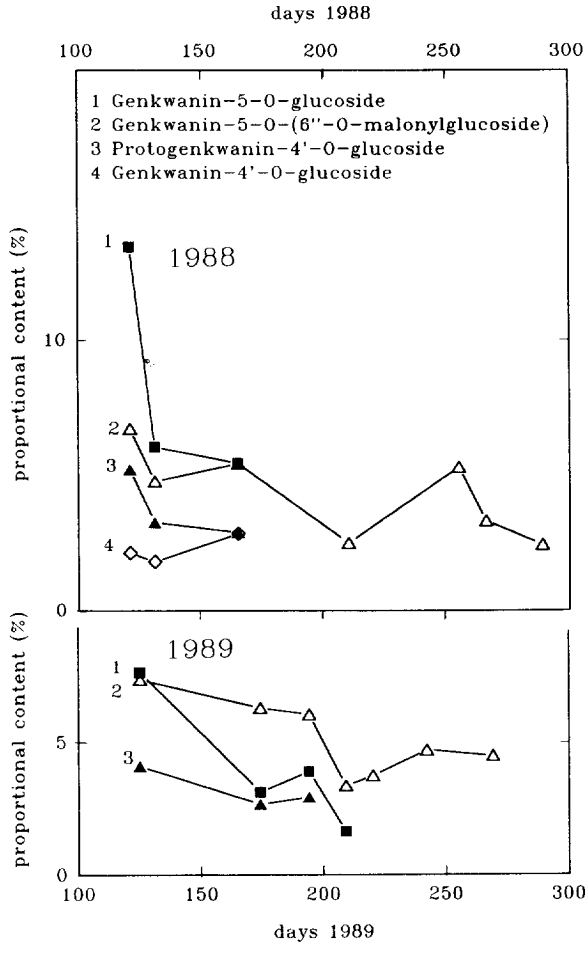

Fig. 3. Accumulation profile of flavone glucosides (proportional content, percent total flavonoids) in *Equisetum arvense* during two growing seasons.

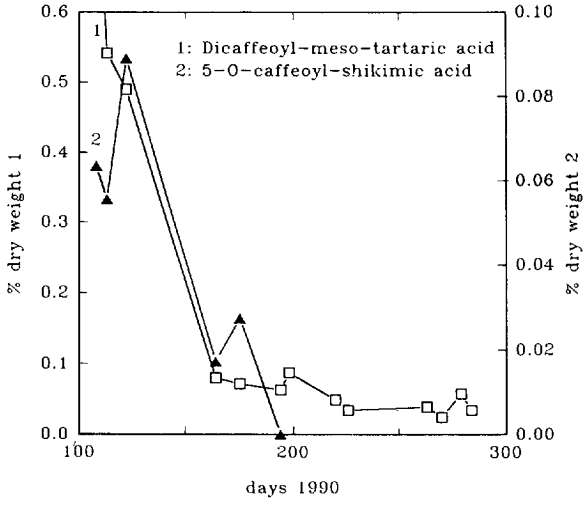

Fig. 4. Accumulation profile of caffeic acid conjugates (absolute content, per cent dry weight) in *Equisetum arvense*.

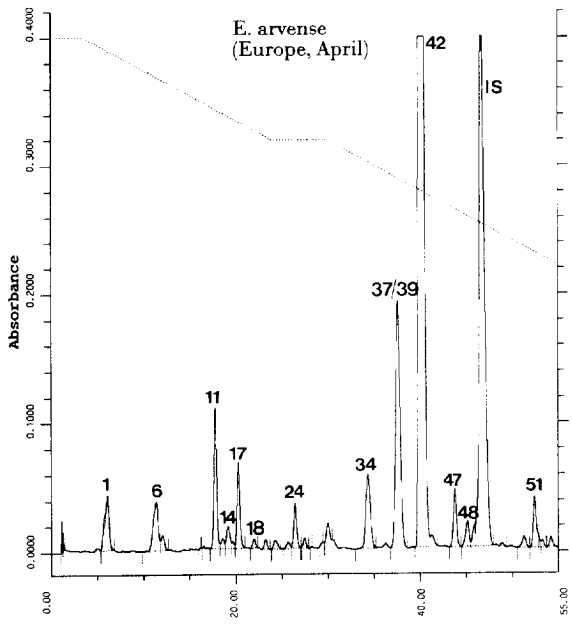

Fig. 5. HPLC trace of a typical methanolic extract from *Equisetum arvense* harvested in spring. Detection 350 nm. Peak numbers refer to Table 1 (conditions, see Experimental).

from year to year. *Equisetum arvense* appeared to be the most variable species, while others like *E. fluviatile* showed only little intraspecific variation. To avoid such intraspecific variations interfering with interspecific variations, for comparison of phenolic patterns (Table 2), the plant material for chemotaxonomic studies was harvested in late Summer or early Autumn (August–October). For quality control of crude drug material and herbal remedies containing *Equisetum*, interspecific variations in that time of the vegetation period are most important, because plants for pharmaceutical properties are collected in the wild during that time. The total flavonoid content of *E. arvense* at this time of the vegetation period varies between 0.15 and 0.3% dry weight for crude drug material from Europe and 0.2 and 0.6% dry weight for material harvested in China (calculated with the calibration data of quercetin 3-*O*-glucoside). Aglycones should be absent from crude drug material. As a lower limit for the content of total flavonoid glycosides, the equivalent to 0.2% dry weight quercetin 3-*O*-glucoside (**34**) is proposed. Compared with other suggestions for standardization [33], it is important to consider the higher contents obtained with spectrophotometric methods used in various pharmacopeias [15–18].

In the subgenus *Equisetum*, so-called monomorphic, dimorphic and semidimorphic species occur. In the monomorphic species, *E. bogotense*, *E. palustre* and *E. fluviatile*, fertile sprouts, bearing sporangia, look similar to the green, sterile sprouts; fertile sprouts (and spores) are produced over the entire growing season. The dimorphic species, *E. telmateia* and *E. arvense*, in Spring first produce unbranched, fleshy fertile stems lacking chlorophyll. They normally wither and die after spores are shed.

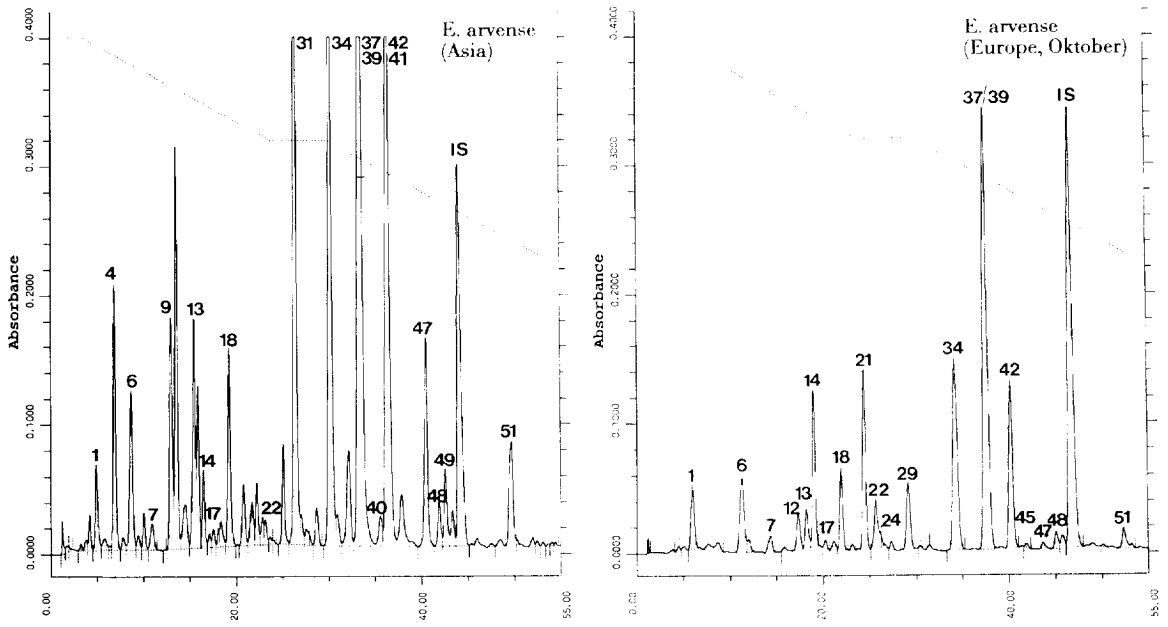

Fig. 6. HPLC trace of a typical methanolic extract from *Equisetum arvense* harvested in autumn. Detection 350 nm. Peak numbers refer to Table 2 (conditions, see Experimental).

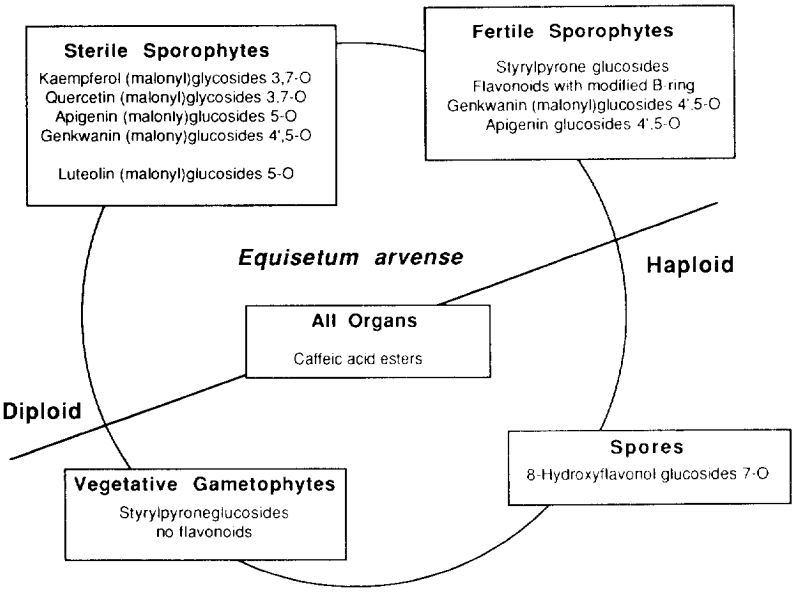

Fig. 7. Organ specific variation of phenolics in *Equisetum arvense*.

In the semidimorphic species, *E. sylvaticum* and *E. pratense*, they persist and become green and branched, and look later, during the vegetation period, like the sterile ones. Some phenolics are typical for the fertile sprouts, e.g. flavones with a modified B-ring, like protogenkwanin 4′-*O*-glucoside (**24**) and protoapigenin 4′-*O*-glucoside (**8**), as well as styrylpyrone glucosides, like equisetumpyrone (**17**). The accumulation of these compounds in the dimorphic and semidimorphic species is confined to very early developmental stages of the sterile shoots. In the monomorphic species they could always be detected in fertile sprouts and were never found in sterile ones.

The spores of all species contain remarkable amounts of gossypetin 7-*O*-glucoside (**25**) and in some cases, trace amounts of herbacetin 7-*O*-glucoside as characteristic compounds. Soon after germination, flavonoids are no longer detectable. Besides several, partly species-specific hydroxycinnamic acid esters, the gametophytes accumulate various styrylpyrone glucosides. Those compounds were also characteristic of the rhizomes of the sporo-

phytes which lack flavonoids [34]. In gametophytes there is a switch from styrylpyrone biosynthesis to flavonoid biosynthesis during sexual formation of sporophytes (Fig. 7). Preliminary results indicate that the enzyme involved in styrylpyrone biosynthesis in gametophytes is closely related to chalcone synthase. Hispidine as precursor of equisetumpyrone (**17**) was formed from caffeoyl-CoA as the only efficient substrate and two malonyl-CoA units. Hispidin has been described as an *in vitro* release product in chalcone synthase assays [35]. It could be derived also biosynthetically in higher plants by a reaction very similar to flavonoid biosynthesis from cinnamoyl-CoA derivatives and two acetate units [36]. We never detected any flavonoids in assays with crude and purified enzyme preparations derived from vegetative gametophytes; in malonyl-CoA dependent reactions only styrylpyrones were formed. That is in contrast to the biochemistry of green sporophytic tissues which are able to biosynthesize a wide variety of flavonol and flavone glycosides. A switch from the pure cinnamate pathway in vegetative gametophytes to the chalcone pathway during sexual development of sporophytes has been reported from other cryptograms [37, 38]. In *Equisetum*, however, an alternative, possibly primitive pathway is realized to attach acetate units to cinnamate precursors. In *Psilotum* psilotins (arylpyrones, glucosylated at the aromatic ring) derived from cinnamate-CoA esters and one acetate unit have been detected [39]. Further investigations will show whether there is a relationship between the enzymes involved in these biosynthetic pathways and if in *E. arvense* all enzymes involved in cinnamoyl-CoA dependent reactions accept caffeoyl-CoA as the main substrate.

EXPERIMENTAL

HPLC. Pumps, detectors, autosampler, integration software from Beckman, F.R.G. Chromatographic conditions—temp: 15° thermostated. Column: Merck Superspher RP 18(e); 4 μm; 120 × 4 mm. Guard column: Merck, F.R.G., Lichrochart RP 18 4 × 4 mm. Elution profile: A = 0,15% phosphoric acid in H_2O–MeOH 77:23 (pH = 2); B = MeOH. Isocratic: 0–3.6 min 100% A; gradient: 3.6 min. 100% A-linear-24.0 min; 80.5% A-isocratic-30 min; -linear-60 min; 51.8% A-linear-67.2 min; 100% B. Flow rate: 1.0 ml min^{-1}. Extraction and sample prepn for HPLC: After adding 10.0 ml of int. standard soln (1.0 mg ml^{-1}) 1.0 g pulverized air-dried plant material was extracted (3×) with MeOH by heating to boiling point. The resulting, cooled extracts were paper filtered. The filter paper at each step was re-extracted with the plant residue in the next step. The solvent of the combined filtrates was removed under red. pres. and the residue redissolved in 2.5 ml MeOH (ultra sonic). After adding 0.5 ml of H_2O the soln was membrane filtered and the filtrate was passed through a SEP PAK C_{18}-cartridge (Waters), which had been equilibrated with 3 ml MeOH prior to 3 ml H_2O. The flavonoids were finally quantitatively eluted with 3 ml MeOH–H_2O (85:15). The combined eluates were used for HPLC analysis. Detection and calibration: UV 330 and 350 nm. Peak purity and identity were checked by comparison of the on-line UV spectra (PDA-detctor) and co-chromatography (TLC, HPLC) with ref. substances. The calibration curves were obtained from quercetin 3-glucoside for quercetin glycosides, kaempferol 3,7-diglucoside for all kaempferol glycosides, luteolin 5-glucoside for all flavone glycosides and dicaffeoyl-*meso*-tartaric acid for all caffeic acid conjugates, using naringenin (Roth, F.R.G.) as int. standard. The method has been validated [18].

TLC. TLC was performed on cellulose plates (Merck, F.R.G.), spray reagent: diphenyl-boric acid–ethanolamine complex 1% in MeOH.

Reference compounds. All ref. substances had been isolated previously from *Equisetum* species, and their identities confirmed by spectroscopy (^1H, ^{13}C NMR, mass, UV), chemical transformation and comparison with authentic ref. material [2–14].

Plant material. Number and origin of all analysed samples: (E = Europe, NA = North-America, A = Asia, I = Iceland, A = Azores, SA = South America): *E. arvense* L.: 167; E,NA,A,I; *E. bogotense* HBK: 3; SA; *E. fluviatile* L: 29; E, NA; *E. palustre* L.: 39; E,NA; *E. pratense* Erhr.: 44; E,NA,I; *E. sylvaticum* L.: 117; E, NA *E. telmateia* Erhr.: 18; E,NA,A; crude drug material of *E. arvense* L.: 42; E,A. Voucher specimens have been deposited at the Julius-von-Sachs-Institut für Biowissenschaften, Würzburg, F.R.G.

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