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Short communication

Capillary electrophoretic separation and quantification of flavone-*O*- and *C*-glycosides in *Achillea setacea* W. et K.

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Abstract

A simple method for the rapid separation and quantification of flavone-*O*- and *C*-glycosides in *A. setacea* W. et K. by capillary zone electrophoresis (CZE) with UV detection is described. Using 25 m*M* sodium borate with 20% (v/v) of methanol (pH 9.3) as running buffer sufficient separation of the analytes was achieved within 19 min. For the quantitative determination isorhamnetin-3-*O*-rutinoside was used as internal standard. The method was successfully applied to a rapid characterisation of the flavonoid complex and a precise quantification of the single and total amount of the flavonoids in different samples of *A. setacea*.

Keywords: *Achillea setacea*; Flavone glycosides

1. Introduction

Achillea millefolium L. s.l. is a well known medicinal plant, widely used in folk medicine against gastrointestinal disorders, lack of appetite and internal as well as external inflammations [1–3]. The drug in the European market is usually collected in south-eastern parts of Europe and only rarely derives from cultivated plants. Due to that, mixtures of the very similar taxa of the *A. millefolium* group and related species, respectively, occur.

Most of the taxa belonging to the *A. millefolium* group contain flavonol- and flavone-*O*-glycosides, mainly luteolin-7-*O*-glucoside, luteolin-7-*O*-glucuronide, luteolin-7,4′-*O*-diglucoside, luteolin-4′-*O*-glucoside, apigenin-7-*O*-glucoside and rutin [4–7]. In contrast, one of these taxa, *A. setacea* W. et K., differs in the composition of the flavonoid complex with predominantly flavone-*C*-glycosides such as schaftoside, isoschaftoside and vicenin-2 [4,8,9]. Due to the possibility of allergenic reactions to sesquiterpenes occurring in *A. setacea* [10–12] this taxon should not be used as herbal medicine. For unambiguous identification of this taxon, the flavonoids as easy accessible components are very useful.

Capillary electrophoresis is a frequently used, simple and rapid separation technique [13–16]. It is employed for the determination of the flavonoids in different taxa of the *Achillea millefolium* group or samples of Millefolii Herba [5–7]. Therefore, in this paper, a simple and reliable CE method for the separation of the flavonoid complex in *A. setacea* is described. Quantification of the compounds was achieved by internal standardisation. The method was applied to the analysis of five samples of *A.*

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setacea from different regions in Austria. The total amount of flavonoids and the qualitative composition of the flavonoid pattern were compared. The established method furthermore supplies a system for the separation of the characteristic compounds of *A. setacea* from the flavonoids occurring in other taxa of the *A. millefolium* group. Thus this method gives the possibility to differentiate between *A. setacea* and other taxa of the *A. millefolium* group and to detect adulterations with this taxa in Millefolii Herba.

2. Experimental

2.1. Materials and reagents

The reference compounds apigenin (96.1%), apigenin-7-*O*-glucoside (95.3%), vitexin (100%), luteolin-7-*O*-glucoside (96.2%), orientin (98.5%), isoorientin (97.7%), rutin (96.8%), isorhamnetin-3-*O*-rutinoside (100%) and chlorogenic acid (96.0%) were purchased from Roth (Karlsruhe, Germany). Vicenin-2, schaftoside, isoschaftoside and luteolin-4′-*O*-glucoside were previously isolated in this laboratory [17,18].

Aerial parts of flowering plants of *A. setacea* were collected at different places in Austria in various years (St. Margarethen, Burgenland, harvested in the years 1995, 1991, 1990; Retz, Lower Austria, 1995; Grafenberg near Retz, Lower Austria, 1990) and air-dried at room temperature. Vouchers are deposited in the Herbarium of the Institute of Pharmacognosy, University of Vienna.

Sodium tetraborate and sodium hydroxide were supplied by Merck (Darmstadt, Germany). Methanol of analytical-reagent grade was purchased from J.T.Baker (Deventer, The Netherlands). Water used for dilution and buffer solutions was deionized, distilled and filtered through 0.8-μm cellulose acetate filters (Sartorius, Goettingen, Germany). All other chemicals were of analytical-reagent grade.

2.2. CE apparatus and conditions

All analyses were performed on a SpectraPHORESIS 1000 (SpectraPhysics, San Jose, CA, USA) capillary electrophoresis system equipped with a highspeed-scanning variable-wavelength detector and Software PC1000, Version 3.0 (ThermoSeparation, Fremont, CA, USA).

Buffer solutions were prepared from sodium tetraborate (25 m*M*) in distilled and deionized water and their pH was adjusted to 9.3 with 1 N NaOH. 20% (v/v) of methanol were added to the buffer solution. Prior to use the buffers were filtered through 0.80-μm membrane filters and degassed. All separations were carried out using an uncoated fused-silica capillary 65.5 cm (58 cm to detector)×50 μm I.D. (J&W Scientific, Folsom, CA, USA) at a voltage of 30 kV and a capillary temperature of 35 °C. Wavelength was set at 270 nm, hydrodynamic injection was performed at the anode for 3 s (1.5 p.s.i.).

At the beginning of each working day, the capillary was washed 3 min with 1 N NaOH, 3 min with 0.1 N NaOH, 5 min with water and 5 min with buffer. Washing steps for 3 min with water, for 2 min with 0.1 N NaOH, again for 2 min with water and further 5 min with running buffer were performed between the runs.

2.3. Thin layer chromatography

System 1: TLC silica gel plate (Merck, Germany), 0.25 mm. Ethylacetate–butanone–formic acid–water (50:30:10:10, v/v). System 2: TLC cellulose plates (Merck, Germany), 0.5 mm. *n*-Butanol–acetic acid–water (4:1:5, v/v), upper phase. Detection: 1% MeOH solution of diphenyl–boric acid–ethanolamine complex (=Naturstoffreagens A; Roth, Germany) and additionally with 5% EtOH solution of PEG 400. After drying the plates were controlled under UV 366 nm.

2.4. Sample preparation

The dried aerial parts of the collected plants were pulverised and homogenised. Then, 0.100 g powdered drug were extracted under reflux with 10 ml methanol–water (40:60, v/v) for 30 min. After decantation of the solvent, the extraction was repeated with 10 ml methanol–water (40:60, v/v) for 30 min. Then, 1.00 ml of the internal standard solution (3.00 mg isorhamnetin-3-*O*-rutinoside in 10.00 ml methanol–water (80:20, v/v)) was added to the combined filtrates. The solution was evaporated

to dryness under reduced pressure, the residue dissolved in 2.50 ml methanol–water (80:20, v/v) and filtered through 1.0 μm filters (Millipore, Dublin, Ireland) prior to injection.

2.5. Calibration graphs, recovery experiments

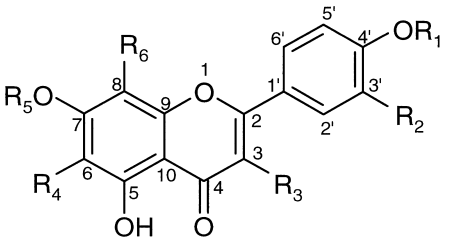
Stock solutions of the flavonoids luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, apigenin, vitexin and isorhamnetin-3-*O*-rutinoside were prepared at a concentration of 1 mg/ml methanol–water (80:20, v/v). These were further diluted and combined to obtain five various reference solutions in the concentration range of 20–200 μg/ml containing isorhamnetin-3-*O*-rutinoside as internal standard at the fixed concentration of 120 μg/ml. These solutions were filtered prior to injection through 1.0 μm filters. Three injections were made for each standard solution. The calibration curves were established between the concentrations of the analytes and the corresponding ratios of the corrected peak areas (area to migration time) of the analytes to the internal standard. For the determination of the recovery three extractions of one sample of *A. setacea* were performed. Two of the extract solutions were spiked with 50 μg (1.00 ml of standard solutions of 1.00 mg in 20.00 ml methanol–water (80:20, v/v)) of luteolin-7-*O*-glucoside and apigenin-7-*O*-glucoside, respectively. The spiked samples and the unspiked sample were analysed each for three times.

3. Results and discussion

3.1. Separation and identification of the flavonoids by CE

Analyses concerning the composition of the flavonoid complex in *A. setacea* were performed in earlier studies: Using paper chromatography the main flavonoids were isolated, identified and the infraspecific variability investigated [4,8,9].

For the analyses of the flavonoids employing CE, variation of the buffer system resulted in an optimum separation of the analytes using 25 m*M* sodium tetraborate with 20% (v/v) of methanol (pH 9.3) as running buffer. Identification of the component peaks was performed by comparison of the UV spectra with reference substances and co-migration with authentic substances using 25 m*M* sodium tetraborate with 20% (v/v) methanol at different pH values (pH 9.0, 9.3, 9.7) as buffer. Furthermore, the main flavonoids in *A. setacea* were identified by re-chromatography with authentic compounds in two different TLC systems. Eight previously known compounds in *A. setacea*—vicenin-2, isoschaftoside, schaftoside, vitexin, orientin, isoorientin, rutin and luteolin-7-*O*-glucoside—were identified. Additionally apigenin-7-*O*-glucoside, apigenin and luteolin-4′-*O*-glucoside (Fig. 1) were unambiguously assigned and three flavonoids, the structure elucidation of which is in progress, were defined as aset X1, aset X2 and aset X3. Besides the flavonoids, chlorogenic acid was identified in the electropherograms of the methanolic extracts. Further caffeoylquinic acid derivatives were



Name	R1	R2	R3	R4	R5	R6
apigenin-7-O-glucoside	H	H	H	H	glu	H
luteolin-7-O-glucoside	H	OH	H	H	glu	H
vicenin-2	H	H	H	glu	H	glu
schaftoside	H	H	H	glu	H	ara
isoschaftoside	H	H	H	ara	H	glu
rutin	H	OH	-O-rut	H	H	H
luteolin-4'-O-glucoside	glu	OH	H	H	H	H
isoorientin	H	OH	H	glu	H	H
vitexin	H	H	H	H	H	glu
orientin	H	OH	H	H	H	glu
apigenin	H	H	H	H	H	H

glu: glucose, ara: arabinose, -O-rut: -O-rutinose

Fig. 1. Structures of the investigated flavonoids.

detected, the structures of those are not completely elucidated yet.

All the flavonoids were sufficiently separated within 19 min, except orientin that co-migrated with aset X2. A typical electropherogram of an extract of a sample of *A. setacea* is shown in Fig. 2.

3.2. Calibration

For the quantification calibration curves were established using isorhamnetin-3-*O*-rutinoside as internal standard. A linear correlation from 20 to 200 μg/ml was found for luteolin-7-*O*-glucoside, vitexin,

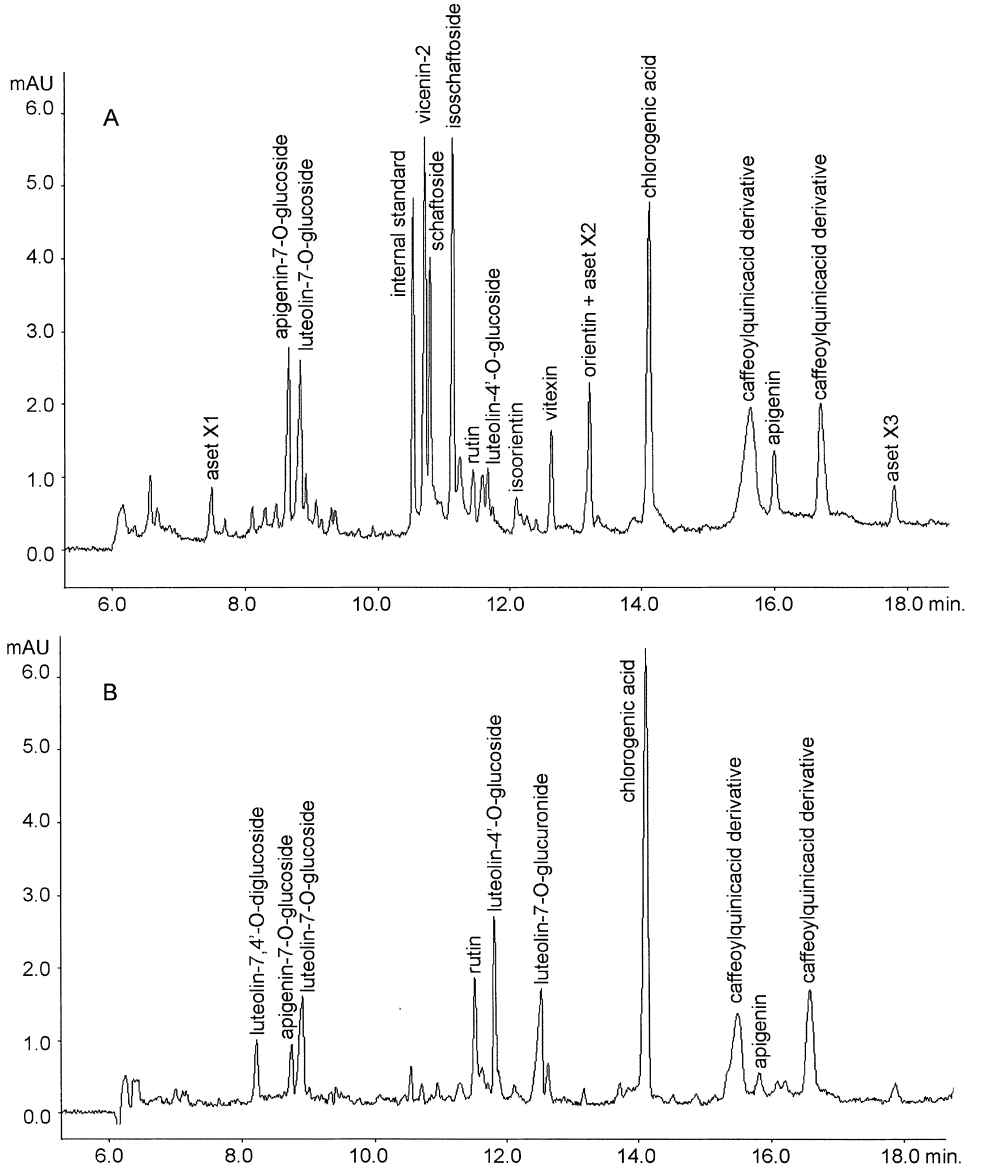


Fig. 2. (A) Typical electropherogram of an extract of a sample of *A. setacea*. (B) Electropherogram of an extract of a sample of *A. pratensis*. Running buffer: 25 m*M* sodium tetraborate containing 20% (v/v) of methanol (pH 9.3); capillary: 65.5 cm×50 μm I.D.; separation voltage: 30 kV; column temperature: 35 °C; UV detection: 275 nm.

Table 1
Regression equations and correlation coefficients

Compound	Regression equation[a]	Correlation coefficient R^2
Apigenin	$y = 1.3776x + 0.0114$	0.992
Apigenin-7-*O*-glucoside	$y = 0.9806x + 0.0025$	0.996
Luteolin-7-*O*-glucoside	$y = 1.1420x + 0.0015$	0.997
Vitexin	$y = 1.1714x - 0.0009$	0.999

[a] In the regression equation: x = the concentration of the analyte (mg/ml); y = the relative peak area of the analyte × concentration of the internal standard (mg/ml)/relative peak area of the internal standard.

apigenin-7-*O*-glucoside and apigenin. The regression equations and correlation coefficients are given in Table 1. For the quantification of aset X1, aset X2 (in combination with orientin), aset X3 and rutin a calibration factor of 1 was used. The luteolin derivatives luteolin-4′-*O*-glucoside and isoorientin were calculated using the calibration curve of luteolin-7-*O*-glucoside. The calibration factors for the commercially not available apigenin derivatives schaftoside, isoschaftoside and vicenin-2 were calculated as follows: calibration factor of *v*itexin/molecular mass of *v*itexin × molecular mass of the component.

The detection limits of the CE method corresponding to a signal-to-noise ratio of three determined for apigenin-7-*O*-glucoside and luteolin-7-*O*-glucoside were 9.1 and 9.6 μg/ml (corresponding to a content of 0.023 and 0.024% in the drug).

To verify the precision of the proposed CE method one sample of *A. setacea* was extracted five times. All five obtained sample solutions were analysed five times each. The run-to-run reproducibilities ($n = 5$) of the single and total flavonoid contents in one sample solution varied between 1.50 and 4.44%. Concerning the reproducibility of the five performed extractions, the relative standard deviation for the single components was usually less than 5%. The total amount of flavonoids varied very little with a relative standard deviation of 3.31%. In conclusion, the method proved to be very reproducible.

Reproducibility of the migration time was also satisfactory with coefficients of variation ranging from 0.34 to 0.45% for five repeated injections.

The accuracy of the method was examined by recovery studies conducted as described in Section 2.5. The recovery of luteolin-7-*O*-glucoside was 98.3% with a relative standard deviation of 5.03% and of apigenin-7-*O*-glucoside 99.6% with a relative standard deviation of 2.63%.

3.3. Quantification of flavonoids in *A. setacea*

The established CE method was applied to the quantification of the flavonoids in five different samples of *A. setacea*. For each sample two extractions were performed and the extract solutions analysed twice. The mean values are given in Table 2. The total amounts of the flavonoids varied within a small range from 1.32 to 1.56% except sample aset 2 with 2.15%. The flavonoid patterns were very similar except small differences in the single concentrations. Vicenin-2, isoschaftoside and schaftoside were the main compounds besides apigenin-7-*O*-glucoside or luteolin-7-*O*-glucoside. Isoorientin, apigenin and aset X3 were detected in all samples, but in very small amounts close to the detection limit. Concerning the *A. millefolium* group, vicenin-2, isoschaftoside and schaftoside can be defined as the substances characteristic for *A. setacea*, as they were not detected in the other taxa of the *A. millefolium* group or just in trace amounts [5–7]. The main flavonoids of the other taxa such as luteolin-7-*O*-glucoside, luteolin-7-*O*-glucuronide, luteolin-7,4′-*O*-diglucoside, luteolin-4′-*O*-glucoside, apigenin-7-*O*-glucoside, apigenin and rutin do not overlap with the three marker flavonoids as can be seen from an electropherogram of *A. pratensis* Saukel & Länger as a representative of the *A. millefolium* group in Fig. 2. So this CE method offers a simple way to differentiate between *A. setacea* and other taxa of the *A. millefolium* group based on the flavonoids as easy accessible components.

4. Conclusions

The CE method described in this study using 25 m*M* sodium borate with 20% (v/v) methanol (pH 9.3) as running buffer has proven to be adequate for the separation of the flavonoids in *A. setacea* in less than 20 min. Precise quantification can be achieved using isorhamnetin-3-*O*-rutinoside as internal standard. Due to the homogeneity of the flavonoid pattern in *A. setacea*, the method furthermore sup-

Table 2
Results of the determination of the flavonoids in five different samples of *A. setacea*

Compound	Aset 1	Aset 2	Aset 3	Aset 4	Aset 5
Aset X1	0.134±0.003	0.085±0.002	0.113±0.003	0.083±0.003	0.106±0.002
Apigenin-7-*O*-glu[a]	0.168±0.004	0.234±0.011	0.191±0.001	0.150±0.001	0.199±0.008
Luteolin-7-*O*-glu	0.131±0.000	0.179±0.011	0.149±0.001	0.123±0.002	0.175±0.014
Vicenin-2	0.248±0.011	0.437±0.021	0.216±0.003	0.232±0.002	0.227±0.001
Schaftoside	0.135±0.001	0.272±0.008	0.125±0.000	0.136±0.000	0.119±0.000
Isoschaftoside	0.221±0.000	0.448±0.005	0.188±0.005	0.195±0.001	0.200±0.009
Rutin	0.170±0.008	0.080±0.002	0.150±0.003	0.163±0.005	0.135±0.001
Luteolin-4′-*O*-glu	0.099±0.002	0.111±0.004	0.119±0.000	0.064±0.012	0.074±0.003
Isoorientin	<0.05[c]	<0.05	<0.05	<0.05	<0.05
Vitexin	0.061±0.002	0.078±0.003	0.052±0.003	0.052±0.001	0.053±0.001
Aset X2+orientin[b]	0.069±0.003	0.122±0.059	0.106±0.009	<0.05	0.156±0.009
Apigenin	<0.05	<0.05	<0.05	<0.05	<0.05
Aset X3	<0.05	<0.05	0.060±0.014	<0.05	0.084±0.003
Total amount	1.530±0.015	2.150±0.079	1.510±0.021	1.316±0.016	1.563±0.049

The given values represent the mean of two independent extractions, each sample solution analysed twice.
[a] glu: glucose.
[b] Calculated as aset X2 using a calibration factor of 1.
[c] Compound detected, but under limit of quantification.

plies a system for the differentiation of *A. setacea* from other taxa of the *Achillea millefolium* group.

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