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Analysis of flavonoids in *Achillea nobilis* L. by capillary electrophoresis

Elke Marchart, Almut Hattenberger, Liselotte Krenn, Brigitte Kopp*

Institute of Pharmacognosy, PharmaCenterVienna, University of Vienna, Althanstraße 14, A-1090 Vienna, Austria

Abstract

The separation and quantification of the flavonoids occurring in *Achillea nobilis* L. by a CE-method is described. Using 20 mM sodium borate at pH 9.5 with 20 % of methanol as buffer, the flavonoids were sufficiently separated within 11 minutes. Kaempferol-7-O-neohesperidoside was used as internal standard for the quantification. Analysis of six different samples showed the flavon-C-glycosides isoorientin, orientin and vitexin as the characteristic main compounds. The contents of flavon-C-glycosides determined by this CE-method correlated with the results achieved by a spectrophotometric determination.

Keywords

Capillary electrophoresis; flavonoids; C-glycosylflavones; *Achillea nobilis* L.; yarrow

Introduction

Yarrow is a well known medicinal plant used for gastrointestinal disorders, lack of appetite and against internal as well as external inflammations because of the antiphlogistic, spasmolytic and antimicrobial activities [1-3]. The European Pharmacopoeia demands *Achillea millefolium* L. as source for Millefolii herba [4]. The drug in the European market is usually collected in south-eastern parts of Europe. Due to that, adulterations with related species like *Achillea nobilis* L., the

morphological features of which are very similar, can occur [5-7]. One possibility to differentiate between the species of the *A. millefolium* group and *A. nobilis* are the differences in the flavonoid pattern: while species of the *A. millefolium* aggregate contain flavon- and flavonol-O-glycosides such as luteolin-7-O-glucoside, luteolin-7-O-glucuronide, luteolin-7,4'-O-diglucoside, luteolin-4'-O-glucoside, apigenin-7-O-glucoside and rutin, the main compounds in *A. nobilis* are flavon-C-glycosides like isoorientin, orientin and vitexin [8-14]. Therefore a method for the analysis of the flavonoid complex in *A. nobilis* was developed. Capillary electrophoresis was used for that purpose and in this paper a simple and reliable method for the separation of the flavonoids in *A. nobilis* is described. Quantification of the compounds was achieved by internal standardisation. The established method was applied to the analysis of several samples of *A. nobilis* to confirm the homogeneity of the flavonoid pattern and the suitability of the method. Furthermore the content of the flavon-C-glycosides in *A. nobilis* was determined using a spectrophotometric assay and the results were compared with the amounts achieved by the CE-method.

Experimental

Materials and Reagents

The reference compounds luteolin-7-O-glucoside, apigenin-7-O-glucoside, kaempferol-7-O-neohesperidoside, isoorientin, orientin, vitexin and chlorogenic acid were purchased from Roth (Karlsruhe, Germany, purity ≥ 90 %). Isoschaftoside, luteolin-4'-O-glucoside, luteolin-7-O-glucuronide, luteolin-6-C-apiofuranosyl-(1'''→2'')-glucoside, quercetin-3-O-arabinosyl-(1'''→6'')-glucoside, quercetin-3-O-methylether and quercetin-3-O-methylether-7-O-glucoside were previously isolated in this laboratory [12].

Six samples of aerial parts of flowering plants of *A. nobilis* were collected (Korneuburg, 1988; Vintschgau, Italy, 1992; garden of The Josefinum 1991, 1995; garden of the Institute of Pharmacognosy, 1996, 1998) 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). Water used for dilution and buffer solutions was deionized, distilled and filtered through 0.8 µm cellulose acetate filters (Sartorius, Goettingen, Germany). Methanol of analytical-reagent grade was purchased from J.T.Baker (Deventer, The Netherlands). All other chemicals were of analytical-reagent grade.

CE Apparatus and conditions

All analyses were performed on a SpectraPHORESIS 1000 (SpectraPhysics Inc., San Jose, CA, USA) capillary electrophoresis system equipped with a highspeed-scanning variable-wavelength detector and Software PC1000, Version 3.0 (ThermoSeparationProducts, Fremont, CA, USA). Buffer solutions were prepared from sodium tetraborate (20 mM) in distilled and deionized water and their pH was adjusted to 9.5 with 1N NaOH. 20% (v/v) of methanol were added to the buffer solution. Prior to use the buffers were filtered through 0.8 µm membrane filters and degassed. All separations were carried out using an uncoated fused-silica capillary 65.5 cm (58 cm to detector) x 50 µm I.D. (J&W Scientific, Folsom, CA, USA) at a voltage of 30 kV (resulting current of approximately 13.5 µA) and a capillary temperature of 35°C. Wavelength was set at 270 nm, hydrodynamic injection was performed at the anode for 1 second (1.5 p.s.i.).

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

Sample preparation for CE

The dried aerial parts of the collected plants were pulverised and homogenised. 0.100 g powdered drug were extracted under reflux with 10 ml 40% methanol for 30 minutes. After decantation of the solvent, the extraction was repeated twice with 10 ml 40% methanol for 10 minutes. 1.0 ml of the internal standard solution (5.40 mg kaempferol-7-O-neohesperidoside in 10.0 ml 80%

methanol) was added to the combined filtrates. The solution was evaporated to dryness under reduced pressure, the residue dissolved in 2.0 ml 40% methanol and filtered through 1.0 µm filters (Millipore, Ireland) prior to injection.

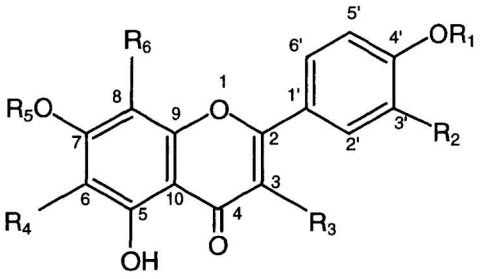
Calibration graphs

Stock solutions of luteolin-7-O-glucoside, isoorientin, orientin, vitexin and kaempferol-7-O-neohesperidoside were prepared at a concentration of 1 mg/ml 80% methanol. These were further diluted and combined to obtain four various reference solutions in the concentration range of 35 - 200 µg/ml containing kaempferol-7-O-neohesperidoside as internal standard at the fixed concentration of 270 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.

Spectrophotometric assay

For the spectrophotometric determination 0.350 g powdered drug were extracted with 10 ml acetone and 1 ml hydrochloric acid for 30 minutes under reflux. After decantation of the solvent the extraction was repeated twice with 10 ml acetone for 10 minutes. The combined filtrates were diluted with acetone to 50.0 ml. 20.0 ml of this solution were mixed with 40 ml of water and washed 3 times with 30 ml portions of hexane. The aqueous phase was then partitioned against ethylacetate (3 x 30 ml) and the combined ethylacetate fractions were washed twice with 50 ml water, each. The washing solutions and the aqueous phase containing the flavon-C-glycosides were combined and diluted with acetone to 250.0 ml (stock solution). 50.0 ml of this stock solution were evaporated to dryness under reduced pressure and the residue dissolved in 11.0 ml of a mixture of methanol/acetic acid (1+10). 10.0 ml of a reagent consisting of 2.5 g boric acid and 2.0 g oxalic acid in 100.0 ml anhydrous formic acid were added and the solution

diluted to 25.0 ml with anhydrous acetic acid (solution 1). For the reference solution the residue of further 50 ml of the stock solution was dissolved in in the same way. 10.0 ml anhydrous formic acid were added and the solution diluted to 25.0 ml with anhydrous acetic acid (solution 2). After 30 minutes the absorption of solution 1 was measured against solution 2 at 405 nm. The amount of the flavon-C-glycosides was calculated as vitexin using the specific absorbance of 617. Absorbances were measured on a Beckman DU 640 spectrophotometer.



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
isoschaftoside	H	H	H	ara	H	glu
luteolin-4'-O-glucoside	glu	OH	H	H	H	H
isoorientin	H	OH	H	glu	H	H
luteolin-7-O-glucuronide	H	OH	H	H	glur	H
orientin	H	OH	H	H	H	glu
vitexin	H	H	H	H	H	glu
luteolin-6-C-apiofuranosyl-(1'''→2'')-glucoside	H	OH	H	apiglu	H	H
quercetin-3-O-methylether	H	OH	O-Me	H	H	H
quercetin-3-O-methylether-7-O-glucoside	H	OH	O-Me	H	glu	H
quercetin-3-O-arabinosyl-(1'''→6'')-glucoside	H	OH	O-araglu	H	H	H

glu: glucose; ara: arabinose; glur: glucuronide; apiglu: apiofuranosyl-(1'''→2'')-glucoside; O-Me: methoxyl; O-araglu: O-arabinosyl-(1'''→6'')-glucoside

Fig. 1. Structures of the flavonoids in *A. nobilis* [8,12-14]

Results and discussion

Separation and identification of the flavonoids by CE

For the characterisation of the flavonoid pattern and for the determination of the amount of these compounds in plant material of *A. nobilis* a CE-method was developed. Using 20 mM sodium tetraborate (pH 9.5) with 20 % methanol added sufficient separation of the flavonoids, which were previously described for *A. nobilis* (Fig. 1) [8,12-14] was achieved within 11 minutes.

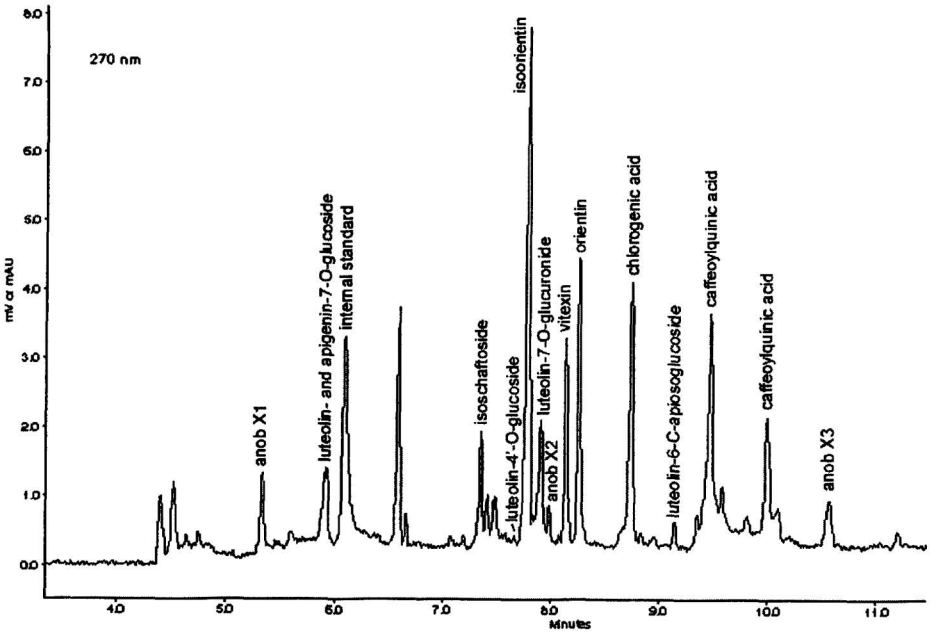


Fig. 2. Electropherogram of an extract of *A. nobilis*. Separation conditions: capillary temperature 35°C; voltage 30 kV; electrolyte buffer 20 mM sodium tetraborate containing 20 % methanol (pH 9.5); UV detection at 270 nm.

Identification of the compounds was performed by comparison of the UV-spectra with reference substances and co-migration with identical substances. Additionally to the compounds described in *A. nobilis*, three flavonoids, the structure elucidation of which is in progress, were defined as anob X1, anob X2 and anob X3. Fig. 2 shows an electropherogram of an extract of *A. nobilis*. All the flavonoids were sufficiently separated, except apigenin-7-O-glucoside and luteolin-7-O-glucoside, which co-migrated. Furthermore chlorogenic acid was detected in all samples besides some caffeoylquinicacid derivatives, the structures of those are not completely elucidated yet.

Calibration

For the quantification calibration curves were established using kaempferol-7-O-neohesperidoside as internal standard. A linear correlation from 35 to 200 µg/ml was found for luteolin-7-O-glucoside, isoorientin, vitexin and orientin. The regression equations of the calibration curves were calulated as $y_{orientin} = 1.6291x - 0.0128$ ($R^2 = 0.989$); $y_{isoorientin} = 1.256x + 0.0041$ ($R^2 = 0.997$); $y_{vitexin} = 1.4028x - 0.0030$ ($R^2 = 0.995$); $y_{luteolin\text{-}7\text{-}O\text{-}glucoside} = 1.0345x - 0.0016$ ($R^2 = 0.991$). For the quantification of anob X1, anob X2 and anob X3 a calibration factor of 1 was used, for luteolin-4'-O-glucoside the one of luteolin-7-O-glucuronide. The co-migrating compounds apigenin-7-O-glucoside and luteolin-7-O-glucoside were calculated together using the calibration curve of luteolin-7-O-glucoside. The calibration factor for luteolin-7-O-glucuronide was calculated: $\frac{\text{Calibration factor}_{\text{luteolin-7-O-glucoside}} * \text{mw}_{\text{luteolin-7-O-glucuronide}}}{\text{mw}_{\text{luteolin-7-O-glucoside}}}$

For luteolin-6-C-apiofuranosyl-(1'''→2'')-glucoside and isoschaftoside the calculation of the calibration factor was performed by analogy using isoorientin and vitexin, respectively. The limits of detection were 10 µg/ml determined for luteolin-7-O-glucoside and isoorientin (corresponding to 0.02 % flavonoid in drug) at a signal to noise ratio of 3:1. The limit of quantitation was set 0.07 % according to the lowest concentrations of the calibration curves.

To verify the precision of the proposed CE-method one sample of *A. nobilis* was

extracted 5 times. The 5 sample solutions were analysed each for 3 times. For the total amount of flavonoids the coefficient of variation was 2.08 %, for the single components 1.35 to 6.81 % (Tab. 1). These results confirmed the good reproducibility of the method.

	extraction							
	1	2	3	4	5	av	sd	rsd
anob X1	< 0.07	< 0.07	< 0.07	< 0.07	< 0.07	< 0.07	-	-
luteolin-, apigenin-7-O-glu	0.112	0.120	0.122	0.117	0.103	0.115	0.008	6.81
isoschaftoside	< 0.07	< 0.07	< 0.07	< 0.07	< 0.07	< 0.07	-	-
isoorientin	0.418	0.430	0.431	0.432	0.430	0.428	0.006	1.35
vitexin	0.225	0.224	0.232	0.232	0.232	0.229	0.004	1.78
orientin	0.203	0.214	0.225	0.214	0.200	0.211	0.010	4.79
luteolin-6-C-apiglu	< 0.07	< 0.07	< 0.07	< 0.07	< 0.07	< 0.07	-	-
anob X3	< 0.07	< 0.07	< 0.07	< 0.07	< 0.07	< 0.07	-	-
total amount	0.958	0.988	1.010	0.995	0.965	0.983	0.021	2.18

av: average; sd: standard deviation; rsd: relative standard deviation; glu: glucoside; apiglu: apiofuranosyl-(1'''→2'')-glucoside; < 0.07 %: compound detected, but under limit of quantitation

Tab. 1. Reproducibility of the CE-method: 5 extracts of one sample were each analysed 5 times. Mean values of the flavonoid concentrations (%) are given in the table

Determination of the flavonoids in different drug samples

Six different samples of *A. nobilis* were analysed to characterise the flavonoid pattern and to determine the homogeneity of the flavonoid complex in this taxon. Each sample was extracted twice and analysed at least three times. The total flavonoid content of the samples varied from 0.98 to 1.81 % (Tab. 2). Isoorientin was the main compound in the analysed samples, except in one sample. High amounts were also detected of vitexin, orientin, luteolin-7-O-glucuronide and the sum of luteolin- and apigenin-7-O-glucoside, respectively. Quercetin-3-O-arabinoglucoside, quercetin-3-

O-methylether and quercetin-3-O-methylether-7-O-glucoside were not detected in the analysed samples. Only two samples contained luteolin-4'-O-glucoside.

The characteristic flavonoids for *A. nobilis* are the flavon-C-glycosides orientin, isoorientin and vitexin, occurring in high amounts. In taxa of the *A. millefolium* group these were not detected or just in trace amounts. The main flavonoids in the *A. millefolium* group are luteolin-7-O-glucoside, luteolin-7-O-glucuronide, luteolin-7,4'-O-diglucoside, luteolin-4'-O-glucoside, apigenin-7-O-glucoside, apigenin and rutin or vicenin-2, schaftoside and isoschaftoside for *A. setacea* [8-11,15]. These compounds do not overlap with the 3 marker flavonoids of *A. nobilis*. So this CE-method allows to differentiate between *A. nobilis* and taxa of the *A. millefolium* group based on the flavonoids as easily accessible components.

	anob 1	anob 2	anob 3	anob 4	anob 5	anob 6
anob X1	0.187	0.137	0.075	< 0.07	< 0.07	< 0.07
luteolin-, apigenin-7-O-glu	0.272	0.464	0.253	0.115	0.126	0.170
isoschaftoside	0.137	0.078	0.099	< 0.07	< 0.07	< 0.07
luteolin-4'-O-glu	n.d.	n.d.	n.d.	n.d.	0.181	0.234
isoorientin	0.547	0.271	0.272	0.428	0.214	0.232
anob X2	< 0.07	0.070	< 0.07	n.d.	n.d.	0.070
luteolin-7-O-glur	0.240	0.256	0.115	n.d.	0.082	0.123
vitexin	0.170	0.150	0.104	0.229	0.100	0.124
orientin	0.257	0.132	0.129	0.211	0.129	0.118
luteolin-6-C-apiglu	< 0.07	< 0.07	< 0.07	< 0.07	< 0.07	< 0.07
anob X3	< 0.07	< 0.07	< 0.07	< 0.07	0.228	0.146
total amount	1.810	1.558	1.047	0.983	1.060	1.217

glu: glucoside; glur: glucuronide; apiglu: apiofuranosyl-(1'''→2'')-glucoside; n.d.: not detected; < 0.07 %: compound detected, but under limit of quantitation

Tab. 2. Concentration (%) of 11 flavonoids in different samples of *A. nobilis*. Each value represents mean of 2 separate extractions, each analysed three times.

Spectrophotometric determination

The results achieved by the new CE-method were compared to a photometric determination of flavonoids according to Glasl [16]. The method involves a complexation with an oxalic-borate reagent under anhydrous conditions for the C-glycosides and the determination of the O-glycosides as aluminium chelates of their aglyca. The author underlines that for samples with equal or higher amounts of flavon-O-glycosides besides flavonol-O-glycosides the results are not precise. In *A. nobilis* mainly flavon-C-glycosides besides flavon-O-glycosides and only small amounts of flavonol-O-glycosides occur. Due to that only the amounts of flavon-C-glycosides as the main and characteristic compounds in *A. nobilis* after separation from the O-glycosides were determined spectrophotometrically. For the calculation of the C-glycosides the specific absorbance of vitexin was used as the flavon-C-glycosides are not hydrolysed under acidic conditions.

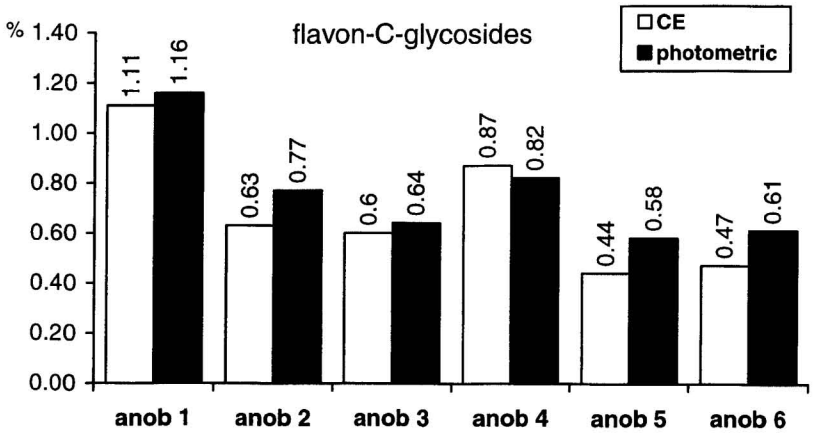


Fig. 3. Amounts of flavon-C-glycosides in samples of *A. nobilis* determined spectrophotometrically (black) in comparison to the contents of flavon-C-glycosides obtained with capillary electrophoresis (white).

The results of the determination of the flavon-C-glycosides by the photometric assay and by the CE-method correlated well. This goes in accordance with the quantification of the flavon-C-glycosides occurring in Passiflorae herba using the photometric determination of the European Pharmacopoeia and a CE-method [17].

Conclusion

The established CE-method allows a rapid characterisation of the flavonoid pattern of *A. nobilis* in less than 11 minutes using 20 mM sodium borate with 20% (v/v) methanol (pH 9.5) as running buffer. Using kaempferol-7-O-neohesperidoside as internal standard good reproducibility is obtained. The method was applied to the analysis of six different drug samples. Due to the homogeneity of the flavonoid pattern in *A. nobilis* this method gives the possibility to differentiate between *A. nobilis* and taxa of the *A. millefolium* group. Besides, the content of flavon-C-glycosides was determined applying a spectrophotometric assay. The satisfactory correlation of the results showed the suitability of the new CE-method.

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