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Short communication

Analysis of rare flavonoid C-glycosides in *Celtis australis* L. by micellar electrokinetic chromatography

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ABSTRACT

This manuscript reports on the first analytical procedure for the determination of flavonoids in *Celtis australis*. The capillary electrophoretic separation of 8 compounds, most of them flavone C-glycosides, was possible using a borax buffer with pH 9.0, which contained 25 mM SDS as detergent and 7.5% of n-butanol as organic modifier. Method validation revealed that the developed assay is repeatable ($\sigma_{rel} \leq 4.0\%$), precise (inter-day $\sigma_{rel} \leq 6.7\%$, intra-day $\sigma_{rel} \leq 3.9\%$), accurate (recovery rates from 96.8 to 102.3%), sensitive (LOD: 2.2–1.6 μg/ml) and linear ($R^2 \geq 0.9996$) within the tested concentration range. The quantitative analysis of several *C. australis* samples showed that isovitexin is the most abundant representative (0.06–0.09%), at a rather uniform content of total flavonoids of approx. 0.3% in all specimens.

1. Introduction

Celtis australis L., an up to 25 m high tree, is frequently found in southern Europe and western Asia [1]. Known as nettle tree or European hackberry this species was previously placed in the Ulmaceae family, but recent molecular and phytochemical data supported its re-classification to the Cannabaceae [2]. Decoctions of young *Celtis* twigs are a traditional remedy in Europe to treat dysentery and colds [3], but the chemical constituents in the plant remain widely unknown. Tannins have been isolated from the bark [4], and only recently two manuscripts reported on the occurrence of rare vitexin and isovitexin glycosides (1–4; Fig. 1) in the leaves [5,6]. These compounds might not only serve as chemosystematic markers but also explain the health benefits of hackberry, as anti-oxidative, spasmolytic and cell-protective activities of structurally related compounds have been described [7–9].

No analytical procedure has been reported for the investigation of *C. australis* so far, and because of the previously described successful separation of vitexin derivatives by CE, we considered this approach a promising one for *Celtis* too [10,11].

2. Materials and methods

2.1. Standards, plant material and reagents

Reference compounds (2″-α-L-rhamnopyranosyl-7-*O*-methylvitexin (1); cytisoside (2); and 2″-α-L-rhamnopyranosyl-vitexin (4)) were isolated from methanolic *C. australis* leaf extracts as described previously [5,6]. Isovitexin (3) was purchased from Roth (Karlsruhe, Germany). Purity (≥95%) and identity of the standards were assured by chromatographic means (HPLC) as well as MS and NMR-data. Plant material for quantitative studies (CA-1 to CA-3) was collected in southern Tyrol 2008 and 2009; its correct identification was assured by one of the authors (C. Zidorn). Voucher specimens are deposited at the herbarium of the Institute of Pharmacy, University of Innsbruck, Austria.

All solvents (water, methanol, n-butanol) and reagents (sodium tetraborate, sodium dodecylsulfate, phosphoric acid, sodium hydroxide) were of p.a. or HPLC grade and purchased from Merck (Darmstadt, Germany).

2.2. Sample preparation

Plant material (1.00 g of powdered *C. australis* leaves) was extracted 3-fold with 3 ml of 20% aqueous methanol by sonication at room temperature (10 min each). After centrifugation (4000 rpm, 5 min) the clear supernatants were combined in a 10 ml volumetric flask. Prior to analysis the flask was filled up to the final volume with extraction solvent, and the samples were membrane filtered (0.45 μm Phenex RC, Phenomenex, Torrance, CA, USA).

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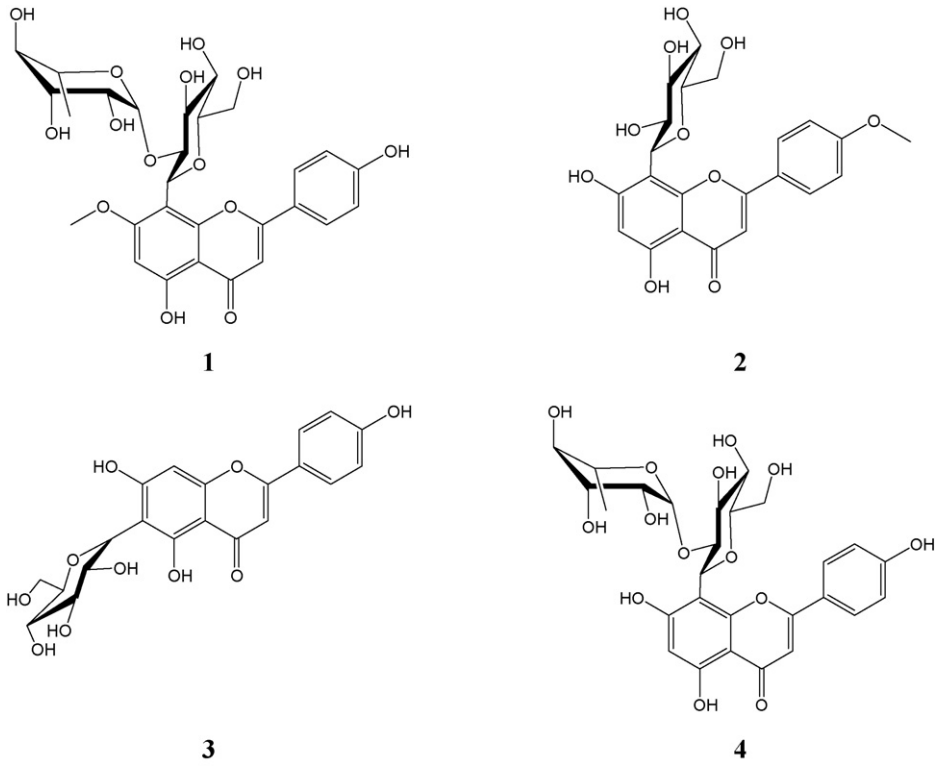

Fig. 1. Structures of the assigned compounds in *Celtis australis*.

2.3. Analytical conditions

An Agilent G1600A capillary electrophoresis system (Agilent, Waldbronn, Germany) was used throughout the study. Separations were conducted using 50 μm I.D. bare fused silica capillaries from Polymer Technologies (Phoenix, AZ, USA), which had an effective length of 84 cm.

Optimum results were obtained with a 40 mM borax buffer pH 9.0 (value adjusted with 1% phosphoric acid in water), containing 25 mM SDS as detergent and 7.5% n-butanol as organic modifier (required run-time 25 min). Separation temperature, detection wavelength and voltage were set to 30 °C, 210 nm and 20 kV, respectively. Samples were injected in hydrodynamic mode by applying 50 mbar for 4 s at the inlet position. Prior to each analysis the capillary was flushed with 0.01N NaOH solution (3 min), and water (2 min), followed by an equilibration step with running electrolyte (4 min).

2.4. Validation

Required validation parameters (Table 1) were determined using compounds 2 and 3, as sufficient amounts of them were available. A standard stock solution was prepared by dissolving 1.00 mg of 2 and 3 in 5.00 methanol, by consecutive serial dilution with methanol (ratio 1:1) six calibration levels were obtained. Limits of detection and quantitation were evaluated by establishing concentrations at signal-to-noise ratios of 3 (LOD) and 10 (LOQ), respectively. For the determination of accuracy recovery experiments at three spiking levels were performed (high, medium and low), and intermediate precision was assured by independently assaying (extraction and analysis) sample CA-1 five-times on each of three consecutive days. Peak purity could be confirmed by comparing available DAD-data of the signals of interest, with the peak purity threshold in the operating software (Chemstation Rev. B.02.01) set to 975.

3. Results and discussion

In contrast to free solution CE micellar electrokinetic chromatography (MEKC) enables the separation of charged and uncharged analytes at the same time. Detergents like SDS or sodium-cholate are added to the electrolyte, in which, above the critical micellar concentration (CMC), charged micelles are formed ("pseudo-stationary phase"). They migrate in the electric field, and due to hydrophobic interaction enable the separation of compounds regardless of their charge. MEKC is therefore commonly applied for the analysis of neutral analytes such as coumarins, terpenes or flavonoids [11].

3.1. Optimization of CE assay

Prerequisites for a successful separation of *Celtis* flavonoids by CE were deduced from literature, including the use of borate based electrolyte solutions (favorable complex formation with flavonoids

Table 1
Performance characteristics of the developed assay.

Parameter	Compound 2	Compound 3
Regression equation	$y = 0.661x$	$y = 1.744x$
Correlation coefficient	$R^2 = 0.9998$	$R^2 = 0.9996$
Range*	5–177	6–202
LOD*	2.2	1.6
LOQ*	7.3	6.3
Accuracy (high spike)**	102.3%	96.6%
Accuracy (medium spike)**	100.8%	98.4%
Accuracy (low spike)**	97.6%	96.8%
Precision (intra-day)***	1.3%	6.7%
Precision (inter-day)****	1.1%	3.9%

* μg/ml.
** Expressed as recovery rates.
*** Maximum deviation within 1 day based on peak area in percent ($n = 5$).
**** Deviation within 3 days based on peak area in percent.

and/or saccharides possessing 1,2- or 1,3-diol structure; [12]) with high pH-value (high EOF), and the need for adding SDS as tenside [13]. Owing to the samples complex flavonoid pattern it was not sufficient to observe the standards 1–4 alone, but also to consider the separation of unknown constituents (a–d; Fig. 2). Based on their UV-spectra the latter were tentatively assigned as flavonoids. Most relevant for a successful separation of the extract were buffer pH, type of electrolyte and its concentration, and organic modifier (see supplementary information for figures); other settings like temperature and applied voltage only had an impact of separation time but not peak resolution.

The influence of pH was studied in detail from 8.5 to 9.5, outside this range certain peak pairs merged (e.g. a-1 at pH 8.5 and b-c at pH 9.5). A divergent trend was noticed depending on the compounds observed. Some peak pairs showed increased resolution at higher pH values, others just the opposite. This is explainable by the phenolic character of the analytes, resulting in varying electrophoretic mobility and interaction with the micelles at different pH-values. At pH 9.0 all compounds of interest were separated with satisfactory resolution ($R_S \geq 1.0$). The same inconsistent trend was observed when increasing the borax concentration from 10 to 70 mM. Migration times increased due to increasing interaction of borate and flavonoids and most compounds showed an improved resolution [14]; nevertheless, b–c merged at higher values. The finally selected concentration of 40 mM represented an acceptable compromise. Some of the investigated compounds are sparingly soluble in aqueous media, therefore an organic modifier had to be added to the buffer system. The addition of 7.5% n-butanol (other solvents like methanol, acetonitrile or acetone were tested as well) was advantageous not only because of solubility issues, but it also resulted in enhanced resolution. This rather high n-butanol content partially explained the observations made during optimizing of SDS concentration. Below 10 mM not all compounds were resolved as the CMC of this tenside was not reached, at values of 50 mM and above stability of the micelles gradually declined (see supplementary information).

3.2. Method validation

All relevant validation parameters are summarized in Table 1. Respective data indicates that the developed assay is linear within the tested concentration range of 5–200 μg/ml ($R^2 \geq 0.9996$), combined with sensitivities typical for CE (LOD: 2.2–1.6 μg/ml, LOQ: 7.3–6.3 μg/ml). Recovery experiments were performed at three levels by spiking plant material with known amounts of standards. Afterwards the samples were extracted and analyzed as proposed. Resulting recoveries between 96.8 and 102.3% assured accuracy of the developed procedure. Repeatability of the assay was confirmed by relative standard deviations for multiple injections of the same solution below 4.0% (Table 2), intermediate precision showed to be below 7.0% (intra-day ≤ 6.7%, inter-day ≤ 3.9%). Selectivity was concluded based on the available UV-spectra and peak purity values.

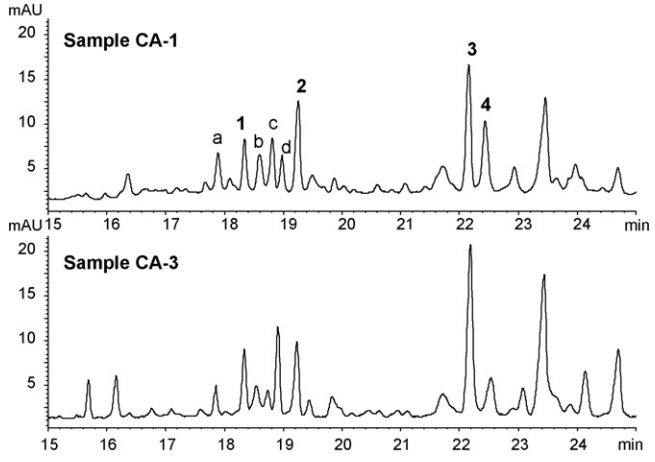

Fig. 2. Separation of samples CA-1 and CA-3 under optimized electrophoretic conditions. Peak assignment according to Fig. 1, for experimental conditions see Section 2.3.

Table 2
Percentage of flavonoids in different *C. australis* samples; relative standard deviation in parenthesis ($n = 3$).

Compound	CA-1	CA-2	CA-3
a*	0.019 (3.55)	0.013 (3.25)	0.012 (2.35)
1	0.027 (3.19)	0.034 (0.94)	0.033 (1.00)
b*	0.017 (1.88)	0.019 (1.61)	0.014 (2.04)
c*	0.029 (3.06)	0.017 (3.98)	0.013 (2.93)
d*	0.015 (2.23)	0.035 (1.70)	0.043 (2.18)
2	0.052 (1.74)	0.065 (2.05)	0.036 (2.18)
3	0.061 (1.81)	0.092 (1.92)	0.089 (0.76)
4	0.049 (2.66)	0.027 (2.22)	0.021 (1.79)
Σ of flavonoids	*0.296*	*0.302*	*0.261*

* Tentatively identified as flavonoid and quantified based on calibration data of 3.

3.3. Quantitative analysis

Prior to the analysis of flavonoids in *C. australis* leaves the suitability of the extraction procedure was evaluated. After repeating the extraction step three-times as proposed, the plant material was extracted one more time, and the resulting extract analyzed for remaining compounds. A maximum residue of 3.5% was found for compound 1, thus an exhaustive extraction was assured.

Typical CE separations obtained under optimized conditions are shown in Fig. 2, in which compounds 1–4 were identified by spiking with standards, and compounds a–d were tentatively assigned based on their UV-spectra. Later on all compounds except 2 were quantified based on the calibration data of 3 (this compound is commercially available). The total amount of flavonoids showed to be comparable in all samples investigated (0.26–0.30%), with isovitexin (3) being the dominant flavonoid (0.06–0.09%), followed by cytisoside (2; 0.04–0.07%). Most of the other flavonoids were present between 0.02 and 0.03%, except compound 4 in sample CA-1 (0.05%) and compound d in sample CA-3 (0.04%).

4. Conclusion

Despite the fact that *C. australis* is a traditionally used medicinal plant no procedure for its analysis can be found in literature. The here presented CE procedure showed to be well suited to study rare and pharmacologically interesting C-glycosidic flavonoids in the plant for the first time. Method validation confirmed that the obtained quantitative results are accurate, precise and true, and a practical application of the assay revealed a complex flavonoid pattern in the samples. Isovitexin was found to be the major compound, but at least seven other flavonoids are present as well. Owing to the high separation efficiency of CE all of them could be baseline separated in less than 23 min at basically no sample or solvent consumption.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2009.11.028.

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