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Capillary electrophoresis as a fast and efficient alternative for the analysis of *Urceola rosea* leaf extracts

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ABSTRACT

Urceola rosea, a plant whose leaves are used as food and for medical purposes, is a climbing liana found in many south-east Asian countries. Main polar compounds are flavonoids (kaempferol and quercetin glycosides) and phenolic acids. As an alternative to the established HPLC method their analysis by capillary electrophoresis is described for the first time. It was possible in < 8 min with a 25 mM sodium tetraborate decahydrate solution with pH 8.5, at a capillary temperature of 40 °C and an applied voltage of 25 kV. Up to five compounds could be quantified in different methanolic *U. rosea* extracts, which showed to be of variable composition; e.g. the content of total flavonoids ranged from 0.29 to 1.08%. In respect to quantitative results as well as validation parameters (e.g. $R^2 \geq 0.994$, recovery rates from 95.5 to 103.6%, inter-day precision ≤ 4.5%) the CE method was well comparable to HPLC. However, in terms of required analysis time and environmental sustainability capillary electrophoresis is definitely advantageous.

1. Introduction

The genus *Urceola*, which belongs to the Apocynaceae family, comprises 15 species commonly found in south-east Asian countries like China, Vietnam, Thailand or India. One typical representative is *Urceola rosea*, an up to 20 m long climbing liana with pink flowers, elliptic leaves (max. 7 × 4 cm in size) and dark brown stems [1]. Especially in China the synonym *Ecdysanthera rosea* is often used for this species [2]. All of its parts are a traditional remedy to treat infections of the endosteum, injury and rheumatism [1], and several recently filed Chinese patents describe multi-herb formulations containing *U. rosea* leaves/stems against different infectious diseases (e.g. mastoiditis [3], asthma [4] or stomatitis [5]). It also should be mentioned that the leaves are locally consumed as food because of their sour taste [6].

Phytochemical data on *Urceola rosea* is scarce. It is mainly related to constituents found in the stems, describing the isolation of antimicrobial and cytotoxic pregnane saponins [7,8], of one pregnane aglycon [9], several sesquiterpenoids [10,11] and hydroquinone diglycoside acyl esters [12]. Among the more polar constituents identified are caffeoylquinic acid derivatives (including chlorogenic acid), as well as tartaric and malic acid [13]. Just recently we have investigated *Urceola rosea* leaves in detail, resulting in the isolation of several phenolic compounds and the development of an HPLC assay for their determination [14]. Major constituents showed to be flavonoids, more precisely derivatives of kaempferol and quercetin. Flavonoids are known antioxidants [15] and thus most likely relevant for the anti-oxidant properties of *U. rosea*. If they are quantified by HPLC all validation criteria are met, however > 25 min are required per analysis. CE on the other hand is known for separation speed and efficiency; it also has been utilized for the analysis of flavonoids and phenolic acids [16]. Thus, from a methodological point of view it was interesting to see how two complementary techniques, HPLC and CE, would compare in terms of validation parameters and quantitative results. The determination of phenolic compounds in *U. rosea* leaves was a practically relevant application to do so.

2. Experimental

2.1. Samples, standards and reagents

Three batches of *Urceola rosea* leaves were analyzed in this study. They were collected in North Vietnam 2016 (UR-1: Ban Thi region, September; UR-2: Ba Vi Park, Hanoi, November) and 2017 (UR-3: Ba Vi Park, Hanoi, May), and authenticated by Duc Trong Nghiem, Department of Botany, Hanoi University of Pharmacy. Voucher specimens of all samples are deposited at the University of Innsbruck, Institute of Pharmacy, Pharmacognosy, Austria.

Reference compounds **1** to **4** were previously isolated in-house from sample UR-1 by column chromatography; detailed information on their purification and structural elucidation can be found in [14]. They all

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had a purity ≥ 95% as determined by HPLC and NMR. Compound **5** (chlorogenic acid) was purchased from Sigma-Aldrich (St. Louis, MO, USA); its purity was also higher than 95%. All reagents required for the preparation of buffers or washing solutions had p.a. quality and came from Merck (Darmstadt, Germany). Ultrapure water was produced by a Sartorius arium 611 UV (Göttingen, Germany) system.

2.2. Sample preparation

The dried, finely powdered leaf material (600 mg) was extracted with 3 mL methanol by sonication (Bandelin Sonorex 35 KHz, Berlin, Germany) for 10 min. After centrifugation (1400 g, 5 min) the supernatant was placed in a 10 mL volumetric flask and the extraction step repeated two more times. All resulting solutions were combined and the flask filled to volume with methanol; directly before analysis each sample solution was membrane filtered (0.45 µm, cellulose acetate, VWR, Vienna, Austria). Sample solutions were stable for at least 2 weeks if stored at 4 °C.

2.3. Analytical conditions

For all experiments an Agilent 3D-CE system (Waldbronn, Germany), equipped with autosampler, diode array detector (DAD) and temperature controlled column compartment, was used. Fused-silica capillaries (PolymicroTechnologies, Phoenix, AZ, USA) with an internal diameter of 50 µm and an effective length of 58.5 cm were filled with 25 mM sodium tetraborate decahydrate (borax) solution; its pH was adjusted to 8.5 with 1% phosphoric acid. During analysis applied voltage, temperature and detection wavelength were set to 25 kV, 40 °C, and 254 nm, respectively. Samples were injected in hydrodynamic mode (50 mbar for 4 s) and after 8 min each run was completed. New capillaries were rinsed with 0.1 N NaOH, 0.01 N NaOH and water (30 min each) prior to initial use; between runs they were flushed with 0.01 N NaOH solution, water and buffer for 3 min each. All samples, buffers and washing solutions were membrane filtered and sonicated for approx. 1 min before being used for CE; buffers were replaced with fresh ones after 4 analyses.

2.4. Method validation

Linearity, the limits of detection (LOD) and quantification (LOQ), selectivity, accuracy and precision were confirmed for the developed CE-assay. A stock solution containing all five standard compounds (concentration 1.0 mg/mL each) was prepared in methanol and further calibration levels obtained by serial dilution with the same solvent in the ratio of 1:3. LOD and LOQ were evaluated visually corresponding to concentrations equivalent to signal-to-noise ratios of 3 (LOD) and 10 (LOQ). Selectivity was assured by utilizing the peak-purity option in the operating software (Agilent chemstation version RevB.04.04-Sp2). Accuracy was investigated by spiking three portions of sample UR-2 with known amounts (high spike: 75 µg/mL; medium spike: 50 µg/mL; low spike: 25 µg/mL) of compounds **2**, **4** and **5**. After extraction and analysis the determined concentrations were compared to the theoretical present ones and expressed as recovery rates in percent. Short term precision (repeatability) was deduced by the relative standard deviation from multiple analyses of the same solution, and reproducibility studied by assessing five individually prepared solutions of UR-2 on day one (intra-day). The same experiments (i.e. extraction and analysis) were repeated on two consecutive days in order to calculate inter-day variation. Table 1 compiles all validation results.

3. Results and discussion

3.1. CE-method development and optimization

Capillary electrophoresis is an analytical method known for high selectivity and efficiency. It can be utilized with aqueous and non-aqueous buffers and facilitates the separation of charged as well as non-charged analytes. The latter is possible either by adding micelle-forming tensides to the electrolyte solution (MEKC, micellar electrokinetic chromatography) or by using borate buffers, which enable the separation of neutral compounds like flavonoids due to complex formation [16]. Hence, such a buffer was also the first choice in the current study, and after evaluating all relevant separation parameters the standard compounds (four flavonoid glycosides and chlorogenic acid, see Fig. 1 for structures) could be baseline resolved in < 8 min (Fig. 2). Comparable results were not possible with other buffer systems in the same pH range (e.g. phosphate or TRIS) and the addition of SDS (sodium dodecyl sulfate) as surfactant was not advantageous either.

The impact of individual parameters on the separation of **1** to **5** is shown in figures provided as supporting material. As can be seen the most influential settings were, not surprisingly, buffer pH and concentration. The feasible pH-range was 8 to 9, however lower values were less favorable for the resolution of **1–2** and **2–3**, and higher ones for **3–4** and **4–5**. A good compromise was reached at pH 8.5, where the resolution between all relevant compounds was higher than 3.5. Buffer concentration had an effect on resolution and analysis time. The electroosmotic flow (EOF) decreases with ion strength, thus using a 35 mM borax buffer resulted in an analysis time of > 10 min without significantly improving the separation. As with 15 mM borax the resolution of **2–3** was below 2, 25 mM was considered to be the optimum. Applied voltage and temperature had less impact on the resolution and they were selected considering aspects of analysis time as well as baseline and current stability. Another option to improve separation efficiency in CE is the addition of organic modifiers like short-chain alcohols to the buffer. They modify solubility and viscosity, but in the current case did not improve the results overall. Most of the solvents added resulted in an enhanced resolution of peak pairs **3–4** and **4–5**, however the effect on the most critical substance pair **2–3** was contrarious. As a consequence, a 25 mM borate buffer with pH 8.5, at 40 °C and + 25 kV was selected for all further experiments. Detection was achieved at 254 nm and samples were injected hydrodynamically (4 s at 50 mbar).

3.2. Comparison of CE and HPLC

3.2.1. Method validation

As required for any newly developed analytical procedure the CE method was validated; respective results are summarized in Table 1. To forestall the results, all respective indicators were well within generally accepted limits. A comparison to reported validation parameters resulting from the analysis of exactly the same compounds/sample solutions by HPLC [14] showed a large degree of conformity. This applied to linearity (both techniques were linear over two orders of magnitude, e.g. for HPLC in a range from approx. 5 to 600 µg/mL), determination coefficients (CE: $R^2 \geq 0.994$; HPLC: $R^2 \geq 0.999$) and accuracy. The latter was determined in spiking experiments using three compounds that were available in sufficient amount. Following ICH guidelines [17] the matrix was augmented with three concentrations prior to extraction and analysis, and the determined recovery rates varied from 95.5% for CE/97.3% for HPLC to 103.6% for CE/102.5% for HPLC. Also in terms of intermediate assay precision both approaches were within accepted limits, even if the deviations in HPLC were generally lower (e.g. intra-day variation by HPLC ≤ 4.3% versus ≤ 8.4% by CE).

Parameters which differed considerably between both techniques were LOD and LOQ. Considering that always a DAD was used, in CE they were much higher than those observed for HPLC (e.g. LOD for **1** 3.6 µg/mL, 0.02 µg/mL by HPLC); however, they were typical for capillary electrophoresis and can easily be explained by its instrumental peculiarities such as short optical path length due to on-capillary detection and injected sample volumes in the nanoliter range. The latter also explain slightly higher σ_{rel} values concerning repeatability and