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Separation and determination of coumarins including furanocoumarins using micellar electrokinetic capillary chromatography

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ABSTRACT

The conditions of micellar electrokinetic capillary chromatography for separation and simultaneous measurement of coumarins (coumarin, scoparone, isoscopoletin, esculin, esculetin, umbelliferone) including furanocoumarins (xanthotoxin, byakangelicin, isopimpinellin, bergapten, phellopterin, xanthotoxol) have been elaborated. The influence of different parameters, such as the pH of the buffer, sodium cholate (SC) or methanol concentration in the buffer, on the migration time, peak resolution, peak asymmetry, and number of theoretical plates was investigated. The optimum separation of the compounds was achieved using 50-μm i.d. capillaries with a total length of 64.5 cm (56 cm effective length) and a buffer system at pH 9.00 consisting of 50 mM sodium tetraborate, 45 mM SC, and 20% of methanol (v/v). The developed method ensured good repeatability of corrected peak areas and migration times (the relative standard deviations were in the range of 2.8–6.1% and 0.8–4.0%, respectively). The average limit of detection for all studied compounds was below 1.3 μg mL. Moreover, good linearity of the relationship between the peak corrected area and the concentration of the compounds was observed (correlation coefficient > 0.99). The method was successfully applied in the quantitative analysis of two different types of samples, i.e. *Heracleum sphondylium* herb and *Aesculus hippocastanum* cortex.

1. Introduction

Despite the rapid development of analytical techniques for direct analysis, e.g. direct mass spectrometry or voltamperometry, determination of metabolites in samples of plant origin is difficult due to the accompanying matrix and structural similarity of numerous compounds. In such a case, initial separation is required to facilitate identification and quantification of components and, therefore, development of separation techniques is still necessary. Different variants of chromatography, e.g. high-performance liquid chromatography (HPLC), ultra-high performance liquid chromatography (UHPLC), and gas chromatography (GC), are most commonly applied for analysis of plant samples. However, capillary electrophoresis (CE) is gaining increased researchers' attention due to its numerous advantages, e.g. a relatively low cost of equipment and single analysis, a small amount of samples required for investigation, high selectivity, and the possibility of separating a wide variety of compounds, both organic and inorganic, charged and uncharged [1–3]. Moreover, the low consumption of solvents makes CE an environmentally friendly technique considered as a promising alternative to chromatography in analysis of samples of plant origin [4].

As one of the groups of secondary metabolites present in higher plants, coumarins (benzo-α-pyrones) arouse great interest due to their biological and therapeutic activity. In a few plants families, e.g. Moraceae, Rutaceae, Apiaceae, and Fabaceae, prenylation of the precursor of furanocoumarins – umbelliferone at C6 or C8 followed by cyclization and closure of the furan ring leads to generation of furanocoumarins constituting a sub-family of coumarin compounds [5]. Both simple coumarins and furanocoumarins exhibit a wide range of biological activities, including anticancer, antioxidant, or anti-inflammatory activity [6,7]. Additionally, furanocoumarins are used in leucoderma and psoriasis therapy as a skin repigmentation stimulator

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[8]. However, it should be noted that furanocoumarins exhibit phototoxic properties resulting in skin burns or erythema [9]. Moreover, coumarins exert a hepatotoxic and carcinogenic effect in some mammal species [10].

Coumarins in plant material have mostly been determined using normal or reverse phase high-performance liquid chromatography (HPLC) [8], thin-layer chromatography (TLC) [11], and gas chromatography (GC) [12]. There are also some literature data on application of different variants of capillary electrophoresis such as zone capillary electrophoresis (CZE) [13–16], pressurized capillary electrochromatography (pCEC) [17,18], or non-aqueous capillary electrophoresis [19] for separation of coumarins. Micellar electrokinetic capillary chromatography (MEKC) has also been applied [20–23]; however, the investigations were mostly focused on separation of only a few compounds. Since the MEKC system is suitable for analysis of uncharged or partially charged agents, appears to be suitable for separation of furanocoumarins [24].

Regardless of the CE variant, the optimization of analysis conditions is quite complicated because many variables should be taken into consideration such as voltage, temperature, capillary length; however, the background electrolyte (BGE) composition (pH, type and concentration of buffer and additives) are an important factor too. It has the strongest effect on the resolution between the neighboring peaks, system efficiency expressed as the theoretical plate number, and peak symmetry.

In this study, the MEKC method was developed and validated for simultaneous determination of 6 coumarins (coumarin, scoparone, isoscopoletin, esculin, esculetin, umbelliferone) and 6 furanocoumarins (xanthotoxin, byakangelicin, isopimpinellin, bergapten, phellopterin, xanthotoxol). Moreover, the impact of various parameters such as the pH of the BGE, concentration of sodium cholate (surfactant) and methanol influencing the system efficiency, selectivity, resolution, and peak shape were investigated. Finally, the optimized method was applied to analyze coumarins in two different plant samples, i.e. *Heracleum sphondylium* L. herb and *Aesculus hippocastanum* L. cortex.

2. Material and methods

2.1. Chemicals

The standard compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA). The stock solutions of coumarins standards were prepared in methanol (Merck Millipore, Bedford, MA, USA) in the concentration of 1 mg mL^{-1}, stored at 4 °C, and used at an appropriate concentration on the same day. The background electrolytes (BGE) were prepared from sodium tetraborate, sodium cholate (SC) (Sigma-Aldrich, St. Louis, MO, USA), and methanol (Merck Millipore, Bedford, MA, USA), and the pH was adjusted with 1 M NaOH (Agilent Technologies, Santa Clara, CA, USA). All solutions were prepared with deionized water purified using a MilliQ water purification system (Merck Millipore, Bedford, MA, USA). All chemical reagents were of analytical grade.

2.2. Apparatus and electrophoretic separation conditions

The capillary electrophoresis analyses were performed on an Agilent 7100 capillary electrophoresis system equipped with a diode array spectrophotometric detector (190–600 nm) (Agilent Technologies, SantaClara, CA, USA). Separations were carried out using 50-μm i.d. capillaries (Agilent Technologies, SantaClara, CA, USA) with a total length of 64.5 cm (56 cm effective length). Before daily operation, the capillaries were rinsed with 0.1 M NaOH (Agilent Technologies, Santa Clara, CA, USA) under pressure (approx. 1 bar) for 10 min, with deionized water for 10 min, and finally with BGE for 10 min. Before introduction of each sample, the capillary was rinsed using BGE. The samples were injected by applying a pressure of 50 mbar for 4.0 s, followed by BGE for 4.0 s with 50-mbar pressure. The separations were performed at 30 kV and capillary temperature of 27 °C. The compounds were detected using a diode array detector (DAD) in the range of 192–500 nm (Suppl. Fig. 1). Different methanol concentrations (0%, 5%, 10%, 15%, 20%, 25%), SC concentrations (45 mM, 55 mM, 65 mM, 75 mM), and pH values (9.00, 9.15, 9.30, 9.45) of the BGE were tested.

The results were analyzed using the ChemStation software (3D-CE ChemStation, Agilent Technologies, Santa Clara, CA, USA). The following parameters: peak asymmetry (As), number of theoretical plates (N), resolution (Rs) were calculated using EZChrom Elite software (Merck, Darmstadt, Germany).

2.3. Samples

Both the *Heracleum sphondylium* L. (herb) and *Aesculus hippocastanum* L. (cortex) samples were dried at 50 °C for 12 h and ground into fine powder. 1.0 g of the powder was extracted in an ultrasonic bath (Sonorex Digitec DT100/H, Bandelin, Berlin, Germany) with 10 mL of 80% methanol for 30 min. The extract was centrifuged (Eppendorf 5804 R, Hamburg, Germany) at 4500 × *g* for 5 min. Prior to the CE analysis, the supernatant obtained was filtered through 0.22-μm nylon filters (Kinesis, Cambridgeshire, United Kingdom).

3. Results and discussion

3.1. Optimization of the separation

Optimization of the capillary electrophoresis separation of the examined compounds was performed for the pH of the BGE, the SC concentration, and the methanol concentration, and related technical parameters (As, N, Rs) were calculated. Additionally, the apparent solute mobility was calculated (μ_a) according to the equation [25]:

$$\mu_a = \frac{l \times L}{t \times V}$$

where l – is effective capillary length, L – total capillary length, t – migration time, and V – voltage.

3.2. Effect of buffer pH

Generally, coumarins are slightly charged in acidic or alkaline medium; hence, the influence of the pH of BGE on coumarin separation is mainly based on changing the EOF speed. Previously, various authors indicated that changing pH hardly influenced coumarin migration [15,17,18]. Ketai et al. (2001) [22] noted a rapid increase in the migration time in the BGE at pH above 9.50.

The results presented in this study indicated that pH below 9.45 did not influence the migration time of most coumarins (the differences were only statistically significant for compounds no 5, 10, 11, and 12) (Suppl. Fig. 2a, Suppl. Table 1, Suppl. Table 2) and had no effect on the apparent mobility (Suppl. Fig. 3a) of the examined coumarins. In turn, a significant increase in the migration times at pH 9.45 of BGE was observed, especially in the case of bergapten, phellopterin, xanthotoxol, esculetin, and umbelliferone. Moreover, the shape of the phellopterin, xanthotoxol, and umbelliferone peaks were distorted, making integration thereof impossible. The Rs values for selected critical pairs of coumarins at four different pH values are shown in Suppl. Fig. 4a. Separation of critical compounds was achieved at all the tested pH values (Rs values above 0); however, poor resolutions of pairs 5–7 and 6–7 were observed at pH 9.30 and 9.45, respectively. The changes in pH in the range of 9.00–9.30 had a minor impact on system efficacy for most of the investigated compounds, but a significant decrease in the theoretical plate number at pH > 9.00 was observed for isoscopoletin and esculetin (peak number 5 and 9; Suppl. Fig. 5a and Suppl. Table 1). In the tested pH range, the peaks were symmetric (asymmetry from 0.99 to 1.22); however, the peak of esculetin had a tendency towards tailing