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Separation of kaempferols in *Impatiens balsamina* flowers by capillary electrophoresis with electrochemical detection

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Abstract

Capillary electrophoresis with wall-jet amperometric detection was used to detect kaempferol and its derivatives kaempferol-3-glucoside, kaempferol-3-glucosylrhamnoside and kaempferol-3-(*p*-coumaroyl)glucoside. The influence of buffer pH on separation was investigated and optimized. With a phosphate buffer at pH 7.5, nearly complete separation of the four kaempferols was achieved according to their different electrophoretic mobilities. The detection potential was also evaluated and optimized. At detection potential of +0.80 V vs. saturated calomel electrode, an amperometric response with high sensitivity and stability was obtained for these four compounds. Detection limit estimated for all the kaempferols examined was less than 1.4 fmol, based on $S/N=3$. The use of this method for the separation and detection of these compounds present in balsam flowers (*Impatiens balsamina*) is reported.

Keywords: *Impatiens balsamina*; Plant material; Electrochemical detection; Detection, electrophoresis; Kaempferols; Flavonols

1. Introduction

Due to their vast presence both in edible plants, foods and beverages, flavonols are vital constituents of the non-energetic part of the human diet. Most importantly, many flavonols containing species are known for their use as traditional medicines for a long time. Various studies reveal that flavonols acting as antioxidants in biological systems are very efficient free radical scavengers in vitro [1,2]. The analysis of flavonols in medicinal plants plays an essential part of any research involving the efficacy, the safety, and therapeutical reproducibility of preparations from these plants [3,4]. During the past years much attention has been devoted to the analysis of flavonol-containing plants for identification and quantification purposes. These analyses have been performed using different techniques, from paper chromatography to HPLC [2,5]. However, due to the inherent structural similarity of most flavonols and the complex characteristics of the sample matrices, gradient elution is often required to provide the adequate efficiency for HPLC. Capillary electrophoresis (CE), as a new technique of increasing popularity, has been shown to be a very powerful and

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efficient tool for the separation of a wide variety of analytes [6,7]. Previously, CE has been reported to apply for the separation of flavonoids based on a UV detection method [8,9]. However, the UV assay method lacks sensitivity due to the small diameter of the separation capillaries.

Electrochemical detection (ED), based on the electrochemical reaction of analytes on an electrode surface, provides one of the most sensitive detection methods for CE. Recently, CE–ED has been applied for the determination of a variety of compounds with different electrode–capillary alignments [10–15]. The carbon-based electrodes have been commonly used in amperometric detection due to its low background current, high stability and resistance to passivation. We recently demonstrated the ability to couple CE separation with amperometric detection at sol–gel carbon composite electrodes (CCEs) [16–18]. The development of such CCEs typically has been based on mixing graphite powder with a suitable sol–gel precursor monomer, typically alkyltrimethoxysilane in the presence of a suitable catalyst, either a strong acid or base. The versatile sol–gel technique allows for the flexible electrode configuration needed for any particular experimental requirement. Generally, such CCEs have been reported to have various desirable properties suitable for electrochemical applications: low background current, good chemical stability, easy preparation, good mechanical stability and wide operating potential [19,20].

In this paper, we first demonstrate the capability of CE–ED to facilitate the separation of a balsam flower of *Impatiens balsamina* extraction sample containing structurally similar kaempferols. An electrochemical detector is described based on the sol–gel technique, which is useful for the detection of these naturally occurring compounds. Identification and quantification of four kaempferol-based compounds in balsam flower extract were also performed. The methanol extract was diluted and then injected directly in the CE system.

2. Experimental

2.1. Reagents and solutions

Methyltrimethoxysilane (MTMOS) was obtained from Merck–Schuchardt. Graphite powder (extra pure) was obtained from Merck. Kaempferol, kaempferol-3-glucoside, kaempferol-3-glucosylrhamnoside and kaempferol-3-(*p*-coumaroyl) glucoside were obtained from dried balsam flowers after a series of extraction, isolation, purification and characterization steps in our laboratory and the Institute of Plant Biochemistry, Halle, Germany. These compounds were identified by diverse assay methods such as HPLC–online-UV–visible spectrometry, MS, NMR and hydrolytic analysis. The supporting electrolyte was 0.02 *M* phosphate buffer (pH 7.5). Stock solutions of kaempferol and its derivatives were prepared in methanol and stored at 4°C. All other reagents were used as received without further purification, and aqueous solutions were prepared in distilled water.

2.2. CE–ED system

Electrophoresis in capillary was driven by the high-voltage supply (Model CZE1000R, Spellman, New York) capable of delivering 0 ± 30 kV. The high-voltage input was placed in a Plexiglas box with an interlock switch on the access door. The outlet end of the capillary was always maintained grounded through a platinum wire in the amperometric detection cell. Experiments were carried out using fused-silica capillary of 25-μm I.D and 360-μm O.D. (Polymicro Technologies, Phoenix) cut to 62 cm in length. Before use, new capillaries were washed with 0.1 *M* NaOH followed by distilled water and the separation buffer. Each washing step was carried out with a syringe pump (flow-rate of 0.1 ml h^{-1}) for ca. 5 min. All separations were performed in 0.02 *M* sodium phosphate buffer at pH 7.5 using a 12 kV separation voltage. Sample introduction was accomplished hydrodynamically (by gravity) for 10 s at a height of 15 cm and the injection volume was estimated to be ca. 0.23 nl.

Amperometric detection at a constant potential with CE was performed with a BAS LC-4CE amperometric detector (West Lafayette, IN, USA) using a laboratory-made wall-jet cell configuration which was similar to that described previously [21,22]. A conventional three-electrode system was used with the sol–gel carbon composite working electrode at the end of the separation capillary. The reference electrode was a saturated calomel electrode

(SCE) and the auxiliary electrode was a platinum wire. The electropherograms were monitored with a strip-chart recorder (Model 8376-20, Cole-Parmer, Chicago). The holding potential applied onto the working electrode was typically +0.80 V vs. SCE, unless stated otherwise.

2.3. Electrode preparation

First a sol–gel solution of the following composition was prepared by mixing 0.5 ml of MTMOS, 20 μl of 6 *M* HCl and 200 μl of distilled water. The mixture was sonicated for 2 min to ensure uniform mixing. Methanol was not added into the sol–gel solution because the sonication was sufficient to ensure complete homogenization. Subsequently, 0.5 g of graphite powder was mixed thoroughly with the above sol–gel solution in a mortar to form a uniformly dispersed carbon–sol–gel paste. Then the carbon–sol–gel paste was packed to ca. 2 cm at one end of a glass capillary (ca. 200 μm I.D., 1 mm O.D.). A copper wire was inserted through the opposite end of the glass capillary to establish an electrical contact. The electrode was allowed to polymerise and left to dry for 4 days under ambient laboratory conditions.

Finally the electrodes were polished with 600-grit polishing paper (dry conditions) and subsequently rinsed thoroughly with distilled water [16]. The optimised silica carbon monoliths did not shrink on drying and exhibited good adhesion onto the glass capillary wall. The resulting electrodes were black, rigid, and porous, though the porosity was not visible to the naked eye. After polishing, the surfaces of the electrodes were smooth, shiny and glassy carbon like.

2.4. Sample preparation

2.4.1. Plant material

The sample, fresh pink balsam flowers naturally grown in the garden of the National Institute of Education, one of the Nanyang Technological University campuses, without any chemical fertilizer, was collected during June 1999 and dried by air under natural ventilation.

2.4.2. Extraction and isolation

A certain amount (ca. 4 g) of dried balsam flowers was ground into fine powder with dry ice (ca. 15 g) in a mortar. Then accurately weighed 2.0639 g of the powdered sample was extracted two times with ca. 20 ml of 80% methanolic aqueous solution at room temperature under continuous stirring for 5 h. The extracts were combined and diluted to 50 ml, and the stock solution was stored at 4°C.

3. Results and discussion

The separation of kaempferol-based compounds [i.e. (1) kaempferol (K), (2) kaempferol-3-glucoside (KG), (3) kaempferol-3-glucosyl-rhamnoside (KGR), and (4) kaempferol-3-(*p*-coumaroyl)glucoside (KCG); see Fig. 1] was based on their acid–base equilibrium properties. As can be seen from Fig. 2, the resolution of kaempferol-based compounds is strongly pH dependent with a buffer concentration of 20 m*M* phosphate and an applied voltage of 12 kV. At pH 8.0, KCG and KG could not be resolved completely. The selectivity for kaempferol and its derivatives exhibit a maximum at pH 7.5 (Fig. 2B). However, as the pH is lowered further, the flavonols became less dissociated, leading to a lower resolution for last three peaks and a rather pronounced tailing for K (Fig. 2A). This tailing phenomenon may be attributed to the increased electrostatic interactions between the negatively charged capillary wall and the greater proportion of cationic species of flavonols present at lower pH. This is similar to those observed in the separation of neurotransmitters and proteins [17,23,24]. Thus, pH 7.5 phosphate buffers were employed for adequate dissociation and separation of four kaempferol-based compounds.

The selection of detection potential is critical for amperometric detection. In order to determine the optimum potential for electrochemical detection, hydrodynamic voltammograms (HDVs) were acquired by making several injections of the same solution into CE system and recording the peak current among varying applied detection potential between CE runs. As shown in Fig. 3, typically the anodic current starts at ca. +0.60–0.65 V, and rises rapidly. Then the currents reach a plateau at +0.80 V except for K. The mediator Cu_2O appeared to have high catalytic activity for the oxidation of all the kaempferols examined in alkaline solutions. This might be associated with the Cu (III) species par-

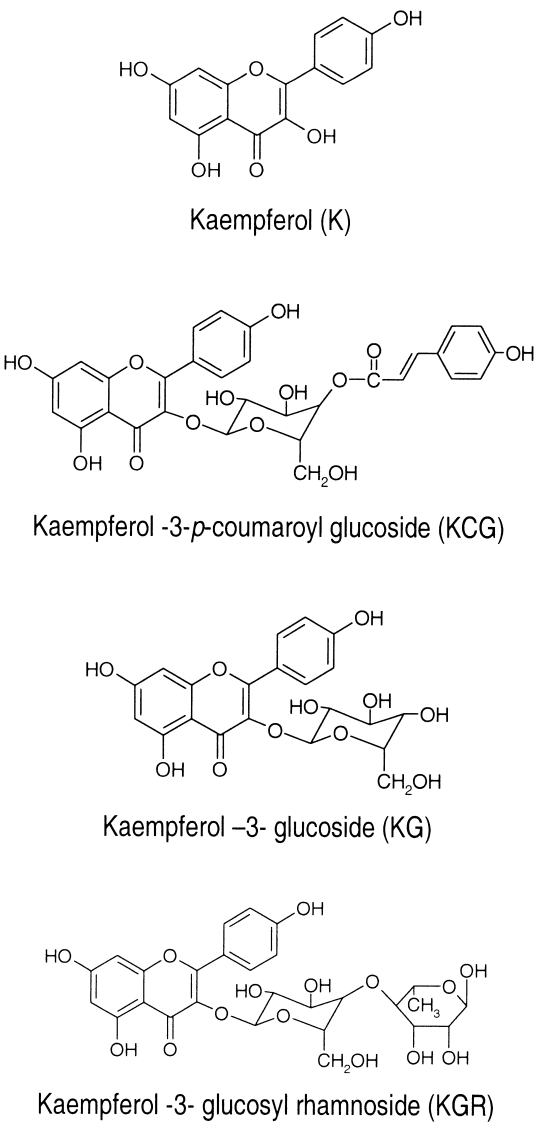

Fig. 1. Chemical structures of kaempferols in *Impatiens balsamina* flowers.

ticipating in the catalytic oxidation process, which is similar to the reported oxidation of carbohydrates at Cu metal and Cu_2O modified electrodes under basic conditions [25,26]. Electrochemical detection at higher potential usually leads to large background currents for CE–ED analysis. For a suitable compromise of high sensitivity and low background current, a value of +0.80 V was usually selected as a suitable potential for the subsequent experiments.

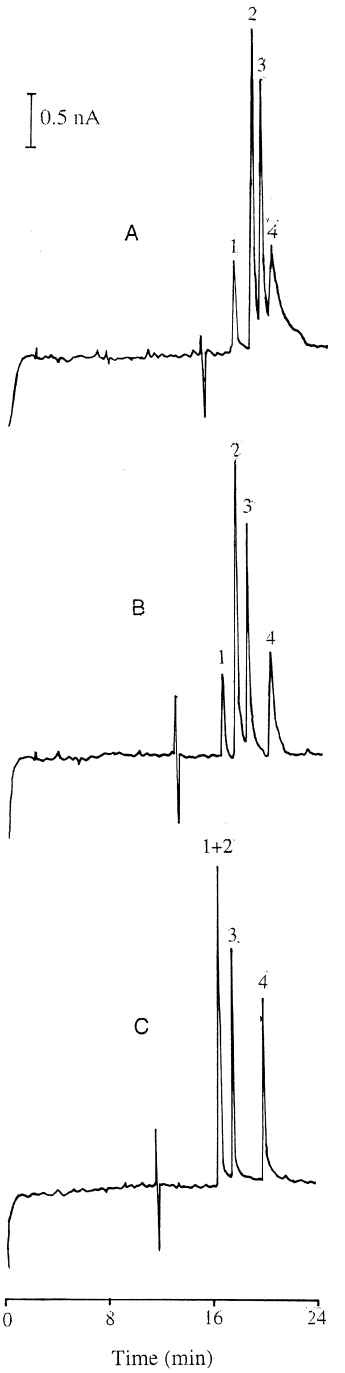

Fig. 2. Electropherograms of four flavonols in different pH buffer: (A) pH 7.0, (B) pH 7.5, and (C) pH 8.0. The flavonols are (1) KCG, (2) KG, (3) KGR, and (4) K (concentrations between 30 and 68 μ*M*). Buffer: 0.02 *M* phosphate; Separation voltage: 12 kV; Injection by gravity: 15 cm for 10 s; Applied electrode potential: +0.80 V vs. SCE.

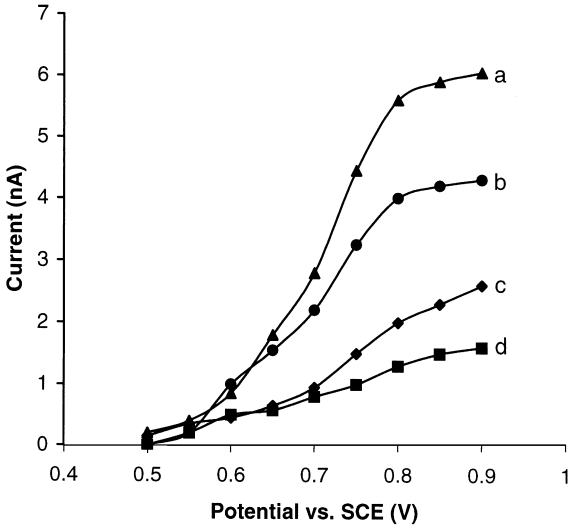

Fig. 3. Hydrodynamic voltammograms of: (a) KG; (b) KGR; (c) K; and (d) KCG (concentrations between 30 and 68 μ*M*) at a CCE in a 0.02 *M* phosphate (pH 7.5). Other conditions are as in Fig. 2.

The reproducibility of amperometric current response was investigated by repeated injection of 20 μ*M* standard solutions and measuring peak heights. Under conditions of manual hydrodynamic injection, the stability of the electrode was shown in Fig. 4 for 10 successive injections. The activity of the CCE used in CE–ED system was observed to decrease slightly for the initial injections of KG, but for KCG it remained stable through all injections. No specific treatment of the electrode was carried out between the injections. The electrode lost 25% of its initial activity for KCG after more than 6 days measurements. The loss in long-term reproducibility was probably attributed to the slightly adsorption of electrochemical products of flavonols onto the electrode surface. The electrode was left in the buffer when not in use. The reproducibility of the migration time was determined using methanol as a marker compound for the electroosmotic flow (EOF). Typically, the methanol peak is the first small peak (without label) in electropherograms shown in Fig. 2. The RSD for the migration time of EOF was evaluated to be only 1.8% for 10 successive CE runs. After extended measuring periods of several hours the EOF became slightly slower probably due to the slightly adsorption of flavonols onto the internal capillary wall. For example, 8 h of continuous CE experiment resulted in the 7.2% longer migration time for EOF than at the first injection. Detection limits for kaempferol and its derivatives were assessed at the detection potential of +0.80 V and were estimated to be 4.8 μ*M* (1.1 fmol), 2.1 μ*M* (0.48 fmol), 1.2 μ*M* (0.28 fmol) and 6.1 μ*M* (1.4 fmol) for KCG, KG, KGR and K respectively, based on $S/N=3$.

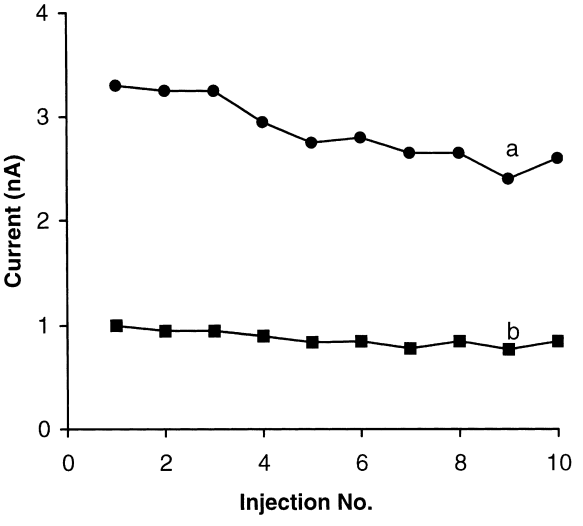

Fig. 4. Stability investigation of the current response at a CCE for: (a) 68 μ*M* KG and (b) 45 μ*M* KCG. Other conditions are as in Fig. 2.

The proposed method was used for the determination of kaempferol and its derivatives in balsam flower. These components possess suitable chemical or similar structure as antioxidants and the potential to act independently or in combination as anti-cancer or cardiac protective agents. Fig. 5A depicts a typical electropherogram for a standard mixture of four flavonols (concentration in the range of 60 and 140 μ*M*). Electropherograms of a methanol extract obtained from balsam flower powder are shown in Fig. 5 (B and C). The only sample preparation needed prior to injection into the CE system was the simple dilution step needed, either 1:1 (Fig. 5C) or 1:9 (Fig. 5B) ratio. The electrode was less sensitive for other constituents in flower sample. Owing to the low concentration of KCG in extract solution, further dilution resulted in the peak disappearance of KCG. The presence of kaempferol and its derivatives in flower sample was identified by means of spiking

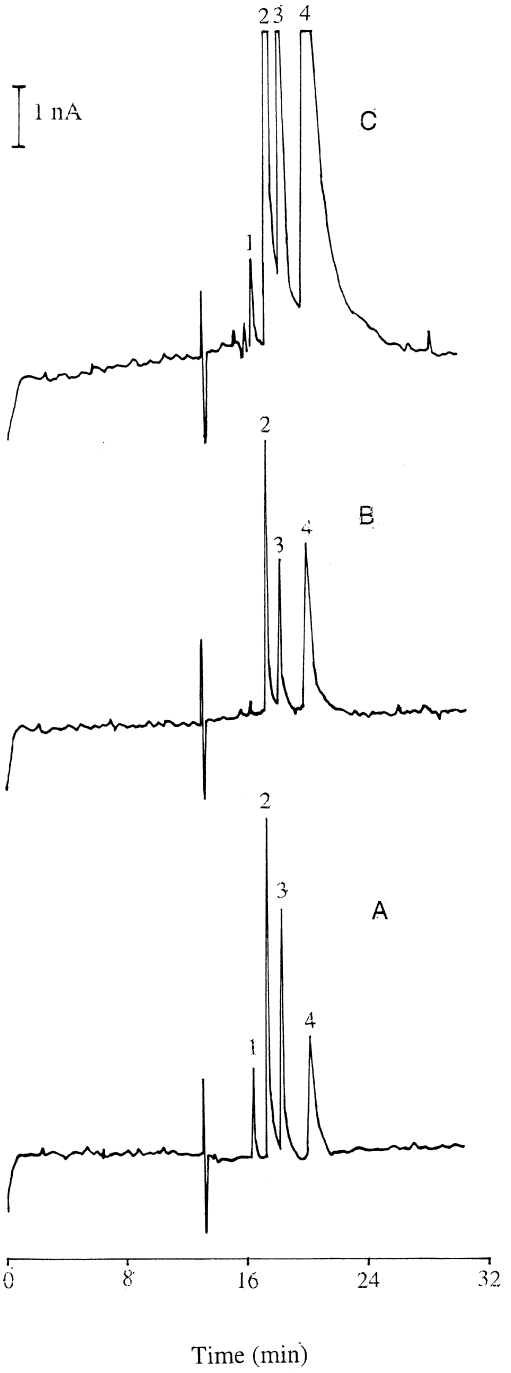

Fig. 5. Electropherograms of (A) a standard sample mixture (concentration in the range of 60 and 140 μ*M*) and flower extract samples diluted in (B) 1+9, and (C) 1+1 ratio. The flavonols are as: (1) KCG; (2) KG; (3) KGR; and (4) K. Other conditions are as in Fig. 2.

each standard solution into the extract sample. The concentrations of the four flavonols, as determined by comparison to calibration data, were 95.4 μ*M* for KCG following dilution of 1:1 and 104 μ*M*, 38.8 μ*M* and 167 μ*M* for KG, KGR and K following dilution 1:9, respectively. The actual amount of the KCG, KG, KGR, and K were 0.27, 1.1, 0.56 and 1.2% (g/g) respectively on a dry mass basis of the powdered balsam flower sample.

4. Conclusions

A CE separation coupled with electrochemical detection based on the sol–gel CCE provides a powerful tool for the determination of redox active compounds in balsam flowers. The CE separation of kaempferol and its derivatives was highly dependent on the pH of the separation buffer. The CE–ED system showed good stability for the separation and detection of these compounds. An application of this simple and sensitive method was successfully carried out for the analysis of a real sample. Further studies would involve the improvement of long-term response stability and the extension of this approach to other plant flower samples.

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