times poorer than the theoretical value. The reason is still the poor coupling of the LED beam with the small-diameter capillary, which cannot assure that all the light rays pass through the center of the capillary.

4. Conclusions

Diode-laser- and LED-based double-beam absorption detection systems for CE using digital cancellation were demonstrated. The background noise was reduced substantially. Better optical coupling of the laser beam with the small capillary is achieved for the diode-laser-based system. Both systems also benefited from the excellent intensity stability of the diode laser and the LED. The detectability is improved over the commercial double-beam CE system. The many wavelengths available for different LEDs make them versatile. Although LEDs are not strictly monochromatic, the linear correlation coefficient for the calibration curve can still be 0.999. Indirect detection can make the system more universally applicable. With the availability of shorter-wavelength diode lasers in the future, we believe these systems will find even broader application.

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Micellar electrokinetic capillary chromatography of limonoid glucosides from citrus seeds

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Abstract

This report presents the results of an investigation into the application of micellar electrokinetic capillary chromatography (MECC) for the analysis of limonoid glucosides in a citrus seed extract. MECC based on sodium dodecyl sulphate (SDS) was used to provide highly efficient separations of the closely related structures. A phosphate–borate buffer containing SDS was used to optimize the separation conditions for a mixture containing standard compounds of the glucosides of limonin, obacunone and nomilin. The study showed that MECC analysis of limonoid glucosides requires minimal sample clean-up steps and achieves excellent separations within a relatively short analysis time. The technique developed was used to quantify two limonoid glucosides in a methanol extract of the seeds of *Citrus nobilis* (nartjie) and *Citrus limon* (lemon).

1. Introduction

Limonoids are a group of chemically related triterpene derivatives which are isolated from plants of the *Meliaceae, Rutaceae* and *Cneoraceae* families. These limonoids are one of the two major bitter principles found in *Citrus*. Thirty seven limonoid aglycones have been isolated from *Citrus* and its hybrids and of these limonin is the major cause of the bitterness in citrus juices. It has been shown recently that limonoids also occur in fruit tissues and seeds of *Citrus* as nonbitter glucoside derivatives [1]. The names and structures of the major limonoid glucosides which have been isolated from *Citrus* are shown in Fig. 1. This discovery has explained the natural debittering process that occurs in fruit during the late stages of fruit growth and maturation. Limonoids have been shown to exhibit anticancer activity in laboratory animals [2–4] and antifeedant activity against insects and termites [5,6]. Citrus seeds contain a high concentration of limonoid glucosides and could therefore be utilized as a source of these important compounds.

Analysis of limonoid glucosides in citrus juices using thin layer chromatography (TLC) [7] and high-performance liquid chromatography (HPLC) [8] have been reported. Limonoid glucosides in citrus seeds have also been analyzed recently using TLC and HPLC [9]. However, the use of HPLC is restricted by the longer clean-up steps required, interference from flavonoids, the lengthy analysis time (at least 35 min) and unsatisfactory resolution between two of the five major limonoid glucosides. The purpose of

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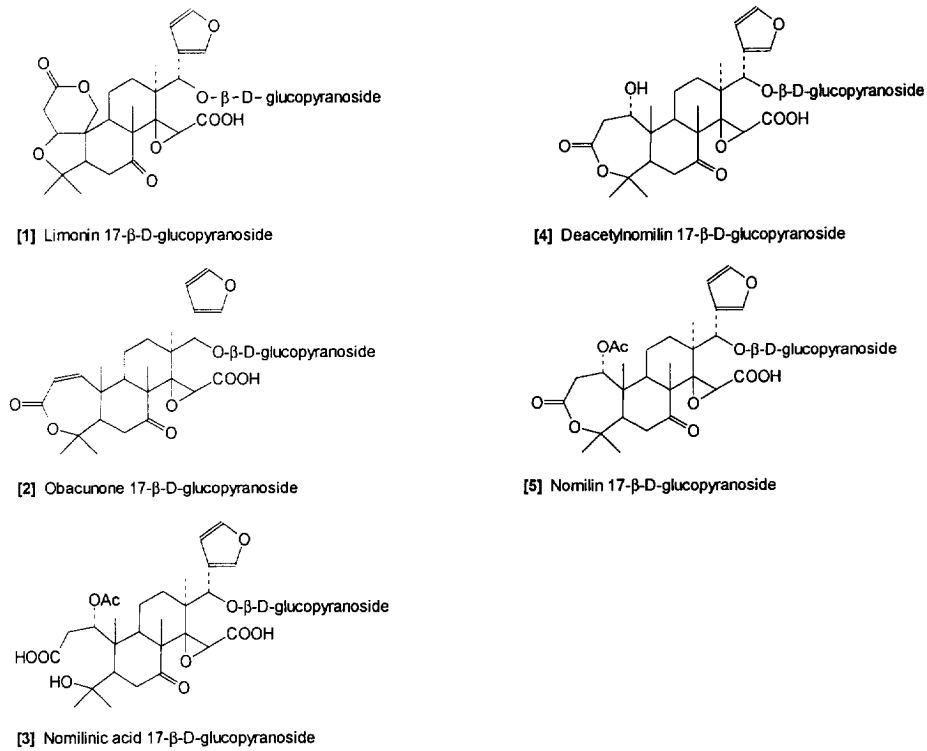

Fig. 1. Structures of the major limonoid glucosides present in *Citrus*.

this study was to establish a simple capillary electrophoretic (CE) procedure for the separation and quantitation of limonoid glucosides in citrus seeds.

Capillary electrophoretic techniques offer very high separation efficiencies, relatively short analysis times and represent an attractive alternative to HPLC methods [10]. Although CE has been applied to the analysis of a wide variety of complex mixtures [11–13], it is limited to the separation of ionic substances. However, in micellar electrokinetic capillary chromatography (MECC), a buffer solution that contains a surfactant (e.g., SDS) at, or above, its critical micelle concentration (cmc) is used as the electrophoretic medium. As a result, a pseudo stationary phase is produced, enabling the separation of both charged and neutral solutes in a single analysis according to differential distribution of the solutes between the aqueous and micellar phases. MECC based on micellar solubilization was first reported in 1984 by Terabe et al. [14]. Since then the technique has been well documented for a wide variety of applications [15–18]. We report for the first time a novel MECC method for the rapid, reproducible and efficient analysis of limonoid glucosides in citrus seeds.

2. Experimental

2.1. Materials

Citrus nobilis and *Citrus limon* were obtained locally. The seeds were dried in a refrigerator for 3 days and then ground finely in a coffee grinder.

2.2. Sample extraction

The seed meals of *Citrus nobilis* (12.0 g) and *Citrus limon* (9.0 g) were extracted for 24 h with refluxing *n*-hexane in a Soxhlet extractor to remove oils and non-glucosylated limonoids. The seed material was thereafter extracted for a further 24 h with refluxing methanol in a Soxhlet

extractor. The methanol extract was evaporated to dryness and the residue was re-extracted with 6 ml of methylene chloride–water (1:1). The water fraction contained the limonoid glucosides and was analyzed directly.

2.3. Reagents and chemicals

The separation buffer consisted of 20 m*M* sodium dihydrogen phosphate (BDH, Poole, UK), 12 m*M* disodium tetraborate (BDH) and 35 m*M* sodium dodecyl sulphate (Sigma, St. Louis, MO, USA) and was prepared in doubly deionised water (Millipore Corp., USA). All samples and standards were filtered through a 0.45-μm syringe filter (Lida, Kenosha, USA). All chemicals were of analytical-reagent grade.

2.4. Standards of limonoid glucosides

In this study, standards of the 17-β-D-glucopyranosides of limonin, obacunone and nomilin were used. They were isolated and characterised by nuclear magnetic resonance, infra-red and mass spectroscopic techniques.

2.5. Instrumentation

The qualitative and optimization experiments were carried out on a laboratory built CE system and the quantitative experiments were performed on a Beckman P/ACE System 2000 (Beckman Instruments, Palo Alto, CA, USA). The laboratory built system consisted of a 0–30 kV high voltage power supply (Series 230, Bertan High Voltage, Hicksville, NY, USA) and a variable-wavelength UV detector (Spectrasystem UV 1000, Spectra-Physics, San Jose, CA, USA) that was fitted with an on-column capillary cell (Model 9550-0155, Linear Instruments, NV, USA). The platinum electrodes were housed in a perspex box which was equipped with an interlock for operator safety. Fused-silica capillaries (50 μm I.D., 220 μm O.D.) were obtained from SGE (Melbourne, Australia). The capillary length was 68 cm (43 cm from the injection end to the detector window). The separations were carried out at ambient temperature and detection was at 210 nm. The electropherograms were recorded with an electronic integrator (Model SP 4200, Spectra-Physics). Hydrodynamic injections were made by lowering the vial at the detector end by 4 cm below its normal position and inserting the upstream end of the capillary into the sample vial for 5 s.

The Beckman system consisted of a 0–30 kV high-voltage built-in power supply, a selectable fixed-wavelength UV detector, a temperature-controlled column cartridge (75 μm I.D.) and an autosampler. The capillary length was 50 cm (43.5 cm from the injection end to the detector window). All experiments were carried out at 25°C at a constant voltage of 25 kV. Injections were made using the pressure mode for 2 s each and the peaks were monitored at 214 nm. The Gold software for system controlling and data handling was used. The electropherograms were recorded on an IBM PS/2 Model 55 SX computer.

2.6. Calibration graphs

The calibration graphs were obtained from standard solutions containing limonin glucoside and nomilin glucoside in the concentration range 0.15–1.5 mg/ml in doubly deionised water. Since only a small amount of obacunone glucoside standard was available it was excluded from the quantitative study.

3. Results and discussion

Preliminary experiments were conducted to separate the limonoid glucosides using capillary zone electrophoresis (CZE) conditions. CZE was unable to resolve limonin and obacunone glucoside in the pH range 6.0–7.5. The upper limit of the pH was set at 7.5 because nomilin glucoside is converted to obacunone glucoside at a pH greater than 7.5 [19].

The three limonoid glucoside standards were estimated from pH measurements to have pK_a values close to 4.3. The difference in ionization of the carboxylic group in the limonoid skeletons was not great enough to allow differentiation

among the electrophoretic mobilities of limonin and obacunone glucosides. However, nomilin glucoside had a sufficiently different charge-to-mass ratio to be well separated from the other two (Fig. 2A).

The technique which gave successful separations of these compounds was MECC (Fig. 2B,C). The separation mechanism is primarily based on differences in hydrophobic interactions for all of the compounds, as well as differences in their electrophoretic mobilities. The effect of SDS concentration on the separation of the three limonoid glucoside standards is shown in Fig. 3. The overall decrease in the migration time values of limonin and nomilin glucosides in the concentration range 0–50 m*M* SDS may indicate an increase in the temperature of the capillary [20,21] or perhaps less interaction between the SDS micelles and these two glucosides. However, obacunone glucoside showed increased migration times as the concentration of SDS was increased, and was increasingly separated from limonin glucoside but tended to migrate closer to nomilin. The increase in migration time of obacunone glucoside relative to the other two is probably due to the increased hydrophobicity of obacunone glucoside compared with limonin glucoside or nomilin glucoside. As can be seen in Fig. 3, the best separation was obtained at an SDS concentration of 32 m*M*.

The migration times obtained for the components present in the *Citrus limon* extract at different pH values are shown in Fig. 4. As expected, the migration times decrease with an increase in buffer pH due to an increase in the electroosmotic flow velocity. At a pH of 6.0 the migration times were excessively high and the peaks were broadened. The peak shape improved as pH was increased from 6.5 to 7.5.

The separation of the limonoid glucosides in *Citrus limon* is shown in Fig. 5 at three different pH values. As can be seen excellent baseline separations were obtained at pH 6.5 and 7.0. Since the analysis time was approximately 2 min shorter at pH 7.0, this condition was chosen for subsequent analysis of the citrus extracts. Furthermore, during pH optimization studies at 25 kV, current fluctuations were experienced on

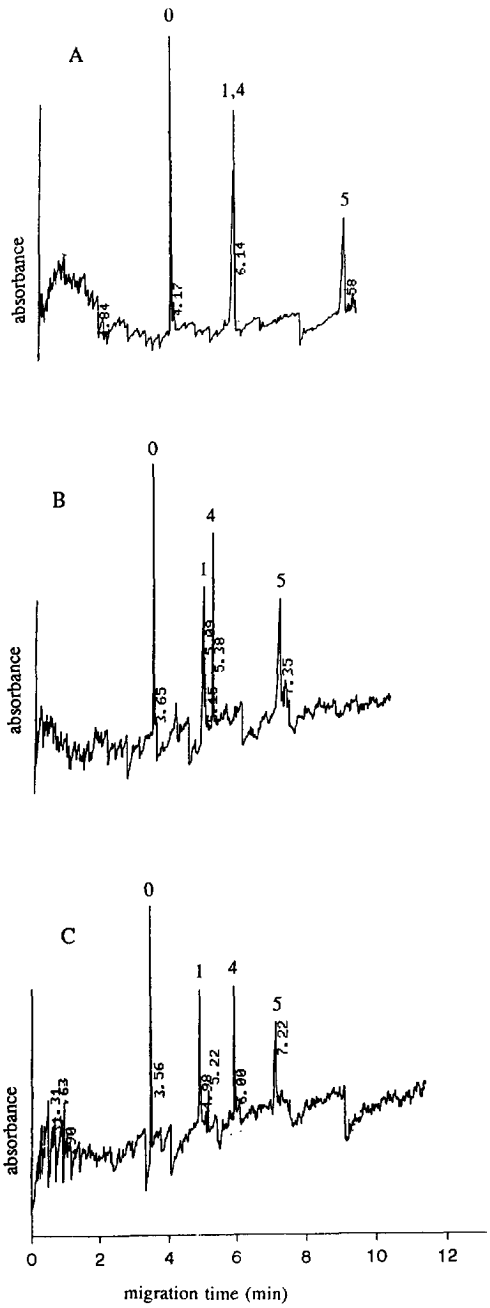

Fig. 2. Electropherograms of a mixture of limonoid glucoside standards showing the separation at (A) 0 m*M* SDS, (B) 10 m*M* SDS, (C) 30 m*M* SDS. Conditions: pH 7.0; 20 m*M* NaH_2PO_4; 12 m*M* $Na_2B_4O_7$; 25 kV; 43 cm (detection point); 68 cm (total length) × 50 μm I.D. untreated fused-silica capillary. Peaks: 0 = formamide (neutral marker); 1 = limonin glucoside; 4 = obacunone glucoside; 5 = nomilin glucoside.

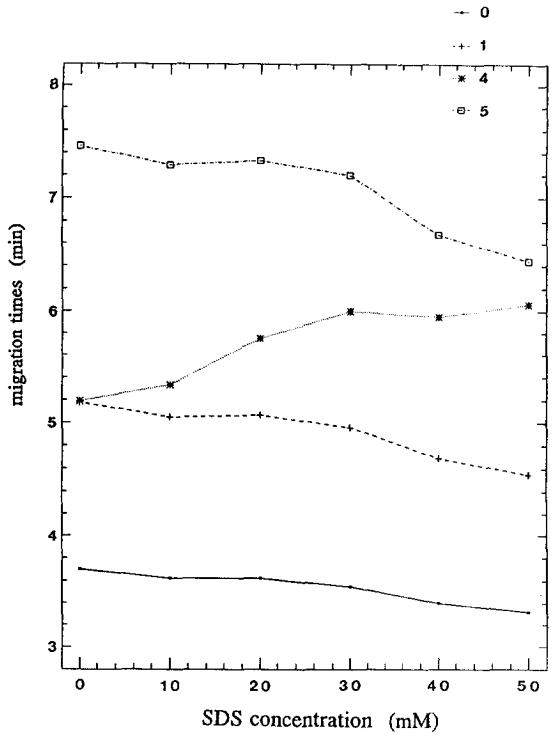

Fig. 3. Variation of migration time of limonoid glucoside standards with SDS concentration. Conditions and numbers as in Fig. 2.

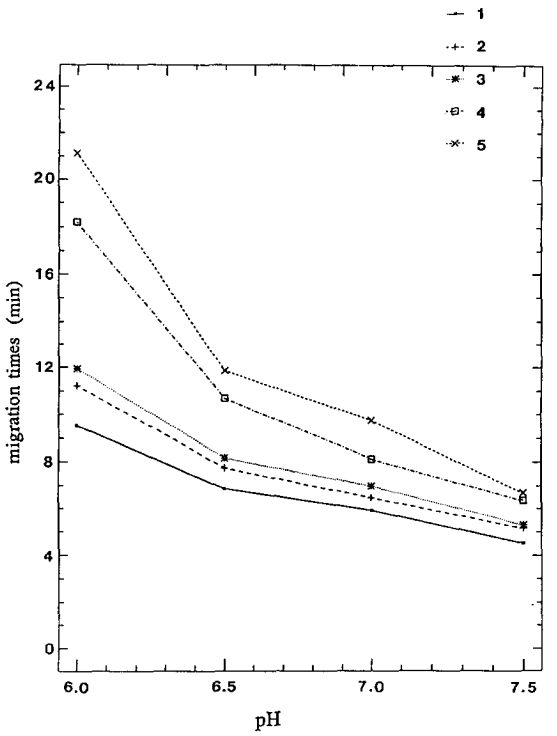

Fig. 4. Effect of pH on the migration time of limonoid glucosides in *Citrus limon*. Conditions: 32 m*M* SDS; 22 kV; other separation conditions as in Fig. 2. Peaks: 1 = limonin glucoside; 2 = unidentified; 3 = unidentified; 4 = obacunone glucoside; 5 = nomilin glucoside.

the laboratory built system at pH 7.5. It was therefore necessary to lower the applied voltage to 22 kV when separations were done at a pH of 7.5. Peaks 1, 4 and 5 were identified by comparison of the migration times with the three standards that were available. The separation of the limonoid glucosides in *Citrus limon* and *Citrus nobilis* is shown in Fig. 6 together with the separation of a standard solution containing limonin, obacunone and nomilin glucosides. Good separations were obtained for both extracts and except for the relative amounts of the glucosides, the electropherograms are very similar.

The water fraction of the methylene chloride–water extract of *Citrus limon* and *Citrus nobilis* was analyzed for limonin glucoside and nomilin glucoside. Quantitative information was obtained by using the external standard method. The peak areas were normalized by dividing the peak area by the migration time. The calibration graphs for limonin glucoside and nomilin glucoside showed good linearity in the concentration range 150–1500 μg/ml.

For the regression equation $y = ax + b$, where x is the concentration of limonoid glucosides (μg/ml) and y is the normalized peak area, the correlation coefficients (r) were as follows: for limonin glucoside, $y = 12.2167 \cdot 10^{-5}x + 283.3000 \cdot 10^{-5}$ ($r = 1.000$); for nomilin glucoside, $y = 11.3359 \cdot 10^{-5}x - 62.3250 \cdot 10^{-5}$ ($r = 0.999$). The results of the quantitation for limonin glucoside and nomilin glucoside are shown in Table 1. The results are within the expected limits of these compounds in citrus seeds found by other methods [19].

The recovery of limonoid glucosides was tested by adding known amounts of limonin and nomilin glucosides to a sample water fraction of