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Analysis of sugarcane flavonoids by capillary zone electrophoresis

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ABSTRACT

The quantitative analysis of flavonoids by capillary zone electrophoresis (CZE) was investigated. Analytical conditions were varied to optimise the separation of the major flavone class of flavonoids in sugarcane. Separation of flavonoids from sugarcane was most influenced by the running buffer pH and addition of methanol. Best separation of sugarcane flavones was obtained with a running buffer of 25 m*M* borate, pH 9.5 with 20% methanol added. Using a simple acetonitrile–water extraction, CZE could be used to quantify flavonoids in sugarcane. R.S.D. values for the flavonoids detected in two cultivars of sugarcane ranged from 2.1 to 7.7, mean 4.8.

INTRODUCTION

Flavonoids are a widely studied class of plant secondary metabolites, widespread throughout the plant kingdom. They have a variety of roles in plants including: involvement in pest [1] and disease [2] resistance, contribution to flower colour, etc. [3], protection against harmful UV radiation [3], and involvement in the association of nitrogen fixing bacteria with legumes [3]. The pattern of flavonoids has been use for determining the chemotaxomony of plants [4].

Smith and Paton [5] have reviewed the flavonoids of sugarcane. All reported from sugarcane or its products belong to the five flavonoid classes; flavones, flavonols, anthocyanins, catechins and chalcones. The major flavonoids in sugarcane are the flavones, of which twenty have been reported. All are derivatives of either tricin, apigenin or luteolin. The structures of some of these flavones are given in Fig. 1. No physiological role has been identified for flavonoids in sugarcane, but two unidentified flavones are present in sugarcane resistant to red rot disease of roots and absent in susceptible varieties [6]. The pattern of flavonoids found in sugarcane leaves varies with sugarcane species and has been used to investigate the origin of the *Saccharum* complex [7]. Flavonoids are also present in raw sugar made from sugarcane where they make up a significant portion of the contaminating colorants.

Analysis of flavonoids is usually performed by paper chromatography (PC), thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) [8], with gas chromatography (GC) being used less commonly. Selectivity of PC and TLC is very good [9], but the separated flavonoids are difficult to quantify. Quantification is more easily achieved using HPLC but separation of structurally similar flavonoids is often difficult and each analysis is time consuming, typically greater than 80 min. Solvent gradients are necessary for good separation, requiring relatively sophisticated HPLC systems.

Capillary electrophoresis (CE) is a relatively new technique with considerable potential for

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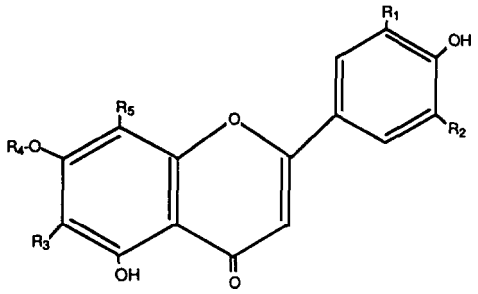

	R_1	R_2	R_3	R_4	R_5
apigenin	H	H	H	H	H
schaftoside	H	H	Glu	H	Ara
isoschaftoside	H	H	Ara	H	Glu
luteolin	OH	H	H	H	H
swertiajaponin	OH	H	Glu	CH_3	H
orientin	OH	H	H	H	Glu
is0 orientin	OH	H	Glu	H	H
tricin	OCH_3	0CH3	H	H	H
tricin 7-glucoside sulphate	OCH_3	OCH_3	H	Glu	H
tricin 7-rhamnosylgalacturonide	OCH_3	OCH_3	H	Rha-galur	H

Fig. 1. Structures of some flavones found in sugarcane.

analysing charged compounds. The application of micellar electrokinetic capillary chromatography (MECC) to the analysis of flavonol-3-O-glycosides and the comparison with reversed-phase HPLC have been reported [10]. The analysis of anthocyanin flavonoids by CE has been reported in a commercial application note [11], but individual flavonoids were not identified. Recently the analysis of pharmaceutically important flavonoids by isotachophoresis [12] and capillary zone electrophoresis (CZE) [13] in plant extracts has been reported.

This report presents the results of investigations into the application of CZE for the analysis of flavone flavonoids in sugarcane extracts. CZE separation conditions were optimised for sugarcane flavones and the technique used to analyse flavones in two commercial sugarcane varieties.

EXPERIMENTAL

Reagents

Borate buffers were prepared at a concentration of 25 m*M* over a pH 9.0-10.5 range. Boric acid was obtained from Bio-Rad (Richmond, CA, USA). Methanol (HiPerSolv grade), glycerol, acetonitrile, sodium tetraborate were all obtained from BDH (Poole, UK). Hydroxypropylenemethylcellulose (HPMC) was obtained from Sigma (St. Louis, MO, USA). Milli-Q (Millipore, Bedford, MA, USA) water was used in all buffer preparations.

The flavonoids apigenin, quercetin and rutin were obtained from Sigma. All other flavonoids were extracted and partially purified from sugarcane (cultivars Q96 and Q123). Identification of these flavonoids was by HPLC with reference flavonoids, provided by N.H. Paton, CSR Ltd. (Sydney, Australia), which had previously been purified and identified [14].

The purchased flavonoids were dissolved in methanol at a concentration of 200 μg/ml. A mixture of sugarcane flavones was prepared by combining several partially purified flavones together. This mixture contained several flavonoids of unknown identity.

Sugar-cane extraction

Rind was removed from sugarcane stalks with

a household vegetable peeler and chopped to a homogeneous powder in a Waring blender. Subsamples (0.5 g) were extracted with 2.5 ml 80% acetonitrile in milli-Q water for 24 h. The extraction comprised of 4 h at 50°C, followed by 20 h on a wrist-action shaker. A small portion (200 μl) of extract was clarified by centrifugation (17 000 *g*, 5 min) before analysis by CZE.

Capillary electrophoresis

CE was performed on an Applied Biosystems capillary electrophoresis system Model 270A-HT. Unless otherwise stated in the text or captions, the conditions were: the capillary was uncoated and 72 cm x 50 μm I.D. (50 cm from autosampler to detector), detection was at 395 nm, applied voltage was 30 kV (417 V/cm), the cathode was at the sample end of the capillary and anode at the detector end, and samples were introduced by vacuum injection of 1.5 s at 5 in.Hg (1 in.Hg = 3386.38 Pa). The manufacturer's recommendations were followed for capillary conditioning and flushing. Electropherograms were recorded on a Spectra-Physics SP4270 computing integrator. Flavonoid concentrations were calculated as apigenin equivalents.

An aqueous solution of benzene was used to measure the electroendosmotic flow in the capillary.

RESULTS AND DISCUSSION

In the CE system used, anions move towards the anode at the sample end of the capillary. Movement of anions towards the detector is a result of greater bulk electroendosmotic flow (EOF) of buffer through the capillary towards the cathode at the detector end of the capillary. Therefore highly charged small flavonoid molecules will have a longer total migration time compared with molecules of less charge and of greater size, due to different phenolic and sugar substitution patterns. Polyphenolics such as flavonoids are weak acids with pK_a values in the range of pH 9-10 [15], and will be charged at high pH. The molecular size of flavonoids also increases when phenolic groups are glycosylated at various positions.

Electropherograms of a synthetic flavone mixture analysed at different pH are shown in Fig. 2. The synthetic mixture contained flavone glycosides present in sugarcane, plus the aglycone flavone apigenin. These flavone glycosides elute in a narrow retention time window with reversed-phase HPLC. Typical of CZE, the buffer pH had a marked effect on total migration time and separation of all flavones in this mixture. Fig. 2 clearly shows that migration times increase with increasing pH. The net charge of flavonoid molecules increases due to the progressive removal of phenolic hydrogens. Additional-

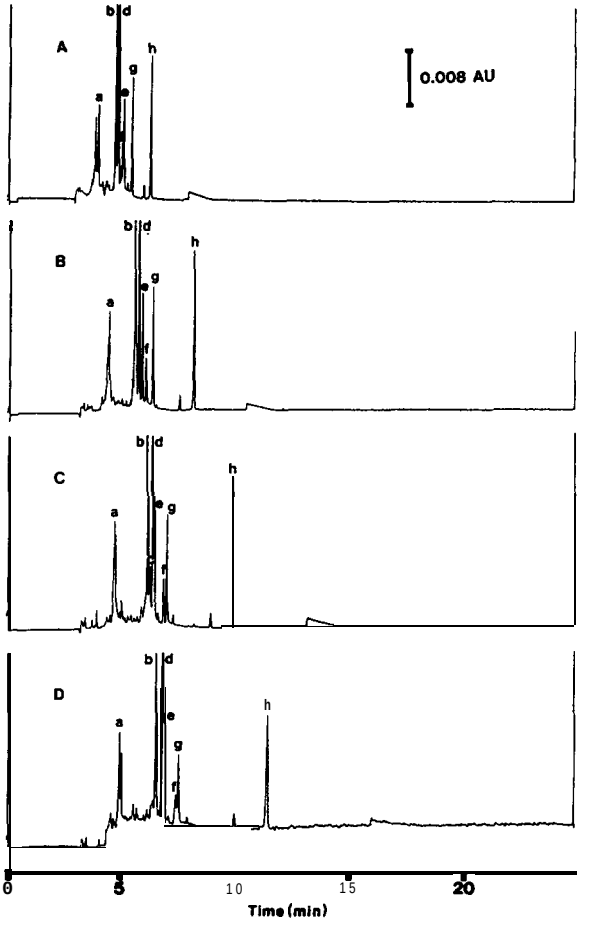

Fig. 2. Electropherograms of a flavone mixture analysed by CZE at different pH values. (A) pH 9.0; (B) pH 9.5; (C) pH 10.0; (D) pH 10.5. Peaks: a =swertiajaponin; b = schaftoside; c = tricin 7-rhamnosylgalacturonide; d = isoschaftoside; e = unidentified flavonoid; f = unidentified flavonoid; g = orientin; h = apigenin. Experimental conditions were: fused-silica capillary, total length 72 cm, length to detector 50 cm; buffer, 25 m*M* borate at indicated pH values; voltage, 30 kV (417 V/cm); detection, 395 nm; temperature, 35°C; injection time 1.5 s.

ly, at higher pH the width of some flavonoids peaks increased, possibly indicating chemical breakdown of these flavonoids. The rate of increase of migration time varied between flavonoids resulting in changes in the separation of flavonoids. For example, at pH 9.5 isoschaftoside (peak d) and the two unidentified flavonoids (peaks e and f) were well separated. At pH 10.5 the first unidentified flavonoid (peak e) is only just separated from isoschaftoside. Generally, EOF increases with increasing pH, but at these higher pH values EOF did not change appreciably between pH 9.0 and 10.5. For the separation these flavonoids a pH of 9.5 was chosen for further optimisation.

Addition of organic modifiers can modify the EOF and separation in both CE and isotachophoresis [12,16]. Addition of modifiers such as methanol and methylcellulose decreases EOF, whereas acetonitrile increases EOF. The effect of adding various amounts of methanol on the separation of the synthetic mixture of flavonoids is shown in Fig. 3. Increasing the amount of methanol increased the migration time and improved the separation of these flavonoids. Increased migration time resulted from decreasing EOF, whereas changes in migration order are probably caused by changes in flavonoid-borate complex solvation or stability due to the added methanol. The best separation was obtained with 20% methanol, particularly for flavonoids that have short migration times similar to swertiajaponin. However, total analysis time was more than three times longer than without added methanol. The addition of hydroxypropylenecellulose at concentrations of 0.01, 0.05 and 0.1%, to increase buffer viscosity, had little effect on the separation of these flavones although EOF was reduced slightly.

An investigation of the effects of borate concentration on migration time showed that higher concentrations of borate increased migration times as shown in Fig. 4. This increase of migration time is partly a result of decreasing EOF but relative migration times also changed possibly due to changes in stability of the flavonoid-borate complexes with increasing borate concentration as suggested by Sietz *et* al. [13]. Current increases dramatically at higher

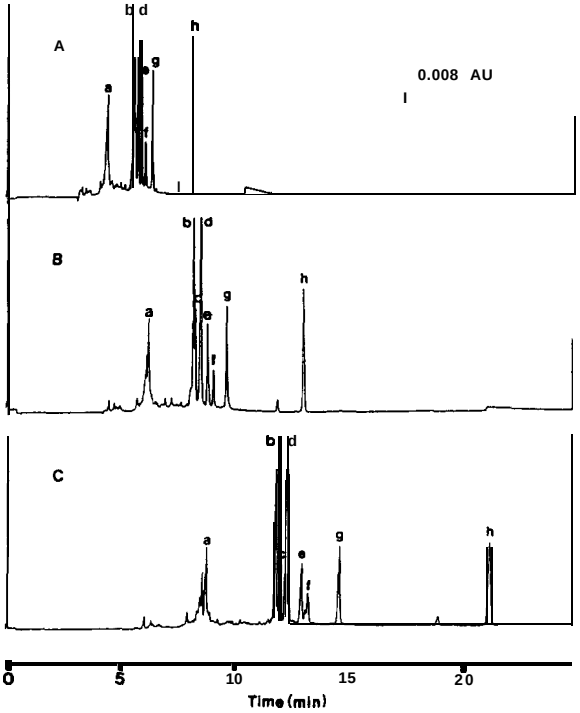

Fig. 3. Electropherograms of a flavone mixture analysed by CZE at pH 9.5 with different methanol concentrations. (A) 0%; (B) 10%; (C) 20% methanol. Peaks: a= swertiajaponin; b = schaftoside; c = tricin 7-rhamnosylgalacturonide; d = isoschaftoside; e = unidentified flavonoid; f = unidentified flavonoid; g = orientin; h = apigenin. Experimental conditions were: fused-silica capillary, total length 72 cm, length to detector 50 cm; buffer, 25 m*M* borate at pH 9.5, methanol concentration as indicated; voltage, 30 kV (417 V/cm); detection, 395 nm; temperature, 35°C; injection time 1.5 s.

borate concentrations and at an applied voltage of 30 kV the currents obtained were 36 and 120 μA for 10 and 60 m*M* borate buffers respectively. In CZE the number of theoretical plates reaches a maximum with increasing electric field strength [16] then decreases with increasing heat production resulting from high currents. Therefore, it is desirable to use a low concentration of borate and add methanol to obtain optimum flavone separation. This maximises theoretical plate numbers through the use of high electric field strengths and avoids excessive heat generation from high currents.

The temperature of the capillary also affects EOF. At 50°C EOF increases by 21% compared with EOF at 30°C. Increasing the capillary tem-

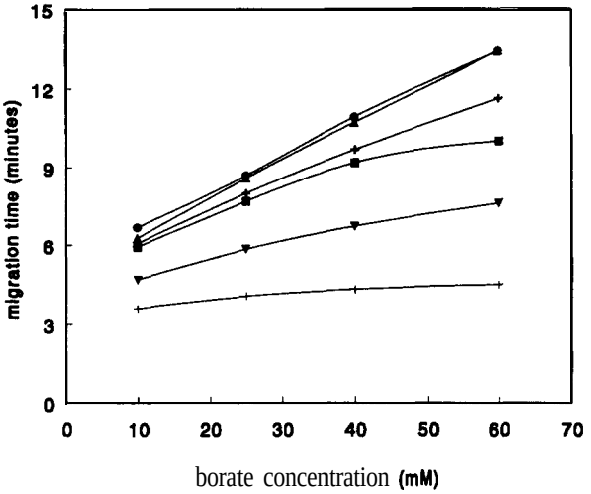

Fig. 4. The effect of increasing borate concentration on flavonoid migration time. + = EOF; ▼ = swertiajaponin; ■ = isoschaftoside; + = schaftoside; A = unidentified flavonoid f; ● = orientin. Experimental conditions were: fused-silica capillary, total length 72 cm, length to detector 50 cm; buffer, 10, 25, 40 and 60 m*M* borate at pH 9.5, 20% methanol; voltage, 30 kV (417 V/cm); detection, 395 nm; temperature, 35°C; injection time, 3.0 s.

perature decreased overall migration times and reduced the time for each analysis with little affect on the separation of flavonoids.

Based on the preceding data the following operating conditions were chosen. The operating buffer was composed of 20% methanol in 25 m*M* borate at pH 9.5, voltage was 30 kV (417 V/cm) and the temperature of the capillary was maintained at 35°C. Using these conditions an operating current of 28 μA was obtained.

Selection of the absorption maxima for flavones increases selectivity for flavonoids. The optimum wavelength for detecting flavones at pH 9.5 was experimentally determined to be 395 nm for apigenin. The wavelength selected for detection of flavones must allow for the bathometric shift of polyphenolic compounds at high pH and will be approximately 400 nm for flavones, but different for other flavonoids. The optimum wavelength for detecting flavonoids is greater than that used for HPLC detection where the eluant if usually acidic.

A calibration curve was prepared for apigenin (lo-200 μg/ml) based on peak area and height. The calibration curve for peak area was linear between 10 and 200 μg/ml (r = 0.999815, equation: y = 3.615~ − 6.643), whereas the best fit for peak height was a second order polynomial curve (r = 0.99956, equation: y = 0.2732 + 0.2578.x − 0.00037x^2), confirming that CZE can be used to quantify flavonoids using either peak area or peak height. Using the described instrumental conditions, the minimum detectable concentration of apigenin was 1.5 μg/ml (3 × SIN).

To demonstrate the application of CZE for the analysis of flavonoids in sugarcane, two commercial cultivars were analysed as described. Electropherograms are shown in Fig. 5. Previous analysis by PC and HPLC has shown that these varieties have a different flavonoid content [17] which is also evident in this analysis. The results of replicated analyses (Table I) show the good reproducibility of acetonitrile-water extraction followed by CZE analysis for flavonoids. Sample flavonoid concentrations were calculated as apigenin equivalents as analytical standards were not available for the flavones present in sugarcane.

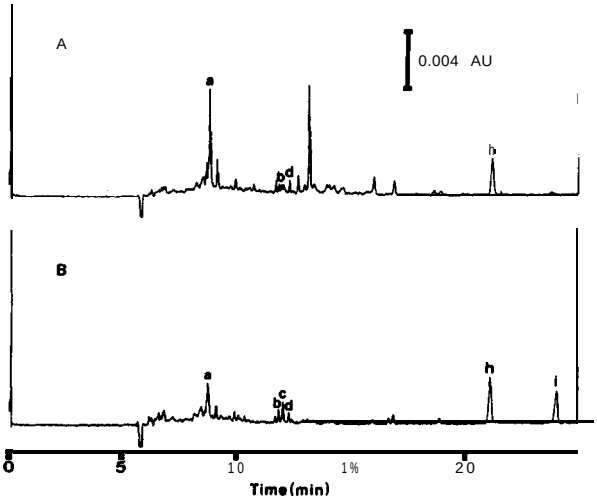

Fig. 5. Electropherograms of sugarcane extracts, cultivars Q96 (A) and Q123 (B). Peaks: a = swertiajaponin; b = schaftoside; c = tricin 7-rhamnosylgalacturonide; d = isoschaftoside; h = apigenin; i = tricin 7-glucoside sulphate. Experimental conditions were: fused-silica capillary, total length 72 cm, length to detector 50 cm; buffer, 25 m*M* borate at pH 9.5, methanol 20%; voltage, 30 kV (417 V/cm); detection, 395 nm; temperature, 35°C; injection time 1.5 s.

TABLE I

ANALYTICAL RESULTS SHOWING THE REPRODUCIBILITY OF CZE OF FLAVONOIDS IN SUGARCANE

Concentrations have been calculated as equivalent mg/kg apigenin. n.d. = Not detected.

Flavonoid	Q96		Q123	
	Concentration (mg/kg)	R.S.D. (%) ($n = 5$)	Concentration (mg/kg)	R.S.D. (%) (n = 4)
Swertiajaponin	164	2.1	53	3.4
Schaftoside	15	7.0	18	3.8
Isoschaftoside	19	7.0	15	7.7
Orientin	132	3.3	n.d.	–
Tricin 7-rhamnosylgalacturonide	n.d.	–	26	4.1
Tricin 7-glucoside sulphate	n.d.	–	43	4.9
Apigenin (internal standard)	64	6.5	61	2.7

These results demonstrate that analysis of flavonoids by CZE is a viable alternative to analysis by PC and HPLC. Separation of flavonoids by electrophoretic mobility is complementary to separation by reversed-phase HPLC and can be optimised for a particular set of flavonoids. Additionally, CZE is quantitative and analysis times are much shorter compared with either pc or HPLC.

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