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CAPILLARY ZONE ELECTROPHORESIS (CZE) OF FLAXSEED PHENOLIC CONSTITUENTS – A SHORT REPORT

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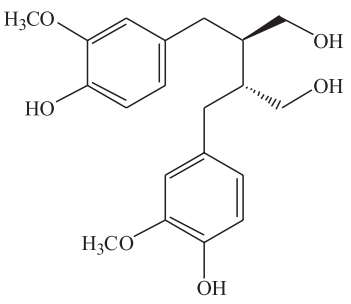
Phenolic compounds were extracted from flaxseed using 80% (v/v) methanol. The resultant crude extract was hydrolysed under basic conditions. Secoisolariciresinol diglucoside (SDG) was separated from the prepared extract using semi-preparative HPLC. Secoisolariciresinol (SECO) was liberated from SDG by acid hydrolysis. The chemical structures of SDG and SECO were confirmed by ESI-MS. Capillary zone electrophoresis (CZE) was employed for the separation of phenolics from the crude extract, the extract resulting after base hydrolysis, and the two lignans. CZE was found to offer a quicker analysis of SDG with higher separation efficiency than with a conventional HPLC methodology. The retention time for SDG was noted at 5.33 min. The migration times of SDG and SECO were close together; a base line separation of both lignans was not achieved. The electropherogram of the crude flaxseed extract showed only one broad peak with a retention time of 6.02 min.

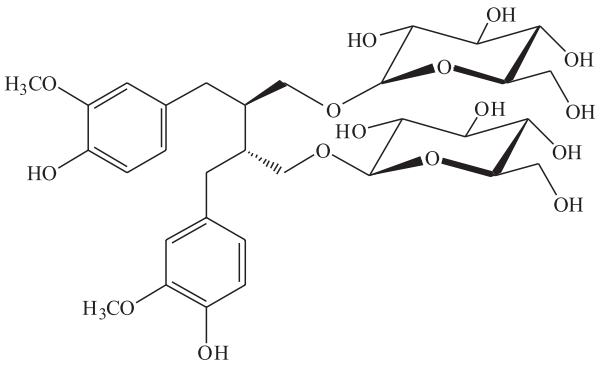
INTRODUCTION

Flaxseed (*Linum usitatissimum* L.) is the richest known natural source of α-linolenic acid, an *ω*-3 fatty acid, and lignans. Omega-3 fatty acids suppress the production of interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α) and leukotriene B_4 (LTB_4). Furthermore, the *ω*-3 quench reactive oxygen species (ROS) produced by polymorphonuclear leukocytes (PMNLs) and monocytes, possess anti-platelet activating factor (PAF) activity, and are antioxidants. PAF, IL-1, TNF-α, and LTB_4 are known to stimulate PMNLs to produce ROS. Dietary flaxseed supplementation can, therefore, prevent hypercholesterolemia-related heart attack and strokes [Prasad, 1997; Edralin *et al.,* 2004].

Secoisolariciresinol diglucoside (SDG) (Figure 1) is the predominant phenolic constituent of flaxseed [Meagher *et al.*, 1999]. SDG is a lignan, and its content in defatted flaxseed approaches 1-3% [Johnsson *et al.*, 2000]. This lignan exerts a phytoestrogenic effect by acting as a precursor of "mammalian" lignans: enteroradiol and enterolactone [Axelson *et al.*, 1982]. A reduced breast cancer risk has been reported for subjects with high urine and plasma levels of enteroradiol and enterolactone [Boccardo *et al.*, 2004]. SDG may also protect men against prostate and colon cancer [Jenab & Thompson, 1996; Adlercreutz, 2002; Lin *et al.,* 2002]. The significant antioxidant activity of SDG was reported by Niemeyer & Metzler [2003]. According to Hu *et al.* [2007], the antioxidant efficacy of SDG and its aglycone secoisolariciresinol (SECO – Figure 1) against AAPH-induced lipid peroxidation in liposome systems was attributed to the 3-methoxy-4-hydroxyl substituents.



Secoisolariciresinol
(SECO)



Secoisolariciresinol diglucoside
(SDG)

FIGURE 1. Chemical structure of secoisolariciresinol diglucoside (SDG) and secoisolariciresinol (SECO).

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The proposed mechanism underlying the anticarcinogenic and antiatherogenic effects of flaxseed and SDG involves the antioxidant activities of SDG and its mammalian lignan metabolites, enterodiol and enterolactone [Yuan *et al*., 1999]. The high content on lignans in flaxseed makes the seeds of this crop a healthful choice of raw material for food applications under the umbrella of functional foods [Oomah & Mazza, 1998].

RP-HPLC is a typical method employed for the analysis of SDG and other flaxseed phenolic compounds [Johnson *et al.,* 2000, 2002; Eldin-Kamal *et al.*, 2001; Charlet, 2002]. In a previous publication [Zduńczyk *et al*., 2004], micellar electrokinetic chromatography (MEKC) was used for SDG analysis. The present study, however, reports on a facile capillary zone electrophoretic method for the separation of SDG, SECO, and other phenolic components from the crude and hydrolysed extracts of flaxseed and compares the findings to those from RP-HPLC and MEKC.

MATERIALS AND METHODS

Plant material

Flaxseeds (*Linum usitatissimum* L.) from the current crop year were obtained from a local drug store in Olsztyn and used in this study.

Extraction

Seeds were ground in a commercial coffee mill and then defatted with petroleum ether in a Soxhlet apparatus for 6 h. Phenolic constituents were extracted from the defatted meal with 80% (v/v) methanol at a material-to-solvent ratio of 1:10 (w/v) at 50°C for 30 min [Amarowicz *et al.*, 1995]. Extraction was carried out in dark-coloured flasks using a constant--temperature shaking water bath. The extraction process was repeated two more times; supernatants were combined and methanol was evaporated off under vacuum at 40°C. The prepared extract was stored at 4°C until further investigated.

Base hydrolysis and RP-18 column chromatography

SDG is present in flaxseed in the form of a polymeric powder [Eldin-Kamal *et al.*, 2001; Johnsson *et al.*, 2002]; therefore before analysis, a hydrolysis must be carried out. In this study, the extract was subjected to base hydrolysis (1 g of extract suspended in 20 mL of 0.3 mol/L aqueous NaOH) for 2 days at room temperature followed by acidification to pH 3 using 2 mol/L HCl [Johnson *et al.*, 2000]. The hydrolysed extract was applied onto a chromatographic column (14 × 2 cm) packed with RP-18 gel (40-63 μm, Merck, Darmstad, Germany). Sugars and salts were removed from the column using 200 mL of distilled water. Then, phenolics were eluted with 200 mL of anhydrous methanol and the eluate was concentrated to dryness using a rotary evaporator.

Semi-preparative HPLC of SDG

Pure SDG was separated from the base hydrolysed fraction material using a semi-preparative HPLC method. A Shimadzu chromatographic system (Kyoto, Japan) consisting of a LC-10A pump, SPD-M10A UV-VIS diode array detector, SCL-10A system controller, semi-preparative LUNA C_{18} column (250 × 10 mm, 5 μ; Phenomenex, Torrance, CA, USA) was employed. The mobile phase comprised water-acetonitrile-acetic acid (88:10:2; v/v/v) [Amarowicz & Weidner, 2001] administered at a flow rate of 4 mL/min. An injection volume of 500 μL was delivered, and the UV detector was set at 280 nm.

SECO preparation

SECO was liberated from SDG after acid hydrolysis. Briefly, SDG (50 mg) was dissolved in 10 mL of 2 mol/L HCl and hydrolysed for 1 h at 100°C. Then, the sample was neutralised using 3.33 mL of 2 mol/L NaOH, and water was evaporated using a rotary evaporator. The dry residue was dissolved in 5 mL of methanol and subjected to semi preparative HPLC separation. The protocol used for SDG was employed for SECO. Separation was achieved with gradient elution using acetonitrile-water-acetic acid (5:93:2, v/v/v) [solvent A] and acetonitrile-water-acetic acid (40:58:2, v/v/v) [solvent B], 0-50 min solvent B from 0 to 100%. The flow rate was 4 mL/min and an injection volume of 500 μL was used. The UV detector was set at 280 nm.

Mass spectrometry

Electrospray ionization (ESI) mass spectra of SDG and SECO were collected in the negative mode using a PE Sciex API1^{Plus} system (Thornhill, ON, Canada) in the Department of Chemistry, The University of Georgia, Athens, GA. Conditions of employment were as follows: samples were infused into the system using methanol:water (1:1, v/v); scan range was from 200-1200 *m/z*; dwell time was 3.0 msec; the negative potential on the ESI needle was -3500 volts.

Capillary zone electrophoresis (CZE)

Capillary zone electrophoresis of the crude extract, the extract recovered after base hydrolysis, pure SDG, and pure SECO was performed using a Beckamn CE 5500 system with a UV-VIS diode array detector. The buffer employed for CZE contained 100 mmol/L boric acid (pH 9.3). An uncoated fused-silica capillary (50 μm *i.d.* × 72 cm) was used to deliver the sample to the instrument. Separation was carried out at 15 kV and the capillary temperature was maintained at 40°C. The photodiode detector was set at 280 nm.

Before injection into the CZE system, the samples were first dissolved in buffer (2 mg of crude extract, hydrolysed extract, and SDG per 1 mL, and 1 mg of SECO per 1 mL) and passed through a 0.45 μm filter.

RESULTS AND DICUSSION

The ESI mass spectra of the two lignans purified from flaxseed (Figures 2 and 3) were characterised by ions with *m/z* of 685 and 385, assigned to $[M_{SDG}\text{-}H]^-$ and $[M_{SECO}\text{-}H]^-$, respectively. The mass spectra confirm the fact that the isolated compounds were really secoisolariciresinol diglucoside and secoisolariciresinol.

The capillary zone electropherogram of the crude flaxseed extract (Figure 4A) showed only one broad peak from a phenolic complex. Its migration time was noted at 6.02 min. The same sample after base hydrolysis (Figure 4B) was charac-

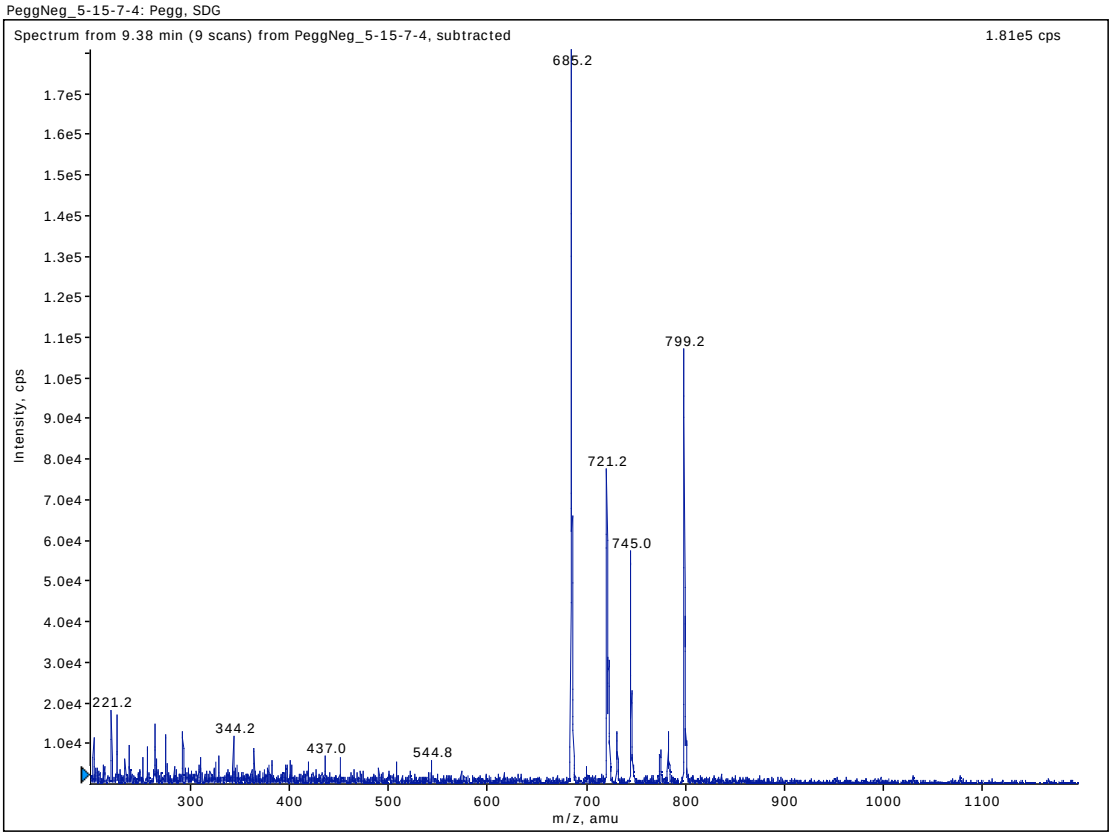

FIGURE 2. ESI mass spectrum of SDG.

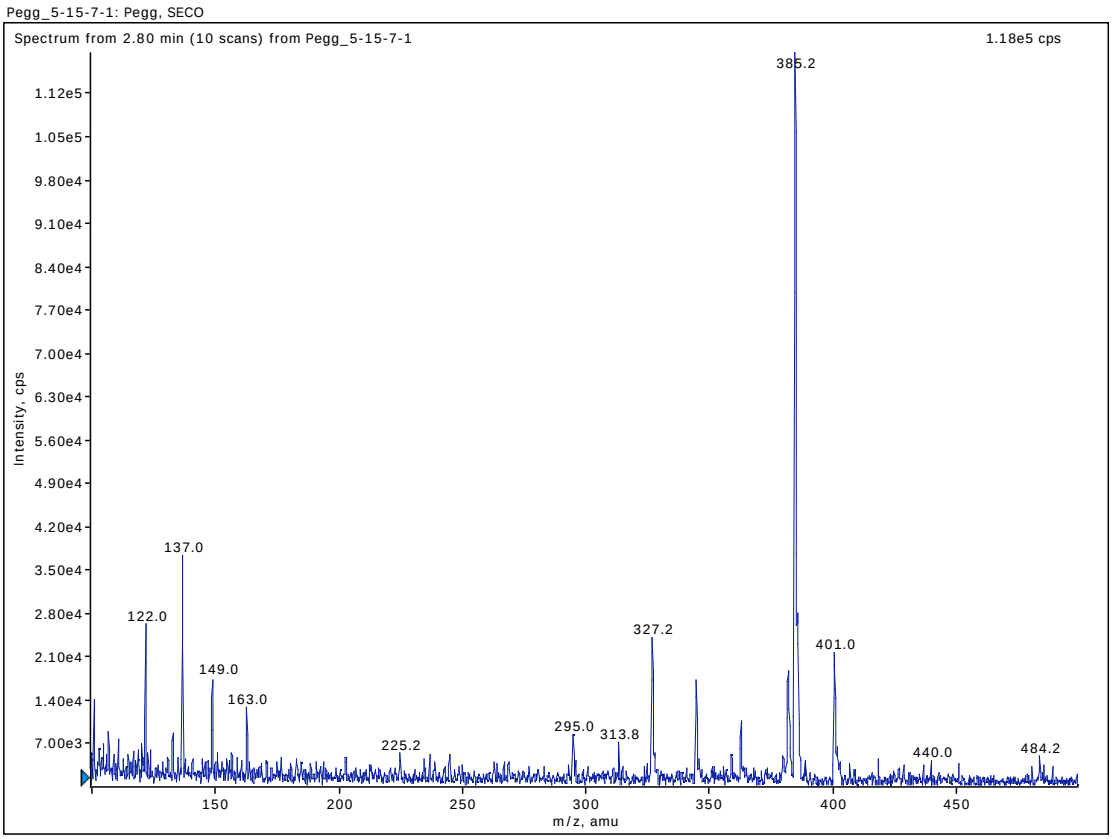

FIGURE 3. ESI mass spectrum of SECO.

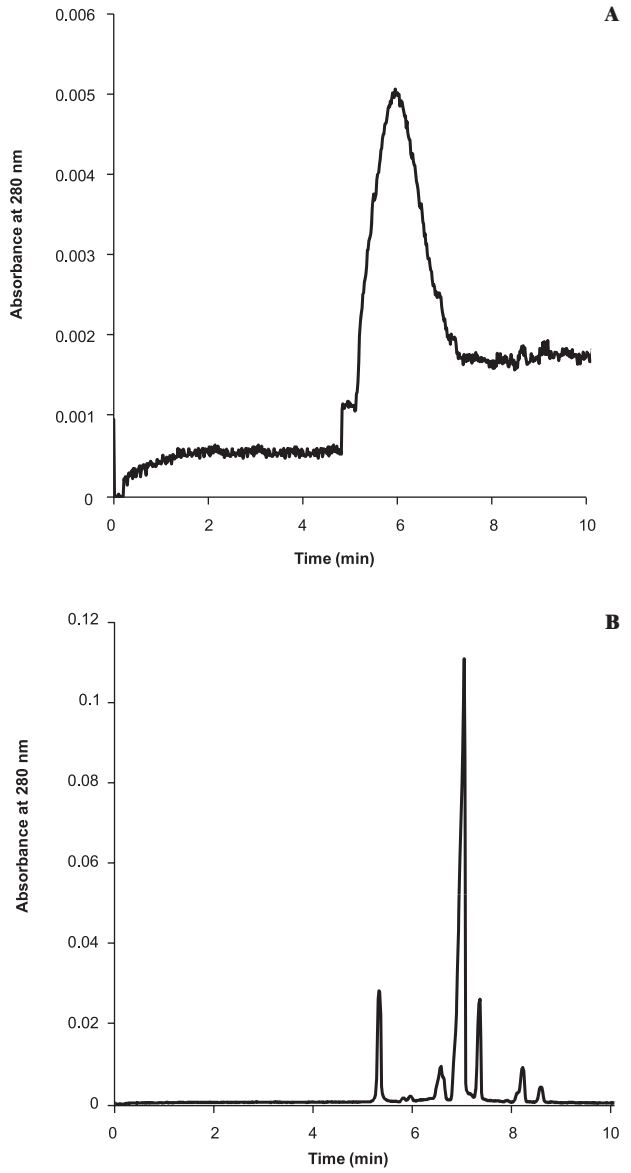

FIGURE 4. Electropherogram of flaxseed crude extract (A) and the extract after base hydrolysis (B).

terised on the electropherogram by six peaks with migration times of 5.33, 6.50, 6.66, 7.33, 8.25, and 8.65 min.

The electropherogram of SDG and SECO (Figure 5) shows the high purity of both lignan standards separated from the hydrolysed samples by semi-preparative HPLC. The migration times of SDG and SECO were recorded at 5.33 and 5.25 min, respectively. Seeing that the migration times of both lignans were close together, a base line separation of both compounds could not be achieved when a mixture of SDG and SECO was subjected to CZE (Figure 6).

Comparing the migration times of phenolic compounds on the electropherogram for the hydrolysed sample (Figure 4B) with that of pure SDG (Figure 5A), the first peak in Figure 4B is believed to be derived from SDG. Other peaks originated from phenolic acids and their glucosides. Based on the conditions employed, the negative charges on SDG arising from the two –OH groups of the molecule made it elute as the first compound. The largest peak in Figure 4B originated

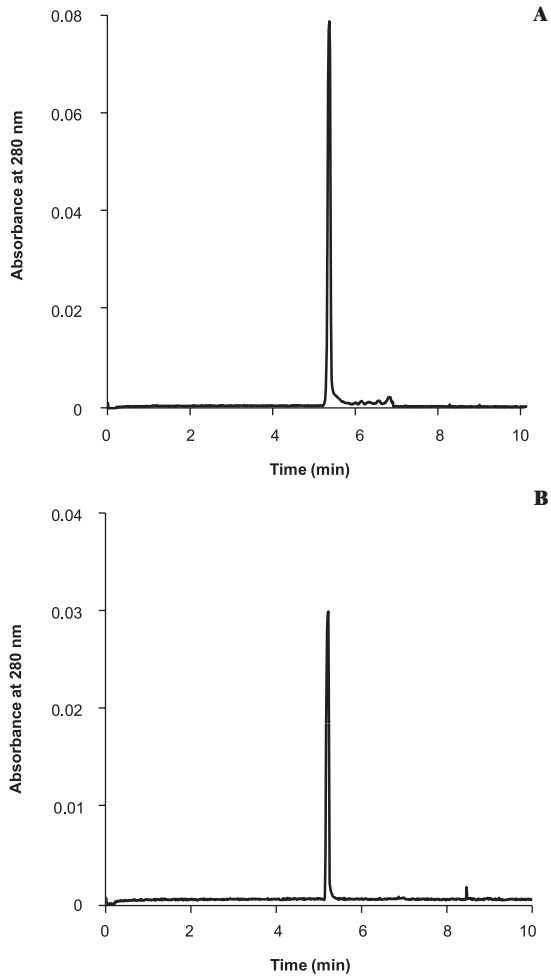

FIGURE 5. Electropherogram of SDG (A) and SECO (B).

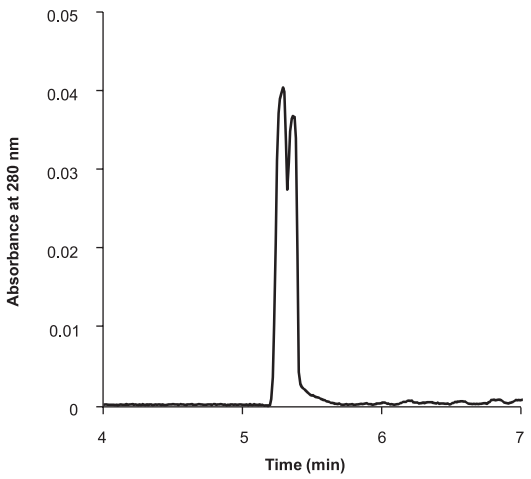

FIGURE 6. Electropherogram of a mixture of SDG and SECO.

from a phenolic acid glucoside; this phenolic acid derivative is without a hydroxyl group on the aromatic ring, and therefore its migration time is longer than that of SDG.

The retention time of SDG obtained under CZE conditions used in this study was relatively short. Johnsson *et al.* [2000] reported a baseline RP-HPLC separation in a gradient system with a migration time of SDG at 19.5 min. The re-

tention time for SDG separated using a MEKC method was noted at 12.25 min [Zduńczyk *et al.*, 2004].

CONCLUSIONS

In conclusion, the migration time required to separate SDG from a hydrolysed flaxseed extract was shorter than that reported with the use of other chromatographic techniques. Capillary zone electrophoresis afforded a quicker analysis with high separation efficiency and less sample handling than the conventional RP-HPLC method.

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