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Analytical Methods Section

Chromatographic techniques for preparation of linustatin and neolinustatin from flaxseed: standards for glycoside analyses

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Cyanogenic glycosides, which are major antinutrients of flaxseed, were extracted from the meal using 80% (v/v) ethanol. After silicic acid and subsequent RP-8 chromatography, cyanogenic glycosides along with soluble sugars were separated. Two cyanogenic glycosides, namely, linustatin and neolinustatin, were subsequently separated on a silica gel column with chloroform/methanol/water (65 : 35 : 10, v/v/v). Cyanogenic compounds so prepared may be used as chromatographic standards for glycoside analyses.

INTRODUCTION

Flaxseed is the third major oilseed crop of Canada (Anon., 1989). Flaxseed oil (commonly referred to as linseed oil) is used mainly as a drying oil in paints and varnishes. However, food-grade, low-linolenic acid linseed oil may soon become available through genetic manipulation (Dorrel, 1972; Prentice, 1992). The meal left behind after defatting contains cyanogenic glycosides, which are regarded as antinutrients. Linustatin and neolinustatin are known glycosides of flaxseed and flaxseedling tops. Linustatin is also found in green tissues of different species of *Passiflora* (Spencer *et al.*, 1986) and in seeds of *Hevea brasiliensis* (Selmar *et al.*, 1987, 1988). Therefore, it is important to have standards of cyanogenic glycosides available for analytical purposes.

The aim of this study was to obtain linustatin and neolinustatin of high purity for their possible use as standards for analysis of cyanogenic glycosides by chromatographic techniques.

MATERIALS AND METHODS

Figure 1 provides a flow diagram for the preparation of linustatin and neolinustatin from flaxseed. Defatted flaxseed (*Linum usitatissmum* L., 100 g) was extracted with 80% (v/v) ethanol at a seed-to-solvent ratio of 1 : 5 (w/v) at 65 to 70°C (Nabisan & Sundaresan, 1984). After gravity filtration on Whatman No. 4 filter paper, ethanol was evaporated under vacuum. The solid residue was transferred with water to a 250 ml volumetric flask and filled to the mark. The solution was then transferred to a 500 ml separatory funnel and was shaken with an equal volume of *n*-butanol to eliminate the phenolic compounds. After standing overnight to allow complete separation, the bottom aqueous layer was recovered and then lyophilized.

The lyophilized residue (6·35 g) was dissolved in methanol and a sufficient quantity of chloroform was added to it to provide a 2 : 1 (v/v) solution of $CHCl_3/CH_3OH$ (Smith *et al.*, 1980). After removal of precipitates, solvents were evaporated under vacuum. The solids (0·85 g) were applied to a silicic acid column (15 × 2·5 cm; 100 mesh; Mallinckrodt, Chesterfield, MO, USA) and eluted sequentially with $CHCl_3$ (400 ml), $CHCl_3/CH_3OH$ (4 : 1, v/v) (600 ml) and finally $CHCl_3/CH_3OH$ (2 : 1, v/v) (1000 ml). The last eluate, after evaporation under vacuum, gave 0·24 g of product, which was dissolved in 5 ml of methanol and was then injected on to a LiChroprep RP-8 prepacked column (31 × 2·5 cm; 40–63 μm; Merck, Elmsford, NY, USA). Elution was effected with CH_3OH/H_2O (15 : 85, v/v). Fractions were collected (4 ml per tube) using a 2112 Redirac fraction collector (LKB, Rockville, MD, USA). The separation efficiency of the column was monitored with precoated TLC silica gel plates (Sigma, St Louis, MO, USA) and a mobile phase $CHCl_3/CH_3OH/H_2O$ (65 : 35 : 10, v/v) (Amarowicz *et al.*, 1993). Spots were visualized by spraying with 10% sulphuric acid followed by heating in a forced-air convection oven at 120°C for 10 min. Fractions containing components with R_F values of 0·57 and 0·50 were combined. Methanol was removed under vacuum and the aqueous residue was freeze-dried.

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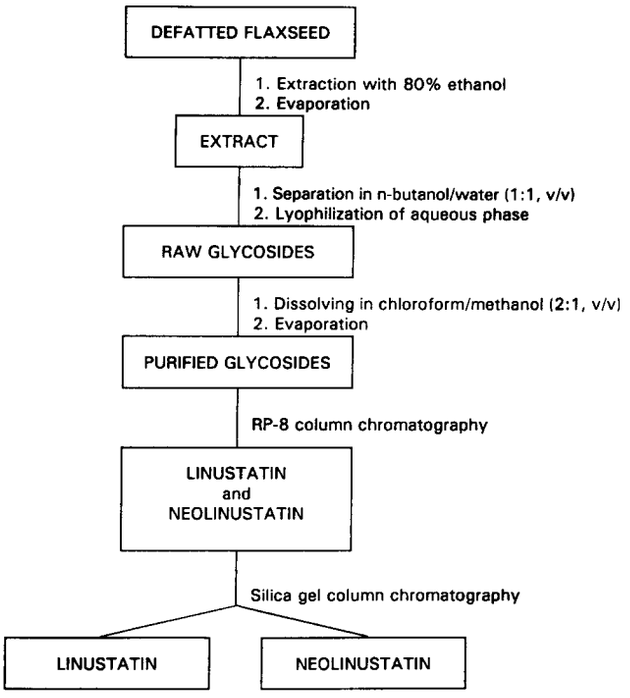
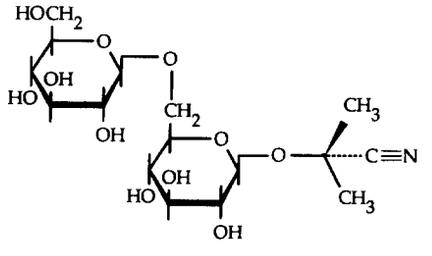
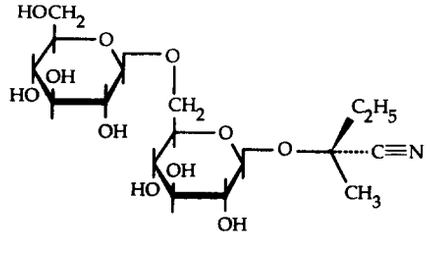


Fig. 1. Flowsheet for preparation of neolinustatin and linustatin from flaxseed.

The lyophilized material (105 mg) was dissolved in methanol (2 ml) and was then applied to a silica gel column (40 × 1 cm; Silica gel 60; 70–230 mesh; Merck) to separate the individual cyanogenic glycosides. The elution was conducted with $CHCl_3/CH_3OH/H_2O$ (65 : 35 : 10, v/v/v) and 4 ml fractions were collected. To a portion of each fraction (0·10 ml) 80% phenol (0·05 ml) and concentrated sulphuric acid (5·00 ml) were added at room temperature. The optical density of the resulting complex in each fraction was



LINUSTATIN



NEOLINUSTATIN

Fig. 2. Chemical structures of neolinustatin and linustatin.

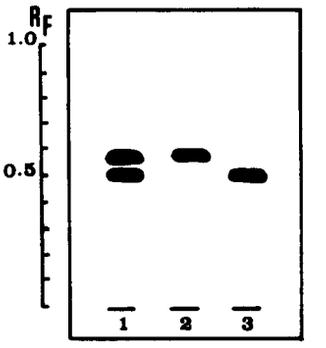


Fig. 3. TLC chromatogram of cyanogenic glycosides: 1, neolinustatin and linustatin after RP-8 column; 2, neolinustatin; 3, linustatin after silica gel column.

measured colorimetrically at 490 nm (Dubois *et al.*, 1956). The purity of the compounds (45 and 30 mg, respectively) after recrystallization from ethanol was determined by HPLC. The equipment consisted of a Shimadzu (Kyoto, Japan) LC-6A pump, SCL-6B system controller, SIL-6B auto injector, CSC–Spherisorb–ODS 2 column (10 μm, 4·5 × 240 mm) (Chromatography Sciences Co. Inc., Montreal, Canada), differential refractometer R401 detector (Waters, Milford, MA, USA) and a CR501 Chromatopac integrator. The solvent employed was 15% CH_3OH (v/v) at a flow rate of 0·8 ml min^{-1}, and 20 μl of sample was injected per run.

RESULTS AND DISCUSSION

The chemical structures of the two isolated cyanogenic glycosides, namely, linustatin and neolinustatin, are presented in Fig. 2. These compounds were efficiently extracted into 80% (v/v) ethanol and had R_F values of 0·57 and 0·50 on silica gel TLC when using a chloroform plates methanol/water mixture, respectively

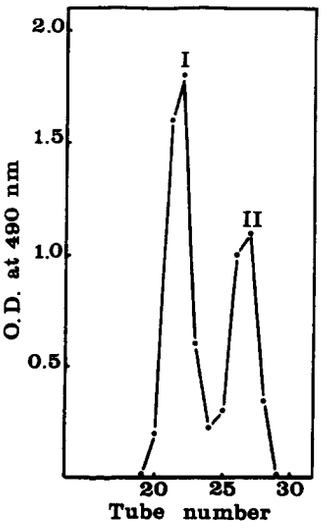


Fig. 4. Separation of neolinustatin (I) and linustatin (II) on the silica gel column.

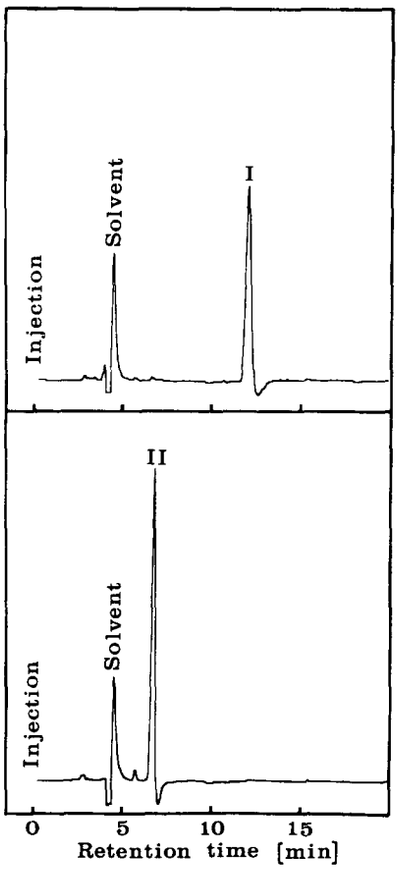


Fig. 5. HPLC chromatograms of neolinustatin (I) and linustatin (II).

(Fig. 3). The molecular weight of the isolated compounds, determined by ionspray mass spectrometry (Covey *et al.*, 1988), corresponded with the molecular structures given in Fig. 2. Neolinustatin was eluated from the column in fractions 20–24 while linustatin was present in fractions 25–29 (Fig. 4). The isolated compounds were of high purity (>99%) and could be used as standards for the HPLC analysis of cyanogenic glycosides. The retention times of linustatin and neolinustatin were 6·3 and 11·8 min, respectively (Fig. 5).

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