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Note

Semi-preparative isolation of crepenynic acid, a potential inhibitor of essential fatty acid metabolism

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Crepenynic (octadec-*cis*-9-en-12-ynoic) acid has been identified as a major component (25%) of the fatty acids of the oil from seeds of *Ixiolaena brevicompta* F. Muell. (Compositae)[1]. The mature seeded plant is considered responsible for causing muscular degeneration and extensive mortalities in sheep on the riverine flood plains of western New South Wales and Queensland[2].

A number of eighteen and twenty carbon acetylenic fatty acids have been found to interfere with complex lipid metabolism in animals. These effects include inhibition of essential fatty acid synthesis[3,4] and selective inhibition of cyclooxygenase and lipoxygenase enzyme pathways[5–7]. Previous studies[5,8] have given no indication that crepenynic acid could be sufficiently toxic to cause deaths in sheep although our recent studies[9] suggest that crepenynic acid is indeed the toxic component in *I. brevicompta* seed oil.

The application of high-performance liquid chromatography (HPLC) to the preparation of individual fatty acids (or esters) in quantity for metabolic studies has received little attention. HPLC has been used for the isolation of fatty acid methyl esters using a dedicated preparative instrument[10], but the capital expenditure required for such machines would be unwarranted where limited amounts of material are required.

This report describes a simple reversed-phase HPLC technique for the isolation of the methyl ester of crepenynic acid in gram quantities necessary for small-scale feeding trials with laboratory animals. The technique involves the use of conventional analytical HPLC equipment and a micro-particulate semi-preparative column and could be applied to the isolation of other unsaturated fatty acid esters from complex mixtures.

EXPERIMENTAL

Preparation of crude methyl esters

The mature seeds of *I. brevicompta* (5 kg) were crushed to a fine flour in a Reitz mill (0.3-mm screen), soaked and extracted three times with 10 dm^3 hexane. Transmethylation of the oil was carried out by scaling-up an analytical method[11], in which the oil was reacted with a 1 *M* solution of sodium propoxide in propanol

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at room temperature for 1 h, followed by acidification with 1 *M* hydrochloric acid and extraction using the procedure of Bligh and Dyer[12]. Column chromatography on Florisil[13] was used to clean up the methyl ester fraction. The fatty acid composition of the methyl esters was obtained by gas–liquid chromatography (GLC) with a Carlo Erba HRGC 5160 gas chromatograph, using a bonded-phase BP20 fused-silica column (SGE, Australia).

Preliminary enrichment of methyl esters

Removal of pigments with activated charcoal was carried out to reduce the risk of column deterioration and blockage. The orange-coloured fatty acid methyl ester mixture (234 g) was dissolved in hexane and clarified by passage through a column (500 × 55 mm I.D.) containing two layers of Florisil (2 × 100 g) separated by a mixed bed of activated carbon and Florisil (50:50) (100 g).

The methyl ester mixture was further fractionated by urea inclusion to remove long-chain saturated esters which may be strongly retained on a reversed-phase HPLC column. The clarified methyl esters (230 g) were boiled with a mixture of methanol (2 dm^3) and urea (250 g).

The urea adduct which formed at room temperature was filtered off and the filtrate, monitored by GLC was found to be enriched in the desired unsaturated esters. A further treatment with 50 g urea produced a filtrate which was greatly enriched in unsaturated esters and was devoid of saturates. The remaining filtrate was acidified with 1 dm^3 1 *M* hydrochloric acid, extracted twice with 1 dm^3 hexane, dried over anhydrous sodium sulphate and filtered through a 0.45-μm filter. The final filtrate after evaporation yielded 160 g of an enriched unsaturated methyl ester fraction for HPLC separation.

HPLC solvents

Methanol (analytical grade) and water were passed through a 0.45-μm filter before use.

HPLC apparatus

A Waters Model 590 pump, Altex Model 500 injector, Dupont Model 845 refractometer and Linear Instruments Model 282 chart recorder were used. A semi-preparative column, Regis 250 × 10 mm I.D., packed with 10-μm particles coated with ODS phase was selected.

The methyl ester mixture (100 mg/cm^3 or 400 mg/cm^3 in methanol) was injected onto the column, using a 1-cm^3 injection loop. When the 100 mg/cm^3 mixture was used the mobile phase was 100% methanol. However, with the larger injection the mobile phase was altered to methanol–water (95:5) to maintain adequate resolution. The flow-rate in both cases was 2.5 cm^3/min.

Identification of fatty acid methyl esters

Fatty acid methyl esters from HPLC runs were identified by comparison of GLC retention times with authentic standards. Methyl crepenynate standard was available from *I. brevicompta* seed oil[1].

RESULTS AND DISCUSSION

Figs. 1 and 2 show HPLC chromatograms of the fatty acid methyl ester mixtures before and after urea enrichment, respectively. The fatty acid composition of the components eluted by HPLC (Figs. 1 and 2) was determined by collection and GLC analysis.

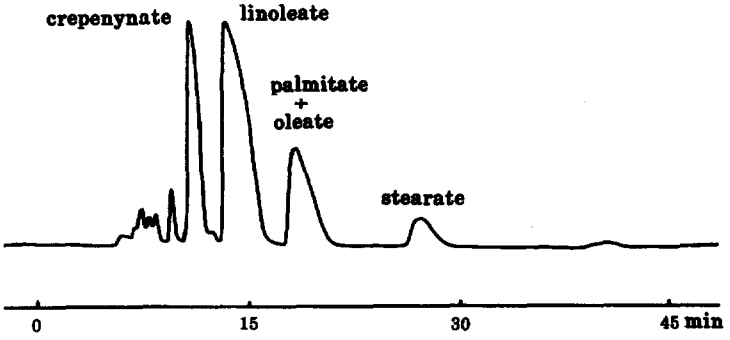

Fig. 1. HPLC trace of methyl esters prepared from the oil extract from *I. brevicompta* seed; 100 mg injection with a flow-rate of 2.5 cm^3/min methanol.

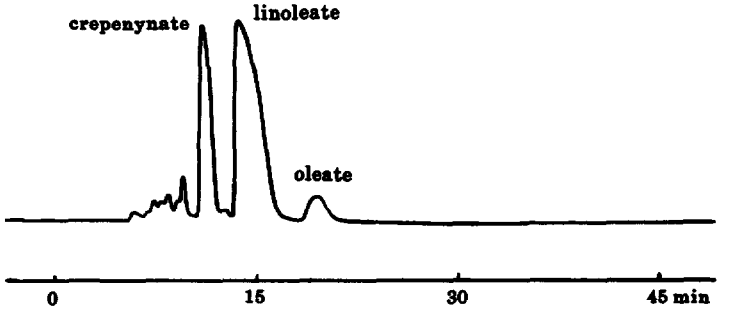

Fig. 2. HPLC trace of methyl esters following enrichment of unsaturated esters using urea inclusion; 100 mg injection with a flow-rate of 2.5 cm^3/min methanol.

The original fatty acid composition of the oil extract (5%) from this batch of mature seeds of *I. brevicompta* was palmitic (8%), stearic (5%), oleic (8%), linoleic (53%) and crepenynic (22%) acids (Fig. 1). Urea inclusion was used to remove most of the saturates and some of the mono-unsaturated fatty ester components leaving a fraction which was comprised of oleic (6%), linoleic (67%) and crepenynic (27%) acids (Fig. 2).

HPLC separation of the enriched fraction, using sample loads up to 400 mg (methanol–water, 95:5, and flow-rate of 2.5 cm^3/min) could be completed in less than 50 min with near-baseline separation between methyl crepenynate and methyl linoleate. The cycle time to re-injection could be further reduced to 30 min by flushing the column with methanol at 6 cm^3/min for 5 min immediately after the elution of methyl crepenynate (20 min), followed by re-equilibration with methanol–water

(95:5) at 5 cm^3/min for 5 min. At this stage, the flow-rate was reduced to 2.5 cm^3/min and the sample injection and HPLC separation repeated as described above.

Using repetitive injection, a total of 15 g of methyl crepenynate (98% pure by GLC) was conveniently isolated for use in animal feeding trials. Subsequently, similar amounts of methyl linoleate and linolenate were isolated by this procedure from safflower oil and linseed oil respectively[14]. The technique could be applied to the isolation of a wide variety of unsaturated esters, and could be readily scaled-up for preparative isolations.

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