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TRIACYLGLYCEROLS OF *VERNONIA GALAMENSIS* SEED OIL

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Key Word Index—*Vernonia galamensis*; Compositae; seed oil; triacylglycerols; trivernolin; divernoloylglycerides; monovernoloyl glycerides; vernolic acid.

Abstract—Separation methods using silica gel column chromatography and preparative thin layer chromatography were employed to prepare pure triacylglycerol fractions of the oil from *Vernonia galamensis* seeds. Pure vernolic acid (12,13-epoxy-octadec-*cis*-9-enoic acid) was prepared by enzymatic hydrolysis of the chromatographically purified major triacylglycerol, trivernolin, using a commercially available immobilised lipase in a non-polar organic solvent. Analysis of the divernoloyl and monovernoloyl triacylglycerols with porcine pancreatic lipase indicated that vernolic acid is found mainly as the secondary ester in the divernoloyl triacylglycerol but not the monovernoloyl triacylglycerol.

INTRODUCTION

The oil extracted from *Vernonia galamensis* seeds contains up to 80% of vernolic acid (12,13-epoxy-octadec-*cis*-9-enoic acid), a fatty acid that has wide applications in the polymer and coatings industry [1]. Vernolic acid is also found in the triacylglycerols of *Euphorbia lagascae* and *Stokesia laevis*, which are potential industrial crops [2, 3]. *Vernonia* oil has been used in the synthesis of toughened elastomers [4] and various polymers [5–7]. Reaction of the oil with diamines yields fatty *bis*amides, which can be used as difunctional epoxy monomers in polymer synthesis [8]. The epoxy group of vernolic acid has been utilised in the synthesis of deuterium-labelled methyl linoleate and its geometric isomers for use in study of fat metabolism [9]. Cleavage of the alkyl chain of vernolic acid at the epoxy group can be used to produce dibasic acids, which are used as precursors in various industrial applications [10]. The preparation of pure vernolic acid and its derivatives is of interest to us in the preparation of fine chemicals and for studying the mechanisms of plant lipases [11].

Vernolic acid and the other fatty acids, linoleic, oleic, palmitic and stearic present in the seed oil, occur mainly as triacylglycerol esters. These triacylglycerols can be divided into four classes, which are non-epoxy or normal triacylglycerols, monovernoloyl triacylglycerols, divernoloyl triacylglycerols and trivernolin; these four classes can be separated using TLC [12]. However, TLC analysis of *Vernonia* oil in the presence of non-epoxy fatty acids and vernolic acid has not yet been reported.

The present paper describes a TLC method designed to analyse the lipids of *Vernonia* oil in the presence of both normal fatty acids and vernolic acid, which may be products of autolysis of the oil, and a column chromatography method devised for the preparation of pure trivernolin, divernoloyl and monovernoloyl triacylglycerols. An enzymatic structural analysis of the divernoloyl and monovernoloyl triacylglycerols is also described.

RESULTS AND DISCUSSION

The presence of an epoxy group in the major fatty acid in the *Vernonia galamensis* oil, makes normal separation methods for preparing pure triacylglycerols and analysis of oil constituents inappropriate for the study of this oil.

Purification of trivernolin

The concentration of esterified vernolic acid in the oil extracted in this case was 80%. The other major fatty acids in the oil were linoleic 11.2%, oleic 3.4%, stearic 0.8% and palmitic 2.1%. The triacylglycerol constitution of the oil was determined by removing the triacylglycerol spots from TLC plates that had been sprayed with phosphomolybdic acid and eluting the blue complex using 50% ethanol. The concentration of the blue complex was determined by

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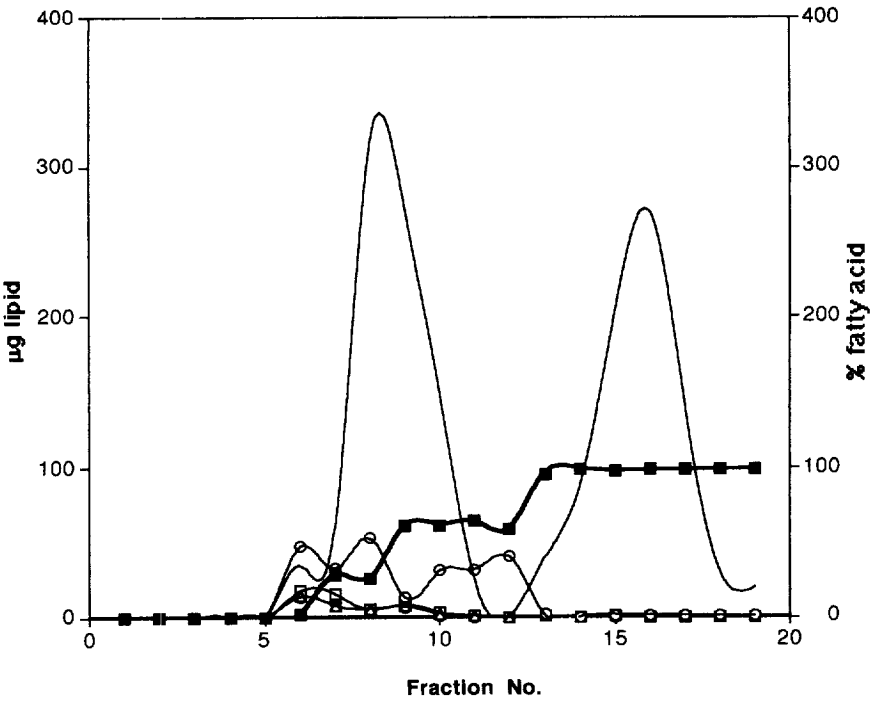

Fig. 1. Fractionation of triacylglycerols of *Vernonia* oil on a silica gel column (25 × 3 cm). The solid line shows the amount of lipid in the eluent and the fatty acids in the triacylglycerols of the eluted fractions are shown as follows: vernolic (■), linoleic (○), oleic (□), palmitic (△) and stearic (●). Triacylglycerols were eluted using hexane–Et_2O (7 : 3).

measuring the absorbance of the solution at 730 nm. The triacylglycerols of *Vernonia* oil consisted of a small amount of non-epoxy triacylglycerols (9%), monovernoloyl triacylglycerols (15%), divernoloyl triacylglycerols (38%) and trivernolin (38%). Most of the work on epoxy triacylglycerols and epoxy fatty acids of *Vernonia* oil has been done using crude oil, in which the percentage of the esterified vernolic acid can be as high as 80%. For industrial applications, the crude oil is adequate as a source of vernolic triacylglycerols. However, for biochemical purposes, such as characterisation of lipases and fatty acid metabolism studies, or for use as a reference standard, confusing results can be obtained if the crude oil is used as a triacylglycerol source of vernolic acid. The polar nature of trivernolin which causes it to be the slowest migrating triacylglycerol on silica gel was exploited to purify trivernolin from the crude oil (Fig. 1). A semi-continuous monitoring of the fatty acid constitution and an estimate of total lipid content of the fractions eluted from the silica gel column was done by taking small samples (ca 1 ml) every 5 min. The variation in fatty acid constitution of the eluent is given in Fig. 1. Elution of the triacylglycerols from the column using hexane–Et_2O (7 : 3) gave two major peaks, an early peak for the mixed triacylglycerols (monovernoloyl and divernoloyl triacylglycerols) and a later peak for trivernolin. A small peak rich in non-epoxy fatty acids preceded these two peaks. This peak is the peak for the normal triacylglycerols.

Structural analysis of divernoloyl and monovernoloyl triacylglycerols

Preparative TLC was used to obtain monovernoloyl and divernoloyl triacylglycerols from the mixed triacylglycerol fractions. The fatty acid esterified to the 2-position of the monovernoloyl and divernoloyl triacylglycerols of *Vernonia* oil was determined by hydrolysing the triacylglycerols using porcine pancreatic lipase and analysing the fatty acids in the 2-monoacylglycerols by GC. The fatty acid compositions of the 2-monoacylglycerols and the enrichment of the individual fatty acids by lipolysis are summarised in Table 1. Vernolic acid was enriched in divernoloyl triacylglycerols, whereas linoleic and oleic acids were enriched in the monovernoloyl triacylglycerols. The saturated fatty acids (16 : 0 and 18 : 0) are the least enriched in the 2-position of these triacylglycerols. The physical properties of a heterogeneous triacylglycerol are greatly influenced by intra-triacylglycerol distribution of the constituent fatty acids. These results show a preference for vernolic acid at the 2-position in the divernonyl triacylglycerols but not the monvernonyl triacylglycerols. This raises a question on the nature of

Table 1. Structural analysis of monovernoloyl and divernoloyl triacylglycerols of *Vernonia* oil. Triacylglycerols were separated by TLC and scraped off. Triglycerides obtained were then hydrolysed using porcine pancreas lipase and the fatty acid in the 2-monoacylglycerol analysed by GC

Triacylglycerol	Fatty acid 16:0	18:0	18:1	18:2	Ve
XVe_2 Triacylglycerol	7.11	4.87	6.76	20.18	61.90
XVe_2 Monoacylglycerol	2.50	1.23	2.93	10.52	82.82
Enrichment	**0.35**	**0.25**	**0.43**	**0.52**	**1.34**
X_2Ve Triacylglycerol	9.24	9.74	14.74	36.86	29.43
X_2Ve Monoacylglycerol	3.57	2.81	19.91	53.93	19.78
Enrichment	**0.39**	**0.29**	**1.35**	**1.46**	**0.67**

X = Linoleic, oleic, stearic or palmitic acids.
Ve = Vernolic acid.

the specificity of a 1-acyl-*sn*-glycerol-3-phosphate acyl transferase with a specificity for vernoloyl CoA as the acyl donor in the triacylglycerol biosynthesis [13].

TLC analysis of lipids of Vernonia *oil and their derivatives*

Development of TLC plates with hexane–Et_2O–HOAc (80:20:1) resulted in the separation of most of the constituent lipids of *Vernonia* oil (Table 2). In the TLC of triacylglycerols of mixed fatty acid content using relatively non polar mobile phases, the order of migration is non-epoxy triacylglycerols, followed by monoepoxy triacylglycerols, followed by diepoxy triacylglycerols, then triepoxy triacylglycerols. Trivernolin being the most polar of the triacylglycerols is the least mobile on silica gel TLC. Vernolic acid is more mobile than trivernolin in hexane–Et_2O–HOAc (80:20:1), whereas in hexane–Et_2O (3:2), trivernolin is more mobile than vernolic acid.

The divernoloyl triacylglycerols ($R_f = 0.23$) and vernolic acid ($R_f = 0.22$) co-migrate if developed using a solvent system of hexane–Et_2O–HOAc (80:20:1) and, if this is done using a solvent system of hexane–Et_2O (3:2), then trivernolin ($R_f = 0.43$) and non-epoxy fatty acids ($R_f = 0.45$) co-migrate (Table 2). Free fatty acids in vegetable oils are considered undesirable because they change the physical and chemical properties of the oil. The presence of a lipolytic enzyme in *Vernonia* seeds [11, 14] means that the quality of oil extracted may be subject to variation due to poor handling, poor processing or poor storage of the seed. TLC analysis methods are helpful to agricultural stations or seed producers in the monitoring of the oil quality from *V. galamenis* or similar plant oils, such as those from *Euphorbia lagascae* and *Stokesia laevis*.

Table 2. TLC of lipids of *Vernonia* oil and their derivatives. Plates were developed using hexane–Et_2O–HOAc (80:20:1) and hexane–Et_2O (3:2), and visualised using phosphomolybdic acid

Lipid	R_f value[a]	R_f value[b]
Non-epoxy triacylglycerols	0.58	0.92
Monovernoloyl triacylglycerols	0.31	0.74
Divernoloyl triacylglycerols	0.23	0.59
Trivernolin	0.15	0.43
Vernolic acid	0.22	0.28
Divernolin	0.09	0.13
Non-epoxy fatty acid	0.34	0.45
Ethyl vernolate	0.46	0.81
Non-epoxy fatty acid ethyl ester	0.77	0.99

[a] Hexane–Et_2O–HOAc (80:20:1).
[b] Hexane–Et_2O (3:2).

The common solvent system, hexane–Et_2O–HOAc (80:20:1), is adequate as a quality control method for detection of lipolysis in most vegetable oils. In *Vernonia* oil, the differential migration of triacylglycerols makes it difficult to achieve separation of fatty acids from all the possible lipids of the oil using one dimensional TLC. A two dimensional method on silica gel 60 plates (10 × 10 cm) using hexane–Et_2O (3:2) in the first dimension and hexane–Et_2O–HOAc (80:20:1) in the second dimension was found to be suitable for the separation of all the lipids (Fig. 2). In the first dimension, trivernolin and normal fatty acids co-migrate but are separated in the second dimension. This two-dimensional TLC analysis made it suitable for analysis of partially hydrolysed oils in which both epoxy and non-epoxy fatty acids may be present. It could be used to determine the composition of other plant oils with vernolic acid derivatives [2, 3].

Trivernolin has potential in the chemical synthesis of fine chemicals and polymers. In addition the study of the metabolism of the triacylglycerol, or its fatty acid, by various microorganisms could lead to the bioconversion of the vernolic acid into industrially important compounds. The availability of pure trivernolin prepared by a method such as that reported here would greatly improve the quality of the products that are synthesised from vernolic acid and make the study of the bioconversions by microorganisms easier.

EXPERIMENTAL

Materials

Vernonia galamensis seeds were a gift from the Agricultural Research Station, Chiredzi, Zimbabwe. Lipozyme was a gift from Novo, Novo Nordisk, Denmark. Porcine pancreas lipase (EC No 3.1.1.3, Pancreatin) was obtained from Sigma. Phosphomolybdic acid, silica gel 60 (particle size 0.040–0.063 mm) and silica gel 60 coated plates were obtained from Merck.

Oil extraction and purification of trivernolin

Seeds (50 g) were extracted by homogenising in 200 ml cold Me_2CO (−20°) [14]. The dissolved oil was collected by vacuum filtration and the solvent evapd

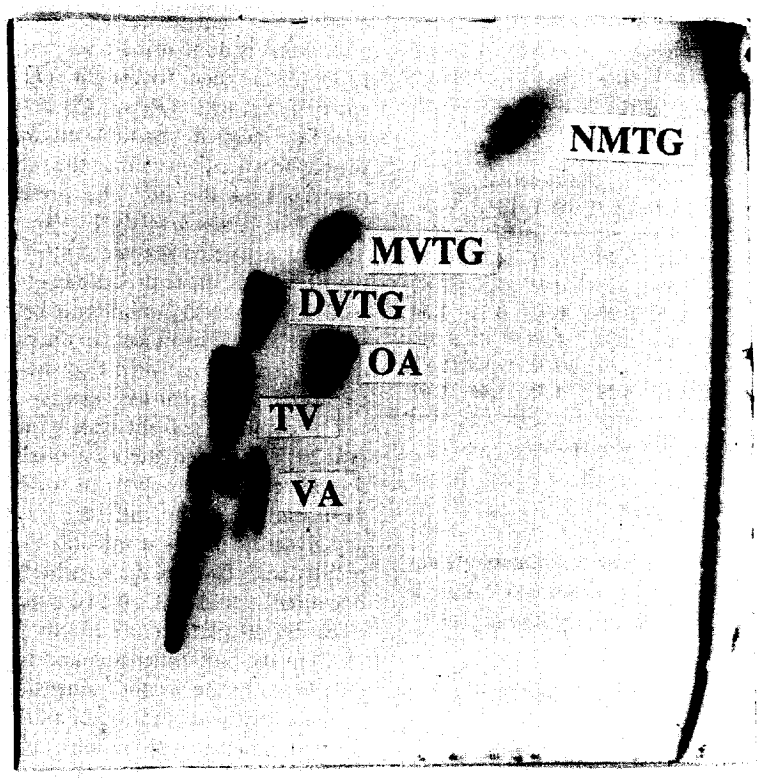

Fig. 2. Two-dimensional analysis of lipids from *Vernonia* oil. NMTG-non-epoxy triacylglycerols, MVTG-monovernoloyl triacylglycerols, DVTG-divernoloyl triacylglycerols, TV-trivernolin, VA-vernolic acid, OA-oleic acid. Plates were developed using hexane–Et_2O (3 : 2) in the first dimension and hexane–Et_2O–HOAc (80 : 20 : 1) in the second.

under N_2 gas at 50°. The oil obtained (15 g) was dissolved in 50 ml hexane and centrifuged to remove ppted material. Fatty acids were removed from the oil by washing with an equal vol. of MeOH–bicarbonate buffer (pH 10) (1 : 1). Hexane was removed from the oil by evapn under N_2. Oil (2 g) was loaded onto a silica gel 60 column (25 × 3 cm), that had been equilibrated with hexane, and washed into the column with hexane. Non polar material was eluted with 200 ml hexane–Et_2O (49 : 1). Total triacylglycerols were eluted with hexane–Et_2O (7 : 3). Bulk fractions of ca 25 ml were collected and portions of 100 μl withdrawn from the frs for GC analysis of fatty acids. The flow rate was 4 ml min^{-1}. Small frs of ca 1 ml were collected every 5 min for monitoring total lipid content and fatty acid constitution. Frs that contained pure trivernolin were pooled and the solvent evapd under N_2 to obtain pure trivernolin as a clear oil at room temp.

Recrystallisation of divernolin

The diacylglycerol, divernolin, was recrystallised × 3 from a soln of crude oil in hexane (5%, w/v). The first recrystallisation was done at room temp. overnight and subsequent recrystallisations at 4° for a few h. Divernolin was obtained after evapn of solvent under N_2, as a dry white solid at room temp.

Preparation of vernolic acid by enzymatic hydrolysis of trivernolin

Trivernolin (300 mg) was dissolved in 1 ml of H_2O-satd *iso*octane and 100 mg of Lipozyme™ was added. The mixt. was incubated on a shaking water bath at 45° and 150 rpm. The extent of hydrolysis was followed by silica gel TLC using hexane–Et_2O–HOAc (80 : 20 : 1). After complete hydrolysis of trivernolin, the soln was removed from the Lipozyme™ and added to 2 ml MeOH–$CHCl_3$ (1.4 : 1.2). Vernolic acid was extracted into aq. MeOH by addition of 1 ml of 200 mM $NaHCO_3$ buffer pH 10. The aqueous MeOH phase was removed and the vernolic acid extracted by adding 1 ml of *n*-hexane followed by 100 μl of 6 M HCl. The hexane layer was withdrawn and the hexane evaporated in a steam of N_2 gas to obtain pure vernolic acid as a white solid at room temp.

Quantification of total lipid

A sample (400 μl) was withdrawn from the samples collected during the elution. The solvent was evaporated under a stream of N_2 gas. The total lipid was quantified by adding 200 μl of 10% phosphomolybdic acid sol. in EtOH and heating for 30 min at 80°. The mixture was then dissolved in 5 ml of 50% EtOH

and the absorbance read at 730 nm. Mixed fatty acid triacylglycerols of *Vernonia* oil were used to obtain a lipid standard curve. Lipids from TLC plates were quantified by scraping off the spots and eluting the coloured complex using 50% EtOH and measuring the absorbance of the sol. at 730 nm.

TLC

The analysis of lipids of *Vernonia* oil and the derivatives of the lipids using TLC was done on silica gel 60 plates. When the separation was done using one dimensional TLC, hexane–Et_2O–HOAc (80 : 20 : 1) or hexane–Et_2O (3 : 2) was used. In a two dimensional separation the latter solvent system was used first in one dimension followed by the more polar solvent system in the second dimension. The lipid spots were visualised by spraying with 10% phosphomolybdic acid in EtOH and heating at 80° for 10 min.

GC

Fatty acids in triacylglycerols were converted to the corresponding Me esters using NaOME [15]. Analysis of Me esters was done on a SP-2380 (stabilised *biscy*anopropylpolysiloxane) thin film capillary column (30 m × 0.32 mm) (Supelco). The vol. injected was 1 μl, with a 1 : 10 split ratio. The initial column temp. was 170° for 2 min, then raised to 190° at 5° min^{-1} and held at this temp. for 8 min. Peak integration was done electronically.

Structural analysis of triacylglycerols

The triacylglycerols mixt. containing monovernoloyl and divernoloyl triacylglycerols (50 mg) was spotted as a band on a silica gel TLC plate (20 × 20 cm) and separated using hexane–Et_2O (3 : 2). The separated triacylglycerols were visualised by spraying with 2,7-dichlorofluorescein, viewing under UV light and the individual bands scraped off and extracted with hexane–Et_2O (1 : 1). After removing solvent with N_2, the triacylglycerols (22 mg divernoloyl and 14 mg monovernoloyl) were emulsified by the addition of 0.25 ml of a soln of 10% gum arabic in Na–Pi buffer pH 8, 0.25 ml taurocholate soln (1.0%), 0.1 ml calcium soln (2.2%) and vortexing vigorously. Lipolysis was started by the addition of 15 mg of porcine pancreatic lipase dissolved in 0.5 ml Na–Pi (100 mM, pH 8) and incubation at 37°. After 10 min, the emulsion was broken by the addition of 1 ml $CHCl_3$–MeOH (2 : 1) and 50 μl of 6 M HCl. The organic phase was removed and the solvent evapd under N_2. The lipid was redissolved in 100 μl of hexane–Et_2O (1 : 1) and spotted to TLC plates and developed with hexane–Et_2O–HOAc (80 : 20 : 1). Monoacylglycerols were scraped off and converted to Me esters for GC analysis.

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