Direct Analysis of Trivernolin by High-Performance Liquid Chromatography[1]

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ABSTRACT

A rapid liquid chromatographic method for the quantitation of trivernolin in epoxy seed oils has been developed. A weighed sample of the oil is dissolved in chloroform (~100-300 mg/ml), and a 1-20 μl portion of the oil is chromatographed on a 2 ft x ¼ in. OD C_{18} μ-Bondapak column with acetonitrile-acetone (2:1, v/v). The area of the trivernolin peak from the differential refractometer recorder tracing is converted to μg trivernolin by use of a calibration curve of peak area vs. trivernolin weight.

INTRODUCTION

Seed oils containing large amounts of vernolic acid (*cis*-12,13-epoxyoleic acid) have generated interest as potential sources of a valuable industrial raw material. The epoxy acids in some oils, such as that from *Veronia anthelmintica,* are primarily present as trivernolin (1), while other high epoxy oils, such as *Euphorbia lagascae* seed oil, have the epoxy acids more randomly distributed in the triglycerides (2). Since fractional crystalization of trivernolin from high vernolic seed oils has been proposed as a simple method of preparing pure vernolic acid, a method to measure trivernolin in seed oils could be valuable. However, quantitation of trivernolin and of di- and monovernoly triglycerides has been a difficult or time-consuming procedure. The epoxy triglycerides do not quantitatively elute during gas chromatography (GC). Derivatizing the epoxyglycerides to 1,3-dioxolanes improves their GC characteristics, but large correction factors are still required to obtian quantitative data (3). The trivernolin content of the seed oil can be determined by preparative thin layer chromatography (TLC) (2), but this procedure is quite time consuming. During our recent work with high-performance liquid chromatography (HPLC) of triglycerides with reverse-phase C_{18} octadecyl silane columns (4), we noted that triglycerides containing epoxyoleic groups had retention times identical to those of saturated triglycerides seven carbon atoms shorter. Therefore, trivernolin elutes from the column before di- and monovernoyl triglycerides and much before normal triglycerides. Thus, the amount of trivernolin in a seed oil can be rapidly and directly determined by HPLC.

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EXPERIMENTAL PROCEDURES

HPLC chromatograms were run with a Waters Associates ALC-201 liquid chromatograph on a μ-Bondapak C_{18} column (2 ft x ¼ in., 10 μ particle size) (Waters Associates, Inc.). The samples were injected with a U6K septumless loop injector. The solvent was acetonitrile-acetone (2:1, v/v), and peaks were detected with a Waters differential refractometer. Typical sample sizes for analysis were about 20-500 μg of trivernolin in 1-20 μl $CHCl_3$ solution. Peak areas were determined by multiplying peak height by width at half height.

To obtain pure trivernolin and to determine the retention volumes of other components likely present in these oils, 1.0 g of *Vernonia anthelmintica* seed oil was chromatographed on a 25-g column of 60/120 mesh Florasil. The column was eluted with hexane followed by mixtures of hexane-diethyl ether and finally by pure diethyl ether. Progress of the separation was monitored by TLC, and pure trivernolin was isolated from the resulting fractions by preparative TLC. The remaining fractions were analyzed by HPLC to verify that no other seed oil components eluted at the same volume as trivernolin.

HPLC peaks were identified by collecting, transesterifying, and analyzing the effluent by GLC on a 12 ft x ¼ in. 5% LAC-2-R 446 column and a 6 ft x ¼ in. 5% Apiezon L column.

A plot of peak area vs. weight of trivernolin was made by injecting known amounts of trivernolin from 30 μg to 1300 μg and measuring the peak areas.

RESULTS AND DISCUSSION

The HPLC chromatogram of two high epoxy seed oils, *Vernonia anthelmintica* and *Euphorbia lagascae,* and a normal triglyceride seed oil, namely soybean oil, are shown in Figure 1. Trivernolin (A) eluted at ~11 ml, followed by the triglycerides with two vernoyl acyl groups and one linoleic (B) or one oleic acyl (or palmitoyl) (C) group at ~13 ml and ~15 ml, respectively. The other divernoyl triglyceride with stearic acid as the third acyl group (D) eluted at ~18 ml among the monovernoyl triglycerides. The chromatogram of soybean oil, run under these same conditions, shows peaks for C_{54} triglycerides with from eight to two double bonds per molecule. Triolein (three double bonds) eluted at ~30 ml under these conditions. Retention

TABLE I

Analysis for Trivernolin Content in Several High Vernolic Oils from eight Different Samples of Our Seed Collection

	Percent trivernolin by HPLC analysis no.				
	1	2	3	Avg. (%)	Std. dev.
1. *Vernonia anthelmintica*	48.8	48.2	43.0	46.7	2.5
2. *Vernonia anthelmintica*	39.1	41.0	37.7	39.3	1.1
3. *Vernonia anthelmintica*	40.0	39.0	42.8	40.6	1.6
4. *Euphorbia lagascae*	12.2	19.2	15.0	15.5	2.9
5. *Euphorbia lagascae*	18.9	17.2	18.6	18.2	0.7
6. *Stokesia laevis*	1.9	2.0	3.2	2.4	0.6
7. *Stokesia laevis*	2.7	3.1	4.0	3.3	0.6
8. *Stokesia laevis*	3.4	3.9	4.9	4.1	0.6

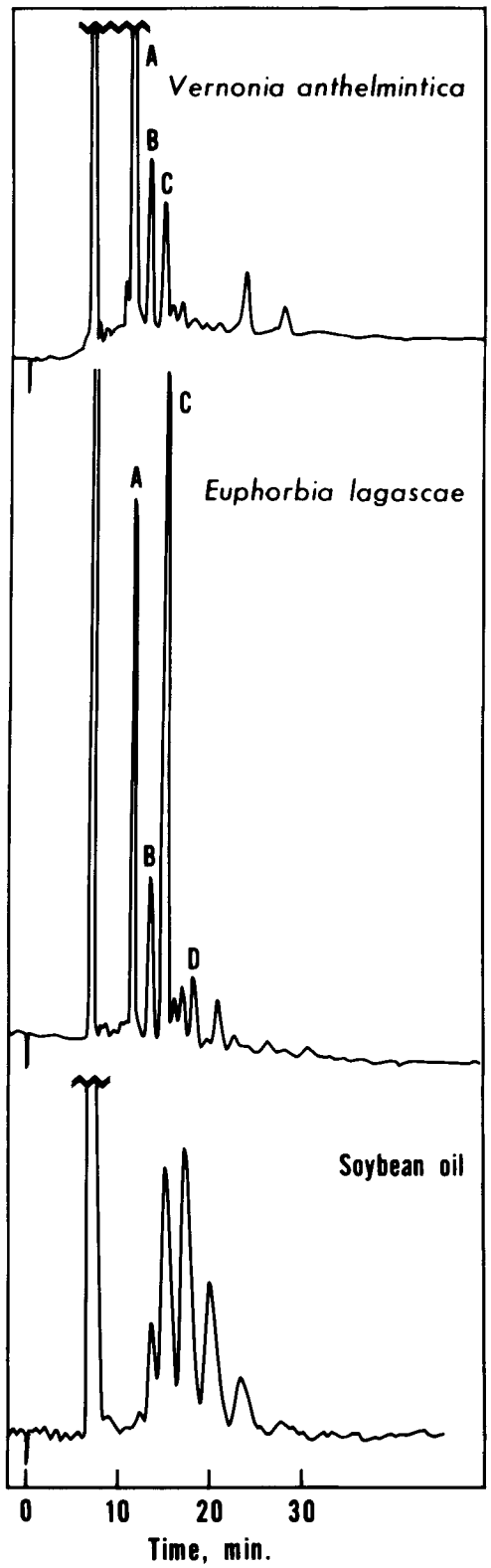

FIG. 1. HPLC chromatograms of three seed oils, *Vernonia anthelmintica, Euphorbia lagascae,* and soybean oil using a 2 ft x ¼ in. μ-Bondapak C_{18} column with acetonitrile-acetone (2:1, v/v) as the eluting solvent and a differential refractometer detector. Component identification: (A) trivernolin, (B) divernoyl linolein, (C) divernoyl olein, (D) divernoyl stearin.

volumes were reproducible to ±0.5 ml, and the trivernolin peak was 1 ml wide.

The plot of peak area vs. trivernolin weight (Fig. 2) was linear over the range of 33 μg to 1.3 mg of trivernolin injected. Better reproducibility was obtained at the lower

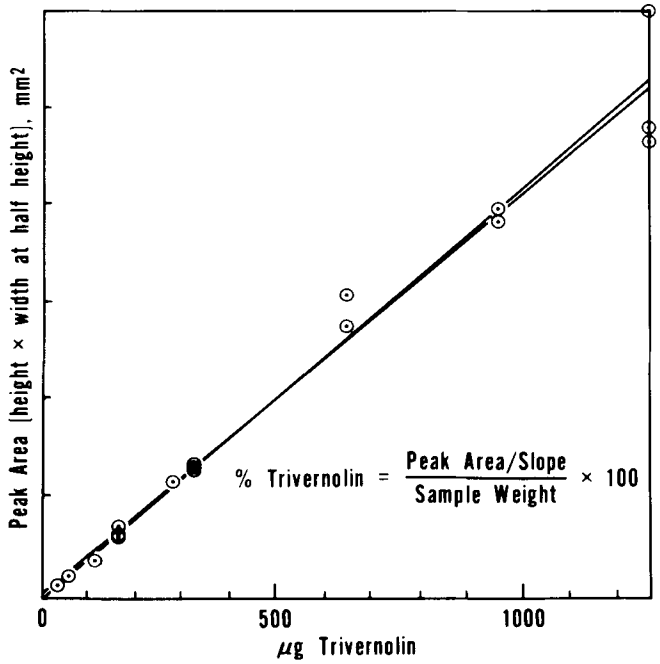

FIG. 2. A plot of peak area vs. μg trivernolin injected for determination of differential refractometer response.

concentrations, probably because attenuation was not necessary to keep the peak on scale. Some broadening of peaks was noted at higher sample loads, indicating some overloading of the column. A plot of peak height vs. trivernolin was therefore not linear.

The percentage of trivernolin in the seed oil can be calculated from Figure 2. The slope of the line was determined by linear regression, and the following equation was used:

$$\% \text{ Trivernolin} = \frac{\text{area of trivernolin peak/slope}}{\text{weight of oil injected}} \times 100$$

Table I shows a triplicate analysis for trivernolin in several samples including three different seed oils. The *Vernonia anthelimintica* seed oils ranged from 39 to 48% trivernolin. The *Euphorbia lagascae* seed oils ranged from 12 to 19%. The *Stokesia laevis* seed oils contained large amounts of vernolic acid; however, because the oils contained large amounts of free acids and partial glycerides, their trivernolin content only ranged from 1.9 to 4.9%. The *Stokesia* oils, like *Euphorbia lagascae,* had significant amounts of divernoyl and monovernoyl triglycerides, indicating a more random distribution of their triglyceride.

This method for quantitation of trivernolin by direct analysis of the oil by HPLC could provide a rapid procedure for use in plant-breeding studies or for comparison of natural epoxy oils from different species. Good reproducibility is obtained for samples which contain primarily triglycerides. In the *Stokesia* oils, when large amounts of free acids are present, the standard deviation for the 2-5% trivernolin were nearly as large as for the *Vernonia* oils with about 39-48% trivernolin. This indicates that it is more difficult to detect statistically significant differences in trivernolin content when it is present in small amounts. The method could potentially be extended for use in analysis of industrial epoxidized oils.

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