Rapid Transesterification and Mass Spectrometric Approach to Seed Oil Analysis

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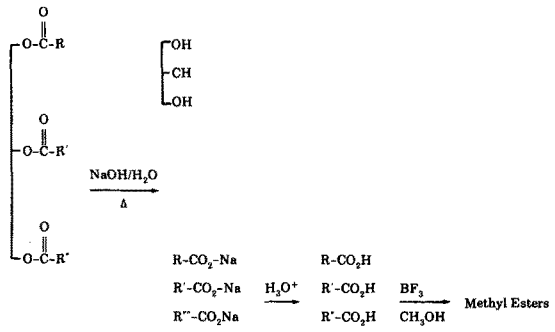
Fatty acid composition of seed oil is determined in less than one hr using a quantitative one-vial technique. The method of analysis requires alcoholic solutions of sodium methoxide mild enough for epoxy and polyunsaturated oils. Separation and characterization of component fatty acids were accomplished by high resolution gas chromatography-mass spectrometry.

Using this method, *Vernonia galamensis* seed oil is shown to contain 79–80% vernolic (*cis*-12,13-epoxy-*cis*-9-octadecenoic) acid. *Amaranthus cruentus*, a West African vegetable crop, is shown to contain 17.3% palmitic acid, 3.2% stearic acid, 22.7% oleic acid, 54.7% linoleic acid and 2.1% linolenic acid.

Most seed oils that are extracted with nonpolar organic solvents consist of 90–100% glycerides, 5–10% free fatty acids (FFA) and 5–10% fat soluble organic compounds. Thus the established method of seed oil analysis involves basic hydrolysis of the crude oil (1,2), followed by acidification and methylation with an appropriate reagent (e.g., BF_3-CH_3OH, H_2SO_4-CH_3OH, CH_2N_2-ether) to obtain methyl esters (Scheme 1).



SCHEME 1.

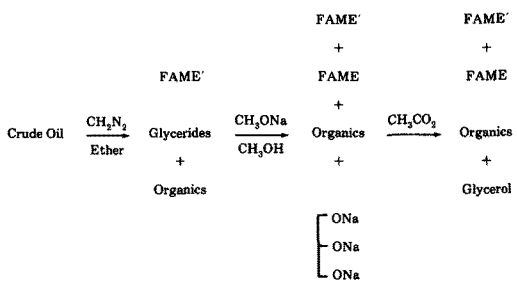
The methylated oil sample is subsequently analyzed by gas chromatography for fatty acid methyl esters (1,2). This procedure is time consuming, particularly when screening a large number of samples. In addition, the procedure could result in isomerization of unsaturated molecules and partially or completely destroy reactive functionalities that may be present in the alkyl chain of the constituent carboxylic acids (3). It should be noted that such functional group transformations may be desired by the investigator (4); otherwise, this process is not suitable for seed oil containing epoxy, hydroxy, cyclopropenyl and cyclopropyl acids (3).

Recent and renewed interest in seed oils as sources of industrial chemicals (5,6) fostered a need to develop a fast, yet efficient and mild method for the determination of fatty acid composition of seed oils. To this end, research groups at the Northern Regional Research Center, ARS/USDA, Peoria, Illinois, developed a transesterification process using methanolic sodium methoxide solution that enables direct qualitative screening of ground seed for the component triglyceride acids (J.F. Cavins, personal communications). However, this method, though very quick and dependable for fatty acid analysis of a large number of samples, is only qualitative and may not be appropriate for seed samples that contain significant levels of free fatty acids (FFA). In such cases, the FFA will not be esterified and thus may pose a problem during the gas chromatographic analysis.

The present study describes a related method that is designed to analyze quantitatively and quickly a large number of seed oils under mild conditions. A detailed account of this approach will be presented using seed oils from *Vernonia galamensis* and *Amaranthus cruentus* as model samples. *V. galamensis* seed oil previously has been reported to contain 72–78% vernolic (*cis*-12,13-epoxy-*cis*-9-octadecenoic) acid (4,5), while species of *Amaranthus* have been reported to contain 37–51% linoleic acid, 19–34% oleic acid, 20% palmitic acid, other minor acids and 4–8% squalene (7).

EXPERIMENTAL

Preparation and analysis of fatty acid methyl esters (Scheme 2). The process is begun from the crude seed oil: Transfer ca. two mg crude seed oil to a 5- 10-ml glass vial, add one ml diazomethane-ether solution (see preparation below). Shake thoroughly and let stand for one min; add ca. 0.2 mg squalane as internal standard. Inject 0.2–1.0 μl of sample into a gas chromatograph to quantitate the FFA composition and any fat soluble organic constituent. After a minimum of triplicate analysis, add 16 μl 3.33M CH_3ONa/CH_3OH solution (slowly dissolve 15.3 g sodium metal in methanol to make 200 ml solution). Shake thoroughly for a few seconds and let stand for 5–10 min, then add 10 μl acetic acid. Allow the suspension to settle, and then inject between 0.2–1.0 μl clear solution into a gas chromatograph for the analysis of total fatty acid composition.



SCHEME 2.

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TABLE 1

Percent Seed Oil in *Vernonia galamensis* and *Amaranthus cruentus*

	Seed origin	Color	% Oil[a]
Vernonia galamensis	Kenya, 1978 crop	Dark brown	38.05
Vernonia galamensis	Zimbabwe, 1985 crop	Light brown	37.57
Amaranthus cruentus[b]	Nigeria	Dark	7.90

[a]Percent dry weight.
[b]From National Horticultural Research Institute, Ibadan, Nigeria.

In order to demonstrate the potential of the above procedure, we analyzed seed oils from two samples of *Vernonia galamensis*. The samples were from trial plantings in Kenya and Zimbabwe, obtained through Robert Perdue, U.S. Department of Agriculture, Beltsville, Maryland. *Vernonia galamensis* is an annual herbaceous plant, rich in epoxytriglyceride, that recently has been shown to have great potential as a cultivable crop in Asia and in tropical and subtropical Africa (5,8). Also analyzed was a sample of *Amaranthus cruentus* obtained from the National Horticultural Research Institute, Ibadan, Nigeria. Each seed sample was ground with mortar and pestle and extracted with glass distilled pentane in a 10-hr Soxhlet extraction. The extracts subsequently were concentrated over a gentle stream of nitrogen gas in a hood to obtain the crude seed oil (Table 1). Each crude seed oil sample was then esterified as described above (Scheme 2), and the ethereal solution of the methylated seed oil was analyzed by direct injection into a gas chromatograph-mass spectrometer. It should be noted that the present procedure (Scheme 2) can be completed in less than one hr, whereas, the established method (Scheme 1) may take several hours even though both procedures have the same number of steps.

Combined gas chromatography-mass spectrometry (GC/MS). A Finnigan gas chromatograph (Model 9611) equipped with a spitless injector and interfaced with a Finnigan MAT 4500 automated mass spectrometer with a SUPERINCOS data system was used for the identification and quantitation of the various components. The interface oven and transfer line were maintained at 300 C, ionizer temperature setting was 140 C and electron energy was 70 eV. The MS was operated in the EI mode with emission current 0.27 mA and electron multiplier 1300 V. The scan rate was set at one scan/sec with scan to scan settling time 0.05 sec. High resolution capillary gas chromatography was obtained with the use of Supelco fused silica SPB-5 (30 m, 0.25 mm I.D., 0.25 n m film) temperature programmed from 50 C to 300 C. Helium was used as carrier gas with a head pressure of 10 psi. Known quantities of squalane (99%, Aldrich Chemical Company, Milwaukee, Wisconsin) were used as internal standards. Quantitation was achieved by using the relative GC peak areas and appropriate conversion factors. The conversion factors were determined by using mixtures containing known quantities of methyl palmitate, methyl stearate, methyl oleate, methyl linoleate, methyl vernolate and squalane. Identification of the methyl esters and squalene were made by comparison of the GC retention times and mass fragmentation data with those of authentic samples (Fig. 1).

Preparation of diazomethane solution. A 500-ml Erlenmeyer flask is charged with 100 g 40% aqueous potassium hydroxide, 200 ml diethyl ether and a mag-

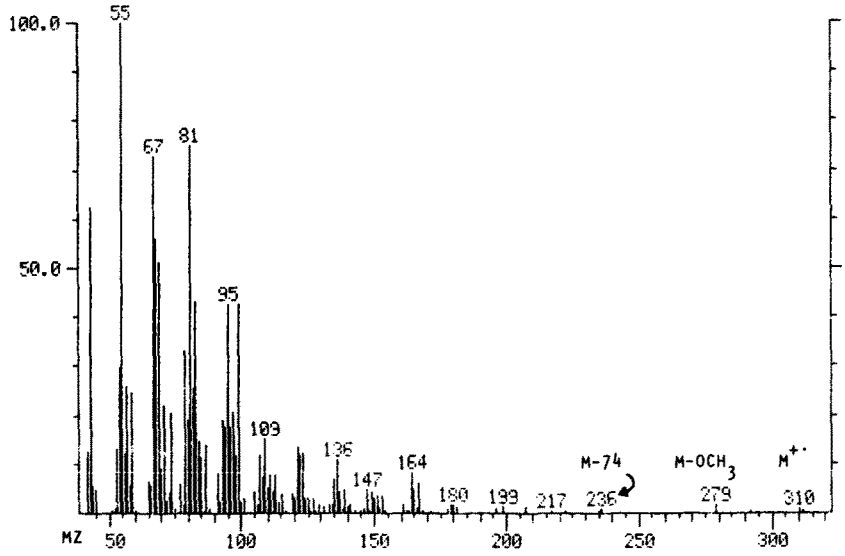

FIG. 1. Mass spectrum of methyl vernolate from *Vernonia galamensis* seed oil. The molecular ion (M^{+}.) lost a methoxy group to give rise to an ion at m/z 279.

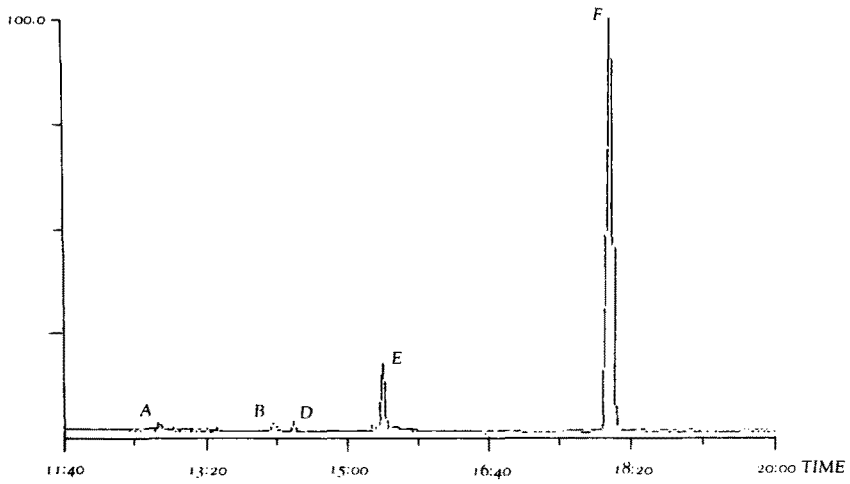

FIG. 2. Reconstituted ion chromatogram of methylated crude seed oil from *Vernonia galamensis* (Kenya 1978 crop), indicating the free fatty acids. A, C16:0; B, C18:2; D, C18:0; E, vernolic acid (12,13-epoxy-*cis*-9-octadecenois); F, squalane (internal standard).

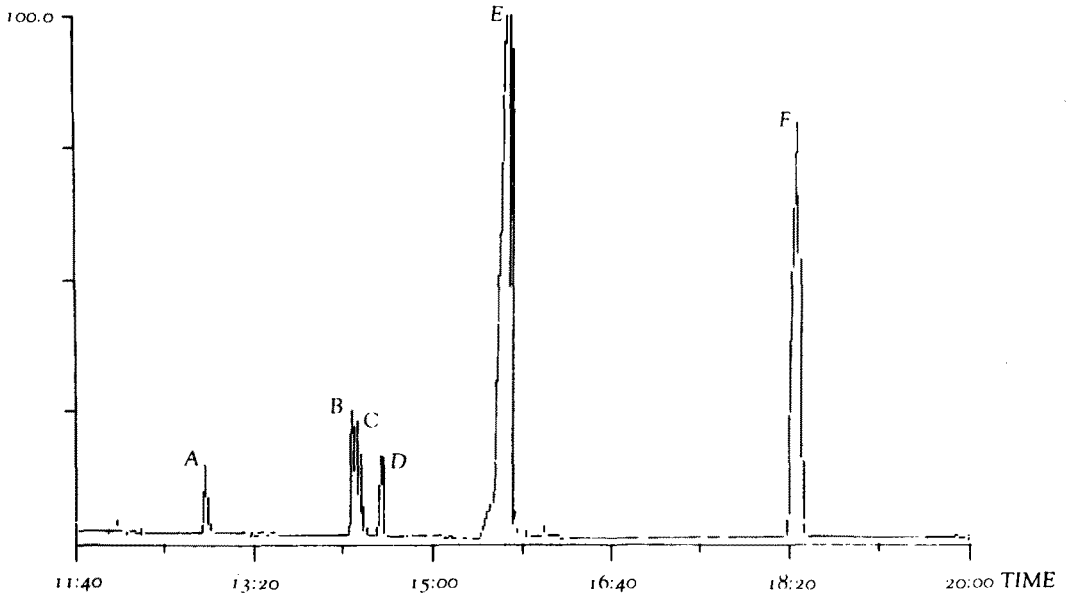

FIG. 3. Reconstituted ion chromatogram showing total fatty acid methyl esters from *Vernonia galamensis* (Kenya 1978 crop). A, C16:0; B, C18:2; C, C18:1; D, C18:0; E, methyl vernolate; F, squalane (internal standard).

netic stirrer. The mixture is cooled to about 10 C. Then 5.0 g 1-methyl-3-nitro-1-nitrosoquanidine (MNNG, from Adlrich Chemical Company, Milwaukee, Wisconsin) is added slowly with vigorous stirring. The mixture is allowed to stand until the ether layer separates. The bright yellow ether solution is then transferred into a flask with a cork stopper and stored in the refrigerator for subsequent use. (Caution: diazomethane is sensitized by ground glass joints and can explode).

RESULTS AND DISCUSSION

The seed oil analysis procedure described in this paper (Scheme 2) provides a very simple, yet efficient means for routine investigation of seed oils for fatty acids without destroying or transforming any reactive functionalities. Additionally, it offers a means for the quantitative transformation of glycerides to the constituent fatty acids, with minimum handling of the sample, thus enhancing the reliability of the quantitation. Shown in Figure 2 is the reconstituted ion chromatogram of a methylated *V. galamensis* seed oil indicating the FFA relative to the internal standard, while Figure 3 shows the chromatogram of the same sample after transesterification (Scheme 2). Listed in Tables 2 and 3 are the percent FFA and percent total acids, respectively, in *V. galamensis* and *A. cruentus*.

TABLE 2

Weight Percent of FFA in *Vernonia galamensis* and *Amaranthus cruentus* Seed Oil

	Vernolic acid[a]	C16:0	C18:0	C18:1	C18:2
Vernonia galamensis[b]	4.58	0.11	0.10	0.13	0.26
Vernonia galamensis[c]	5.81	0.26	0.23	0.51	1.67
Amaranthus cruentus[d,e]	—	1.30	Trace	1.33	.57

[a]Contains trace amount of an unidentified isomer.
[b]From Kenya 1978 crop.
[c]From Zimbabwe 1985 crop.
[d]From National Horticultural Research Institute, Ibadan, Nigeria.
[e]Also contains 3.19% squalene.

TABLE 3

Relative Weight Percent of Fatty Acids in *Vernonia galamensis* and *Amaranthus cruentus*

	Vernolic acid[a]	C16:0	C18:0	C18:1	C18:2	C20:0
Vernonia galamensis[b]	79.10	2.58	2.88	6.43	9.64	.37
Vernonia galamensis[c]	79.81	2.37	2.22	5.37	10.23	—
Amaranthus cruentus[d,e]	—	17.30	3.23	22.66	54.71	—

[a]Also contains 2–6% of an unidentified isomer.
[b]From Kenya 1978 crop.
[c]From Zimbabwe 1985 crop.
[d]From National Horticultural Research Institute, Ibadan, Nigeria.
[e]Also contains 3.19% squalene.

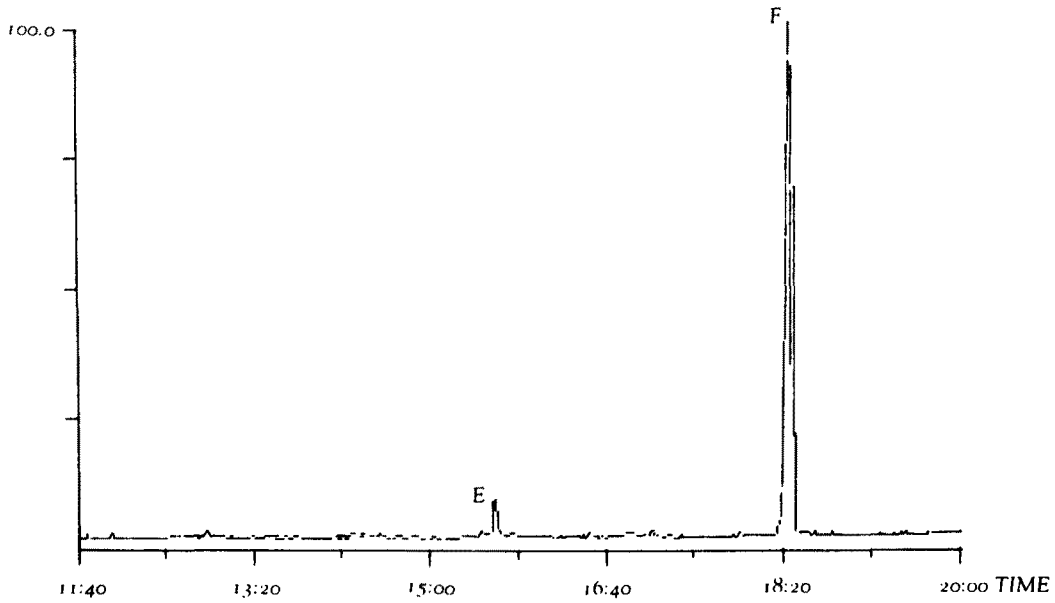

FIG. 4. Reconstituted ion chromatogram of methylated crude seed oil obtained from preheated (100 C for 4 hr) ground seed of *Vernonia galamensis* (Kenya 1978 crop). E, methyl vernolate (1.07% of oil).

These data are consistent with previously reported values (4,7). The data also indicate that crude *V. galamensis* seed oil contains significant levels of FFA. This is in agreement with the recent study by Carlson and co-workers, in which they demonstrated that *V. galamensis* seeds contain an active lipase which catalyzes the hydrolysis of triglycerides to FFA if the seed is crushed without pretreatment with steam (4). We therefore felt it might be of interest to immediately preheat the ground seed at 100 C for four hr prior to extraction. Figure 4 shows the chromatogram and percent FFA (1.07%) of oil extracted from the preheated ground seed, indicating a result comparable with Carlson's findings of 0.78% FFA from steam pretreated whole

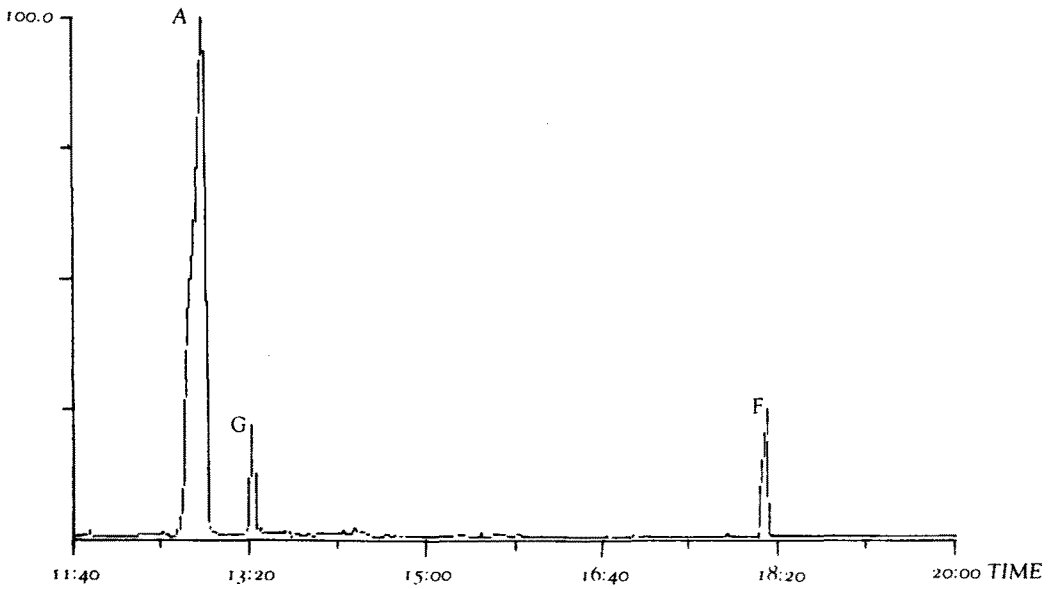

FIG. 5. Reconstituted ion chromatogram of commercially purchased tripalmitin after transesterification with alcoholic sodium methoxide. A, methyl palmitate; G, methyl 2-methylhexadecanoate; F, squalane (internal standard).

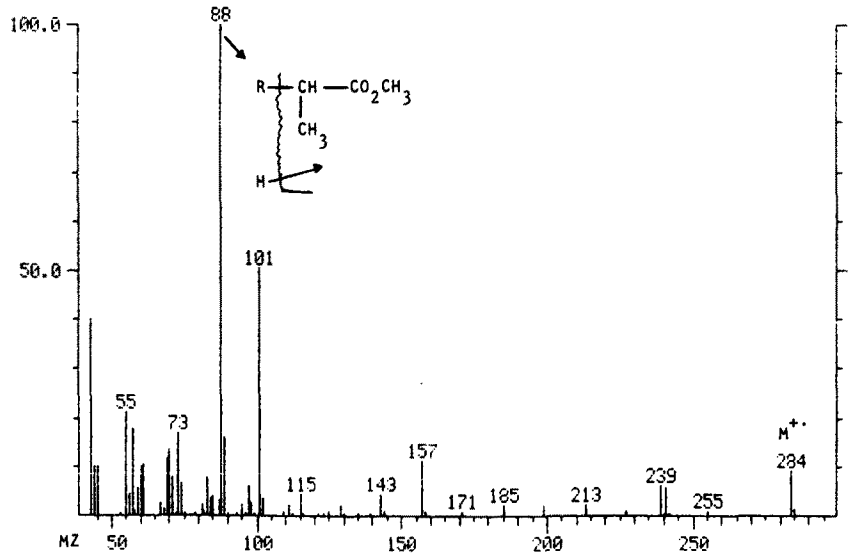

FIG. 6. Mass spectrum of methyl 2-methylhexadecanoate. The ion at m/z 88 results from C_2–C_3 cleavage accompanied by C_4-hydrogen transfer to the carbonyl group.

seeds. Currently we are undertaking further investigation to determine the optimum heat treatment conditions that would deactivate the lipase action in ground *V. galamensis* seed. If this preliminary finding can be confirmed further, this might constitute a viable means of lipolyptic enzyme deactivation in *Vernonia* seed for commercial processing.

Finally, in order to demonstrate the usefulness of the approach, some commercially purchased triglycerides were analyzed. The reconstituted ion chromatogram of tripalmitin (99+%, Supelco, Inc., Bellefonte, PA) is shown in Figure 5, indicating about 4% of 2-methylhexadecanoic acid (MS in Fig. 6) with trace amounts of oleic acid. Thus, the approach described in this study constitutes a simple, reliable and fast method for the routine analysis of seed oils.

ACKNOWLEDGMENTS

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