Bis(trimethylsilyl)acetamide in the silylation of lipolysis products for gas–liquid chromatography

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SUMMARY When bis(trimethylsilyl)acetamide is used to silylate lipolysates for gas–liquid chromatography, the pyridine solution containing the sample and silylating agent can be injected directly onto the column. Conversion of free fatty acids to methyl esters before silylation is unnecessary, since silyl esters elute as sharp gas–liquid chromatographic peaks.

KEY WORDS silylation · trimethylsilylation · silyl esters · bis(trimethylsilyl)acetamide · hexamethyldisilazane · trimethylchlorosilane · gas–liquid chromatography · pancreatic lipase · intraglyceride distribution patterns · vernolic acid · dimorphecolic acid · α-eleostearic acid · *Cephalaria joppica* · *Dimorphotheca sinuata* · *Centranthus macrosiphon*

Within the past few years a number of unusual fatty acids from seed oils have been discovered in this laboratory and others. A 1966 review lists 35 examples (1). We have undertaken the determination of intraglyceride distribution patterns of some of these unusual acids, for example, vernolic acid (2). Until recently, for such determinations we employed a sequence including methylation, silylation (more correctly, trimethylsilylation), and temperature-programmed GLC of unfactionated products from treatment of seed oils containing unusual acyl groups with pancreatic lipase (3). The mole percentages of various components in the monoglycerides can be calculated from the areas of peaks in the monoglyceride region of the chromatogram obtained.

The excellent GLC separations of Krebs' cycle acids as silyl esters reported by Horii, Makita, and Tamura (4) suggested a further simplification of our method—namely, elimination of the treatment with diazomethane to convert free acids to methyl esters. Unfortunately, silylation with the combination of reagents we used (HMDS plus TMCS) resulted in the appearance of extraneous peaks on gas chromatograms of lipolysis products containing acid-sensitive groups (e.g., epoxide rings). Apparently, ammonium chloride that formed as a by-product in the silylation reaction was responsible, and the decomposition was avoided by an aqueous wash included in our previous procedure (3) to remove the inorganic salt. However, silyl esters, being much more easily hydrolyzed than silyl ethers, would be destroyed by the washing.

Use of an excess of a highly reactive silyl donor, BSA (5), has made it possible to simplify our method of analyzing seed oil lipolysates. Mono(trimethylsilyl)acetamide is formed as the by-product. It causes no decomposition, is eluted from the GLC column with the solvent, and therefore eliminates the need for an aqueous wash. The pyridine solution containing the sample to be analyzed and the silylating agent can be injected directly onto the column. Avoiding the aqueous wash in turn makes methylation of free fatty acids before silylation of the sample unnecessary, since silyl esters give sharp peaks on chromatograms. In this note we illustrate the advantages of BSA over HMDS plus TMCS as a silylating agent by analysis of lipolysates of two oils containing acid-sensitive acyl groups.

Materials. Pancreatic lipase (EC 3.1.1.3) (steapsin) came from Nutritional Biochemicals Corporation,[1] Cleveland, Ohio, and was extracted with acetone and ether as described by Luddy, Barford, Herb, Magidman, and Riemenschneider (6). Bacto bile salts were from Difco Laboratories, Detroit, Mich. Methyl vernolate was prepared from *Cephalaria joppica* oil (2). All other reference compounds were acquired from The Hormel Institute, Austin, Minn. BSA reagent was prepared by dissolving 1 ml of the pure silylating agent from Perco Supplies, San Gabriel, Calif. in 5 ml of Fischer reagent grade pyridine (No. 214H, Eastman Organic Chemicals, Rochester, N.Y.), further purified by distillation from barium oxide. HMDS plus TMCS reagent, a mixture of HMDS and TMCS in pyridine (Tri-Sil), was purchased from Pierce Chemical Co., Rockford, Ill.

Characterizations of unusual acids from the seed oils selected for this study have been reported as follows: vernolic (*cis*-12,13-epoxy-*cis*-9-octadecenoic) acid from *Cephalaria joppica* oil (2); dimorphecolic (9-hydroxy-*trans*-10,*trans*-12-octadecadienoic) acid from *Dimorphotheca sinuata* (formerly *D. aurantiaca*) oil (7); and α-eleostearic (*cis*-9,*trans*-11,*trans*-13-octadecatrienoic) acid from *Centranthus macrosiphon* oil (8).

Lipolysis. A sample of oil (100 mg) was suspended in 10 ml of distilled water to which we added 1 ml of 22% calcium chloride solution and 2 ml of 0.1% Bacto bile salt solution. The mixture was magnetically stirred and maintained at 40°C. The pH was adjusted to 8.0 by addition of 0.1 N sodium hydroxide. A suspension of 80 mg of the extracted steapsin in 2 ml of distilled water was added, and the pH was maintained at 8.0 with a

Abbreviations: GLC, gas–liquid chromatography (chromatographic); BSA, bis(trimethylsilyl)acetamide; HMDS, hexamethyldisilazane; TMCS, trimethylchlorosilane.

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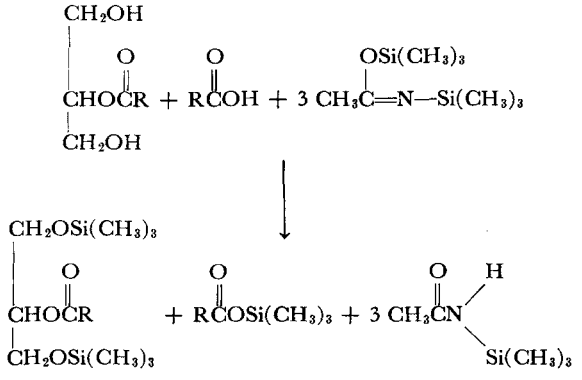
[1] Mention of trade products or firm names does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over similar products or other firms not mentioned.

Radiometer pH-stat. When the volume of 0.1 N sodium hydroxide consumed corresponded to the desired extent of lipolysis, the reaction was stopped by pouring the mixture into a separatory funnel that contained crushed ice, 1 ml of 1 N hydrochloric acid, and 20 ml of diethyl ether. The lipolysis products and any unhydrolyzed oil were extracted with diethyl ether, and the organic solvent was removed in vacuo. The final product was dried in a vacuum desiccator over phosphorus pentroxide.

Silylation and GLC. The silylating reagent, 0.2–0.3 ml, was added to 2–3 mg of the lipolysis product in a centrifuge tube. The centrifuge tube was immediately stoppered, the reaction mixture was shaken for 1 min, and 3 μl of it was injected directly onto the GLC column. Conditions for GLC were the same as previously described (3), except that the column was packed with 3% OV-1 on 60–80 mesh Gas-Chrom Q (Applied Science Laboratories Inc., State College, Pa.). OV-1 is claimed to be similar to SE-30 in partitioning properties but to have better thermal stability.

Percentages of components were computed from GLC results as previously described (3).

Results and Discussion. The silylation of a monoglyceride and a fatty acid with BSA are illustrated as follows:



Gas chromatograms from our evaluation of BSA as a silylating agent for lipolysis products are shown in Fig. 1 (curves *1–6*). In contrast to the stationary phase employed in our previous work (3), the OV-1 now used gives partial separation of α- from β-monoglycerides and of saturated from unsaturated C_{18} silyl esters.

GLC after silylation with BSA of a standard mixture containing an epoxy acid produced a chromatogram (curve *1*) with no extraneous peaks. Table 1 shows the agreement of results calculated from this chromatogram with the actual amounts of the components used to make up the mixture. Such agreement would not have been achieved if there had been significant decomposition of the epoxide ring in vernolic acid.

The chromatogram for a lipolysate of an epoxy oil silylated with BSA (curve *2*) has a smooth base line and no significant peaks other than those for expected components. For comparison, the same lipolysate was again analyzed in exactly the same manner except that HMDS plus TMCS was used as the silylating agent. In the resulting chromatogram (curve *3*), the many small peaks around the silyl vernolate peak and the deviation from the base line after the emergence of monovernolin apparently represent decomposition products formed as a result of the presence of ammonium chloride during

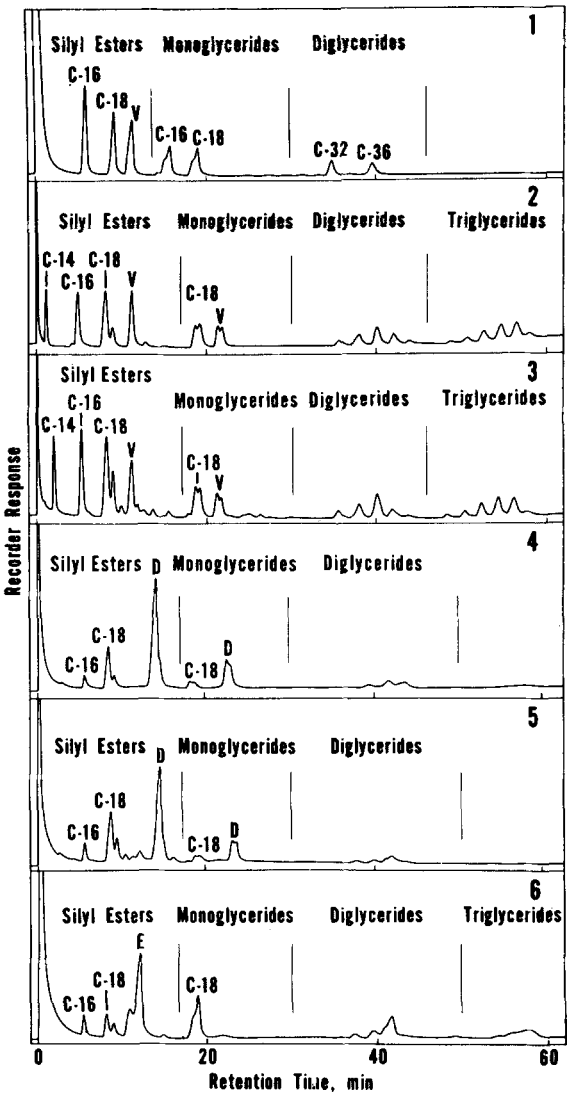


FIG. 1. Temperature-programmed (4°C/min) GLC of silylated samples. Conditions were as given in Ref. 3 except that a 3% OV-1 on 60–80 mesh Gas-Chrom Q column was used. Peaks representing silyl esters and monoglycerides are identified by the chain length for nonoxygenated acyl groups. Silylated dipalmitin and distearin in curve *1* are designated C-32 and C-36, respectively. V, silylated vernolic acid and monovernolin; D, silylated dimorphecolic acid and monodimorphecolin; E, silyl α-eleostearate. Curve *1*, standard mixture silylated with BSA; curves *2* and *3*, lipolysis product of *Cephalaria joppica* oil silylated with BSA and with HMDS plus TMCS, respectively; curves *4* and *5*, lipolysis product of *Dimorphotheca sinuata* oil silylated with BSA and with HMDS plus TMCS, respectively; curve *6*, lipolysis product of *Centranthus macrosiphon* oil silylated with BSA.

TABLE 1 GLC OF STANDARD MIXTURE SILYLATED WITH BSA

Component	% of Total	
	Actua	Found
Palmitic acid	20	22
Stearic acid	17	19
Vernolic acid	20	20
Monopalmitin	13	13
Monostearin	14	13
Dipalmitin	8	7
Distearin	7	6

TABLE 2 DETERMINATION OF UNUSUAL ACYL GROUPS IN MONOGLYCERIDES AFTER SILYLATION WITH BSA AND WITH HMDS PLUS TMCS

Source of Seed Oil	Acyl Group*	Moles % in Oil	Moles % in Monoglycerides	
			BSA	HMDS + TMCS†
Cephalaria joppica (Spreng.) Beg.	Vernoloyl	27	47	37
Dimorphotheca sinuata DC.	Dimorphecoloyl	71	88	72
Centranthus macrosiphon Boiss.	α-Eleostearoyl	65	~0	—

* Vernoloyl, *cis*-12,13-epoxy-*cis*-9-octadecenoyl; dimorphecoloyl, 9-hydroxy-*trans*-10,*trans*-12-octadecadienoyl; α-eleostearoyl, *cis*-9,*trans*-11,*trans*-13-octadecatrienoyl.

† As in Ref. 3 except no water wash to remove NH_4Cl.

GLC. The effect of this decomposition on the calculated monovernolin content of monoglycerides from the *Cephalaria joppica* oil can be seen in Table 2.

The 47 mole % monovernolin content in monoglycerides from *Cephalaria joppica* oil calculated from curve *2* agrees very well with the 46 mole % previously found (3). In this study we deliberately limited the extent of lipolysis of this oil to 35% instead of the 51–54% used before. This change was made to secure further evidence against a possible slower rate of hydrolysis of vernoloyl glycerides by the lipase (see pertinent comments in Ref. 2). If such discrimination occurred, the monoglyceride composition would be expected to change with the extent of lipolysis as a result of selective hydrolysis of α-monoglycerides arising from isomerization of the β-monoglycerides initially formed (9).

Another oil investigated in our comparison of BSA and HMDS plus TMCS as silylating agents for lipolysates was one containing dimorphecoloyl groups. The conjugated dienol system in these groups is very sensitive to acid as it is readily dehydrated to a conjugated triene (7). Freedman (10) obtained a gas chromatogram of the silyl ether of methyl dimorphecolate showing only a slight amount of dehydration to triene. We see no evidence of this dehydration on the chromatogram for the BSA-silylated lipolysate of *Dimorphotheca sinuata* oil (curve *4*); but when HMDS plus TMCS was used for the silylation, significant decomposition occurred (curve *5*). Again, the effect of this decomposition on monoglyceride composition calculated from GLC results is shown in Table 2. Curve *6* was obtained for comparison with curve *5*. Methyl α-eleostearate is known to give multiple peaks due to *cis,trans*-isomerization and movement of the conjugated triene system up and down the chain during GLC (11, 12). Consequently, the appearance of two peaks in the silyl ester region of curve *6* is not surprising. That these peaks line up very well with two of the minor peaks in the silyl ester region of curve *5* supports the assumption that conjugated triene forms during GLC of the lipolysate of the *Dimorphotheca* oil silylated with HMDS plus TMCS. The absence of a significant mono-α-eleostearin peak in curve *6* indicates a strong 1,3-preference for the α-eleostearoyl group. Gunstone, Hamilton, Padley, and Qureshi (13) found a similarly strong preference for the primary glycerol hydroxyls by the 9-*trans*,11-*trans*,13-*cis*-conjugated C_{18} trienoic acid in *Catalpa bignonoides* seed oil but a much weaker 1,3-preference by α-eleostearoyl groups in tung oil.

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