Hydrazinolysis of lipids and analysis of their constituent fatty acids

Vishwanath P. Agrawal[1]

Federal Center for Lipid Research, Piusallee 68/76, D-4400 Münster, Federal Republic of Germany[2]

Summary Treatment of *O*-acyl lipids with hydrazine degrades them to hydrazides of the constituent fatty acids. These hydrazides react with acetone to yield less polar and more volatile *N'*-isopropylidenealkanohydrazides, $R-CO-NH-N=C(CH_3)_2$, which strongly absorb ultraviolet light and are amenable to thin-layer and gas–liquid chromatographic analyses. In presence of cyclopentene, reduction of double bonds in unsaturated fatty acids with hydrazine does not occur. The method of hydrazinolysis followed by acetonization has been used for fatty acid analysis of different classes of lipids.—**Agrawal, V. P.** Hydrazinolysis of lipids and analysis of their constituent fatty acids. *J. Lipid Res.* 1983. **24:** 216–220.

Supplementary key words acid hydrazides • *N'*-isopropylidenealkanohydrazides • lipid analysis

Degradation of biopolymers (1, 2) and synthetic fibers (3) by hydrazinolysis has been described, but the hydrazinolysis of lipids has not been reported. This communication describes a method for the quantitative conversion of *O*-acyl lipids to acid hydrazides. Reaction of the hydrazides with acetone yields the less polar and more volatile *N'*-isopropylidenealkanohydrazides, $R-CO-NH-N=C(CH_3)_2$. These isopropylidene derivatives can be resolved by gas–liquid chromatography. Moreover, they should be amenable to high performance liquid chromatography using a UV detector, as they absorb light at 229 nm.

MATERIALS AND METHODS

All chemicals were purchased from E. Merck A.G., D-6100 Darmstadt, Germany, except 98% hydrazine hydrate which was from Fluka A.G., CH-Buchs S.G., Switzerland. Glycerylphosphorylcholine and α-glycerophosphate were obtained from Sigma Chemie GmbH, D-8028 Taufkirchen, Germany. Ratfish (*Hydrolagus colliei*) liver oil was a gift from Dr. D. C. Malins, Seattle, WA 98112. 1-*O*-Hexadecylglycerol was prepared by Mrs. H. Becker of this laboratory (4). Phosphatidylcholine from egg (E. Merck A.G.) was purified by TLC on silica gel G using chloroform–methanol–water 65:35:4 (v/v) for development and chloroform–methanol 2:1 (v/v) for elution. Tetrahydrofuran (THF) was refluxed with $LiAlH_4$ and then distilled. Ethanol and THF were degassed before use.

Adsorption-TLC was carried out on activated layers (0.3 mm) of silica gel H. Chromatograms were visualized either by charring with H_2SO_4 or, when the products had to be recovered, by spraying with 0.2% 2',7'-dichlorofluorescein in ethanol. Hydrazinolysates were chromatographed with chloroform–methanol 9:1 (v/v) as developing solvent. TLC of isopropylidenealkanohydrazides was done using chloroform as developing solvent and eluant. Solvent systems for TLC of phosphatidylcholine and 1-*O*-alkylglycerols were chloroform–methanol–water 65:35:4 (v/v) and ethyl ether–water 100:0.5 (v/v), respectively (5).

Argentation-TLC of isopropylidenealkanohydrazides was carried out on activated layers of silica gel H, impregnated with 2% (w/w) $AgNO_3$, using chloroform–methanol 85:15 (v/v) for development. For the recovery of the derivatives, silica gel from the appropriate region was scraped off and extracted thrice with 10 ml of 90% methanol containing 1% NaCl. The combined methanol extracts were extracted with chloroform and dried over Na_2SO_4.

Analytical GLC was carried out using a Perkin-Elmer Gas Chromatograph F-22, equipped with a flame ionization detector and coupled to Spectra-Physics Autolab System IVb Chromatographic Data Analyser. Methyl esters and isopropylidenealkanohydrazides were analyzed on glass columns (2 m × 6.3 mm) packed with 10% and 3% Silar 10 C on Gas Chrom Q (Applied Science Laboratories Inc., State College, PA 16801) at 185°C and 210°C, respectively; N_2 was used as the carrier gas at a flow rate 40 ml/min. Peaks were identified using compounds of known structure.

Preparative GLC was carried out using a thermal conductivity detector. Helium was used as the carrier gas at a flow rate 40 ml/min, and hexane at −20°C was used to trap effluents from the gas chromatograph.

Spectrophotometric measurements were carried out using a Pye Unicam SP8-200 UV/VIS Spectrophotometer and methanol as solvent. In all experiments appropriate blanks were used.

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

[1] Present address: Department of Biochemistry and Biophysics, University of California, Davis, CA 95616. Permanent address: Department of Chemistry, Kirtipur Campus, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

[2] Address reprint requests to the Federal Center for Lipid Research, Piusallee 68/76, D-4400 Münster, Federal Republic of Germany.

Acid-catalyzed transesterification of lipids was done as described elsewhere (6).

For the preparation of *N'*-isopropylidenealkanohydrazides of individual fatty acids, a mixture of hydrazine hydrate–ethanol–cyclopentene 2:9:1 (v/v), 3 ml, was added to a solution of methyl esters (50 mg) in 1 ml of ethanol. The reaction mixture was flushed with N_2 for 1 min and the tube was tightly closed with a Teflon-lined screw cap and heated at 50°C for 2 hr. After addition of 5 ml of acetone, the mixture was heated at 50°C for a further 30 min. The solvents were removed by a stream of N_2 and the resulting isopropylidenealkanohydrazides were purified by TLC on silica gel H with chloroform as the developing solvent.

Procedure for the hydrazinolysis of lipids followed by acetonization

To 50 mg of lipid in 0.5 ml of THF placed in a tube (100 × 16 mm) is added 4.0 ml of hydrazine hydrate–THF–ethanol–cyclopentene 1:3:3:1 (v/v) and N_2 is bubbled through the reaction mixture for 2 min. After tightly closing the tube with a Teflon-lined screw cap, the reaction is allowed to proceed either at 40°C for 4 hr or at 50°C for 2 hr. Acetone (5 ml) is added and the mixture is heated at 50°C for 30 min. After drying a 0.1 ml aliquot of the reaction mixture, 1 ml of methanol is added and the solvent is evaporated off with N_2. This process is repeated once more and the residue is dissolved in 0.1 ml of methanol.

Procedure for the methanolysis of isopropylidenealkanohydrazides

The acetonized hydrazinolysate (2 ml) is dried under reduced pressure, dissolved in 5 ml of methanol, and dried again. The residue is transferred with 5 ml of 6% H_2SO_4 in methanol into a tube, flushed with N_2, and heated at 80°C for 3 hr. In order to recover methyl esters, the reaction mixture is extracted with chloroform (10 ml × 3), washed with 10% sodium bicarbonate, and dried over Na_2SO_4.

For the analysis of water-soluble deacylated products obtained by hydrazinolysis of phosphatidylcholine, the acetonized hydrazinolysate (5 ml) was dried in a stream of N_2, dissolved in 5 ml of methanol and dried again. This process was repeated once more. To the residue was added 1 ml of methanol followed by 1 ml of water. The mixture was extracted with chloroform (10 ml × 3) and 25–50 μl of the aqueous layer was analyzed by paper chromatography on SS no. 2040 b paper (Carl Schleicher & Schull, D-3354 Dassel, Germany) using phenol–water 100:38 (w/v) for development and sodium periodate–o-tolidine and 5-sulphonosalicylic acid–ferric chloride for detection (5).

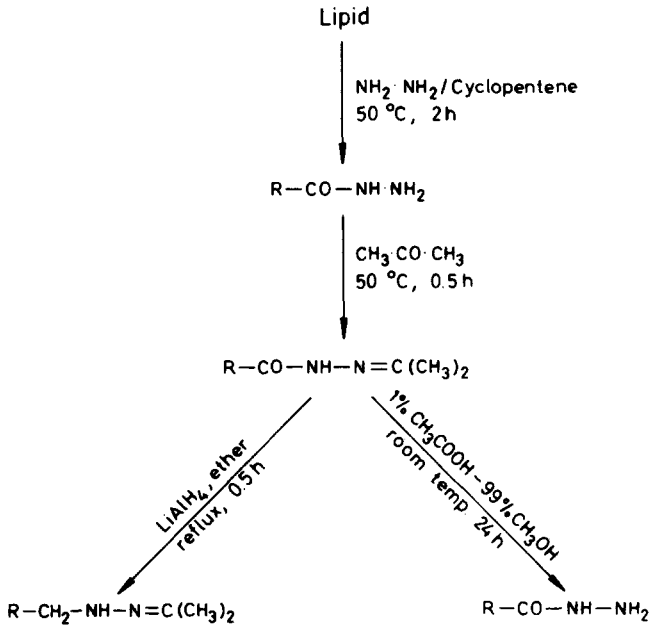

Fig. 1. Scheme for preparation and reactions of *N'*-isopropylidenealkanohydrazides.

RESULTS AND DISCUSSION

The observation that hydrazine converts methyl esters of fatty acids to the corresponding hydrazides (7) motivated the author to examine whether hydrazine can cleave the ester linkage of glycerolipids as well. Initially, soybean oil was treated with hydrazine and the degradation of its triacylglycerols to hydrazides of constituent fatty acids was suggested by the observation that TLC of the hydrazinolysate showed one major spot with a mobility similar to that of palmitic acid hydrazide. Cyclopentene was added to the reaction mixture to protect the double bonds in fatty acids from hydrazine reduction (8) by competing for diimide produced in situ through oxidation of hydrazine with traces of oxygen, peroxides, or any other oxidant. Cyclopentene was chosen because it is easily reduced by hydrazine, and has a low boiling point permitting its easy removal (**Fig. 1**).

In order to circumvent the problems that could arise in the analysis of the hydrazides due to their high reactivity, polarity and low solubility, they were converted to the corresponding isopropylidene derivatives by treatment of the hydrazinolysate with acetone. TLC of the acetonized hydrazinolysate of soybean oil showed one major fraction that had the same mobility as palmitic acid isopropylidene hydrazide (**Fig. 2**). This indicated that the triacylglycerols were degraded in quantitative yields to a class of compounds that were identified as $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$ isopropylidene hydrazides by GLC of the TLC fraction (**Fig. 3**). The structures of the various constituents were substantiated

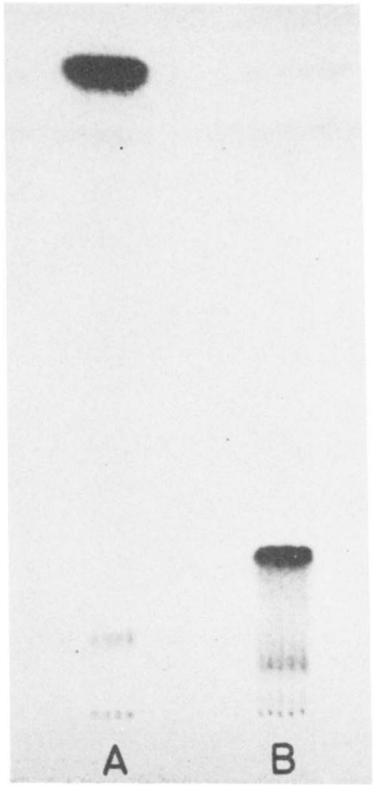

Fig. 2. Thin-layer chromatogram of *N'*-isopropylidenealkanohydrazides. A, soybean oil; B, acetonized hydrazinolysate of soybean oil. Chromatograms were developed with chloroform as described in the Materials and Methods section.

by mass spectrometry of individual compounds obtained by argentation TLC and preparative GLC. The spectra showed the characteristic mass ions (70 eV): M^+, 2–32% of the base peak at m/e 72; M^+-CH_3, 3–11%; m/e 127, 7–20%; and m/e 114, 20–30%. The intensities depended upon the degree of unsaturation of the acid.

Similarily, TLC of acetonized hydrazinolysate of ratfish liver oil indicated that 1-*O*-alkyl-2,3-diacylglycerols were deacylated by hydrazine to 1-*O*-alkylglycerols and fatty acid hydrazides.

Paper chromatography of the water-soluble fraction of the acetonized hydrazinolysate of phosphatidylcholine revealed only one spot, which migrated between glycerylphosphorylcholine and glycerophosphate. A prior treatment of the water-soluble fraction with an equal volume of 1 N NaOH had no effect on the mobility of this spot. On the other hand, when this fraction was treated with an equal volume of 1 N HCl at room temperature for 30 min and then neutralized to pH 7 with NaOH, the R_f of the spot was similar to that of glycerophosphate. These results, along with the consideration of the high pH (>11) of the hydrazinolysis mixture and nucleophilic nature of hydrazine suggested that this fraction might be due to the *N'*-isopropylidene derivative of glycerophosphoric acid hydrazide, which could be formed by nucleophilic attack of hydrazine on phosphorus of cyclic glycerophosphate.

These results, as well as those obtained by TLC of the acetonized hydrazinolysate of phosphatidylcholine, indicated that it was fully degraded by this method to isopropylidene hydrazides of constituent fatty acids and glycerophosphoric acid. Attempts to hydrazinolyse wax esters at 50°C were not successful.

In order to test the feasibility of using hydrazinolysis followed by acetonization for fatty acid analysis, acetonized hydrazinolysates of various lipids were prepared and subjected to GLC, without prior purification. The results of fatty acid analyses of different lipids as isopropylidene hydrazides were found in excellent agreement with those obtained by transesterification (**Table 1**). The observation that the results obtained by methanolysis of acetonized hydrazinolysates of lipids to methyl esters were very close to those obtained by transesterification showed the practicability of analyzing fatty acid isopropylidene hydrazides after converting them to the corresponding methyl esters. When hydrazinolysis was carried out in the absence of cyclopentene, values obtained for $C_{18:3}$ were about 10% lower than those obtained in its presence (data not shown). This result indicates that cyclopentene protects polyunsaturated fatty acids from reduction.

Since the method described here does not involve any strong acid treatment, and since the unreacted hydrazine is destroyed by acetone while other reaction

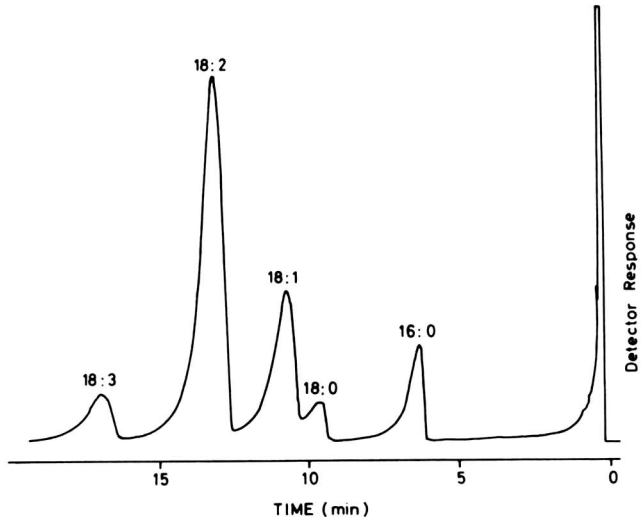

Fig. 3. Gas–liquid chromatogram of *N'*-isopropylidenealkanohydrazides derived from soybean oil by hydrazinolysis–acetonization method. GLC was done on 3% Silar 10 C at 210°C, as described in the Materials and Methods section.

TABLE 1. Gas–liquid chromatographic analyses of constituent fatty acids in lipids after hydrazinolysis and acetonization

		Percent Composition (w/w)				
		$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
Soybean oil	A)	10.1	4.5	22.1	53.8	9.4
	B)	10.1	4.5	21.8	53.8	9.8
	C)	10.3	4.5	21.8	54.3	9.1
Linseed oil	A)	5.1	3.0	15.8	16.2	59.5
	B)	5.0	3.2	16.4	15.7	59.6
	C)	5.1	3.2	16.3	15.8	59.5
Olive oil	A)	10.5	2.7	78.0	7.6	0.5
	B)	10.9	2.7	76.1	8.6	0.9
	C)	11.1	2.6	77.8	7.5	0.8
Egg lecithin	A)	33.1[a]	18.1	26.5	16.1	
	B)	33.5	17.9	27.8	15.7	
Ratfish liver oil	A)	15.1[b]	5.2	52.3		
	B)	15.3	4.5	51.9		

Experimental procedures are described in the Materials and Methods section.

A, Isopropylidene hydrazides obtained by the hydrazinolysis–acetonization method.

B, Methyl esters obtained by transesterification of the lipids.

C, Methyl esters obtained by methanolysis of acetonized hydrazinolysate of lipids.

[a] The other component representing about 5% could not be identified.

[b] Components representing the remaining 27% could not be identified, except $C_{16:1}$ which comprised 9.1% of the total acids.

products do not interfere with GLC analysis, the acetonized hydrazinolysate need not be purified by TLC before GLC. Moreover, fatty acid isopropylide hydrazides were stable for at least 6 months when the crude reaction mixture containing them was stored at 4°C.

Argentation chromatography on silica gel H impregnated with 2% (w/w) $AgNO_3$ allowed a satisfactory separation of saturated, mono-, di-, and tri-unsaturated isopropylidene hydrazides from each other. When chloroform was used as a developing solvent, the isopropylidene hydrazides of saturated and unsaturated fatty acids did not migrate at all on layers of silica gel containing 1% (w/w) or more $AgNO_3$. This indicated that silver ions, in addition to binding to olefins, also complex with the C=N bond of the isopropylidene hydrazides. This property can be utilized for purification of isopropylidene hydrazides by column chromatography. A polar solvent, such as 90% methanol containing 1% NaCl, is required to elute isopropylidene hydrazides from the absorbent; chloroform or methanol alone does not elute them in good yields. Use of a higher percentage of $AgNO_3$ in argentation TLC does not improve the resolution of isopropylidene hydrazides, but adversely affects their elution.

Hydrolysis of isopropylidenealkanohydrazides to fatty acids can be carried out by refluxing with 3 N HCl in 90% methanol for 30 min. For their conversion to hydrazides, isopropylidenealkanohydrazides are treated at room temperature with 1% acetic acid in 99% methanol for 24 hr. They are converted to methyl esters upon heating with 6% H_2SO_4 in methanol. Their reduction is carried out by refluxing with $LiAlH_4$ in either for 30 min; excess $LiAlH_4$ is decomposed with water and the resulting hydrazones $R-CH_2-NH-N=C(CH_3)_2$ are extracted with chloroform.

Spectrophotometric measurements showed that fatty acid isopropylidene hydrazides strongly absorb UV light in the 210–240 nm range, with a maximum at 229 nm. The molar extinction coefficient of palmitic acid isopropylidene hydrazide was calculated to be about 10,500 $M^{-1}cm^{-1}$ at 229 nm. The absorbance of $C_{16:0}$ isopropylidene hydrazide at 229 nm was rapidly eliminated by the addition of dilute HCl, but was unaffected by dilute NaOH. Addition of dilute acetic acid slowly decreased the absorbance by about 80%; the residual absorbance was due to palmitic acid hydrazide.

CONCLUSIONS

Based on the results described, it can be concluded that *O*-acyl lipids are degraded by hydrazine to fatty acid hydrazides that are amenable to gas–liquid chromatographic analysis after conversion to their isopropylidene hydrazides. The validity of this method for fatty acid analysis by GLC has been demonstrated by a good agreement of the results with those found by transesterification.

The high absorbance of isopropylidene hydrazides at 229 nm makes it possible to use a UV detector in high performance liquid chromatographic analysis of fatty acids in lipids, using the present method.

Since hydrazinolysis can be carried out at relatively lower temperatures and does not require any kind of acid catalyst, this method could be used in situations where acid- or heat-labile moieties are involved.

Adequate precautions should be observed in using hydrazine hydrate, because some hydrazines are suspected to be carcinogenic (9).

The author expresses his gratitude to the Alexander von Humboldt Foundation, D-5300 Bonn, for awarding a fellowship, and to Tribhuban University, Kathmandu, Nepal, for granting a sabbatical leave. He is thankful to Professor H. K. Mangold for providing research facilities and critically reading the manuscript. He also thanks Dr. H. Schiller for mass spectral analyses, and Drs. K. D. Mukherjee and R. J. Blagrove for helpful discussions.

Manuscript received 2 June 1982 and in revised form 9 September 1982.

REFERENCES

1. Takemura, S. 1959. Hydrazinolysis of nucleic acids. I. The formation of deoxyribo-apyrimidinic acid from herring sperm deoxyribonucleic acid. *Bull. Chem. Soc. Japan.* **32:** 920–926.
2. Fraenkel-Conrat, H., and C. M. Tsung. 1967. Hydrazinolysis (in end-group analysis of proteins). *Methods Enzymol.* **11:** 151–155.
3. Nissen, D., V. Rossbach, and H. Zahn. 1974. Determination of carboxyl endgroups and comonomers in poly(ethylene terephthalate) with hydrazine. *J. Appl. Polym. Sci.* **18:** 1953–1958.
4. Baumann, W. J., and H. K. Mangold. 1964. Reactions of aliphatic methanesulfonates. I. Syntheses of long-chain glyceryl-(1)ethers. *J. Org. Chem.* **29:** 3055–3057.
5. Kates, M. 1972. Techniques of Lipidology. Isolation, Analysis and Identification of Lipids. North-Holland Publishing Co., Amsterdam. 539, 559–564.
6. Chalvardjian, A. 1964. Fatty acids of brown and yellow fat in rats. *Biochem. J.* **90:** 518–521.
7. Pajari, K. 1944. Über die Hydrazide einiger Fettsäuren und über die hydrierende Wirkung des Hydrazinhydrates auf Öl- und Linolsäure. *Fette Seifen.* **9:** 347–351.
8. House, H. O. 1972. Modern Organic Reactions. 2nd edition. W. A. Benjamin, Inc., Menlo Park, CA. 248–254.
9. Toth, B. 1980. Actual new cancer-causing hydrazines, hydrazides, and hydrazones. *J. Cancer Res. Clin. Oncol.* **97:** 97–108.