Lochmuller (1). As the temperature rises, the alkyl chains apparently become vertical to the silica base, affording increased adsorption sites for the larger solutes and reducing solute retention. As indicated in Figure 7, as the temperature was elevated, the larger solute probe was affected to a greater extent. Regeneration of the column returns the bonded alkyl chains to their original geometry (7).

Another technique that can be used effectively for chromatographing a complex solute matrix on reverse-phase columns is temperature programming. Figure 8 compares temperature programming and an isocratic run of a natural oil triglyceride sample. The temperature program was performed ballistically, in increments, as shown in Figure 8. Temperature programming resulted in very little loss in resolution, but significantly reduced analysis time. Even though this technique has not been widely explored, it has been used with some interesting effects (7,8).

The analysis of triglycerides on reverse-phase columns in this study shows that $CHCl_3$ was superior to other lipid solvents when used as the sample solvent. A mobile phase using absolute ethanol as the polar modifier resulted in better resolution of triglycerides compared with methanol. A ballistic temperature program was used effectively to reduce analysis time of triglycerides. The above parameters resulted in adequate resolution for triglyceride groups of natural oils on reverse-phase columns.

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[Received February 18, 1983]

A Novel Method for Spectrophotometric Determination of Triglycerides

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ABSTRACT

A new method for the spectrophotometric determination of triglycerides at microgram levels has been developed. The method relies on the quantitative degradation of *O*-acyl lipids with hydrazine and the subsequent conversion of the fatty acid hydrazides to the corresponding *N'*-isopropylidenealkanohydrazides by reaction with acetone. Results are presented for triolein and some oils of plant origin.

INTRODUCTION

Triglycerides are determined most frequently by analyzing the glycerol generated by their saponification or transesterification. Although a number of methods, including an enzymatic one, have been used for the determination of glycerol, the preferred method involves oxidation of the glycerol with periodic acid to yield formaldehyde, which can be assayed spectrophotometrically after condensation with chromotropic acid to give a violet compound (1,2).

Recently, we have described the quantitative degradation of *O*-acyl lipids with hydrazine to their constituent fatty acid hydrazides (3). These hydrazides, on reaction with acetone, are converted into their corresponding fatty acid *N'*-isopropylidenealkanohydrazides, R—CO—NH—N = $C(CH_3)_2$, which strongly absorb ultraviolet light at 229 nm. Because these derivatives can be resolved by gas chromatography (GC) (3) and by high performance liquid chromatography (HPLC) (4), the hydrazinolysis-acetonization method can be used for fatty acid analyses of acylglycerols.

In this communication, we have modified the method of hydrazinolysis, followed by acetonization, for the spectrophotometric determination of triglycerides. The procedure has been designed to eliminate interference caused by incomplete removal of reactants. It has been tested with a number of triglycerides of plant origin.

EXPERIMENTAL PROCEDURES

All chemicals were purchased from E. Merck (Darmstadt, West Germany), except 98% hydrazine hydrate, which was from Fluka AG, Buchs, Switzerland. Triolein was obtained from Sigma Chemical Co., St. Louis, MO.

Preparation of Palmitic Acid Isopropylidene Hydrazide

Ca. 1 g of methyl palmitate was refluxed with 20 mL of 50% hydrazine hydrate in ethanol for 3 hr. To the reaction mixture, 50 mL of water was added and the resulting precipitate was filtered, washed with water and crystallized from 90% ethanol to yield pure palmitic acid hydrazide (mp 112-113 C). About 100 mg of the hydrazide was refluxed with 30 mL of acetone for 2 hr. Acetone was removed under reduced pressure and the *N'*-isopropylidenealkanohydrazide was crystallized from acetone (mp 71-72 C).

Preparation of Acetonized Hydrazinolysates of Triglycerides

Triglyceride (20-120 μg) was heated at 60 C with 0.1 mL

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[1]This research was undertaken at the Federal Center for Lipid Research, Piusallee 68/76, D-4400 Münster, Federal Republic of Germany.

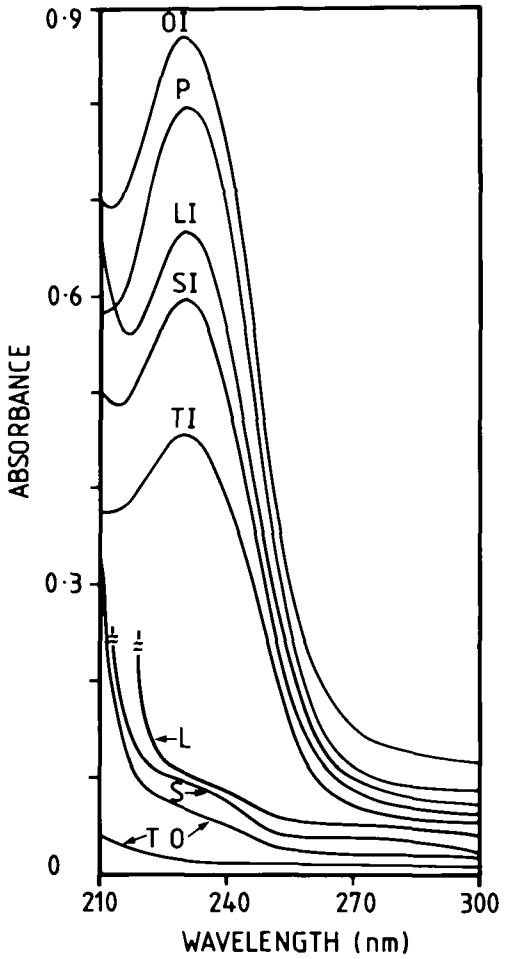

FIG. 1. Absorption spectra of acetonized hydrazinolysates of triglycerides. OI, LI, SI and TI represent olive (109 μg), linseed (82.7 μg) and sesame (74.5 μg) oils, and triolein (57 μg), respectively. O, L, S and T represent untreated samples in the amounts given above. P represents palmitic acid isopropylidene hydrazide. Absorbance at 210 nm for sesame and linseed oils was 0.76 and 2.31. In all cases, the absorbance at 300 nm was less than 0.01—the baseline for each curve has been drawn at a different level for clarity.

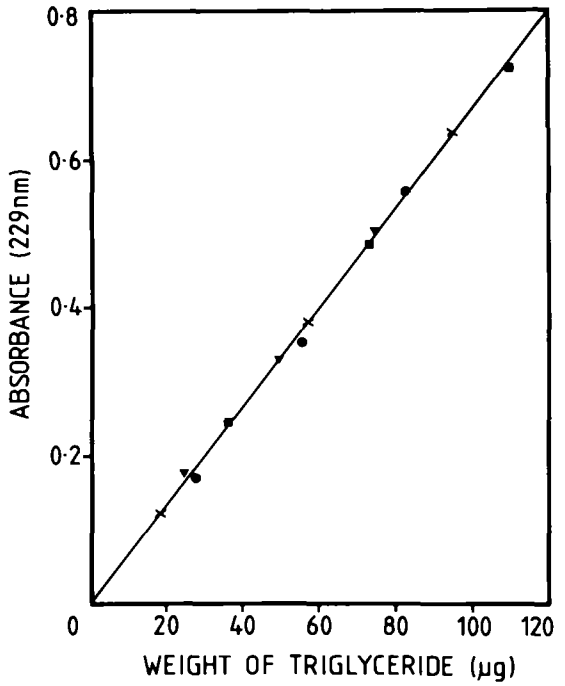

FIG. 2. Graph of absorbance at 229 nm vs weight of triglyceride analyzed. Absorbance of the blank varied between 0.02-0.04. Olive oil ■—■; linseed oil ●—●; sesame oil ▲—▲; triolein x—x.

of hydrazine hydrate/tetrahydrofuran/ethanol/cyclopentene (1:3:3:1, v/v) in a closed tube for 2 hr. After adding 1 mL of chloroform, the reaction mixture was washed with water (5 mL × 4) to remove excess hydrazine. Chloroform was removed from the organic layer and the remaining aqueous residue was heated with 2 mL of acetone at 60 C for 30 min. The acetone was evaporated and 1 mL of methanol was added. After removing this methanol, the aqueous residue was diluted to 5 mL with methanol and its ultraviolet absorbance was measured. For the preparation of a blank, the procedure described above was followed, except the triglyceride was omitted. In all cases, the removal of solvent was carried out at 60 C in a gentle stream of N_2.

Absorbance measurements were made in quartz cells of 1 cm path length, using a Pye Unicam SP9-200 UV/VIS Spectrophotometer and methanol as solvent. In all experiments, appropriate blanks were used.

RESULTS AND DISCUSSION

In order to examine the feasibility of using the hydrazinolysis-acetonization method for quantitation of triglycerides, triolein and some triglycerides of plant origin were tested. These oils showed only weak absorbance near 230 nm at the levels subsequently used for quantitation, but their absorption varied widely in the range of 210-220 nm (Fig. 1). On the other hand, the absorption spectra of their acetonized hydrazinolysates all showed strong maxima at 229 nm and were similar in shape to each other. They closely resembled the spectrum of palmitic acid isopropylidenehydrazide (Fig. 1). The spectral differences observed below 216 nm for the various samples were presumably caused by minor constituents of the oils.

In the range of 20-120 μg, a linear relationship was found between absorbance at 229 nm, caused by the acetonized hydrazinolysates of the triglycerides, and the weight taken for analysis (Fig. 2). A common increment of 6.6_9 absorbance units/mg was found for triolein and the other triglycerides. Considering the difference in composition of the oils used, we conclude that the molar absorption of fatty acid isopropylidene hydrazides at 229 nm does not depend significantly on the degree of unsaturation of the fatty acid. In addition, any differences in the average molecular weights of the fatty acid moieties in these samples is apparently not sufficient to measurably influence the absorbance increment observed.

The value of the molar absorption of palmitic acid isopropylidene hydrazide at 229 nm was found to be 10,100 M^{-1} cm^{-1}. The experimental absorbance increments observed above for triolein and the various plant oils were only 2.5% lower than the value calculated using 10,100 M^{-1} cm^{-1} for the molar absorption in the case of triolein. Consequently, this value can probably be used for all triglycerides with a similar distribution of fatty acid chain lengths. If a purified sample of the particular oil for analysis is available as a control, then uncertainty regarding the appropriate absorbance increment will not arise.

These results show that for triglyceride determination, our modified hydrazinolysis-acetonization method provides a faster, more convenient, but equally sensitive, alternative

to techniques that measure the release of glycerol. As hydrazinolysis can be carried out at relatively lower temperatures, and does not require any kind of acid catalyst, this approach will have a particular advantage over transesterification where acid- or heat-labile moieties are involved.

That degradation with hydrazine, followed by reaction with acetone, quantitatively converts phospholipids as well as other lipids to isopropylidene hydrazide derivatives of their constituent fatty acids has been shown (3). Therefore, we can assume that the present method should work for quantitation of *O*-acyl lipids other than triglycerides. Moreover, a combination of spectrophotometry and GLC/HPLC should enable accurate determination of total fatty acids and their individual proportions in a lipid sample (4). To use a hydrazinolysis value, determined by absorbance at 229 nm, in the manner that iodine or saponification values are used for characterization of oil samples, should be possible. The method can also be used for analysis of the triglyceride content of blood samples.

ACKNOWLEDGMENTS

Vishwanath P. Agrawal thanks the Alexander von Humboldt Foundation, D-5300 Bonn, for the award of a fellowship and the Tribhuvan University, Kathmandu, Nepal, for granting leave of absence. We thank H. K. Mangold for providing research facilities.

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[Received June 28, 1983]

♣Effect of Moisture Content of Oil Type Sunflower Seed on Fungal Growth and Seed Quality During Storage

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ABSTRACT

Oil-type hybrid sunflower seed exposed to relative humidities of 65%, 84% and 93% in environmental chambers at 10 C attained equilibrium moisture contents (mc) of 7.5±0.2%, 10.1±0.2% and 13.4±0.5% and were stored under these conditions for up to 60 weeks (wk). At 7.5% mc, germinability of seed changed very little during storage, but at 10.1% mc and 13.4% mc, germination significantly decreased during storage. At 7.5% mc, free fatty acid (FFA) levels in extracted oil did not change significantly during 60 wk of storage. However, at 10.1% mc, FFA increased significantly during 40 wk of storage and were significantly correlated with the invasion of seed by the storage fungus *Aspergillus* (r = 0.81). At 13.4% mc, FFA increased significantly during storage and were positively correlated with the invasion of seed by *Aspergillus* and *Penicillium* and negatively correlated with germination percentage. Invasion of surface-disinfected seed by fungi decreased from 83% to ca 66% of total seed during storage at 7.5% mc. The predominant fungus was *Alternaria alternata* (Fr.) Keissler. A previously unreported *Alternaria* sp., morphologically similar to *A. ricini* (Yoshii) Hansford and *A. macrospora*, was isolated from 9% of the seed. At 10.1% mc, fungal invasion also decreased for 24 wk and then began increasing again. At 24 wk of storage, *Aspergillus* began invading the seed. At 13.4% mc, 100% of the seed were invaded with fungi within 8 wk of storage. Total *Alternaria* rapidly decreased during storage; and after only 4 wk of storage, the seed were invaded by both *Aspergillus* and *Penicillium*. After 24 wk of storage, the predominant genus was *Aspergillus*, followed by *Penicillim* and *Alternaria*. Other fungi invading the seed were *Cladosporium, Phoma, Mucor, Rhizopus* and several unidentified fungi.

INTRODUCTION

Sunflower seeds are sometimes harvested at high moisture content and stored without adequate drying. Fungal invasion, seed-mass heating, high levels of free fatty acids (FFA) in extracted oils and a decrease in seed germinability are problems encountered when sunflower seeds are stored under high moisture conditions (1-7). Fungal invasion and decreased germinability are proportional to increased moisture content, elevated seed-mass temperature and length of storage (1). Poisson et al., (4) reported that molds begin growing on whole sunflower seed above 65% relative humidity (RH) and 6.5% moisture content (mc), whereas yeast and bacteria required seed moistures above 10%. Christensen (8) reported that 100% of open-pollinated sunflower seed (cv. Peredovik) stored at moisture contents of 9.5% and 11% were invaded by storage fungi within 82 days and the decrease in germinability was approximately proportional to increased moisture content. Baudet (9) reported that sunflower seed with 12% moisture will maintain seed quality for 4 months when stored at 5 C and only 3 months when stored at 10 C. He also found that sunflower seed at 10% moisture will maintain seed quality for ca. 5 months when stored at 10 C and about 3 months at 20 C. No information about maintaining quality was given for seed moisture contents of less than 10%.

Conflicting information exists on the optimum and safe storage conditions for sunflower seed and no research has been reported on storage of the new hybrid seed. A wide range of "safe" moisture levels are currently recommended (6-10%) that supposedly minimize microfloral growth and invasion and maintain good seed quality during storage (7,10,11). Scientists at North Dakota State University recommended that seed to be stored for up to 6 months should be at 10% moisture or less whereas seed to be stored up to a year should be 8% or less (7). Christensen (10)