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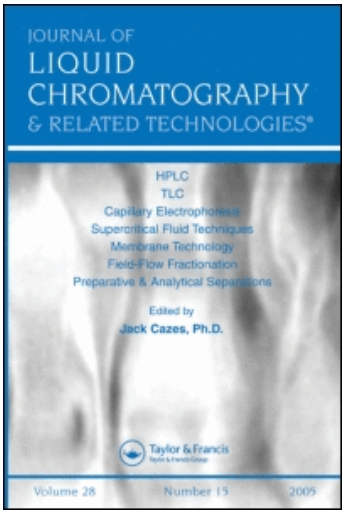

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Densitometric Determination of C-18 Fatty Acids as Phenacyl Esters Following Reversed-Phase Thin Layer Chromatography

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DENSITOMETRIC DETERMINATION OF C-18 FATTY ACIDS AS PHENACYL ESTERS FOLLOWING REVERSED-PHASE THIN LAYER CHROMATOGRAPHY

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ABSTRACT

A simple method of quantitatively determining C-18 fatty acids in an aqueous oxidized polyethylene emulsion is presented, utilizing the formation of phenacyl esters in the presence of crown ether as catalyst, followed by separation by reversed-phase thin layer chromatography, and subsequent densitometry of the strongly ultraviolet absorbing separated compounds.

The method has good sensitivity, permitting the detection of as little as 5 nanomoles or less of fatty acids, and is applicable to various biological mixtures and to acids of different chain lengths.

INTRODUCTION

Long chain fatty acids differing only in degree of unsaturation are not readily separated by adsorption chromatography on silica gel (1), but are well suited to separation by reversed-phase partition thin layer chromatography (2). The problem encountered here is that the organically bonded layer cannot withstand the high temperatures necessary for detection of fatty acids by the

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commonly used charring techniques, particularly in the case of the unsaturated, less reactive fatty acids. The method of Gübitz (1), employing p-hydroxybenzaldehyde/sulfuric acid as a reagent has the disadvantage of having a crucial reaction step which is time and temperature dependent. In addition, the sulfuric acid used in the reagent results in a deterioration of the background, due to the degradation of the hydrocarbon chains.

Due to their relatively low reactivity, there are virtually no non-destructive visualization methods applicable to fatty acids detection in thin layer chromatography. The ultraviolet quench technique, which is well suited for densitometric work and requires no destructive reagents, does not apply to free fatty acids, since most fatty acids do not absorb ultraviolet radiation, at least not sufficiently to allow acceptable levels of detection. Formation of suitable ultraviolet absorbing derivatives is an ideal solution, allowing for ready visualization and optimum quantitation of the compounds on a thin layer plate by densitometry.

The formation of phenacyl esters in the presence of a crown ether catalyst, as described by H.D. Durst (3) and applied to liquid chromatography (4), forms the basis for this method. Crown ethers are known for their ability to complex metal salts and aid the dissolution of these salts in non-polar, aprotic solvents (5).

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Stoichiometric concentrations of the crown ether are not necessary, thus the phase transfer of carboxylate salts may be catalyzed by crown ethers in molar ratios of from 1:10 to 1:100. A 1:10 ratio was used in this procedure.

The reaction can take place satisfactorily in almost any nonpolar aprotic solvent. The solvent used in this method was benzene, but acetonitrile, cyclohexane, methylene chloride or carbon tetrachloride are reported to be equivalent and can be substituted to meet solubility requirements. Traces of water have not been shown to affect the completeness of reaction, thus rendering rigorous anhydrous conditions unnecessary (3). The reaction takes place rapidly and under extremely mild conditions, giving quantitative yields and good reproducibility. The p-bromophenacyl esters of fatty acids thus obtained exhibit high molar absorptivity and can, therefore, be detected at very low concentrations on a thin layer place with fluorescent indicator. This technique was applied to the quantitative determination of fatty acids presence in polyethylene emulsions.

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MATERIALS

α, p-Dibromoacetophenone and 18-Crown-6 (1,4,7,10,13,16-hexaoxacyclooctadecane) were obtained from Aldrich Chemical Co.

Stearic acid, oleic acid and linoleic acid of 99.0% or better purity were obtained from Chemical Dynamics.

All organic solvents used were commercial A.R. grade and were used without further purification.

Thin Layer Plates- Whatman KC-18F, reversed phase plates containing a fluorescent indicator excited at 254nm, 20 x 20 cm., 0.25mm thickness (#4803-800) were used.

Instrumentation - Densitometric measurements were performed using a Schoeffel Model SD-300 Spectrodensitometer.

METHODS

Standard Stock Solution - approximately 60mg each of stearic, oleic and linoleic acids are dissolved in chloroform and diluted to 50.0ml.

Alkylating Solution - 555mg of dibromoacetophenone and 53mg of 18-Crown-6 ether are dissolved in 20ml of benzene, yielding a 10:1 molar ratio of alkylating agent/crown ether. This solution may be stored in a refrigerator for two to three weeks.

Sample Preparation - a 10.0ml sample of oxidized polyethylene emulsion was precipitated by the addition of 1.0ml of 6N hydrochloric acid. The precipitate was suction filtered on a coarse porosity sintered glass funnel, repeatedly washed with 25.0ml portions of distilled water, followed by a final rinse of 50% methanol/water. Following suction-drying to remove residual

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water, the precipitate was redissolved in chloroform, transferred to a 100ml volumetric flask and diluted to volume with chloroform.

Alkylation

For the preparation of standard curves, 2.0ml, 3.0ml, 4.0ml, 5.0ml and 6.0ml aliquots of Standard Stock Solution (concentration range of total acids from 0.025mM - 0.075mM) are pipeted into five appropriately marked 10ml volumetric flasks held in specially designed rack for ease of handling.

A 4.0ml aliquot of sample solution (equivalent to approximately 0.05mM total acids) is pipeted into a 10ml volumetric flask.

All solutions are neutralized to a phenophthalein end-point by dropwise addition of 0.5% methanolic potassium hydroxide solution and are then evaporated to dryness under nitrogen in a 45° - 50°C water bath.

Into all flasks,1.5ml of Alkylating Solution are added to suspend residues. Flasks are immersed in an 80°C water bath and heated for 20 minutes with frequent agitation. Solutions must not be allowed to dry out. If necessary, 0.5ml of benzene may be added to all flasks.

At the end of the heating interval, flasks are cooled and diluted to volume with benzene. Solutions are now ready for thin layer chromatography.

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Thin Layer Chromatography

Using disposable, open-end capillaries (Drummond Microcaps), 5 lambda (μl) of each standard and three 5-lambda spots of sample solution are applied at two centimeter intervals to a 20 x 20cm Whatman KC-18F reversed phase TLC plate.

When dry, the plate is placed in a filter paper lined chamber, equilibrated for at least 30 minutes, containing the solvent system acetonitrile:chloroform (90:10).

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Plates are developed to a distance of 12cm. Development time is approximately 15 minutes. Plates are immediately dried at room temperature while protected from light.

After development and drying, the chromatograms are viewed under short wave ultraviolet light. The fatty acids appear as follows:

Compound	R_f
Behenic acid	0.31
Erucic acid	0.37
Stearic acid	0.43
Oleic acid	0.50
Linoleic acid	0.56
Linolenic acid	0.61
Excess reagent	0.75

The chromatograms are scanned in the reflectance mode using a Schoeffel Spectrodensitometer (Fig. 1). Peak areas of standards and samples are determined, the

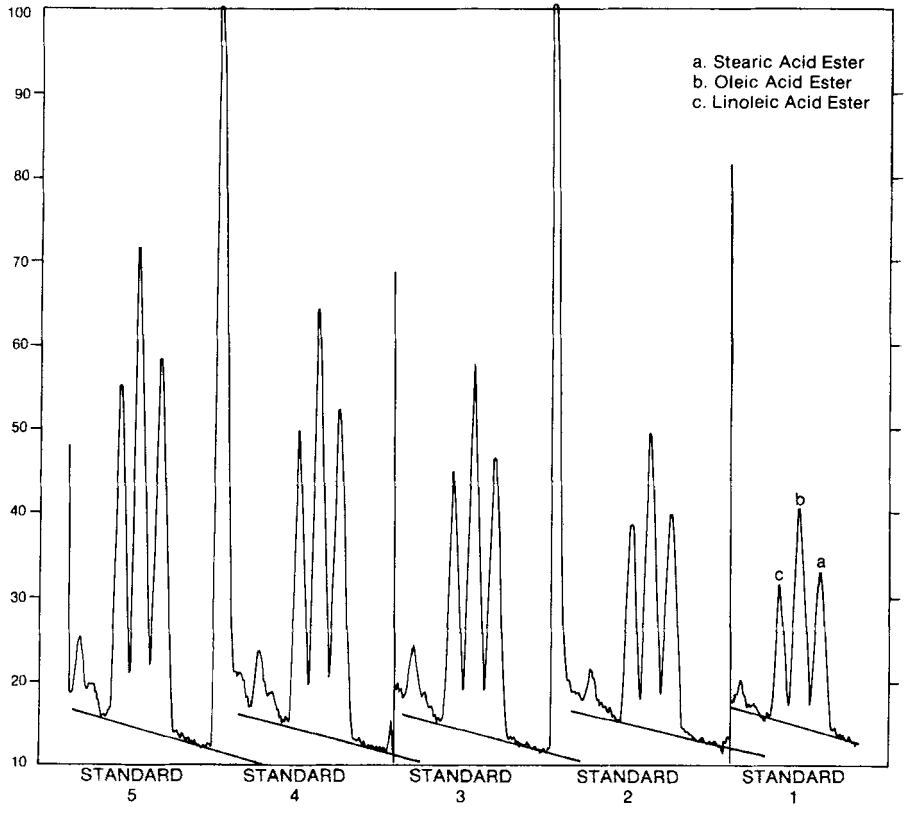

Figure 1. Densitometric Scans for Standard Curves.

average of triplicate scans are plotted vs. corresponding weight of fatty acid in mg., to obtain a standard curve for each acid (Figs. 2, 3 and 4). It is essential for good precision to scan in triplicate.

Fatty acid contents in samples are then calculated using these standard curves.

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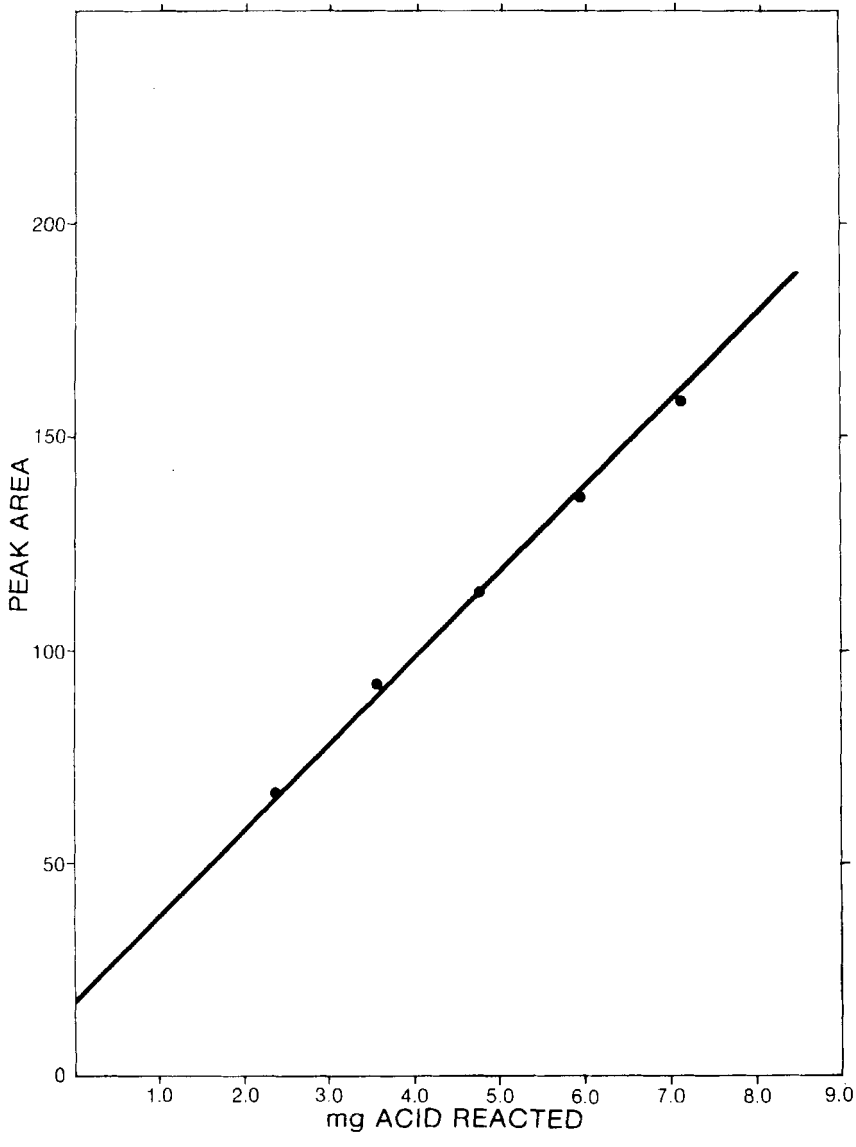

Figure 2. Standard Curve for Stearic Acid.

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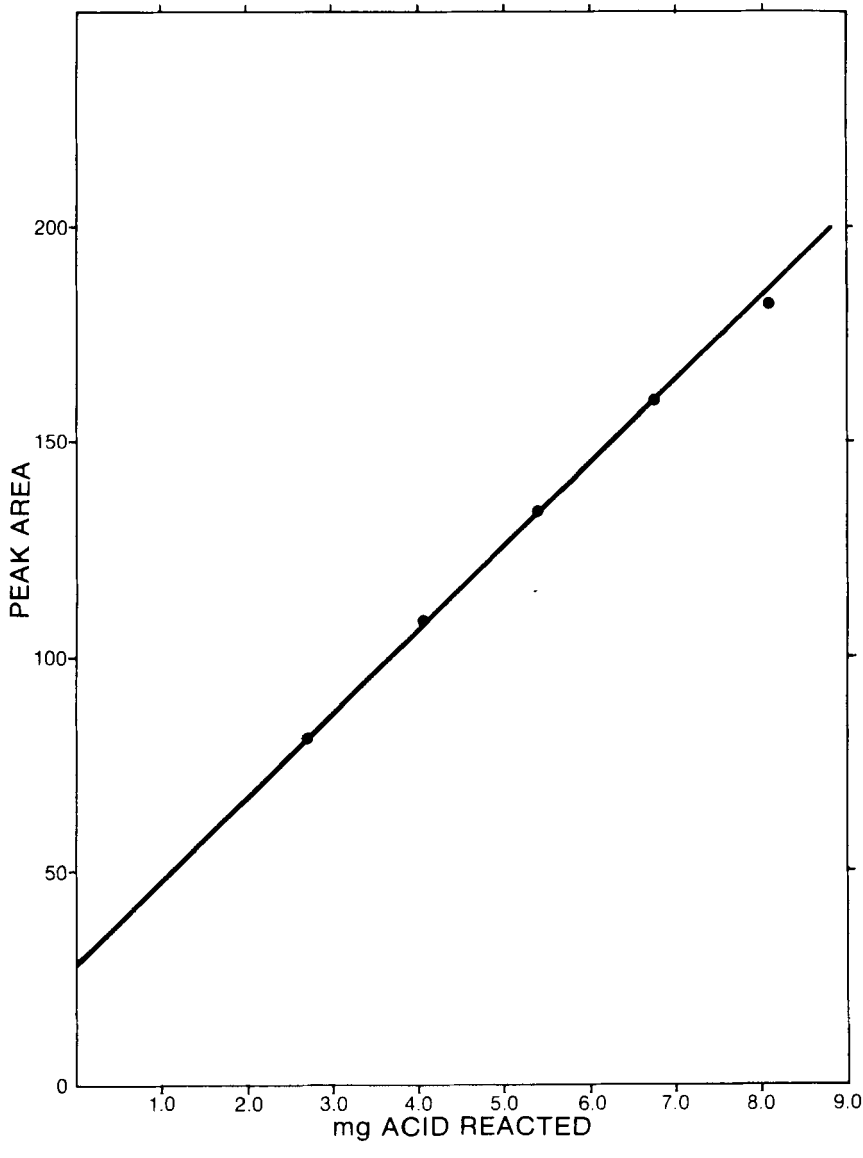

Figure 3. Standard Curve for Oleic Acid.

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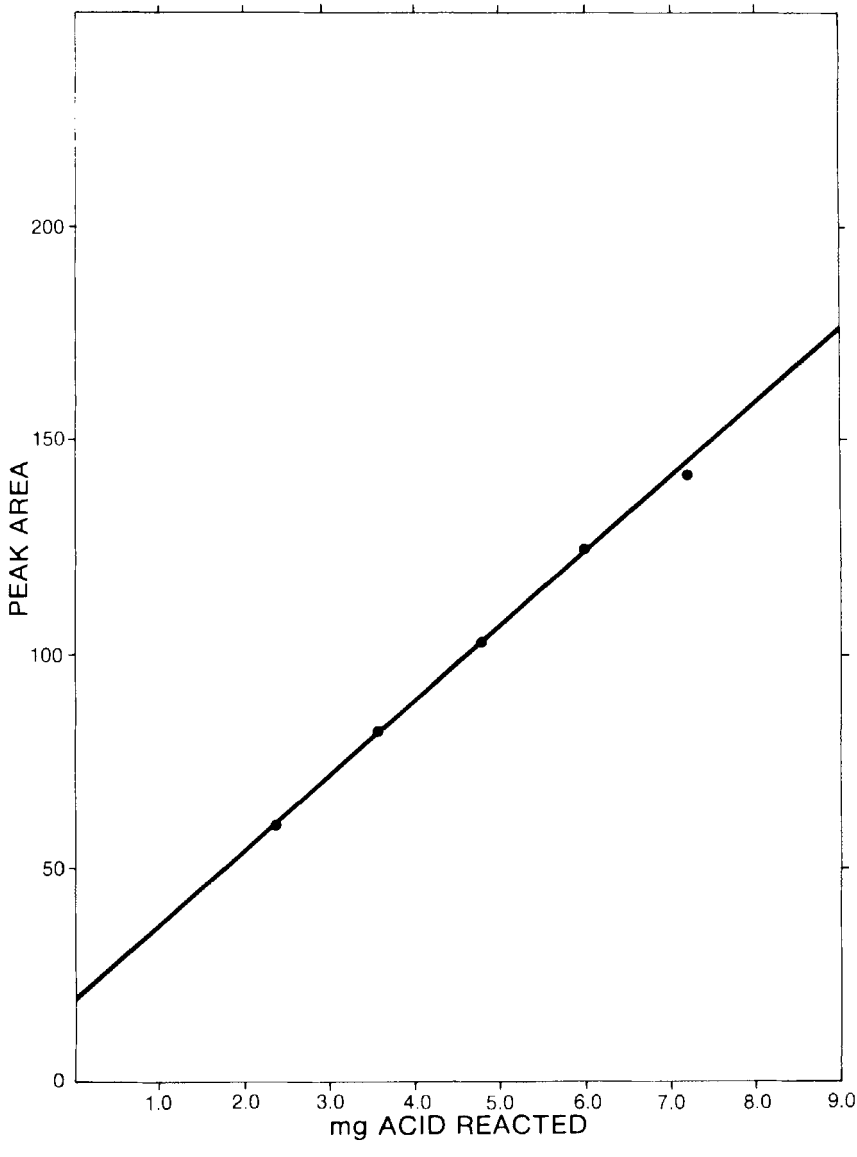

Figure 4. Standard Curve for Linoleic Acid.

CONCLUSIONS

A simple and stable method for simultaneous quantitative determination of 18-carbon fatty acids in a mixture by derivatization, reversed phase TLC separation, and densitometry of the chromatogram has been presented.

The method shows coefficient of variations in the range of 2.0 to 6.5% for the six fatty acids studied. The reproducibility of the method was determined by measuring the peak areas for 20 developed spots of each fatty acid. The Relative Standard Deviation was 1.0 to 2.3%.

The ability to determine fatty acid content in emulsions of the nature described, we believe, make it applicable to a wide variety of biological samples, where fatty acid determinations are considered to be of great diagnostic value. Future work will be conducted in this area.

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