A Simple Photometric Method for Microdetermination of Fatty Acids in Lipids

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Abstract

A single step photometric micromethod for determination of fatty acids in lipids in benzene solution, using rhodamine 6G reagent, has been developed. The method eliminates the disadvantage of formation of a biphasic system and is applicable in the presence of glycerides, sterols, epoxy compounds, hydrocarbons and long chain hydroxy compounds such as long chain fatty alcohols. The long chain fatty acids from C_{12} to C_{22}, both saturated and unsaturated, can be determined with reasonable accuracy in the concentration range of 0.08–0.25 μmole/ml. The method is simple, rapid, and requires relatively inexpensive chemicals.

Introduction

A number of micromethods based either on titrimetric or on colorimetric determination of fatty acids in lipids has been reported. In 1956, Ayers (1) described a procedure for colorimetric determination of long chain fatty acids in the form of copper or cobalt soap extracted into chloroform layer. Iwayama (2) increased the sensitivity of the copper soap method by means of triethanolamine buffer. Duncombe (3) further modified the procedure by incorporation of diethyldithiocarbamate as a reagent for copper detection. The copper soap method thus modified gained much popularity and has been in wide use since. Novak (4) reported a colorimetric procedure based on conversion of the fatty acids into cobalt soap and subsequent determination of the cobalt by α-nitroso β-naphthol. However, the efficiency of both of these procedures is limited by the necessity of using a more or less cumbersome separation procedure of the chloroform phase (in the case of the copper soap method) or chloroform-heptane phase (in the case of the cobalt soap method) from the respective biphasic system and the consequent, somewhat incomplete, recovery of fatty acids. Recently, another colorimetric method (5) has been reported, based on Feigl's observation (6) that benzene soluble acidic compounds such as higher monobasic fatty acids on addition to a benzene solution of rhodamine B, followed by shaking with a 1% aqueous solution of uranyl salts, develop a red or pink color, the shade depending on the amount of the acids present. In the method described, the benzene layer was separated from the aqueous layer and the optical density of the solution measured. The method thus involves separation of the benzene layer from the biphasic system and introduces a possible source of error. Palit and Ghosh (7) observed that aqueous solutions of some dyes on extraction with benzene at an appropriate pH yield extracts that are sensitive towards acids, and reported analysis of some carboxylic acids, based on photometric estimation of color developed, using rhodamine 6GX reagent.

The present paper makes use of Palit and Ghosh's observation and reports a simple photometric micromethod for fatty acids in lipids in benzene solution, using rhodamine 6G reagent. The method is a single step procedure that eliminates the disadvantage of formation of a biphasic system and utilizes readily available and inexpensive chemicals.

Experimental Procedure

Apparatus and Reagents

The spectrophotometer used was the Carl Zeiss visible spectrophotometer. The glassware consisted of glass-stoppered test tubes (10 ml capacity), glass-stoppered conical flasks, etc. All glassware used was scrupulously cleansed with cleaning powder, washed with water, rinsed with warm chromic acid, then thoroughly washed with distilled water, and finally dried in a hot air oven. The reagents used were: (a) benzene, purified with cold concentrated sulfuric acid (AR), washed free of mineral acid, dried over anhydrous sodium sulfate, and distilled; (b) standard solutions in benzene (concentration, ca. 1 μmole/ml) of lauric, palmitic, behenic and oleic acids (Nutritional Biochemicals Corporation/Hormel Institute, 99+ % pure) and tripalmitin (Hormel Institute, chromatographic grade), and solutions of known but varying concentrations of lipids, such as coconut oil, peanut oil, etc.; (c) phosphate buffer solution (0.2 M KH_2PO_4 + 0.2 M NaOH), pH 10–12; (d) methanol, ethanol, propanol, isopropanol, butanol-1, butanol-2, tertiary butanol, dodecanol, cholesterol, and hydrocarbon solvents such as petroleum ether, normal hexane, liquid paraffin (the materials were all of reagent grade); (e) Rhodamine 6G (Harleco, USA) 0.005% solution in benzene.

Preparation of Rhodamine 6G Reagent

Twenty-five milligrams of rhodamine 6G were dissolved in 25 ml of phosphate buffer and immediately extracted with 500 ml of benzene. The aqueous layer was discarded and the clear orange-yellow colored benzene layer transferred into a thoroughly cleaned and dried amber-colored glass bottle and preserved over solid caustic soda in the dark.

Procedure for Microanalysis

A suitable volume of the test sample in benzene solution was added from a pipette into a test tube provided with a glass stopper. The volume was made up to 4 ml with purified benzene, 2 ml of rhodamine 6G reagent was then added, the tube stoppered and shaken for a few seconds to ensure uniform mixing of the contents. A pink color developed almost instantaneously. After 30 min (although a shorter time interval may be allowed) the optical density of the solution was measured in the spectrophotometer at 535 mμ. A reagent blank without the sample was run under exactly identical conditions and used to adjust the instrument to a transmittance of 100%.

All estimations reported were performed at least in triplicate and the results calculated with reference to a calibration curve constructed with palmitic acid as the reference acid (Fig. 1).

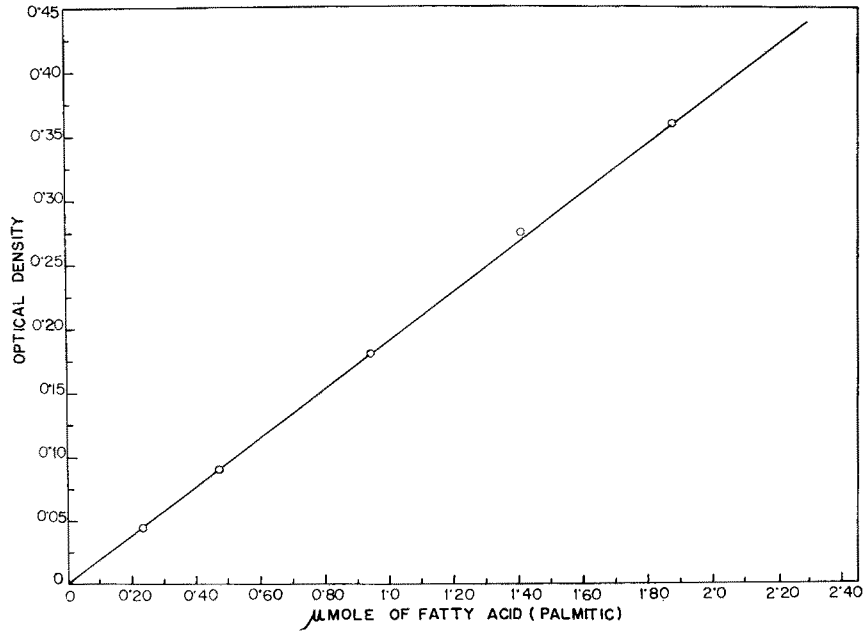

FIG. 1. Optical density vs. micromoles of palmitic acid.

Estimation of Some Standard Fatty Acids

The fatty acids selected for estimation were those occurring in lipids. Of these, one was lauric acid $C_{12:0}$—a relatively low molecular weight fatty acid; one palmitic acid ($C_{16:0}$)—a typical fatty acid of intermediate molecular weight; one behenic acid ($C_{22:0}$)—a high molecular weight fatty acid; and oleic acid ($C_{18:1}$)—a long chain monounsaturated fatty acid. Varying concentrations of these acids in benzene were determined, following the procedure previously described. Results are recorded in Table I.

Estimation of Added Palmitic Acid in Tripalmitin and Some Neutral Lipids (Peanut and Coconut Oils)

Solutions of tripalmitin as well as of the neutral lipids in benzene were fortified with graded amounts of palmitic acid, and the concentrations of palmitic acid in the respective solution were determined by the reported method. Results are presented in Table II.

Application to Free Fatty Acids Determination in Several Vegetable Oils of Varying Acid Values

Appropriate quantities depending on the free fatty acids (FFA) contents of the oils were dissolved in benzene to give a workable solution concentration. The FFA contents of the oils were next determined photometrically and expressed in terms of acid values. The acid values were compared with the values obtained by classical alkali titration (8). Results are included in Table III.

TABLE I

Estimation of Some Standard Fatty Acids

Sample	Amount taken, μmoles/6 ml	Amount found (mean of three determinations) μmoles	Mean deviation, %
Lauric acid	0.5250	0.50	4.54
	0.7875	0.76	3.49
	1.0500	1.02	2.85
	2.1000	2.20	4.76
Palmitic acid	0.5220	0.50	4.21
	0.7830	0.76	2.94
	1.0440	1.07	2.49
	1.8780	1.81	3.61
Behenic acid	0.5050	0.47	6.93
	0.7575	0.72	4.95
	1.2625	1.29	1.86
	2.0200	1.83	9.40
Oleic acid	0.6050	0.57	5.78
	0.9075	0.93	2.47
	1.2100	1.18	2.47
	2.0740	2.19	5.58

Interference

Possible interferences by some reagents were investigated, using model mixtures. Results are discussed later.

Results and Discussion

Construction of Calibration Curve

Extrapolation of optical density values vs. micro moles of palmitic acid as reference acid shows that the relationship is linear up to 1.9 μmoles of palmitic acid in a solution of 6 ml taken initially (Fig. 1). Beyond this range the optical density does not increase in linear proportion to the increase in concentration of the solution. The molar extinction coefficient measured at 535 mμ in a cell of 1 cm in length was found to be 1.15×10^4.

The slopes of the fatty acid curves in the fatty acid series from C_{12} to C_{22} (investigated in our laboratory) do not show any significant variation. This is supported by the data in Table I, where the results of estimation of a few acids have been recorded. The optical densities of the acids were ascertained at three different known concentrations and the corresponding values in μmoles of FFAs were

TABLE II

Estimation of Added Palmitic Acid in Some Lipids

Lipids	Per cent of palmitic acid added in lipid	Per cent of palmitic acid found in lipid (mean of five determinations)	Standard deviation
Tripalmitin	33.58	32.05	2.63
	50.27	48.95	1.75
Refined peanut oil (*Arachis hypogaea*)	4.80	4.30	0.62
	9.60	9.06	0.61
Refined coconut oil (*Cocos nucifera*)	2.98	2.94	0.18
	5.96	5.71	0.31

TABLE III
Determination of Acid Values of Some Lipids (Vegetable Oils)

	Present method		Alkali titration	
	Acid values (mean of five determinations)	Standard deviation	Acid value	Ratio present method/ titrimetric method
Oils with low acid values				
Peanut oil (*Arachis hypogaea*)	0.55	0.06	0.5	1.1
Refined kusum oil (*Schleichera trijuga*)	0.55	0.04	0.5	1.1
Coconut oil (*Cocos nucifera*)	1.35	0.06	1.3	1.0
Mahua seed oil (*Madhuca latifolia*)	3.03	0.06	2.4	1.2
Safflower oil (*Carthamus tinctorius*)	3.45	0.18	2.9	1.2
Sesame oil (*Sesamum indicum*)	4.38	0.16	3.8	1.1
Tung oil (*Aleurites fordii*)	7.91	0.13	7.1	1.1
Oils with medium acid values				
Tobacco seed oil (*Nicotiana tabacum*)	14.76	0.40	12.9	1.1
Castor oil (*Ricinus communis*)	15.62	0.39	15.1	1.0
Oils with high acid values				
Crude kusum oil (*Schleichera trijuga*)	61.76	0.40	55.9	1.1
Crude *Vernonia anthelmintica* oil	83.25	0.97	67.4	1.2

calculated from the graph constructed with palmitic acid standard (Fig. 1). Palmitic acid was chosen as the reference acid for the construction of calibration curve on the following grounds: (a) Palmitic acid is the truly representative fatty acid ($C_{16:0}$) occupying a middle position in respect to carbon chain length in the fatty acid series; (b) It is the major fatty acid synthesized biologically; and (c) it is invariably present in almost all oils and fats.

Workable Range of the Method

Consistent and reliable results are obtained for fatty acids in the concentration range of 0.08–0.25 μmole/ml with highest accuracy at mid-range. Values obtained within this workable range of concentration by the present photometric method were in good agreement with those obtained by the conventional alkali titration method (Table III).

Color Stability

The color developed by interaction of Rhodamine 6G with the acid samples is stable for a considerable length of time. No decrease in color intensity was noted in the time interval studied (180 min). This is evident from Figure 2, where color intensity (optical density) vs. time (minutes) has been plotted.

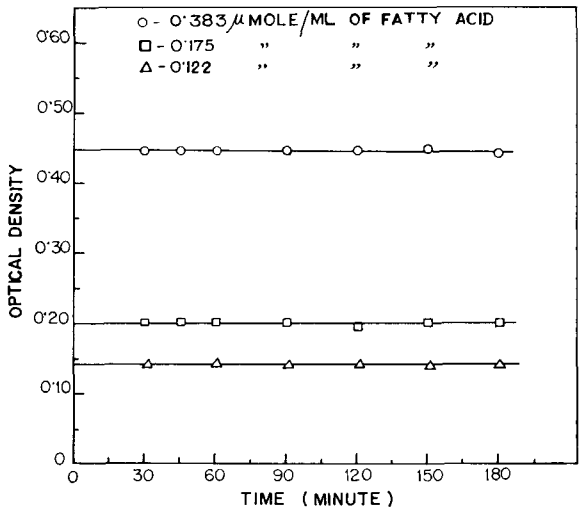

FIG. 2. Effect of time on the stability of color intensity.

Interference

At the micro- or near-micronormal range of concentrations, hydrocarbon solvents such as petroleum ether, normal hexane, liquid paraffin, sterols, primary, secondary or tertiary hydroxyl group in long chain compounds such as long chain fatty alcohols, epoxy groups and glycerides do not interfere. Short chain alcohols such as methanol, ethanol, propanol, butanol, etc., do interfere. In the alcohol series, the interference decreases with increase in carbon chain length. Again, for alcohols with the same carbon number, the order of interference is primary > secondary > tertiary. Interference is also caused by mineral acids, acetone, etc.

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