J. Lipid Research, July, 1962
Volume 3, Number 3

The determination of esterified fatty acids in glycerides, cholesterol esters, and phosphatides

WESLEY D. SKIDMORE and C. ENTENMAN

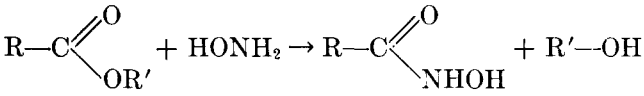
U. S. Naval Radiological Defense Laboratory, San Francisco 24, California

[Received for publication January 24, 1962]

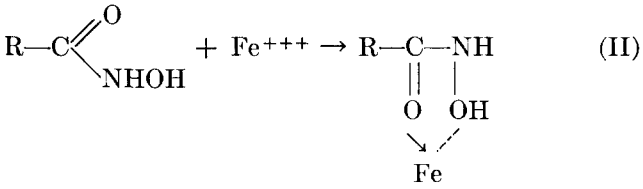
SUMMARY

The conditions for the hydroxamic acid reaction for determining esterified fatty acids have been modified to control the variable factors involved and to obtain molar absorptivities per ester group for triglycerides, cholesterol esters of long-chain fatty acids, and phosphatides that are equivalent for amounts up to 8 μEq of ester. Control of the amount of water present during the formation of hydroxamates was the most important single factor in obtaining equivalent color values. The accuracy and precision of the method have been well defined by showing that the optical density values for five different ester standards were on the identical straight-line curve. The colored end-products gave identical spectral curves between the wavelengths of 410 mμ and 700 mμ whether they were derived from standard carboxylic acid esters, Folch extracts of rat serum, rat liver, or human serum. A long-chain cholesterol ester must be used as one of the standard esters because of its special solubility characteristics. Cholesteryl acetate *cannot* be used as a reliable representative in place of a long-chain cholesterol ester.

A number of methods are available in the literature for the determination of fatty acid esters using hydroxamic acid formation coupled with ferric ion complex production (Bauer and Hirsch (1), Stern and Shapiro (2), Hack (3), Rapport and Alonzo (4), Weller (5), and Eggstein (6)). None of these methods has been shown to yield quantitative results for cholesterol esters of long-chain fatty acids. All of the methods gave good color yields



(I)



(II)

when used with cholesteryl acetate as a standard. In most instances cholesterol esters of long-chain fatty acids were not tested nor used as standard. Weller (5), however, made an extensive study of the conditions required to give equivalent color yields with different types of long-chain fatty acid esters, and concluded that the extinction of the color complex depended on added water, on the temperature of reaction I, and on the added amount of alkali. He placed the alkali sensitivity in the center of consideration, but also stressed water sensitivity in reactions I and II.

In this laboratory, attempts to determine fatty acid esters by Weller's techniques were abandoned for the following reasons: (*a*) the length of time for reaction I was too long and too variable, (*b*) blank readings were frequently high, (*c*) the color yield for cholesteryl stearate was often lower than that for trimyristin, and (*d*) it was difficult to prevent the formation of interfering yellow-colored products during solvent evaporation in reaction I. Accordingly, a study was made of some of the factors influencing the technique and means whereby the above disadvantages could be overcome.

With the techniques reported below, the color yield was found to be directly proportional to the amount of ester up to 8 μEq (glycerides, phosphatides, and cholesterol esters of long-chain fatty acids). It was further shown that the optical density (o.d.) values obtained from a Folch (7) lipid extract of rat serum, rat liver, and human serum were also directly proportional to the amounts of extract used. In order to obtain equivalent color yields for the different types of esters, rigorous control of the amount of water present in reaction I seemed to be the most critical

requirement although other factors in both reactions I and II profoundly affected the color yield.

REAGENTS

Absolute Ethanol (aldehyde-free). Mix 2 liters of reagent grade absolute ethanol with 200 g barium oxide and let stand one day. Pour the solvent into an all-glass distillation apparatus containing a few potassium hydroxide pellets and some boiling chips (carborundum). Reflux the mixture for an hour, then collect the distillate. Protect the reflux and distillation processes from moisture with the aid of a calcium chloride drying tube attached to a side-arm vent between the condenser and the collecting flask. Store at room temperature in a glass-stoppered Pyrex bottle. *This reagent must be anhydrous.*

Ethyl Ether (anhydrous and peroxide-free). Redistill anhydrous reagent grade diethyl ether from hydroxylamine hydrochloride using a calcium chloride tube as described above. Store in a glass-stoppered Pyrex bottle in the refrigerator over sodium wire or use immediately. *This reagent must be anhydrous.*

Chloroform. Redistill reagent grade chloroform and add a volume of absolute ethanol, equivalent to 0.4 per cent of the chloroform volume, to retard decomposition.

Stock 50% (w/w) Aqueous Carbonate-Free Sodium Hydroxide (saturated).

Stock Sodium Hydroxide–Ethanol Reagent (1.13 N; 4.2% water by volume). With a 10-ml syringe-pipet,[1] eject rapidly 5.60 ml of stock 50% NaOH into approximately 90 ml of absolute ethanol in a glass-stoppered 100-ml graduated cylinder, stopper, and quickly mix vigorously. Cool to room temperature and dilute to 100 ml with absolute ethanol. Store in a polyethylene-stoppered Pyrex bottle and keep refrigerated to increase the stability of the reagent. The reagent is stable and colorless for at least one week and probably as long as it is not diluted with moisture. A slight white precipitate settles out on standing.

Test standard triglyceride and cholesteryl stearate to see if they produce identical optical densities when carried through the method described later. If the optical density values for the standards are not identical, discard the reagent and use a different volume of stock NaOH to prepare a new reagent. The correct volume of stock NaOH to use can be determined by constructing a plot of o.d. values of the standards as illustrated in Figure 1 for CS and TM. For example, if the o.d. value for CS is lower than the o.d. value for TM, select a smaller volume of stock NaOH than 5.60 ml. If the o.d. value of CS is higher than the o.d. value for TM, select a volume of stock NaOH greater than 5.60 ml. In this way a fine control of *water* in the reagent is accomplished.

Stock 1.1 N Sodium Ethylate. Add 2.5 g pure metallic sodium to about 90 ml absolute ethanol in a glass-stoppered flask fitted with a calcium chloride drying tube. Cool in an ice bath until the sodium has dissolved. Adjust the volume to 100 ml with absolute ethanol. Keep tightly stoppered and store in the refrigerator. This reagent is stable for at least one week.

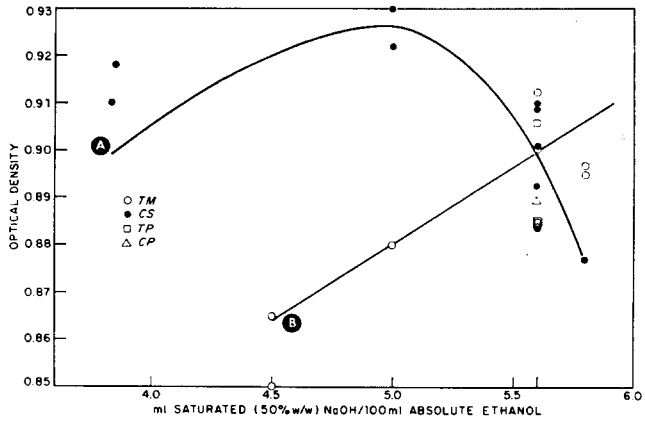


FIG. 1. Plot to determine amount of saturated sodium hydroxide (50% w/w) to dilute to 100 ml with absolute ethanol. Reaction I contained 75 μEq hydroxylamine, variable base, and variable water. Reaction II contained 20 μmoles ferric ion, 1,000 μEq perchloric acid, and 0.06 ml water. The standards used were 8 μEq TP, CP, TM, and CS. All points were read with a Beckman DU at a wavelength of 530 mμ after 30 min color development. Curves A and B were obtained in a single experiment using 8 μEq CS and 8 μEq TM, respectively. The other 10 points at the 5.6-ml sat. NaOH level were obtained from replicate determinations performed on different days with different reagents.

Stock Hydroxylamine Hydrochloride–Ethanol Reagent (2.61%, w/v; 0.375 N).

1) Dissolve 2.61 g $HONH_2 \cdot HCl$ (J. T. Baker's hydroxylamine–HCl recrystallized from ethanol)[2] in about 75 ml of absolute ethanol while heating the mixture and shaking it vigorously. Cool the solution to room temperature and dilute to 100 ml with absolute ethanol. This reagent contains 75 μEq $HONH_2 \cdot HCl$ and *no water* in 0.2 ml. Store in a glass-stoppered Pyrex bottle and keep it refrigerated. It is stable for at least one month.

[1] A syringe with a Chaney adapter. Available from Hamilton Co., Whittier, Calif.

[2] J. T. Baker Chemical Co., Phillipsburg, N. J.

2) As an alternate method, dissolve 2.61 g reagent grade $HONH_2 \cdot HCl$ in exactly 4.88 ml distilled water and dilute to 100 ml with absolute ethanol.

Working Alkaline Hydroxylamine Reagent.

1) Add equal volumes of stock sodium hydroxide–ethanol and stock hydroxylamine hydrochloride–ethanol (Reagent No. 1) to a screw-cap tube. Cap the tube, mix by inversion, let the mixture stand for about 5 min, and then centrifuge for 5 min at 2,000 rpm. Pour off the alkaline hydroxylamine supernatant fluid from the precipitated sodium chloride into another screw-cap tube and immediately cap. This reagent contains 75 μEq $HONH_2$, 150 μEq NaOH, and 0.00975 ml water (0.00135 ml water of neutralization included) in 0.40 ml.

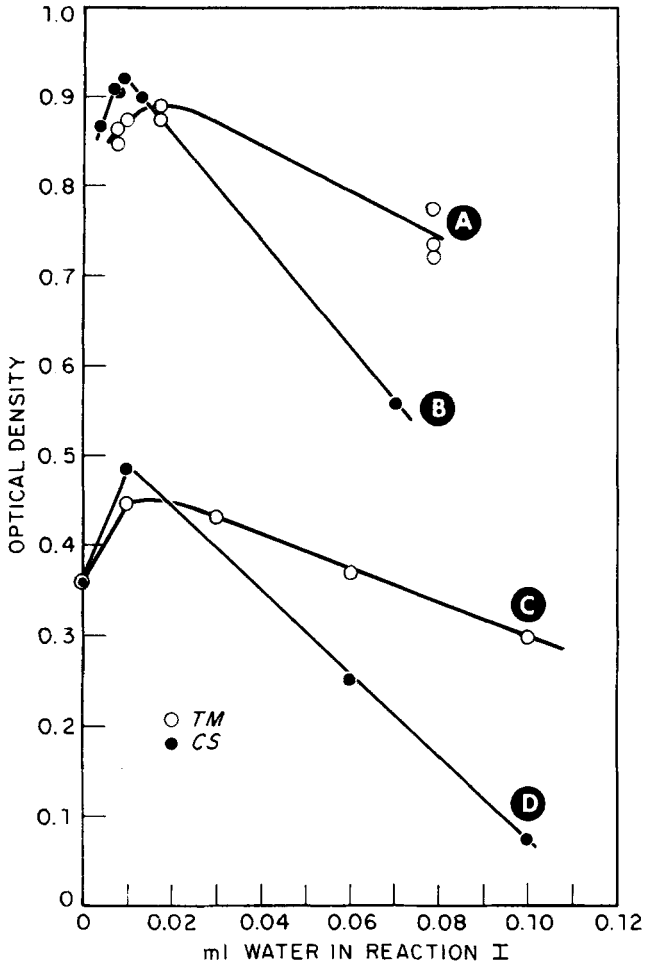


FIG. 2. Effect of water on reaction I. Reaction I contained 72 μEq hydroxylamine, 128 μEq excess sodium ethylate, and variable water. Reaction II contained 20 μmoles ferric ion, 1,000 μEq perchloric acid, and 0.06 ml water. Curve A was obtained with 8 μEq TM, curve B with 8 μEq CS, curve C with 3 μEq TM, curve D with 3 μEq CS. Curves A and B were read with a Beckman DU and curves C and D were read with a Coleman Jr. at a wavelength of 530 mμ after 30 min color development.

2) As an alternate method, mix sodium ethylate (Reagent No. 6) and stock hydroxylamine hydrochloride–ethanol (Reagent No. 2) using the techniques just described.

Stock Ferric Perchlorate Reagent (0.20 M $Fe(ClO_4)_3$, 10 M $HClO_4$, and 60% water). Dissolve 1.12 g of 99.8% pure iron wire in 81 ml 70–72% reagent grade perchloric acid (specific gravity 1.8, 12.5 *N*) by heating the mixture at low temperature on a hot plate in an Erlenmeyer flask covered with a glass bubble stopper. Cool the yellow solution and add, with swirling and continued cooling, about 15–18 ml distilled water. Transfer the reagent to a 100-ml volumetric flask and dilute to the mark with distilled water. Store in a glass-stoppered Pyrex bottle. This reagent is stable for at least three weeks.

Working Ferric Perchlorate–Ethanol Reagent. Dilute 1 ml stock ferric perchlorate to 100 ml with absolute ethanol in a glass-stoppered graduated cylinder, stopper, and mix by inversion. Prepare fresh daily. This reagent contains 20 μmoles ferric ion, 1,000 μEq perchloric acid, and 0.06 ml water in 10 ml.

Standard Esters (μEq/ml).

1) Trimyristin (TM) (mol. wt. 723.15). Transfer quantitatively 48.2 mg of four-times-recrystallized trimyristin to a 100-ml volumetric flask and dilute to the mark with redistilled chloroform. Mix well by inversion. Transfer 1-, 2-, and 3-ml aliquots to screw-cap tubes and dry at 60° under a stream of nitrogen. Cap tightly with Teflon-lined screw caps and store in the refrigerator marked as 2, 4, and 6 μEq ester, respectively.

2) Cholesteryl Stearate (CS) (mol. wt. 653). Transfer quantitatively 130.6 mg of cholesteryl stearate[3] to a 100-ml volumetric flask and continue as described in 1) above.

3) Tripalmitin (TP) (mol. wt. 807.3). Transfer quantitatively 53.8 mg of four-times-recrystallized tripalmitin to a 100-ml volumetric flask and continue as described in 1) above.

4) Cholesteryl Palmitate (CP) (mol. wt. 625). Transfer quantitatively 125 mg of cholesteryl palmitate[3] to a 100-ml volumetric flask and continue as described in 1) above.

5) Alpha-Cephalindipalmitoyl (C) (mol. wt. 673 ethanolamine cephalin; Mann Laboratories). Transfer quantitatively 67.3 mg to a 100-ml volumetric flask and continue as described in 1) above.

[3] A gift from Dr. David Kritchevsky, Wistar Institute, Philadelphia, Pa.

METHODS AND RESULTS

Final Esterified Fatty Acid Procedure

Into clean, dry 16 x 150-mm Teflon-lined screw-cap culture tubes, pipet aliquots of a purified lipid extract containing 1–8 μEq of ester. For standards, select tubes containing corresponding ester equivalents as previously prepared. For a reagent blank, add the equivalent volume of pure solvent mixture to an empty tube. Place the tubes (12–24 at one time) in a tube heater[4] at 60°, and evaporate the solvent completely under a stream of nitrogen delivered through a glass manifold. To each tube, add 1 ml of ethyl ether using a syringe pipet.[1] Cap the tubes *tightly* with Teflon-lined screw caps, and heat in a tube heater at 60° for 2 min to insure complete solution of the esters (cholesterol esters are more difficultly soluble). Swirl to mix,[5] and cool to room temperature. Prepare the working alkaline hydroxylamine–ethanol reagent. Uncap the tubes and, to each tube, add *immediately* 0.40 ml of alkaline hydroxylamine reagent using a 1-ml syringe-pipet[1] adjusted to deliver exactly 0.40 ml. Mix briefly by slight swirling. A slight turbidity develops in all tubes. Place all tubes in a 60° tube heater, direct a gentle flow of nitrogen evenly into each tube about an inch above the surface of the reaction mixture so that the solvent will evaporate within a 10-min period. (Evaporation should be carried out as uniformly as possible and conditions adjusted in order to evaporate to dryness in 10 min.) Prepare the working ferric perchlorate–ethanol reagent. Remove all tubes from tube heater (after the 10-min timer signal) and check for complete dryness by tilting the tubes and looking directly at the white precipitate through the bottom of the tubes. If the tubes are not dry, return them to the heater and increase the flow of nitrogen until they are dry. Add 10 ml working ferric perchlorate color reagent to the dry residue in each tube using a 10-ml syringe-pipet. Swirl the mixture thoroughly[5] for about 10–15 seconds until the color of the solution becomes homogeneous. The purple color is stable from 30 min to several hours after mixing. Read the optical density of the unknowns and standards against the reagent blank in a spectrophotometer at a wavelength of 530 mμ.

Effect of Water on Reaction I. Previous investigations have indicated that the hydroxamic acid reaction for esters was very sensitive to water. Preliminary studies showed that this was, indeed, true. Hence a systematic evaluation of the effect of water on the formation of the hydroxamates in reaction I was undertaken. For this purpose, different volumes of water were added to ether solutions containing either 3 or 8 μEq of trimyristin (TM) or cholesteryl stearate (CS) prior to the addition of alkaline hydroxylamine–ethanol reagent. All other conditions remained constant as described in the legend to Figure 2.

The results obtained (Fig. 2) indicate that with a low amount of water the o.d. values for TM were lower than for CS and at a higher amount of water the o.d. values for TM were higher than those for CS. However, the data also show that with a certain amount of water available for the reaction, the curves for the two types of esters intersected. *Thus, it is obvious that the amount of water in reaction I must be controlled precisely in order to obtain identical o.d. values for the different esters.*

In view of the above findings, studies were carried out to define more precisely the amount of water essential to reaction I in order to obtain identical o.d. values for the different esters. To accomplish this, an attempt was made, not only to control the water in the hydroxylamine–ethanol and the sodium hydroxide–ethanol reagents, but also to include the water formed by the neutralization reaction between these two reagents. Variable results between different preparations of reagents, however, were obtained due to the extreme sensitivity of the reaction to water. The final method evolved was obtained by the use of anhydrous hydroxylamine reagent and precise control of the amount of water in the sodium hydroxide–ethanol reagent. A typical example of the results obtained is shown in Figure 1. The data were found to be reproducible with different batches of reagents when 0.009 to 0.01 ml of water was present in reaction I. The mean o.d. values and the standard deviation at the 8-μEq ester level using the standard conditions of the method with different standard esters as determined from 12 analyses was 0.892 ± 0.013. This represents a coefficient of variation of ±1.5%. The errors of pipetting, weighing, and all general manipulations contributed to this variability. It should be noted that similar precision can be obtained when alkaline hydroxylamine reagent, prepared from sodium ethylate and hydroxylamine (Reagent No. 2), are substituted for the above reagent.

Effect of Alkalinity on Reaction I. The alkalinity of reaction I does not appear to play a sensitive role in obtaining equivalent o.d. values for different esters. The effect of base was shown by holding the amount of hydroxylamine hydrochloride constant at 75 μEq and by increasing the amount of anhydrous sodium ethylate

[4] Research Specialties Co., 200 S. Garrard Blvd., Richmond, Calif.

[5] Vortex Jr. mixer. Scientific Industries, Inc., Springfield, Mass.

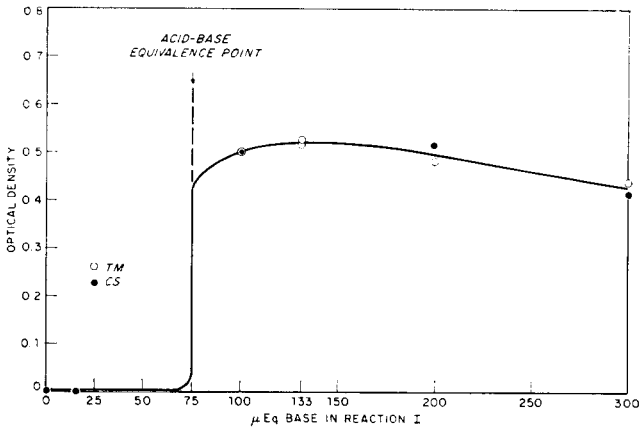


FIG. 3. Effect of alkalinity on reaction I. Reaction I contained 75 μEq hydroxylamine hydrochloride, variable sodium ethylate, and about 0.008 ml water. Reaction II contained 15 μmoles ferric ion, 1,500 μEq perchloric acid, and 0.14 ml water. The standards used were 3 μEq TM and CS. The curve was read with a Coleman Jr. at a wavelength of 530 mμ after 30 minutes color development.

added in the preparation of alkaline hydroxylamine reagent. All other variables were held constant as described in the legend to Figure 3.

It was observed that at the 3-μEq level, cholesteryl stearate (CS) and trimyristin (TM) were essentially on the same optical density curve over the range of 0–300 μEq of sodium ethylate. Before the hydroxylamine hydrochloride was neutralized by the base used, reaction I did not occur. However, just after the equivalent point was reached there was a very sharp increase in o.d. values to a plateau level. The optimum amount of base required for hydroxamate formation in reaction I was between about 100 and 200 μEq (Fig. 3).

Effect of Hydroxylamine on Reaction I. Very little information has been reported on the amount of hydroxylamine necessary for reaction I to occur quantitatively with different esters. A requirement seemed to be that an excess of hydroxylamine be present (Table 1). It was decided to study this effect at the 8-μEq level of TM and CS because this amount of ester produced o.d. values near the upper range of a Beckman DU spectrophotometer, and because it was observed during preliminary studies that certain conditions would work well for 3-μEq ester levels but when higher ester levels were used the o.d. values for different esters would not be identical.

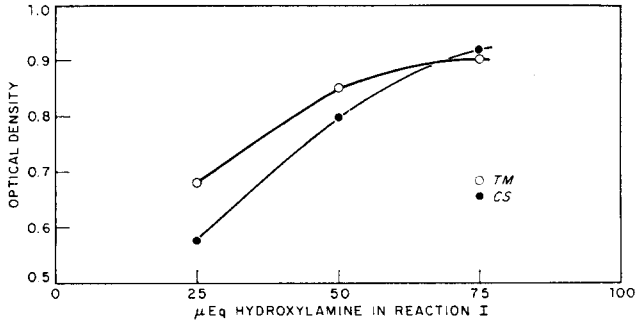


FIG. 4. The effect of hydroxylamine on reaction I. Reaction I contained variable hydroxylamine, 226 μEq sodium hydroxide, and 0.00975 ml water. Reaction II contained 20 μmoles ferric ion, 1,000 μEq perchloric acid, and 0.06 ml water. The standards used were 8 μEq TM and CS. The curves were read at a wavelength of 530 mμ with a Beckman DU after 30 min color development.

The amount of hydroxylamine was varied by diluting stock hydroxylamine hydrochloride with absolute ethanol. All other variables were held constant as described in the legend of Figure 4. When 25 μEq of hydroxylamine was used, TM and CS gave values that differed from each other. Both esters gave low color yields. When 50 μEq of hydroxylamine was used, TM and CS o.d. values were at higher levels but they were still different. If, however, 65–75 μEq of hydroxylamine was used, equivalent color values were obtained for both TM and CS with quantities up to 8 μEq ester (Fig. 4).

Effect of Water on Reaction II. When 0.06 ml water was added to the color reagent, the 8-μEq TM and CS standards were near the maximum o.d. values and the o.d. values were together. When the amount of water in the color reagent was increased to 0.2 ml, there was a decrease in o.d. values and a separation of the TM and CS values. A minimum quantity of water was needed in reaction II, so that the purple color developed was stable for several hours and also so that the maximum identical o.d. values for TM and CS at the 8-μEq level were attained.

Effect of Acidity on Reaction II. An acid medium was found to be necessary for the solution of a brown ferric hydroxide precipitate which formed in alkaline medium. The purple color appeared quickly after the excess base from reaction I had been neutralized. When there was a large excess of perchloric acid present, the color tended to fade out. When the amount of excess base in reaction I was changed in any way, the acidity of reaction II had to be correspondingly changed so that an optimum amount of perchloric acid was present for proper color development. The effect of acid was demonstrated by adding specific quantities of 71% perchloric acid before dilution with absolute ethanol to obtain the color reagent for each different point. All other components were as indicated in Table 1. There is an acceptable range of perchloric acid, between 600 and 1,500 μEq acid, that will provide for identical o.d. values for TM and CS at the 3-μEq level. The optimum values for maximum color development were

TABLE 1. HYDROXAMIC ACID METHODS FOR THE DETERMINATION OF ESTERIFIED FATTY ACIDS

Method for Esterified Fatty Acids (EFA)	Reaction I—Hydroxamic Acid Formation								Reaction II—Color Development								Absorptivity per Ester Group‖
	EFA Solvent		Alkaline Hydroxylamine Added						Ferric Reagent Added								
				Microequivalents			Total Vol. Water‡ (ml)	Reaction Conditions			Microequiv.		Total Vol. Water (ml)	Final Color Vol. (ml)	Color Stability	
	Vol. (ml)	Type	Vol. (ml)	Hydroxylamine	Excess Base				Vol. (ml)	Solvent	Ferric Ion	Excess Acid				
Bauer and Hirsch (1)	15	Ether	0.6	108	79	NaOH	0.031	To dryness at 60°	10.0	Ethanol	70	4,100	0.83	10.0	20 min.	...
Thompson (9)	5	Ethanol–ether	1.5	540	1,800	NaOH	0.0097	30 min., 25°	12.5	Water	18	12,000	0.725	18.5	...	...
Stern and Shapiro (2)	3	Ethanol–ether extract†	1.0	1,000	750	NaOH	1.26+§	20 min., 25°	1.1	70% Ethanol	185	1,700	2.35	5.1	...	...
Goddu, et al. (8)	5	Absolute ethanol	3.0	2,700	2,000	NaOH	0.485	5 min., 72°	42.0	2B Anhyd Ethanol	240	4,800	0.386	50.0	Several hours	900 to 1,100
Hack (3)	0*	(Footnote*)	0.7	108	140	NaOH	0.037	15 sec., b.p.	9.3	Ethanol	9.4	4,000	0.484	10.0	4% loss in 2 hr.	...
Rapport and Alonzo (4)	3	Ether	0.1	21.5	16	NaOH	0.0054	Dryness, 65°	6.0	Ethanol	63	1,900	0.77	6.0	Several hours	1,000 to 1,100
Snyder and Stephens (10)	0*	(Footnote*)	1.0	287	700	NaOH	0.055	2 min., 65°	2.5	Absolute Ethanol	11	250	0.16	3.5	>1 hr.	...
Weller (5) [Eggstein] (6)	5	Ether	0.4	72	128	Sodium Ethylate	0.008	5 min., 0° Dry in vacuo up to 60° (2 hr.)	10.0	96% Ethanol	58	4,100	0.57	10.0	...	1,040
Antonis (11)	3	Iso-Propyl ether†	1.0	1,000	700	NaOH	0.07+§	30 min., 25°	6.0	Absolute Ethanol	20	1,200	0.26	10.0	...	...
Connerty, et al. (12)	5	Iso-Propanol extract†	1.0	575	900	NaOH	0.26+§	15 min., 37°	2.0	Absolute Methanol	21	800	0.04	8.0	30 min.	800 to 900
Skidmore and Entenman	1	Ether	0.4	75	150	NaOH	0.00975	To dryness in 10 min. at 60° with nitrogen	10.0	Absolute Ethanol	20	850	0.06	10.0	Several hours	1,150

* The dry EFA extracts were dissolved in hydroxylamine reagent containing ethanol.

† The EFA extracts were not taken to dryness.

‡ The water of neutralization was included.

§ The water present in the lipid extracts was not included.

‖ Sample calculation:

$$\text{Absorptivity per ester group} = \frac{\text{Optical density}}{\frac{\text{g ester used}}{\frac{\text{MW}}{\text{No. of ester groups per mole}}} \times \frac{1{,}000 \text{ ml/l}}{\text{Volume of colored solution}} \times \text{light path (cm)}} = \frac{0.91}{8 \times 10^{-6} \times \frac{1{,}000}{10} \times 1.0}$$

= 1,150 for cholesteryl stearate, cholesteryl palmitate, trimyristin, tripalmitin, and cephalin.

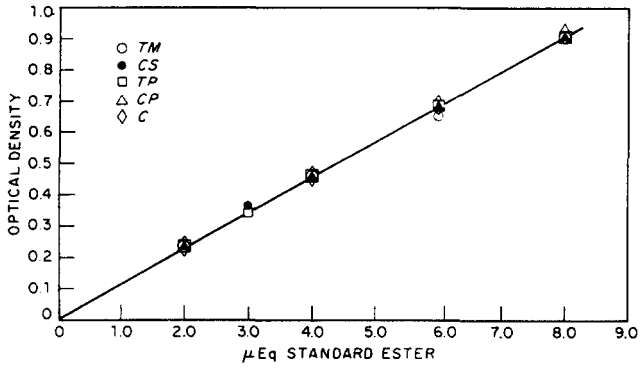


FIG. 5. Straight-line curves for five different ester standards. Reaction I contained 75 μEq hydroxylamine, 150 μEq excess sodium hydroxide, and 0.00975 ml water. Reaction II contained 20 μmoles ferric ion, 1,000 μEq perchloric acid, and 0.06 ml water. The standards used were different μEq of TP, CP, TM, CS, and cephalin (C). All points were read on a Beckman DU at a wavelength of 530 mμ after 30 min color development.

between 700 and 1,000 μEq perchloric acid in 10 ml color reagent.

Effect of Ferric Ion on Reaction II. The results were obtained by adding specific quantities of stock ferric ion reagent before dilution with absolute ethanol to obtain the color reagent for each different point. All other components were held constant as indicated in the last line of Table 1. At the 3-μEq level of TM and CS, a range of 5–25 μmoles ferric ion per 10 ml gave identical o.d. values for TM and CS. The o.d. values were low at ferric ion levels less than 5 μmoles. As the amount of ferric ion was increased above 25 μmoles, the initial color was brown and an increasing amount of time of standing was required to develop the typical purple color. The development of the purple color occurred in coincidence with the disappearance of a yellow color in the blank. It was observed at the 8-μEq level of TM that 20 μmoles of ferric ion was necessary to bring the o.d. values up to the standard straight-line curve at a wavelength of 530 mμ. A similar observation of the requirement of ferric ion for a straight-line curve was reported by Goddu, et al. (8).

Results with Five Different Ester Standards and with Folch Lipid Extracts

In order to determine if the method gave color yields proportional to the amount of ester present, different standards were selected so that the optical densities of the resulting colored solutions would fall between 0.1 and 1.0. Between 2 and 8 μEq of ester, the method described gave identical straight-line curves for five different ester standards (Fig. 5). The synthetic cephalin standard used did not completely dissolve in the ether, but it could have dissolved at a later stage after the ether evaporated and before the ethanol and water evaporated in order for it to have reacted as well as it did in the method. The triglycerides dissolved in ethanol and reacted well without any ether present. Nevertheless, ether was necessary to dissolve the cholesteryl stearate.

In order to show the applicability of the method to lipid extracts of biological samples, the technique described was applied to different amounts of extracts obtained from rat serum, rat liver, and human serum. Accordingly, lipid extracts from these tissues were prepared by chloroform–methanol extraction followed by partition-dialysis against 0.2 volumes of water (7) and esters were determined. The o.d. values of the respective Folch extracts fell on a straight line that passed through the origin when plotted against the volume of extract.

One representative sample was chosen from each standard and each lipid extract, and the o.d. values were measured between the wavelengths of 410 mμ and 700 mμ with a Beckman DK-2 ratio-recording spectrophotometer. The Folch extracts gave a ferric hydroxamate with observed spectrum indistinguishable from that given by pure standards. The 4-μEq ester curve had an o.d. value twice that obtained from 2 μEq of a different ester, and so forth. The wavelength of 530 mμ was in the optimum area of maximum color development for readings.

DISCUSSION

It becomes apparent, when this method is compared with the others (1–6, 8–12) listed in Table 1, that the most important single factor is the concentration of water in reaction I. If the amount of water present in reaction I is not accurately controlled as in the method described, the molar absorptivities of the esters determined may not be identical. The effect of water is so great that in calculating the amount of water in reaction I, even the water of the neutralization of hydroxylamine hydrochloride with sodium hydroxide must be taken into consideration. By controlling the volume of one reagent (i.e., stock NaOH), the water in reaction I could be adequately controlled. The spread of values in Figure 1 offers some indication of the difficulty of the precise control of the water. Figure 2 shows the marked effect of water, and Figure 3 shows the less significant effect of alkalinity on obtaining identical o.d. values for TM and CS.

It is possible that water plays an important role in controlling the polarity of the medium during reaction I. Hydroxylamine is a polar molecule that is water- and ethanol-soluble. If no water is present in the

ethanol-ether mixture in reaction I, the solution is clear; if water is present as in the final method, a turbidity appears; and when there is excess water present, a precipitate forms. The desired results are obtained only when the reaction mixture is turbid.

The triglycerides are nonpolar molecules that are soluble in ethanol and ether. The long-chain cholesterol esters are nonpolar molecules that are soluble in ether and only slightly soluble in ethanol at room temperature. During the drying process at 60°, the relatively polar hydroxylamine reacts with the relatively nonpolar triglycerides and cholesterol esters. In reaction I, when the turbid mixture is taken to dryness, the ether evaporates first. As this happens, CS becomes less soluble and hydroxylamine becomes more soluble. The hydroxamates from CS probably form during this stage. After the ether evaporates, the triglycerides and hydroxylamine become more soluble in the ethanol-water solution. The reaction of these compounds then probably proceeds quantitatively. After the ethanol evaporates, the remaining aqueous solution offers opportunity for the reaction of esters that are more soluble in water than the triglycerides or cholesterol esters. By taking the reaction mixture to dryness, one reaction product, which is an alcohol, either evaporates or precipitates. Thus reaction I is forced quantitatively to the right. Also, hydrolysis of the hydroxamates formed is prevented by drying the products of reaction I, which eliminates water.

Weller (5) showed an effect of sodium ethylate different from that presented here. Weller concluded that the sodium concentration was the critical factor for equalizing the extinctions of the different esters. However, it is pointed out in this investigation that trimyristin and cholesteryl stearate have identical optical densities over a rather broad range of sodium hydroxide or sodium ethylate concentrations, and that it is the amount of water in reaction I that is critical in bringing the extinctions of the different esters together.

In reaction II, it was also observed that when the composition of the reaction mixture was held within certain narrow limits, the ferric-hydroxamate complex would form properly and remain stable. Thus, it was found that (*a*) a minimum of water was necessary in reaction II for proper color development, and an excess of water gave different o.d. values for equivalent amounts of TM and CS; (*b*) a plateau in the curve of o.d. values could be obtained by varying the ferric ion concentration in reaction II (the upper limit for the amount of ferric ion could very possibly have been near 200 μmoles, although that amount was not tested); and (*c*) about 1,000 μEq of acid per 10 ml of colored solution gave good stability of the colored complex. The amount of ferric ion used by other investigators to develop the color (Table 1) varied from about 10 to 200 μmoles. The variation of excess acid used by other workers, as summarized in Table 1, was from about 750 to 6,000 μEq per 10 ml color solution. The methods that indicated about 1,000 μEq acid per 10 ml color solution reported good stability of the color complex, in agreement with that reported here. Some methods showed good color stability and high molar absorptivities, but only the method described here showed identical molar absorptivities for all esters tested.

REFERENCE

1. Bauer, F. C., Jr., and E. F. Hirsch. *Arch. Biochem. Biophys.* **20**: 242, 1949.
2. Stern, I., and B. Shapiro. *Brit. J. Clin. Pathol.* **6**: 158, 1953.
3. Hack, M. H. *Arch. Biochem. Biophys.* **58**: 19, 1955.
4. Rapport, M. M., and N. Alonzo. *J. Biol. Chem.* **217**: 193, 1955.
5. Weller, H. *Klin. Wochschr.* **37**: 951, 1959.
6. Eggstein, M. *Proceedings of the 3rd International Conference on Biochemical Problems on Lipids*, Brussels, 1956, 150.
7. Folch, J., M. Lees, and G. H. Sloane-Stanley. *J. Biol. Chem.* **226**: 497, 1957.
8. Goddu, R. F., N. F. Le Blanc, and C. M. Wright. *Anal. Chem.* **27**: 1251, 1955.
9. Thompson, A. R. *Australian J. Sci. Research* **3A**: 128, 1950.
10. Snyder, F., and N. Stephens. *Biochim. et Biophys. Acta* **34**: 244, 1959.
11. Antonis, A. *J. Lipid Research* **1**: 485, 1960.
12. Connerty, H. V., A. R. Briggs, and E. H. Eaton, Jr. *Clin. Chem.* **7**: 37, 1961.