Technical

# A Simple and Rapid Colorimetric Method for Determination of Free Fatty Acids for Lipase Assay

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**A simple and rapid colorimetric method was developed to determine the lipase activity for fat splitting. Free fatty acids produced by lipase from triacylglycerols were determined by observing the color developed using cupric acetate-pyridine as a color developing reagent. This modified method requires only a few minutes to determine the free fatty acids, whereas it takes over 20 min by the conventional methods which require solvent evaporation and centrifugation steps. The sensitivity and reproducibility of the method were good for caproic, caprylic, capric, lauric, myristic, palmitic, stearic and oleic acids.**

Lipases or acylglycerol-acylhydrolases (EC 3.1.1.3) are the enzymes which hydrolyze the esters of long chain aliphatic acids from glycerol at oil/water interface (1). Recently, lipases have been used extensively in the hydrolysis of lipid under mild conditions (2) and synthesis of new triacylglycerols by interesterification (3). Many research papers on fat splitting using microbial lipases in the substrate emulsion system (4), or in the two-phase system (5) have been published. We reported the effect of organic solvents on the stability and catalytic activity of the lipases from *Candida rugosa* for fat splitting, and isooctane was recommended as the most suitable solvent in the two-phase system (6).

There is a need to determine the lipase activity by measuring the fatty acids produced. Fatty acids produced by lipase can be determined by titrimetry, copper soap colorimetry, chromophore spectrophotometry, isotopic methods, gas liquid chromatography, enzymatic methods and immunological methods (7). Jensen (7) reported that the most practical methods are titrimetry and copper soap colorimetry for the study of enzymatic fat splitting and that copper soap colorimetry is better than titrimetry.

Copper soap colorimetry measures color after fatty acids are converted to copper soaps with color reagents. This colorimetric method, originally developed by Duncombe (8) using $Cu(NO_3)_23H_2O$ and triethanolamine as a copper reagent and a color reagent, respectively, was later modified and improved by many workers for their specific research purposes (9-14). Lowry and Tinsley (15) developed a rapid colorimetric determination of free fatty acids with a good sensitivity and reproducibility using cupric acetate-pyridine. This method is again modified by replacing benzene with hexane for determination of free fatty acids formed in the enzymatic hydrolysis of olive oil in the solvent system (6).

The purpose of this paper is to report a further simplification of the method of Lowry and Tinsley (15) for determination of free fatty acids by eliminating the solvent evaporation and centrifugation steps for lipase assay.

## EXPERIMENTAL

*Materials.* Oleic, stearic, palmitic, myristic, lauric, capric, caprylic, caproic and butyric acids, specially manufactured as fatty acid standards, were purchased from Sigma Chemical Co. (St. Louis, Missouri). Benzene, isooctane and other solvents were purchased from Tokyo Kasei Chemical Co., Ltd. (Tokyo, Japan). All other chemicals and reagents used were of analytical grade.

Copper reagent was prepared according to Lowry and Tinsley (15). A 5% (w/v) aqueous solution of cupric acetate was prepared and filtered, pH being adjusted to 6.1 using pyridine. The solution does not require any other color reagent.

*Standard Curves of Free Fatty Acids.* Samples containing 2.0-50.0 μmole free fatty acids, butyric, caproic, caprylic, capric, lauric, myristic, palmitic, stearic and oleic acids each, were prepared by dissolving them in test tubes with 5 ml of isooctane. Slight warming was necessary to make solution for solid stearic, palmitic and myristic acids. Then 1.0 ml of cupric acetate-pyridine reagent was added and the two phases thus formed were mixed vigorously for 90 sec using a vortex mixer. The mixture was allowed to stand still for about 10-20 sec until the aqueous phase was sedimented clearly from the solution of isooctane and fatty acid. The standard curves of free fatty acids vs. absorbancy were determined by measuring the absorbance of isooctane solution at 715 nm against the control which contains no free fatty acids.

*Determination of Lipase Activity.* The enzyme reaction in the emulsion system (4) was stopped by adding 6N HCl and isooctane, followed by mixing and boiling the reaction mixture for 5 min. The 5 ml of upper isooctane layer containing the fatty acids was drawn off to a test tube for analysis. The lipase activity in the two-phase system (16) was determined by adding 6N HCl at the very end of the reaction, and the mixture was agitated vigorously for 30 sec. after which 5 ml of the upper layer was taken in a test tube. Free fatty acids dissolved in isooctane were determined by spectroscopy according to the method described above. Lipase activity was determined by measuring the amount of

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free fatty acids from the standard curves of free fatty acids.

## RESULTS AND DISCUSSION

To determine the lipase activity according to the conventional colorimetric methods (15) for an emulsion system (4), we must selectively extract the free fatty acids from the reaction mixture with the solvent, evaporate the solvent, replace that with benzene or other solvents, and then add the color reagents followed by mixing the color reagent mixture vigorously. This mixture must be centrifuged in order to separate the solvent phase from the aqueous phase. Lipase activity was determined by observing the color development of free fatty acids in the solvent phase. For a two-phase system (6), lipase activity was determined by taking the supernatant containing free fatty acids from the reaction mixture and evaporating the solvent which was replaced by benzene or other solvents, followed by centrifugation of the color reagent mixture. Then the lipase activity was determined by assaying the fatty acids. All of these colorimetric methods required extraction, evaporation and centrifugation.

This modification of the colorimetric method of free fatty acid determination is based on the fact that the density and water miscibility of benzene are greater than those of isooctane, which was selected as the most suitable solvent for fat splitting in the two-phase system (6). The density of isooctane and benzene is 0.69 and 0.88 at 20 C, respectively, and water immiscibility of isooctane is greater than that of benzene (Table 1). When isooctane was used we were able to determine the free fatty acids for assaying the lipase activity rapidly while elimating the centrifugation step.

The spectrum of the color developed by reaction of free fatty acids with cupric acetate-pyridine at various wavelengths of spectrophotometer was investigated as a preliminary step. The result showed that the absorbance in the range of 710 nm and 720 nm was maximum, as reported by Lowry and Tinsley (15). They reported that the optimum wavelength was 715 nm. The effect of the pH of cupric acetate solution on the color development of cupric acetate-oleic acids soaps in isooctane was investigated by varying the pH with pyridine and comparing the pH dependency of absorption in this solvent with that in benzene. The absorbance of this color in isooctane was maximum at pH 5.8-6.4 (Fig. 1), whereas the absorbance in benzene was maximum at pH 6.0-6.2 (15). This broad optimum pH range for an isooctane system is considered to be due to the higher water immiscibility of isooctane. Broad spectrum of the pH dependency in isooctane is one of the advantages of this method for reproducibility.

To study the effect of carbon numbers of fatty acids on their color development, the absorbance was measured at 715 nm with 30 μeq free fatty acid/5ml of isooctane, unless otherwise specified. The color developments of caproic, caprylic, capric, lauric, myristic, palmitic, stearic and oleic acids are shown in Figure 2. The results suggest that this method was suitable for

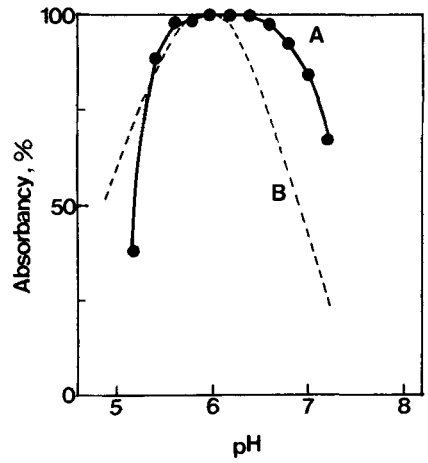


Fig. 1. pH dependency of absorption cupric acetate-oleic acid soaps in 5 ml isooctane at 715 nm. 5% (w/v) aqueous solution of cupric acetate was used as a color reagent. Relative absorbancy of color development using 30 μeq oleic acid was represented by varying the pH of cupric acetate solution (1 ml) with pyridine. Curve A, isooctane; curve B, benzene. Curve B represents the data reported by Lowry and Tinsley (15). Tests were run in duplicate.

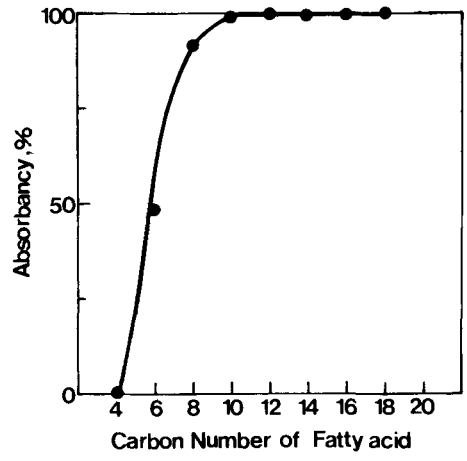


Fig. 2. Effect of carbon numbers of fatty acid chain on color development. Relative absorbancy at 715 nm of color developed for each 30 μeq butyric ($C_4$), caproic ($C_6$), caprylic ($C_8$), capric ($C_{10}$), lauric ($C_{12}$) and oleic acids ($C_{18:1}$) in 5 ml isooctane were represented. 10 μeq stearic ($C_{18}$), palmitic ($C_{16}$) and myristic acids ($C_{14}$) in 5 ml isooctane were used for calibration. Tests were run in duplicate.

estimation of the fatty acids produced when triacylglycerol having fatty acids of carbon numbers larger than $C_{10}$ was used as a substrate. However, each caproic and caprylic acid also could be determined with good reproducibility.

Standard curves for various free fatty acids were prepared using caproic, caprylic, capric, lauric, myristic, palmitic, stearic and oleic acids. The regression equation of the standard curves for capric, lauric, myristic, palmitic, stearic and oleic acids were almost the same. Figure 3 shows the standard curves for oleic acid (line A) and caproic acid (line B). The data show good reproducibility and sensitivity up to 50 μmole of free fatty acid/5 ml of isooctane without centrifugation

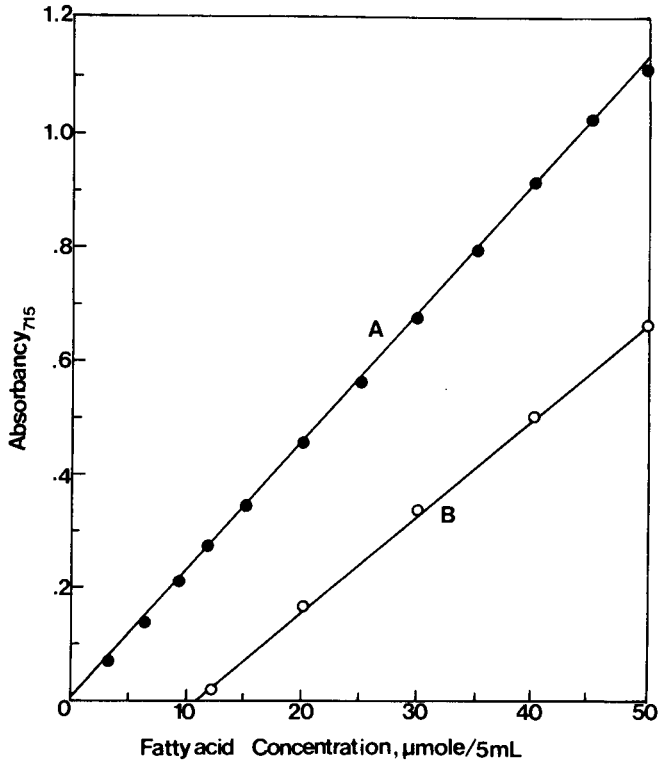


**Fig. 3. Standard curves of fatty acids in isooctane. Absorbancy at 715 nm with the pyridine-cupric acetate reagent was represented. line A, oleic acid; line B, caproic acid. The regression equation for line A is y = 0.0227x + 0.002, and for line B is y = 0.0180x − 0.203, where y = absorbancy at 715 nm and x = μeq free fatty acid/5 ml of isooctane. Each point represents the mean value of three determinations.**

and solvent evaporation steps. In case of caproic acid (line B), color did not develop up to 10 μmole caproic acid/5 ml of isooctane, although the color was developed in linear proportion to the fatty acid concentration at above 10 μmole/5 ml. Because the free caproic acid is slightly soluble in water, some part of the caproic acid which is not extracted readily by the solvent may remain in aqueous phase so that the color does not develop up to 10 μmole of caproic acid/5 ml of solvent. As shown in Table 2, the data for the butyric acid seem to be in agreement with this fact. For butyric acid, which is relatively soluble in water, copper soap color did not develop up to 50 μmole of butyric acid/5 ml of solvent because of its low extractability to the solvent. Table 2 shows the standard curves of such saturated fatty acids as stearic, palmitic and myristic acids were constructed only up to 18 μmole of stearic, 22 μmole of palmitic and 30 μmole of myristic acids/5 ml of solvent. Over these concentrations, soap-like emulsion developed probably due to the limited solubility of saturated fatty acids in solvent (18).

For the study of lipase assay for both the emulsion system and the two-phase system, using the isooctane as an extraction solvent excludes laborious solvent evaporation and centrifugation steps. Adding the concentrated hydrochloric acid helps not only for stopping the lipase reaction but also for increasing the extractability of free fatty acids to the solvent. HCl

**TABLE 1**

**Comparison of Density and Water Miscibility (Water Solubility in Solvent, Solvent Solubility in Water) of Solvent (17)**

| Solvent | Density at 20 C g/ml | Water solubility of solvent, % (w/w) at 25 C | Solvent solubility of water, % (w/w) at 25 C |
|---|---|---|---|
| Iso-octane | 0.692 | 0.0055[a] | 0.00024 |
| Benzene | 0.879 | 0.063 | 0.1780 |

[a]Water solubility at 20 C.

**TABLE 2**

**The Concentration Range for Determination of Absorbancy of Various Free Fatty Acids[a]**

| Free fatty acids[b] | Concentration (μmole FFA/5 ml) | |
|---|---|---|
| | Lower range | Upper range |
| Butyric | >50[c] | |
| Caproic | 10 | >50[d] |
| Myristic | 0 | 30 |
| Palmitic | 0 | 22 |
| Stearic | 0 | 18 |

[a]Experiment was carried out up to 50 μmole of FFA in 5 ml of isooctane.

[b]The data for caprylic, capric, lauric and oleic acids are excluded in this table, because the color of copper soap of these fatty acids develops well over 50 μmole FFA with good sensitivity.

[c]Color does not develop up to 50 μmole of FFA.

[d]No limitation was detected up to 50 μmole of FFA.

associated with an ionized free fatty acid ($RCOO^-$) to form a nonionized free fatty acid (RCOOH), and the nonionized form ($RCOO^-$). In the mixture of isooctane-cupric acetate-pyridine, only free fatty acids form a cage-like complex with cupric acetate and the color development is not interfered by monoacylglycerols, diacylglycerols, triacylglycerols, or another lipid (15). In fact, we calculated the lipase activity by this method to investigate the solvent effects on the lipase stability and lipase activity (19), and compared the method with the conventional methods (6, 15). Previous reports (6) detected the lipase stability by determining the residual activity after incubating in organic solvents, and the lipase activity in organic solvent. The present method has the advantage of eliminating evaporation and centrifugation, which are required in conventional methods. As a result, the present method takes only a few minutes to determine the free fatty acids in contrast to the conventional methods of 20 min. The result of the method agreed with results of the conventional methods in determining the solvent stability of lipase; it also shows better reproducibility. On the other hand, in determining the lipase activity the two phase system was recommendable, because the reproducibility of the data in

the two phase system was better than that of the emulsion system.

The results indicate that the present method is a very simple and rapid one to determine free fatty acids, and is suitable for the determination of lipase activity.

## REFERENCES

1. Brockman, H. L., in *Lipases*, edited by B. Borgström and H. L. Brockman, Elsevier Sci. Pub., Amsterdam, 1984, pp. 3-46.
2. Linfield, W.M., R.A. Barauskas, L. Sivieri, S. Serota and R.W. Stevensor Sr., *JAOCS 61*:191 (1984).
3. Macrae, A.R., *JAOCS 60*:291 (1983).
4. Kwon, D.Y., and J.S. Rhee, *Korean J. Chem. Eng. 1*:153 (1984).
5. Blain, J.A., M.W. Akhtar and J.D.E. Patterson, *Pak. J. Biochem. 10*:41 (1976).
6. Kim, K.H., D.Y. Kwon and J.S. Rhee, *Lipids 19*:975 (1984).
7. Jensen, R.G., *Lipids 18*:650 (1983).
8. Duncombe, W.G., *Biochem. J. 88*:7 (1963).
9. Hron, W.T., and L.A. Menahan, *J. Lipid Res. 22*:377 (1981).
10. Sahasrabudhe, M.R., *JAOCS 59*:354 (1982).
11. Bowyer, D.E., J.S. Cridland and J.P.King, *J. Lipid Res. 19*:274 (1978).
12. Radding, W., G.G. Mayer and J.W. Correll, *J. Lipid Res. 24*:100 (1983).
13. Shipe, W.F., G.F. Senyk and K.B. Fountain, *J. Dairy Sci. 63*:193 (1980).
14. Bains, G.S., S.V. Rao and D.S. Bhatia, *JAOCS 41*:831 (1964).
15. Lowry, R.R., and I.J. Tinsley, *JAOCS 53*:470 (1976).
16. Leuenberger, H.G.W., in *Biotechnology*, edited by H.-J. Rehm and G. Reed, Verlag Chemie, Weinheim, 1984, Vol. 6a pp. 5-29.
17. Riddick, J.A., and W.B. Bunger, in *Organic Solvents*, edited by A. Weissberger, 3rd edn., John Wiley & Sons, New York, 1970, pp. 95-108.
18. Singleton, W.S., in *Fatty Acids*, edited by K.S. Markley, Interscience Pub. Inc., New York 1960, pp. 609-678.
19. Kwon, D.Y., and J.S. Rhee, *Korean J. Food Sci. Technol. 17*:490 (1985).

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# Addition of Phthalimidonitrene to Acetylenic Fatty Acid Esters: Synthesis of Long-Chain 2-Phthalimido-2H-Azirines

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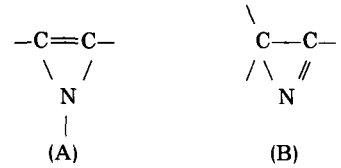
**Lead tetraacetate (LTA) oxidations of N-aminophthalimide in the presence of acetylenic fatty acid esters have resulted in the formation of corresponding 1H-azirines that spontaneously rearranged to give 2H-azirines in moderate yields. 2H-Azirine derivatives (IV, V and VI) of acetylenic fatty acid esters, methyl 10-undecynoate (I), methyl 9-undecynoate (II) and methyl 9-octadecynoate (III), respectively, have been prepared and characterized with the help of spectral and micro analyses.**

To continue our studies on the synthesis of long chain N-aminoaziridines (12) by the addition of aminonitrene intermediate to olefins, we focused on monounsaturated analogs of aziridine, i.e., 1H-azirine (A) and 2H-azirine (B).



(A) (B)

No azirine (A) has been isolated yet or even demonstrated clearly to be a reaction intermediate. However, it is believed that A is formed first and then rearranges very rapidly to B. The rearrangement may be due to the high antiaromatic (3,4) nature of A. A number of methods (5-8) to prepare azirines are described in the literature, but the addition of nitrene to acetylenes is a relatively new method that gave a fairly good yield of azirine in one step. Anderson et al. (9) first described the addition of aminonitrene to acetylenes and reported the formation of B, which probably occurred by the rearrangement of A. We report here the synthesis of chain-substituted 2H-fatty azirine by the reaction of acetylenic fatty acid esters (terminal, penultimate, internal) with the nitrene intermediate generated in situ by the LTA oxidation of N-aminophthalimide.

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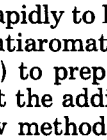
## RESULTS AND DISCUSSION

The oxidation of N-aminophthalimide in the presence of methyl 10-undecynoate (I) (Scheme 1), using LTA as an oxidant at room temperature on final work up and column chromatographic fractionation, gave an inseparable isomeric B (IV). Its infrared (IR) spectrum showed a characteristic sharp band at 1775 $cm^{-1}$, which has been assigned to the highly strained carbon nitrogen double bond vibration of the azirine ring (10). A broad band in the region of 1740-1680 revealed the presence of carbonyl functions of ester and phthalimido groups. Bands at 1600 and 1455 $cm^{-1}$ accounted for C=C stretching of the benzene ring, a band at 1070 $cm^{-1}$ accounted for C—H bending and one at 705 $cm^{-1}$ accounted for an out-of-plane ring by sextants of the benzene ring. Its NMR spectrum gave a sharp multiplet at $\delta$8.57 showing long range coupling (  ) and a multiplet at $\delta$7.82 for four protons of the benzene ring along with usual signals of fatty methyl ester. These data confirmed the structure of product IV as 2-(8-carbomethoxyoctyl)-2-phthalimido-2H-