Rapid Colorimetric Determination of Free Fatty Acids[1,2]

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ABSTRACT

In 1964, a method was described for the determination of free fatty acids (FFAs) in vegetable oil. This paper describes an expansion of that work, improving the sensitivity and reproducibility of the method, as well as examination of solubilities of the copper soaps as a function of chain length and unsaturation. Involvement of the micellar structure was reviewed. Finally, a procedure is described that permits very rapid determination of FFA at the 2.0-14.0 μmol (0.5-4.0 mg oleic acid) level, and the results with several oils are given. Particular attention was given to evaluation of solvent systems which would extract the copper complexes.

INTRODUCTION

The analysis of free fatty acid (FFA) levels is a continuing concern of workers in the lipid field. Originally carried out by titration, the need for speed and/or increased sensitivity led to the development of a number of colorimetric procedures, beginning with the work of Ayers in 1956 (1). Since then, more than 20 additional papers have been published based on modifications of her procedure.

A much simpler, but less sensitive, procedure was developed by Baker, first for determining fat acidity in

[1]Presented in part at the AOCS Meeting in San Francisco, April 1969.

[2]Technical Paper No. 4036, Oregon Agricultural Experiment Station.

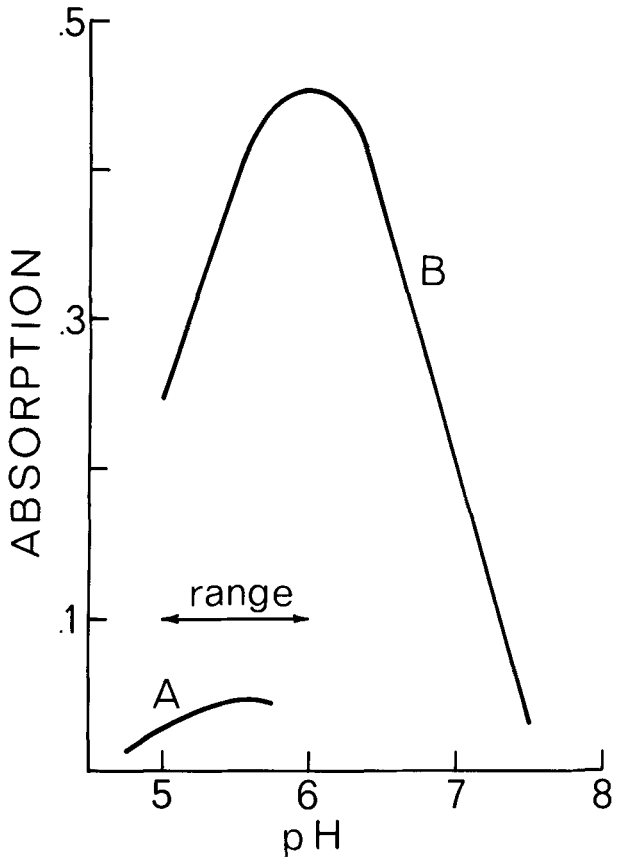

FIG. 1. Absorption maximums of cupric acetate-oleic acid soaps in benzene. Curve A without pyridine, 640 nm; curve B with pyridine, 715 nm. The normal range of pH expected from reagent grade cupric acetate (5% aqueous solution) is also indicated.

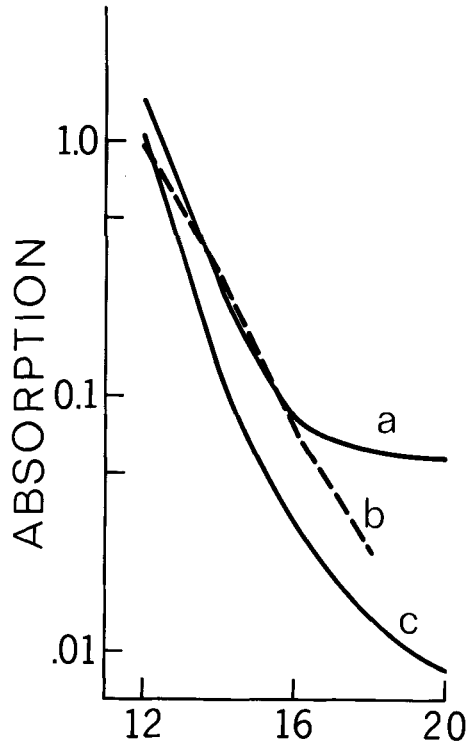

FIG. 2. Interrelationships of chain length and the solubility of cupric soaps of saturated free fatty acids as measured by absorption at 680 nm in ethyl acetate (a) and benzene (c). The solubilities of FFAs in benzene (x 10^{-1} g/100 g) are shown by (b). The abscissa represents the number of carbons in the chain.

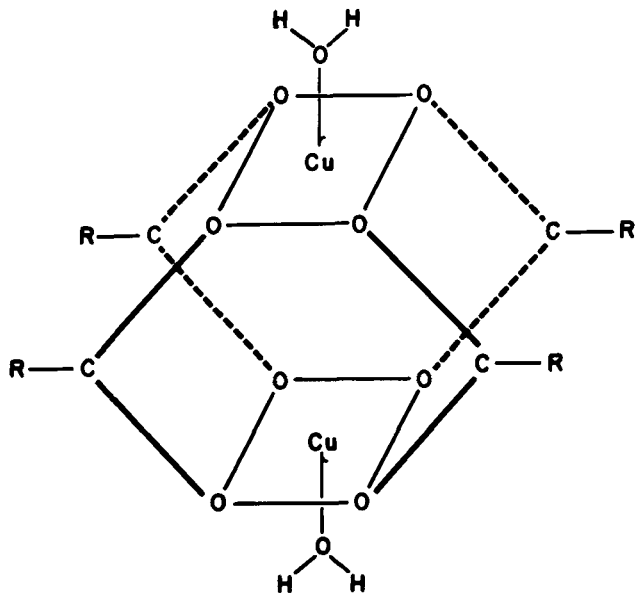

FIG. 3. Structure of cupric soaps showing the cage-like complex formed. R = 12-20 carbon chains on the fatty acids.

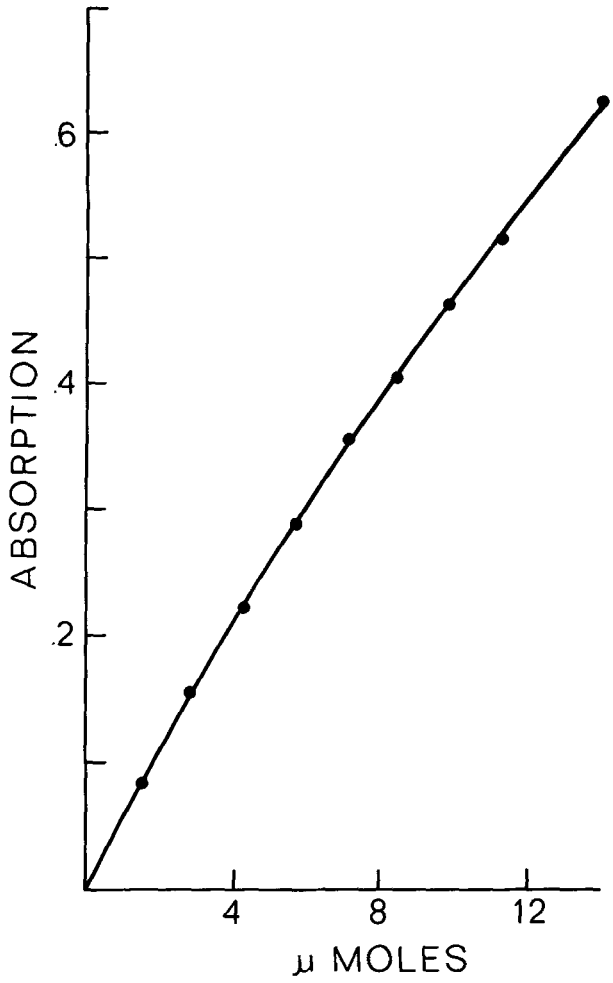

FIG. 4. Absorption curve for oleic acid standard at 715 nm, with the pyridine-cupric acetate reagent described herein.

grains (2) and then for FFA in vegetable oils (3). The work reported herein is an expansion and modification of this procedure and that described by Bains and co-workers (4). It retains the advantages of a single stable reagent and of analysis of the upper phase while adding 10-fold sensitivity, stability to the complexes formed, and uniform response with chain length for saturated FFAs.

TABLE I

Sensitivity of Procedure to Amount of Reagent Used[a]

Reagent (ml)	Absorption of blank	Absorption of standard
1.0	.117	.443
3.0	.090	.376
5.0	.069	.333

[a]All samples read against pure benzene; n=3=number of replicates.

MATERIALS AND METHODS

Apparatus and Reagents

Spectrophotometric measurements were made on a Cary Model 11 or Coleman Model 44 at 715 mμ. Samples were shaken on a Burton Model 1450 shaker equipped with a holder modified to accept 16 x 125 mm screw cap culture tubes. Tubes were scrupulously cleaned.

Centrifugation was carried out using an International Clinical centrifuge with a 221 head operated at full speed (1,470 x g at the tip). The optimum running time for separation was 5 min.

A 5% (w/v) aqueous solution of reagent grade cupric acetate was made and filtered, then the pH was adjusted to 6.0-6.2 using pyridine. The reagent was stable for more than 2 years. All solvents used were either reagent grade or redistilled before use in an all glass apparatus. Oleic acid of >99% purity obtained from Nu-Chek-Prep (Elysian, MN), was used as the FFA standard. Other pure fatty acids and triglycerides were obtained from the same source.

Procedure

Place a portion of the sample containing 2.0-14.0 μmol of FFA in a screw cap culture tube and remove any solvent present at 50 C using a nitrogen jet. Accurately add 5.0 ml of benzene and swirl to dissolve the sample. Slight warming may be necessary to effect solution. Then add 1.0 ml of cupric acetate-pyridine reagent and shake the biphasic system for 2 min. After centrifugation for 5 min, the upper layer is read at 715 nm.

EXPERIMENTAL PROCEDURES

The first parameter examined was pH. The ACS standards listed for reagent grade cupric acetate permit a pH range from 5.0 to 6.0 for a 5% solution. It was found that different solutions made up from the same bottle of reagent gave pH values ranging from 5.3 to 5.5. Such slight changes

TABLE II

Analysis of Some Oils to Ascertain Interference Levels and Linearity

Pure triglyceride[a]			Wheat extract[b]		Corn[c] oil		Cottonseed[c] oil		Pecan oil[d]		Spiked pecan oil		
TG (mg)	FFA (μmol)	Abs. n=2[e]	Oil (mg)	Abs. n=3	Oil (mg)	Abs.	Oil (mg)	Abs. n=3	Oil (mg)	Abs. n=3	Oil (mg)	FFA (μmol)	Abs. n=3
0.00	7.32	.360	12.0	.184	50.0	.010	10.0	.186	19.1	.131	19.3	0.00	.126
1.01	7.32	.361	24.0	.327	100.0	.015	15.0	.275	28.7	.189	19.3	1.42	.198
2.02	7.32	.358	36.0	.459	150.0	.020	25.0	.440	38.2	.239	19.3	2.83	.269
3.03	7.32	.353	48.0	.591	200.0	.026	35.0	.595	57.3	.341	19.3	4.25	.334
4.04	7.32	.352											
5.05	0.00	.006											
Intercepts[f]		—		.050		.005		.025		.024			—

[a]99.9% pure tripalmitin. TG = triglyceride, FFA = free fatty acid.

[b]Ground wheat (63.3 g) extracted with benzene in a Soxhlet for 16 hr; made up to 100 ml (12.0 mg lipid/ml).

[c]Commercially available food grades.

[d]Pecan meats homogenized and extracted with chloroform:methanol; oil dissolved in benzene and filtered.

[e]Abs. = absorption; n = number of replicates.

[f]Intercepts were obtained by plots of the above values and are a measure of the interference from the oil.

in pH will cause the results to be inconsistent, since values observed at pH 5.5 were 10% higher than those at pH 5.3. Also confirmed at this time was the observation of Bains et al. (4) that the maximum absorption occurs in the region of 670-690 nm for Baker's method.

Three bases were examined that permitted alteration of pH of cupric acetate solutions over the range shown in Figure 1, each utilizing acetic acid for the acid source in the system as needed. Curve A represents the range obtained using sodium hydroxide, the upper pH limit reflecting the formation of insoluble cupric hydroxides. Ammonium hydroxide prevented the formation of cupric salts over the entire range. Pyridine not only permitted the formation of cupric soaps over a wider range but also displayed a marked increase in absorbancy. A constant level of oleic acid was used throughout, and the absorption values reflect differences introduced by the bases used.

The effect of chain length on solubility of the copper soaps in benzene is particularly important, as reflected by the absorbancy of saturated solutions (Fig. 2). In going from lauric to arachidic, the solubility decreases over 100-fold. The values for the solubilities of the FFAs in benzene as reported in Markley (5) are given for comparison. The solubility of the copper soaps are greater in ethyl acetate as shown, but this solvent has several unfavorable properties which will be discussed later.

Increased unsaturation increases the solubility of both fatty acids and cupric soaps in benzene; oleate soaps are 100 times more soluble than stearates. This property and the structure portrayed in Figure 3 account for the "intersolubility of different soaps in benzene" referred to by Bains (4). The basic complex contains four fatty acids; therefore, any mixture of FFAs containing saturated and unsaturated acids will form complexes containing both, with a resultant "intersolubility."

This structure is capable of forming a larger complex involving 40 or more fatty acid molecules to become micellar, which subsequently can aggregate and become colloidal and particulate. A second advantage in using pyridine appears here as the pyridine displaces the water molecules shown (6), thus increasing the solubility of the complex in benzene tremendously and decreasing the transition to the micellar form. Experiments have shown that samples containing the pyridine are stable over at least a 3-day period. With the increased solubility induced by pyridine, pure lauric, palmitic, or arachidic soaps gave essentially equal absorption values on a mol basis; i.e., they would plot as a horizontal line in Figure 2.

The procedure is somewhat sensitive to the amounts of reagent used, as shown in Table I. The amount of standard and volume of benzene (5.0 ml) were kept constant. Since the absorption change of the FFA sample was proportionately greater than that of the blank, the overall sensitivity of the procedure is increased by using smaller volumes of the reagent.

Several other solvents were examined as potential replacements for benzene. Hexane, isobutyl alcohol, and 2-chloro-propane proved to be poor solvents for the copper soaps formed from cupric acetate solutions. Ethyl acetate, while a good solvent for the soaps, is a weak solvent for cupric acetate. Chloroform, although a good solvent for copper soaps, is cumbersome to use in a biphasic system (1,7,8). Benzene permits the direct aspiration of the sample into a flow cell of a spectrophotometer, permitting the rapid reading of samples.

The final conditions for analysis, as given under Materials and Methods, were used to obtain the absorption curve given in Figure 4, oleic acid being used as the FFA source. Up to an absorption of 0.350, the curve is sufficiently linear to permit assuming this for calculating values from the slope. The maximum error induced this way is $< 5\%$. Pure triglycerides appear to have no effect on the determination of FFA at the levels shown in the first column in Table II. Additionally, the FFA levels of four oils were determined at various levels and their intercepts obtained through plotting. The values suggest that a minimum of interference can be expected from nontriglyceride materials in food grade oils.

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