Automated Free Fatty Acid Determination Using Flow Injection Analysis Solvent Extractions

John S. Canham and Gilbert E. Pacey*
Department of Chemistry, Miami University, Oxford, Ohio 45056

A simplified, automated, colorimetric method is described for the determination of free fatty acids (FFA) in solution using solvent extraction flow injection analysis (SEFIA). A membrane separator utilizing a microporous Teflon membrane is used for a clean phase separation. A newly designed segmentor is used for segmenting the reagent and solvent streams. A linear relationship exists between normality and absorbance from 6×10^{-5} to greater than 3×10^{-2} N for oleic acid and from 4.4×10^{-5} to 6×10^{-3} N for stearic acid. The correlation between concentration and absorbance in this method is better than in previously advanced copper soap colorimetric methods. The sample throughput is 130 injections/hr.

Copper soap colorimetry has been used for the determination of free fatty acids (FFA) in various sample matrices since Ayers developed the method in 1956 (1). Many variations have been developed which use different copper(II) complexes to increase the molar absorptivity of the extracted copper-fatty acid species (2-4). The most commonly used complex is tetrapyridine copper(II), which is more lipophilic than aqueous copper(II) ion and exhibits a higher molar absorptivity. These characteristics make it a good choice for the copper soap colorimetric methods.

The tetrapyridine copper(II) soap method is usually performed as a batch process in which the FFA sample is mixed with an organic solvent and then with an aqueous copper reagent. After mixing, the aqueous and organic phases are separated and the absorbance of the organic phase is measured at about 716 nm. The absorbance of 716 nm is almost entirely due to the extracted copper soap. The copper soap batch method is stated as being faster and more practical than other methods commonly used for determination of FFA concentration (4). Factors limiting the rate of sample analysis in the batch methods are mixing, separation, sample transfer and cuvette cleaning. These steps affect both the speed and the precision of the method because human factors often increase the uncertainty in the measurements.

In order to decrease analysis times and increase the precision and accuracy of the copper soap spectrophotometric analysis method, mixing, separation, sample transfer and cuvette cleaning are rapidly and reproducibly accomplished using solvent extraction flow injection analysis (SEFIA). A SEFIA method developed by Ekstrom in 1981 (5) is more complex than necessary for FFA determination in most matrices. The present method was developed in order to incorporate some of the recent refinements in SEFIA including membrane separator and segmentor that produce smaller segments. The availability of commercial SEFIA manifolds such as those made by Tecator AB makes this technique extremely practical.

*To whom correspondence should be addressed.

EXPERIMENTAL

Materials. The chemicals used were oleic acid (U.S.P. by Matheson Coleman and Bell), copper(II) acetate (ACS certified by Fischer, Cincinnati, Ohio), pyridine (spectrometric grade by Matheson Coleman and Bell), stearic acid (Eastman), deionized glass distilled water, and Kroger brand vegetable oil. Teflon membranes of 0.45 micron pore size (W.L. Gore and Associates) with and without polypropylene scrim backing were used for the separation. The copper reagent was prepared according to Lowry and Tinsley, adjusted to pH 6.1 with pyridine (6). The FFA standards were titrated and the equivalent weights determined in order to correct for any impurities of these materials. Standard solutions of oleic and stearic acids were prepared in toluene volumetrically using serial dilutions. Vegetable oil solutions were prepared volumetrically.

Equipment. A Tecator 5020 flow injection analyzer was used for all of the determinations. Connections between system components were made with 0.5 mm i.d. teflon tubing. Polyvinyl chloride (pvc) peristaltic pump tubes were used for delivering solutions. A displacement bottle was used to deliver the toluene carrier stream because the pvc tubes are not resistant to toluene. Samples were aspirated through the injection valve using pvc pump tubing which was discarded when flow rates decreased due to tubing degradation. Detection was achieved using a Tecator 5023 spectrophotometer operated at 699 nm with a bandwidth of 19 nm. Both the segmentors and membrane separator were fabricated in our laboratory. A Beckman DU spectrophotometer was used for the linearity study at 716 nm and a bandwidth of 5 nm.

The SEFIA manifold system was set up as shown in Figure 1. Standard response curves of FFA were determined using 100 µl sample injection volumes. One of the segmentors used was of a new design which will be discussed later. The extraction coil was made from 0.5 m of 0.5 mm i.d. teflon tubing; the tubing was coiled into 3-cm diameter coils. The membrane separator had

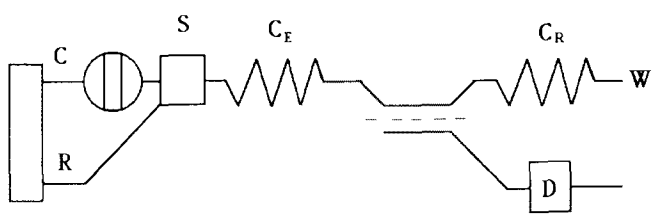

FIG. 1. The toluene carrier (C) and reagent (R) are pumped at 1.4 ml/min. The sample (S) is injected into the carrier stream, which is then segmented with the reagent stream. The segmented stream enters the 0.5-m extraction coil (C_E) where extraction occurs. The segmented stream then enters the membrane separator where the phases are separated. The aqueous phase enters the restriction coil (C_R), and the toluene phase enters the detector (D). The effluents from the detector and restriction coil are then collected in separate waste (W) containers.

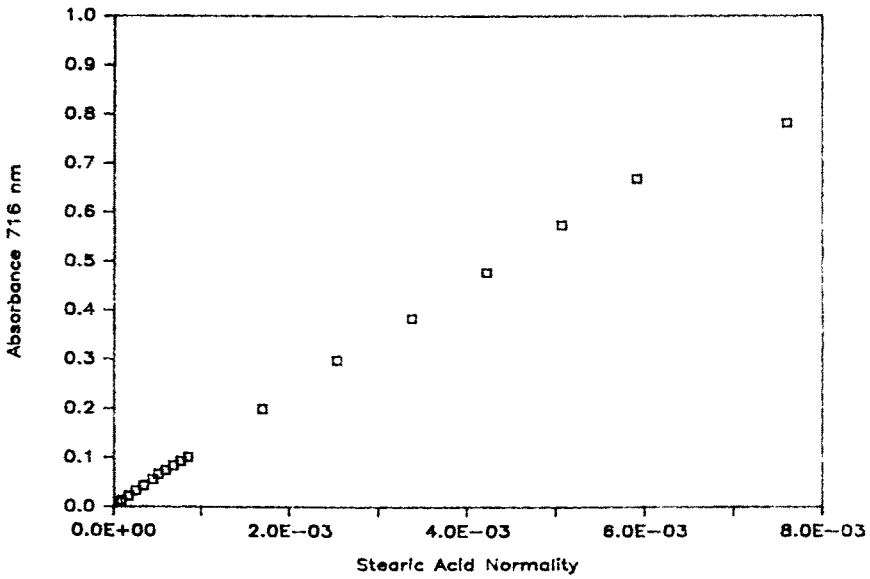

FIG. 2. Stearic acid concentration versus absorbance peak height at 716 nm.

channel lengths of 3.5 cm and widths of 0.2 cm. A minimal length of tubing was used to connect the output of the membrane separator to the detector flow cell, to minimize dispersion of the sample.

A minimum of five injections were made for each data point. The mean and standard deviations were calculated, and these values were used for all subsequent data manipulations. The membranes were changed in the separator daily because the membrane eventually became permeable to the aqueous reagent. This presumably is due to pyridine associating with the membrane, which decreased the barrier for transportation of the aqueous solution through the membrane. After changing the membrane, standards were run to obtain new standard response curves.

RESULTS

This SEFIA system has a sample throughput of 130 samples/hr, allowing the analyst to perform four analyses with blank measurements in two min. By comparison, the batch method might allow 30 analyses/hr without a blank determination between each sample.

With the Tecator 5023 detector set at 699 nm, the linear detection range was 6×10^{-5} to 6×10^{-3} N oleic acid. The precision is about 1%, except where limited by the baseline noise of 0.004 AU. Analyses of a series of 18 standards of stearic acid were used to determine the linear range of the method. The standards ranged from 4.42×10^{-5} to 7.59×10^{-3} N. The resultant concentration absorbance curve showed a point at the highest concentration that was greater than three standard deviations from the regression line (Fig. 2). Elimination of this point from the data significantly increased the linearity of the plot. The regression line calculated from the remaining 17 points is $A = 116.3N + 0.013$. The standard deviation in Y is 2.7×10^{-3} AU, in the slope is 0.35 AU/N, and in the intercept is 8.7

TABLE 1

Copper Soap Colorimetric Methods, Detection Ranges and Analysis Times

Reference number	Color reagent	Detection range (N)	Analysis time
1	–	$8 \times 10^{-3} - 4 \times 10^{-2}$	>5 min
2	Diethyldithiocarbamate	$2 \times 10^{-5} - 1 \times 10^{-3}$	>5 min
4	–	$4 \times 10^{-4} - 1 \times 10^{-2}$	>2 min
5	EDTA	$3.5 \times 10^{-4} - 2 \times 10^{-2}$	3-5 min[a]
6	–	$4 \times 10^{-4} - 2.8 \times 10^{-3}$	>7 min
10	Diphenylcarbazide	4.6×10^{-4}	>30 min[b]
11	–	$3.5 \times 10^{-3} - 2.8 \times 10^{-2}$	>45 min[c]
12	1.5 Diphenylcarbazide	$5 \times 10^{-4} - 2.5 \times 10^{-3}$	1.5 min
13	1.5 Diphenylcarbazide	$2 \times 10^{-5} - 4 \times 10^{-4}$	1.5 min
This work	–	$4 \times 10^{-5} - 6 \times 10^{-3}$	<0.5 min

[a]Re-extracts copper into an aqueous phase.

[b]Nonlinear.

[c]Not applicable for high 18:0 concentrations.

$\times 10^{-4}$ AU. All of the points correlate within two standard deviations of the line.

The reproducibility of this method is as good as or better than that reported for the batch method. At 699 nm the data points all agreed with the regression line within experimental uncertainty. The regression line for normality absorbance at 699 nm is A = 158N − 0.003, where A is the absorbance and N is the normality of the acid, and the standard deviation is 0.3 in the slope and 0.00077 in the intercept. Deviations from the line were most pronounced at values near 1.0 AU and 0.000 AU, as would be expected due to the inherent lack of precision at extreme absorbance values. However, absorbance values in these ranges are nearly impossible to measure precisely in batch methods due to cell alignment, sample carryover and other variations. Because the flow through cell is not moved between samples, and all of its other characteristics remain the same, higher precision readings may be made at absorbance extremes than with batch methods assuming the same detector limitations. Blank measurements are made automatically in FIA and SEFIA, before the sample arrives at the detector.

When series of dilutions of vegetable oil from 2% to 80% were examined, samples containing more than 40% showed that a significant portion of the sample did not pass through the membrane. Below this concentration, the response was linear. In oils, response is not entirely due to FFA; there is an interference that is probably due to phospholipids. These compounds can be removed (9) or, by performing a standard addition curve using oleic acid as the standard, one can determine the sensitivity of a linear sample response curve. We first determined the concentration of the FFA in the oil used for the standard addition titrimetrically and then calculated the response curve using this known concentration. We then used this sample response for determination of similar samples. The 20% vegetable oil sample resulted in a linear sample response curve where A_{716} = 72.53 N + .0847 AU, the standard deviation in Y is 3.141 $\times 10^{-3}$ AU, in the slope .56 AU/N, and in the intercept 0.0025 AU.

DISCUSSION

The detection limit for this method is lower than that of all of the other copper soap colorimetric methods which do not use an added color reagent for copper. The method is only about a factor of 2-4 less sensitive than the methods which add a color reagent (Table 1).

SEFIA was chosen for this method because several advantages are realized over batch and other automated methods (7). SEFIA uses a minimum of reagents, which lowers the cost per analysis. The system is closed, so reagent and solvent fumes are held to a minimum. The rate at which determinations are made is high, as is the precision of the measurements. This is due to the basic FIA characteristics of sample injection, reproducible timing and controlled dispersion which allow for nonsteady state observations.

Membrane separators as described by Karlberg and Thelander (8) allow rapid, clean separation of liquid phases and operate with little or no dependence upon solution density. These separators use a microporous membrane, generally teflon, to separate the phases. To ensure nearly complete transport of the organic phase across the membrane, a slight pressure differential is required. A restriction valve is often used to achieve this, but such valves are not easy to control. In this SEFIA method a restriction coil of 0.5 mm teflon tubing was used, and reproducible backpressure was obtained. This coil induced sufficient backpressure to achieve greater than 99% transfer of the toluene across the membrane.

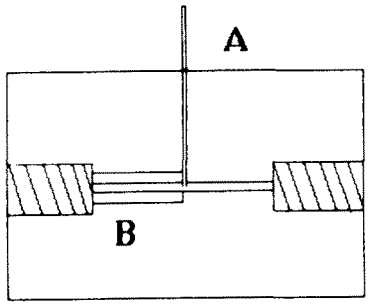

FIG. 3. A classical affinity segmentor, the organic phase enters through the small metal tube (A). The aqueous phase enters through the glass tube (B). The flow of the segmented stream continues out in the direction of the original aqueous stream flow.

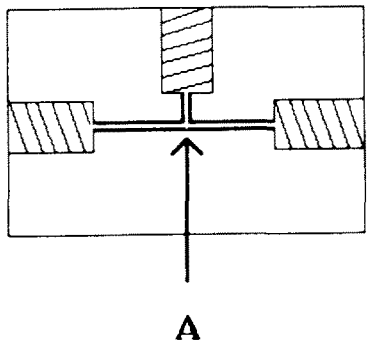

FIG. 4. The all Teflon segmentor used for this method's development. The organic and aqueous phases enter the segmentor from opposite directions, merging at point A. The small volume of this mixing point permits only small segments to form. By maintaining the position of the segmentor and the flow rates, a reproducible segmentation pattern can be achieved.

Classically, segmentors of the type used in the Technicon AutoAnalyzer that operate on an affinity principle have been used in SEFIA systems (Fig. 3). The segmentor which was used in this SEFIA system is only slightly dependent upon the affinity of the solvent for the segmentor material. This specially constructed all teflon segmentor which was made in our laboratory produces the segments due to turbulence. Segment size is limited by the small volume of the mixing area (Fig. 4). A 2-mm "T" shaped channel was bored into a block of teflon, and short lengths of 0.5-mm i.d. PTFE tubes were flared on one end and inserted into the ends of the "T" in order to decrease the internal volume. Although the segment size was not as uniform as in the affinity systems, due to the decrease in the segment size, extraction was more efficient and the degree of extraction more uniform through the individual seg-

ments. The increase in the uniformity of reaction and extraction should lead to better reproducibility.

This is a generalized method and may be modified for a specific type of FFA analysis. By adding a sample dilution step, one could readily monitor the FFA content of oils during processing, either by collecting discrete samples or by sampling directly from the processing equipment. The rapidity of this method would allow an almost continuous monitoring. The method also could be used in monitoring the concentration of FFA in waste water from acidulation of soapstock and other processors of fats and oil, such as oil refineries. The instrumentation used in this method is capable of automatic injection, which would allow the instrument to monitor FFA concentrations over long periods of time without the operator being present. The method is also quite applicable to the determination of FFA in laboratory samples such as lipase activity studies.

REFERENCES

1. Ayers, C.W., *Anal. Chim. Acta 15*:77 (1956).
2. Duncombe, W.G., *Biochem. J. 88*:7 (1963).
3. Iwayama, Y., *J. Pharm. Soc. Japan 79*:552 (1959).
4. Kwon, D.Y., and J.S. Rhee, *J. Am. Oil Chem. Soc. 63*:89 (1986).
5. Ekstrom, L.G., *Ibid. 58*:935 (1981).
6. Lowry, R.R., and I.J. Tinsley, *Ibid. 53*:470 (1976).
7. Nord, L., and B. Karlberg, *Anal. Chim. Acta 164*:233 (1984).
8. Karlberg, B., and S. Thelander, *Ibid. 98*:1 (1978).
9. Rouser, G., G. Kritchevsky, G. Simon and G. Nelson, *Lipids 2*:37 (1967).
10. Jensen, R.G., *Lipids 18*:650 (1983).
11. Hron, W.T., and L.A. Menahan, *J. Lipid Res. 22:* 377 (1981).
12. Bains, G.S., S.V. Rao and D.S. Bhatia, *J. Am. Oil Chem. Soc. 41*:831 (1964).
13. Bowyer, D.E., J.S. Cridland and J.P. King, *J. Lipid Res. 19*:274 (1978).
14. Radding, William, and Gerda G. Mayer, *Ibid. 24*:100 (1983).

[Received August 4, 1986]