An Automatized Method for Determination of Free Fatty Acids

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ABSTRACT

An automated colorimetric method is described for determining free fatty acids (FFA) in vegetable oils using the flow injection analysis (FIA) technique. In this procedure, an almost linear relationship exists between the peak height and the FFA concentration. Liquid samples can be poured directly into the sample cups on the sampler for an automatic analysis of the FFA content. The dynamic range of this method is from 0.01 to almost 5%. Samples with higher FFA content must be diluted before analysis. The sample capacity is 12-20 injections/hr. No evidence of the existence of the earlier proposed cage-like complex $(Cu(II)(FFA)_2)_2$ in the organic phase was observed in this study.

INTRODUCTION

Determinations of the amount free fatty acids (FFA) are quite common in lipid chemistry. If a large number of determinations of FFA are to be done, it may be desirable to automate these determinations, although the AOAC official manual method is fairly quick. An attempt in this direction is described, in which the startling point is the manual procedure for FFA determinations by Lowry and Tinsley (1) and Bains et al. (2). These workers have used a colorimetric method in which the sample first is dissolved in benzene and copper(II) ions, from a copper(II) acetate solution, is then extracted to the organic phase as a copper(II) acetate solution, is then extracted to the organic phase as a copper(II) FFA complex. The complex formed is measured colorimetrically.

If circumstances are favorable, it may be possible to automate a manual method directly without any changes in the working procedure. In the present case, however, this was not directly applicable, since both the sample and the formed copper(II) FFA complex are in the same phase. For absorbance measurements when flow cells are used, the measured absorbance is indeed very sensitive for sudden variations in the density, because light scattering phenomena are created at surface boundaries and the oil in the sample volume creates this variation in density. Therefore, we extended the method of refs. 1 and 2 such that the copper(II) ions, from the formed copper(II) FFA complex in the organic phase, are extracted to an aqueous phase containing a strong chelating agent. Thereby, the difficulties are avoided and an automation is possible.

The main advantages of the method are the high analytical capacity and the relative simplicity in handling; it can be easily implanted as a routine method. However, the method is addressed to those who are doing a large number of FFA determinations.

EXPERIMENTAL

Method

If the sample is a liquid at room temperature, it can be transferred directly to sample cups in polyethene on a sampler, but if the sample is a solid it must first be dissolved in toluene or benzene. Liquid samples, which are not dissolved in either of these solvents, require a somewhat different measuring system.

The sample is automatically injected in a carrier stream of toluene. From a copper(II) acetate $(Cu(II)(OAc)_2)$ solution, copper(II) ions are extracted to the organic phase, probably by formation of the Cu(II)(FFA)(OAc) complex. From the organic phase, Cu(II) ions are then extracted back to another aqueous phase containing the strong chelating agent ethylendiaminotetraacetic acid, EDTA (H_4Y), and the formed copper(II) EDTA Complex (CuY) is measured spectrophotometrically. The extraction scheme is shown in Figure 1.

Apparatus

The basic units used in the Bifoc, Upplands-Väsby, Sweden, FIA system are: sampler for 40-100 samples, FIA 07; peristaltic pump with 8 channels and continuous speed control, FIA 08; basic unit, FIA 05; injection valve is made of Teflon and PVDF, which has one channel with variable sample volume; extraction units consist of a segmentor, an extraction coil and a separator; filterphotometer is equipped with a 18 µL flow cell; FIA 06. A general description of the FIA system is found in ref. 3. The internal diameter (id) of the transport tubes, of Teflon, is 0.5 mm. The tubes which have been used for the coils have a slightly larger id, 0.7 mm, to prevent a pressure drop. The transport tube

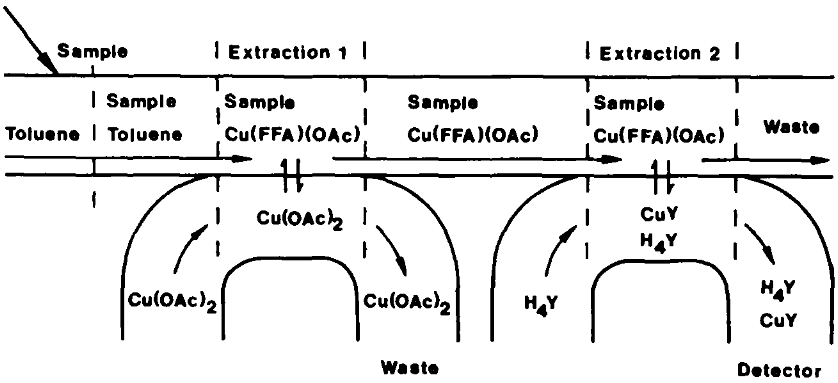

FIG. 1. Extraction procedure for the determination of FFA.

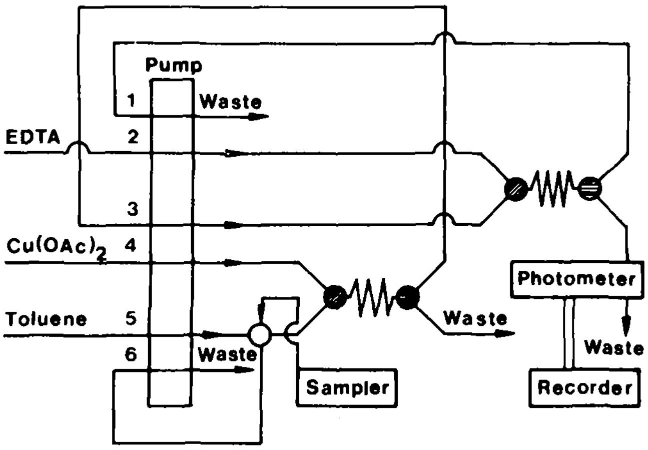

FIG. 2. Flow diagram of the FIA instrument for determination of FFA in lipid products, when the sample is dissolved in toluene. The symbol "○" refers to an injection valve, the symbol "⊘" to a segmentor and "⊖" to a separator. Pumping tubes are according to the Technicon coding systems: 1. Acidflex, red/red; 2. silicone, orange/orange; 3. Acidflex, white/white; 4. silicone, red/red; 5. Acidflex, red/red; 6. Acidflex, red/red.

which leads from the separator unit to the waste is lengthened with a constriction tube with an id of 0.3 mm. The reason for having this restrictor is to prevent degasing and siphon effects in the separator. At the tube joints and the tube entrances, "Kem Inert" fittings of polypropylene are used. The flow diagram of the FIA instrument for determination of FFA in lipid products when the sample is dissolved in toluene is shown in Figure 2. The corresponding flow diagram for liquid samples added directly is shown in Figure 3.

Manual spectrophotometric measurements were done on a Shimadzu spectrophotometer UV-110-02, single beam, and the spectrum was taken with a Varian UV-visible spectrophotometer series 653, double-beam. Centrifugations were done on a Heraeus Labofuge 1 with swinging-out head, 8 × 15 mL.

Reagents

We used 0.05 M aqueous solution of EDTA disodium salt (Merck, Titriplex III. pa); 5% (w/v) aqueous solution of copper(II) acetate (Merck, pa) in which the pH was adjusted to 6-6.2 with pyridine (Merck, pa); and oleic acid (Kebo, purum), minimum 98% purity.

PROCEDURES

To optimize the method, the id of the pumping tubes were first varied, and the sample volume was then adjusted to the existing conditions (10-150 µL).

A coil length of 1.8 m was found to give an acceptable extraction yield. Lengthening of the tubes in the coils to 2.3 m increased the relative absorbance by only 4%. But, longer coil length gave rise to an increased analysis time and an increased back-pressure in the tube. This back pressure increased the risk of leakage in tube fittings. Therefore, all measurements were performed with a coil length of 1.8 m.

By increasing the flow rate (pumping rate) in the system, the extraction yield is increased, although the connection time between the phases is decreased. This is shown in Figure 4 in which the relative absorbance is plotted as a function of the pumping rate, where the sample concentration is held constant (2.0 mg FFA/mL). The graph shows that there is an almost linear relationship between absorbance and pumping rate.

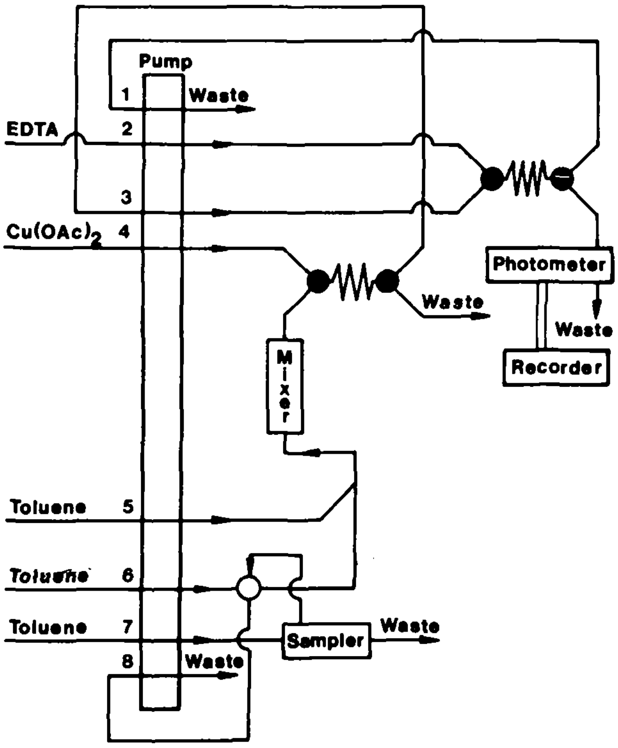

FIG. 3. Flow diagram of the FIA instrument for determination of FFA when the sample is a liquid. The sample is directly injected into the instrument without any pretreatment. The symbols "○", "⊘" and "⊖" and the pumping tubes numbered 1-4 are the same as described in Fig. 2. The mixing chamber is Spectra-Physica HPLC equipment. The mixer has a dead volume of 0.9 mL. The speed of the magnetic stirrer, in the chamber, can be varied in a broad range without affecting the result. The mixer is connected to the transport tubes via Swagelok tube fittings. Pumping tubes are according to the Technican coding system: 5. Acidflex, black/black; 6. Acidflex, black/black; 7. Acidflex, red/red; 8. Acidflex, red/red.

A too-high flow rate in the system decreases reliability in the separation unit. For this reason, a pumping rate that is 70% of the maximal pumping rate has been chosen as a compromise. The maximal pumping rate (100%) is a flow rate of 4 mL/min with a red/red Tygon pumping tube (Technicon) for the pump used.

RESULTS

An almost linear relationship is found between the peak height and the FFA concentration. The dynamic range for the method when the equipment shown in Figure 2 is used is 0.1 to 6.0 mg FFA/mL, with a 150-µL sample loop.

The dynamic range can be extended to higher concentrations by decreasing the sample loop. An increase of the range to 50 mg FFA/mL is obtained if the equipment shown in Figure 3 is used. The absorption curve for oleic acid standards with this method deviated less than 3% from linearity.

The absorption maximum for Cu(II)EDTA is at 735 nm, but as the filter photometer used in the automatized system lacks a suitable filter, those measurements were done at 660 nm. If the measurements had been done at 735 nm, the peak heights would have increased by about 50%.

The loss of solution at the extraction steps, and thus reduced yield, depends on the relative size of the id of the pumping tubes for the transport of solution back and forth to the extraction units. Pumping tubes with id that are too similar have very uncertain separation. Since our method contains 2 extraction steps, the method will be sensitive to changes in the id of the pumping tubes. The pumping tubes used for the extraction units are Acidflex (Technicon), which, unfortunately, are very sensitive to mechanical deformation. For this reason, every 20th injection should be a standard.

The time between consecutive injection must be 3 min for the equipment according to Figure 2 and 5 min for the equipment according to Figure 3, to ensure that an overlapping of peaks is avoided. Thus, the maximal capacity of these systems is 20 and 12 injections/hr, respectively.

For this automatic method, using repeated injection, the relative coefficient of variation is found to be about 2%.

For some samples, standard solutions of FFA were added in such a large amount that the signal was almost doubled. From this standard addition procedure, it appeared that the yield of FFA was almost 100%.

To test for carry-over effect in the system, standards of high concentration of FFA (6 mg/mL) and respectively low concentration (0.2 mg/mL), with each standard injected twice, were alternatively injected. No carry-over effect was, however, observed.

If benzene is used as organic solvent instead of toluene, a better yield in the extraction step is obtained. Thus, the peak height is increased by about 20% if benzene is used. For health reasons, however, toluene is preferred. The vapor pressure for benzene is 3 times higher than that for toluene at 20 C and, furthermore, the safety limit for hygienic exposure is 15 times higher for toluene compared to benzene, according to Swedish regulations.

In order to make a comparison between the present method, with the equipment according to Figure 2, and the manual method of Lowry and Tinsley (1), some measurements were made of FFA in frying fats with different degrees of aging. These fats came from local street kitchens (fast-food restaurants). The results in Table I showed good agreement between the manual benzene method with the present FIA method using either benzene or toluene as solvent. From the investigation of these samples, it also became evident that the upper limit of the amount of sample per mL solvent is about 0.7 g; otherwise, the separator will not function.

The present method, with the equipment according to Figure 3 in which the sample is added directly in liquid form without any dilution with benzene or toluene, was also compared with the manual method (1). Some liquid vegetable oils from local shops were tested. The results are shown in Table II. Again, good agreement was found between the 2 methods.

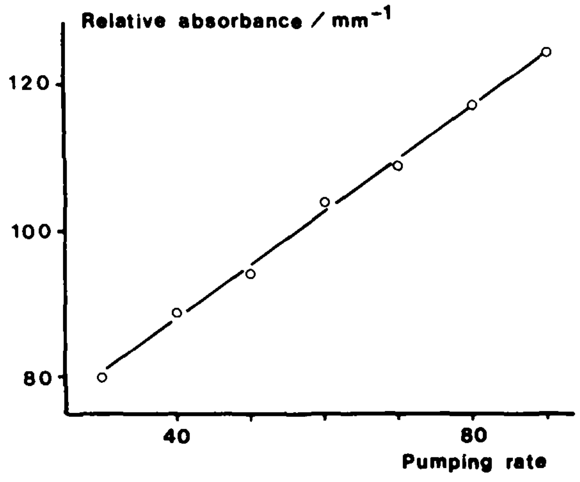

FIG. 4. Relative absorbance (peak height) as a function of pumping rate, for a sample concentration of 2 mg/mL. The figures on the X-axis refer to percentage of maximal pumping rate.

TABLE I

Percentage (by wt) FFA in 4 Samples of Frying Fats from Local Street Kitchens

Sample no.	Manual method: benzene	FIA Benzene	FIA Toluene
1	1.01 (3)[a]	1.01 (2)	1.02 (2)
2	2.23 (3)	2.30 (2)	2.28 (2)
3	0.25 (3)	0.25 (1)	0.25 (2)
4	0.72 (3)	0.66 (1)	0.74 (3)
5	1.35 (4)	1.23 (1)	1.35 (4)

[a] The presented values are the means; the figures in parentheses give the number of measurements.

TABLE II

Percentage (by wt) FFA in 5 Samples of Liquid Vegetable Oils from Local Shops

Sample	%(w) FFA FIA	%(w) FFA Manual method
Sunflower oil	0.71[a]	0.72
Olive oil (I)	0.33	0.33
Olive oil (II)	0.38	0.41
Wheat germ oil	3.94	4.14
Sesame oil	0.03	0.00

[a] The presented values are means of 2 measurements.

The cage-like complex $(Cu(II)(FFA)_2)_2$ in the organic phase (benzene) proposed by Lowry and Tinsley (1) using manual preinvestigations to determine FFA by means of double extraction did not agree with our findings. This was the background for the following measurements.

The absorbance of some copper standard solutions was measured as a function of the concentration of Cu(II) $(0<Cu(II)<3 \cdot 10^{-3}$ M) at a constant concentration of EDTA (=0.05 M, disodium salt) in an aqueous solution at 735 nm. As a reference solution, 0.05 M EDTA was used.

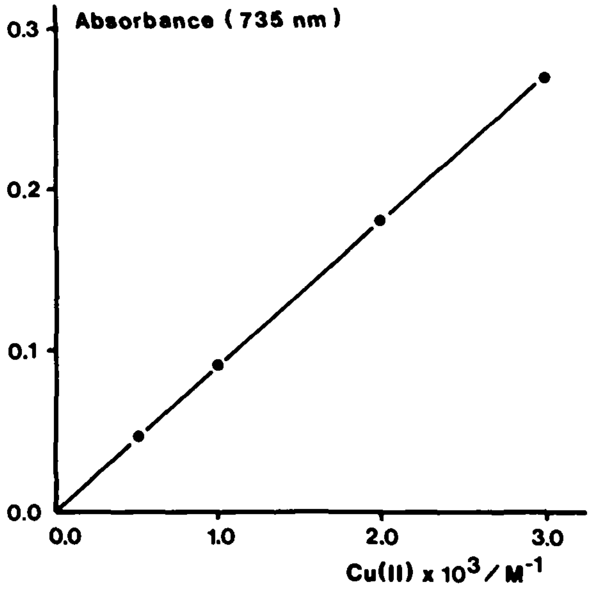

FIG. 5. Absorbance of Cu(II)EDTA as a function of Cu(II) at 735 nm. The total concentration of EDTA (=0.05 M) is constant.

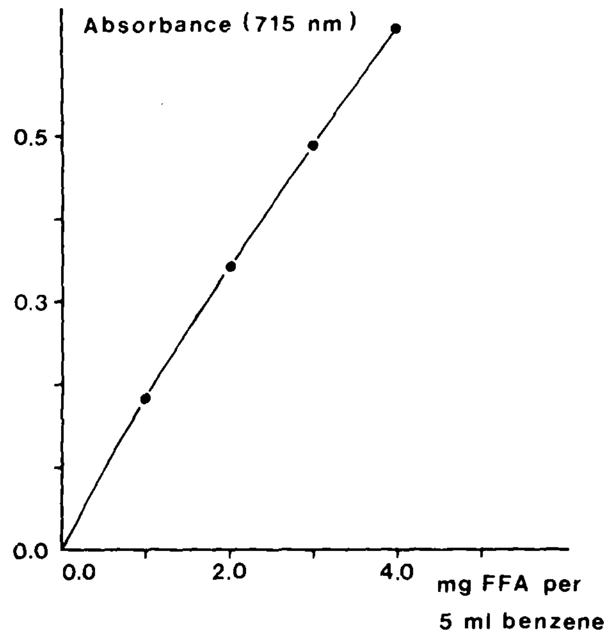

FIG. 6. The absorbance of the copper(II) FFA complex in the benzene phase as a function of FFA concentration.

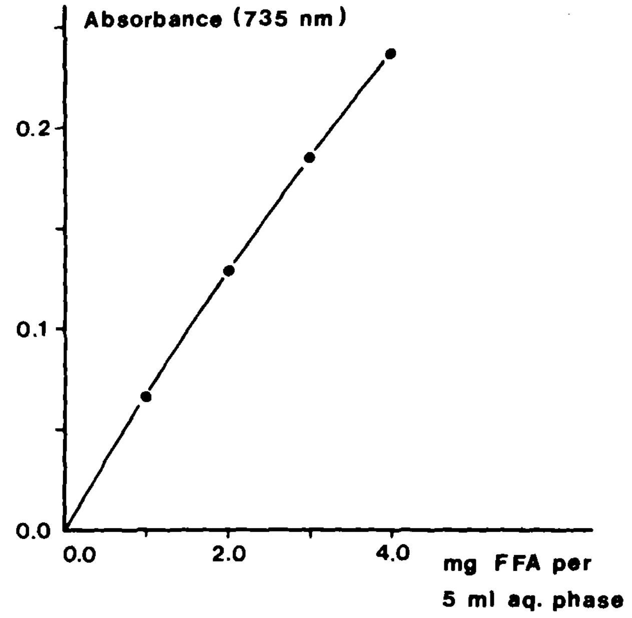

FIG. 7. The absorbance of the Cu(II)EDTA complex after extraction with the benzene phase, as a function of the FFA concentration.

TABLE III

Mole Ratio between Cu(II) and FFA Obtained from Absorbance Measurements

mg FFA/5 mL	Absorbance of of Cu(II)EDTA in the aq. phase	Conc. Cu(II)/M^{-1} obtained from the graph in Fig. 5	Mole ratio Cu(II)/FFA
0.98 (0.69 10^{-3})[a]	0.066	0.72 10^{-3}	1.04
1.96 (1.39 10^{-3})	0.128	1.42 10^{-3}	1.02
2.94 (2.08 10^{-3})	0.185	2.05 10^{-3}	0.98
3.92 (2.78 10^{-3})	0.236	2.62 10^{-3}	0.94

[a] The figures in parentheses give the concentration FFA in mol/dm^3 (M).

The results are shown in Figure 5. The absorbance of the Cu(II)EDTA solutions was not influenced if the solutions were shaken together with pure benzene.

Standard solutions of FFA containing 1-4 mg FFA/5 mL benzene were shaken with 1 mL 5% $Cu(II)(OAc)_2$ for 2 min. After centrifugation for 5 min, the absorbance of the copper(II) FFA complex was measured in the organic phases at 715 nm compared to pure benzene. These values are presented in Figure 6. Four mL of the organic phase from each concentration was transferred to new test tubes and 4 mL 0.05 M EDTA was added to each tube. The test tubes were shaken for 2 min and centrifuged 5 min. The absorbance of the Cu(II)EDTA complex in the aq. phase was measured at 735 nm. These absorbance values are given in Table III in the second column and the absorbance is plotted as a function of the FFA concentration in Figure 7. The absorbance of the organic phase measured at 715 nm after extraction with the EDTA solution was found to be equal to 0.000. This indicated that at least Cu(II) from the Copper(II) FFA complex is totally extracted from the organic to the aqueous phase. The absorbance values obtained from the measurements of the aq. phase, the Cu(II)EDTA complex, can be transformed via the graph in Figure 5 to the corresponding Cu(II) concentrations. These Cu(II) concentrations are given in the third column in Table III. In the fourth column of Table III, the mole ratios between Cu(II) and FFA are given. The mean mole ratio achieved is 1.00 (± 0.05) indicating that the Cu(II) to FFA maintains the ratio 1:1 in the organic phase. As the copper ion has a charge of +2, it is very likely that an additional anion is connected to the Cu(II) complex; one possibility is that the Cu(II)(FFA)(OAc) exists in the organic phase.

ACKNOWLEDGMENTS

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