JOURNAL OF
AGRICULTURAL AND FOOD CHEMISTRY

Modified Method for the Analysis of Free Fatty Acids in Fish

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The objective of this study was the development of a method for the quantification of free fatty acids (FFA) using less aggressive reactants against the handler and the environment than those used in the classic method of Lowry and Tinsley. The modified procedure is a variation of the Lowry and Tinsley method employing cyclohexane in place of benzene. The use of benzene is prohibited in certain work processes and laboratories, and the competent authority in each country is actively promoting research into harmless or less harmful products that could replace benzene. A comparison with the traditional AOCS titration method for oil analysis was performed. FFA content in mackerel frozen at −10 °C was measured according to the three methods over a 12 month period. The results showed similar values, and good correlations were obtained.

KEYWORDS: Benzene; cyclohexane; free fatty acid; FFA; analytical technique; carcinogenic; Lowry method; fat fish

INTRODUCTION

Hydrolysis of ester bonds in lipids by enzymatic action or heating in the presence of water liberates free fatty acids (FFA). In animal tissues, once sacrificed, FFA can be liberated by enzymatic action. Lipolysis of triacylglycerol and phospholipid fractions has been shown to occur during frozen storage of nonheated food systems. Moreover, fish enzymes may be active even at temperatures below −20 °C (*1*−*3*).

The accumulation of FFA during the processing or storage of foods influences the quality of the final product and the period of useful life. FFA content in hake and anchovies has been used to establish the grade of deterioration (*4*, *5*). Their presence mainly causes textural alterations by their association with proteins. Numerous studies on frozen fish have related the decrease in protein solubility with accumulation of FFA. FFA are believed to primarily attack the myofibrillar proteins, but the mechanism has not yet been explained (*6*, *7*). Their participation in oxidation processes remains unclear. The source of FFA is critical to whether lipid hydrolysis has an accelerating or inhibiting effect on subsequent rates of lipid oxidation. Triacylglyceride hydrolysis has been suggested to lead to increased oxidation, whereas phospholipid hydrolysis produces the opposite effect (*8*).

Accumulation of these lipids causes disagreeable flavors in foodstuffs. The flavor impairment caused by lipolysis is usually described as “rancidness” or “soapiness”. In some case, the quantification of FFA serves to establish the limits by which the food is not organoleptically acceptable. Studies on frozen fish have shown that lipid hydrolysis plays a key role in sensory deterioration (*9*, *10*).

Various methods have been proposed for the analysis of these lipids according to the primary material and the quantification procedure. Examples include colorimetric determination methods in meat (*11*, *12*), a colorimetric method that utilizes phenol red solubilized in reverse micelles in vegetables oils (*13*), the BDI method for the lactic industry (*14*), and other applications of automated titration (*15*) or thin-layer chromatography (*16*). One of the most general titration methods was proposed by the AOCS (*17*) for fats and oils, with small variations in the concentration and volumes of the reactants, according to the likely quantities of FFA and the sample available (*18*, *19*). The main inconvenience in our case is the minimum quantity of lipid extract necessary and the quantification system which, because it is by titration, implies most variability in the results.

One of the colorimetric methods used in fish is that of Lowry and Tinsley (*20*). This rapid and sensitive method has the inconvenience that it employs a benzene solvent. Contact with the skin and, mostly, inhalation cause severe disorders in the handler, which ought to be avoided. Moreover, benzene is considered to be a human carcinogen on the basis of experimental and epidemiological data. The International Agency for Research on Cancer (IARC) rated benzene as “known to be carcinogenic to humans” (group 1) (*21*).

The objective of this paper was to develop a safe and economic method for FFA analysis avoiding the use of highly toxic solvents. The validity of the proposed method was verified by comparison with the Lowry method and the titration method as described by the AOCS (*17*).

MATERIALS AND METHODS

Apparatus. Spectrophotometric measurements were made on a Perkin-Elmer LS 3B with 3.5 mL glass cell. Centrifugation employed a Hettich Universal 30 RF.

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10.1021/jf040282c CCC: $30.25 © 2005 American Chemical Society
Published on Web 02/19/2005

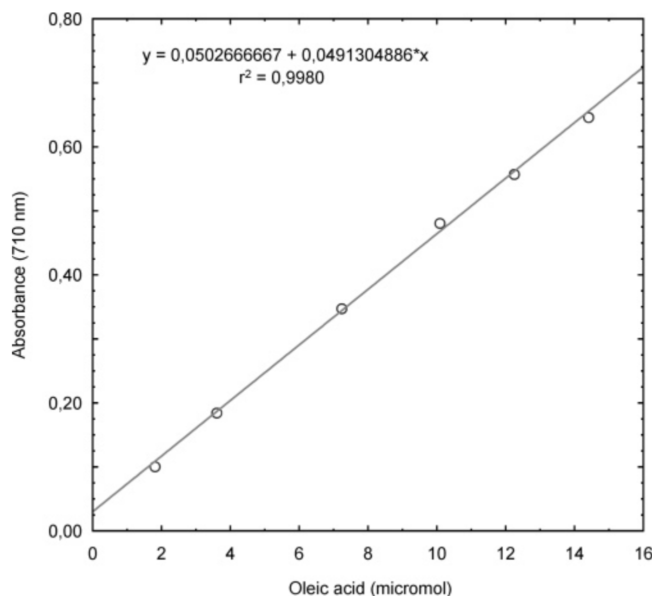

Figure 1. Standard curve of color formation for method A.

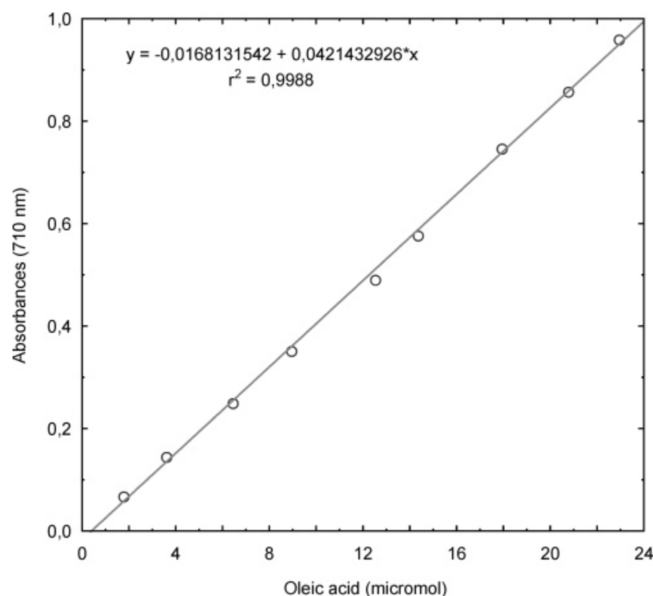

Figure 2. Standard curve of color formation for method B.

Reagents. Dichloromethane (Prolabo), methanol (Prolabo), benzene (Probus), cyclohexane (Carlo Erba Reagenti), pyridine (Merck), and copper(II) acetate 1-hydrate (Panreac) were all of analytical reagent grade. Oleic acid of 99% purity (Merck) was used as a FFA standard.

Samples. Atlantic mackerel (*Scomber scombrus*) with a lipid composition of 5.25% was used. The fish was landed on the Galician coast in March 2002 and maintained on ice until processing in our laboratory.

Processing and Storage. The fish was gutted and beheaded within the first 24 h post-mortem, and fillets were immediately removed, skinned, and minced. The batch was distributed into 50 g samples in individual LDPE bags, which were closed and frozen at −70 °C. The following day the samples were transferred to a freezer at −10 °C.

Lipid Extraction Using the Folch Method. The Folch extractions were performed using the original extraction ratio of 20 parts of 2:1 dichloromethane/methanol to 1 part of tissue. A weak salt solution (e.g, 0.66% NaCl) was then added to achieve a final ratio of 8:4:3 dichloromethane/methanol/water including the water contained within the tissue (*22*).

The dichloromethane phase was concentrated in a rotary evaporator to ∼20 mL at ambient temperature. The flask was totally covered with a black material to avoid light influence. This extract was frozen at −70 °C until determination of FFA in all samples.

The method proposed by Folch uses chloroform for the extraction. We used the less toxic dichloromethane following previous laboratory results. In interlaboratory lipid extraction studies this substitution produces a change in solvent polarity but no change in extraction potential (*23*).

Lowry and Tinsley Method (Method A). The lipid extract was deposited in 14 × 100 Pyrex tubes. All of the solvent was evaporated with nitrogen, and 5 mL of benzene was added, followed by 1.0 mL of cupric acetate−pyridine reagent with agitation of the biphasic system for 2 min. After centrifugation at 3400*g* for 20 min, the upper layer was read at 715 nm. The FFA concentration in the sample was calculated as micromolar oleic acid based on a standard curve spanning a 2−14 μmol range (**Figure 1**).

Proposed Method (Method B). The quantity of sample and the procedure follow method A with three alterations. The 5 mL of benzene was substituted by 3 mL of cyclohexane, and the contact time required for the two phases was ∼30 s. After centrifugation at 2000*g* for 10 min, the upper layer was read at 710 nm. The sample concentration of FFA was calculated as micromolar oleic acid based upon a standard curve spanning a 2−22 μmol range (**Figure 2**).

Titration (Method C). This method was adapted to the quantity and nature of the sample. The lipid extract of the fish was evaporated with nitrogen and redissolved in ethanol previously neutralized with *m*-cresol purple and preheated to 60 °C. Three replicates per sample were titrated to a violet end point. *m*-Cresol purple has been proposed to increase the sensitivity of the method and has been used for the titration of lipid fish extracts (*24*, *25*). In this case, 0.05 N NaOH was used for the titration of the extract, which contained 100 mg of lipids. FFA values are usually reported as percentage oleic acid:

$$\% \text{ oleic acid} = (\text{mL of NaOH} \times \text{NaOH normality} \times 28.2)/\text{weight of sample (g)}$$

Statistical Analysis. The performances of three different FFA determination methods were compared by statistical analysis with a StatView software program. The regression curves for the standards and the correlation between methods were determined using the Statistica 6.0 package.

RESULTS AND DISCUSSION

After obtaining the fish lipid extracts, we determined possible interference of the sample matrix in FFA quantification with the proposed method (B) and the Lowry and Tinsley method (A). Samples frozen for 4−8 weeks at −10 °C were employed. The required centrifugation velocity was higher in method A than in method B because good separation of the two phases was not obtained at the velocity and centrifugation time recommended in the Lowry method. In this case, the lower phase tended to form emulsions with the sample. This effect was not observed with the standard (oleic acid) or method B at any time.

In the Lowry method, the quantification of FFA was carried out spectrophotometrically at 715 nm. In method B, a spectrophotometric scan was made between 400 and 750 nm by using the oleic acid standard. Maximum absorption occurred between 705 and 715 nm, in agreement with the absorbance found in method A.

A linear relationship between absorbance and lipid concentration verified that the quantification of FFA in fish extracts by the method proposed was correct within the range assayed.

It was further necessary to establish the agitation time for the proposed method. We checked whether 30 s was sufficient for contact between the two phases and thus to achieve correct quantification of FFA ($\alpha = 0.05$; $t = -1.3695$; gl = 9).

Oleic acid was used for the calibration curves. Lowry and Tinsley observed no deviations from linearity over a range of 2−14 μmol. For the proposed method a wider range was tested,

Table 1. Increase of Free Fatty Acid (FFA) Content of Mackerel Stored at −10 °C[a]

time (weeks)	benzene		cyclohexane		titration[b]	
	mean[c]	SD	mean	SD	mean	SD
4	45.44	7.70	41.42	1.18	50.58	1.22
6	102.25	2.34	93.59	2.53		
8	106.11	3.28	98.12	1.10		
10	113.81	2.10	110.24	2.77	139.97	7.05
11	124.54	1.24	117.27	1.48		
14	125.46	7.81	116.91	6.18	118.70	2.86
15	135.48	8.91	124.10	7.96	144.24	4.79
18	127.01	3.97	117.86	2.02	126.84	2.75
29	185.36	4.61	178.47	2.83	190.66	5.40
52	190.91	6.69	178.15	2.00	194.20	2.03

[a] Results expressed in g of FFA/kg of lipid. [b] Analysis limited to the quantity of available lipids. [c] All determinations performed on three replicates.

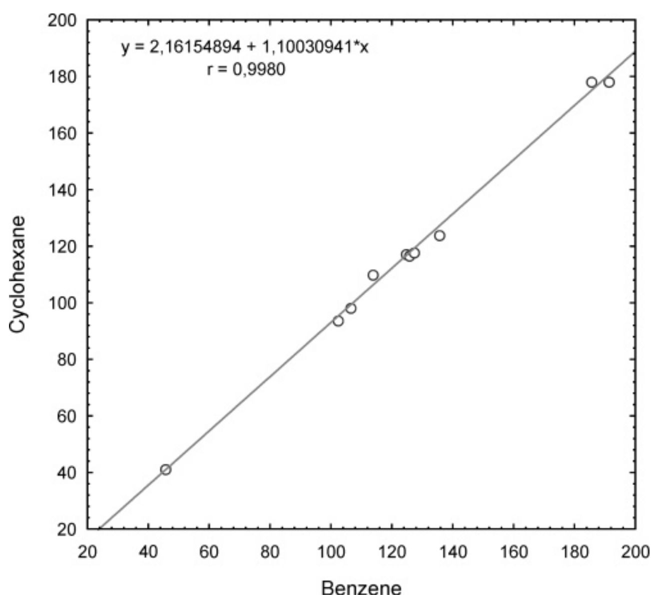

Figure 3. Linear correlation plot between free fatty acid values obtained according to methods A (benzene) and B (cyclohexane).

~2−22 μmol (equivalent to 0.6−6 mg), and no deviations from Beer's law were noted. However, in method A the absorbance for an equal quantity of standard was higher. Nevertheless, the blank values for method A were ~0.140, and in method B the reactants contributed a maximum value of 0.015.

Once these checks were carried out, 10 frozen (−10 °C) extracts of mackerel were studied, corresponding to different weeks over a period of 12 months in agreement with the three methods described (**Table 1**). The results showed differences in FFA concentrations, with highest values in the titration method and similar values in the other two cases.

Excellent agreement was observed between the proposed method and the two conventional methods, with highest correlations with method A (r = 0.9980, **Figure 3**) and lower correlations with method C (r = 0.9828, **Figure 4**). Even so, titration methods are biased toward personal analytical ability at the time the end point is determined. Moreover, the effects of other polar alkali-titratable non-FFA components on the total alkalimetrically determined FFA were checked (*26*). One of the principal interfering compounds are phospholipids. The titration method is therefore more susceptible to the lipid composition of the sample, to utilization of more or less polar solvents, or to variations in the process of lipid extraction that favor phospholipid extraction. This is especially important in the case of lean fish such as cod and pike, for which the majority of the lipids extracted form part of the cellular membranes where phospholipids may constitute >50% of total lipids (*27*). In oily species such as mackerel or herring with a lipid percentage of >5% on a wet weight basis, the percentage of phospholipids is far lower. In our case, the highest FFA values were obtained by the titration method, and it shows that variations in lipid extract composition with respect to phospholipids could be responsible for the lower correlation with the proposed method.

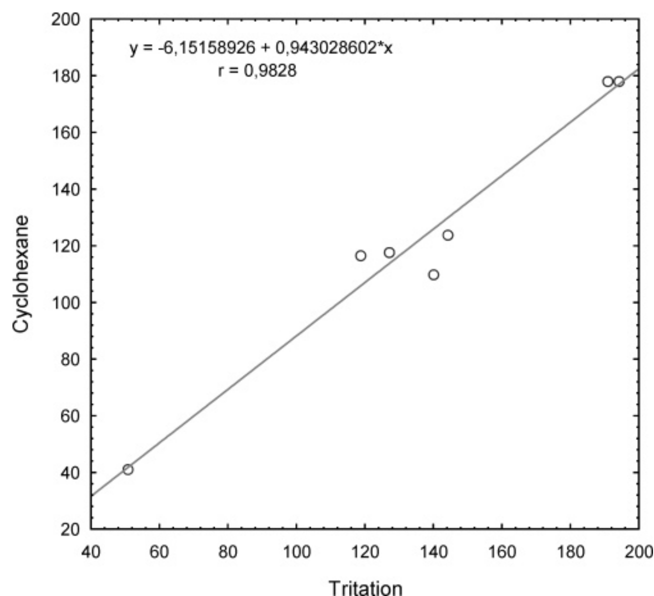

Figure 4. Linear correlation plot between free fatty acid values obtained according to methods B (cyclohexane) and method C (titration).

Table 2. Standard Recovery Percentage[a]

	method A		method B	
sample	0.253	0.077	0.196	0.246
oleic acid	0.215	0.277	0.181	0.284
sample + oleic	0.439	0.356	0.356	0.520
percentage	93.7	100.4	94.5	98.1

[a] Percentage calculated from absorbance at 715–710 nm.

Method C was also not deemed to be a valid alternative for our analysis because the quantity of sample required was 5 times greater than for methods A and B. Considering that the samples were carried out in triplicate, the cost of lipid extraction from a matrix such as fish is the only real drawback. Accordingly, given the cost and quantification procedure, the comparison was limited to methods A and B.

Several analyses were carried out with an internal standard for substantiating the percentage recovery of the method proposed. Recoveries >93% were always obtained (**Table 2**) for different quantities of standard and FFAs. An analysis of the internal standard was also performed as a reference for method A, and a similar percentage recovery (93.7%) was obtained.

Data from our studies in frozen fish stored at −10 °C show an increment of FFA until week 8. Thereafter, the values remained similar until week 18. A later analysis after 29 weeks of storage indicated a significant increment in FFA. The organoleptic analysis indicated a rejection of the sample at weeks 29 and 52 of storage. This behavior was very different from that of other samples analyzed with a higher lipid composition. We note here that mackerel is a species with notable seasonal

variation in type and quantity of lipids (*28*). These aspects will be discussed in a future paper.

Both the Lowry and proposed methods were similar with regard to rapidity, ease, and availability of the necessary equipment. However, the proposed method has the economical advantage of lower solvent requirement. We believe that this motive alone is not decisive for choosing between the two methods. Of more concern is the nature of the reactants employed. Benzene is widely classified as a toxic chemical compound and a proven carcinogenic agent. Aside from the short- and long-term effects, the immediate potential symptoms of its use oblige the employment of fume cupboards or personal protection equipment for laboratory handling.

Various methods have been cited in the literature for the quantification of fatty acids, among others the titration and Lowry methods. One must bear in mind that the values obtained by both methods can differ, with the titration method tending toward higher values as occurred in our analysis. Nevertheless, both methods are acceptable for FFA quantification. The method proposed in this study gave an excellent correlation with the Lowry method. In view of the need to avoid contact between harmful chemicals and people and the environment, method B described in this work is therefore proposed as an alternative for the quantification of free fatty acids.

SAFETY

Benzene is a carcinogen to humans, and therefore precaution must be taken when free fatty acids are determined. Organic solvents should be handled under fume hood conditions.

ACKNOWLEDGMENT

We thank Elsa Silva Caride and Carlos Suárez for technical assistance.

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Received for review June 10, 2004. Revised manuscript received January 3, 2005. Accepted January 13, 2005. This research was supported by the Interministerial Commission of Science and Technology (CICYT) during the Research Project AGL2001-1355.

JF040282C