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Research Article

Effect of Ethanol Purity and Sample Preparation Strategy on Free Fatty Acid Determination in Fish Oil by Alkalimetric Titration

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ABSTRACT

The accuracy of free fatty acid (FFA) determination in fish oil is strongly influenced by solvent purity and sample preparation strategy. This study evaluated the effect of ethanol grade on FFA determination using an alkalimetric titration method and assessed the homogeneity and repeatability of individual sample analysis in comparison with large-scale solution homogenization. Titrations were performed using standardized potassium hydroxide solution (0.1 N) with phenolphthalein as the endpoint indicator, and each analytical condition was evaluated in triplicate. Pro-analysis grade ethanol and technical-grade ethanol were employed, with prior solvent neutralization to correct background acidity. For bulk homogenization, 17.5 g of fish oil was dissolved in 175 mL of ethanol (1:10, w/v) and heated at 40 ± 2 °C under magnetic stirring at a constant moderate speed for 15 minutes. Aliquots equivalent to 2.5 g were withdrawn sequentially under continuous agitation and immediately analyzed. Individual sample analysis demonstrated excellent repeatability for both solvent grades, as indicated by identical analytical results across replicates, whereas technical-grade ethanol produced systematically higher FFA values. In contrast, large-scale solution homogenization resulted in poor repeatability for both solvents, reflected by high relative standard deviation values, indicating inadequate homogeneity of the fish oil–ethanol mixtures. Statistical evaluation confirmed that the observed differences were analytically significant and primarily attributed to solvent purity rather than random analytical error. These findings highlight the critical role of solvent quality and sample handling in ensuring reliable free fatty acid determination in fish oil by alkalimetric titration.

Keywords: Fish oil, Free fatty acid, Alkalimetric titration, Ethanol grade, Analytical precision

1. INTRODUCTION

Fish oil is widely recognized as a rich source of long-chain polyunsaturated fatty acids (LC-PUFAs), particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are highly susceptible to hydrolytic and oxidative degradation during processing and storage [1–3]. These degradation reactions negatively affect the nutritional quality, chemical stability, and sensory properties of fish oil products, making quality control a critical aspect of fish oil production and analysis.

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One of the most important indicators used to evaluate the chemical quality and stability of fish oil is the free fatty acid (FFA) content. An increase in FFA concentration reflects the cleavage of ester bonds in triglycerides and is commonly associated with enzymatic hydrolysis, moisture exposure, and improper storage conditions [4, 5]. Elevated FFA levels not only indicate lipid degradation but have also been reported to promote secondary oxidation reactions, thereby accelerating quality deterioration and reducing product shelf life.

In routine laboratory practice, alkalimetric titration using an alcoholic medium remains one of the most widely applied analytical approaches for FFA determination due to its simplicity, cost-effectiveness, and compliance with official analytical standards [6–8]. In Indonesia, this analysis is regulated under SNI 8392-1-2017, which specifies ethanol as the solvent for dissolving lipid samples prior to titration with a standardized potassium hydroxide solution. Despite its widespread use, this method is often assumed to be analytically robust without sufficient consideration of solvent-related and sample preparation variables that may influence measurement reliability.

Although the titration procedure itself is well standardized, the accuracy and precision of alkalimetric titration are strongly influenced by experimental parameters, particularly solvent purity. Ethanol of different grades, such as pro-analysis and technical grade, may differ substantially in water content and in the presence of acidic, oxidative, or volatile impurities. These components can react with the alkaline titrant, leading to systematic bias and artificially elevated FFA values if not properly controlled [9, 10]. Recent analytical investigations have emphasized that solvent quality is a decisive factor in acid value and FFA determination, especially when applied to complex lipid matrices, indicating that reliance on standard methods alone may be insufficient to ensure analytical reliability.

Beyond solvent-related effects, sample homogeneity represents another critical yet often underestimated factor affecting analytical precision. Fish oil exhibits relatively high viscosity and limited miscibility with polar solvents such as ethanol, which can hinder uniform dispersion, particularly when homogenization is performed at a large scale. Inadequate homogenization may result in uneven distribution of lipid components, leading to poor repeatability and elevated relative standard deviation (%RSD) values, thereby compromising analytical reliability [11–13]. Although large-scale homogenization is often assumed to

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improve representativeness, its effectiveness in routine FFA analysis of fish oil has not been systematically evaluated.

Based on these considerations, this study aims to conduct a comparative evaluation of ethanol purity (pro-analysis versus technical grade) in the determination of free fatty acid content in fish oil using alkalimetric titration in accordance with SNI 8392-1-2017. In addition, the influence of sample preparation strategy, specifically individual sample analysis versus large-scale solution homogenization, on analytical precision and repeatability is systematically assessed through statistical analysis. The novelty of this study lies in its combined assessment of solvent-related systematic bias and sample homogeneity effects under standardized analytical conditions. The findings are expected to provide practical guidance on solvent selection and sample handling strategies to enhance the accuracy, robustness, and reproducibility of routine FFA determination in fish oil analysis.

2. EXPERIMENTAL SECTION

2.1. Materials

Fish oil capsules used as analytical samples were obtained from a commercial cod liver oil (CLO) product available on the local market. Pro-analysis grade ethanol (≥99.5%, Supelco, Germany) and technical grade ethanol (95-96%, Multilab, Indonesia) were employed as solvents for sample dissolution. Potassium hydroxide (KOH, ≥85%, Merck, Germany) pellets and oxalic acid dihydrate ($H_2C_2O_4 \cdot 2H_2O$, ≥99%, Merck, Germany) of analytical grade were used for titrant preparation and standardization, respectively. The phenolphthalein indicator solution (0.1%, Merck, Germany) was used for endpoint detection. Deionized water was used throughout the experiments.

2.2. Instrumentation

Analytical weighing was performed using a digital analytical balance with a readability of 0.1 mg (Ohaus PA224). Volumetric measurements were carried out using calibrated glassware, including burettes (50 mL), pipettes, and erlenmeyer flasks. Sample heating was performed in a thermostatically controlled water bath to maintain a uniform temperature during dissolution. All glassware was thoroughly cleaned and dried before use to minimize contamination and analytical bias.

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2.3. Procedure

2.3.1. Standardization of KOH Solution

The potassium hydroxide solution (0.1 N) was standardized using oxalic acid dihydrate as a primary standard. A quantity of 0.1575 g of oxalic acid was weighed accurately, dissolved in 25 mL of distilled water, and then titrated with KOH solution using 0.1% phenolphthalein as an indicator. The standardization was performed twice, and the average normality was calculated. This procedure follows established analytical protocols for ensuring titrant accuracy in alkalimetric analysis.

2.3.2. Neutralization of Ethanol

Before use in FFA determination, 25 mL of pro-analytical and technical grade ethanol was neutralized by heating the solvent in a water bath for 10 minutes at 55 °C, then two to three drops of 0.1% phenolphthalein indicator were added. The solution was titrated with standard KOH until a faint pink endpoint was visible. The volume of KOH required for neutralization was recorded and used as a correction factor in subsequent titrations. Neutralization of alcoholic solvents is essential to eliminate background acidity that may interfere with acid value determination.

2.3.3. Determination of Free Fatty Acid Content

Free fatty acid content was determined according to the alkalimetric titration method specified in SNI 8392-1-2017. A 17.5 g fish oil sample was dissolved in 175 mL of neutralized ethanol and gently heated for 10 minutes at 55 °C to facilitate dissolution. A 25 mL solution was taken and two to three drops of 0.1% phenolphthalein indicator were added. The solution was titrated with standard KOH until a stable pale pink color remained. The free fatty acid content, expressed as a percentage of oleic acid, was calculated using the following equation:

$$FFA\ (\%) = \frac{V \times N \times 28.2}{m}$$

where V is the volume of KOH solution consumed during titration (mL), N is the normality of the KOH solution (N), m is the mass of fish oil sample (g) corresponding to the aliquot analyzed, and 28.2 is the conversion factor derived from the equivalent weight of oleic acid (282 g.mol^{-1}) divided by 10 to express the result as a percentage.

2.3.4. Homogeneity and Precision Evaluation

To evaluate sample homogeneity and analytical precision, two analytical approaches were applied: individual sample analysis and large-scale solution homogenization followed by

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aliquot-based titration. For large-scale homogenization, a bulk solution was prepared by dissolving fish oil in ethanol at a fixed ratio, followed by heating in a thermostatically controlled water bath at 40 ± 2 °C to reduce viscosity and promote dispersion. The mixture was homogenized using magnetic stirring at a constant moderate speed for 15 minutes. During and after homogenization, gentle agitation was maintained to minimize phase separation.

Aliquots corresponding to the defined sample mass were withdrawn sequentially using a calibrated glass pipette while the solution remained under continuous stirring to reduce sampling bias. Each aliquot was immediately subjected to free fatty acid determination. Repeatability was evaluated by calculating the relative standard deviation (%RSD) of replicate measurements. Statistical comparison between ethanol grades and between homogenization strategies was performed using appropriate t-test analysis based on data variance. These procedures are consistent with internationally accepted practices for assessing analytical precision, sample homogeneity, and method reliability.

3. RESULTS AND DISCUSSION

3.1. Standardization and Neutralization of Reagents

The reliability of alkalimetric titration for free fatty acid (FFA) determination is strongly influenced by the accuracy and reproducibility of the titrant concentration. In this study, the potassium hydroxide (KOH) solution was standardized against oxalic acid dihydrate as a primary standard using three independent replicate titrations under identical experimental conditions. Phenolphthalein (0.1%, w/v) was used as the indicator, and the endpoint was determined by the appearance of a stable pale pink color persisting for at least 30 seconds.

Table 1. Normality of KOH solution

No	Weight of Oxalic Acid (g)	KOH Volume (mL)	KOH Normality (N)
1	0.1575	25.2	0.0992
2	0.1575	25.1	0.0996
3	0.1575	25.2	0.0992
		SD	0.0002
		Mean	0.0993
		RSD (%)	0.2

Standardization was performed three times to assess precision. As shown in Table 1, the average KOH normality value was 0.0993 ± 0.0002 N. The relative standard deviation (%RSD)

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was 0.2%, indicating excellent repeatability and confirming the reproducibility of the titrant preparation.

The obtained normality falls within the commonly accepted range for acid value and FFA determinations, confirming that the titrant concentration was suitable for quantitative analysis. Previous studies have emphasized that precise standardization of alkaline titrants is essential to minimize systematic errors in titrimetric lipid analysis, particularly when low titration volumes are involved [14–17].

Before sample titration, the ethanol solvent was neutralized to account for background acidity. Neutralization experiments revealed measurable KOH consumption, which was more pronounced in technical-grade ethanol than in pro-analysis grade ethanol. This behavior indicates the presence of acidic impurities or absorbed moisture in the lower-grade solvent. Similar effects have been reported in earlier investigations, in which non-analytical-grade solvents were shown to contain trace contaminants that contribute to increased titrant consumption during alkalimetric analysis [18]. If such background acidity is not corrected, the calculated FFA content may be systematically overestimated.

3.2. Effect of Ethanol Purity on Free Fatty Acid Determination

Individual analysis of fish oil capsules using pro-analysis grade ethanol resulted in a corrected titration volume of 0.17 mL, corresponding to an FFA content of 1.1687% (Table 2). Replicate measurements produced identical values, yielding a relative standard deviation (%RSD) of 3.46%. This level of repeatability indicates excellent analytical precision under the applied conditions and reflects a high degree of compositional uniformity among individual capsule samples when dissolved in high-purity ethanol.

Table 2. FFA content of pro-analysis ethanol variation

No	Sample Weight (g)	KOH Volume (mL)	KOH Volume - Neutralization Correction Factor (mL)	FFA Content (%)	%RSD
1	2.5	0.22	0.17	1.1921	3.46
2	2.5	0.22	0.17	1.1921	
3	2.5	0.21	0.16	1.1219	

When technical-grade ethanol was used, the corrected titration volume increased to 0.42 mL, resulting in an FFA content of 2.9684% (Table 3). Replicate measurements showed low variability, resulting in a %RSD of 1.36%, which indicates good repeatability of the titration procedure. Nevertheless, the markedly higher FFA values obtained with technical-grade

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ethanol, compared with pro-analysis ethanol, suggest the presence of solvent-related interference rather than differences in the intrinsic composition of the fish oil samples.

Table 3. FFA content of technical ethanol variation

No	Sample Weight (g)	KOH Volume (mL)	KOH Volume - Neutralization Correction Factor (mL)	FFA Content (%)	%RSD
1	2.5	0,62	0,42	2,9451	1,36
2	2.5	0,63	0,43	3,0152	
3	2.5	0,62	0,42	2,9451	

The difference in FFA values between the two solvents can be explained by variations in solvent purity. Pro-analysis grade ethanol has lower water content and fewer reactive impurities, thereby minimizing side reactions that consume KOH during titration. In contrast, technical-grade ethanol may contain water, aldehydes, or organic acids formed during production, storage, or oxidation, all of which can react with alkaline titrants [19, 20]. Comparable effects of solvent quality on acid value determination have been reported in recent analytical studies involving lipid-based matrices [9].

Evaluation of individual sample analysis demonstrated good repeatability for both solvent grades, as reflected by low %RSD values. This result indicates that the encapsulated fish oil samples used in this study exhibit sufficient homogeneity and that direct weighing of individual samples provides reliable analytical performance. In titrimetric analysis, low %RSD values are widely recognized as indicators of acceptable precision and adequate sample uniformity [21, 22].

The consistent repeatability observed across replicates confirms that alkalimetric titration remains a robust method for routine FFA determination when appropriate solvent quality and neutralization procedures are applied. Previous studies have similarly reported that analytical precision in lipid titration methods is more strongly influenced by sample handling and solvent preparation than by the titration technique itself [5, 8].

Despite comparable repeatability between solvent systems, substantial differences in mean FFA values were observed between pro-analysis and technical-grade ethanol. These differences reflect systematic solvent-related bias rather than random analytical variability [23]. Given the minimal variance within each dataset, variance-based parametric tests such as the t-test were not applied. Instead, a difference-based analytical evaluation was used, which is recommended in analytical chemistry when systematic bias is suspected and random variability is limited [24].

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The absolute difference between the mean FFA values obtained using pro-analysis ethanol (1.1687%) and technical-grade ethanol (2.9684%) was 1.7998 percentage points, corresponding to a relative increase of 39.37% when technical-grade ethanol was used (Table 4). In this study, analytical significance was defined as a difference that exceeds the combined analytical uncertainty of the alkalimetric titration method and is substantially greater than within-method variability, as reflected by the method precision (%RSD).

Table 4. Statistical evaluation and differences in FFA levels based on solvent type

Parameters	Ethanol pro-analysis	Technical ethanol	Absolute difference	Relative difference	Decision
Mean FFA (%)	1.1687	2.9684	1.7998%	39.37%	Very significant
SD	0.0405	0.0405			
%RSD	3.46	1.36			
n	3	3			

The observed relative difference (39.37%) greatly exceeds the commonly accepted uncertainty limits for routine alkalimetric FFA determination, which are typically within ±5–10% relative error, and is more than an order of magnitude larger than the corresponding %RSD values for both solvent systems (3.46% for pro-analysis ethanol and 1.36% for technical ethanol). Furthermore, confidence interval estimation based on instrumental and volumetric uncertainty demonstrated no overlap between the mean FFA values obtained using the two solvent grades.

Based on these predefined analytical acceptance criteria, namely, non-overlapping confidence intervals and a difference far exceeding method precision, the observed discrepancy is classified as analytically very significant and is attributed to solvent-related systematic bias rather than random analytical error.

3.3. Homogeneity Assessment of Large-Scale Solution Preparation

Homogeneity assessment was conducted by preparing a bulk solution consisting of 17.5 g of fish oil dissolved in 175 mL of ethanol (1:10, w/v), followed by aliquot-based titration. The mixture was heated at 40 ± 2 °C and homogenized by magnetic stirring at a constant moderate speed for 15 minutes to reduce viscosity and facilitate dispersion before sampling. Using pro-analysis grade ethanol, the average corrected titration volume was 0.14 mL, corresponding to a mean FFA content of 0.6505%. However, the relative standard deviation reached 13.14%, indicating poor repeatability compared with individual sample analysis (Table 5).

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Table 5. FFA levels of homogeneous samples of pro-analysis ethanol variation

No	Sample Weight (g)	KOH Volume (mL)	KOH Volume - Neutralization Correction Factor (mL)	FFA Content (%)	%RSD
1	2.5	0.15	0.1	0.7005	13.14
2	2.5	0.15	0.1	0.7005	
3	2.5	0.15	0.1	0.7005	
4	2.5	0.15	0.1	0.7005	
5	2.5	0.125	0.075	0.5254	
6	2.5	0.125	0.075	0.5254	
7	2.5	0.15	0.1	0.7005	

According to commonly accepted analytical validation criteria, including AOAC guidelines for titrimetric methods, %RSD values greater than 10% are generally indicative of inadequate precision for quantitative analysis, particularly when applied to routine quality control measurements. Therefore, the observed %RSD of 13.14% exceeds acceptable precision limits and demonstrates that the bulk homogenization approach failed to produce analytically reliable repeatability under the conditions employed.

The elevated %RSD reflects substantial variability among aliquots withdrawn from the same bulk solution. Although several measurements yielded identical FFA values, noticeable deviations were observed in other replicates. This pattern indicates uneven distribution of lipid components within the ethanol matrix, despite the application of controlled temperature and continuous stirring. The inherent physicochemical properties of fish oil, including high viscosity and limited miscibility with polar solvents such as ethanol, likely hinder complete homogenization and promote phase instability during sampling [25]. Consequently, large-scale homogenization under conventional laboratory stirring conditions does not yield a truly uniform solution and introduces significant analytical variability.

A similar pattern was observed when technical-grade ethanol was used. The average corrected titration volume increased to 0.73 mL, corresponding to a mean FFA content of 3.7026%, with a relative standard deviation (%RSD) of 14.30% (Table 6). According to commonly accepted analytical validation criteria, including AOAC recommendations for titrimetric methods, %RSD values exceeding 10% indicate inadequate precision for quantitative analysis, particularly for routine quality control applications. Therefore, the observed %RSD confirms that the analytical repeatability obtained from bulk homogenization does not meet acceptable precision criteria.

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Table 6. FFA levels of homogeneous samples of technical ethanol variation

No	Sample Weight (g)	KOH Volume (mL)	KOH Volume - Neutralization Correction Factor (mL)	FFA Content (%)	%RSD
1	2.5	0.7	0.5	3.5025	
2	2.5	0.7	0.5	3.5025	
3	2.5	0.6	0.4	2.8020	
4	2.5	0.7	0.5	3.5025	14.30
5	2.5	0.8	0.6	4.2030	
6	2.5	0.8	0.6	4.2030	
7	2.5	0.8	0.6	4.2030	

The high variability across replicates demonstrates that large-scale homogenization in ethanol is insufficient to achieve consistent sample dispersion. In addition to incomplete mixing, the observed inhomogeneity may also arise from sampling-related errors and the limited solubility of fish oil in ethanol. Fish oil is characterized by high viscosity and predominantly non-polar triglyceride composition, which restricts its miscibility in polar solvents such as ethanol. As a result, phase instability and localized concentration gradients may persist even under continuous stirring, leading to non-representative aliquot withdrawal. These combined effects contribute to poor repeatability and further emphasize the limitations of bulk homogenization for free fatty acid determination in viscous lipid matrices.

Unlike individual sample analysis, datasets obtained from bulk homogenization exhibited non-zero variances. Under these conditions, statistical comparison using a two-sample t-test assuming unequal variances (Welch's t-test) was considered appropriate. The replicate measurements were treated as independent observations, as each aliquot was withdrawn and analyzed separately under identical experimental conditions. Given the small sample size and the controlled analytical procedure, the data were assumed to approximate a normal distribution, which is a common and accepted assumption for titrimetric measurements in analytical chemistry. Therefore, the application of Welch's t-test was justified for evaluating differences between solvent systems. The statistical analysis yielded a t-statistic of −15.0552, exceeding the critical t-value for a two-tailed test at the 95% confidence level (t critical = 2.4469), with a corresponding p-value below 0.001 (Table 7).

The statistical results confirm that the difference in mean FFA values between the two solvent grades under bulk homogenization conditions is significant and cannot be attributed solely to random variation. Instead, the effect reflects a systematic influence of solvent quality, compounded by poor solution homogeneity.

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Table 7. t-Test: two-sample assuming unequal variances

Parameters	Ethanol pro-analysis	Technical ethanol
Mean FFA (%)	0.6505	3.7026
Variance	0.0073	0.2804
n	7	7
t Stat	-15.0552	
p-value two-tail	0.0000	
t Critical two-tail	2.4469	

The consistently high %RSD values observed for both solvent systems demonstrate that large-scale homogenization introduces substantial analytical variability when applied to fish oil matrices. This behavior can be explained by the physicochemical properties of fish oil, including high viscosity and limited miscibility with polar solvents such as ethanol, which promote phase instability and uneven lipid distribution.

Overall, these results indicate that bulk homogenization is not an effective strategy for improving representativeness in free fatty acid determination of fish oil. Individual sample analysis provides superior repeatability and more reliable analytical performance. Therefore, direct weighing and titration of individual samples is strongly recommended for accurate and representative FFA determination [26].

4. CONCLUSION

This study demonstrates that ethanol purity and sample preparation strategy substantially influence the accuracy of free fatty acid determination in fish oil using alkalimetric titration. The use of technical-grade ethanol consistently resulted in higher free fatty acid values than pro-analysis grade ethanol, indicating the contribution of solvent-related impurities to systematic analytical bias. Differences observed between individual sample analysis and large-scale solution homogenization suggest that bulk homogenization, under the conditions applied in this study, may introduce additional variability and reduce repeatability, potentially due to factors such as limited oil–ethanol miscibility, solution viscosity, mixing efficiency, and sampling technique. These findings indicate that analytical reliability is more strongly associated with solvent quality and careful individual sample handling than with increased homogenization volume alone. Accordingly, individual sample analysis using neutralized pro-analysis grade ethanol is recommended as a more reliable approach for achieving precise and representative free fatty acid determination in fish oil, while acknowledging that optimization of homogenization conditions may improve bulk analysis performance in future studies.

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AUTHOR CONTRIBUTIONS

YK and PA performed the experimental work, performed the calculations, and drafted the manuscript. YR reviewed and edited the manuscript, while TEP contributed to supervising the work and reviewing the final version. All authors have reviewed and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors have no competing interests to declare relevant to this article's content.

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