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Evaluation of Ethanol Grade on the Robustness of Acid Number Determination in Fish Oil

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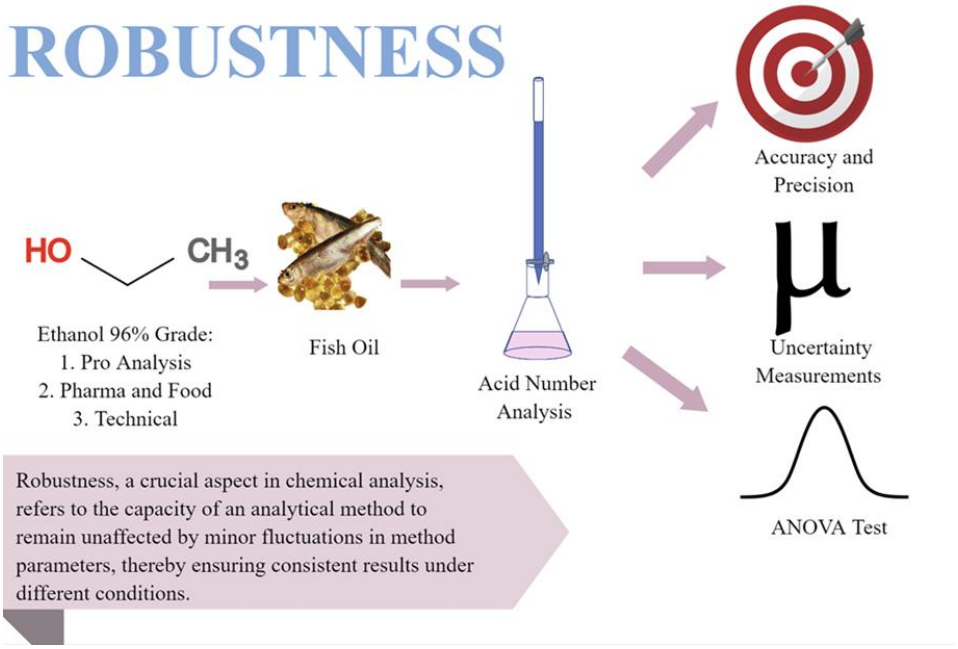
GRAPHICAL ABSTRACT



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ABSTRACT

The robustness evaluation of the acid number analysis method in fish oil was carried out to see the effect of the type of ethanol used as a solvent in the titration process. The types of ethanol used in this study were pro analysis, pharma, food, and technical. The acid number values (mg KOH/g) in fish oil with various solvents obtained were 2.3955±0.3211 for pro analysis grade, 2.7932±0.2983 for pharma and food grade, 2.7812±0.3362 for technical grade 1; 2.7031±0.3405 for technical grade 2. The acid number for all types of ethanol has a value following the requirements of SNI 8467: 2018, where the acid number value must be less than 3 mg KOH/g. However, suppose the measurement uncertainty value is included in the calculation. In that case, only the pro-analysis grade ethanol solvent has an acid number value following the requirements of SNI. The results of the ANOVA test also showed a significant difference in the

variation of ethanol types for determining acid numbers because the calculated F value (13.9004) was greater than the F table (3.0984). Therefore, the solvent that must be used to determine acid numbers in fish oil is pro-analysis grade ethanol.

1. INTRODUCTION

Evaluating the quality of fish oil products necessitates examining several physicochemical parameters. Among these are the acid, peroxide, and saponification values, which can be precisely determined using titration. This method involves a chemical reaction between free fatty acids and the reagent, resulting in precise measurements of these values [1]. The acid number is a crucial measure in evaluating the quality of fish oil. A high acid number indicates an increased amount of free fatty acids, which often stem from the hydrolysis or degradation of the oil. This can result in poor taste, a reduced shelf life, and a lower nutritional quality, particularly impacting the concentration of beneficial omega-3 fatty acids. It is essential to monitor and control the acid number to ensure the efficacy and safety of the fish oil for consumption [2].

In analyzing acid number (AN), ethanol serves as a solvent to dissolve the sample, including fatty acids, and facilitate their even distribution throughout the sample. During titration, ethanol facilitates the homogeneous distribution of the sample, allowing the titrant, typically a base such as potassium hydroxide or KOH, to react effectively with the free fatty acids present in the oil. Accurately quantifying the acid number, which measures the free fatty acid content and overall oil quality, is essential for this process. Ethanol is a preferred solvent due to its ability to dissolve non-polar and polar compounds, making it suitable for oils and fats. Additionally, ethanol is less toxic than other solvents, such as methanol, making it a safer choice for laboratory personnel and more suitable for food-related applications [3].

Ethanol is available in various grades based on purity and application. The highest purity level of ethanol is known as pro analysis, commonly used in laboratory settings for analytical purposes, such as chromatography and spectroscopy. It contains minimal impurities, ensuring accurate and reliable results in sensitive analytical procedures. Pharmaceutical-grade ethanol is used in the pharmaceutical industry and meets the strict standards of pharmacopoeias. It is used in the production of medicines and disinfectants and as a solvent in the preparation of drugs. The purity level is high, but it may contain small amounts of water and other permissible impurities. Ethanol food grade is suitable for human consumption and is used in food and beverage production, such as in making extracts, flavorings, and alcoholic beverages. It must comply with food safety regulations, ensuring it is free from harmful contaminants. Technical-grade ethanol is used in industrial applications and has a lower purity level than pro-analysis or pharmaceutical grade. It is used in cleaning, as a solvent in manufacturing, and as a fuel additive. Technical-grade ethanol may contain higher levels of impurities, which are acceptable for non-critical applications where high purity is not required [4].

In this research, we aim to investigate the impact of various types of ethanol on acid number analysis. Robustness, a crucial aspect in chemical analysis, refers to the capacity of an analytical method to remain unaffected by minor fluctuations in method parameters, thereby ensuring consistent results under different conditions. This is essential for the credibility and reproducibility of analytical findings across various laboratories and conditions. We can determine its stability and reliability under varying experimental conditions by subjecting the method to slight alterations in parameters such as temperature, pH, solvent composition, and flow rate. Robustness is an integral part of method validation, which involves rigorously testing the method under diverse conditions to guarantee that it produces dependable results. This process often entails testing the method using different analysts, instruments, and environments. It is also valuable for employing experimental designs, such as factorial designs, to systematically vary parameters and evaluate their impact on the analytical outcome. This approach enables us to pinpoint critical parameters that must be tightly controlled. Statistical tools such as Analysis of Variance (ANOVA) can be employed to assess the robustness of the method further. ANOVA helps determine whether variations in method parameters significantly impact the results. Robustness guarantees that the technique is dependable enough to

be incorporated into standard operating procedures, allowing for consistent application in routine analysis [5].

2. EXPERIMENTAL METHODS

The acid number analysis in this study is based on SNI 8392-1:2017 [6] which is a standard method that applies in Indonesia.

2.1. Material

The materials used in this research include fish oil (channa fish, from marketplace), ethanol 96%: pro analysis (Merck), pharma & food grade (PT. Rofa), technical grade 1 (CV. Cipta), technical grade 2 (CV. Sarana), phenolphthalein indicator 1%, potassium hydroxide (KOH) 0.1 N, oxalic acid dihydrate (Merck), and distilled water. While the equipment used is a set of Iwaki glassware.

2.2. Standardization of KOH 0.1N

The solid oxalic acid dihydrate was carefully weighed at as much as 0.6303 g and then dissolved with 50 mL of distilled water in a beaker. The solution was transferred to a 100 mL measuring flask and calibrated to the appropriate mark (concentration 0.1N). A 0.1 N oxalic acid solution was carefully pipetted to a volume of 10 mL, followed by the addition of 3 drops of 1% phenolphthalein indicator. The mixture was then titrated using 0.1 N KOH until a pink color persisted for 30 seconds. The volume of KOH solution used during the titration was recorded, and the KOH concentration was determined to calculate the normality of KOH, equivalent to the normality of oxalic acid at the end of the titration.

2.3. Determination of acid number in fish oil

A mixture of 12.5 mL neutral ethanol and a 1 g fish oil sample was heated using a water bath for 10 minutes. Three drops of 1% phenolphthalein indicator were added to the solution. The solution was then titrated using 0.1 N KOH until it turned pink, lasting 30 seconds. The titration volume was recorded, and the titration was repeated six times. The acid number determination was performed using four different types of 96% ethanol: pro analysis, pharma & food grade, and two technical grades using the formula in Equation 1.

$$Acid\ number\ = {V_{titration} \times N_{KOH} \times Mr_{KOH}}/{mass\ sample} \quad (1)$$

2.4. Accuracy analysis of acid number

A mixture of 12.5 mL neutral ethanol, 1 g sample of fish oil and 0.1 g oleic acid standard was heated using a water bath for 10 minutes. Three drops of 1% phenolphthalein indicator were added to the solution. The solution was then titrated using 0.1 N KOH until it turned pink, lasting 30 seconds. The titration volume was recorded and repeated six times to obtain the acid number, referred to as C_{spike}. The C_{target} value was determined from the titration results for a mixture of 12.5 mL of ethanol and 0.1 gram of oleic acid standard. The accuracy was calculated as the difference between C_{spike} and C_{sample} divided by C_{target} (Equation 2), and this process was carried out for all types of ethanol.

$$\%Recovery\ = {[C_{spike} - C_{sample}]}/{C_{target}} \times 100 \quad (2)$$

3. RESULTS AND DISCUSSIONS

3.2. Acid Number Analysis in Fish Oil

In this research, the acid number indicates the quantity of free fatty acids in fish oil. It is expressed as the number of milligrams of KOH required to neutralize the free fatty acids in one gram of oil or fat. The outcomes of determining the acid number are presented in Table 1. Based on Table 4.1, it is known that if the uncertainty estimate value is included, only the ethanol pro analysis meets the quality requirements according to SNI 8467:2018 [7], which states that the acid number value in fish oil must be less than 3 mg KOH/g.

TABLE 1. The results of determining the acid number in fish oil.

Ethanol Grade	Density (g/mL)	Acid Number (mgKOH/g)	SNI Requirements [7]	Decision
Pro analysis	0.7619	2.3955±0.3211*	Less than 3 mg KOH/g	Suitable
Pharma & food grade	0.7801	2.7932±0.2983*		Not suitable
Technical grade 1	1.0061	2.7812±0.3362*		Not suitable
Technical grade 2	0.9107	2.703±0.3405*		Not suitable

* This value is obtained by calculating measurement uncertainty

When the density of the ethanol solvent is plotted as the independent variable (x), and the acid number of the sample as the dependent variable (y) based on Table 1, a correlation coefficient of 0.6211 is obtained. This result indicates a moderate positive correlation between the density of the solvent and the acid number value. Solvent density may influence the outcome of titration by affecting the solubility and reactivity of the titrant or analyte, as well as the buoyancy of the droplets in methods such as electrospray deposition [8]. In the context of enhanced oil recovery, solvent density can impact the sweep efficiency of the process. For instance, solvent-based nanofluid flooding followed by waterflooding has demonstrated an increase in sweep efficiency, which is partially attributed to the density profiles of the fluids involved [9].

3.3. Precision

Precision is a term that refers to the degree of consistency or reproducibility in a series of tests. It can be quantified in various ways, such as through standard deviation, average deviation, or range, the largest and smallest difference in the measurement results. A smaller deviation indicates a higher level of precision. Three types of evaluations can be conducted with precision parameter methods: repeatability, reproducibility, and intermediate precision [10]. The precision values for acid number analysis in the fish oil can be seen in Table 2. The findings from the RSD analysis were assessed against the 0.67 or 2/3 CV Horwitz benchmark [11], as there are currently no established precision requirements within the SNI. According to the AOAC Official Methods of Analysis [12], the permissible %RSD limit for a mass fraction of 10^{-3} is 3.7%. The outcome is due to the deficiency of alcoholic potassium hydroxide, which exhibits greater reactivity and effectiveness in neutralizing the acidic constituents present in the sample, thereby resulting in more precise and dependable outcomes [13].

TABLE II. The precision analysis.

Ethanol Grade	%RSD	2/3 CV Horwitz	Result
Pro analysis	5	3.3231	Not precision
Pharma & food grade	3.45	3.2540	Not precision
Technical grade 1	4.42	3.2493	Not precision
Technical grade 2	4.76	3.2632	Not precision

3.4. Accuracy

Accuracy is defined as the degree of closeness between an analysis's results and the analyte's actual concentration. It is typically expressed as a percentage of the recoverable analyte. To determine accuracy, the measured levels are compared to the theoretical levels with the aid of standard additions. Table 3 contains the results of accuracy testing for various procedure variations. Table 3 shows that the average % recovery value for all types of ethanol falls short of the AOAC Official Methods of Analysis requirement [12], which mandates a recovery range of 95-105%. The reason for this is the absence of alcoholic KOH in the titration process. Nevertheless, when calculating t for accuracy uncertainty, it is determined that the $t_{calculated}$ is smaller than the $t_{criteria}$, thus

eliminating the need to include the accuracy value in the acid number reporting process or uncertainty calculation. Despite this shortcoming, the accuracy value remains acceptable [14].

TABLE III. The accuracy analysis.

Ethanol Grade	C_{sample}	C_{spike}	C_{target}	Accuracy					Result
	(mg KOH/g)			Ave. % Rec.	% RPD	$\mu_{recovery}$	$t_{calculated}$	$t_{criteria}$	
Pro analysis	2.3955	208.5445	186.5315	110.70	10.70	0.0757	1.4144	12.7062	$t_{calculated} < t_{criteria}$ a correction factor (1/Recovery) is not being applied and therefore recovery is not explicitly included in the calculation of uncertainty measurement
Pharma & food grade	2.7932	213.1788	186.2706	112.97	0.00	0.0000	0.0000		
Technical grade 1	2.7812	203.9102	189.5668	106.13	8.17	0.0578	1.0607		
Technical grade 2	2.703	224.76465	192.76765	115.41	15.29	0.1081	1.4252		

$\mu_{recovery} = standar\ deviation/\sqrt{repititions}$
$t_{calculated} = [1 - rec]/\mu_{recovery}$
$t_{criteria} =$ the $2 - tailed\ critical\ value, for\ n-1\ degrees\ of\ freedom\ at\ 95\ \%confidence$

3.5. Uncertainty Measurements

The term "uncertainty" signifies the restricted comprehension of a specific value. The uncertainty of measurement does not imply doubt about the validity of a measurement; on the contrary, knowledge of the uncertainty suggests increased confidence in the validity of a measurement result. Determining uncertainty estimates involves several steps. Firstly, the measure of uncertainty must be specified, along with the sources of uncertainty. Subsequently, the individual components of uncertainty must be quantified. Finally, the combined and expanded uncertainty must be calculated [14]. The relevant uncertainty sources are shown in Figure 1. These uncertainty sources can be further analyzed to better understand their impact on the overall system.

The uncertainties of each source in Figure 1 are determined using a specific formula [14]. Subsequently, the combined uncertainty of all sources is calculated. The reporting of uncertainty values is done by expressing them as expanded uncertainties, obtained by multiplying the combined uncertainty by a coverage factor based on the confidence interval (factor of 2 for a 95% confidence interval). The outcomes of the uncertainty estimation calculation are depicted in Table 4. According to Table 4, it can be discerned that there are three primary contributors to the most significant uncertainty value in descending order, namely test repeatability, titration volume, and KOH normality. Repeatability uncertainty originates from the test precision value, while titration volume uncertainty stems from burette calibration and temperature factors. The source of uncertainty in KOH normality is the KOH standardization process. Hence, when determining the acid number, it is crucial to consider these three aspects.

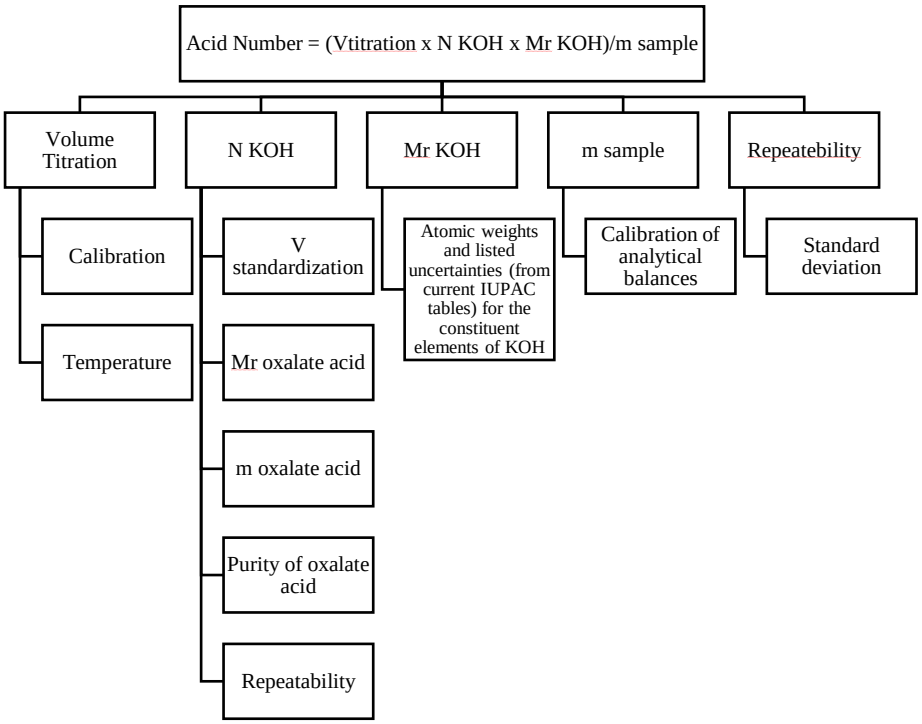

Figure 1. The identification of the uncertainty sources in acid number analysis.

TABLE IV. The quantifying uncertainty measurement.

Ethanol Grade	Standard Uncertainty					Combined standard uncertainty	Expanded uncertainty
	V titration	N KOH	Mr KOH	Mass sample	Repeatability		
Pro analysis	0.0204	0.0018	0.0010	0.0327	4.9868	0.1605	0.3211
Pharma & food grade	0.0204	0.0018	0.0010	0.0327	3.4508	0.1492	0.2983
Technical grade 1	0.0204	0.0018	0.0010	0.0327	4.4175	0.1681	0.3362
Technical grade 2	0.0204	0.0018	0.0010	0.0327	4.7592	0.1703	0.3405

3.6. ANOVA

The Analysis of Variance (ANOVA) test is conducted by calculating the variance between population distribution and other variables, as well as the variance within the samples. ANOVA is classified into two categories: one-way and two-way. One-way ANOVA involves a single independent variable, while two-way ANOVA involves two independent variables [15]. The results of a one-way ANOVA test for an ethanol grade variation are shown in Table 5, and these results indicate that the null hypothesis of no difference in the means can be rejected at a significance level of $p < 0.05$. Therefore, it can be concluded that there is a statistically significant difference in the means between the two groups. It is essential to consider the implications of this finding for future research and potential interventions. Additionally, it is crucial to explore possible explanations for the observed phenomenon and assess the effectiveness of existing interventions in addressing the issue.

TABLE V. The result of ANOVA one-way.

Source of Variation	SS	df	MS	F	P-value	F crit.
Between Groups	0.5729	3	0.1910	13.9004	0.0000	3.0984
Within Groups	0.2748	20	0.0137			
Total	0.8476	23				

4. CONCLUSIONS

Characterization of the acid number of various ethanol grades is essential for maintaining product quality and complying with regulatory standards. By integrating measurement uncertainty in the acid number value, it is evident that only the pro-analysis grade ethanol satisfies the SNI requirement. Employing aqueous KOH leads to precision and accuracy values that fail to meet the AOAC criteria. The main reason for this is that alcoholic KOH is more reactive and efficient in neutralizing the acidic components in the sample, resulting in more precise and dependable outcomes. The results of the ANOVA test also showed a significant difference in the variation of ethanol types for determining acid numbers. Therefore, it is essential to utilize ethanol pro analysis as a solvent for samples and reagents when determining acid numbers.

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