Food Research 7 (6) : 229 - 234 (December 2023)
Journal homepage: https://www.myfoodresearch.com

FOOD RESEARCH

METHOD DEVELOPMENT

Modification of free fatty acid test for food products containing fat and organic acid

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Article history:
Received: 3 March 2022
Received in revised form: 5 April 2022
Accepted: 5 November 2022
Available Online: 22 December 2023

Keywords:
Determination,
Fat,
Free fatty acids,
Modification,
Organic acids,
Yogurt

DOI:
https://doi.org/10.26656/fr.2017.7(6).060

Abstract

The free fatty acid test in food products containing fat (insoluble water) and organic acid (soluble water) usually uses the titration method with ethanol solvent. However, the free fatty method with 1 step titration detected both free fatty acid and non-fatty acid. Therefore, this study aimed to modify the free fatty acid test method in food products containing fat and organic acid. This research used free fatty acid methods with two steps of titration. Oil and acetic acid were used as a sample in the first step, while yogurt was used in the second titration step. The results showed that the free fatty acid method in food products containing fat and organic acids should be done with two-step titration using ethanol and distilled water solvent in steps 1 and 2, respectively. The amount of free fatty acids in the first and the second titration differed. The two-step titration method exhibited a free fatty acid content of about 0.27%, which was higher than the one-step titration. The different titration results between ethanol and distilled water were in NaOH volume used by free fatty acids. Therefore, the analysis of free fatty acid in food containing fat/oil and water-soluble organic acids were more precise using the two-step titration than one-step titration.

1. Introduction

A titration method commonly measures the free fatty acid (FFA) concentration in fat and oil food. The AOAC method is the most common analytical method to validate free fatty acids in cooking oil, margarine, coconut milk, fish oil, and others. This method starts with the oil or fat sample dissolved in 95% alcohol and neutralized by adding phenolphthalein solution. The sample was then titrated with NaOH solution until it turned pink (AOAC International, 2019). Nurhasnawati *et al.* (2015) also measured cooking oil's free fatty acids levels using AOAC methods. However, this method cannot be used in food materials containing fat, oil, and organic acids. In the free fatty acids test, ethanol is frequently used to dissolve the free fatty acids in the cooking oil because it serves as a penetrating chemical by dissolving NaOH. Because the NaOH solution is created with water as a solvent, this is the case. Ethanol has a non-polar component called alkyl (R) and a polar group called hydroxyl (OH). Non-polar free fatty acids will be dissolved by the alkyl group, while polar water will be dissolved by the hydroxyl group (OH) (Marnoto *et al*., 2012).

The degree of polarity is used to determine the type of solvent. The polarity of the extracting solution must meet the polarity of the substance to be extracted. Non-polar chemicals can dissolve well in non-polar solvents, but polar compounds dissolve better in polar solvents. Ethanol is an effective semipolar solvent to extract colour from red fruit. Most red fruit pigments are carotenoids, with non-polar characteristics and lengthy carbon chains, making them difficult to dissolve in ethanol (Sirwutubun *et al*., 2016). According to Marnoto *et al*. (2012), methanol and ethanol produce OH-ions which are easier to interact with polar functional groups, such as tannins. Acetone is an aprotic polar solvent that cannot produce OH-ions. As a result, acetone yields less tannin extract (0.016 g/mL) than protic polar solvents (methanol and ethanol). The extract yield in the non-polar solvent n-hexane is the lowest, at 0.0031 g/mL (Marnoto *et al*., 2012). The total acid levels in food products, which contain organic acids (water-soluble), including acetic acid, lactic acid, citric acid, and malic acid, were measured using a polar solvent such as water.

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eISSN: 2550-2166
Published by Rynnye Lyan Resources

Water is used as a solvent because organic acids in food are dissolved in water.

Coconut milk yogurt (Nurminabari *et al*., 2018), mixed fruit yogurt (Riana *et al*., 2018), fermented soybean juice, and probiotic cheese (Rahayu *et al*., 2010) are kinds of food products that contain oil as well as organic acids. Cheese is produced from fermented milk and a cheese starter, which is acid-producing lactic acid bacteria. Streptococcus is a lactic acid bacterium commonly used in cheese starter cultures. *Rhizopus oryzae*, in addition to lactic acid bacteria, may produce lactic acid. Lactic acid is a soluble acid that can be found in milk. The fat percentage of cow's milk cheese is 35.02%, while the total acidity (pH 4.4) is 4.4 (Estikomah, 2017). Processed cheese has a fat content of 35.78% and a total acid content of 1.17% (Dewi, 2007). The measurement of free fatty acids in food products should be modified due to an error in the test results. The ethanol dissolves free fatty acids and organic acids (lactic acid), resulting in a mix of the free fatty acid and organic acid content in the final titration result.

Many studies have analyzed the FFA levels in foodstuffs using 1 step of titration with ethanol, such as yogurt (Irawan *et al*., 2013) and kefir (Setyawardani *et al*., 2017). The test results are not precise because ethanol can dissolve both fatty acids and non-fatty acids. Therefore, this study aimed to modify the free fatty acid test method in food products containing fat and organic acid. A modification procedure with two-step titration will be used in this study. The first titration counts all of the acids contained in the substance (free fatty acids and organic acids). Only the organic acids sample (water-soluble) is measured in the second titration. The amount of free fatty acids present in the sample differs between the first and second titrations. The first titration dissolves all of the acids in the sample, both free fatty acids and organic acids, using hot neutral alcohol as the solvent.

2. Materials and methods

2.1 Research time and location

The research was carried out from January until August 2021 in the Laboratory of Chemistry and Laboratory of Processing, Widyagama University, Malang, Indonesia.

2.2 Research design

This research was conducted in two steps of titration. Oil and acetic acid were used as a sample in the first step, while yogurt (a mixture of milk and coconut milk) was used in the second titration step.

For step 1, there were four samples, including:

a. Acetic acid as a sample + distilled water as a solvent
b. Cooking oil as a sample + ethanol as a solvent
c. The mixture of acetic acid and cooking oil as a sample + distilled water as a solvent
d. The mixture of acetic acid and cooking oil as a sample + ethanol as a solvent.

For step 2, there were two groups. The first group used yogurt made from a mixture of cow milk and coconut milk with different ratios and solvents. This experiment consisted of six samples:

a. Yogurt from 100% cow's milk + distilled water as a solvent
b. Yogurt from 100% cow's milk + ethanol as a solvent
c. Yogurt from 75% cow's milk and 25% coconut milk + distilled water as a solvent
d. Yogurt from 75% cow's milk and 25% coconut milk + ethanol as a solvent
e. Yogurt from 50% cow's milk and 50% coconut milk + distilled water as a solvent
f. Yogurt from 50% cow's milk and 50% coconut milk + ethanol as a solvent

The second group, used yogurt from a mixture of cow's milk and coconut milk (1:1) with different incubation times and solvents. These experiments consisted of six samples, including:

a. Yogurt from cow's milk + coconut milk with incubation for 4 hrs + distilled water as a solvent
b. Yogurt from cow's milk + coconut milk with incubation for 4 hrs + ethanol as a solvent
c. Yogurt from cow's milk + coconut milk with incubation for 8 hrs + distilled water as a solvent
d. Yogurt from cow's milk + coconut milk with incubation for 8 hrs + ethanol as a solvent
e. Yogurt from cow's milk + coconut milk with incubation for 12 hrs + distilled water as a solvent
f. Yogurt from cow's milk + coconut milk with incubation for 12 hrs + ethanol as a solvent

2.3 Methods

2.3.1 Step 1

Approximately 5 mL of sample was added to 10 mL of solvent followed by 3 - 5 drops of phenolphthalein indicator. Then the sample was titrated with 0.1 N NaOH until it changed to pink colour (stable for 10 s). The required amount of NaOH was recorded.

2.3.2 Step 2

2.3.2.1 Coconut milk preparation

The old coconut was peeled off the husk, outer shell

https://doi.org/10.26656/fr.2017.7(6).060

and epidermis. Then, the coconut was grated and added to water (1:1) and squeezed to produce coconut milk. The coconut milk was ready to be used (Su'i *et al.*, 2020).

2.3.2.2 Yogurt preparation and titration

2.3.2.2.1 First experiment

Cow milk and coconut milk were pasteurized at 80° C for 20 mins. The cow milk and coconut milk were mixed in a 1:1 ratio after it was cold (40°C) and then stirred until homogeneous. The mixture was added with 10% plain yogurt as a starter. Then it was incubated at 55°C for 5 hrs until it produced coconut milk yogurt. Approximately 5 mL samples from each treatment were added with solvent according to treatment (distilled water or ethanol) and stirred until homogeneous. Furthermore, the sample was added with 3-5 drops of phenolphthalein indicator and titrated with 0.1 N NaOH until it changed to pink colour. The required NaOH volume was measured.

2.3.2.2.2 Second experiment

Cow milk and coconut milk were pasteurized at 80°C for 20 mins. The cow milk and coconut milk (room temperature) were mixed in a 1: 1, then stirred until homogeneous. The mixture was added with 10% plain yogurt as a starter. Then it was incubated at 40°C for 4, 8 and 12 hrs to produce coconut milk yogurt. The procedure of yogurt preparation refers to Fatmawati *et al.* (2013). A 5 mL sample from each treatment was then added with appropriate solvent (distilled water or ethanol) and stirred until homogeneous. Then the sample was added with 3-5 drops of phenolphthalein indicator and titrated with 0.1 N NaOH until it changed to pink colour. The required NaOH volume was measured.

3. Results

3.1 Step 1

The first step of the study analyzed the acetic acid sample, cooking oil, and the mixture of acetic acid and cooking oil using alcohol and water as solvents. Table 1 shows that the acetic acid sample dissolved in distilled water required 8.5 mL of NaOH. It indicated that the amount of NaOH needed to react with the acetic acid in the sample was 8.5 ml. At the same time, the cooking oil sample dissolved in ethanol required 1.5 mL of NaOH. It was indicated that 1.5 mL NaOH was required to react with free fatty acids in cooking oil. The mixture of acetic acid and cooking oil dissolved in ethanol required 9.7 mL of NaOH. This amount of NaOH was the total of NaOH required to react with free fatty acids and acetic acid.

Table 1. Titration results in acetic acid and cooking oil.

Sample	Solvent	NaOH Volume (mL)
Acetic Acid	Distilled water	8.5
Cooking Oil	Ethanol	1.5
Acetic Acid + Cooking Oil	Distilled water	8.3
Acetic Acid +	Ethanol	9.7

3.2 Step 2

This research analyzed the free fatty acids contained in the food materials, which consist of organic acids (polar) and free fatty acids (non-polar). Yogurt was made from cow's milk and coconut milk, containing free fatty acids from coconut milk and lactic acid and fermented by *Lactobacillus bulgaricus*. The second step in the first (Table 2) and second (Table 3) experiments showed that ethanol required a higher NaOH volume than distilled water. If the sample is given distilled water solvent, NaOH only reacts with lactic acid (water-soluble organic acid). While yogurt was given ethanol solvent, NaOH

Table 2. Titration results in yogurt with different milk and coconut milk ratios and various solvents.

Sample	Solvent	NaOH Volume (mL)	Difference
Yogurt (100% cow's milk)	Distilled Water	7.05	0.05
Yogurt (100% cow's milk)	Ethanol	7.10	
Yogurt (75% cow's milk + 25% coconut milk)	Distilled Water	7.80	0.55
Yogurt (75% cow's milk + 25% coconut milk)	Ethanol	8.35	
Yogurt (50% cow's milk + 50% coconut milk)	Distilled Water	6.35	1.05
Yogurt (50% cow's milk + 50% coconut milk)	Ethanol	7.40	

Table 3. Titration results in yogurt (50% cow's milk + 50% coconut milk) at different long incubation times and solvents.

Sample	Solvent	Volume NaOH (mL)	Difference
Yogurt incubated at 40°C, 4 hrs	Distilled Water	13.30	1.40
Yogurt incubated at 40°C, 4 hrs	Ethanol	14.70	
Yogurt incubated at 40°C, 8 hrs	Distilled Water	13.95	1.55
Yogurt incubated at 40°C, 8 hrs	Ethanol	15.50	
Yogurt incubated at 40°C, 12 hrs	Distilled Water	16.15	4.05
Yogurt incubated at 40°C, 12 hrs	Ethanol	20.20	

https://doi.org/10.26656/fr.2017.7(6).060

reacted with lactic acid and free fatty acids. The difference between the titration results was the amount of NaOH that reacted with free fatty acids.

4. Discussion

Several studies have modified the free fatty acid test method, such as in soft cheese products using the method developed by Godinho and Fox (1981). The previous study by Amar *et al.* (2017) revealed that the higher the titration volume, the higher the concentration of free fatty acids in cheese. This method used non-polar diethyl ether to dissolve free fatty acids (Amar *et al.,* 2017). In soft cheese, diethyl ether will only dissolve free fatty acids, not organic acids (lactic acid). This procedure has a limitation because diethyl ether is highly expensive and limited. Furthermore, this method is complex since the non-polar portion must be separated first. Therefore, the development of a simpler and less costly method is needed.

The present study used ethanol as a solvent in the material that contained non-polar free fatty acids and polar organic acids. According to Sirwutubun *et al.* (2016), ethanol is an effective semipolar solvent to extract dyes from red fruit in the form of carotenoids. This compound had non-polar properties and a long carbon chain to be dissolved in ethanol. According to Marnoto *et al*. (2012) methanol and ethanol were protic polar solvents, which could provide OH-ions and easily interact with polar functional groups in tannins.

In this study, the titration results required 8.3 mL of NaOH on acetic acid and cooking oil mixture sample with distilled water as a solvent. The required NaOH volume was almost the same as NaOH in the acetic acid sample with distilled water as a solvent. The free fatty acids and organic acids with distilled water solvent were titrated by NaOH only organic acids. This was because distilled water (solvent) was polar, which only interacted with acetic acid, which was polar too. According to Sirwutubun *et al.* (2016), polar compounds only dissolve well in polar solvents, and non-polar compounds could dissolve well in a non-polar solvent.

The current study also showed that using ethanol solvent in samples containing oil and organic acids exhibited a mixture of free fatty acid and organic acid. The titration must be done twice by counting the total NaOH volume for free fatty acid. In the first and second titration, the solvent was ethanol and distilled water, respectively. The difference between NaOH volume in alcohol and distilled water solvent was NaOH which reacts with free fatty acids.

In the first experiment (Table 2), the more coconut milk was used, the difference in titration results was higher because the fat content in coconut milk was higher than in cow's milk. The fat content in coconut milk was 24.33% (Su'i *et al*., 2021) and 25.05% (Cahyono and Yuwono, 2015). In contrast, the fat content in cow's milk was 3.4% (Anindita and Sovi, 2017). The results of the second experiment (Table 3) showed that the longer incubation, the amount of free fatty acids in yogurt was higher. There was a decomposition of fat in coconut milk and cow's milk into free fatty acids during incubation; therefore, the free fatty acids in yogurt were high. According to Tamime and Robinson (1989), fat hydrolysis could occur by lipase enzymes produced by yogurt bacteria. *Lactobacillus acidophilus* and Bifidobacterium exert lipase enzyme activity of about 9.00 units, while *Lactobacillus bulgaricus* and *Streptococcus thermophillus* exhibit about 4.05 units of lipase activity (Adriani *et al*., 2008). Nurminabari *et al*. (2018) revealed that lactic acid bacteria hydrolyze fat in coconut milk into various organic acids (free fatty acids) in yogurt.

The second step is followed by the results of the first research step. The use of ethanol as a solvent required a higher NaOH than distilled water. Ethanol dissolves free fatty acid compounds and organic acids such as acetic acid and lactic acid. The difference between NaOH volume using ethanol solvent and NaOH volume using distilled water solvent was counted. The difference between ethanol and distilled water was the amount of NaOH that was reacted with free fatty acids also be measured by the following formula:

$$\text{mL NaOH FFA} = \text{mL NaOH (ethanol)} - \text{mL NaOH (distilled water)}$$

$$\text{FFA content} = \frac{\text{mL NaOH} \times \text{N NaOH} \times \text{BM FFA}}{\text{Sample (g)} \times 1000} \times 100$$

The titration results in the second step (Table 2) required about 7.05 mL of 0.1 N NaOH, which is equivalent to 1.27% of lactic acid. Fatmawati *et al*. (2013) revealed that the level of lactic acid in yogurt is 1.125 % with the same titration methods. The lactic acid levels in yogurt, according to SNI, ranged from 0.5%-2%. It was indicated that the analysis of lactic acid in yogurt is better to use titration with distilled water. In this study, yoghurt's free fatty acid levels from cow milk and coconut milk (Table 3) required about 15.55-13.95 = 1.55 mL NaOH or equivalent to 0.27%. While Irawan *et al*. (2013) revealed that free fatty acid levels from yogurt from cow milk are 3.22% with 3.66% of fat content, which is higher than our results. The high fatty acid levels in the report of Irawan *et al.* (2013) are due to one step of titration using ethanol solvent, which detects both free fatty acid and lactic acid. It was supported by the fat content results, which were almost the same as the free fatty acid content level. Thus, our study has proven that the free fatty acid method on materials containing fat/oil

https://doi.org/10.26656/fr.2017.7(6).060

and water-soluble organic acids should be done in two steps of titration.

4. Conclusion

The free fatty acid test method for food products containing organic acids and oil or fat must be carried out in two-step titration. The first step used ethanol and the second step used distilled water. The different titration results with ethanol solvent and distilled water solvent were NaOH volume required to react with free fatty acids.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The authors thank Widyagama Malang University for facilitating this research.

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