

## Evaluation of a colourimetric method for the measurement of oxidation in butter

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### Abstract

Butter is an emulsion of water in oil containing approximately 25% unsaturated fat. It is used in various types of cooking because of its delectable flavour. However, oxygen absorption by the unsaturated fatty acids alters its quality. Therefore, oxidation measurement is essential for the quality evaluation of butter. Although titrimetric analysis is mainly used for oxidation measurement in butter, it needs a large quantity of samples and reagents, and the results lack reproducibility. In this study, the colourimetric method was evaluated to measure oxidation levels in butter. A comparative analysis of the standard titrimetric methods and the colourimetric method revealed an increase in the oxidation levels with an increase in time. The findings demonstrated a positive correlation between the titrimetric and colourimetric methods. Furthermore, the measurement accuracy of the colourimetric method was less than 10% (1.7–9.5%) on all examination days. Overall, these findings suggest the efficiency of the colourimetric method to measure oxidation in butter with increased accuracy.

## 1. Introduction

Butter is a dairy product made by kneading fat grains of raw milk or cream. It has a cooking characteristic of shortening and a creamy property (Sugiyama *et al.*, 2008) and is used worldwide in various dishes, mainly sweets. Butter comprises approximately 17% water, 80% fat, and other components, including carbohydrates and ash (Asaoka and Habara, 2012). The total fatty acids in butterfat are approximately 72% saturated, 25% monounsaturated, and 3% polyunsaturated (Ministry of Education, Culture, Sports, Science and Technology, 2015). In oil, unsaturated fatty acids with more than three double bonds are oxidized much faster than those with one or two double bonds (Misharina *et al.*, 2019), whereas unsaturated fatty acids are more easily oxidized than saturated fatty acids. Several studies have also revealed that autoxidation of lipids is a free radical chain reaction (Yeo *et al.*, 2010). During this process, the hydrogen present in the methylene group next to the double bond is pulled out, consequently forming a lipid hydroxyl radical, which then extracts hydrogen from another lipid to form lipid peroxide, continuing the chain reaction (Kishimoto *et al.*, 2011). An accumulating body of evidence has shown that oxidation is a prime cause of the quality deterioration in butter. Therefore, it is necessary to suppress the oxidation of unsaturated fatty

acids to maintain the quality of butter, which could be achieved by accurately evaluating its extent.

Currently, in the quantification of primary and secondary products of oxidation (Yi *et al.*, 2013), peroxide value (POV), acid value, carbonyl value, and anisidine value are used as indices of deterioration of fats and oils (Ichikawa, 2009). Of these, POV, a basic index, is commonly used to determine the degree of oxidative changes in fats and oils (Chiba, 2017) and quantify the amount of lipid hydroperoxide—a primary oxidation product of fats and oils (Totani, 2001). This titration-based method of measuring POV has been widely adopted by the Japan Oil Chemists' Society, the American Oil Chemists' Society, and the International Union of Pure and Applied Chemistry as an official method (Totani, 2001). However, it requires relatively large volumes of samples and reagents and involves complicated and time-consuming steps. Moreover, a study using a modified ferric thiocyanate method with increased sensitivity has indicated the potential of colourimetric methods (Ichikawa *et al.*, 1996) to overcome the limitations of the widely used titrimetric method. However, the study did not explore the relationship between the oxidation time and the degree of oxidation.

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Therefore, in this study, a colourimetric method was evaluated for POV measurement in butter and the relationship between the oxidation time and the degree of oxidation was investigated. Additionally, we compared the efficiencies of the titrimetric method and the proposed colourimetric method to determine the measurement accuracy of the latter. This work is the first detailed comparison of titrimetric and colourimetric methods to measure oxidation in butter.

## 2. Materials and methods

### 2.1 Materials

Butter (Yotsuba butter) used in this study was obtained from Yotsuba Dairy Co., Ltd. One hundred grams of the sample were softened using a food processor (Panasonic Corporation) and kept in a state containing a certain amount of air. The processed sample was transferred into three transparent containers of 26 g each, wrapped, and allowed to stand in natural light at room temperature (approximately 25°C) for up to six days. The measurements were performed four times every two days—on days 0, 2, 4, and 6. Day 0 was the day of preparation, and the butter from the sample was collected after being uniformly stirred with a spoon.

### 2.2 Titrimetric method

The POV was estimated by the titrimetric method following the procedures described by Totani (2001). Briefly, approximately 5 g of the sample was precisely weighed in an Erlenmeyer flask and dissolved in 30 mL of a 2:3 chloroform: acetic acid mixed solution. Subsequently, 0.5 mL of a saturated potassium iodide solution was added. The mixture was shaken for 1 min and then left in the dark at room temperature (25°C) for 5 mins. Then, 30 mL of pure water was added, and the mixture was shaken, followed by the addition of 0.5 mL of 1% starch solution. Titration was performed with 0.01 M sodium thiosulfate standard solution. POV was calculated using the following equation:

$$\text{POV} = 10 \times (V - v) \times F/C \quad (1)$$

where V is the total volume of 0.01 M sodium thiosulfate standard titration (mL) used in this experiment, v is the 0.01 M sodium thiosulfate standard titration (mL) used in the blank test, and C is the sampling amount (g); F was set as a factor (F = 1.0) of 0.01 M sodium thiosulfate standard solution. The collected data were plotted on a graph with POV (meq/kg) on the vertical axis and the number of days elapsed (days) on the horizontal axis.

### 2.3 Colourimetric method

The colourimetric analysis was performed by

partially modifying the simple method for determining the POV of edible oils reported by Ichikawa *et al.* (1996). Approximately 1 g of the sample was weighed and dissolved in 10 mL of a 6:4 ethanol: diethyl ether solvent. Subsequently, 0.1 mL of a 30% aqueous ammonium thiocyanate solution was added and stirred, and 0.5 mL of an aqueous solution of 0.02 M iron (II) chloride and 3.5% hydrochloric acid was added and stirred to develop a red colour. After standing for 10 mins, the butter residue was precipitated by centrifugation (16,104×g, 5°C, 2 mins), and the absorbance was measured (495 nm) (A). The absorbance of the solvent was used as a blank (B), and the difference between the absorbances (A–B) was determined. The results were plotted on a graph with absorbance on the vertical axis and the number of days elapsed (days) on the horizontal axis.

### 2.4 Correlation between the titration and the colourimetric methods

The correlation between the titrimetric and colourimetric methods was analysed by estimating the correlation coefficient between the absorbance: the absorbance was plotted on the vertical axis and the POV on the horizontal axis for each elapsed day. Furthermore, the relationship between the POV obtained by the two methods was also investigated by plotting the POV value obtained by the colourimetric method against the POV value obtained by the titration method.

### 2.5 Investigation of measurement accuracy of the colourimetric method

The standard deviation of the measured absorbance on each elapsed day (n=4) was calculated to estimate the measurement accuracy of the colourimetric method using the following formula:

$$\text{Measurement accuracy (\%)} = \text{Standard deviation of } \quad (2)$$

### 2.6 Statistical analyses

Data were subjected to one-way analysis of variance using the least significant difference method, and t-tests were used for pairwise comparisons. A p-value of <0.05 was considered statistically significant.

## 3. Results and discussion

### 3.1 Relationship between oxidation time and degree of oxidation in titrimetric and colourimetric methods

The relationship between POV and elapsed days is shown in Figure 1. The POV of the samples obtained on days 0, 2, 4, and 6 increased quadratically with time (0.51, 1.8, 2.5, and 6.6 meq/kg, respectively) and were significantly different (p<0.05). A similar trend is also

observed in Figure 2. Absorbance values of the samples increased quadratically (0.26, 0.45, 0.83, and 1.7 on days 0, 2, 4, and 6, respectively) and were significantly different ( $p < 0.05$ ). The least-square curves fitting POV for the titrimetric method and absorbance for the colourimetric method against the elapsed days were quadratically high fitting ( $R^2 = 0.99$  and  $0.995$ , respectively), indicating the best fit model of the relationship in both the methods. Kanematsu *et al.* (1973) have reported that the POV of butter stored in a refrigerator at  $5^\circ\text{C}$  remained at  $0.60$  meq/kg for 12 months, similar to the day 0 value in this study. However, it was  $350$  meq/kg after 100 hrs in butter oxidized by air while heating at  $70^\circ\text{C}$  (Otake, 1961), which was higher than what was found in this study, which could be due to the differences in temperatures between the two studies (Zhao *et al.*, 2018). Furthermore, similar tendencies of increasing POV with time have been reported in olive oil rich in oleic acid (Kato *et al.*, 2008; Ahamad *et al.*, 2020), corn oil high in linoleic acid (Iritani *et al.*, 1978), and flaxseed oil high in linolenic acid (Nagasaka *et al.*, 1975; Kubo *et al.*, 2008). In addition, Figure 1 shows that POV tends to increase quadratically, and this tendency has also been reported in egg yolk lecithin (Fujita and Yamanaka, 1991), bonito and sardines (Miki *et al.*, 1994), and fish oil (Zhao *et al.*, 2018).

It has been shown that in radical oxidation of unsaturated fatty acids such as linoleic acid and  $\alpha$ -linolenic acid, POV depends on the amount of hydroperoxide produced in the early stages of fat oxidation, which increases with time (Oku *et al.*, 2005). Moreover, the oxidation of fats and oils occurs due to autoxidation, which begins when hydrogen is extracted from highly unsaturated fatty acids, and the free radical chain reaction proceeds (Kishimoto *et al.*, 2011). The hydrogen abstraction in autoxidation has been shown to occur with active methylene groups between the double bonds of unsaturated fatty acids, such as linoleic acid,  $\alpha$ -linolenic acid,  $\gamma$ -linolenic acid, arachidonic acid,

eicosapentaenoic acid, docosahexaenoic acid, and their esters (Schneider, 2009). Taken together, it can be inferred that the major unsaturated fatty acids in butter, including oleic acid, linolenic acid, and linolenic acid, are involved in its oxidation (Brat and Pokorny, 2000). Reportedly, autoxidation of fats and oils progresses in three stages: a slowly increasing start phase, a rapidly increasing chain reaction phase, and a decreasing end phase (Fukumoto and Iibuchi, 2000). Of these, hydroperoxide accumulates during the chain reaction phase (Fukumoto and Iibuchi, 2000). Moreover, it has been reported that hydroperoxide catalyzes the oxidation reaction (Xu *et al.*, 2021). Based on these studies, it is concluded that the quadratic increase in the degree of oxidation is regulated by the catalytic action of hydroperoxide.

The absorbance, which indicates the degree of oxidation in butter, increased over time (Figure 2). The modified ferric thiocyanate method used in this experiment is based on the principle that  $\text{Fe}^{2+}$  is oxidized to  $\text{Fe}^{3+}$  by hydroperoxide and develops a red colour by the reaction of  $\text{Fe}^{3+}$  and ammonium thiocyanate. Therefore, the amount of hydroperoxide has a positive effect on absorbance. The method of oxidizing  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$  for colourimetric measurement has also been used for measuring serum lipid peroxide (Yamamoto, 1991) and *in vivo* lipids (Miyazawa, 1992).

### 3.2 Investigation of the correlation between the titrimetric and colourimetric methods

As shown in Figure 3, POV increased with increasing absorbance and showed a high positive correlation ( $R^2 = 0.996$ ). Similarly, the POV calculated by the colourimetric method (calculated value) and the titrimetric method (true value) were also positively correlated ( $R^2 = 0.997$ ) (Figure 4). Furthermore, the regression equation obtained from the correlation between POV and absorbance was deciphered as follows:

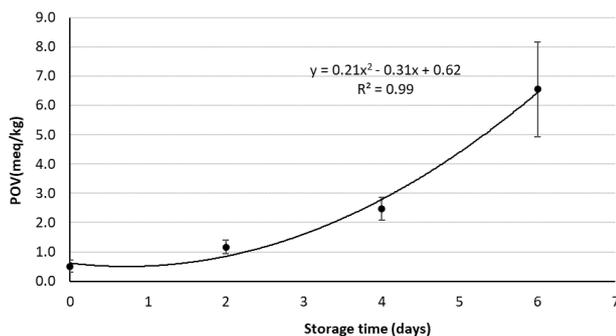


Figure 1. Changes in peroxide value (POV) of butter over storage time until six days. Bars denote the standard deviation of the mean ( $n = 3$ ). Mean POV differed significantly over time ( $p < 0.05$ ).

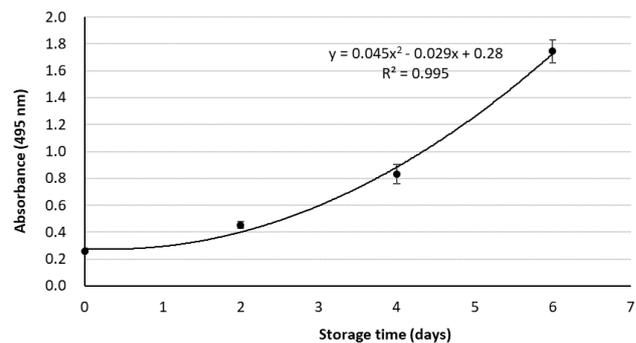


Figure 2. Changes in absorbance in the butter over storage time until six days. Bars denote the standard deviation of the mean ( $n = 4$ ). Mean absorbance differed significantly over time ( $p < 0.05$ ).

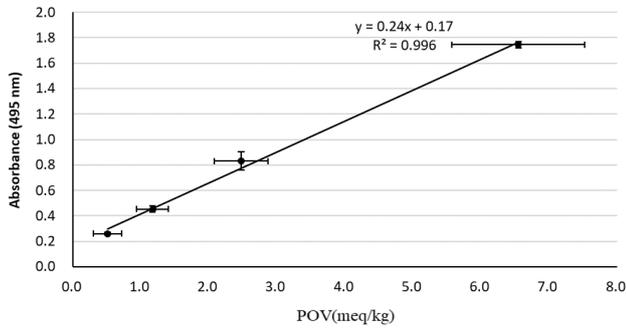


Figure 3. Correlation between peroxide value (POV) and absorbance. Bars denote standard deviation of the POV mean ( $n = 3$ ), and absorbance mean ( $n = 4$ ). Values from days 0, 2, 4, and 6 were used.

$$\text{Absorbance} = 0.24 \times \text{POV} + 0.17 \quad (3)$$

Using equation (3), the formula for estimation of POV was estimated as follows:

$$\text{POV} = (\text{Absorbance} - 0.17)/0.24 \quad (4)$$

Subsequently, by substituting the POV and absorbance from equations (1) and (2), we obtained the following:

$$10 \times (V-v) \times F/C = \{(A-B) - 0.17\}/0.24 \quad (5)$$

Intriguingly, the POV obtained through these regression equations was relatively well in agreement with the POV shown in Table 1. Taken together, these results suggest that the POV can be obtained from the absorbance value. The correlation between the colourimetric method using a xylenol orange (FOX) assay and the titrimetric method using iodometric assay has been reported in several fruit and vegetable species (DeLong *et al.*, 2002). This correlation has also been reported in refined vegetable oils, lard, and hydrogenated fats (Osawa *et al.*, 2007). Our study findings agree with the findings of these studies based on the same principle and using the same colourimetric method to assess the oxidizing action of hydroperoxide.

### 3.3 Investigation of measurement accuracy of the colourimetric method

Table 1 shows the standard deviation and measurement accuracy of the POV calculated from the absorbance on each elapsed day. The measurement accuracy was 9.1% on day 0, 8.7% on day 2, 9.5% on day 4, and 1.7% on day 6. These were well in agreement with the measurement accuracy of POV reported in previous studies for cooking oil (9.5%; Custodio-Mendoza *et al.*, 2020), low and high oxide oils (4.9% and 0.16%; Hafer *et al.*, 2020), and mayonnaise (5.9%; Merx *et al.*, 2018). Taken together, these findings indicate the high measurement accuracy of the colourimetric method used in this study.

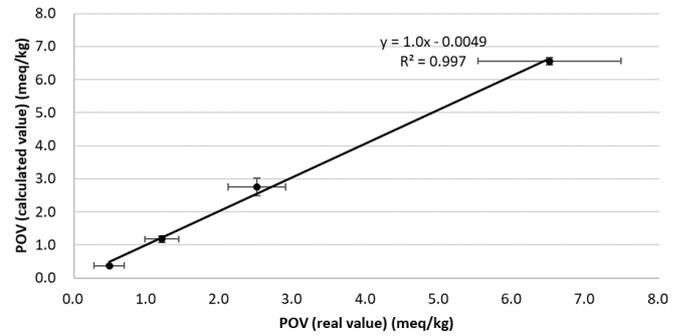


Figure 4. Correlation between peroxide value (POV; calculated value) and POV (real value). Bars denote standard deviation of the POV mean (calculated value;  $n = 3$ ) and POV mean (real value;  $n = 3$ ). Values from days 0, 2, 4, and 6 were used.

Table 1. The data on POV, standard deviation, and accuracy of measurement obtained through the colourimetric method

Storage time	POV	Standard	Accuracy of
0	0.38	0.034	9.1
2	1.2	0.10	8.7
4	2.8	0.26	9.5
6	6.6	0.11	1.7

## 4. Conclusion

In this study, we investigated a simple method for measuring POV, one of the indicators of alteration of lipid oxidation in butter. The measurement of oxidation using the widely used titrimetric method and the colourimetric method showed that the degree of oxidation increased with time. The study also demonstrated a high correlation between the two methods and the calculated and true values of POV in the colourimetric method. Furthermore, the estimation of POV from the absorbance values confirmed the measurement accuracy of the colourimetric method, which varied from 1.7% to 9.5%. Overall, from the findings of this study, it is evident that the colourimetric method is an efficient and easy method for the estimation of POV of butter and, therefore, could be useful for the food industry.

## Conflict of interest

The authors declare no conflict of interest.

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