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Analysis of Melamine on Powdered Milk-Based Diazoation Reactions with Eugenol Reagent By Spectrophotometry

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Abstract. The analysis of melamine on the milk-based diazotization reaction with eugenol was carried out. The presence of primary aromatic amine on melamine was reacted to sodium nitrite, while chloride acid formed diazonium salt, and then diazonium salt reacts with eugenol to form a colored azo compound. The absorbance was measured spectrophotometrically at a maximum wavelength of 470 nm. Analysis of melamine on the sample milk was determined by using a standard addition method. In this research, the optimization of concentration and incubation time was observed at the maximum formation of azo dyes. The optimization results were obtained at a concentration of eugenol of 9.10^{-3}M, $NaNO_2$ of 9.10^{-4} M, HCl of 0.25 M, and an incubation time of 5 minutes. The content of melamine in the milk sample was $2.513.10^{-3}$ M.

Keywords: Melamine, Reaction Diazotization, Eugenol, Spectrophotometric

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1 Introduction

According to information from the WHO (World Health Organization) 2008, in China, there was protein enrichment with the addition of melamine in baby milk, which was fatal because it caused the death of several babies due to kidney failure. Melamine is found in dairy-based foods such as yogurt, biscuits, and canned drinks. Based on information on the World Health Organization website, mixing melamine in milk begins with the mixing of milk with water. As a result of this dilution, the protein content of milk decreases. The protein content in milk raw materials is usually checked by determining the nitrogen content, the addition of melamine is intended to increase the protein content in order to achieve the specified standard.

BPOM (Food and Drug Supervisory Agency) has taken samples of imported products, especially from China through distribution facilities. Based on the test results of 19 imported products from China, 6 types of products containing melamine were found. All samples of

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imported products from China were found to be positive for melamine with melamine levels ranging from 8.51 mg/kg to 945.86 mg/kg (BPOM RI, 2008)

Various methods have been used to detect melamine levels in milk, including GC – MS (Litzau et al. 2008), LC-MS/MS (Andersen et al. 2007), Electrospray Ionization Mass Spectra (EESI – MS) (Zhu et al. 2008), HPLC-Diode Array (Sun et al. 2009), capillary electrophoresis (Cernova et al. 2009), LC-MS (Fligenzi et al. 2007) (Huang et al. 2008). Some of these methods require sophisticated equipment and are expensive and require a long time for sample preparation such as derivatization or extraction.

Spectrophotometry is a simple method that is widely available in the laboratory and has several advantages, namely it can be widely used in various quantitative measurements for organic compounds, high sensitive, selective, easy, fast, and uses color formation reactions. In previous studies, the use of spectrophotometry with color formation by diazotization reaction has been carried out, only the reaction time for color formation is still relatively long and needs to be increased by using a new coupling agent (Ida et al. 2012).

The coupling agent is a strongly activated aromatic ring, for example, phenols and aromatic amines (Hart, 2003). Eugenol is one of the derivatives of phenolic compounds which is a component of clove oil. Eugenol is easily isolated with NaOH and then neutralized with mineral acids. Clove oil is obtained by steam distillation from the leaves of the clove tree. The main product of the clove tree is the clove flower which contains essential oil, so its availability is affordable (Sastrohamidjojo, 2004). Therefore, there was a desire to investigate the use of eugenol as a coupling agent for the determination of melamine in milk powder by spectrophotometric method.

2 Materials and Methods

2.1 Equipments

Electric Scales, Spektronic 20, Glass Beaker, Dropper Pipette, Volume Pipette, Pipette Mat, Measuring Cup, Stirring Rod, Hot Plate, Funnel, Bottle of Water, Thermometer, Rubber Ball, Filter Paper, Micropipette.

2.2 Materials

$NaNO_2$, NaOH, Aquades, Melamine, $HCl_{(p)}$, Eugenol 99%, Glacial Acetic Acid, Milk powder, Ice cubes.

2.3 Determination of the optimum wavelength of melamine

5 mL of 4.10^{-3} M melamine solution was put into a 25 mL volumetric flask and cooled at 0 to 5ºC. Next, it was added by 3 mL of 0.25 M HCl, 3 mL of 9.10^{-3} M $NaNO_2$, 6 mL of 9.10^{-3} M

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eugenol, and then dissolved in distilled water up, then homogenized. The % transmittance was measured using a spectrophotometer at wavelengths of 430, 440, 450, 460, and 470 nm. The same procedure was carried out for aquadest as blank.

2.4 Determination of the optimum concentration of $NaNO_2$

5 ml of 4.10^{-3} M melamine solution was put into a 25 mL volumetric flask and cooled at 0-5°C. Next, it was added by 3 mL of 0.25 M HCl, 3 mL of 9.10^{-3} M $NaNO_2$, 8 mL of eugenol 9.10^{-3} M, and then diluted with distilled water, then homogenized. The % transmittance was measured at a wavelength of 470 nm. The same procedure was carried out for $NaNO_2$ with a concentration of 9.10^{-4} M and 9.10^{-5} M.

2.5 Determination of the optimum concentration of eugenol

5 mL of 4.10-3 M melamine solution was put into a 25 mL volumetric flask and cooled at 0-5°C. Next, it was added by 3 mL of 0.25 M HCL, 3 mL of 9.10^{-3} M $NaNO_2$, 8 mL of eugenol 9.10^{-3} M, and then diluted with distilled water, then homogenized. The % transmittance was measured using a spectrophotometer at a wavelength of 470 nm. The same procedure was carried out for eugenol with a concentration of 9.10^{-4} M and 9.10^{-5} M.

2.6 Determination of the optimum concentration of HCl

5 mL of 4.10^{-3} M melamine solution was put into a 25 mL measuring flask and cooled at 0-5°C. Next, it was added by 3 mL of 0.1 M HCL, 3 mL of 9.10^{-3} M $NaNO_2$, 8 mL of eugenol 9.10^{-3} M, and then diluted with distilled water, then homogenized. The % transmittance was measured at a wavelength of 470 nm. The same procedure was carried out for HCl with a concentration of 0.2; 0.25; 0.3; 0.35 M.

2.7 Determination of time optimization

5 mL of 4.10^{-3} M melamine solution was put into a 25 mL measuring flask and cooled at 0-5°C. Next, it was added 3 mL of 0.1 M HCL, 3 mL of 9.10^{-3} M $NaNO_2$, 8 mL of eugenol 9.10^{-3} M, cooled at 0-5°C for 2,3,4,5,6 and 7 minutes, and then diluted with distilled water, further homogenized it. The % transmittance was measured using a spectrophotometer at a wavelength of 470 nm. The same procedure was carried out for distilled water as a blank.

2.8 Calibration curve

Standard series solution of 4.10^{-4} M; 8.10^{-4} M; $1.2.10^{-3}$M; $1.6.10^{-3}$M was prepared by adding 5 mL of 2.10^{-3}M and put into a 25 mL volumetric flask, cooled it at 0 to 5ºC. Next, it was added by 4 mL of 0.25 M HCl, 3 mL of 9.10^{-4} M $NaNO_2$ and cooled at 0-5°C, further added 8 mL of eugenol 9.10^{-3} M. Then it was cooled at 0-5ºC for 5 minutes and diluted with distilled water and homogenized it. The % transmittance was measured at a wavelength of 470 nm. The same thing was done for distilled water as a blank.

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2.9 Analysis of melamine in milk

1 g of powdered milk was dissolved in 10 mL of warm water in a 50 mL glass beaker and transferred to a 100 mL volumetric flask. Next, it was added by 2 mL of 5% acetic acid and then diluted with distilled water, shaken, and allowed to stand until a precipitate is formed. Then filtered, the filtrate was pipetted 5 mL and put each into 25 mL volumetric flasks, then 0.1 M melamine was added as much as 1 mL, 2 mL, and 3 mL, respectively in 1, 2, and 3 volumetric flasks. Then added 4 mL of 0.25 M HCl, 3 mL of $NaNO_2$ 9.10^{-4} M, cooled it at 0-5°C, then added 8 mL of eugenol 9.10-3M, cooled at 0-50 for 5 minutes, and then diluted with distilled water, homogenized. The % transmittance was measured with a spectrophotometer at a wavelength of 470 nm.

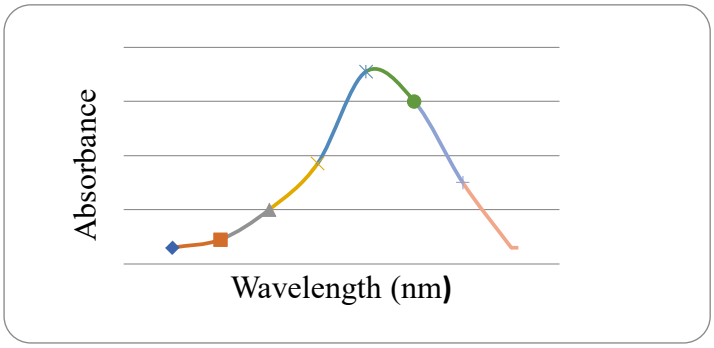
3 RESULT AND DISCUSSION

From Table 1 below, a plot of wavelength against absorbance was made to obtain the optimum absorbance spectra of melamine.

Table 1. The results of determining the optimum wavelength of melamine

Wavelength (nm)	Absorbance
430	0,366
440	0,369
450	0,380
460	0,397
470	0,431
480	0,420
490	0,390
500	0,366

From figure 1 below, it can be seen that the optimum absorbance of melamine was at a wavelength of 470 nm.



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Figure 1. The results of determining the optimum wavelength of melamine

The optimum concentration of $NaNO_2$ is 9.10^{-4} M, and it can be seen more clearly in Table 2 and figure 2 below.

Table 2. The results of determining the optimum concentration of $NaNO_2$

Concentration of $NaNO_2$	**(-Log Concentration of $NaNO_2$)**	**Absorbance**
9.10^{-5} M	5	0.3766
9.10^{-4} M	4	0.3979
9.10^{-3} M	3	0.3872

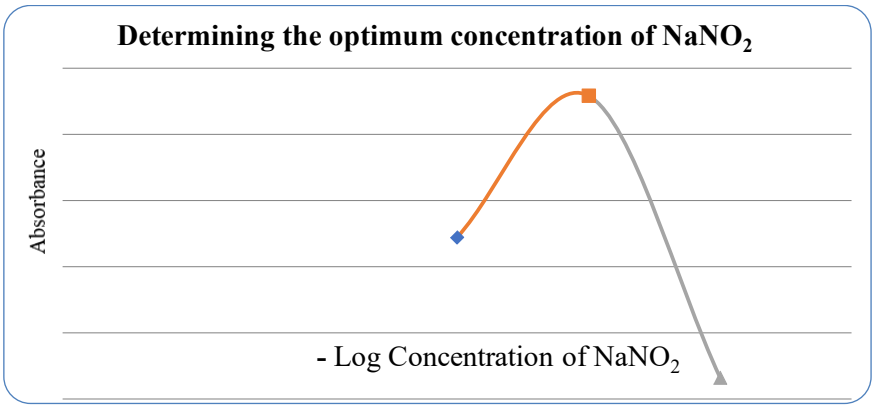


Figure 2. The results of determining the optimum concentration of $NaNO_2$

From Table 3 and Figure 3 below, a plot of wavelength against absorbance was made to obtain the optimum concentration of HCl. The optimum concentration of HCl was obtained at 0.25 M HCl with an absorbance of 0.394.

Table 3. The results of determining the optimum concentration of HCl

Concentration of HCl	**Absorbance**
0.10 M	0.369
0.20 M	0.373
0.25 M	0.394
0.30 M	0.390
0.35 M	0.373

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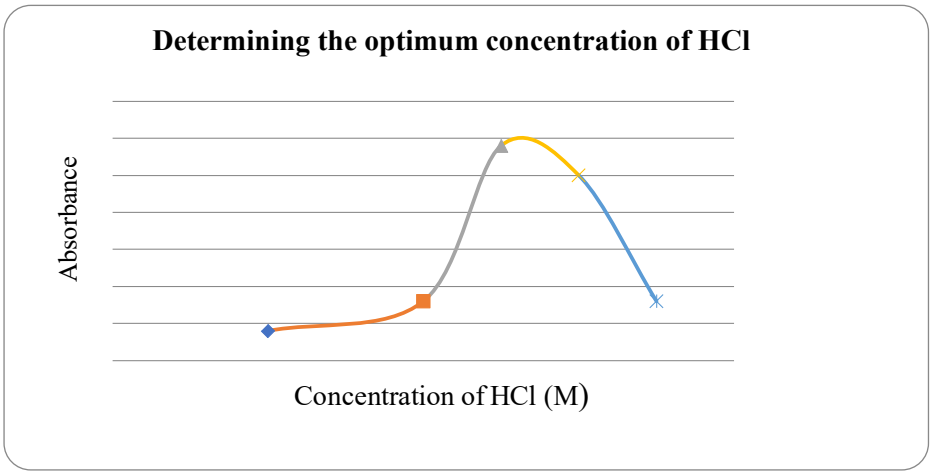


Figure 3. The results of determining the optimum concentration of HCl

The optimum concentration of Eugenol is 9.10^{-4} M, and it can be seen more clearly in Table 4 and figure 4 below. The optimum concentration of Eugenol was 9.10-3 M with an absorbance of 0.394.

Table 4. The results of determining the optimum concentration of Eugenol

Concentration of Eugenol	(-Log Concentration of Eugenol)	Absorbance
9×10^{-5} M	5	0.380
9×10^{-4} M	4	0.390
9×10^{-3} M	3	0.394

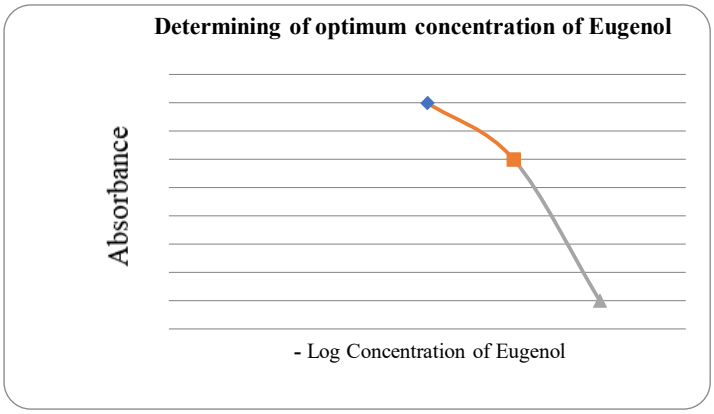


Figure 4. The results of determining the optimum concentration of Eugenol

Table 5. The results of determination of the time optimization

Time (minutes)	Absorbance
2	0,387
3	0,390
4	0,405
5	0,416
6	0,397
7	0,383

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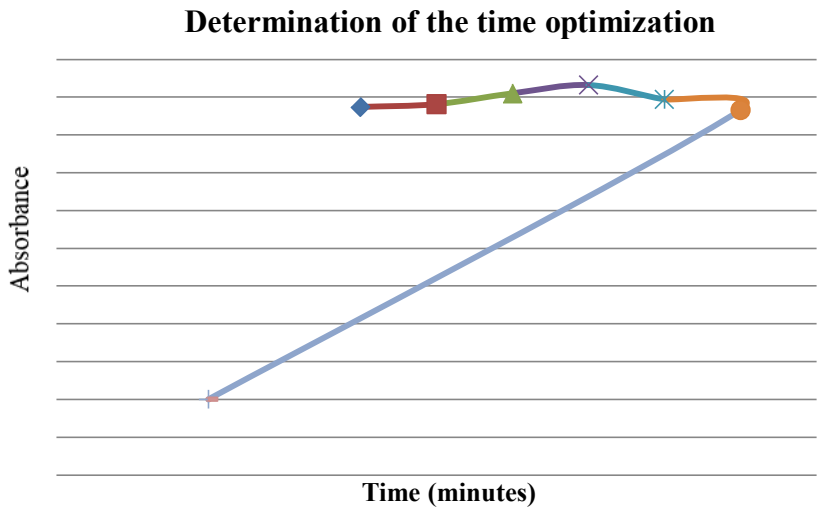


Figure 5. The results of the determination of the time optimization

From Table 5 and figure 5 above, the optimization of time is obtained at 5 minutes with an absorbance of 0.416. After the optimum concentration of the reagent is known, a calibration curve for the melamine standard solution is made using the least square method.

The regression line equation for the calibration curve is derived using the least square method, as shown in Fig. 6, the linear regression line equation Y = 163.92 x + 0.006, with a slope value which is a sensitivity of 163.92 and an intercept value of 0.007. Furthermore, the value of the correlation coefficient is determined, which is to estimate how well the point corresponds to a straight line. In this study, the correlation coefficient (r) was 0.995.

In this study, the standard addition method was carried out by adding standard solutions to samples with different volumes, so that the matrix in the sample with the matrix in the standard solution was the same, the only difference was the concentration, this method was carried out to minimize the matrix (component other than analyte) that may interfere with the response of the instrument to the analyte. While on the calibration curve of the matrix - the matrix does not exist.

The measurement of the concentration of melamine in milk samples was carried out by the standard addition method, 10 L, 20 L, and 30 L of 0.1 M melamine solution were added to the sample solution, respectively, and the absorbance of the sample was also measured as shown in Table 6.

Table 6. Results of measurement of sample concentration with the standard addition method

Concentration (M)	Absorbance

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0	0.022
2.10^{-5} M	0.033
4.10^{-5} M	0.054
6.10^{-5} M	0.065

Next, a plot of absorbance versus concentration is made and a standard addition calibration curve is obtained through negative extra polarization as shown in Figure 6. The melamine content was obtained at $2{,}513.10^{-5}$ M. With the dilution treatment, the final concentration of melamine was obtained at $1.25.10^{-4}$ M.

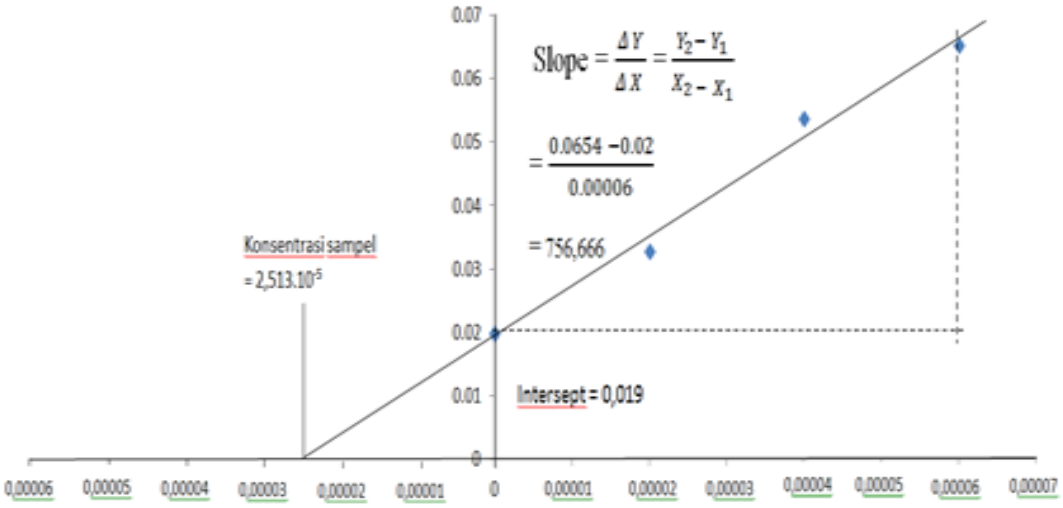


Figure 6. Measurement of sample concentration with standard addition method

4 Conclusion

From the data obtained in this study, it can be concluded that eugenol can be used as a coupling agent in the diazotization reaction, the azo compound formed is brownish orange in color and is absorbed in a wavelength of 470 nm. The results were obtained for the sample concentration of $1.25.10^{-4}$M. It is recommended for future researchers to use other couplings, and determine the limit of detection, precision, and accuracy of this study.

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