Determination of Formaldehyde in Tofu by Sequential Injection Analysis Using Acetoacetanilide as Hantzsch Reaction Reagent

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Abstract. Formaldehyde is a toxic and dangerous compound that is still often used freely by irresponsible traders or producers as food additives. At low concentrations, formaldehyde can cause eye, nose, throat and skin irritations. Exposure to large amounts of formaldehyde can cause severe pain, vomiting, coma, and even death. According to the International Program on Chemical Safety (IPCS), the threshold for formaldehyde in food that can still be tolerated in adult bodies is 1.5 mg to 14 mg per day while in a drinking water is 0.1 ppm. Determination of formaldehyde generally employ a titration method, which requires a large amount of sample and reagent, and long analysis time. This work aims to determine the formaldehyde in tofu commercially available in traditional markets of Malang City by Sequential Injection Analysis method using acetoacetanilide (AAA) at optimum conditions. Determination of formaldehyde is based on the Hantzsch reaction, which involves a cyclization between AAA and formaldehyde in the presence of ammonia so that it will produce a yellow dihydropyridine derivative product detected at a wavelength of 359 nm. The optimum condition for the formation of the reaction product occurred at an AAA concentration of 0.03 M, an ammonium acetate concentration of 1.5 M, a reaction time of 50 s and a flow rate of 75 µL/s. Excellent sensitivity and accuracy could be attributed to our developed formaldehyde detection method as the detection limit of 4.5 ppb and the recovery values of > 95 were achieved. Low reagent consumption, less waste production, and high throughput analysis are other advantages of our proposed method.

Keywords: Formaldehyde, Acetoacetanilide, Sequential Injection Analysis, Tofu.

1. Introduction

Formaldehyde is a toxic and dangerous compound that is still often used freely by irresponsible traders or producers. Formaldehyde is commonly used as a germ killer so that it is used for cleaning, preservatives, cosmetics and food additives. The ban on the use of formaldehyde as food additives has been stated in the minister of health regulation of Indonesia (Permenkes RI) No. 033/2012 [1]. The regulation does not make a number of people obey it because the price of formaldehyde is relatively cheap and easy to obtain. Through a number of surveys and laboratory examinations conducted by National Agency of Drug and Food Control of Indonesia (BPOM), a number of food products were found using formaldehyde as a preservative. Some examples of products that often contain formaldehyde such as salted fish, fresh fish, chicken, wet noodles and tofu are on the market.

The use of formaldehyde in food additives by manufacturers is intended to extend the life of storage, because formaldehyde is a versatile antimicrobial compound that can kill bacteria, fungi, and even viruses. In addition, the interaction between formaldehyde and protein in food produces a non-
brittle texture [2]. This longer storage is very beneficial for producers and traders. However, it is harmful to human safety. The danger posed by the consumption of formaldehyde itself is very serious and not beneficial to human health.

The impact of consuming formaldehyde is indeed not immediately visible but will be felt for years to come after levels of formaldehyde accumulate in the body. At low concentrations, formaldehyde can cause eye, nose, throat and skin irritation. Exposure to large amounts of formaldehyde can cause severe pain, vomiting, coma, and even death [3]. Therefore, ACGIH (American Conference of Governmental and Industrial Hygienists) sets the threshold for formaldehyde with in the body is 0.4 mg/L. Meanwhile, according to the IPCS (International Program on Chemical Safety), a special institution from three UN organizations namely ILO, UNEP and WHO cares about the safety of the use of chemicals, that in general the safe threshold of formaldehyde in food can still be tolerated in adult bodies is 1.5 mg to 14 mg per day and formaldehyde in drinking water preparations is 0.1mg/L [4].

This formaldehyde abuse is needed for the analysis of formaldehyde in food on the market. Previously, analysis of formaldehyde could be carried out by several methods such as volumetric titration, spectrophotometry, and chromatography. However, titration and spectrophotometry methods require long analysis times and use large amounts of reagents. In addition, the chromatographic method requires relatively expensive and complicated instrumentation. In the spectrophotometric method there are various kinds of reagents that can be used to test formaldehyde in food, including KMnO₄, K₂Cr₂O₇, FeCl₃, chromotropic acid, Schiff’s, Nash’s, Fehling and AgNO₃ reagents. In a previous study conducted by Yasri et al [5], the spectrophotometric analysis of formalin in rain water, wood products, and total cigarette smoke samples using tryptamine in a sulfuric acid medium produced a high error value so that the method was less sensitive. Additionally, the amount of waste produced was quite large. Therefore, a new alternative method is needed for the determination of formaldehyde using more environmentally friendly chemical reagent.

Currently, the popular methods for determining formaldehyde are Flow Injection Analysis (FIA) and Sequential Injection Analysis (SIA). The advantages of using a flow system are faster analysis time, less reagent consumption, less waste production, and high sensitivity. Qiong [6] developed the formaldehyde determination in waste water samples using the Flow Injection Analysis (FIA) method by employing acetoacetanilide (AAA) reagent. This method is based on the Hantzsch reaction; AAA is reacted with formaldehyde in the presence of ammonia to form a cyclic compound of dihydropyridine derivate which can be detected spectrophotometrically or fluorophotometrically. High sensitivity due to the largest molar absorptivity of AAA in comparison to other beta-diketone compounds and no need any heating procedures could be attributed as the benefits of this method. However, special attention should be paid since in the FIA system the reagent solution continuously flows during the analysis process which may contribute to large amount of waste resulted.

According to aforementioned facts, the Sequential Injection Analysis (SIA) was chosen in this study for formaldehyde analysis because the amount of reagents and samples could be taken as needed, resulting in less waste production in comparison to the FIA system. More sensitive analytical results could be achieved by the use of a mixing-tip in the proposed SIA system. In this work, all system is controlled by a computer using the laboratory-made SIA MVP Lite 2 software prepared by a visual basic program. The Hantzsch reaction reagents such as AAA and ammonia, and formaldehyde samples are dispensed into the mixing-tip, then a yellow product of dihydropyridine derivate is detected at a wavelength of 359 nm. Some parameters affected sensitivity of the proposed method which include concentration of AAA and ammonium acetate, reaction time, and flow rate were studied in detail. Using the optimized parameters, the developed SIA method was successfully applied to the determination of tofu samples in the traditional market of Malang city.
2. Material and Method

2.1 Apparatus and instrumentation

Some glassware, PTFE bottles, and micropipettes commonly used for analysis are used in this work. A set of Laboratory-made SIA system was constructed using a syringe pump (SP; Hamilton, Reno, Nevada USA) with a volume of 2500 μL, eight valve selection valve (SV; Hamilton, Reno, Nevada USA) and computer-controlled UV-Vis spectrophotometer detectors equipped with a flow cell (Shimadzu 1600 series), capillary tubing (PTFE 0.75 mm i.d) and holding coil (PTFE 1.8 mm i.d). The SIA system was controlled by SIA MPV LITE 2 software written with Visual Basic Program.

2.2 Reagents

All chemicals used in this work were of analytical reagent grades, and distilled water was used throughout the experiments for the preparation of all solutions. A 0.2M acetoacetanilide stock solution was prepared by dissolving 3.544 g of acetoacetanilide (Wako Pure chemicals, Osaka) in 100 mL of ethanol 30%. An ammonium acetate stock solution was prepared by dissolving 23.125 g of ammonium acetate in 100 mL of the distilled water. A 10 mg/L standard solution of formaldehyde was prepared by diluting 3.1 μL of 40% formaldehyde (HCHO) with the distilled water till the final volume of 100 mL. The working standard solutions were daily prepared by accurate dilution of the standard stock solution.

2.3 Sequential-injection procedure for the determination of HCHO in tofu

The manifold of the sequential-injection system used in this work is shown in Fig.1. In the proposed method, syringe valve is set in “out” position. Then, a syringe pump is set to aspirate 100 μL of AAA-ammonium acetate mixture reagent from the port 3 to the holding coil at a flow rate of 10 μL/s. Afterward, a syringe pump is set to aspirate 50 μL of formaldehyde/sample solution from the port 3 to the holding coil at a flow rate of 10 μL/s. Similar procedures were repeated to aspirate AAA-ammonium acetate mixture reagent and formaldehyde/sample solution from port 5 and port 6, respectively so that the segmentation of mixture reagent-sample-mixture reagent-sample formed in the holding coil. This segmentation is then dispensed into mixing tip (MT) via port 1 (flow rate: xx μL/s). The mixture is left to stand for 50 s in the MT. After complete reaction, the mixture is sent back to the holding coil. Then, a syringe pump is set to aspirate 1850 μL of distilled water at flow rate of 200 μL/s. Finally, the mixture of solution (reagent and sample) is dispensed into the detector at flow rate of 75 μL/s for measurement of dihydropyridine derivative product at the wavelength of 359 nm.

![Figure 1. SIA system for the determination of HCHO using acetoacetanilide as a reagent.](image-url)
3. Result and Discussion
This research was conducted to detect the formaldehyde content in the sample of know-trade using the Sequential Injection Analysis (SIA) method. The detection of formaldehyde is based on the formation of dihydropyridine derivatives from the yellow Hantzsch reaction. Before the detection of formaldehyde, the maximum wavelength measurements were carried out first and optimization of the concentration of AAA reagents, ammonium acetate concentration, reaction time and product flow rate towards the detector. Based on the results of scanning the maximum wavelength of the product using a UV-Vis spectrophotometer with a measurement range of 200-800 nm, the maximum wavelength is 359 nm. The molar extraction value is greater than 10000 dm$^3$ mol$^{-1}$ cm$^{-1}$ so that it can be said that the measurements made are sensitive. The results of scanning the maximum wavelength of the product can be seen in Figure 2.

![Figure 2](image_url)

**Figure 2.** The results of scanning the maximum wavelength of the reaction product at 200-800 nm

3.1 Optimization of reagent concentrations for SIA-spectrophotometric determination of formaldehyde
The AAA concentration needs to be optimized because to meet the adequacy it reacts based on the stoichiometry of the reaction that occurs to form the reaction product. Based on the Hantzsch reaction that occurs, AAA functions as a reagent or reagent which binds to formaldehyde and ammonia to form dihydropyridine derivative. Based on Figure 3, the absorbance values increased from AAA concentrations from 0.01 to 0.03 M. However, absorbance decreased from 0.04 to 0.06 M.

![Figure 3](image_url)

**Figure 3.** Effect of the concentration of AAA and the absorbance of the reaction product. Conditions: 50 µL formaldehyde, 50 µL ammonium acetate 2 M, variation of AAA concentration 0.01-0.06 M, reaction time 60 s and product flow rate to detector 50 µL/s.

The high AAA concentration causes the AAA number to increase so that the excess AAA that does not react with formaldehyde will react with ammonium acetate. So that at higher AAA
concentrations it does not increase the possibility of sensitivity because in the system there is a backlash and the product formed becomes small. From the measurement results, the optimum AAA reagent concentration is 0.03 M because it produces the maximum absorbance. The optimum AAA concentration value is used to perform further parameter optimization.

Ammonium acetate concentration needs to be optimized because to meet the adequacy it reacts based on the stoichiometry of the reaction that occurs to form derivatives of dihydropyridine. Based on the Hantzsch reaction that occurs, ammonium acetate serves as a buffer and source of ammonia to form dihydropyridine derivative products which are used as the basis of measurement. Based on Figure 4, the absorbance value increases from the concentration of ammonium acetate 0.5 to 1.5 M. However, the absorbance decreases from 2 to 2.5 M. This is probably because the reaction did not take place perfectly because there is still excess ammonium acetate. The excess ammonium acetate causes a shift in the equilibrium of the chemical reaction that occurs and produces fewer reaction products. From the measurement results, the optimum concentration of ammonium acetate is 1.5 M because it produces the maximum absorbance. The optimum ammonium acetate concentration value is used to perform further parameter optimization.

![Figure 4. Effect of the concentration of ammonium acetate and the absorbance of the reaction product. Conditions: as in Figure 3 with variations in the concentration of ammonium acetate 0.5 - 2.5 M.](image)

3.2 Optimization manifold parameters for SIA-spectrophotometric determination of formaldehyde

The reaction time is the time required by one reagent with another reagent to react at the mixing coil. This optimization aims to determine the length of reaction between one reagent and other reagents so as to produce the optimum absorbance value for measuring formaldehyde levels. The results of this optimization measurement are shown in Figure 5. Based on Figure 5, the absorbance increases from the reaction time of 10 to 50 s. However, at 50 to 70 s, absorbance tends to be constant. Reaction time needs to be optimized because to know that the system has reached equilibrium reaction or not. Therefore, the addition of reaction time that occurs can be seen from the effect on the formation of the reaction product.
In determining the optimum reaction time, the time used is 50 s because at 50 s the system has reached the equilibrium of the reaction so that the addition of the reaction time above 50 s does not affect the reaction product formation and there is no significant increase in absorbance statistically the t-test results with comparing the two average values at the time of 50 s and 60 s obtained by the value of \( t_{\text{count}} = 0.008 \) and the value of \( t_{\text{table}} = 2.78 \) means the value of \( t_{\text{count}} > t_{\text{table}} \) \((\alpha = 0.05)\) then the results obtained are not significantly different. In addition, to make the analysis time efficient the optimum reaction time is 50 s because at the time of the reaction one reagent with another reagent can react perfectly and increase absorbance. However, the longer the reaction time, the greater the dispersion of the product by the carrier solution. The optimum reaction time of 50 s is used to perform further parameter optimization.

**Figure 5.** Effect of the reaction time and absorbance of the reaction product. Condition: as in Figure 3 with a reaction time variation of 10 - 70 s.

Determination of the optimum flow rate was done by using AAA 0.03 M 50 μL, ammonium acetate 1.5 M 50 μL, formaldehyde 50 μL, reaction time 50 seconds and flow rate varied 50; 75; 100; 125; 150 μL / s. Flow rate optimization is done to see the detector's ability to detect the absorbance of the product. Apart from absorbance, determining the flow rate can be seen from the peak profile produced. The optimum flow rate will produce a peak that is tapered and not tailed. The results of this optimization measurement are shown in Figure 6.

**Figure 6.** Effect of the flow rate and the absorbance of the reaction product. Conditions: as in Figure 3 with variations in the flow rate of 50-150 μL/s.

Based on Figure 6, the absorbance values increase from the flow rate of 50-75 μL/s. However, with a flow rate above 75 μL/s the absorbance value decreases. This is because the use of a fast flow rate can cause a large back pressure. That way, it is possible to cause product dispersion by a carrier.
solution to produce a low absorbance value. From the measurement results that can be seen from the graph the optimum flow rate optimization is obtained at the flow rate of 75 μL/s because the absorbance produced is the greatest and produces a peak that is tapered and not tail so that it can increase sensitivity.

3.3 Calibration graph and analytical figure of merit

The sensitivity of the SIA method is determined by making the formaldehyde standard curve first and then measuring the LOD value from the blank with 10 measurements. The standard curve for determining formaldehyde is made by measuring formaldehyde concentration 0-0.15 ppm. The measurement of the standard curve uses all the optimum conditions parameters. The standard curve is made based on the results of measurements of the absorbance of each concentration of formaldehyde. The results of the measurement of the standard curve can be seen in Figure 7.

Based on Figure 7, shows that the concentration of formaldehyde is directly proportional to absorbance so that it has fulfilled the law of Lambert-Beer. From the standard curve obtained the line equation y = 2.3709x + 0.0128 with a coefficient of determination (R²) of 0.9909 where y is the measured formaldehyde absorbance and x is the concentration of formaldehyde. Detection limit (S / N = 3) from blank solution (n = 10) obtained based on a standard curve that is equal to 4.5 ppb.

![Figure 7. The Relationship Curve of Formaldehyde Concentrations and Absorbance.](image)

3.4 Application of the proposed method to tofu samples

In this work, the measurement of formaldehyde in tofu samples was carried out using the SIA method. The use of the SIA method for measuring formaldehyde levels in tofu trade samples uses three samples of tofu trade which are sold in traditional markets in Malang City. The sample absorbance that has been measured using a UV-Vis spectrophotometer detector is included in the standard curve equation y = 2.3709x + 0.0128 with a coefficient of determination (R²) of 0.9909.

| Samples | Concentration added (ppm) | Concentration recovered (ppm) | SD | %RSD | Recovery |
|---------|---------------------------|-----------------------------|----|------|----------|
| Tofu A  | 0.000                     | 0.096                       | 0.001 | 2.632% | -        |
|         | 0.120                     | 0.098                       | 0.004 | 3.784% | 96 %     |
| Tofu B  | 0.000                     | 0.110                       | 0.001 | 2.273% | -        |
|         | 0.120                     | 0.110                       | 0.003 | 2.542% | 95%      |
| Tofu C  | 0.000                     | 0.153                       | 0.001 | 1.639% | -        |
|         | 0.120                     | 0.157                       | 0.007 | 4.307% | 99%      |
Based on Table 1, it can be seen that know from markets A, B and C have very low levels of formaldehyde. In fact, the presence of formaldehyde in food is not always due to intentional addition but is naturally present in the range of 1 mg/Kg (ppm) as a result of the deterioration process. Deterioration process is a process that leads to damage or deterioration in the quality of food by bacteria during the storage period, for example lipid oxidation which causes rancidity. The deterioration of tofu by bacteria results in the formation of various proteins, amino acids, fats, and several types of aldehydes including formaldehyde [7].

The results of the formaldehyde measurements in the tofu samples were tested for accuracy using validity tests. From Table 1 it is shown that from the results of the validity test, the measurement of formaldehyde using SIA has high accuracy, with% recovery of each sample, namely 96%, 95% and 99%. Based on the results of the formaldehyde measurement test in the tofu sample, the SIA method can be recommended as a method of measuring formaldehyde. This is because this method is carried out with fast analysis time, high sensitivity and little sample volume and accurate measurement results, as evidenced by the results of % recovery reaching> 95%.

4. Conclusion
In this work, the optimum condition of formaldehyde measurement with acetoacetanilide reagent (AAA) using SIA in the tofu trade sample is at the AAA concentration of 0.03 M, ammonium acetate concentration of 1.5 M, reaction time of 50 s and product flow rate to the detector 75 μL/s. The SIA method is recommended as a method of measuring formaldehyde due to fast analysis time (160 s), high sensitivity (LOD=4.5 ppb) and accurate results (average %recovery=97%). Measurement of formaldehyde levels using the SIA method can be applied to samples of trading knowledge. Formaldehyde levels obtained in the three samples know A, B, and C in a row of 0.096 ppm; 0.110 ppm and 0.153 ppm.

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