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Analysis of Chloramphenicol in Shrimp Using Standard Addition Method Based on Diazotization

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ABSTRACT: This research aimed to determine the concentration of chloramphenicol in shrimp using the standard addition method based on the diazotization reaction using Zn powder as a reducing agent of chloramphenicol, followed by the use of N-(1-naphthyl) ethylenediamine dihydrochloride as a coupling agent and measured at 565 nm. Based on the test, the shrimp sample was found to contain 1964.91 µg/kg of chloramphenicol and it exceeded the requirements set by the European Commission which was 0,15 µg/kg. The limit of detection (LOD) value is 0.19 µg/mL and the limit of quantitation (LOQ) value is 0.64 µg/mL. The correlation coefficient (R^2) was 0.9991 for the concentration range of 0-50 ppm. The analysis of the results showed that the %recovery in shrimp analysis using this method was 87.41%-107.73% with an average of 109.38%.

KEYWORDS: Chloramphenicol, Shrimp, Diazotization Reaction, %recovery, LOD

INTRODUCTION

Due to Indonesia's status as one of the world's largest exporters of shrimp, shrimp is a valuable commodity in Indonesia's aquaculture. Production of farmed shrimp would reach 911.200.00 tons in 2020 [1]. Along with the development of shrimp farming, farmers are faced with several obstacles in producing errors such as diseases caused by *Aeromonas hydophila* which can be treated with antibiotics [2]. Chloramphenicol (CAP) is an antibiotic compound with strong antibiotic power and is active against both aerobic and anaerobic microbes. This antibiotic works by inhibiting the protein synthesis process of an organism, so it is widely used as a drug in aquacultures such as shrimp and fish to control the disease by increasing growth and production. The chloramphenicol structure is shown in Figure 1.

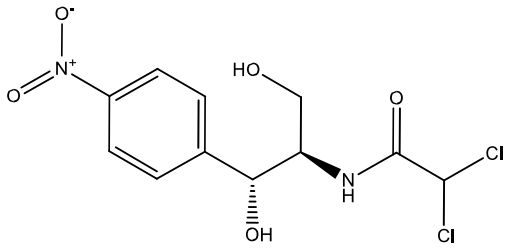

Figure 1. Chloramphenicol structure [3]

The use of CAP in aquaculture can cause accumulation and residue left in the tissue, organs, and flesh of these animals. Consumption of food products contaminated with chloramphenicol can cause allergic reactions for people with antibiotic allergies, decreased immunity, digestive tract disorders, and aplastic anemia. With several harmful effects of using chloramphenicol, the European Commission in 2019 set a minimum limit of chloramphenicol content in food of animal origin at 0,15 µg/kg [4]. In addition, in Indonesia the use of CAP antibiotics is prohibited from being added as a food additive and is regulated in the Indonesia National Standard (SNI) 01-6366-2000 in 2000 [5].

Several studies shows that numerous techniques have been employed to determine chloramphenicol, such as Enzyme-Linked Immunisorbent Assay (ELISA), High-Performance Liquid Chromatography (HPLC), and Liquid Chromatography-Mass Spectrometry (LC-MS) [6]–[8]. Each of these methods has advantages, but these methods require a log time analysis and expensive, so there is a possibility of degradation, which causes a decrease in the concentration of CAP. So, an easy, fast, and accurate method of analyzing CAP is needed. This research was conducted based on diazotization process in which the colourless chloramphenicol

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has been change into colour substances, then was analyzed using spectrophotometer UV-Vis. This method was quite fast and easy as the colour can be seen directly after process.

In the analysis of CAP in shrimp samples, it is necessary to do protein precipitation. Precipitation protein can be done by several methods, such as dialysis which removes the interfering substances but is not able to concentrate protein. In addition, organic reagents, Sodium Dodecyl Sulfate (SDS), and salts such as ammonium sulphate can also be used [9]. Ammonium sulphate has the ability to fractionate protein, so it is added salt to precipitate protein because it is able to maintain protein stability. However, the addition of this salt is required in high concentrations, so it is less effective. In addition, it can also be done with the addition of acids such as trichloroacetic acid (TCA). TCA is often used to precipitate proteins and purify protein from complex matrixes. [9]. The use of TCA was chosen because it is able to precipitate protein with the addition of TCA in small concentrations. Then it can be continued with the next stage of analysis.

In this research, chloramphenicol in shrimp was analyzed using the standard addition method, which is more accessible, cheaper, and more accurate in detecting CAP content using UV-Vis spectrophotometric analysis based on diazotization reactions. The standard addition method was chosen because the sample is added to a certain quantity of standard solution whose concentration is known accurately so that the sample's matrix does not influence the analysis. The diazotization reaction was chosen because it is simpler, more accurate, and cheaper in detecting CAP by producing an intensely colored azo dye solution that produces a maximum absorption at a wavelength at 575 nm [10]. Ravisankar [11] analyzed azo dye at a wavelength at 550 nm. So in this research, the absorbance of azo dye solution was measured at a wavelength at 550-575 nm.

MATERIALS AND RESEARCH METHODS

Tool and Material

The tools used in this study were UV-Vis Spectrophotometer (Shimadzu 1800), vortex (Wizard IR Infrared Vortex Mixer), 50 mL volumetric flask (Iwaki Asahi Glass), 500 mL beaker glass (Iwaki Asahi Glass), measuring cylinder (Iwaki Asahi Glass), and whatmann filter paper.

The materials used in this study were shrimp, chloramphenicol (CAP) (Sigma Aldrich), ethanol (Merck) 99,9%, methanol (Merck) 99,9%, trichloroacetic acid (TCA) (Merck), formic acid (Merck), HCl (Merck), aquabidest, N-(1-naphthyl) ethylenediamine dihydrochloride (Sigma Aldrich) 98%, Sodium nitrite ($NaNO_2$) (Sigma Aldrich) 97%, ammonium sulfamate (Sigma Aldrich) 98%, and Zn powder (Merck).

Research Methods

Application of CAP in Shrimp

Application in shrimp begins with 250 g of shrimp washed and separated from the shell and mashed. In a beaker glass, 100 g of mashed shrimp are placed. 100 mL of TCA 15% was added to stand for one night. Separate the filtrate and the residue. 10 mL of the filtrate should be divided among three 50 mL volumetric flasks. Each volumetric flask was added with 1 mL, 2,5 mL, and 5 mL of a standard solution of CAP 500 ppm. Methanol was added up to the mark. Thus, concentration of 10 ppm, 25 ppm, and 50 ppm were attained. The same procedure was used to generate blanks without the addition of 500 ppm standard solution of CAP. Then, the filtrate is separated from the residue.

Reduction of CAP

5 mL of sample filtrate was added with 2,5 mL of aquabidest, 2,5 mL of formic acid, and 0,3 g of Zn powder. Then it was stirred for 40 minutes with a rotation speed of 350 rpm. The solution was filtered.

Formation an Azo Dye

7 mL of the reduced filtrate was pipetted. Added 1 mL HCl, 1 mL $NaNO_2$ 0,2%, and 1 mL ammonium sulfamate 0,5%. Each addition was vortexed and allows to be kept at a cold temperature. The solution was added with ethanol and 0,5 mL of 0,1% N-(1naphthyl) ethylenediamine dihydrochloride and kept at a cold temperature for 20 minutes to form an azo dye.

RESULT AND DISCUSSION

Application of CAP in Shrimp

The first step in preparing shrimp samples was to precipitate protein from shrimp using a 15% TCA solution. This protein precipitation was carried out because CAP is relatively not bound to protein [12]. TCA was used to inactivate protease enzymes at

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low pH and modify the conformation of proteins so that proteins can precipitate and remove contaminants like salts and polyphenols [13]. As a result of the protein's isoelectric point, the addition of acid can result in changes in pH that change the protein's structure. Proteins become positively charged when acid is added because the amino groups on the protein capture protons and the pH decreases. Therefore, precipitation results from an imbalance in the protein structure [14]. The addition of strong acids such as TCA can trigger strong protein-protein interactions, as opposed to protein-solution, resulting in precipitation [15]. TCA 15% (w/v) is used because TCA concentration between 5%-45 % (w/v) is the most optimal for precipitating protein [14]. The photograph before and after addition of TCA shown in Figure 2. The obtained aliquots were then added to methanol. Methanol is used because CAP is highly lipid-soluble, and lipids are easily soluble in polar solvents such as methanol, ethanol, and chloroform. Additionally, methanol is a chloramphenicol solvent [12]. Methanol was employed to eliminate the remaining lipid interferences in the extracts by precipitation and it showed the highest lipid removal efficiency up to 97% [16]. Therefore, methanol is needed to draw CAP in aliquots and eliminate the lipid from the extracts.

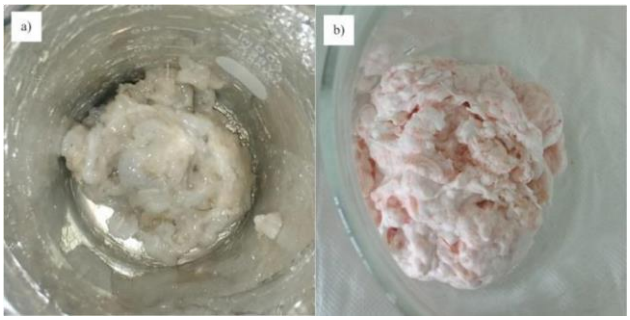

Figure 2. Shrimp sample a) before and b) after the addition of TCA

Reduction of CAP

The purpose of the reduction procedure is to convert the nitro group into a primary amine group. The formic acid solution is used because it produces high sensitivity, which is optimal for reducing the NO_2 group in CAP to N-H groups [17]. The mechanism of the chloramphenicol reduction reaction is shown in Figure 3.

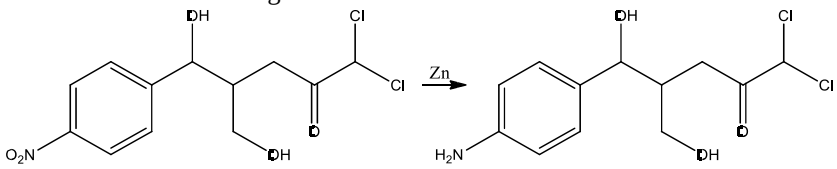

Figure 3. Reduction reaction mechanism [18]

Formation an Azo Dye

In the subsequent step, a solution of sodium nitrite ($NaNO_2$) and hydrochloric acid (HCl). is added to the reduced CAP. When sodium nitrite and hydrochloric acid react, nitrosonium ions and water are produced. Furthermore, the addition of ammonium sulfamate was carried out and did not affect the color intensity of the coupling reaction can reduce the excess sodium nitrite required for the reaction [19]. The nitrosonium ion then will react with reduced chloramphenicol to form a diazonium salt. All additions were stored at a cold temperature. This is due to diazonium salt's poor thermal stability, which requires low temperatures at 0-5°C [18]. The mechanism of diazonium salt formation is shown in Figure 4.

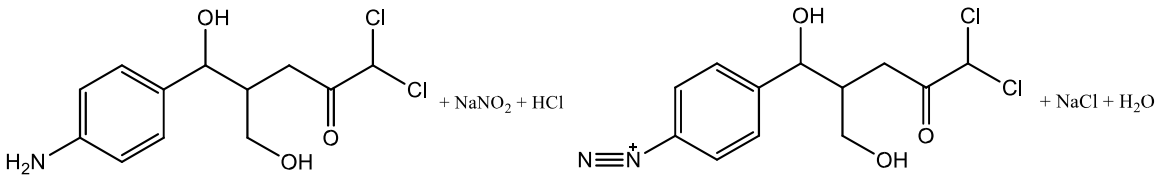

Figure 4. The mechanism reaction for the formation of diazonium salt [18].

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Diazonium cations react with N-(1-naphthyl) ethylenediamine dihydrochloride (NEDA) through electrophilic substitution, with diazonium salts as electrophiles to produce the purple azo dye shown in Figure 5. Purple-colored azo compounds can be identified by spectrophotometry at the maximum absorption wavelength of 565 nm. NEDA is used because it is highly sensitive, can be used in acidic solutions, and is widely used as a reagent for determining samples containing primary amine groups [11], [20]. The mechanism of formation of azo compounds is shown in Figure 6.

Figure 5. Azo dye from chloramphenicol

The results of the study on determining the content of CAP in blank shrimp samples obtained from the traditional market in Surabaya found the content of 1964.91µg/kg of CAP. The results of this study indicate that the sample of CAP in shrimp exceeds the European Union's minimum limit for CAP content which was 0.15 µg/kg. This indicates the sample does not meet the requirements for consumption. Based on the findings of this study, the agency in charge of food supervision should supervise the distribution of food more closely, and the consumer should be more selective in their food selections. Based on previous research, no CAP content was found in shrimp using the HPLC method. Table 1 shows a comparison of the analytical methods.

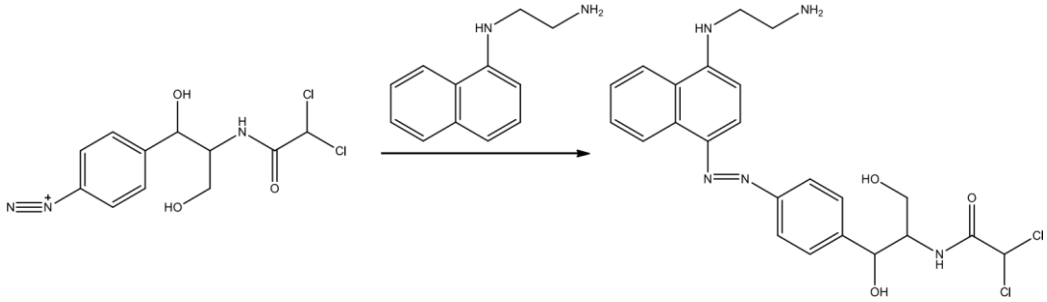

Figure 6. The mechanism of azo compounds formation [18].

Table 1. Comparison of HPLC Method with Diazotization on Shrimp Sample

Method	Area	Absorbance	CAP
HPLC	0	-	-
Diazotization	-	0.016	1964.91 µg/kg

High accuracy is one of the requirements for a good analytical procedure. This study used %recovery to determine the accuracy of the research. The calculation of %recovery addition was derived from the concentration of CAP added to the sample and analyzed using a diazotization reaction with a standard CAP concentration of 500 ppm before the shrimp were added. The results are shown in Table 2. The percentage recovery produced is very good, with a range between 87.41 to 107.73%, with an average of 109.38%. This value falls within the 80-110% permitted by AOAC in 2016, so the validation results based on the %recovery value satisfy the requirements [21]. This indicates that the recovery test for CAP in shrimps using the standard addition method based on the diazotization reaction has good accuracy. In order to determine the result of an analysis, a detection limit (LOD) and a quantization limit (LOQ) are required. In this study, the LOD was 0.19 µg/mL, and the LOQ was 0.64 µg/mL. Linearity in the study gave good results with the correlation coefficient (R^2 = 0.991) shown in Figure 7.

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Table 2. Percent Recovery

Code	%Recovery	%Recovery average
Blank	0	
10 ppm	87.41	109.38
25 ppm	107.21	
50 ppm	102.73	

Wafi, et al., [22] described a spectrophotometric method with a diazotization reaction at room temperature to analyze shrimp with a LOD of 0.36 µg/mL and a LOQ of 1.19 µg/mL. Sharma, et al., [10] detected chloramphenicol residues in milk with a diazotization reaction at room temperature resulting in a %recovery of 98.66-101.12%. Hussein, et al., [23] analyzed using an oxidative coupling reaction to produce LOD, LOQ, and %recovery of 0.241 µg/mL, 0.804 µg/mL, and 99.77-100.5%, respectively. The present study yielded a higher percentage of recovery and a lower detection limit based on prior research. This indicates that the CAP sample analysis using the standard addition method based on diazotization has greater accuracy, linearity, lower LOD, and LOQ. This study demonstrates that the standard addition method by diazotization reaction is an excellent method for enrichment and detection of CAP in shrimp samples and a quantitative and qualitative CAP analysis method.

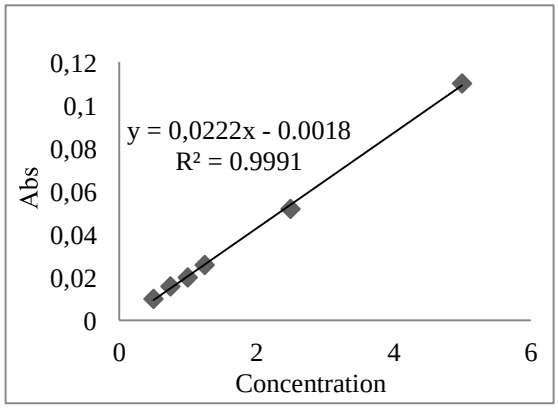

Figure 7. CAP calibration curve

CONCLUSION

Based on the test results, the shrimp sample did not meet the standards set by the European Commission, which found content 1964.91 µg/kg of CAP. The spectrophotometric method for CAP analysis using the addition method based on the diazotization reaction has good accuracy shown by %recovery was 87.41%-107.73% with an average of 109.38% and it has good linearity as shown by the correlation coefficient (R_2) was 0.9991. The limit of detection (LOD) value is 0.19 µg/mL and the limit of quantitation (LOQ) value is 0.64 µg/mL.

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