Method Validation of Chloramphenicol Analysis in the Shrimp Based on Diazotization Reaction

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Abstract
A simple, rapid and precise spectrophotometric method has been developed and validated for the determination of Chloramphenicol (CAP) in the shrimp based on diazotization reaction at room temperature. The CAP was reduced by zinc powder and the diazotization reaction was carried out in the presence of NaNO2, bismuth nitrate pentahydrate as catalyst. The 2-napthol used as coupling agent to form a red-violet solution and the absorbance of azo dye solution was measured by UV-Vis spectrophotometer at 554 nm. The method validation parameters including linearity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ) have been investigated. The correlation coefficient (R2) was 0.996 for concentration range 0.70 – 4.65 µg/mL. The LOD and LOQ were 0.36 µg/mL and 1.19 µg/mL. Accuracy and precision of the method were performed by spiking of CAP in the shrimp sample at concentration 1.16; 2.33; 3.49 µg/mL. Analysis result showed that the accuracy and precision of the method were 92.77-97.37 % and 0.21-2.39 % respectively.

Keywords: Chloramphenicol, diazotization, method validation, shrimp, spectrophotometry

Validasi Metode Analisis Kloramfenikol pada Udang Berbasis Reaksi Diazotasi

Abstrak
Sebuah metode yang sederhana, cepat dan presisi telah dikembangkan untuk penentuan kloramfenikol (CAP) pada udang berdasarkan reaksi diazotasi pada suhu kamar secara spektrofotometri. CAP direduksi dengan menggunakan serbuk seng (Zn) dan reaksi diazotasi dilakukan dengan mereaksikan NaNO2, bismut (III) nitrat pentahidrat (Bi(NO3)3.5H2O) sebagai katalis, dan β-naftol sebagai agen pengkopling untuk membentuk senyawa azo yang berwarna merah-ungu dan absorbansi diukur dengan spektrofotometer UV-Vis pada panjang gelombang 554 nm. Parameter validasi yang ditentukan antara lain linearitas, akurasi, presisi, batas deteksi (LOD) dan batas kuantifikasi (LOQ). Koefisien korelasi (R2) yang diperoleh dalam penelitian ini sebesar 0,996 untuk rentang konsentrasi 0,70-4,65 µg/mL, LOD dan LOQ masing-masing sebesar 0,36 µg/mL dan 1,19 µg/mL. CAP yang ditambahkan (spiking) ke sampel udang Litopenaeus Vanamie dan Litopenaeus Monodon sebesar 1,16; 2,33; 3,49 µg/mL. Hasil analisis menunjukkan akurasi dan presisi masing-masing berkisar 92,77-97,37 % dan 0,21-2,39 %.

Kata Kunci: Kloramfenikol, reaksi diazotasi, spektrofotometri, udang, validasi metode
1. Introduction

Chloramphenicol (CAP) is a bacteriostatic anti-microbial compound originally derived from the bacterium Streptomyces venezuelae. It is now synthesized chemically and has an antibacterial effect by interfering with protein synthesis in microorganisms. CAP has been commonly used in the treatment of bacterial disease in the aquaculture production such as shrimp and ornamental fish. The use of CAP antibiotic can promote growth and improve the production of aquatic products. However, the excessive use of CAP will lead to the existence of antibiotic residues in aquatic products. These residues may cause a risk for human consumption. It is well-known that CAP antibiotic may lead to bone marrow suppression, leukemia, and aplastic anemia in human beings. Hence, the residue of CAP in aquaculture products is strictly controlled.¹

Indonesia shrimp production had reached 785,900 tons in 2015 and most of them were exported to several countries such as Japan, the European Commission, and the United States. Those countries have strictly implemented zero tolerance of CAP residue in aquatic products. Furthermore, Indonesia also has banned the use of CAP as mentioned in Permenkes No. 722/Menkes/Per/IX/88. However, the illegal use of CAP still exists due to its low price and consistent antibiotic effectiveness. Therefore, it is still needed to develop different or simple approaches.

Spectrophotometric method is a very popular method or equipment owing to its easy-to-operate, specificity, and low cost. In this work, an analytical protocol for the determination of CAP in shrimp based on diazotization reaction was established. The diazotization reaction occurred at room temperature in the presence of Bi(NO₃)₃·5H₂O as a catalyst. 2-napthol was used as a coupling agent to form azo dye solution and the absorbance of azo dye solution was measured by UV-Vis spectrophotometer. The validation parameters such as linearity, precision, accuracy, LOD and LOQ of this method have been studied.

2. Method

2.1. Material

CAP reference standard was purchased from Sigma Aldrich, Singapore. Bismuth nitrate pentahydrate (Bi(NO₃)₃·5H₂O) was purchased from Merck, Germany. Ethanol, sodium nitrite, ethyl acetate, concentrated hydrochloride acid, 2-napthol, zinc powder (Zn) were pure analytical grade. Litopenaeus vanamei and Litopenaeus monodon shrimp were obtained from the local market in Surabaya, Indonesia.

2.2. Preparation of reagent

CAP 970µg/mL: CAP powder (0.097 g) was weighed quantitatively and dissolved in ethanol. The solution was transferred to a 100 mL volumetric flask and the same diluent was used to mark.

NaNO₂ 8x10⁻² M: NaNO₂ powder (0.5520 g) was weighed quantitatively and dissolved in distilled water. The solution was transferred to a 100 mL volumetric flask and made up with the same diluent to mark.

2-napthol 4x10⁻³ M: 2-napthol powder (0.0576 g) was weighed quantitatively and dissolved in 50 mL ethanol. The solution was
transferred to a 100 mL volumetric flask and made up with distilled water to mark.

2.3. Reduction of CAP

A 2.50 mL of CAP solution 970 µg/mL was added with 1 mL distilled water, 1 mL of concentrated hydrochloride acid and 0.15 g zinc powder, subsequently allowed for 15 minutes for reduction process. The solution was filtered and transferred quantitatively to a 25 mL volumetric flask and made up with distilled water to mark to obtain the reduced CAP 97 µg/mL.

2.4. Diazotization reaction and formation of azo dye

A 0.15 g of Bi(NO3)3.5H2O was added with 2.0 mL NaNO2 8x10⁻² M. The solution was then added with 3 mL of reduced CAP solution and 3 mL 2-napthol 4x10⁻³ M, allowed at room temperature for 8-9 minutes to form azo dye solution.

2.5. Spectrophotometric calibration curve

The standard solutions for calibration curve were prepared by transferring 1.5; 3.0; 5.0; 8.0 and 10.0 mL of reduced CAP 97 µg/mL in five different 25 mL volumetric flask and diluted with distilled water. The obtained standard solutions were 5.82; 11.63; 19.39; 31.02; 38.78 µg/mL.

Furthermore, the diazotization reaction was carried out for the five standard solutions. Subsequently, the obtained azo dye solution of each concentration was transferred to a 25 mL volumetric flask and diluted with distilled water to obtain CAP 0.70; 1.40; 2.33; 3.72 and 4.65 µg/mL. The absorbance of azo dye solutions were measured with UV-Vis spectrophotometer Shimadzu-1800 at 554 nm.

3. Results

3.1. Formation of azo dye

The principle reaction of this method is diazotization and following by coupling reaction to form red-violet azo dye solution as shown in Figure 2.

3.2. Method validation

A. Linearity

The linearity was tested with the standard CAP concentration range of 0.70 – 4.65 µg/mL as shown at Table 1.

The calibration curve, linear regression equations, and correlation coefficient (R) are shown in Fig. 3. The result showed good linearity with correlation coefficient (R²)
The sensitivity of this method was 0.0749.

B. Limit of detection and quantification
The limits of detection (LOD) and the limit of quantitation (LOQ) of the proposed method were 0.36 µg/mL and 1.19 µg/mL, respectively as shown at Table 2.

C. Accuracy and precision
The accuracy and precision of the proposed method were determined at three different concentration of standard CAP including 1.16; 2.33; 3.49 µg/mL. The results of accuracy and precision were 92.77-97.37% and 0.21-2.39 as shown at Table 3.

3.3. Application in shrimp
The standard CAP was spiked in the shrimp sample at concentrations 1.16; 2.33; 3.49 µg/mL, respectively. The result showed that the recoveries of CAP spiked in Litopenaeus vanamei and Litopenaeus monodon were 92.64-97.37 % and 93.83-104.25 % respectively. The relative standard deviations (RSD) of CAP spiked in Litopenaeus vanamei and Litopenaeus monodon were 0.39-1.96 % and 0.68-1.68 % respectively as shown at Table 4.

4. Discussion
In this study, the development of method for CAP analysis based on the diazotization
reaction has been performed by reducing of nitro group to amine group on CAP structure using zinc powder. Reduced CAP acts as primary aro-matic amine for establishing the diazonium salt on diazotization reaction. Generally, diazonium salt has poor thermal stability and must be han-dled around 0-5°C. But in this study, it kept sta-ble at room temperature because it was synthe-sized in presence of Bi(NO₃)₃.5H₂O as catalyst.

Generally, the reduced CAP, NaNO₂, and Bi(NO₃)₃.5H₂O were mixed homogeneously for 1 minute to form a diazonium salt. Further, the 2-napthol as a coupling agent was added to the diazonium salt and mixed homogeneously for 8-9 minutes to form a red-violet solution. The absorbance was measured by UV-Vis spectro-photometer at 554 nm. The proposed reaction and optimization of each analytical parameter had been reported.

In order to evaluate the performance of this method, the validation parameters such as linearity, precision, accuracy, LOD and LOQ have been evaluated. The result showed a good correlation coefficient (R² = 0.996) and sensitivity (0.0749). Alshirifi and Alhameedi reported a spectrophotometric method of CAP analysis base on condensation reaction. The result showed an identical correlation coefficient (R² = 0.9983) and lower sensitivity (0.0577). However, the LOD and LOQ were lower compared with the present work with nearly 0.068 and 0.207 µg/mL, respectively. Al-Abachi, et al., also proposed the determination of CAP in eye drop by using diazotization reaction in low tem-perature. The result exhibited a lower sensitivity compared with present method with nearly 0.0288. However, it had a better (lower) LOD of 0.1334µg/mL. From this point of view, the present method exhibited a higher sensitivity and LOD.

The accuracy of this study was 92.77-97.37 %. The result showed a slightly lower accuracy compared with previous method with nearly 98.5-104.4% and 98.6-100 %. In addition, the precision of this study was 0.21-2.39%. Alshirifi and Alhameedi; Al-Abachi, et al., have reported the precision of their methods were 0.23-0.67 % and 0.67-0.91 %. The com-parison of some parameters for CAP analysis in the proposed method and

**Table 4.** The recoveries and RSD of CAP spiked in shrimp sample

| Shrimp type       | Spiked levels (µg/mL) | Detected (µg/mL) (n=3) | Recovery (%) | RSD (%) |
|-------------------|-----------------------|------------------------|--------------|---------|
| *Litopenaeus vanamie* | 1.16                  | 1.08                   | 92.77        | 1.96    |
|                   | 2.33                  | 2.24                   | 96.12        | 1.19    |
|                   | 3.49                  | 3.40                   | 97.37        | 0.39    |
| *Litopenaeus monodon* | 1.16                  | 1.21                   | 104.25       | 1.68    |
|                   | 2.33                  | 2.18                   | 93.83        | 1.22    |
|                   | 3.49                  | 3.38                   | 96.98        | 0.68    |

**Table 5.** The comparison of CAP analysis in the proposed method and other literatures method

| Analytical parameters | Proposed Method | Literature Method 8 | Literature Method 13 | Literature Method 13 | Literature Method 14 |
|-----------------------|-----------------|---------------------|----------------------|----------------------|----------------------|
| Type of method        | Diazotization   | HPLC                | Diazotization        | Diazotization        | Diazotization        |
| Reagent               | 2-naphthol      | -                   | Imino dibenzyl       | 3-amino phenol       | N-methyl aniline     |
| λₘₕₐₓ (nm)            | 554             | 225                 | 590                  | 470                  | 504                  |
| Color of dye          | red-violet      | -                   | violet               | orange               | orange               |
| Temperature (°C)      | room temperature| 25                  | 0-5                  | 0-5                  | 0-5                  |
| Recovery (%)          | 92.77-97.37     | 81.1                | -                    | -                    | -                    |
| LOD (µg/mL)           | 0.36            | 0.024               | -                    | -                    | 100                  |
other previous method is shown at Table 5. The standard additions method on the shrimp samples (Litopenaeus vannamei and Litopenaeus monodon) was investigated to confirm the direct procedure. As shown in Table 4. The method exhibits a good accuracy (% recovery) and precision (%RSD) with nearly 92.77-104.25% and 0.39-1.96%. It indicated no interferences appeared during preparation of samples. On other word, the method is reliable enough on the determination of CAP in the shrimp samples.

5. Conclusions
A spectrophotometric method validation of CAP analysis based on simple diazotization reaction has been successfully performed. From the present study it can be concluded that the proposed method was simple, rapid, precise and accurate.

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