Method Validation of Contact and ImmersionTLC-bioautography for Determination of Streptomycin Sulfate in Shrimp

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ABSTRACT

Contact and immersion Thin Layer Chromatography (TLC)-bioautography have been developed for identification and quantification of streptomycin sulfate in shrimp. The TLC of streptomycin sulfate standard solution has been carried out by using silica gel F_{254} and 7.5 % of KH_2PO_4 solution as stationary and a mobile phase respectively. Retardation factor (Rf) of the streptomycin sulfate standard was 0.51 and the selectivity of streptomycin sulfate was 4.1 with the presence of kanamycin sulfate in the shrimp. The bioautograpy was performed by *Escherichia coli* ATCC 8739 as a test bacterial. Limit of Detection (LOD) of streptomycin sulfate obtained by the contact and immersion TLC-bioautography were 0.24 µg and 0.16 µg, respectively. Both methods showed good linearity with the r-value more than 0.999 and the Vxo value less than 2%. Accuracy of the contact and immersion TLC-bioautography were done using standard addition method and the obtained percentage recovery were 86.93±1.60% and 96.42±0.65%, respectively. Coefficient variation of the contact and immersion TLC-bioautography were 2.39±1.79% and 0.53±0.17 %, respectively. The immersion TLC-bioautography was more sensitive with better recovery than contact TLC-Bioautography. In addition, immersion TLC-bioautography method was successfully employed for determination of streptomycin sulfate in shrimp.

Key words : Streptomycin sulfate, contact TLC-bioautography, immersionTLC-bioautography, shrimp**.**

INTRODUCTION

Shrimp is one of Indonesia export commodities generated significant impact on the Indonesian economy. Highly exported demands sometimes makes uncontrolled cultivation because farmers generally use antibiotics to prevent the fish disease[1,2]. As regulated by the Minister of Maritime Affairs and Fisheries in the PER regulation number 02/MEN/ 2007[3], the fishery product must be free from drug residues, chemicals, biological materials and other contaminants. One of the antibiotics used by farmers for disease prevention is streptomycin. Streptomycin is aminoglycosides that used for treatment of infections caused by aerobic Gram-negative and also effective against Gram-positives such as *Staphylococcus aureus*[4]. In Indonesia, streptomycin usually use for overcoming bacterial disease in shrimp and ornamental fish[5]. According to the Codex Alimentarus, the maximum residue limit of streptomycin is 600µg/kg[6]. Antibiotic residues in food can be at risk to human health because the residues can

contribute to antibiotic resistance through the food chains[7]. Therefore, a fast and perfect analysis method is needed to detect antibiotic residues especially streptomycin sulfate in shrimp.

The TLC-bioautography method is used for determination level of antibiotics in complex samples based on the microbiological activities. In the TLC-bioautography, determination of antimicrobials level initiate by applying antimicrobial analytes on the TLC plate and eluted with suitable mobile phase. Contact TLC-bioautography method was performed by put the TLC chromatogram plate on the surface of the agar medium inoculated with the test bacteria and left in contact with the agar medium for a certain time for the diffusion process[8]. Subsequentially, the chromatogram plate is removed and incubated for 16-24 hours for the growth range, but can be reduced to 5-6 hours by spraying 2,6-dichlorophenol-indofenol or 2,3,5-tetrazoliumchloride on the surface of the test media. The antimicrobial activity was devoted by the inhibitory zone around the reservoir hole on the surface agar media or the spot position on the TLC-bioautogram plate, corresponds to the spots on the TLC chromatogram plate[9].

Immersion bioautography is a combination of direct and contact bioautography. The chromatograms are sprayed until the plate is covered by test media containing the test bacteria at temperature of 45^{o}C. The plate then cooled to condense and allow for diffusion process. Furthermore, the plate is incubated at a certain temperature for a certain time, then sprayed with tetrazolium salt to visualize the inhibitory zone.

Antibiotic analysis in the shrimp matrix such as kanamycin[10], oxytetracycline[11] and streptomycin sulfate[12] with the TLC-bioautography contact method have been reported. However, comparison of contact and immersion TLC-bioautography methods in determining the levels of streptomycin in frozen shrimp has never been reported, so it is necessary to conduct research to select a more effective method and provide results that meet the validation parameters.

MATERIALS AND METHODS

Chemicals

Streptomycin sulfate and kanamycin sulfate were obtained from PT Meiji, shrimp obtained from local market, *Escherichia coli* ATCC 8739, KH_2PO_4, nutrients broth and nutrient agar (Oxoid), sodium chloride p.a., methanolp.a., TLC silica gel plate GF $_{254}$ (Merck), Methyl thiazole tetrazolium (Sigma Aldrich), and distilled water (Otsuka),

Microliter syringe (Hamilton), Chromatographic vessel (10 × 10 × 6 cm^3), incubator (Memmert), caliper (Tricle brand), autoclave (Huxley HV-340 Speedy), Spectrophotometer Genesis 20, Shaker incubator (Thermo Fisher Scientific) were used in this study.

Preparation of growth media:

Eighteen grams of agar, 8 g of nutrient broth powder and 1000 mL of distilled water were mixed and heated until dissolved and homogeneous. The liquid medium were poured into the test tube (10, 15, and 20 mL) then covered with fat cotton. The media was sterilized by autoclaving at 121ºC for 15 minutes[13].

Preparation of Bacterial Test

Escherichia coli ATCC 8739 were inoculated on agar slant media and incubated at 35 - 37 ºC for 24 - 48 hours. The bacterial suspension was prepared by adding 10 mL of sterile saline (NaCl 0.9%) solution to a 24-hour culture and shaking with vortex until the entire colony is removed from the surface of the agar media. A 25% transmittance of bacteria was measured with Spectrophotometer at wavelength of 580 nm.

Loss on drying of shrimp samples

Loss on Drying was done according to Indonesian Pharmacope 5th edition[14]. Sample containers are heated at temperature of 105ºC for 30 minutes. Weighing the container until it reaches constant weight. One gram of the samples were weighing carefully and put into the constant container. Samples then put down in the oven with an open lid. Samples and the lid were heated at 105ºC until constant weight obtained. Loss on Drying was calculated using equation below:

Loss on Drying = (initial sample weight – final sample weight)/initial sample weight x 100%

Validation method of contact and immersion TLC-bioautography

The methods of analysis were validated for the parameters of selectivity, limit of detection, linearity, accuracy and precision. The accuracy was done using standard additon method.

Analysis using contact TLC-bioautography

A 8-µL of test solution was bottled to the silica gel TLC plate F_{254}, then eluted with 7.5% KH_2PO_4 solution as the mobile phase. Subsequently, the TLC plate was dried and attached to the surface of agar inoculated with *Escherichia coli* in a sterile petri dish. The TLC plate then stored in the fridge for an hour to let the diffusion and stain process of the compound to the media. Marks were made on the side of the plate followed by incubation of the TLC plate at 37ºC for 24 hours. Finally, the inhibitory zone was observed and its diameter was measured.

Analysis using immersion TLC-bioautography

Eight microlitres of test solution was applied to the silica gel TLC plate F_{254}, then eluted with 7.5% KH_2PO_4 solution as the mobile phase. Followed by drying the TLC plate and coated with 15 mL of inoculated - *Escherichia coli* media until a thin layer was formed. The TLC plate is stored in sterile petri dishes then incubated at 37ºC for 16-18 hours. The plates were sprayed with methyl thiazoletetrazolium (2.5 mg / mL) and finally observed the white-yellow inhibitory zone[15].

RESULTS AND DISCUSSION

The mobile phase 7.5% KH_2PO_4 solution used to eluate streptomycin sulfate, was based on the previous research.[13] The R_f results of contact TLC-bioautographic of streptomycin was presented in Table 1. The Rf values has met the requirement range of 0.2 - 0.8[17]. The loss on drying of the shrimp was 9.44 ± 1.85 % (Table 2).

The selectivity was done by spotting of the streptomycin sulfate, kanamycin sulfate standard solution, and shrimp on the F_{254} silica gel TLC plate. The elution was carried out by the 7.5% KH_2PO_4 solution. Selectivity test results of the contact TLC-bioautography method depicted in Figure 1 and Table 3. The data showed the Rf and Rs value of the streptomycin and kanamycin sulfate analyzed simultaneously using the contact TLC-bioautography method. The Rs value was 4.1 (Rs ≥ 1.5), which means that both analytes can separate well.

The detection limit was determined by antibiotic concentration in which still showing activity. The minimum inhibitory concentration (MIC) of streptomycin sulfate analyzed using contact TLC-bioautography was 30.4 mg/L with an amount of 8 µL of sample solution (equivalent to 0.24 µg of streptomycin). Whereas the MIC of the streptomycin analyzed by immersion TLC-bioautography was 20.3 mg/L (equivalent with 0.16 µg of streptomycin) (Table 4).

The linearity test of streptomycin in contact and immersion of TLC-bioautography was carried out in concentration range 100-250 mg/L. The linearity of the streptomycin analyzed using contact and immersion TLC-bioautography were y = 14.7212x – 23.2398 (r-value = 0.9992) and y = 12.6655x – 18.5557 (r-value = 0.9994), respectively (Figure 2 and 3).

Accuracy and precision were done by spotting three different concentration of streptomycin sulfate. The accuracy and precision results of streptomycin sulfate analyzed by two methods of TLC-bioautography were shown in Table 5 and Table 6, respectively.

The contact and immersion TLC-bioautography method developed for the determination of streptomycin sulfate and kanamycin sulfate were precise and reliable by only using single, cheap and hazardless solvent. Based on the TLC-bioautogram, the regression linier equation is capable of reliably predicting the analytes concentration in the range of 5–100 mg/mL and 0.1–100 mg/mL for streptomycin sulfate and kanamycin sulfate, respectively.

CONCLUSION

The method validated was successfully and can be used to simultaneously determine the streptomycin sulfate and kanamycin sulfate in a common market frozen shrimp. Those simple methods are recommended for monitoring antibiotics abused in frozen foods, especially for streptomycin at the concentration of 0.16 µg in the present of kanamycin sulfate.

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REFERENCES

1. Serrano P. H. Responsible use of antibiotics in aquaculture (1st ed). Rome; FAO; 2005: 469.
2. I. Dananjaya and A. Wahyujati. Peningkatan Daya Saing Produk Lokal Dalam Upaya Standardisasi Memasuki Pasar Global (Standardisasi Mutu dan Kualitas Udang Windu). UG Journal.2013; 6(2); 9-15.

3. Minister of Marine and Fisheries of The Republic of Indonesia. Control of Fish Drug Residues, Chemicals, and Contaminants in Consumption of Fish Cultivation Activities. Regulation of The Minister of Marine and Fisheries of The Republic of Indonesia. 2015; 1; 1-19
4. United States of Pharmacopeia.Aminoglycosides. Drugs for Animal Use. Veterinary-Systemic. United States of Pharmacopeia Convention. 2007; 1-30
5. K. Holmström, S. Gräslund, A. Wahlström, S. Poungshompoo, B.-E. Bengtsson, and N. Kautsky.Antibiotic use in shrimp farming and implications for environmental impacts and human health. Journal of Food Science and Technology. 2003; 38 (3); 255-266.
6. World Health Organization. Dihydrostreptomycin/Sterptomyicin. In : Maximum Residue Limits (MRLs) and Risk Management Recommendations (RMRs) for Residues of Veterinary Drugs in Foods (2nd ed). USA; FAO. 2018; 2; 18
7. R. C. Okocha, I. O. Olatoye, and O. B. Adedeji. Food safety impacts of antimicrobial use and their residues in aquaculture.Public Health Rev.2018; 39:21.
8. I. Choma and E. Grzelak.Bioautography detection in thin-layer chromatography. Journal of Chromatography A. 2011;19; 2684-2691.
9. S. Dewanjee, M. Gangopadhyay, N. Bhattacharya, R. Khanra, and T. K. Dua. Bioautography and its scope in the field of natural product chemistry.J. Pharm. Anal.2015; 5 (2); 75–84.
10. Mufida FA. Perbandingan Metode KLT-Densitometri Dan Bioautografi Untuk Penetapan Kadar Kanamisin Dalam Matriks Udang. Final Report. 2010.
11. Anggraeni FY. Perbandingan Validasi Metode KLT-Densitometridan KLT-Bioautograf iuntuk Penetapan Kadar Oksitetrasiklin dalam Udang. Final Report. 2010.
12. JahanbakhshiA.Determination of some engineering properties of snake melon (Cucumis melo var. flexuosus) fruit.Agric. Eng. Int. CIGR J., 2018; 20 (1); 171–176.
13. Astuti A. Validasi Metode Bioautografi untuk Determinasi Streptomisin Sulfat.Final Report. 2006.
14. Anonim. Penetapan Susut Pengeringan In : Farmakope Indonesia V. Jakarta : Departemen Kesehatan RI. 2014; 1043–1044.
15. Kusumaningtyas D. E, Astuti E. Sensitivitas metode bioautografi kontak dan

agar overlay dalam penentuan senyawa antikapang. J. Ilmu Kefarmasian Indonesia. 2008; 6(2): 75–79.

Table 1. Retardation factor (R_f) of streptomycin sulfate standard analyzed by contact TLC-bioautography

Mobile phase	Concentration of streptomycin sulfate (mg/L)	Rf
7.5% KH_2PO_4solution	50.75	0.53
	101.50	0.51
	152.25	0.50
	203.00	0.50
	253.75	0.53
Mean of Rf		**0.51**

Rf: Retardation factor

Table 2. Loss on drying of shrimp

Sample Name	Replicate	Initial weight (g)	Final weight (g)	LOD (%)	Mean of LOD (%)
Shrimp	1	1.0134	0.9187	9.34	9.44 ± 1.85
	2	1.0187	0.9204	9.65	
	3	1.0172	0.9240	9.32	

LOD: Loss of Drying

Table 3. Resolution (rs) value of streptomycin (s), kanamycin (k), and shrimp (u)

Compound Name	Rf (S)	Rf (K)	Rs
Streptomycin Sulfate (S)	0.51	-	-
Streptomycin + Shrimp (SU)	0.52	-	-
Kanamycin + Streptomycin + Shrimp (KSU)	**0.50**	**0.21**	**4.1**
Kanamycin (K)	-	0.24	-
Kanamycin + Shrimp (KU)	-	0.22	-
Kanamycin + Streptomycin (KS)	0.52	0.24	3.9

Table 4. Detection limit of streptomycin analyzed using contact and immersion TLC-bioautography method

Method of TLC-bioautography	Concentration (mg/L)	Inhibitory zone	Diameter of inhibitory zone (mm)
Contact	20.3	-	-
	30.4[(a)]	**+**	**4.90**
	40.6	+	6.10
	50.8	+	6.70
	60.9	+	8.10
Immersion	5.1	-	-
	10.2	-	-
	15.2	-	-
	20.3[(b)]	**+**	**3.50**
	30.4	+	4.60

(a) : detection limit of streptomycin sulfate in contact TLC-bioautography

(b) : detection limit of streptomycin sulfate in immersion TLC-bioautography

Table 5. Accuracy of streptomycin sulfate analyzed by contact and immersion TLC-bioautography

Method	Replicate	Added amount (µg)	Inhibitory Zone (mm)	Obtained amount (µg)	% Recovery	Mean of % Rec (± SD)
Contact TLC-bioautography	I	1.2992	8.50	1.1459	88.20	86.93 ± 1.60
	II	1.4616	9.20	1.2785	87.47	
	III	1.6240	9.70	1.3825	85.13	
Immersion TLC-Bioautography	I	1.2992	9.20	1.2432	95.69	96.42 ± 0.65
	II	1.4616	9.90	1.4119	96.60	
	III	1.6240	10.50	1.5746	96.96	

SD: Standard deviation

Table 6. Precision of streptomycin sulfate analyzed by contact and immersion TLC-bioautography

Method	Conc. (µg/L)	Inhibitory Zone (mm)			%CV	Mean of % CV
		I	II	III		
Contact TLC-bioautography	121.8	5.70	6.10	6.20	4.41	2.39
	162.4	8.30	8.60	8.45	1.78	
	203.0	10.10	10.30	10.20	0.98	
Immersion TLC-Bioautography	160.0	8.25	8.20	8.30	1.24	0.53
	180.0	8.60	8.65	8.65	1.41	
	200.0	9.10	9.00	9.10	1.57	

% CV: percent coefficient of variation

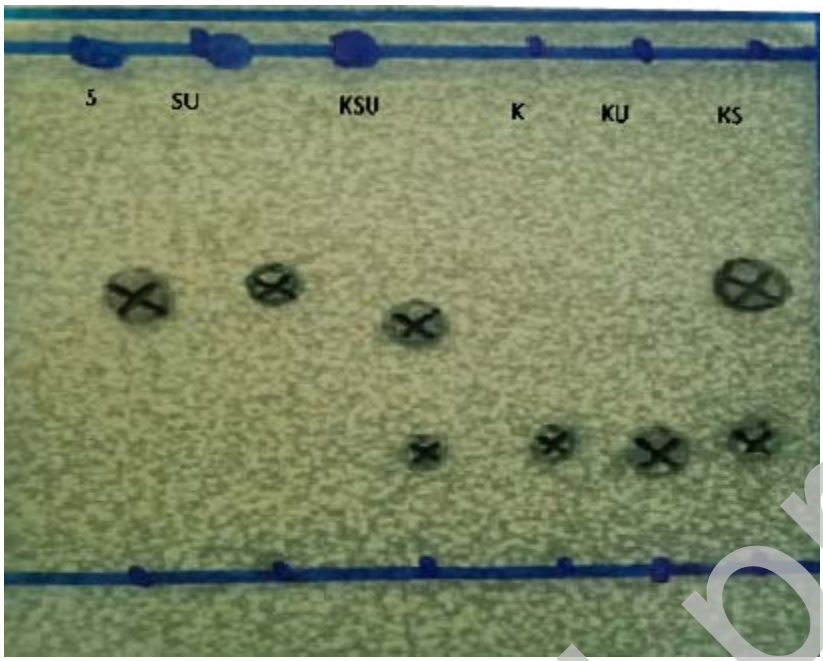

Figure 1: The retardation factor (R_f) of streptomycin sulfate (S), kanamycin sulfate (K) in shrimp (U) for the determination of resolution (R_s) value.

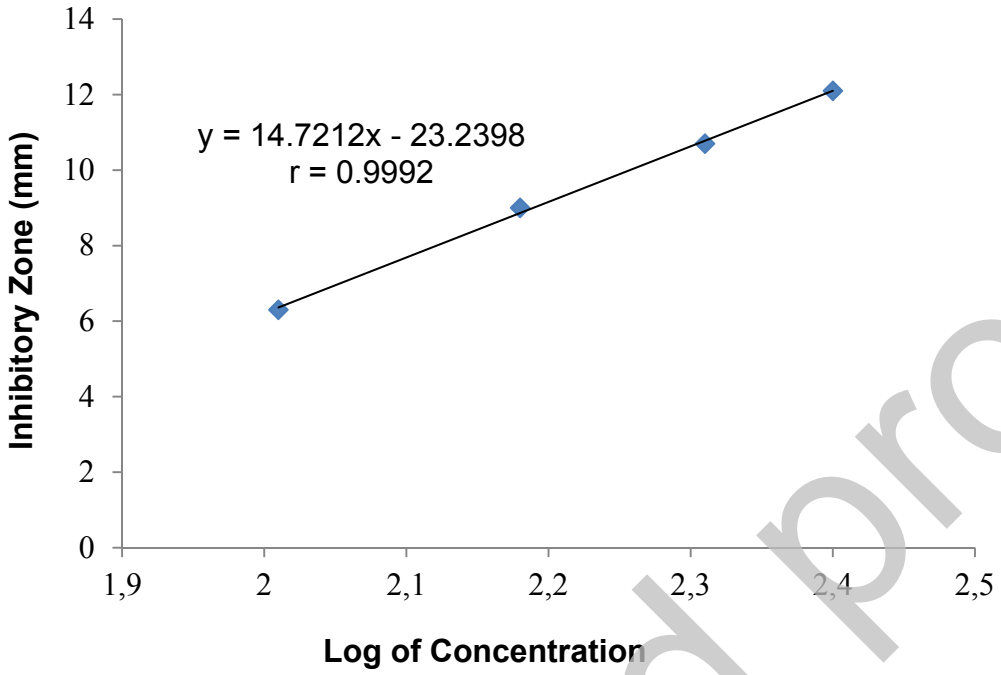

Figure 2: Linear regression of streptomycin sulfate analyzed using contact TLC-bioautography

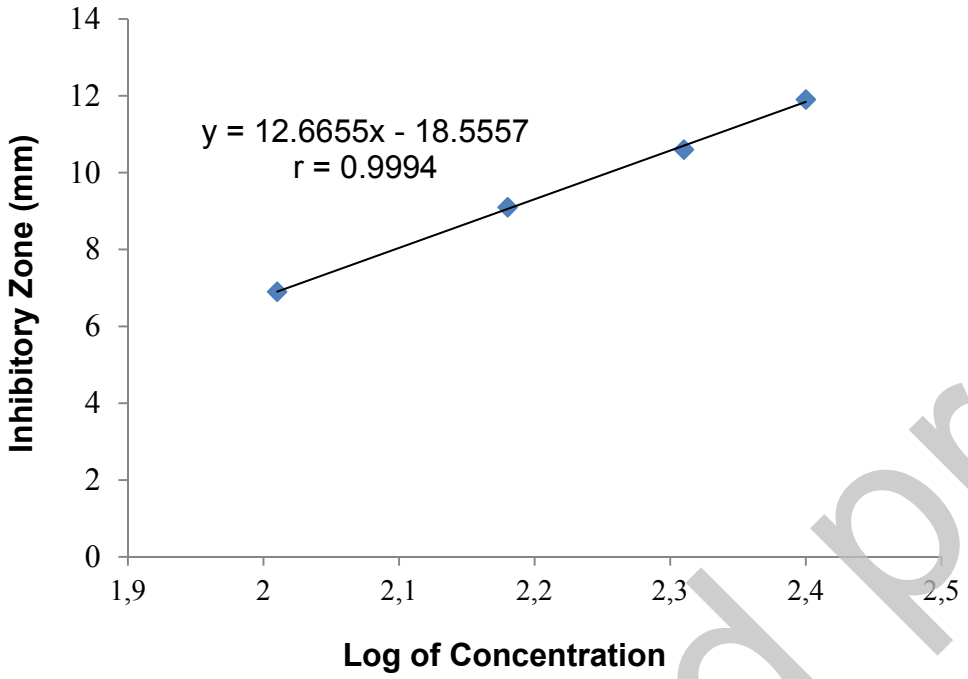

Figure 3: Linear regression of streptomycin sulfate analyzed using immersion TLC-bioautography