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Determination of Tetracycline Antibiotic Residue Levels in Goldfish (*Cyprinus carpio*) using High Performance Liquid Chromatography (HPLC)

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ABSTRACT. The method for determining residual levels of tetracycline antibiotics in goldfish (*Cyprinus carpio*) was validated using High Performance Liquid Chromatography (HPLC). The HPLC method has advantages, including high accuracy, efficiency, ease of operation, and high selectivity and sensitivity. This research aims to develop a method for determining tetracycline residues and tetracycline levels in goldfish meat obtained from eight markets in Bandar Lampung City. The analytical method carried out was optimized for the composition and flow rate of the mobile phase. Optimization results show a composition of acetonitrile:water = 35:65 and a flow rate of 0.5 mL/minute. The method validation parameters carried out are linearity, precision, accuracy, selectivity, Limit of Detection (LoD), and Limit of Quantitation (LoQ). The linearity results obtained from this research were R^2 = 0.9980. This method has repeatability precision with a Relative Standard Deviation (%RSD) value of 1.35%, and reproducibility precision with %RSD values of 0.96% and 1.14%, respectively. The %recovery method value is 101.05%. The LoD and LoQ values for this method are 0.27 and 0.90 ppm. Measurement of tetracycline antibiotic residue levels in carp samples from 8 market locations in Bandar Lampung showed values of 21.77 – 38.58 ppm, with uncertainties of 0.87 – 1.42. The method validation results show that the method can be used for routine analysis of tetracycline antibiotic levels in the laboratory.

INTRODUCTION

Goldfish cultivation is a freshwater fish cultivation that is currently growing rapidly in Indonesian society. Goldfish is widely cultivated because it offers advantages such as a good meaty taste, a savory flavor, a high protein content, and easy adaptation to Indonesian water environments. However, during development, carp farming often faces obstacles, including disease outbreaks, which necessitate the use of antibiotics (Preena *et al.,* 2021; Preena *et al.,* 2019). Antibiotics are antimicrobial drugs that can suppress or stop bacterial growth, thereby increasing fishery production (Lastauskienė *et al.,* 2021; He *et al.,* 2017).

Antibiotics commonly used in aquaculture include tetracycline, at 570 – 2790 µg kg^{-1} in the commercial feed sample bags used (Zhou *et al.,* 2021). Tetracyclines are a group of antibiotics produced by *S. aureofaciens* or *S. rimosus*. This tetracycline antibiotic offers advantages such as easy availability, low cost, high quality, and broad antimicrobial activity, making it widely used in human medicine, the livestock industry, aquaculture, and agriculture (Li *et al.,* 2021; Daghrir and Drogui, 2013).

Excessive use of the antibiotic tetracycline can lead to residues in fish and natural aquatic environments, posing potential human health risks through food consumption (Postigo and Richardson, 2014; Wang and Helbling, 2016; Urbano *et al.,* 2017). Although many countries issue guidelines for the use of antibiotics in aquaculture, inappropriate sales and use of antibiotics are common (Liu *et al.,* 2017). On the other hand, tetracycline residues in fish are derived from other sources, such as wastewater from agricultural areas, which can persist for a long time in the aquatic environment and are readily transported and distributed throughout aquatic

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systems (Alhaji *et al.,* 2021). Therefore, it is necessary to investigate the presence and levels of tetracycline antibiotics in freshwater fish farming, especially in goldfish. The maximum residue limit (MRL) for tetracycline antibiotics in meat, as specified in the Indonesian National Standard (SNI) 01-6366-2000, is 0.1 mg/kg. According to Orlando and Samionato (2013), the MRL of tetracycline in fish meat is 2 mg/kg, and the Codex Alimentarius Commission International Food Standards (2017) determines the MRL for the antibiotic group tetracycline to be 0.2 mg/kg in fish meat.

The presence of antibiotic residues in fish meat has been widely reported in previous studies; for example, Morshdy *et al.* (2022) reported that freshwater fish samples from Segiri Market, Samarinda, East Kalimantan, contained tetracycline residues. The research by Barani and Fallah (2015) showed that 63.1% of 38 trout samples from Iranian trout farms contained tetracycline residues, with the detected concentrations in positive samples ranging from 1.43 to 101.4 µg/kg. In addition, the research by Alarape and Adeyemo (2017) reported the detection of tetracycline residues in 69% samples of fresh and smoked fish, in which tetracycline was found in *Clarias gariepinus* collected from a selected fish farm and market in Ibadan, Nigeria. The average residue level of tetracycline detected (236 µg/g) was higher than the maximum recommended residue limit (200 µg/g) in fish muscle.

Although tetracycline antibiotic residues in fish are considered to be at very low concentrations (Cui *et al.,* 2018), long-term exposure can lead to a variety of human health problems, including increased antibiotic resistance and changes in the metabolism and composition of the gut microbiota (Wang *et al.,* 2016; Caniça *et al.,* 2019). The levels of tetracycline antibiotics in food are very low, so a more accurate, sensitive, effective, and efficient analytical method is needed (Önal, 2011; Abera *et al.,* 2021). The determination of tetracycline residue levels by High Performance Liquid Chromatography (HPLC) needs to be optimized and validated to ensure the method is appropriate and provides reliable results (Shalaby *et al.,* 2011). Therefore, in this study, acetonitrile and water were used as the mobile phase, and the optimization of the mobile-phase composition and flow rate was also conducted.

Optimization of the HPLC system for the determination of tetracycline residues in goldfish will use an acetonitrile:water mobile phase and an optimal C18 stationary phase for method validation. Based on the description, this study will optimize and validate a method for determining tetracycline in carp (*Cyprinus carpio*) meat using High-Performance Liquid Chromatography (HPLC). The validation parameters used in this study are precision, accuracy, linearity, selectivity, limit of detection (LoD), limit of quantification (LoQ), and measurement uncertainty, to ensure the results of the analysis.

RESEARCH METHODS

The tools used in this study include the main and supporting tools. The main tools used are an HPLC (Shimadzu) system with a SPD-M20A detector, a DGU-20A degasser, an LC-20AD pump, a CTO-20A oven, and C18 (Hypersil ODS) columns (5 µm, 4.6 mm × 250 mm). Other supporting equipment used includes an HPLC syringe, a Branson 1510 sonicator, a vacuum pump, a micropipette, an analytical balance, a pH meter, a blender, a magnetic stirrer, a centrifuge, a centrifuge tube, glass tools, and spatulas.

The materials used in this study are standard tetracycline, methanol (p.a.), acetonitrile (p.HPLC), *aquapure*, citric acid monohydrate (E. Merck), anhydrous hydrogen phosphate disodium (E. Merck), disodium EDTA dihydrate (E. Merck), HCl 0.1 N (E. Merck), Whatman filter membrane 0.45 µm *Nylon*, Whatman filter syringe 0.2 µm PTFE, filter paper, and carp meat.

Sample Collection

The sample used in this study was goldfish meat obtained from 8 market locations in Bandar Lampung City, namely Rajabasa Market, Untung Market, Way Kandis Market, Way Halim Market, Cimeng Market, Tugu Market, Yellow Bamboo Market, and Sukarame Market. The samples obtained in fresh conditions are then cleaned separately of their scales, dirt, skin, and bones. Each sample from each market location was first mashed with a blender, yielding a ±50 g sample of fish meat. Random sampling was applied from 8 locations in Bandar Lampung City. Random sampling did not review whether the fish came from the same or different ponds.

Sample Extraction

The mashed sample was weighed at 50 g, and 40 mL of EDTA-McIlvaine buffer (pH 4) was added. Next, the mixture was homogenized using a magnetic stirrer for 10 minutes. The sample was centrifuged at 3500 rpm

for 10 minutes, and the supernatant was collected. The obtained deposits were added to 20 mL of McIlvaine-EDTA buffer and centrifuged at 3500 rpm for 10 minutes. The supernatant was collected, and the sediment was resuspended in 20 m of EDTA-McIlvaine buffer, then centrifuged at 3500 rpm for 10 minutes. Next, the supernatant was collected and centrifuged again at 5000 rpm for 20 minutes. The results obtained were transferred to a 100 mL measuring flask and diluted with HCl 0.1 N to the limit mark; this solution is called the sample solution (Laboratory Quality Assurance Division, 2007).

HPLC Optimization

The HPLC used acetonitrile:water mobile phase (35:65) with a flow rate of 1 mL/min, an injection volume of 12 µL, and a wavelength of 355 nm. The optimization carried out was of the mobile phase composition (40:60, 35:65, and 30:70) and flow rate (0.5, 1.0, and 1.5 mL/min).

Method Validation

Based on the optimal HPLC conditions, the method validation was carried out using the following parameters, including linearity, precision, accuracy, selectivity, LoD, and LoQ. Linearity was determined by preparing a calibration curve of standard tetracycline solutions over a range of 5 concentrations, from 2 to 10 ppm (Dagron, 2014). Precision was determined by adding 0.1 mL of a 250 ppm standard solution to a 5 mL measuring flask and then adding the sample solution to the limit mark. Precision measurement of repeatability was carried out using HPLC with 6 replications on the same day, and a %RSD value was determined, while precision of reproducibility was assessed by repeating the analysis on different days (Eurachem, 2014). Precision was determined by adding 0.1 mL of a standard solution of tetracycline at 250 ppm to a 5 mL measuring flask, then adding the sample solution to the limit mark. Furthermore, 6 replications were conducted, and the %recovery was determined using Equation 1 (Dagron, 2014).

$$\%\text{Recovery} = \left(\frac{\text{Spiked concentration} - \text{Sample concentration}}{\text{Standard concentration}}\right) \text{x } 100\% \quad (1)$$

The determination of selectivity was carried out by making a standard solution of pure tetracycline and a standard solution of mixed tetracycline. Then, selectivity was determined by comparing the retention times of pure tetracycline standard solutions with those of mixed standard solutions (Eurachem, 2014). The LoD and LoQ for the samples were determined using calibration curves with concentrations of 2, 4, 6, 8, and 10 ppm (Dagron, 2014).

Determination of Tetracycline Antibiotic Residue Levels in Samples

The sample solution is pipetted by 1 mL and put into a 10 mL measuring flask, then diluted with methanol to the limit mark. The determination was carried out using 8 goldfish samples prepared with the validated HPLC method. The residual content of tetracycline can then be determined using Equation 1, where C_x is the concentration of analytes measured from the regression equation (mg/L), F_p is the dilution factor, V is the sample volume (L), and B is the sample weight (kg).

$$\text{Residue level (mg/kg)} = \frac{C_x \times F_p \times V}{B} \quad (2)$$

RESULTS AND DISCUSSION

HPLC Optimization

Table 1 shows the optimization results of the mobile phase composition carried out, namely with a ratio of 30:70; 35:65; and 40:60 (acetonitrile:water), where it can be found that the retention time (Tr) obtained from all the mobile phase composition comparisons has met the requirements of good retention time according to the Indonesian Pharmacopoeia Edition IV (1995), which is ≤ 10 minutes. However, the composition that best meets the requirements for column efficiency and tailing factor is 35:65. The chromatogram of the mobile phase optimization results is shown in Figure 1.

Table 1. The value of the mobile phase composition optimization parameters.

Mobile phase (acetonitrile:water)	Tr (minutes)	N (≥ 2000)	HETP	TF(≤ 2)	Compliance
30:70	3.763	3,497	71.5	5.954	No
35:65	3.418	2,638	94.7	1.784	Yes
40:60	3.304	877	284.8	1.191	No

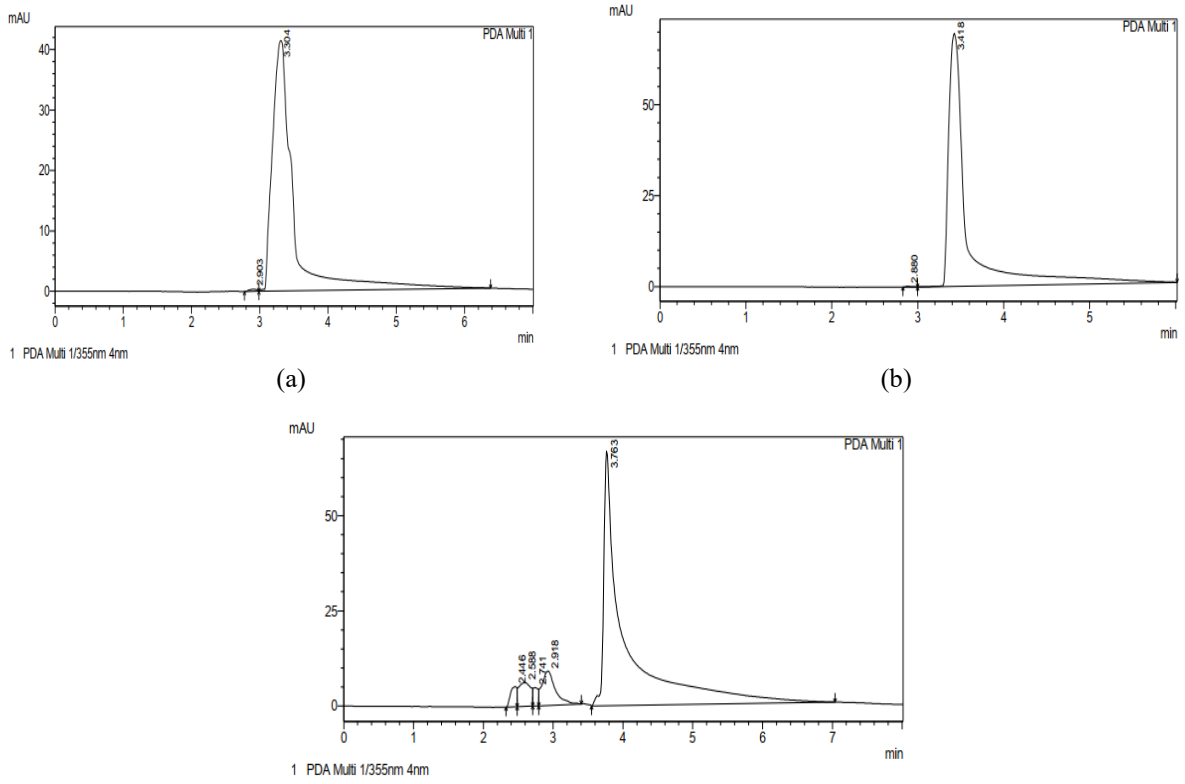


Figure 1. Chromatograms of mobile phase optimization results (a) Acetonitrile:Water (40:60), (b) Acetonitrile:Water (35:65), and (c) Acetonitrile:Water (30:70).

Table 2 shows the results of the flow rate optimization carried out, namely 0.5, 1, and 1.5 mL/min, using the optimal composition of acetonitrile and water mobile phases. It can be seen that the retention time (Tr) obtained across all flow rate variations meets the requirements. A flow rate of 0.5 mL/min has a tailing factor (TF) value that is lower than a flow rate of 1 mL/min, which is 1.2484 (the closer to 1, the more symmetrical), and the number of theoretical plates (N) at a flow rate of 0.5 mL/min is greater than the flow rate of 1 mL/min, which is 4078, and the height equivalent to a theoretical plate (HETP) is low, which is 61.3. The flow rate that best meets the requirements for column efficiency and TF is 0.5 mL/min. The chromatogram is shown in Figure 2.

Table 2. Value the flow rate optimization parameters.

Flow Rate (mL/min)	Tr (minutes)	N (≥ 2000)	HETP	TF (≤ 2)	Compliance
0.5	6.973	4078	61.3	1.248	Yes
1	3.388	2424	103.2	1.906	Yes
1.5	2.253	1790	139.7	2.292	No

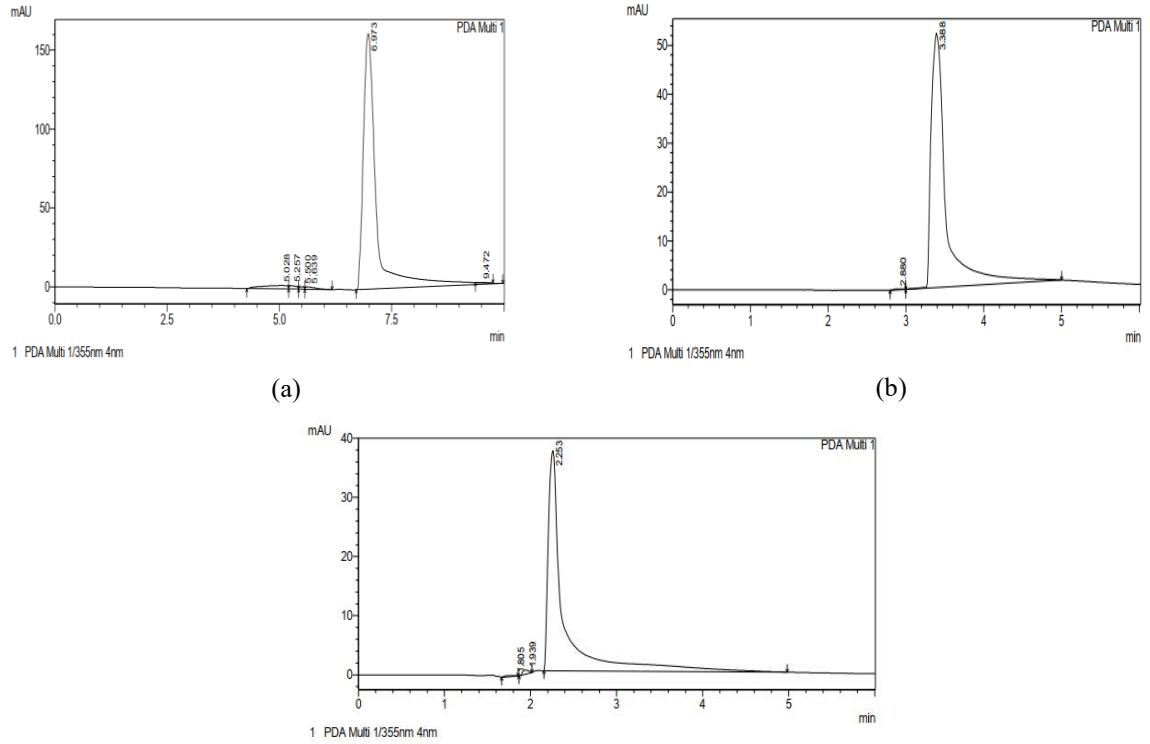


Figure 2. Chromatograms of flow rate optimization results: (a) Flow rate 0.5 mL/min; (b) Flow rate 1 mL/min; and (c) Flow rate of 1.5 mL/min.

Method Validation

The linearity determination showed good results, with a correlation coefficient (R^2) of 0.9980 for tetracycline antibiotics and a regression equation of y = 152.88x + 124.49 (Figure 3). The value of the correlation coefficient is within the conditions of method validation analysis according to Eurachem (2014), with a correlation coefficient value of ≥ 0.990. The linearity test can also be seen from the residual value plot. Figure 4 shows that the residual value of the standard tetracycline solution is randomly distributed on the x-axis.

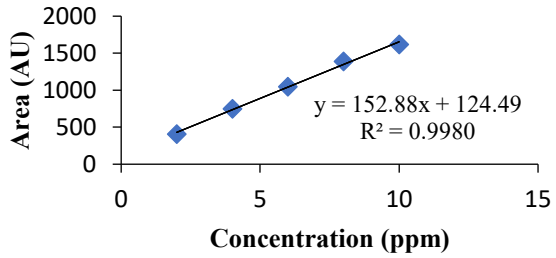


Figure 3. The linear curve of tetracycline antibiotics based on the relationship between concentration and area.

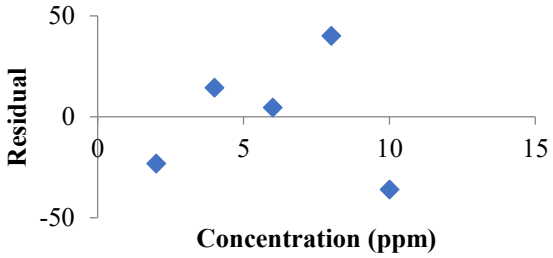


Figure 4. Residual plot linearity.

Based on the results of accuracy measurements on fish samples (Table 3), the % recovery value after 6 replications was 101.05%. The results have met the accuracy requirements, with % recovery for analytes at 1 – 10 ppm ranging from 80 – 110% (Guidelines for Standard Method Performance Requirements Appendix F, 2012).

Based on the results of precision measurements on goldfish samples (Table 4), the %RSD value was 1.35%. The results of the precision of repeatability have met the requirements (Table 6), where the %RSD value for the analysis concentrated 1 – 10 ppm is < 7.5% (Guidelines for Standard Method Performance Requirements, Appendix F, 2012). The acceptable value for repeatability precision can also be determined by Horwitz's rule, where the Horwitz %RSD in the sample is 6.03%. If the value of the %RSD of the sample is compared to the

%RSD of Horwitz, then it has met *the Horwitz* rule because the value of %RSD ≤ ½ of the value of %RSD Horwitz.

Table 3. Accuracy measurement results.

Sample	Concentration Measurable (ppm)	Recovery (%)
1	6.57	101.35
2	6.53	100.69
3	6.61	102.18
4	6.43	98.59
5	6.49	99.84
6	6.68	103.62
Average		101.05
Largest		103.62
Smallest		98.59

Table 4. Results in precision repeatability.

No	Peak Area	Measured Concentration (ppm)
1	1122.294	6.57
2	1117.033	6.53
3	1128.802	6.61
4	1100.513	6.43
5	1110.417	6.49
6	1140.115	6.68
Average		6.55
SD		0.09
%RSD		1.35%
%RSD Horwitz		6.03%

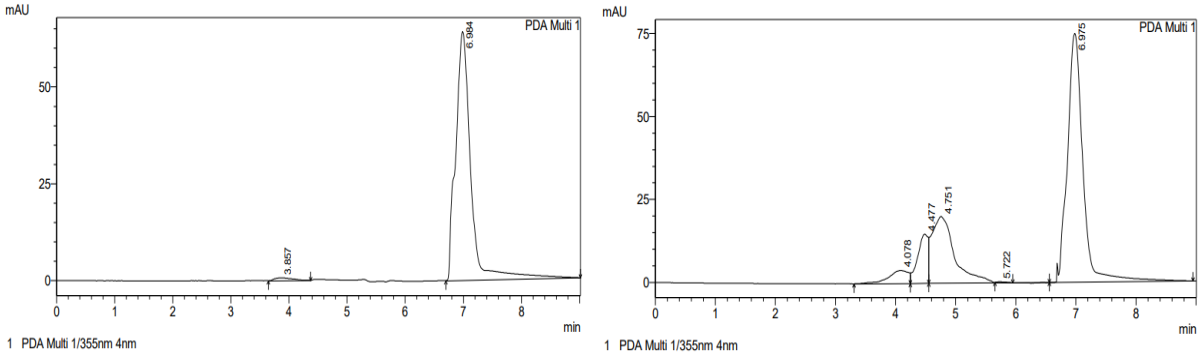
Based on the results of the precision measurement of reproducibility in goldfish samples (Table 5), the %RSD values for three consecutive days were 1.11%; 0.96%; and 1.14%, in which the resulting %RSD value ≤ ⅔ of *Horwitz*'s %RSD. The reproducibility precision results meet the requirements of the Guidelines for Standard Method Performance Requirements Appendix F (2012) and Horwitz*'s rules*.

Table 5. Reproducibility precision results.

Repetition	Day One	Day Two	Day Three
	Concentration (ppm)	Concentration (ppm)	Concentration (ppm)
1	6.54	6.55	6.58
2	6.51	6.64	6.46
3	6.58	6.48	6.60
4	6.62	6.63	6.66
5	6.49	6.64	6.65
6	6.42	6.60	6.65
Average	6.53	6.59	6.60
SD	0.07	0.06	0.08
%RSD	1.11%	0.96%	1.14%
%RSD Horwitz	8.04%	8.03%	8.03%

The selectivity test in this study was performed by comparing the chromatograms of a pure tetracycline standard solution and a mixed tetracycline standard solution. Based on Figure 5, the tetracycline retention time in the two solutions is almost identical, i.e., 6.984 minutes for the pure standard solution and 6.975 minutes for the mixed solution. The similarity in retention times indicates that there is no interference from matrix components, so the HPLC method used meets the selectivity criteria. The chromatogram in Figure 5b also shows a higher peak area at the same retention time after the standard addition to the sample solution. This confirms that the peak is derived from tetracycline and not from other components in the matrix. These findings are consistent with the reports of Rama *et al*. (2015) and Saleh *et al*. (2021), which state that the retention time match between the standard solution and the sample is a strong indicator of the absence of interference. Thus, the HPLC method in this study

has good selectivity for the determination of tetracycline residues. The unknown peak in Figure 5b is suspected to arise from contaminants carried in the HPLC system and does not affect the identification of tetracycline.



(a) (b)

Figure 5. The standard solution chromatogram results are (a) pure tetracycline and (b) mixed tetracycline.

The LoD obtained was 0.27 ppm, the lowest level of tetracycline detectable by the HPLC-based tetracycline analysis method. Sample levels with concentrations below the LoD are considered undetected by the tool. The quantification limit (LoQ) for this method is 0.90 ppm, the lowest level that can still be quantified with accuracy and precision. If a value is below LoQ, it indicates only that tetracycline is detected in a sample; its accuracy and precision are reduced.

Measurement Uncertainity

Measurement uncertainty in this study was calculated by combining all errors into a single range in accordance with the work procedure. Next, each source of the standard uncertainty was combined into an expanded uncertainty. Sources of measurement uncertainty in determining tetracycline antibiotic residue levels in goldfish using HPLC include sample mass uncertainty, volume uncertainty, material uncertainty or tetracycline standard purity, dilution uncertainty, calibration curve uncertainty, and repeatability precision uncertainty (Table 6).

Table 6. Source of uncertainty of the residual levels of tetracycline antibiotics in goldfish samples.

Source	Value	Combined Uncertainty	Units
Weighing (M)	50	0.0000164	g
10 mL measuring flask (V1)	10	0.00167	mL
Measuring flask 5 mL (V2)	5	0.003	mL
Micropipette 1 mL (V3)	1	0.0008	mL
Standard purity (CSTD)	5	0.00001	ppm
Dilution Factor (FP)	10	0.00188	-
Calibration curve (Cplot):			
a. Rajabasa Sample	13.10	0.0187	ppm
b. Untung Sample	13.10	0.0193	ppm
c. Way Kandis Sample	13.10	0.0184	ppm
d. Way Halim Sample	13.10	0.0193	ppm
e. Cimeng Sample	13.10	0.0184	ppm
f. Tugu Sample	13.10	0.0179	ppm
g. Bambu Kuning Sample	13.10	0.0192	ppm
h. Sukarame Sample	13.10	0.0191	ppm
Repeatability (Rep):			
a. Rajabasa Sample	1	0.00051	-
b. Untung Sample	1	0.00006	-
c. Way Kandis Sample	1	0.000073	-
d. Way Halim Sample	1	0.00084	-
e. Cimeng Sample	1	0.00044	-
f. Tugu Sample	1	0.00029	-
g. Bambu Kuning Sample	1	0.000009	-
h. Sukarame Sample	1	0.00103	-

The expanded uncertainty value for each sample (Table 7) is acceptable because it remains below the tetracycline concentration in the fish sample, indicating a low analysis error rate. Based on the table, an uncertainty value of ±0.87 to ±1.42 ppm was obtained.

Table 7. The uncertainty (U) of each sample.

Sample	Market Location	U (±)
1	Rajabasa	1.12
2	Untung	1.07
3	Way Kandis	1.23
4	Way Halim	0.87
5	Cimeng	1.21
6	Tugu	1.42
7	Bambu Kuning	0.91
8	Sukarame	0.97
	Average	1.10

Determination of Tetracycline Antibiotic Residue Levels in Goldfish

Antibiotic residue levels in goldfish meat samples were measured using the developed method. The determination in this study was carried out by constructing a calibration curve from a tetracycline standard solution at concentrations of 2, 4, 6, 8, and 10 ppm. The measurement results for goldfish samples were obtained using the regression equation y = 149.10x + 177.92.

Residue levels of tetracycline antibiotics were measured in goldfish meat samples obtained from 8 different market locations. Based on Table 8, the residue levels of tetracycline antibiotics were 29.28 ppm, 21.77 ppm, 32.70 ppm, 21.89 ppm, 32.10 ppm, 38.58 ppm, 23.13 ppm, and 24.70 ppm. The tetracycline antibiotic residue level has exceeded the maximum residue limit (MRL). According to the Indonesian National Standard (SNI) 01-6366-2000, the MRL for tetracycline allowed in fish meat is 0.1 mg/kg (1 ppm = 1 mg/kg). Meanwhile, according to Orlando and Simionato (2013), it is 2 mg/kg. Variations in tetracycline levels across these samples are likely due to the goldfish being sourced from 8 different markets in Bandar Lampung. Each market likely has different goldfish cultivation practices, so antibiotic use varies depending on the condition of the goldfish.

Table 8. The results of the measurement of the residue levels of the antibiotic tetracycline in the sample.

Market Location	Rate (ppm)		Average (ppm)
	1	2	
Squirrelly	30.03	28.54	29.28
Profit	21.83	21.70	21.77
There is no such thing as a Kandis	32.58	32.82	32.70
Not Halim	22.81	20.97	21.89
Cimeng	31.39	32.81	32.10
Monument	38.02	39.14	38.58
Yellow Bamboo	23.14	23.12	23.13
Squirrelly	23.43	25.97	24.70
	Average		28.02

CONCLUSION

Determination of tetracycline antibiotic levels by High Performance Liquid Chromatography (HPLC) is optimal at a 35:65 mobile phase composition (acetonitrile:water) with a flow rate of 0.5 mL/min. The parameters of linearity, accuracy, precision, selectivity, limit of detection (LoD), and limit of quantification (LoQ) in the validation of the HPLC method for determining tetracycline residue levels in goldfish (*Cyprinus carpio*) samples have shown good results. The results of the measurement of tetracycline antibiotic residue levels in goldfish samples from 8 market locations in Bandar Lampung were in the range of 21.77 – 38.58 ppm with an uncertainty value of ±0.87 to ±1.42, where the residue level had exceeded the maximum permissible residue limit.

CONFLICT OF INTEREST

There is no conflict of interest in this article.

AUTHOR CONTRIBUTION

RR and AAK: Conceptualization, Methodology, Investigation, Writing–Original Draft; AR, PP, YO: Resources, Writing, Editing; SH and SW: Review and Supervision.

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