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Development of a Simultaneous Sulfadiazine, Sulfamethoxazole, and Tetracycline Antibiotic Level Determination Method with High-Performance Liquid Chromatography

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A B S T R A C T

Sulfonamides and tetracyclines are a group of synthetic antibacterials that are widely used in veterinary practice for the treatment and prevention of infections in animals that can be consumed by humans. The purpose of this study was to determine the optimum conditions for High-Performance Liquid Chromatography for simultaneous analysis of sulfadiazine, sulfamethoxazole, and tetracycline and meet the validation requirements. The research method used is the HPLC method. The instruments used are Agilent 1220 Infinity II low-pressure dual-channel gradient valve, variable wavelength detector (VWD) BI detector, the column used is Agilent 5 TC-C18 (250 x 4.6 mm), motion phase A = acetic acid 0.08%: acetonitrile (9:1) and motion phase B = methanol with a flow rate gradient of 0.5 mL/minute and UV-VIS detector at wavelength 295 ηm. The results showed that the method of determining the levels of sulfadiazine, sulfamethoxazole, and tetracycline met the requirements of selectivity, accuracy, precision, linearity, LOD, and LOQ and could be applied to fish samples. The accuracy parameter test obtained recovery results between 96.99%-103.16% at all sample concentration levels of 80%, 100%, and 120%. Testing the precision parameters showed that all sample concentrations were 100% repeated for 6 times replications, fulfilling the precision requirements, namely, the concentration of ≥10 ppm was < 7.3%. The linearity test showed that the correlation coefficient of sulfadiazine was 0.999, sulfamethoxazole was 0.999, and tetracycline was 0.998. LOD and LOQ values of sulfadiazine 1.15 ppm and 3.84 ppm, sulfamethoxazole LOD 2.51 ppm and LOQ 8.37 ppm, and tetracycline LOD 3.37 ppm and LOQ 11.22 ppm. Applications to fish samples were carried out by addition and obtained levels of 14.79 ppm sulfadiazine, 14.01 ppm sulfamethoxazole, and 45.05 ppm tetracycline.

1. Introduction

Control of food residues of animal origin is very important because it is an effort to produce food that is safe for public consumption. Detecting residual events in fishery products is one method that can be done. The presence of sulfonamide and tetracycline antibiotic residues in food must be detected accurately, effectively, and efficiently. High-Performance Liquid Chromatography is a compound separation technology with high speed, sensitivity, and precision. One study showed that all fishery products use antibiotics to promote growth, increase feed efficiency, and prevent and treat disease. The presence of antibiotic residues in food of animal origin is a public health problem of great concern. Sulfonamides, tetracyclines, and fluoroquinolones are a group of synthetic antibacterials that are widely used in veterinary practice for the treatment and prevention of infections in animals that can be consumed by humans. These three groups of antibiotics are most often used for therapeutic purposes in veterinary practice because of their low cost, easy availability, over-the-counter and broad-spectrum activity.[1.2]

Antibiotic residues are chemical contaminants in food that threaten human health. These threats include toxic effects, poisoning, and digestive diseases. Residual problems are closely related to the uncontrolled and uncontrolled use of antibiotics, resulting in the accumulation of antibiotics in animal tissues or organs. The most frequently detected antibiotic residue in fish is tetracycline. Residues are chemical residues or metabolites in animal tissues or organs. The National Standardization Agency recommends a tetracycline antibiotic residue of 0.1 ppm. According to European Union (EU) regulations, the consumption of sulfonamides in animal tissues is 100 µg/kg, while the Codex Alimentarius Commission (CAC) sets a limit of 25 µg/kg for sulfonamides in cow's milk and animal products.[3-5]

In this case, the control of food residues of animal origin becomes very important because it is an effort to produce food that is safe for public consumption. Detecting residual events in fishery products is one method that can be done. The presence of sulfonamide and tetracycline antibiotic residues in food must be detected accurately, effectively, and efficiently. High-Performance Liquid Chromatography is a compound separation technology with high speed, sensitivity, and precision. The simultaneous determination of analyte content with High-Performance Liquid Chromatography is based on the difference in the interaction or affinity between the analyte and the stationary phase, which results in a different retention time for each substance. If the motion phase is polar and the stationary phase is nonpolar, it is called reverse phase chromatography. In the reverse phase, the more polar analytes elute more rapidly than, the less polar analytes.[6-9]

2. Methods

The materials used in this study included comparison standards of BPFI sulfadiazine, BPFI sulfamethoxazole, and BPFI tetracycline obtained from BPOM, Acetic Acid (Merck), Acetonitrile for HPLC (Merck), Methanol for HPLC (Merck), and fish samples containing obtained from the market.

The tools used in this research are HPLC instrumentation brand Agilent 1220 Infinity II Low-Pressure Dual Channel Gradient Valve, Variable Wavelength Detector (VWD) BIcolumn Agilent 5 TC-C_{18} (250 x 4.6 mm), glass tool, sonicator, centrifuge, digital analytical balance Ohaus, nylon membrane filter 0.45 0,45 µm 13 mm Whatman and 0.45 µm 47 mm filter, and Filtration unit for HPLC.

Preparation of the main standard solution

Carefully weighed the standard raw materials sulfadiazine, sulfamethoxazole, and tetracycline, each with a concentration of 500 ppm in methanol.

Preparation of working standard solutions

The standard working solutions were prepared from the standard parent solutions of 15 ppm sulfadiazine, 40 ppm sulfamethoxazole, and 60 ppm tetracycline.

Determination of the optimum wavelength

The standard working solutions of each sulfadiazine, sulfamethoxazole, and tetracycline seen in the UV spectra were observed at a wavelength of 200-400 nm.

Optimization of HPLC conditions

Effect of the composition of the phase gradient of motion. The standard solution of a mixture of sulfadiazine, sulfamethoxazole, and tetracycline was injected as much as 5µL using the motion phase of acetic acid: acetonitrile: methanol with the composition of motion phase A = 0.08% acetic acid: acetonitrile (9:1) and motion phase B = methanol. Elution in quantitative analysis of sulfadiazine, sulfamethoxazole, and tetracycline simultaneously using a gradient, namely at 0 – 5 minutes, the composition of the motion phase A:B with a ratio of 90:10, at 5 – 12 minutes, the composition of the motion phase A:B is a gradient with a ratio of 60:40 to 12 – 20 motion phase composition A:B gradient with a ratio of 90:10. The results of chromatograms with various motion phase compositions were observed,

and conditions were selected which gave optimum analyte peak resolution. The optimum condition is reached when the value of > α 1; price Rs > 1.5; the number of N >, and fast analysis time.

Effect of motion phase velocity. The standard solution of a mixture of sulfadiazine, sulfamethoxazole, and tetracycline was injected as much as 5 µL using the composition of the motion phase A = 0.08% acetic acid: acetonitrile (9:1) motion phase B = methanol in a ratio of 90:10 with the selected gradient and variations were carried out. motion phase velocity 0.5 mL/minute; 0.7 mL/minute; 1.0 mL/min. The results of chromatograms with varying motion phase velocities were observed, and conditions were selected which gave optimum analyte peak resolution. The optimum condition is reached when the value of α > 1; price Rs > 1.5; Number of N >, and fast analysis time.

Effect of acetic acid concentration in water. The standard solution of a mixture of sulfadiazine, sulfamethoxazole, and tetracycline was injected as much as 5 µL by using the velocity of the motion phase and the composition of the motion phase with the selected gradient and varying the concentration of 0.08% acetic acid; 0.1%; 0.12%. The results of chromatograms with varied compositions of acetic acid were observed, and conditions were selected which gave an optimum resolution of analyte peaks. The optimum condition is reached when the value of α > 1 ; price Rs > 1.5, the number of N >, and fast analysis time.

Effect of ratio of acetic acid: acetonitrile on channel A. A standard stock mixture of sulfadiazine, sulfamethoxazole, and tetracycline was injected as much as 5 µL using the velocity of the motion phase, the composition of the motion phase with a gradient, variations in the concentration of acetic acid in the selected motion phase were carried out by varying the ratio of acetic acid: acetonitrile on channel A (9:1) ; (8:2) ; (7:3). The results of the chromatogram with variations in the concentration of acetic acid were observed, and conditions were selected which gave an optimum resolution of analyte peaks. The optimum condition is reached when the value of α > 1; price Rs > 1.5; Number of N >, and fast analysis time.

Method validation

Selectivity determination. From the mother liquor of each sulfadiazine, sulfamethoxazole, and tetracycline, various concentrations were made. Each solution was filtered through a 0.45 µm porous sieve, and then about 5 µL was injected into the HPLC apparatus under selected conditions. The selected chromatogram was calculated by calculating the value of the resolution (Rs), retention time (tR), selectivity factor (α), and the number of N.

Determination of linearity. From the mother liquor of each sulfadiazine, sulfamethoxazole, and tetracycline, various concentrations were made. Each solution was filtered through a 0.45 µm porous sieve, and then about 5 µL was injected into the HPLC apparatus under selected conditions. The chromatogram obtained is calculated by the value of the correlation coefficient (r) through the equation y = a + bx between the standard concentration and the area. The correlation coefficient (r) is accepted if the value of r > 0.999 or the value of r count is greater than the value of the r table (Mulja M & Suharman, 1995). To be more precise, the parameter V_{XO} (standard deviation of the function) is used if < 5%.

Determination of accuracy. How to make a placebo sample plus analyte with a certain concentration (usually 80-120% of the estimated analyte content), then 3 times replication. Sample preparation was carried out by addition. Namely, the sample was weighed, then added aquabidest in a blender and then filtered through a 0.45 µm Whatman filter, centrifuged at 3500 rpm for 20 minutes, vortexed for 40 minutes, filtered through a 0.45 µm Whatman filter, then injected about 5 µL in the HPLC device with the following conditions: selected. The response area of the chromatogram of each sample is entered into the calibration curve equation so that the levels are obtained. Accuracy is expressed as % recovery from the results of the analysis of the test sample to the actual concentration of analyte added in each sample.

$$\% \text{ recovery} = \frac{measurable\ rate}{actual\ rate} x100\%$$

Determination of precision. Precision was performed by injecting one concentration of a standard solution of sulfadiazine, sulfamethoxazole, and tetracycline 6 times. The resulting chromatogram calculated the coefficient of variation (KV) from the standard area, where the coefficient of variation should not be more than 2%. The precision of the method is done by calculating the coefficient of variation (KV) of the chromatogram produced at the accuracy stage. Precision is considered good if the KV value is ≤ 10%.

$$RSD = \frac{\text{SD}}{X} x\ 100\%$$

Determination of LOD and LOQ. Several concentration solutions were made with each solution using a 0.45 µm Whatman filter membrane, and then about 5 µL was injected under selected HPLC conditions. The obtained chromatogram is calculated by the regression equation between the standard concentration and the analyte area, which is expressed by the equation (y = a + bx) where S is the slope (b), after which the peak height of the analyte is determined. LOD and LOQ prices are calculated using the LOD and LOQ formula equations.

$$Q = \frac{\text{K x Sb}}{\text{Sl}}$$

Description:

Q = LOD (detection limit) or LOQ (quantitation limit)

K = 3 for detection limit or 10 for quantitation limit

Sb = standard deviation of analytical response from blank

Sl = direction of linear line (direction sensitivity) of the curve between response to concentration = slope (b in the line equation y = a+bx

The detection limit (Q)

Because k = 3, standard deviation (Sb) = Sy/x, then:

$$Q = \frac{\text{3Sy/x}}{\text{Sl}}$$

The quantity limit (Q)

$$Q = \frac{\text{10Sy/x}}{\text{Sl}}$$

Sample application. Samples on the market are taken randomly, namely samples. The fish was added to the fish sample was weighed and then added with aquabidest in a blender and then filtered through a 0.45µm Whatman filter, centrifuged at 3500 rpm for 20 minutes, then extracted with chloroform 3 times, vortexed for 40 minutes, filtered through a 0.45µm Whatman filter, then 5µL was injected into the HPLC apparatus with using the selected optimization conditions The results of the chromatogram can be calculated area and interpolated into the standard curve equation to obtain sulfonamide and tetracycline levels of each sample.

3. Results and Discussion

This research was not carried out at one of the maximum wavelengths of compounds because that wavelength is only sensitive to changes in the concentration of one compound. The wavelength was carried out on the overlapping of the 3 compounds showing that the results of each wavelength were then overlapped so that the meeting point of the three antibiotics was obtained at a wavelength of 295nm. The wavelength results are then applied to the HPLC method.

Sulfadiazine, sulfamethoxazole, and tetracycline standards were used, each with a concentration of 500 ppm. From each standard solution, 15 ppm sulfadiazine working standard, 40 ppm sulfamethoxazole, and 60 ppm tetracycline were made. The motion phase was optimized using acetic acid: acetonitrile: and methanol as the motion phase. Based on the optimization carried out, the optimum motion phase results for the analysis of sulfadiazine, sulfamethoxazole, and tetracycline with the composition of the motion phase channel A = 0.08% acetic acid: acetonitrile (9:1) and the motion phase channel B = methanol. Elution in quantitative analysis of sulfonamides and tetracyclines simultaneously uses a gradient, namely at 0 – 5 minutes, the composition of the motion phase A:B with a ratio of 90:10, at minute 5 – 12, the composition of the motion phase A:B is a gradient with a ratio of 60:40 to minute 12 – 20 motion phase compositions A:B with a ratio of 90:10. With a flow rate of 0.5 mL/minutes at a wavelength of 295 nm.

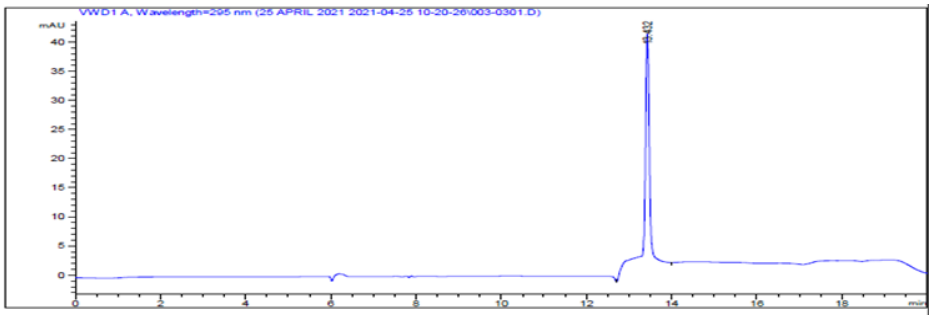


Figure 1. Sulfadiazine chromatogram optimum conditions.

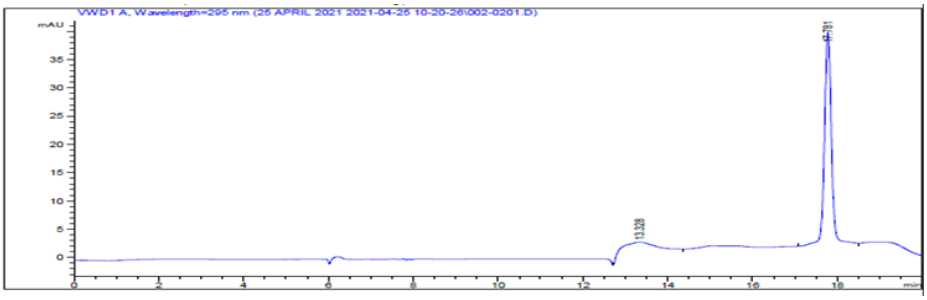


Figure 2. Sulfamethoxazole chromatogram optimum conditions.

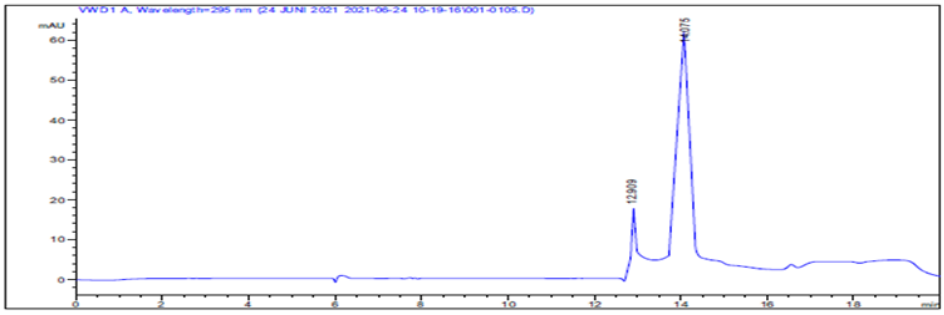
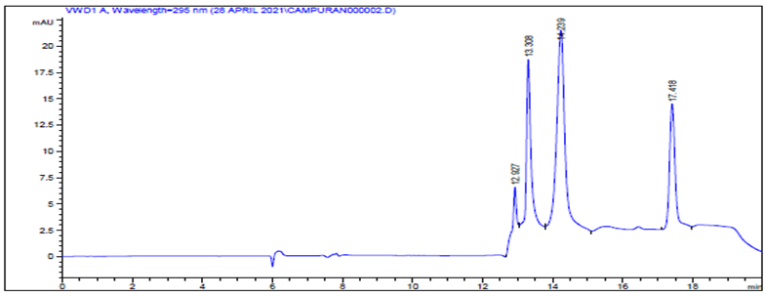


Figure 3. Optimum condition of tetracycline chromatogram.

The sulfadiazine peak chromatogram appeared at a retention time of 13,432. For sulfamethoxazole, the peak appeared at a retention time of 17,782, while for tetracycline, it appeared at a retention time of 14,133. The motion phase used here contains more water so that the retention time obtained is longer. The Ɛº value indicates the eluent strength in the stationary phase. The stronger the solvent to elute, the higher the Ɛº value. When acetonitrile or methanol is mixed with water, it is much easier to control the separation of the analyte than just water being used as the motion phase.[10-12]

The ability of an organic solvent to be able to dilute a compound called eluent strength (Ɛº), the greater the value of the eluent strength, the greater the ability of organic solvent elusion. In order to obtain a good separation, it is necessary to pay attention to eluent strength. This is because a greater the value of Ɛº can cause an overlap between 2 compounds that have an adjacent retention time. While the smaller the value of Ɛº, the more difficult the phase of motion to dilute the compound.[13-15]

Optimization of the motion phase includes variations in the concentration of acetic acid, variations in the ratio of the motion phase in channel A, namely acetic acid: acetonitrile, variations in flow rate, and variations in the ratio of flow rate gradients in channel A and channel B.



Area Percent Report with Performance

Multiplier: : 1.0000
Dilution: : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: VWD1 A, Wavelength=295 nm

RetTime [min]	k'	Area [mAU*s]	Height [mAU]	Symm.	Width [min]	Plates	Resol ution	Select ivity
12.927	-	55.54414	5.93419	1.34	0.1092	77679	-	-
13.308	-	178.12215	17.11966	0.77	0.1250	62781	1.91	1.09
14.239	-	300.46771	18.82849	0.98	0.2239	22529	9.14	1.07
17.418	-	129.94714	11.83974	0.84	0.1617	64298	9.70	1.22

Figure 4. Chromatogram of sulfadiazine, sulfamethoxazole, and tetracyclines simultaneously under optimum conditions.

In this chromatogram, sulfadiazine peaks at 13, 308 minutes, while sulfamethoxazole appears at 17,418 minutes, and tetracyclines appear at 14,239 minutes. The selectivity value of the mixture α >1 and a good resolution value, namely the peak of the analyte compound, was separated from the peaks of other compounds with a value of Rs> 1.5.

The results showed that the method of determining the levels of sulfadiazine, sulfamethoxazole, and tetracycline met the requirements of selectivity, accuracy, precision, linearity, LOD, and LOQ and could be applied to fish samples. The accuracy parameter test obtained recovery results between 96.99%-103.16% at all sample concentration levels of 80%, 100%, and 120%. Testing the precision parameters showed that all sample concentrations were 100% repeated for 6 times replications, fulfilling the precision requirements, namely, the concentration of ≥ 10 ppm was < 7.3%. The linearity test showed that the correlation coefficient of sulfadiazine was 0.999, sulfamethoxazole was 0.999, and tetracycline was 0.998. The LOD values of sulfadiazine were 1.15 ppm

and LOQ 3.84 ppm, sulfamethoxazole LOD 2.51 ppm and LOQ 8.37 ppm, and tetracycline LOD 3.37 ppm and LOQ 11.22 ppm. Applications to fish samples were performed in addition to obtaining sulfadiazine levels of 14.79 ppm, sulfamethoxazole of 14.01 ppm, and tetracycline of 45.05 ppm.

Applications were made on fish samples by addition. An Additional technique is used because this technique can be applied to analyze very small amounts of compounds. Fish samples were added with standard sulfadiazine, sulfamethoxazole, and tetracycline in a measurable manner and then read the chromatogram in HPLC using optimum conditions. The area for each stable sample was only the retention time on tetracycline and sulfamethoxazole was earlier than the retention time on the standard. When done simultaneously on fish samples, only 2 peaks appeared because the retention time of the tetracyclines appeared earlier, so the peaks overlapped between the sulfadiazine peaks and the tetracycline peaks. The overlapping of peaks is due to the concentration of sulfadiazine added, which is smaller than that of tetracycline, so the area looks very small compared to tetracycline, and it looks like they are piling up at first glance. The results of previous studies stated that tetracycline could bind to protein by 65% in fish samples and is able to bind to proteins to form conjugates, so it is difficult to extract from the sample matrix. In addition, the extraction of tetracyclines from meat samples can form chelates between tetracyclines and metal ions contained in the matrix. So for deproteination, use a slightly acidic solvent to liberate tetracycline, which is non-covalently bound to these macromolecules.[16-20]

4. Conclusion

From the results of the study, it was found that the assay methods of sulfadiazine, sulfamethoxazole, and tetracycline met the requirements of selectivity, accuracy, precision, linearity, LOD, and LOQ and could be applied to fish samples. The accuracy parameter test obtained recovery results between 96.99%-103.16% at all sample concentration levels of 80%, 100%, and 120%. Testing the precision parameters showed that all sample concentrations were 100% repeated for 6 times replications, fulfilling the precision requirements, namely, the concentration of ≥ 10 ppm was < 7.3%. The linearity test showed that the correlation coefficient of sulfadiazine was 0.999, sulfamethoxazole was 0.999, and tetracycline was 0.998. LOD and LOQ values of sulfadiazine 1.15 ppm and 3.84 ppm, sulfamethoxazole LOD 2.51 ppm and LOQ 8.37 ppm, and tetracycline LOD 3.37 ppm and LOQ 11.22 ppm. Applications to fish samples were carried out by addition and obtained levels of 14.79 ppm sulfadiazine, 14.01 ppm sulfamethoxazole, and 45.05 ppm tetracycline. To find out other effects, it is necessary to suggest similar research by making different conditions in the motion phase by using variations in pH.

5. References

1. Anastasia Y. Technique for analyzing residues of the tetracycline group in chicken meat using high-performance liquid chromatography. Agricultural Engineering Bulletin, 2011; 16(2): 68–73.
2. Amen LZ. Rational Selection of Antibiotics. Medicinus. 2014; 27(3):40-5.
3. Atabani EAI, El-Ghareeb WR, Elabbasy MT, Ghazaly EI. Oxytetracycline residues in marketed frozen beef livers at Sharkia, Egypt. Benha Vet Med J. 2014; 26(1): 104-112.
4. Bueno EC, Forastero A, Tudela JLR, Estrella MC, Lopez AG. HPLC/UV or bioassay: two valid methods for posaconazole quantification in human serum samples. Clin Microbiol Infect. 2012; 18: 12.
5. Jank L, Martins MT, Arsand JB, Motta TMC, Feijó TC. Liquid chromatography-tandem mass spectrometry multiclass method for 46 residues in milk and meat antibiotics: development and validation. Food Analytical Methods. 2017; 10(7): 2152-64.
6. Kim HJ, Jeong MH, Park HJ, Kim WC, Kim JE. Development of an immunoaffinity

chromatography and HPLC-UV method for determination of 16 sulfonamides in feed. Food Chemistry. 2015; 196: 1144-9.
7. Lekshmi M, Ammini P, Kumar S, Varela MF. The food production environment and the development of antimicrobial resistance in human pathogens of animal origin. Microorganisms. 2017; 5(1): 11.
8. Dewi AAS, Widdhiasmoro NP, Nurlatifah I, Riti N, Purnawati D. Residues of antibiotics in food of animal origin, impact and mitigation efforts. Veterinary Bulletin. 2014; 26(3): 85.
9. Dewi AM, Swacita IBN, Suwiti NK. The effect of differences in muscle type and storage time on the nutritional value of Bali beef. Udayana Veterinary Bulletin. 2016; 8(2): 135-44.
10. Martins MT, Barreto F, Hoff RB, Jank L, Arsand JB. Determination of quinolones and fluoroquinolones, tetracyclines and sulfonamides in bovine, swine and poultry liver using LC-MS/MS. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure, and Risk Assessment. 2015; 32(3): 333-41.
11. Donkor ES, Newman MJ, Tay SCK, Dayle NTKD, Bannerman E. Investigation into the risk of exposure to antibiotic residues contaminating meat and egg in Ghana. Food Control. 2011; 22(3): 869-73.
12. Moudgil P, Bedi JS, Aulakh RS, Gill JPS, Kumar A. Validation of HPLC multi-residue method for determination of fluoroquinolones, tetracycline, sulphonamides, and chloramphenicol residues in bovine milk. Food Analytical Methods. 2019; 12(2): 338-46.
13. Lee MH, Lee HJ, Ryu PD. Public health risks: Chemical and antibiotic residues. Asian-Australasian J Anim Sci. 2001;14(3):402-13.
14. Nurhasnawati H, Siti J. Determination of residual levels of tetracycline HCl in freshwater fish circulating in the markets using ultraviolet spectrophotometric method. Manuntung Scientific Journal. 2016; 2(2): 173-8.
15. Oguz H, Turk E. Investigation of tetracycline residues in fish caught from surrounding fish farms in Mugla district. Eurasian J Vet Sci. 2016; 32(2): 1123-31.
16. Patyra E, Przeniosło-Siwczynska M, Kwiatek K. Determination of sulfonamides in feeds by high-performance liquid chromatography after fluorescamine precolumn derivatization. Molecules. 2019; 24(3): 432-40.
17. Rahayu D, Agus A. Analysis of organochlorine pesticide residues on turmeric rhizomes by UV spectrophotometry. J Pharmacy. 2009; 6(2): 69–75.
18. Rohman A. Chromatography for drug analysis. Graha Ilmu: Yogyakarta. 2009.
19. Vishnuraj MR, Kandeepan G, Rao KH, Chand S, Kumbhar V. Occurrence, public health hazards and detection methods of antibiotic residues in foods of animal origin: A comprehensive review. Cogent Food Agriculture. 2016; 2(1): 1–8.
20. Yu H, Mu H, Hu YM. Determination of fluoroquinolones, sulfonamides, and tetracyclines multi residues simultaneously in porcine tissue by MSPD and HPLC-DAD. Journal of Pharmaceutical Analysis. 2012; 2(1): 76-81.