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Dual Extraction Methods for Nitrofuran Antibiotics Residues; Furaltadone, Furazolidone, and Nitrofurazone from Biotransformation Assay

N.T. Zulkifle[1], N.S. Mohammad[2], M.F. Safian[1] and Z.Z. Ariffin[1*]

[1]*Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia*

[2]*Nano Life Quest Sdn Bhd, 2A, Jalan Sepadu 25/123A, Axis Industrial Park, 40400 Shah Alam, Selangor, Malaysia*

This study aims to extract nitrofuran antibiotics residues, furaltadone, furazolidone, nitrofurazone from biotransformation assays by local *Aspergillus tamarii* KX610719.1. Nitrofuran antibiotics were quantified by using a high-performance liquid chromatography-diode array detector (HPLC-DAD). Liquid-liquid extraction (LLE) and solid phase extraction (SPE) were employed to extract nitrofurans residue for the optimal purification and cleaned up procedures. In this study, a good linearity with the coefficient of determination R^2 value of 0.9951 – 0.9972 mg/L with the concentration range of 1 to 50 mg/L nitrofuran antibiotics was achieved. The relative standard deviation (RSD) for triplicate nitrofurans assay were less than 10.0 %. The limit of detection (LOD) and limit of quantification (LoQ) of furazolidone, furaltadone and nitrofurazone were found to be in the range of 2.37 – 10.56 mg/L and 7.17 – 31.99 mg/L, respectively. Moreover, the recovery of the nitrofuran antibiotics investigated in biotransformation assays ranged from 70.0 % - 88.0 %. The developed technique was effectively employed to determine nitrofurans residue in biotransformation assays and demonstrated remarkable clean-up capacity.

Keywords: furaltadone; furazolidone; nitrofurazone; liquid-liquid extraction; solid phase extraction; high–performance liquid chromatography – diode array detector (HPLC)

I. INTRODUCTION

Veterinary treatments for livestock and aquaculture involve nitrofurans such as furazolidone, furaltadone, and nitrofurazone (Mohammad *et al.,* 2018). Nitrofurans antibiotics have been extensively applied as food additives to enhance animal growth and reduce bacterial infection (Hassan *et al.,* 2013). The usage of nitrofurans in livestock production was prohibited in the European Union in 1995 since the toxic effects of antibiotic residues had a negative impact on human health (Mohammad *et al.,* 2018). Antibiotics used in animal farming have received increasing interest in ensuring healthier farm animals. However, nitrofuran antibiotics that are administered in livestock production cannot be fully metabolised by animals.

According to a previous study, only 20 % of antibiotics absorbed into the biological system, while the remaining 80 % were eliminated (Chee-Sanford *et al.,* 2001). The nitrofurans residues bound to body tissue can potentially cause harm to humans (Pacholak *et al.,* 2020). Nitrofurans may be eliminated as metabolites or as active compounds through stool. However, if not treated, nitrofurans residue from agriculture animal manure may have a negative impact on microorganisms such as soil bacteria. Antibiotic resistance in soil microorganisms could be caused by excess antibiotic residue in the soil (Senwan *et al.,* 2018). The potential of local isolated fungi, *Aspergillus tamarii* KX610719.1 to degrade nitrofurans has been explored in recent work (Mohammad *et al.,* 2018). This filamentous fungal's potential to degrade

*Corresponding author's e-mail: drzaidah@uitm.edu.my

nitrofuran antibiotics revealed the microbe's ability to remediate soil contamination.

Only minimal analytical equipments have been used to analyse residual nitrofurans and their metabolites. According to Tsai *et al.* (2010), in recent years, several laboratories have relied on a high-performance liquid chromatography-diode array detector (HPLC-DAD) to quantify nitrofurans metabolite residues. According to Ryu *et al.* (2016), HPLC is the optimal method for separating and studying nitrofurans metabolites. The most significant benefit is that it can be used with a wide range of analytes, from small organic compounds and ions to large macromolecules and polymers (Pacholak *et al.,* 2020). HPLC is progressively becoming the standard industry platform technology for bioanalytical tests and residual trace analysis in drugs. According to Mohammad *et al.* (2018), prior to HPLC analysis, biotransformation products must be separated to determine the residual component of nitrofurans in the assays. Instrumental measurement typically requires sample pre-treatment procedures. Liquid–liquid extraction (LLE) and solid phase extraction (SPE) are the most widely used procedures to date. According to Senwan *et al.* (2018), LLE and SPE methods have been demonstrated to be the best for extracting target component from biotransformation assay attributed to their ability to clear up with minimal solvent consumption and time. Thus, the aim of the study is to quantify nitrofurans residues by HPLC-DAD.

II. MATERIALS AND METHODS

A. Material

In this study, Potato Dextrose Broth (PDB) (Merck, Germany) was used. Local *Aspergillus tamarii* KX610719.1 was obtained from the School of Biology, Faculty of Applied Sciences, Universiti Teknologi MARA Fungal Culture Collection. Dimethyl sulfoxide (DMSO) (Nacalai Tesque, Japan), ethyl acetate and methanol from System (UK) were also used. All solvents were HPLC-grade such as acetonitrile and water (Merck, Germany). Nitrofuran antibiotics (furazolidone, furaltadone and nitrofurazone) from Sigma – Aldrich (U.S.) were used.

B. Nitrofurans Standard Solution Preparation

The standard furazolidone, furaltadone and nitrofurazone were prepared to obtain a final concentration of 500 mg/L stock solution. About 5 mL of acetonitrile (HPLC grade) was used to dissolve 2.5 mg of nitrofuran antibiotics. A series of working solutions were prepared by using standard solution in the range of 1 to 50 mg/L. The stock was wrapped in aluminium foil and stored in freezer at 4 ^{o}C.

C. Percentage Recovery of Nitrofurans

Percentage recovery is the total amount of purified product recovered from a standard after purification and cleaning compared to the initial amount of non-purified standard. Five hundred mg/L nitrofurans standard solution was prepared and filtrated through a 0.45 µM nylon filter. Then, about 4 mL of nitrofurans standard solution was passed through a SPE C18 column cartridge for the purified product (Mohammad *et al.,* 2018).

Then, by using a 1:1 dilution factor, a concentration of 500 mg/L of both purified and non-purified standard was diluted four times. Finally, diluted nitrofurans standard was injected in the HPLC-DAD instrument. The percentage recovery of nitrofurans was calculated by using Equation (1). Equation (1) shows the amount of recovery of nitrofurans which is the concentration of purified standard solution (a) divided by the concentration of non-purified standard solution (b) multiplied by 100.

$$Percentage\ Recovery\ \% = (a \div b) \times 100\ \% \quad (1)$$

D. Analyses

1. HPLC-DAD Analysis of nitrofurans residual

The HPLC-DAD analysis was conducted by Mohammad *et al.* (2018). Acetonitrile and HPLC grade water were the optimum mobile phases for nitrofurans detection, according to the prior work. The parameters of HPLC – DAD is tabulated in Table 1.

Table 1. HPLC-DAD Parameters for nitrofurans detection

	Furaltadone	Furazolidone	Nitrofurazone
Mobile Phase	A: Water B: Acetonitrile	A: Water B: Acetonitrile	A: Water B: Acetonitrile
Mobile Phase Ratio (A: B) %	(40:60)	(50:50)	(50:50)
Injection Volume	10 µL	20 µL	20 µL
Flow Rate	1.0 mL/min	1.2 mL/min	1.2 mL/min
Run Time	10 mins	10 mins	10 mins
Detector Signal	365 nm	365 nm	365 nm

2. Method validation

The calibration standards (seven levels) were prepared by diluting the nitrofurans standards in acetonitrile (HPLC grade) with a range 1 to 50 mg/L. The analyte's peak arrangement concentration was plotted against the response peak area using linear regression analysis to determine linearity. Precision and repeatability were determined by examining triplicates of each sample over a five-day period and calculating the percentage of relative standard deviation for each compound. The recovery was measured by the concentration of each analyte and was determined in triplicates of purified and non-purified samples. The Limit of Detection (LOD) was calculated to be 3x the signal/noise ratio and the Limit of Quantitation (LoQ) to be 10x the signal/noise ratio on every compound.

E. Biotransformation Assay

Submerged fermentation was used in these assays (Mohammad *et al.,* 2018). Potato Dextrose Broth (PDB) was distributed into four 250 mL Erlenmeyer flasks with 100 mL each. About 100 µL of 1x 10^6 local *Aspergillus tamarii* KX610719.1 was injected. Both furazolidone and furaltadone flasks were incubated at 25 ºC at a 120 rpm agitation speed. The optimum parameters for nitrofurazone were determined based on previous research done by Zulkifle *et al.* (2022). The nitrofurazone flask was at pH 4.80, incubated at 35 ºC, at a 121 rpm agitation speed. Incubation time takes place until the log phase was reached on Day 2. Afterwards, about 10 mL of the prepared nitrofurans solution was spiked into the Erlenmeyer flask filled with culture media to achieve a final concentration of 500 mg/L. The initial concentrations of the three antibiotics are similar. The experiment was carried out for 96 hours. Throughout the study, a 6 mL sample of biotransformation product was taken every 24 hours for further analysis.

F. Preparation of Biotransformation Product

1. Liquid-Liquid Extraction (LLE)

The biotransformation products were extracted by liquid-liquid extraction following one day of incubation. As recommended by Mohammad *et al.* (2018), the extraction process was performed in a separatory funnel. All samples were extracted by using ethyl acetate as the solvent. The separatory funnel was loaded with 25 mL of distilled water, 25 mL of ethyl acetate and 6 mL of the biotransformation products. The nitrofurans residue was obtained by vigorously shaking up the sample with ethyl acetate and distilled water for about one to two minutes while continuously venting the air. After that, the phase separation method was carried out. The extractions were mixed using a rotary evaporator. Concentrated nitrofurans residue was dissolved with dimethyl sulfoxide (DMSO) in a 1.5 mL microcentrifuge tube and stored at 4 ºC.

2. Solid Phase Extraction (SPE)

Solid Phase Extraction (SPE) was used for the cleaning up and purifying steps (Jewell *et al.,* 2016). The SPE column cartridges were preconditioned with 3 mL methanol and 3 mL HPLC grade water to activate the sorbent surface. The sample was loaded into 4 mL cartridges. The cartridges were then washed with 4 mL HPLC grade water without vacuum, followed by 4 mL of HPLC grade methanol for elution. Finally, each tube was fractionally eluted with a 1 mL sample.

The eluted products from the second and third tubes were combined to perform HPLC-DAD analysis. Before being injected into the HPLC-DAD instrument, the eluted products were diluted to 1:10 in acetonitrile and filtrate through a 0.45µM nylon filter.

III. RESULTS AND DISCUSSION

A. Nitrofurans Standard Chromatogram

HPLC-DAD analyses were performed on an Agilent Technologies 1200 series, and HPLC separation was performed by using column reverse phase C18 (ZORBAX SB-C18, 5µM, 4.6 x 250 mm). Figure 1 depicts the elution time of furazolidone (3.62 min), furaltadone (3.87 min), and nitrofurazone (2.48 min) at 50 mg/L in a representative chromatographic curve.

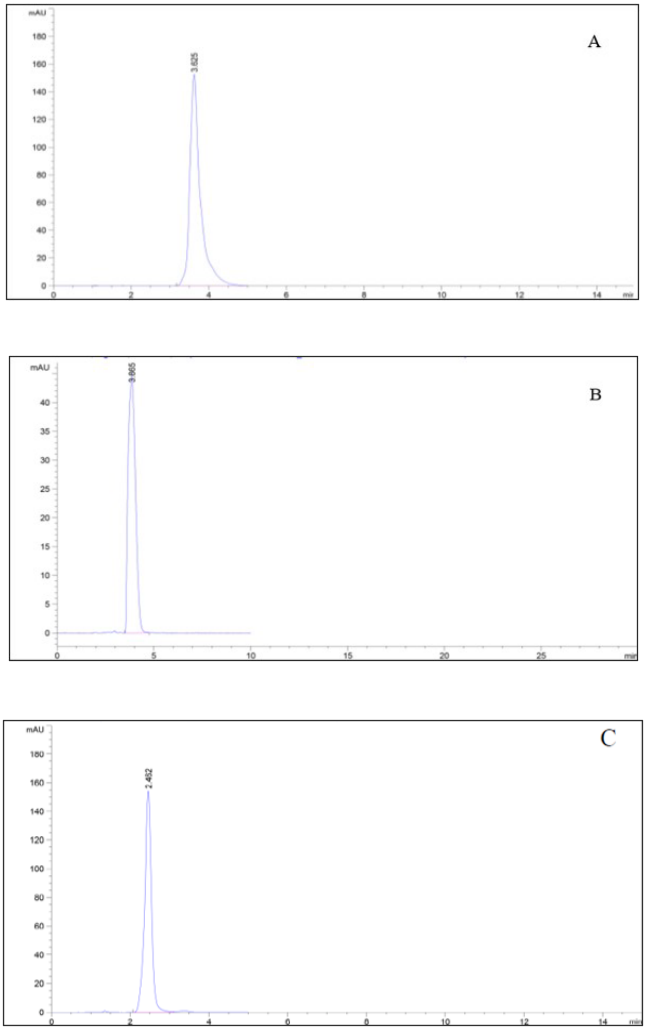

Figure 1. HPLC Chromatogram of (A) Furazolidone, (B) Furaltadone, and (C) Nitrofurazone at concentration 50 mg/L

B. Method Validation

Simple regression analysis was used to describe the concentration peak area correlations. Table 2 shows the correlation coefficient R^2 of the calibration curves for furazolidone, furaltadone and nitrofurazone to be 0.9972, 0.9971 and 0.9951 across the range of 1 – 50 mg/L, respectively. According to Sarrai *et al.* (2016), when the R^2 value was close to 1.00, the model was stronger, and the response predictions were better. The linear range applied in the calibration curve does not exceed 50 mg/L to prevent column degradation and to extend the column's lifespan (Pinto *et al.,* 2000).

Table 2. The parameters of calibration curves

Nitrofurans	Regression Equation	Correlation Coefficient (R^2)	Linear Range (mg/L)
Furazolidone	y = 53.276x + 68.55	0.9972	1.0-50.0
Furaltadone	y = 22.258x + 24.63	0.9971	1.0-50.0
Nitrofurazone	y = 36.011x + 19.207	0.9951	1.0-50.0

The results obtained for the recovery test of nitrofurans are shown in Table 3. According to Mohammad *et al.* (2018), the acceptable range for nitrofurans standard recovery tests was 70 – 80 %. This indicates that nitrofurans recoveries with SPE using a C18 column cartridge were within the acceptable limits.

Table 3. Percentage recovery of nitrofurans

Nitrofurans	Percentage of Recovery (%)
Furazolidone	75.0
Furaltadone	80.8
Nitrofurazone	88.2

Precision and repeatability were obtained by analysing triplicates of each sample over a five-day period under same operating circumstances. The Relative Standard Deviation (RSD %) of nitrofuran residues were calculated between days.

The results were acceptable, with RSD % values below 10% across all samples. Table 4 shows the results of method validation for nitrofurans residue.

Table 4. Results of method validation for nitrofurans residue

Nitrofurans	Relative Standard Deviation (%)		LOD	LOQ (mg/L)	Percentage of Nitrofurans Residue (%)
	0 hour	96 hours			
Furazolidone	1.02	0.81	2.37	7.17	37.49 ± 0.01
Furaltadone	0.97	0.01	10.56	31.99	86.73 ± 0.14
Nitrofurazone	1.09	0.30	4.62	14.01	29.17 ± 0.50

The suggested method was developed and validated using the European Food Safety Authority (EFSA) criteria for LOD and LoQ. Based on the results, the LOD was estimated to be at a signal to noise ratio (S/N) of 3 and furazolidone, furaltadone and nitrofurazone were 2.37 mg/L, 10.56 mg/L and 4.62 mg/L, respectively. The LoQ was estimated to be at signal to noise ratio (S/N) of 10 and furazolidone, furaltadone and nitrofurazone were 7.17 mg/L, 31.99 mg/L and 14.01 mg/L, respectively. The LOD and LoQ reported are greater than those specified in the European Food Safety Authority (EFSA). According to the EFSA Panel on Contaminants in the Food Chain (2016), the reported LOD and LOQ for this method above 1 mg/L was acceptable because in products of poultry meat, the legislated Minimum Required Performance Limit (MRPL) of 1 mg/L for the nitrofuran marker metabolites does not apply.

The discrepancies in LODs and LOQs found in our study could be attributable to a modification in the methodologies or should be investigated further.

C. HPLC Analysis of Nitrofurans Residue

HPLC analysis was applied to determine the sample concentration at various times during the biotransformation process. The results of the percentage of nitrofurans residue are shown in Table 5.

Table 5. Percentage of nitrofurans residue

	Furazolidone (%)	Furaltadone (%)	Nitrofurazone (%)
Control	100.0	100.0	100.0
0 hour	98.17	98.80	96.54
24 hours	82.21	93.67	71.81
48 hours	70.53	90.61	74.72
72 hours	55.59	83.28	52.49
96 hours	37.49	86.73	29.17

The concentration of nitrofurans residue decreases as the incubation time decreases. The nitrofurans degradation rate was estimated based on the HPLC chromatogram. The retention time of furazolidone, furaltadone and nitrofurazone was 3.62 min, 3.87 mins and 2.441 mins, respectively (Figure 2). The retention time for all samples was similar to the nitrofurans standard solution.

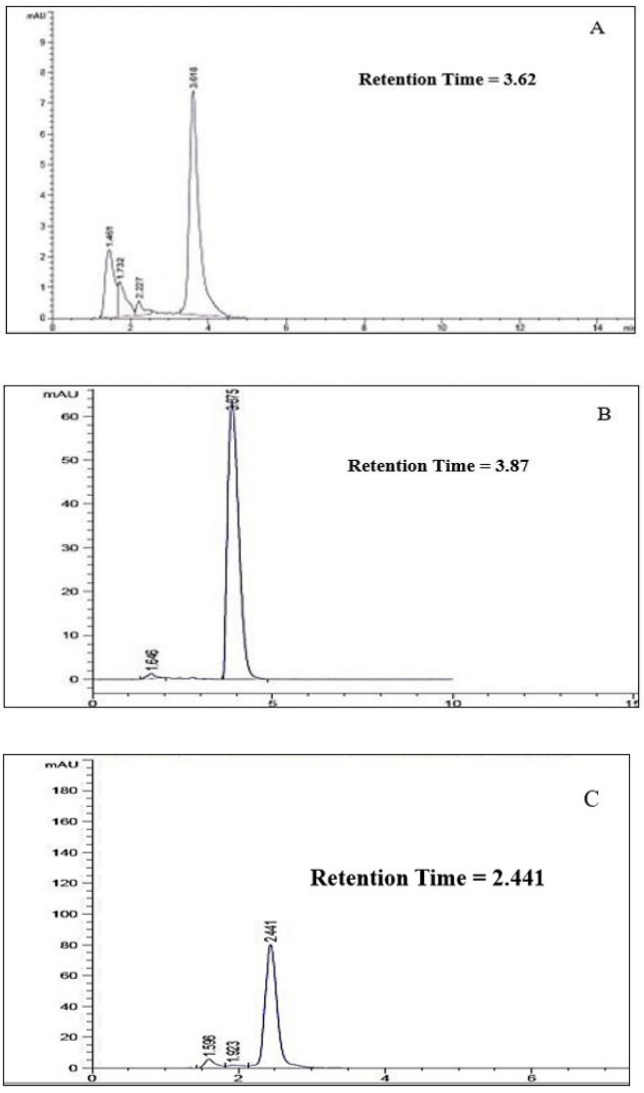

Figure 2. HPLC Chromatogram for Nitrofurans Residue at 96 hours: (A) Furazolidone, (B) Furaltadone, and (C) Nitrofurazone

According to Mohammad *et al.* (2018), prior to HPLC analysis, biotransformation products must be separated by solvent extraction. Liquid-liquid extraction (LLE) and solid phase extraction (SPE) are extensively used methods for nitrofurans. LLE with ethyl acetate and distilled water as a solvent was used to separate the broth (polar) from the nitrofurans residue (non-polar). The process was then carried out by SPE. SPE extraction used a C18 column cartridge that comprised octadecyl silica as a filler, which attracts and retains non-polar components through strong hydrophobic contact. According to Senwan *et al.* (2018), this ensures that interferences such as dimethyl sulfoxide (DMSO) are washed off and not mixed with the nitrofurans residue.

During HPLC analysis, there were several factors that affected resolution, separation and retention time of chromatography. Briefly, about 20 µL of furaltadone was injected into the HPLC system at a flow rate of 1.2 mL/min with a 50:50 (A: B) mobile phase ratio. This resulted in a wide and split peak. A 10 µL injection volume was reduced with 40:60 (A: B) mobile phase ratio and a flow rate of 1.0 mL/min, resulting in a narrow peak and fast elution. According to Mohammad *et al.* (2018), 20 µL of injection volume with a 1.2 mL/min of flow rate were the optimum conditions for furazolidone and nitrofurazone. Acetonitrile was the best solvent for the mobile phase as it triggered the peak to elute quicker (Mohammad *et al.,* 2018). Torre *et al.* (2015) reported that a greater organic solvent ratio such as acetonitrile can reduce the overall retention time.

According to Mohammad *et al.* (2018), the chromatogram of nitrofurans residue in Figure 2 depicted a dimethyl sulfoxide (DMSO) peak that was eluted earlier and has been supported in the standard reference by Agilent Technologies. In general, matrix effects are an issue of concern in nitrofuran metabolite analysis studies (Ryu *et al.,* 2016). Matrix effects occurred due to co-eluting interferences compounds that impact the ionisation of the chromatographic of the target analytes (Panuwet *et al.,* 2016). Therefore, the development of reliable and faster methods is important to ensure food chain and environment safety. Nitrofurans are still widely employed in animal farming despite government restrictions. This research has shown that the method for determining nitrofurans residue in biotransformation assays by local *Aspergillus tamarii* KX610719.1 is cost-effective, sensitive, and efficient.

IV. CONCLUSION

In conclusion, LLE extraction followed by SPE extraction observed a good recovery with HPLC-DAD detection which allows the determination of nitrofurans residue in the biotransformation assays. Thus, quantitative analysis of nitrofurans residue by HPLC-DAD in biotransformation assays by local *Aspergillus tamarii* KX610719.1 was validated. These combined methods offer high sensitivity with both liquid–liquid extraction and solid–phase extraction. Finally, this approach can be used to quantify nitrofuran residues in biotransformation assays by local

Aspergillus tamarii KX610719.1 and can also be widely used in the analysis of other biotransformation assays.

V. ACKNOWLEDGEMENT

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