

Chapter 2

Experimental

2.1 Chemicals and materials

2.1.1 Standard chemicals

- ♦ Nitrofurantoin purity 98.0 % (Sigma, USA)
- ♦ Nitrofurazone purity 99.0 % (Sigma, USA)
- ♦ Furazolidone purity 99.0 % (Sigma, USA)

2.1.2 General chemicals and solvents

- ♦ Acetonitrile, AR grade (Lab-scan, Thailand)
- ♦ Methanol, AR grade (Merck, Germany)
- ♦ Dimethylformamide, AR grade (Lab-scan, Thailand)
- ♦ Potassium hydroxide (Lab-scan, Thailand)
- ♦ Aluminium Oxide active basic (BDH, England)
- ♦ Glass wool

2.1.3 Samples

Animal feed samples were purchased from animal feed stores in Songkhla Province, Thailand. The samples were stored in dark place at room temperature.

2.2 Instruments and apparatus

2.2.1 High performance liquid chromatography (HPLC) system consisted of;

- ♦ High pressure liquid Chromatographic pump model 515 (Waters, USA)

- ♦ Ultraviolet-Visible (UV-Vis) model 2487 Dual wavelength Absorbance Detector (Waters, USA)
- ♦ Auto-sampler injection model 717 plus autosampler (Waters, USA)
- ♦ Millennium³² software (Waters, USA)
- ♦ Ultra Aqueous C-18 column: 250 mm × 4.6 mm. I.D., 5 µm (Restek, USA)

2.2.2 Spectrophotometer system ;

UV-Vis spectra were obtained on UV-Vis spectrophotometer model SPECORD S100 (Analytik Jena AG, Germany)

2.2.3 General Apparatus

- ♦ Glass column, 20 mm I.D. × 300 mm
- ♦ Amber vial 1.0 ml with caps (waters, USA)
- ♦ Filter paper-No.3 (Whatman, Maidstone, England)
- ♦ Microlitre pipette: model P1000, 100-1000 µl; model P200, 50-200 µl; and model P20, 5-20 µl (Gilson, France)
- ♦ General glasswares such as volumetric flask 10, 100, 250 ml; Conical flask 250 ml; test tubes beakers.
- ♦ Syringe Filter, Filter device Polyvinylidene fluoride filter Media with Polypropylene Housing, 13 mm. i.d., 0.2 µm. pore size (Orange Scientific, Belgium)
- ♦ Membrane filter, 47 mm, 0.2 µm pore size, supor[®]-450 membrane (Waters, USA)
- ♦ Syringe for sample filter, Micro-Mate[®] interchangeable (Popper & Son, Inc, ITALY)
- ♦ Shaker Model 75 (Burrell corporation, USA)

2.3 Methodology

This work focused on the analysis of the three most commonly use nitrofurans in Thailand, *i.e.*, furazolidone, nitrofurantoin and nitrofurazone, (Wanishphongphun, 2002) in animal feeds by spectrophotometric and high performance liquid chromatographic technique with UV detection.

2.3.1 Standard stock solutions of nitrofurans

Ten milligrams (10.00 mg) of each standard, *i.e.*, furazolidone, nitrofurantoin and nitrofurazone, was weighed and transferred into a 10 mL volumetric flask. It was completely dissolved and adjusted to a final volume of 10 mL with dimethylformamide. Standard stock solutions of final concentration of 1000 $\mu\text{g mL}^{-1}$ were obtained. They were stored at 4 °C and protected from light.

2.3.2 Working standard solutions of nitrofurans

Working standard solutions, 5 and 10 $\mu\text{g mL}^{-1}$ were prepared in dimethylformamide. These working standard solutions were used to investigate optimum conditions.

2.3.3 Determination of nitrofurans by HPLC technique

2.3.3.1 Degassing systems

Degassing is an important step to eliminate dissolved gases in the mobile phase and to reduce the possibility of air bubbles forming in the pump or detector during the separation process. Elimination of oxygen in the mobile phase is also important to prevent reaction with mobile phase and/ or stationary phase within the analysis. In this work, degassing of the mobile phase was performed under suction through 47 mm Supor[®] membrane filter pore size 0.2 μm . For mobile phase, the vacuum had to be carefully regulated to keep the degassed mobile phase from foaming to such an extent that it entered into the vacuum lines (Horvath, 1980). The degassed mobile phases were kept ready to be used, in the mobile phase reservoir.

2.3.3.2 Optimization of the HPLC-UV conditions for nitrofurantoin analysis

Optimum conditions of the HPLC-UV system were studied for optimum wavelength, composition of mobile phase and mobile phase flow rate. In the optimum condition studies, the interested parameter was varied while the others were kept constants. The preliminary HPLC-UV conditions were set followed the application note of Cieri (1979) as shown in Table 5. Five replications were carried out for each investigation.

Table 5 Preliminary HPLC-UV conditions (Cieri, 1979)

Parameter	Description
Column : Packed column	μ Bondapak C ₁₈ , (30 cm \times 3.9 mm)
Mobile phase	30% Acetonitrile in water
Flow rate	0.8 mL min ⁻¹
Maximum wavelength	365 nm
Injection volume	10 μ L

(1.) Optimum wavelength

In this investigation the composition of mobile phase, flow rate and injection volume were set followed the values in Table 5. Ten μ L of 10 μ g mL⁻¹ nitrofurantoin working standard solution was injected to HPLC-UV system to find the optimum wavelength. The wavelength was tested at 360, 365, 370, 375 and 380 nm. The optimum wavelength was the one that provided the highest absorption (response).

(2.) Composition of mobile phase

The optimization of mobile phase composition was carried out by varying concentration of acetonitrile at 20, 25, 30, 35 and 40% in distilled water. Ten μ L of 10 μ g mL⁻¹, nitrofurantoin working standard solution was used. The wavelength

used was the one obtained in experiment (1) of 2.3.3.2. The mobile phase was filtered and degassed before used as described previously (2.3.3.1). The composition that gave the highest response was then chosen.

(3.) Optimum flow rate

To optimize the mobile phase flow rate, 10 μL of 10 $\mu\text{g mL}^{-1}$ nitrofurans working standard solution was injected to the HPLC-UV system by using the optimum wavelength and mobile phase composition obtained earlier. Optimum flow rate was investigated by varying the flow rate of mobile phase at 0.8, 0.9, 1.0 and 1.1 mL min^{-1} . The retention time and peak area of nitrofurans were obtained from the chromatogram. The height equivalent to the theoretical plate (HETP) was determined from the response, retention time and the height at half width. The optimum flow rate was obtained at the lowest HETP in the Van Deemter graph.

2.3.3.3 Method validation for HPLC method

Parameters were validated according to the Center for Drug Evaluation and Research (CDER) and US-FDA method validation guidance as follows.

(1.) Limit of detection (LOD)

The limit of detection was the lowest concentration that the detector could detect. In this investigation, LOD was determined by diluting nitrofurans standard stock solutions, 10 $\mu\text{g mL}^{-1}$ with dimethylformamide to the concentration range of 1-10 $\mu\text{g L}^{-1}$. Ten μL of each solution was injected into the HPLC-UV system at optimum conditions. Five replications were carried out for each investigation. The minimum nitrofurans concentrations that gave the signal to noise ratio (S/N) of more than three ($S/N > 3$) were the limit of detection values.

(2) Linear dynamic range (LDR, Linearity)

The linear dynamic range was determined from the plot of the peak area versus the concentration of nitrofurans. Standard stock solutions of nitrofurans

were diluted with dimethylformamide to various concentration in the range of 0.01-1000 $\mu\text{g mL}^{-1}$. A 10 μL aliquot of each standard solution was injected into the HPLC-UV system at optimum conditions. Five replications were done for all analysis. The LDR was justified by considering the linear regression coefficient.

2.3.4 Determination of nitrofurans by UV-Vis spectrophotometric technique

The spectrophotometric method was based on colors forming when nitrofurans were reacted with the chemical reagent, methanolic potassium hydroxide (Moffat *et al.*, 1986), followed by measuring the absorbance of the colors at the optimum wavelength of each nitrofurans (Díaz *et al.*, 1994).

2.3.4.1 Optimization of the UV-Vis spectrophotometry conditions

The spectrophotometric optimum conditions of UV-Vis spectrophotometry system were studied for optimum wavelength, color forming time and concentration of potassium hydroxide solution. Optimizations were carried out by varying the interested parameter while the other conditions were kept constance. Preliminary conditions used in the investigation followed the reports of Moffat *et al.* (1986) and Díaz *et al.* (1994) which are shown in Table 6. Five replications were carried out for each investigation.

Table 6 Preliminary conditions of UV-Vis spectrophotometry (Moffat *et al.*, 1986 and Díaz *et al.*, 1994)

Parameters	Condition
Time of color forming	30 seconds
Wavelength scan	200-800 nm
Methanolic potassium hydroxide concentration	2.0 mol L ⁻¹

(1.) Time of color forming

Measuring time was an appropriate time that gave distinct colors of nitrofurans. Three mL of $5 \mu\text{g mL}^{-1}$ of nitrofuran working standard solution was reacted with 0.5 mL of the chemical reagent (methanolic potassium hydroxide). A good measuring time is the shortest period of time where a distinct color was formed. The measuring time was varied at 15, 60, 120, 180 and 300 seconds. In this investigation the wavelength scan and the chemical reagent concentration were set followed Table 6.

(2.) Optimum wavelength

The optimum wavelengths for nitrofurans were investigated by scanning the wavelength from 200 to 800 nm using the optimum color forming time. The wavelength that provided the highest absorbance was selected for optimum wavelength.

(3.) Optimum concentration of methanolic potassium hydroxide solution

To optimize the concentration of methanolic potassium hydroxide solution, nitrofurans working standard solution were reacted with this solution at various concentrations 0.5, 1.0, 1.5 and 2.0 mol L^{-1} . They were detected by UV-Vis spectrophotometer by using the time of color forming and optimum wavelength obtained earlier. Each concentration was done in five replications. The optimum concentration of potassium hydroxide solution was the concentration that gave the highest absorbance.

2.3.4.2 Method validation for UV-Vis spectrophotometry method

For the spectrophotometric technique, the method validation were performed followed the report of Harris (1995) and Morelli (1987).

(1) Limit of detection (LOD)

The detection limit was the lowest concentration of analyte that can be detected and the signal to noise ratio (S/N) was more than three (S/N>3). Nitrofurans, 1000 $\mu\text{g mL}^{-1}$, standard stock solutions were diluted with dimethylformamide to various concentrations in the range of 0.1-1 $\mu\text{g mL}^{-1}$. Each solution was reacted with methanolic potassium hydroxide and detected by UV-Vis spectrophotometric system at the optimum conditions. Five replications were done for all analyses.

(2.) Linear dynamic range (LDR, Linearity)

In this work, linearity was determined by using nitrofurans standard stock solutions, 1000 $\mu\text{g mL}^{-1}$, diluted with dimethylformamide to various concentrations in the range of 0.10-20 $\mu\text{g mL}^{-1}$. Each solution was detected with the UV-Vis spectrophotometric system at the optimum conditions. Five replications were done for all analyses. The linear dynamic range was obtained from the plot of the absorbance *versus* the concentration of nitrofurans.

2.4 Sample preparation

This work focused on the isolation of the nitrofurans from animal feed samples. The sample preparation for this work was based on solvent extraction technique, adapted from Lin and Jeng (2001). Chicken feed was used as a representative in the optimization of extraction conditions. The feed samples were grounded by a blender and passed through a 120-mesh sieve. A 10.00 grams of pulverized animal feed sample was spiked with 100 μL of 1000 $\mu\text{g mL}^{-1}$ standard stock solution before adding 5 mL of water. The mixture was stirred and allowed to stand for 5 minutes. Dimethylformamide 50 mL, was added and shaken for 15 seconds. The mixture was placed in the dark for 1 hour before being filtered through filter paper-No.3.

The extractant was cleaned up using the set up shown in Figure 5

the top of the column. The added extractant was washed by dimethylformamide. The resulting eluant was filtered through 0.2 μm polyvinylidene fluoride filter and analyzed by HPLC and UV-Vis spectrophotometric techniques at the optimum conditions.

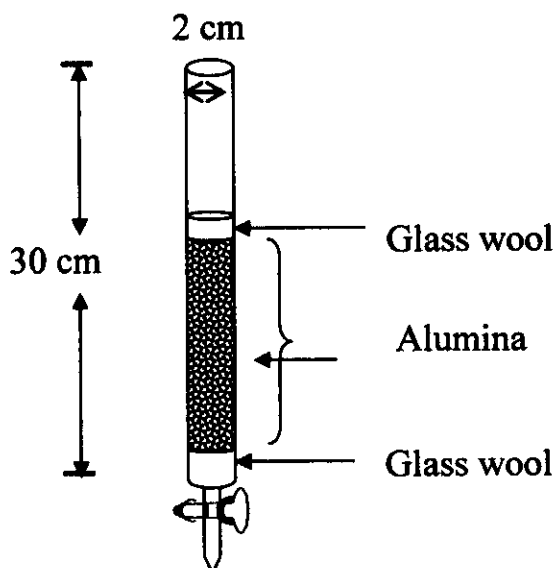


Figure 5 Glass column packed with Alumina used for sample clean up

The best recovery of nitrofurans was obtained by optimizing the extraction conditions, repeating five times with each parameters. Five replications of each extractant were analyzed with HPLC-UV at optimum conditions.

2.4.1 Extraction time

The extraction time necessary to completely extract the nitrofurans from the matrix was varied at 1, 3, 5, 10 and 15 hours. The time was selected at the highest response.

2.4.2 Type of extraction solvent

The type of extraction solvent is an important parameter in the extracting of analytes from the sample. The extraction solvent must be highly

2.4.2 Type of extraction solvent

The type of extraction solvent is an important parameter in the extracting of analytes from the sample. The extraction solvent must be highly selectivity to analyte. Two solvents for nitrofurans extraction were investigated, acetonitrile (McCracken and Kennedy, 1997) and dimethylformamide (Díaz *et al.*, 1994, and Lin and Jeng, 2001). The solvent that provided a better extraction efficiency was chosen.

2.4.3 Extraction (Shaking) time

The extraction, in this case shaking time is another important parameter. Shaking bring the sample into more contact with extraction solvent. The shaking time was varied at 0.25, 1.0 and 3.0 minutes. The shaking time that provided the highest response was then selected.

2.4.4 Type of washing solvent

The washing solvent is another important parameter to be considered in the washing of the analytes from alumina. Washing solvent should be strong with respect to the analyte for the high selectivity. Two washing solvents for nitrofurans extraction were investigated, dimethyformamide (Díaz *et al.*, 1994) and methanol (Cieri, 1979). The solvent that provided the highest response was then selected.

2.4.5 Recovery

The recovery is an important parameter in the expressing the accuracy of the sample preparation. The recovery was carried out by spiking standard nitrofurans at concentration $1 \mu\text{g mL}^{-1}$ into the sample. The spiked sample was extracted and analyzed by HPLC-UV at the optimum conditions. Good sample preparation should provide high recovery.

2.4.6 Matrix interference

Matrix interference was another parameter studied in the determination of nitrofurans in animal feed. The aim of this section was to evaluate the effect of matrix in the sample by spiking known amount of nitrofurans standards in the range of 2 –8 $\mu\text{g mL}^{-1}$ in 10.00 g of sample. The spiked sample was extracted and analyzed by HPLC-UV and UV-Vis spectrophotometer at the optimum conditions. The responses were plotted against the known concentrations. The slope of the standard and the spiked sample were compared for matrix interference.

2.5 Qualitative and quantitative analysis of animal feed sample

2.5.1 Sampling

Three types of animal feed sample, *i.e.* chicken feed, pig feed and shrimp feed, were purchased from stores in Songkhla province and kept in a dark place.

2.5.2 Qualitative analysis

2.5.2.1 UV-Vis spectrophotometric technique

Qualitative analysis was achieved by comparing the maximum wavelength (λ_{max}) of the absorption spectrum of unknown samples to nitrofurans standards (Settle, 1997).

2.5.2.2 HPLC-UV technique

Qualitative analysis was carried out by comparing the retention time (t_R) of the chromatogram of unknown samples to nitrofurans standards. The retention time t_R , is the time which is measured from the time of sample injection to the time of the band maximum leaves the column (Snyder and Kirkland, 1979).

2.5.3 Quantitative analysis

Quantitative analysis of nitrofurans in animal feed samples were

carried out by standard addition method. Animal feed samples were spiked with standard of nitrofurans at different concentrations ($2 - 8 \mu\text{g mL}^{-1}$). Then, the spiked samples were passed through the extraction and clean up steps (experiment 2.4.1-2.4.4). These sample extractants were analyzed using HPLC-UV detector and UV-Vis spectrophotometry at the optimum conditions. The results were plotted, and the response that was proportional to the amount of nitrofurans in the original sample is then determined.