CHAPTER II

EXPERIMENTAL

2.1 Instruments and Apparatus

Instruments and Apparatus	Model	Company	Country
1. Acrylic glass	-	-	Thailand
2. Analytical balance	AB 204 – S	Mettler Toledo	Switzerland
3. Analytical column HPLC	Zorbax C18	-	UK
4. Digital multimeter	UT60F Series	Uni-Trend	Hong kong
5. Centrifuge	1000 Series	Labquip	England
6. Elbow connector	Tubing 1/16"	Cole-Parmer	USA
7. Extraction cartridges	Oasis® HLB	Oasis and Waters	USA
8. Extraction cartridges	Sep-Pak® NH_2	Sep-pak and Waters	USA
9. Flow rate tygon pump tubing	PTFE tube	TACS	Australia
10. High performance liquid Chromatograph (HPLC)	1100 Series	Agilent Hewlett	USA
11. Micropipettor 10 – 100 µL	-	Biohit Proline	Finland
12. Micropipettor 100 – 1000 µL	-	Biohit Proline	Finland
13. Microsyringe (50 µL)	-	Hamilton	USA

Instruments and Apparatus (continued)

Instruments and Apparatus	Model	Company	Country
14. Nylon syringe filter (0.45 μm)	-	Vertical	Thailand
15. pH meter	827 pH lab	Metrohm	Switzerland
16. Photomultiplier tube	9828SB	Electron Tubes	UK
17. Power supply	PM20D	Electron Tubes	UK
18. Rotary vacuum evaporator	Eyela	Tokyo Rikakikal	Japan
19. Six port selection valve	-	Upchurch Scientific	USA
20. Two-channeled peristaltic pump	Miniplus 3	Gilson	France
21. Ultrapure water purification unit	-	Milipore	USA
22. UV/Vis spectrophotometer	2900 U	Hitachi	England

2.2 Chemicals

Chemical	Molecular Formula	Company	Country
1. Acetone	C_3H_6O	Merck	Germany
2. Ammonium acetate	CH_3COONH_4	Ajax	Australia
3. Ammonium hydroxide (30%)	NH_4OH	J.T. Baker	USA
4. Cetyltrimethylammonium bromide (CTAB)	$((C_{16}H_{33})N(CH_3)_3Br$	J.T. Baker	USA
5. Ethanol	C_2H_5OH	Merck	Germany
6. Ethyl acetate	$CH_3COOC_2H_5$	Lab-Scan	Ireland

2.2 Chemicals (continued)

7. Fluorescin	$C_{20}H_{12}O_5$	Merck	Germany
8. Furazolidone	$C_8H_7N_3O_5$	Sigma-Aldrich	Germany
9. Hydrogen peroxide	H_2O_2	Merck	Germany
10. Methanol	CH_3OH	BDH	UK
11. *N,N*-Dimethylformamide	C_3H_7NO	Fisher	UK
12. Nitrofurantoin	$C_8H_6N_4O_5$	Sigma-Aldrich	Germany
13. Nitrofurazone	$C_6H_6N_4O_4$	Sigma-Aldrich	Germany
14. Potassium ferrocyanide	$K_4Fe(CN)_6.3H_2O$	Ajax	Australia
15. Quinnine	$C_{20}H_{24}N_2O_2$	Fluka	USA
16. Rhodamine B	$C_{28}H_{31}ClN_2O_3$	Fluka	USA
17. Sodium dodecyl sulfate (SDS)	$(C_{12}H_{25}SO_4Na)$	J.T. Baker	USA
18. Sodium hexametaphosphate (SHMP)	$(NaPO_3)_6$	Sigma-Aldrich	Germany
19. Sodium hydroxide	NaOH	Merck	Germany
20. Triton X-100	$C_{14}H_{22}O(C_2H_4O)_n$	Fisher	UK
21. Tween 80	$C_{64}H_{124}O_{26}$	Merck	Germany

2.3 Preparation of Standard Solutions and Reagents

All chemicals used in this work were of analytical reagent grade. All solutions were used as recived and were prepared using ultrapure water of resistivity not less than 18.2 MΩ·cm at 25°C from a Milli-Q water purification system (Millipore, France).

2.3.1 Luminol stock solution (10.0 mmol L^{-1})

The stock solution of luminol was prepared by dissolving 0.4429 g of luminol in 2.5 mL of 2.0 mol L^{-1} NaOH and transferred into a 250 mL volumetric flask then sonication until dissolved, and adjusting to mark with ultrapure water.

To prevent the luminol from degrading under UV radiation, the stock solution of luminol was kept in a refrigerator at 4 ºC in an amber container when not in use.

2.3.2 Potassium ferocyanide stock solution (10.0 mmol L^{-1})

The stock solution of potassium ferocyanide was prepared by dissolving 0.4267 g of $K_4Fe(CN)_6.3H_2O$ in ultrapure water in a 100 mL volumetric flask.

2.3.3 Hydrogen peroxide stock solution (1.0 mol L^{-1})

The stock solution of hydrogen peroxide was prepared by diluting 2.55 mL of 30 % (v/v) H_2O_2 in ultrapure water in a 25 mL volumetric flask.

2.3.4 Reagent stream solution

The reagent stream solution consisting of luminol/$K_4Fe(CN)_6$ in alkaline medium. The working mixed-reagent solution were prepared from the appropriate dilution of stock solution of the luminol (0.75 mmol L^{-1}) and $K_4Fe(CN)_6$ (50 μmol L^{-1}) solution, and diluted with 0.40 mol L^{-1} sodium hydroxide solution in a 100 mL volumetric flask.

2.3.5 Oxidant and surfactant streams solution

The oxidant and surfactant stream consisting of H_2O_2/sodium hexametaphosphate surfactant solution. The working mixed-reagent solution were prepared from the appropriate dilution of stock solution of H_2O_2 (0.10 mol L^{-1}) and surfactant (0.1 % w/v) sodium hexametaphosphate solution, and diluted with ultrapure water in a 100 mL volumetric flask.

2.3.6 Standard solutions preparations

Separate stock solutions of each nitrofurans (FZD, NFT and NFZ) at concentration of 100 mg L^{-1} were prepared by dissolving 0.0100 g of each nitrofuran in 5.0 mL *N,N*-dimethyformamide and made up to 100 mL with ultrapure water. All standard Solutions were stored in a refrigerator at 4 °C and protected from light when not in use. (The solutions were found to be stable for at least 3 day, if kept in a refrigerator protected from light.)

Working standard solutions of their stock standard solutions of NFZ, NFT and FZD were prepared daily by making appropriate dilutions of the nitrofurans stock solutions (100 mg L^{-1}) with ultrapure water as carrier stream.

2.3.7 Pharmaceuticals sample preparations

2.3.7.1 NFT and FZD pure tablets

For each preparation, the contents of 20 tablets were accurately weighed and the average weight per tablets was determined. An accurately weighed portion of the finely powdered sample equivalent to 20.0 mg of pure drug and was dissolved in 10 mL of *N,N*-dimethyformamide by sonication, transferred to a 100 mL volumetric flask, filter if necessary and completed to volume with ultrapure water as describe previously by Hassan et al. and Walash [51-52]. Appropriate concentrations of sample solutions were obtained by dilution with carrier solution.

2.3.7.2 FZD blend tablets

Tablet blend samples, containing kaolin and neomycin were weighed and pulverize for 20 tablets. An accurately weighed amount of the powder equivalent to 20.0 mg of blend drug was dissolved in 10 mL *N,N*-dimethyformamide and transferred to a 100 mL volumetric flask, which was then completed to volume with ultrapure water. The SPE cartridge (Oasis® HLB 3cc, 60 mg) was used as a pretreatment for cleaning FZD blend tablets and concentrating the fulazolidone. The SPE cartridge was conditioned with 3 mL of methanol and 2 mL of water. Then 20 mL of sample solution was passed through the cartridge, followed by washing with 10 mL of 10 % (v/v) methanol and eluted with 3 mL of methanol. The eluate was dried under a stream of nitrogen and reconstituted with 1 mL of *N,N*-dimethyformamide, transferred to a 10 mL volumetric flask, filter if necessary and completed to volume with ultrapure water as similarly describe by Tribalat et al. and Angela et al. [53-54].

Appropriate concentration of sample solution was obtained by dilution with carrier solution [55]. The schematic procedure was illustrated in Figure 2.1.

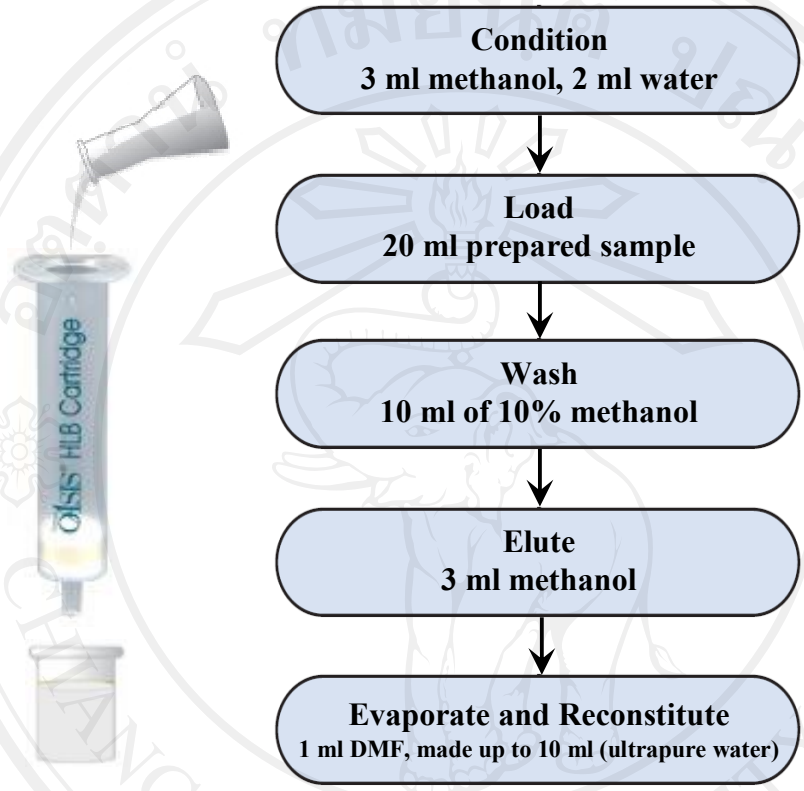


Figure 2.1 The SPE cartridge (Oasis® HLB 3cc, 60 mg) protocol summary for extraction of FZD blend tablets

2.3.7.3 FZD Suspension

An accurate volume of FZD suspension contain kaolin as filler (5 mL) was transferred to a 100 mL volumetric flask and dissolved with 10 mL of *N,N*-dimethyformamide. The solution was adjusted to volume with ultrapure water. Working solution was prepared by dilution with ultrapure water to contain calibration

curve of fulazolidone. The resulting solution was decanted onto a SPE cartridge (Oasis® HLB 3cc, 60 mg) similarly described in section 2.3.7 (b).

2.3.7.4 NFZ ointment

The 2 content ointment tubes were mixed and quantity of mixed ointment equivalent to 20.0 mg of nitrofurazone was accurately weighed and was dissolved in 10 mL of *N,N*-dimethyformamide by sonication for 35 min. The dissolved ointment base and the nitrocompound was then transferred to a 100 mL volumetric flask, filter if necessary and the volume was made up with ultrapure water. This solution should be protected from light as describe previously by Hassan et al. and Walash [51-52]. Appropriate concentrations of sample solutions were obtained by dilution with carrier solution prior to analysis.

2.3.8 Animal feeds sample preparations [56]

2.3.8.1 Extraction

An amount of 5.0 g thoroughly minced feed was weighed into a 250 mL polypropylene copolymer centrifuge flask. Then, 20 mL of ammonium acetate 79 mmol L^{-1} solutions (pH 4.6) were added and the pH was adjusted to 8 with diluted ammonia hydroxide solution. The mixture was allowed to rest for 15 min. A 30 mL of ethyl acetate was added before stirring for 20 min in a rotary shaker and centrifuged for 10 min at 3000 rpm. The organic layer was collected and evaporated to dryness in a rotary vacuum evaporator at 35 ^{o}C and 240 mbar. The resulting extract is reconstituted in 2 mL of a mixture of acetone and methanol 80:20 (v/v).

2.3.8.2 Clean-up

A Sep-Pak® NH_2 cartridge was conditioned with 5 mL of a mixture of acetone and methanol 80:20 (v/v). The reconstituted extract was put onto the cartridge and, then, the nitrofurans were eluted with 5 mL of the previous mixture. The eluate was evaporated to dryness and the residue was reconstituted with 5 mL of 10% (v/v) *N,N*-Dimethylformamide. The resulting solution was filtered through a 0.45 μm PVDF Mini-uniprep™ vial before injected to a microfluidic chemiluminescence system. The procedure was illustrated in Figure 2.2.

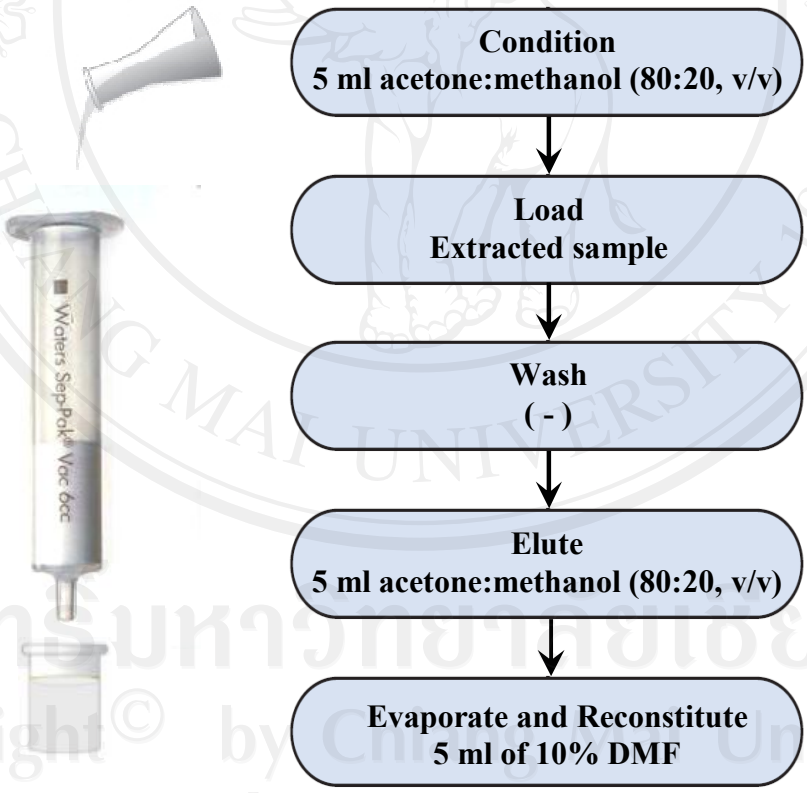
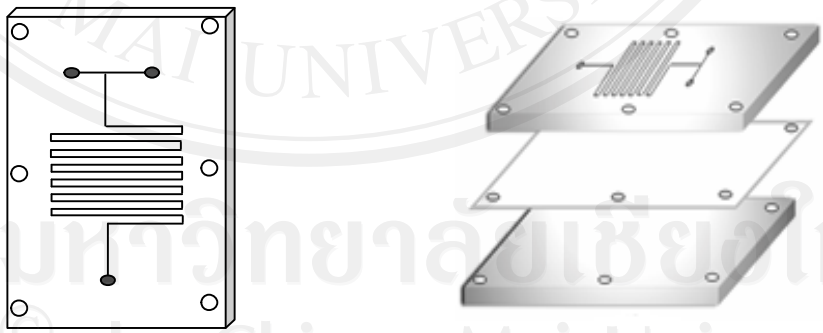


Figure 2.2 The SPE cartridge (NH_2 cartridges) protocol summary for extraction of animal feed samples

2.4 Fabrication of Microfluidic Device

The microfluidic device was designed using *CorelDraw X4* and was fabricated on an acrylic glass by laser engraving. The microfluidic chip was composed of two halves acrylic glasses (one of which having been laser-engraved with flow lines), sandwiching a polydimethylsiloxane (PDMS) plate, altogether clamped using screws and bolts. The PDMS plate was previously molded by thoroughly mixing a 10:1 weight mixture of PDMS prepolymer and curing agent (Sylgard 184 silicone elastomer kit, Dow Corning, Midland, MI). The resulting mixture was poured onto a glass slide template and was allowed to stand at room temperature to let the bubbles rise and come off. As the mixture became free of bubbles, curing was done at 60^{o}C for overnight until the PDMS hardened. The PDMS replica was later peeled off from the glass template and was cut into the desired plate size and shape. An illustration of the microfluidic chip and its flow-through line engravings are shown in Figure 2.3.



(A) (B)

Figure 2.3 Schematic layout of the microchip with three plates reprinted from. Illustration of the (A) laser-engraved flow lines of the (B) microfluidic chip with a PDMS sheet sandwiched between two acrylic glasses to control leakage during the flow of reagents along the flow-through lines

2.5 Flow Injection Chemiluminescence Studies for Determination of Some Nitrofurans

In order to develop a microfluiddic chemiluminescence procedure for nitrofurans determination, the CL intensity was tested for the sensitivity of chemiluminescence reactivity of the selected nitrofurans (furazolidone, nitrofurantoin and nitrofurazone) using the chemiluminescence reaction of interest. This can be performed by using the flow injection chemiluminescence (FI-CL) method prior to miniaturization as a microfluidic chemiluminescence manifold.

Flow manifold

The flow injection manifold as shown in Figure 2.4 consisted of a peristaltic pump. The sample or standard solution was injected via one six-port injection valve with a 100 μL sample loop. PTFE connection tubing (0.5 mm i.d.) was used as flow lines for the chemiluminescence reagent. The chemiluminescence signal was monitored in a custom built flow-through luminometer, which consisted of a 250 μL flat spiral glass flow cell mounted flush against a red sensitive photomultiplier tube (PMT). The operational potential for the PMT was provided by a stable power supply. The detector output was recorded using a portable computer via a USB/RS-232 interfaced to the detector, with a digital multimeter (UNI-T, UT60F).

The designed FI-CL manifold was a three channel FI manifold (Figure 2.4) in which the ultrapure water (C), Triton X-100/H_2O_2 (R1) and luminol/$K_4Fe(CN)_6$ in alkaline medium (R2) were premixed in the Y-pieces PTFE connection tubing. The reaction immediately mixed at the flat spiral glass flow cell, where the chemiluminescence reaction takes place upon merging three streams. Finally, the CL light is there monitored

by means of a intensity of photomultiplier tube (PMT) connects to a personal computer. Preliminary investigations for the optimum FI-CL conditions were conducted by univariate method, which are essential in assessing the influence of individual system parameters on its performance. In the univariate approach, the initial conditions employed and ranges of studied were listed in Table 2.1.

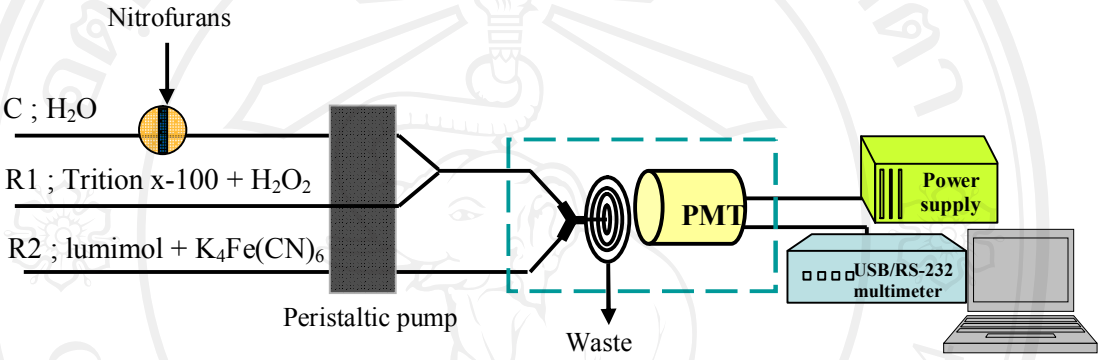


Figure 2.4 The flow injection chemiluminescence (FI-CL) manifold for preliminary studies of nitrofurans determination. C, a ultrapure water (H_2O) stream; R1, a oxidant/surfactant (H_2O_2/Triton X-100) stream; R2, a reagent (luminol/$K_4Fe(CN)_6$) stream solution.

Table 2.1 Preliminary experimental conditions for nitrofurans determination by FI-CL system

Parameter	Initial values	Studied ranges*
PMT voltage (V)	850	600 – 1000
Luminol concentration (mmol L^{-1})	0.80	0.50 – 1.00
$K_4Fe(CN)_6$ concentration (µmol L^{-1})	75	10 – 100

* NFZ was used for parameter studied

Table 2.1 Continued

Parameter	Initial values	Studied ranges*
NaOH concentration (mol L^{-1})	0.40	0.20 – 0.65
H_2O_2 concentration (mol L^{-1})	0.001	0.001 – 2.00
Flow rate C (mL min^{-1})	0.5	0.1 – 1.7
Flow rate R1 (mL min^{-1})	0.5	0.1 – 1.7
Flow rate R2 (mL min^{-1})	0.5	0.1 – 1.7
Triton X-100 concentration (% v/v)	none	0.01 – 0.50

* NFZ was used for parameter studied

2.6 Microfluidic Chemiluminescence (μFI-CL) Systems for Determination of Some Nitrofurans

For a microfluidic chemiluminescence (μFI-CL) system, Figure 2.5 showed the manifold for the on-line microfluidic chemiluminescence determination of some nitrofurans such as nitrofurazone (NFZ), nitrofurantoin (NFT) and furazolidone (FZD), which consisted of the three channels peristaltic pump to propel a carrier stream, reagent streams and a stream of oxidant/surfactant solution. The total flow rate was set at 0.6 mL min^{-1} with equal flow rate for reagent, oxidant/surfactant and carrier streams solution.

2.6.1 Procedure

For the preliminary conditions from session 2.5 provided some general idea for μFI-CL investigations. The three-line microfluidic system manifold used for the determination of some nitrofurans with chemiluminescence detection is shown in

Figure 2.5. The microfluidic chips prepared by the proposed procedure (as depicted in section 2.4), was incorporated into the chemiluminescence detection system. A 100 µL aliquot of sample or standard solution containing each nitrofurans was injected manually into a carrier stream (ultrapure water), which then merged with the oxidant/surfactant (Sodium hexametaphosphate with H_2O_2) and a stream of reagent (luminol/$K_4Fe(CN)_6$) solution. At the total flow rate the combined reaction mixture, flowing at 0.6 mL min^{-1}, was passed through a microfluidic chip where the CL intensity was detected by a PMT put underneath a microchip operated at a fixed voltage of 850 V. The output of the PMT, which is proportional to the CL intensity, was continuously monitored via a digital multimeter connected with a portable computer. Peak height of the chemiluminescence signals corresponding to peak heights were plotted versus various concentrations of each drug.

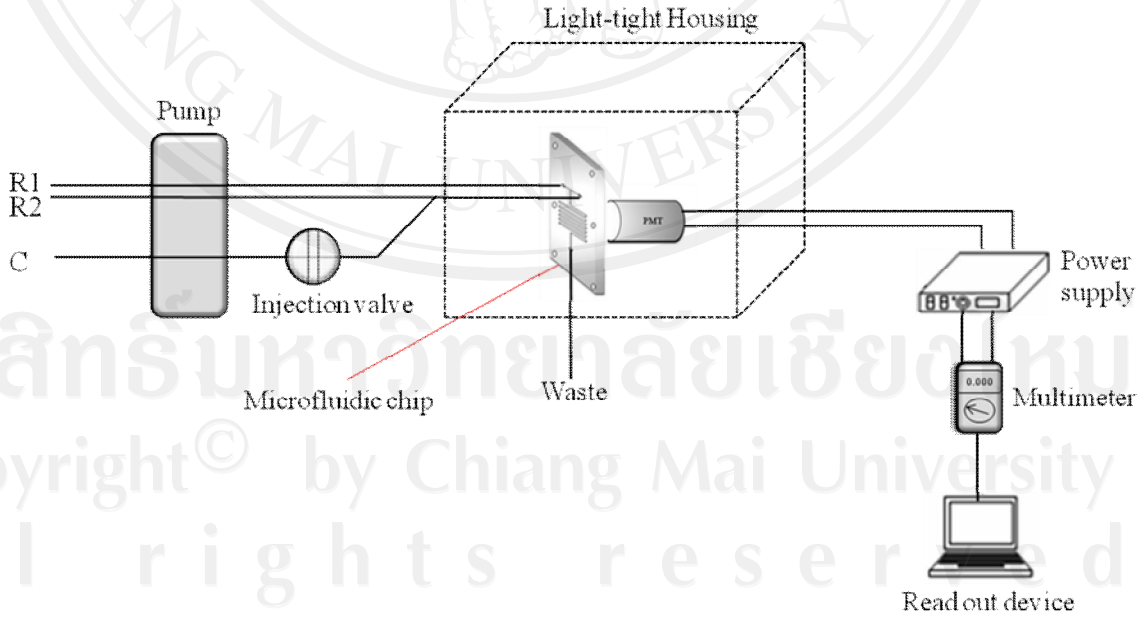


Figure 2.5 Schematic diagram of a microfluidic chemiluminescence system manifold: R1, a reagent stream; R2, a oxidant/surfactant stream; and C, a carrier stream

2.6.2 Optimization of the microfluidic chemiluminescence system by univariation method

The optimizations of experimental conditions were carried out by means of a univariation method. For this feature, a variable was modified maintaining the other variables at their constant values. The NFZ, NFT and FZD were initially used as the working standard in optimizing different parameters in order to obtain optimum results for the determination of each nitrofurans. The other physical and chemical parameters optimized in this study include: the concentration of streams of reagent and oxidant/surfactant solution; individual flow rates; sample injection volume; and PMT applied potential. The appropriate conditions were obtained by measuring from the highest CL intensity of each parameter using the microfluidic chemiluminescence manifold as shown in Figure 2.5. The initial values and their studied ranges for the microfluidic chemiluminescence parameters were presented in Table 2.2.

Table 2.2 The studied range for the microfluidic chemiluminescence parameters

Parameter	Initial values	Studied ranges*
PMT voltage (mV)	850	750 – 1000
Luminol (mmol L^{-1})	0.85	0.50 – 2.00
$K_4Fe(CN)_6$ (µmol L^{-1})	40	10 – 80
NaOH (mol L^{-1})	0.45	0.30 – 0.60
H_2O_2 (mol L^{-1})	0.10	0.01 – 0.70
Flow rate C (mL min^{-1})	0.2	0.05 – 0.40
Flow rate R1 (mL min^{-1})	0.2	0.05 – 0.40

Table 2.2 Continued

Parameter	Initial values	Studied ranges*
Flow rate R2 (mL min^{-1})	0.2	0.05 – 0.40
Types of surfactant	none	Triton X-100, Tween 80, SHMP, SDS and CTAB
Types of sensitizer	none	Quinnine, Fluorescin and Rhodamine B
Surfactant concentration (% w/v)	none	0.05 – 0.50

* NFZ, NFT and FZD was used for parameter studied.

2.6.3 Linearity of calibration graph

Using the microfluidic chemiluminescence manifold (Figure 2.4) under the optimum conditions, linear range of calibration graph was studied from the relationship return the CL intensity and standards of NFZ, NFT and FZD in the concentration ranging from 0.1 - 30.0 mg L^{-1}. CL intensity corresponding to various concentrations of nitrofurans was measured by microfluidic chemiluminescence procedure and recorded as CL intensity (peak heights) as function of time. A typical calibration range was selected by linear of plotting the CL intensity against various concentrations of NFZ, NFT and FZD.

2.6.4 Detection and Quantification limits

The signal-to-noise ratio is given by S/N = 3 and quantification limit as that concentration for which the signal-to-noise ratio is given by S/N = 10. Evidently, detection and quantification limit is dependent only on baseline noise (N) and the signal (S) [57].

2.6.5 Precision

The precision of the proposed method was verified by injecting 15 replicates of 2.0 mg L^{-1} standard of NFZ, NFT and FZD solution, and the % RSD was calculated from the equations as follows;

$$\%RSD = \frac{SD \times 100}{\overline{X}} \quad (2.1)$$

Where $\%RSD$ = percentage relative standard

SD = standard deviation

$\overline{X}$ = mean

2.6.6 Intra-day and Inter-day variations of the method

The intra-day and inter-day variations of the method were determined using three replicate injections of each standard of concentrations and analyzed on the same day and three different days over a period of a week. The data was used to calculate the standard deviation, mean, and correlation value (%RSD).

2.6.7 Excipients and interference studies

The excipient effects of some possible pharmaceutical substances and the interference effects of some possible foreign ions in the microfluidic chemiluminescence system for determination of nitrofurans were studied by the proposed microfluidic chemiluminescence procedure under the optimum conditions. A systematic study to check for the effects of some possible substances (Glucose, Starch, Sucrose, Lactose Fructose, Acacia Bentonite, Methyl cellulose, Carboxyl methyl cellulose, Polyethylene

glycol) and foreign ions (Al^{3+}, Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, Cl^{-}, SO_4^{2-}, NO_3^{-}, HPO_4^{2-}, CO_3^{2-}) on determination of nitrofurans was performed by adding known amounts of each interference to 2.0 mg L^{-1} of NFZ, NFT and FZD standard solution.

2.7 Validation Method

In order to determine the accuracy of the method, the some nitrofurans, including nitrofurazone (NFZ), nitrofurantoin (NFT) and furazolidone (FZD) in pharmaceutical preparations and animal feed samples were determined by using the validation method (the official BP and HPLC method) [58,56] versus the proposed method. The nitrofurans values obtained by both the methods were compared and verified by using student *t*-test at 95% confident level.

2.7.1 The official BP method for determination of nitrofurans in pharmaceuticals sample preparations [58]

2.7.1.1 Nitrofurazone

A sample amount equivalent to approximate 60 mg of nitrofurazone (NFZ) was dissolved into 20 mL of dimethylformamide and diluted to 500 mL with deionized water. Five milliliters of this solution was transferred into a 100 mL volumetric flask and diluted to volume with deionized water and mixed. The absorbance of this solution was measured at 375 nm. The content of NFZ in the sample solution was calculated by reference to the calibration curve.

2.7.1.2 Nitrofurantoin

A sample amount equivalent to approximate 120 mg of nitrofurantoin (NFT) was dissolved in 50 mL of dimethylformamide and diluted to 1000 mL with deionized water. Five milliliters of this solution was transferred into a 100 mL volumetric flask containing 18 g L^{-1} of sodium acetate and 0.14 percent (v/v) of glacial acetic acid.

The absorption of this sample solution was measured at 367 nm, using the sodium acetate solution described above as compensation liquid. The content of NFT in the sample solution was calculated by reference to the calibration curve.

2.7.1.3 Furazolidone

A sample amount equivalent to approximate 80 mg of furazolidone (FZD) was dissolved into 150 mL of dimethylformamide, swirled, then added sufficient water to produce 500 mL. Five milliliters of this solution was transferred into a 100 mL volumetric flask and diluted to volume with deionized water and mixed. The absorbance of this solution was measured at 367 nm. The content of FZD in the sample solution was calculated by reference to the calibration curve.

2.7.2 The HPLC method for determination of nitrofurans in animal feeds [56]

The sample solutions of the studied drugs were cleaned up by SPE as described in earlier section 2.3.8 (b). Each solution was evaporated and reconstituted in 1 mL of 14 mmol L^{-1} ammonium acetate solution (pH 4.6) : acetonitrile (70:30, v/v). A 25 μL of the extracted solution was injected into the Agilent 1100 HPLC automated system. The HPLC analytical conditions were as follows: column, sorbax SB-C18

(4.6×250 mm i.d.5 µL); column temperature, 25 ºC mobile phase, and 70 % acetonitrile-ammonium acetate (pH 4.6) 1 min and then 2 min, 50% acetonitrile - ammonium acetate (pH 4.6), with diode array detection at 375 nm. The flow rate was used as 1.2 mL min^{-1} with a total run time of 12 min summary of HPLC parameters were listed in table 2.3.

Table 2.3 Procedure parameters for HPLC systems

Analytical parameters	**Conditions**
Column	Zorbax C18
Detector	diode array detector (DAD)
Wavelength	375 nm
Injection volume	25 µL
Mobile phase ratio (Gradient)	H_2O : acetate buffer
	Time (min) % acetate buffer
	0 70
	1 50
Flow rate	1.2 mL min^{-1}
Total run time	12 min

2.7.3 Student *t*-test

In order to validate the proposed method for some nitrofurans determination, a comparative determination of nitrofurazone (NFZ), nitrofurantoin (NFT) and furazolidone (FZD) by the BP method was carried out. Results obtained by both methods were verified by using student *t*-test. The calculated t_{cal} value was obtained from the equation as follows [59];

$$t_{calculated} = \frac{\left|\overline{X}_1 - \overline{X}_2\right|}{s_{pooled}} \sqrt{\frac{n_1 n_2}{n_1 + n_2}} \quad (2.2)$$

$$s_{pooled} = \sqrt{\frac{s_1^2(n_1 - 1) + s_2^2(n_2 - 1)}{n_1 + n_2 - 2}} \quad (2.3)$$

where; S_{pooled} = pooled standard deviation

$n_1 + n_2 - 2$ = number of degree of freedom

2.8 Analytical recovery

The recoveries of nitrofurans by the proposed method were verified by spiking two known amounts of nitrofurans standard solution in the pharmaceutical preparations and animal feeds samples. After analysis of the sample solution by the proposed method, the nitrofurans concentrations were calculated from linear regression equation obtained from the calibration graph. Finally, the percentage recovery was calculated from the equation as follows;

$$\%\ Recovery = \frac{(total\ nitrofurans\ conc. - nitrofurans\ conc.\ in\ sample) \times 100}{spiked\ nitrofurans\ conc.} \qquad (2.4)$$