Chapter 3

Results and discussion

3.1 Nitrofurans determination by HPLC technique

3.1.1 Optimization of the HPLC-UV analysis conditions

(1.) Optimum wavelength

An ultraviolet detector was a photometer based on ultraviolet absorption property of sample (Snyder and Kirkland, 1979). Sample must absorb in the UV region 190-400 nm to be detected. In this work, the optimum wavelength for nitrofuran analysis was investigated between 360-380 nm (2.3.3.2) and the results are shown in Table 7 and Figure 6. The wavelength that gave the highest absorption (response), 365 nm, was selected. The optimum wavelength of this work agreed with the reports of Cieri (1979), Smallidge (1985), Kaniou *et al.* (1994), McCracken and Kennedy (1997) and Lin and Jeng (2001).

Table 7 Response of 10 μg mL^{-1}, 10 μL nitrofurans at various wavelengths

Wavelength (nm)	Response × 10^5* (AU×s)		
	Nitrofurazone (NZ)	Nitrofurantoin (NF)	Furazolidone (FZ)
360	1.90	1.80	1.77
365	2.13	1.78	1.86
370	2.12	1.80	1.81
375	2.14	1.76	1.74
380	2.08	1.65	1.64

* 5 replications, RSD < 4%

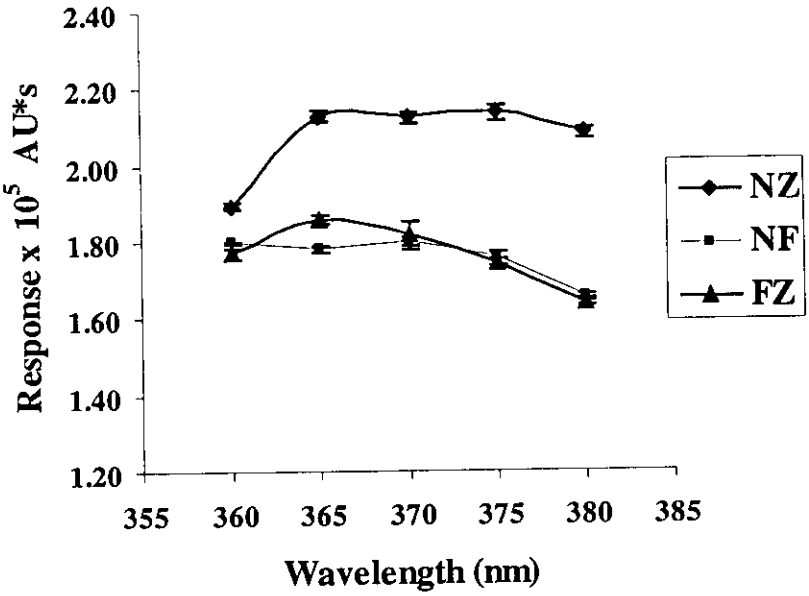


Figure 6 Response of 10 μg mL^{-1}, 10 μl nitrofurans at various wavelengths

(2.) Composition of mobile phase

In reversed phase chromatography, the mobile phase is more polar than the stationary phase, and the most polar compounds elute first from the column with the increased analyte retention depend on the decreasing mobile phase polarity. Normally one of the mobile phase is water, which does not interact with the hydrophobic adsorbent surface and does not compete with the analyte for the adsorption sites. Another mobile phase is the organic modifier which can interact with the adsorbent surface and compete with analyte molecules for the adsorption sites. Increasing of the modifier concentration in the mobile phase leads to decreasing of the analytes retention. So, optimization of the amount of modifier is necessary (Currell, 2000).

In this work the mobile phase was based on the modification of Cieri's report (Cieri, 1979). The modifier is acetonitrile because it has a very low UV cutoff (<190 nm) (Sadek. 1996). Acetonitrile also has a low viscosity (0.358 cP). Viscosity has a significant role in actual operation due to two effects, at a fixed flow velocity of the mobile phase, the pressure drop across a column, ΔP, is proportional to

the viscosity, η, of the mobile phase according to Darcy's law (Horváth, 1980)

$$v_0 = \frac{B^0}{\eta} \cdot \frac{\Delta P}{L} \qquad (1)$$

where L and B^0 are the column length and specific permeability coefficient respectively, and v_0 is the flow velocity of mobile phase. According to the above equation the pressure drop at a given flow rate is directly proportional to the viscosity. So a high viscosity mobile phase may produce an excessively large pressure drop and if pressure drop is held constant, the flow rate is inversely proportional to the viscosity (Horváth, 1980).

The choice of mobile phase composition may also be considered by two factors, *i.e.*, capacity factor and retention time of analyte. The capacity factor (k') is a widely used parameter to describe migration of solutes on a column.

$$k' = \frac{t_R - t_0}{t_0} \qquad (2)$$

Where t_R and t_0 are the retention time for the sample and unretain peak, respectively. High efficiency of separation is realized for analytes with low capacity factor. Normally, the range of capacity factor at optimum are $1 \leq k' \leq 10$ (Snyder and Kirkland, 1979). Another choice is the decreased retention time (t_R) of analyte after increasing of mobile phase composition. The concentration of organic modifier which was added in the mobile phase should give a chromatographic run within an appropriate retention time.

For the analysis of nitrofurans, 4 different compositions of acetonitrile were studied *i.e.* 20, 25, 30, 35 and 40% in distilled water (2.3.3.2). The results showed that 35% acetonitrile in distilled water was the most suitable (Tables 8-10 and Figures 7-9) since it gave the highest response and the appropriate capacity factor value (k' ~ 1 to 2). At 35% of acetonitrile the retention time of nitrofurazone, nitrofurantoin and furazolidone were 5.52, 6.50 and 8.39 minutes, respectively.

Table 8 Responses of nitrofurans 10 μg mL^{-1}, 10 μl at different percentage of acetonitrile in water

Acetonitrile concentration in water (%)	Response × 10^5* (AU×s)		
	Nitrofurazone (NZ)	Nitrofurantoin (NF)	Furazolidone (FZ)
20	5.98	5.29	5.37
25	5.97	5.21	5.31
30	5.98	5.26	5.30
35	6.28	5.42	5.53
40	6.20	5.39	5.48

* 5 replications, RSD < 4%

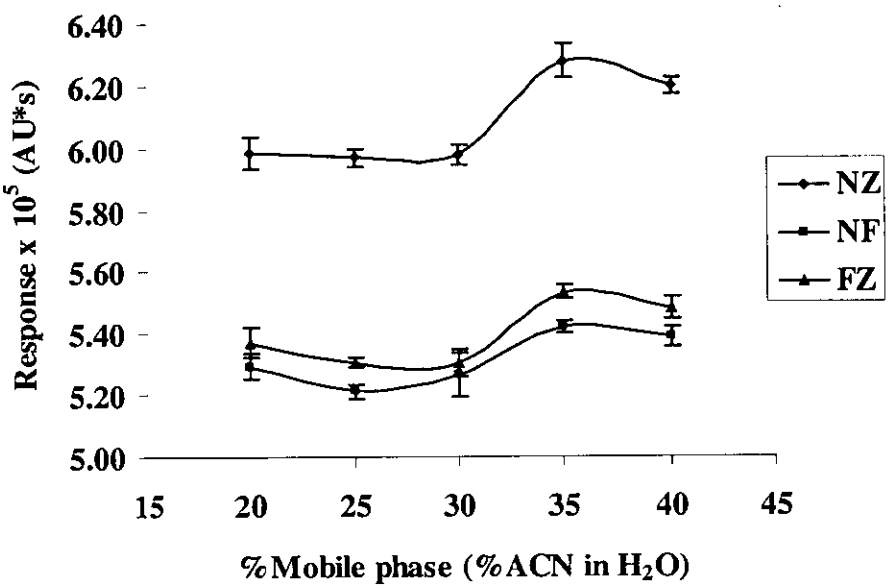


Figure 7 Responses of 10 μg mL^{-1}, 10 μL nitrofurans at different acetonitrile concentration in water (%)

Table 9 Capacity factor of nitrofurans (10 μL, 10 μg mL^{-1}) at different percentage of acetonitrile in water

Acetonitrile concentration in water (%)	Capacity factor (k')		
	Nitrofurazone (NZ)	Nitrofurantoin (NF)	Furazolidone (FZ)
20	3.05	3.67	5.33
25	1.93	2.43	3.66
30	1.17	1.57	2.43
35	0.90	1.24	1.84
40	0.69	0.96	1.55

* 5 replications, RSD < 4%

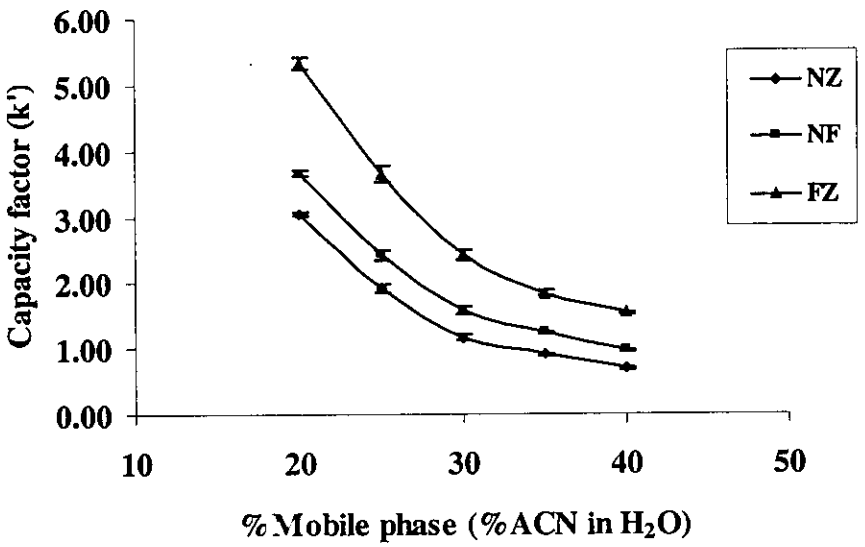


Figure 8 Capacity factor (k') of 10 μg mL^{-1}, 10 μL nitrofurans at different acetonitrile concentration in water (%)

Table 10 Retention time of nitrofurans (10 µl, 10 µg mL^{-1}) at different percentage of acetonitrile in water

Acetonitrile concentration in water (%)	Retention time (t_R)		
	Nitrofurazone (NZ)	Nitrofurantoin (NF)	Furazolidone (FZ)
20	12.61	14.52	19.69
25	8.53	9.99	13.56
30	6.60	7.82	10.45
35	5.52	6.50	8.39
40	4.93	5.73	7.43

*5 replications, RSD < 4%

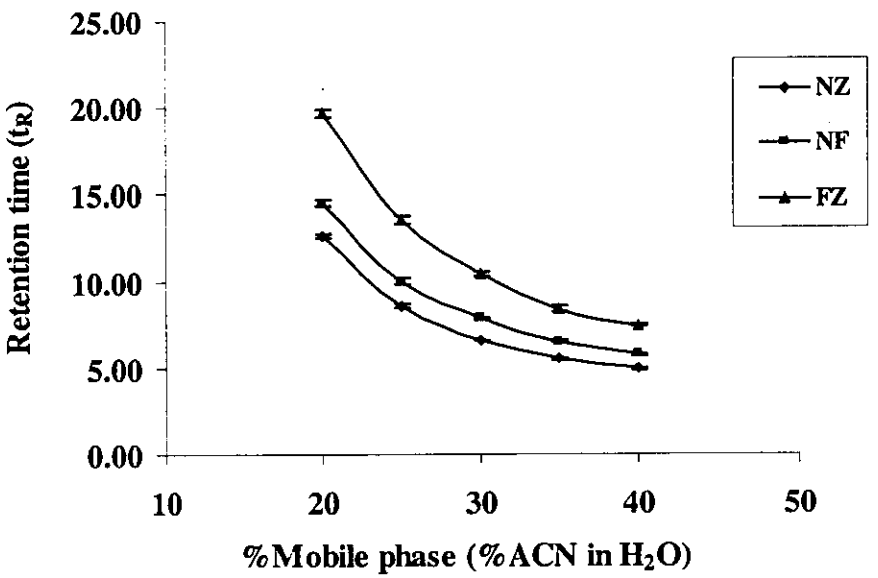


Figure 9 Retention time (t_R) of 10 µg mL^{-1}, 10 µL nitrofurans at different acetonitrile concentration in water (%)

(3.) Optimum flow rate

In HPLC, flow rate is the volumetric rate of flow of a mobile phase through the HPLC column. The optimum flow rate selection relate to the HETP (height equivalent of a theoretical plate or plate height) which measures the efficiency of a column per unit length and can be calculated by

$$H = L / N \qquad (3)$$

Where H is the height equivalent of a theoretical plate or plate height, L is the length of column and N is the theoretical plate number of the column. N can be calculated by

$$N = 5.54 (t_R / W_{1/2})^2 \qquad (4)$$

Where t_R is the retention time of the solute and $W_{1/2}$ is the peak width at the half height

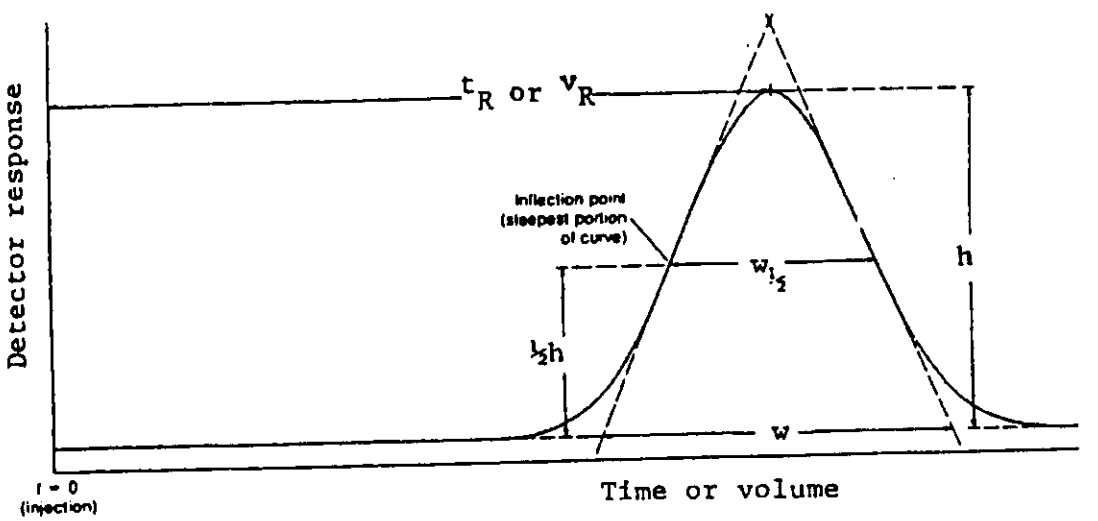


Figure 10 Measurement used in calculating total theoretical plate

From equation (3) and (4), the attainment of the smallest H values means the most efficient column and the largest N value. This point is the optimum flow rate (Snyder and Kirkland, 1979). The relationship between H value and flow rate of mobile phase in HPLC separation is important. A Van Deemter plot is normally used to find the optimum mobile phase flow rate. The general form of the

Van Deemter equation is given by,

$$H = A + \frac{B}{u} + Cu \qquad (5)$$

where A is an eddy diffusion term, represents the multitude of pathways by which a component finds its way through the column. The term B is related to the longitudinal diffusion, the process in which the molecules diffuse from the concentrated center of a zone to the more dilute regions ahead and behind zone and the C term is the mass transfer term which describes the time available for equilibrium of an analyte to be established between the mobile and stationary phases.

Figure 11 shows a Van Deemter plot. This is the plot of plate height (H) vs the flow rate, the minimum of the Van Deemter curve represents the optimum flow rate (Horváth, 1980).

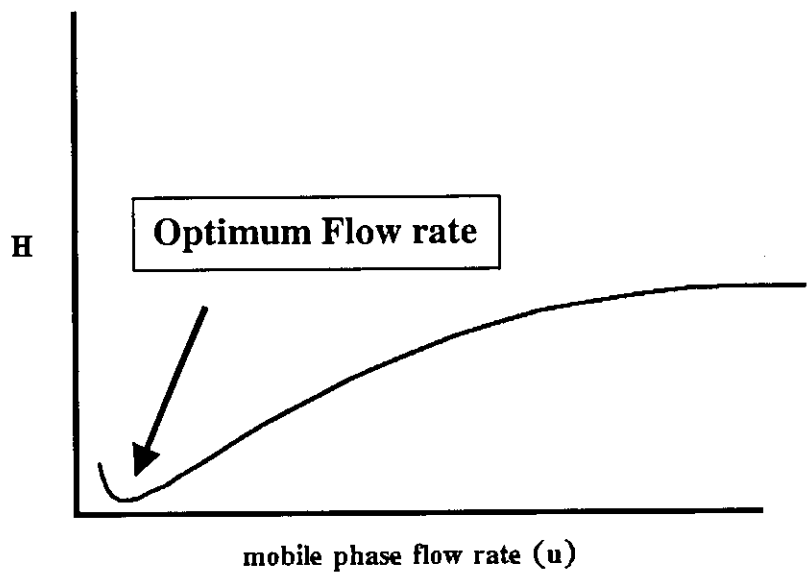


Figure 11 Van Deemter plot for HPLC

In this work, equation (3) and (4) were used to calculate HETP. The relationship between HETP and the mobile phase flow rate for nitrofurans is shown in Table 11 and Figure 12. The optimum mobile phase flow rate was obtained at the lowest HETP of the Van Deemter plot in Figure 12, at 1.0 mL min^{-1}.

Table 12 summarizes the optimum conditions of HPLC-UV

Table 11 The height equivalent to a theoretical plates, HETP, at various flow rate of mobile phase (35% acetonitrile in water)

Flow rate (mL min^{-1})	HETP (cm)*		
	Nitrofurazone (NZ)	Nitrofurantoin (NF)	Furazolidone (FZ)
0.8	0.0169	0.0165	0.0145
0.9	0.0170	0.0172	0.0148
1.0	0.0174	0.0178	0.0143
1.1	0.0183	0.0187	0.0147

* 5 replications, RSD < 4%

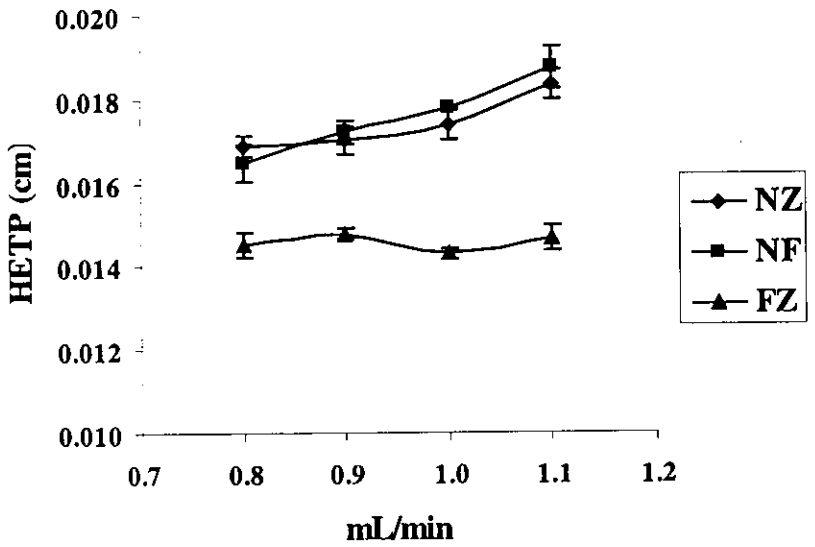


Figure 12 The Van Deemter plot of nitrofurans

Table 12 Optimum conditions of HPLC-UV detection with C-18 column

Parameters	Optimum values
Mobile phase	35% acetonitrile in water
Flow rate	1.0 mL min^{-1}
Maximum wavelength	365 nm
Injection volume	10 μL

3.1.2 HPLC method validation

(1.) Detection limit

The detection limit of an individual analytical technique is the lowest concentration of an analyte in a sample that can be detected, though not necessarily quantitated (Swartz and Krull, 1997). The detection limit is defined as the concentration corresponding to a signal to noise ratio equal or more than 3 (Harris, 1995). The limit of detection of nitrofurans was 5 μg L^{-1} for this work. The limit of detection was lower than Díaz, *et al.*, 6 μg L^{-1} (Díaz *et al.*, 1997) with electrochemical detection. This is because HPLC-UV detection can give higher sensitivity than electrochemical detection (Snyder and Kirkland, 1979).

(2.) Linear dynamic range

Linear dynamic range is the ability of the method to elicit test results that are directly proportional to analyte concentration within a given range (Swartz and Krull, 1997). As indicated in experiment 2.3.3.4, the linear dynamic range was demonstrated experimentally by running various concentrations. Tables 13-15 and Figures 13-18 showed the concentration ranged which were used to study the linear dynamic range of nitrofurans. At higher concentrations the plots were non linear. Figures 14, 16 and 18 show the response and coefficient of determination, R^2 of nitrofurans, and are summarized in Table 16.

Table 13 Response of nitrofurazone (NZ) of various concentrations

Concentration of NZ (mg L^{-1})	Response × 10^4* (AU×s)
0.01	0.05
0.02	0.10
0.03	0.15
0.04	0.19
0.05	0.26
0.1	0.48
0.5	2.38
1	4.33
5	24.69
10	50.55
50	252.73
100	511.86
300	1507.14
500	2474.96
700	3254.64
900	3925.12
1000	4224.89

* 5 replications, RSD < 4%

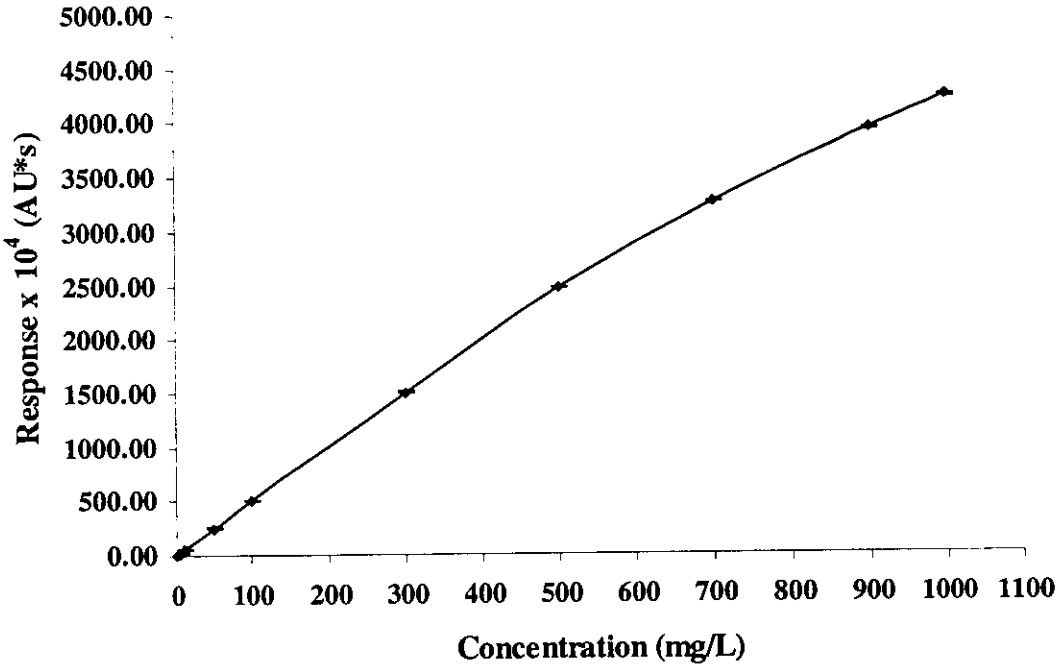


Figure 13 Relationship between response and concentration of nitrofurazone (NZ)

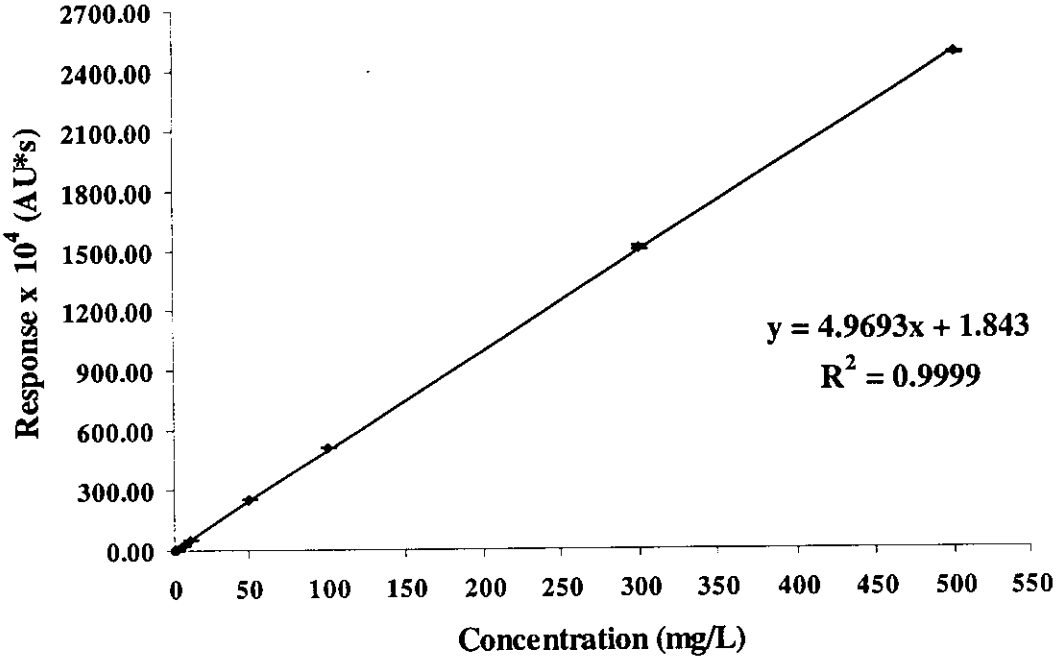


Figure 14 Linear dynamic range of nitrofurazone (NZ)

Table 14 Response of nitrofurantoin (NF) of various concentrations

Concentration of NF (mg L^{-1})	Response × 10^4* (AU×s)
0.01	0.05
0.02	0.09
0.03	0.14
0.04	0.18
0.05	0.25
0.1	0.46
0.5	2.31
1	4.14
5	23.90
10	48.94
50	243.61
100	493.42
300	1456.55
500	2378.13
700	3133.18
900	3829.57
1000	4127.93

* 5 replications, RSD < 4%

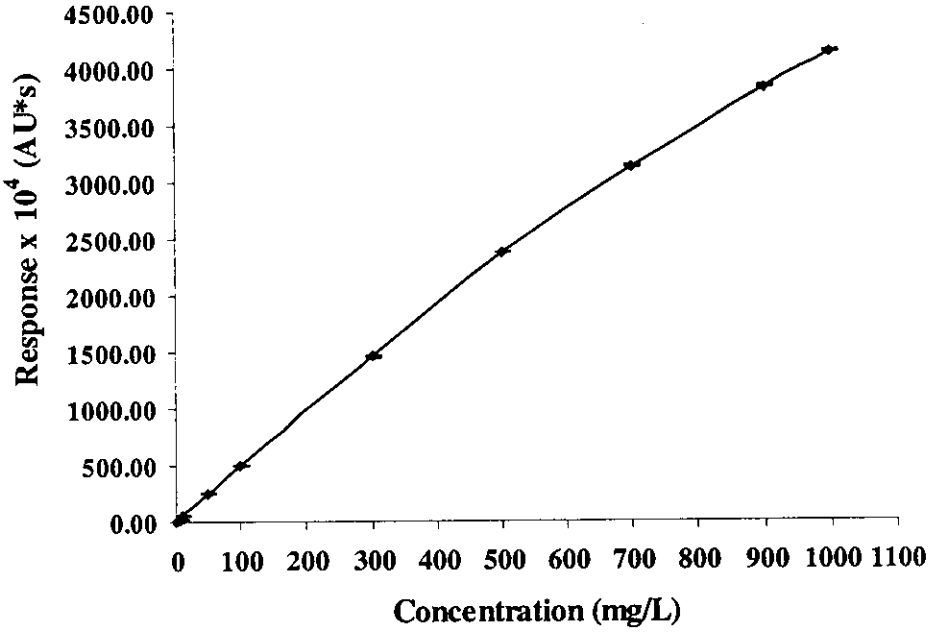


Figure 15 Relationship between response and concentration of nitrofurantoin (NF)

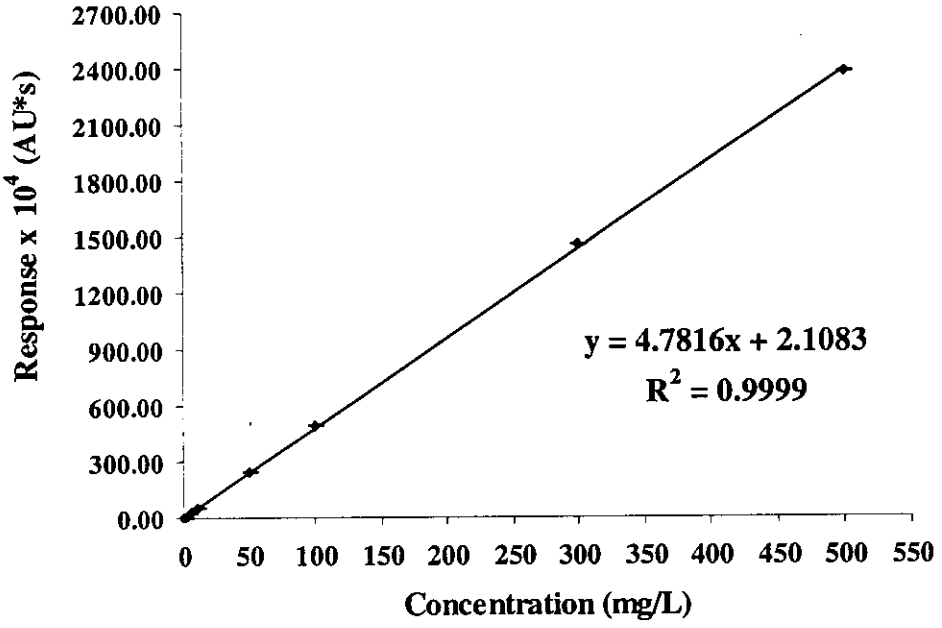


Figure 16 Linear dynamic range of nitrofurantoin (NF)

Table 15 Response of furazolidone (FZ) of various concentrations

Concentration of FZ (mg L^{-1})	Response × 10^4* (AU×s)
0.01	0.04
0.02	0.09
0.03	0.14
0.04	0.19
0.05	0.26
0.1	0.47
0.5	2.40
1	4.29
5	23.94
10	48.11
50	234.20
100	476.40
300	1409.32
500	2314.01
700	3175.05
900	4040.04
1000	4401.83

* 5 replications, RSD < 4%

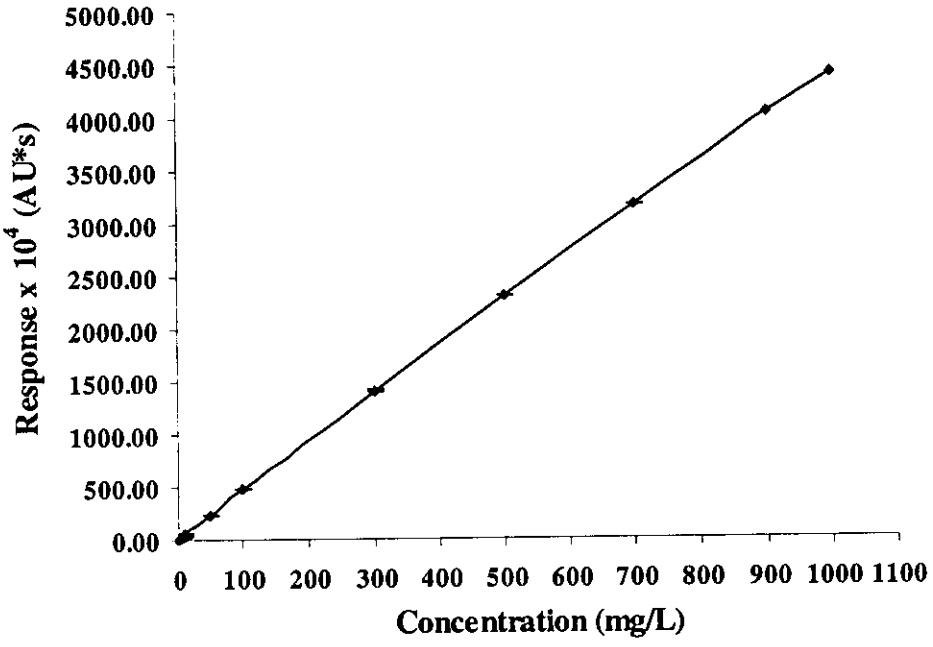


Figure 17 Relationship between response and concentration of furazolidone (FZ)

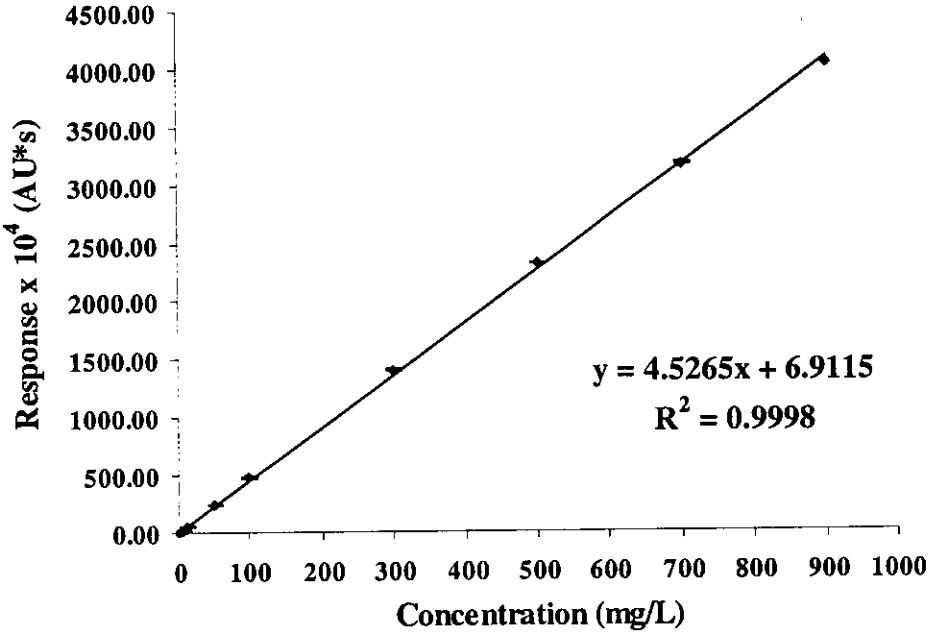


Figure 18 Linear dynamic range of furazolidone (FZ)

Table 16 Summary of linear dynamic range and coefficient of determination (R^2) of nitrofurans

Compounds	Linear dynamic range (mg L^{-1})	Coefficient of determination (R^2)
Nitrofurazone (NZ)	0.01-500	0.9999
Nitrofurantoin (NF)	0.01-500	0.9999
Furazolidone (FZ)	0.01-900	0.9998

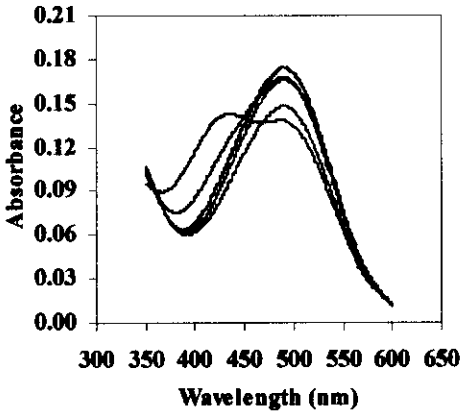
This system provided a wide linear dynamic range which were better than Kaniou *et al.*'s report, 0.1-5 mg L^{-1} (Kaniou *et al.*, 1994). In addition, the coefficient of determination, R^2 is greater than 0.999.

3.2 Nitrofuran determination by UV-Vis spectrophotometric technique

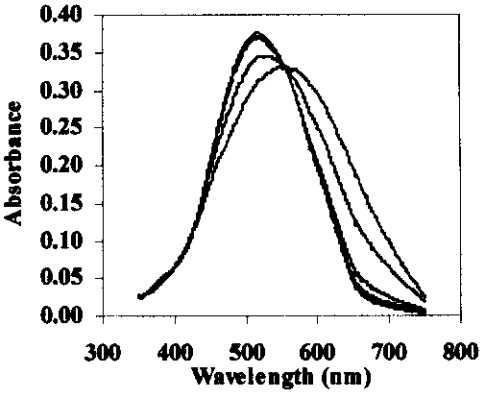
3.2.1 Optimization of the UV-Vis spectrophotometric conditions

(1.) Time of color forming

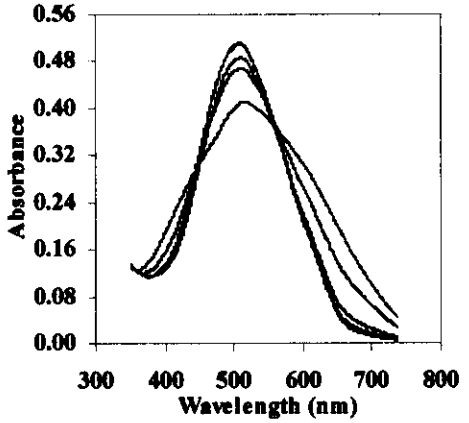
The precise control of the color forming time is required for distinction of nitrofurans when brought into contact with chemical reagent (Díaz *et al.*, 1994). However, some of these colors are unstable and their spectra change with time, as can be seen in Figures 19-20. Therefore, the best measuring time was chosen at 15 seconds.



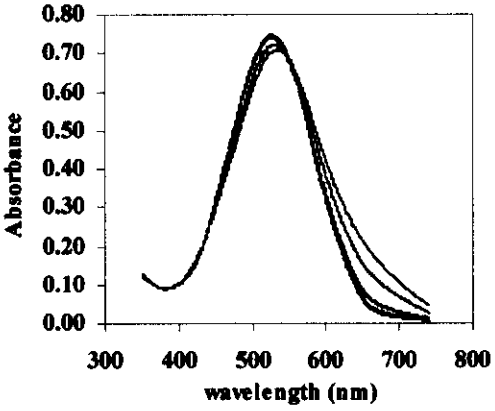
a.) Nitrofurantoin



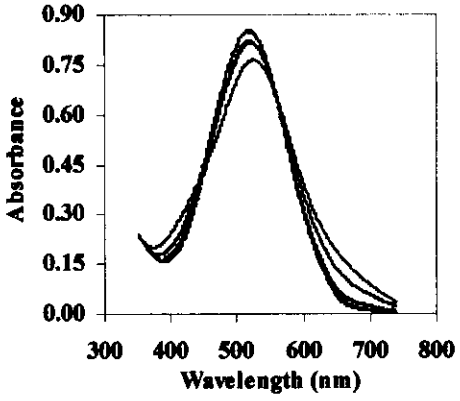
b.) Furazolidone



c.) Nitrofurantoin - Furazolidone



d.) Nitrofurazone - Furazolidone



e.) Nitrofurazone - Furazolidone - Nitrofurantoin

Figure 19 Evolution of the absorption spectrum of a 10 μg mL^{-1} nitrofuran solution at various periods of time

(____ 30 sec, ____ 60 sec, ____ 120 sec, ____ 180 sec, ____ 300 sec)

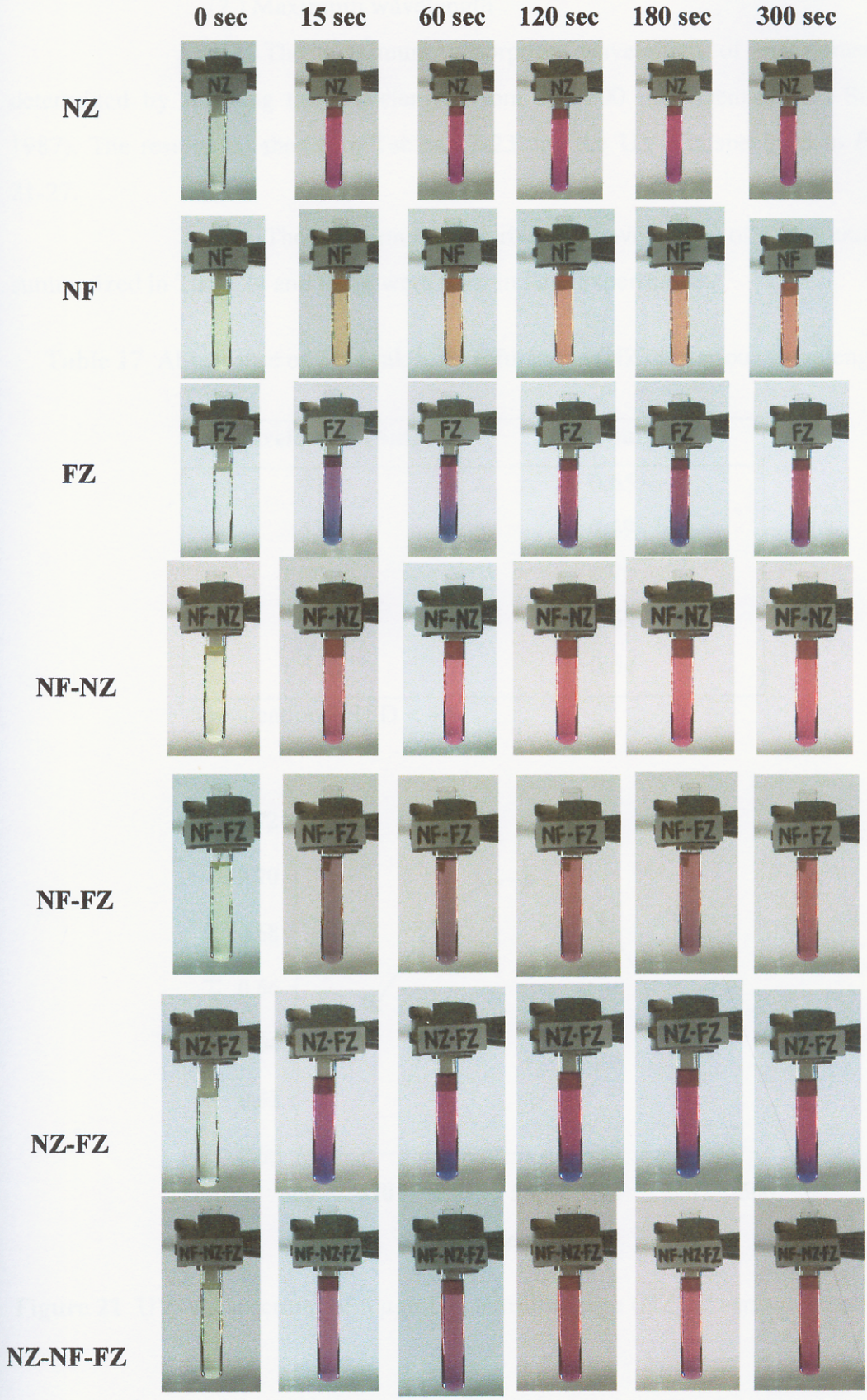


Figure 20 Nitrofuran solution, 10 μg mL^{-1}, contacted with chemical reagent at 0, 15, 60, 120, 180 and 300 sec

(2.) Maximum wavelength

The maximum absorption wavelength of nitrofurans was determined by scanning the wavelength from 200-800 nm (Denney and Sinclair, 1987). The results are shown in Tables 17-23 and the UV-Vis spectrum in Figures 21-27.

The maximum absorbance wavelength of nitrofurans are summarized in Table 24 and these were used in later experiments.

Table 17 Absorbance of 5 μg mL^{-1}, Nitrofurazone (NZ) at various wavelengths

Wavelength (nm)	Absorbance*
510	0.65
520	0.69
530	0.70
540	0.68
550	0.64

* 5 replications, RSD < 4%

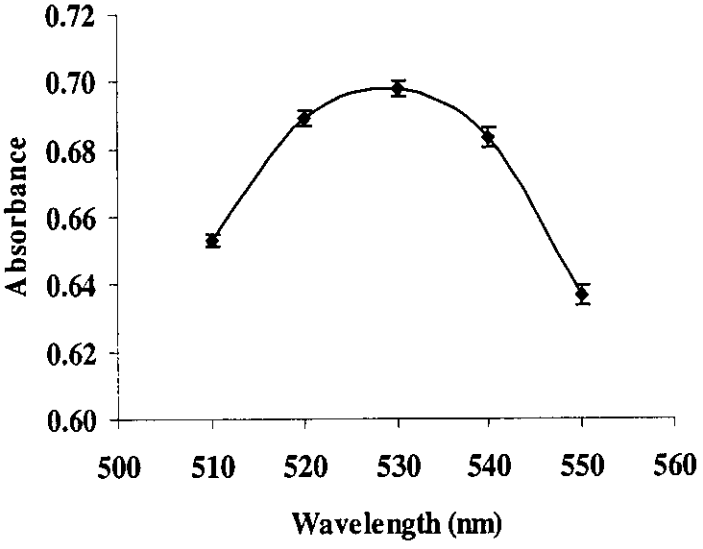


Figure 21 UV-Vis spectrum of 5 μgmL^{-1}, Nitrofurazone (NZ) at various wavelengths

Table 18 Absorbance of 5 μg mL^{-1}, Nitrofurantoin (NF) at various wavelengths

Wavelength (nm)	Absorbance*
390	0.17
410	0.20
430	0.23
450	0.21
470	0.20

* 5 replications, RSD < 4%

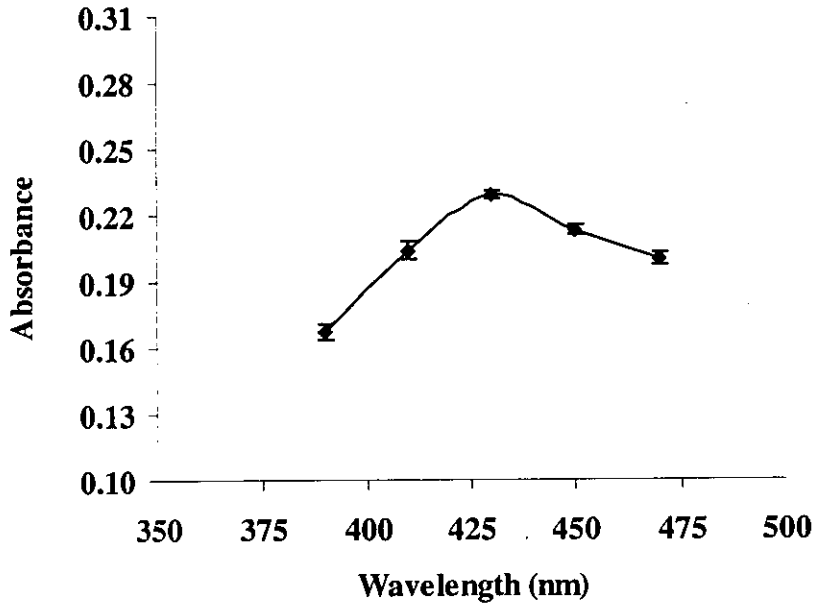


Figure 22 UV- Vis spectrum of 5 μg mL^{-1}, Nitrofurantoin (NF) at various wavelengths

Table 19 Absorbance of 5 μg mL^{-1}, Furazolidone (FZ) at various wavelengths

Wavelength (nm)	Absorbance*
520	0.49
540	0.52
560	0.53
580	0.51
600	0.47

*5 replications, RSD < 4%

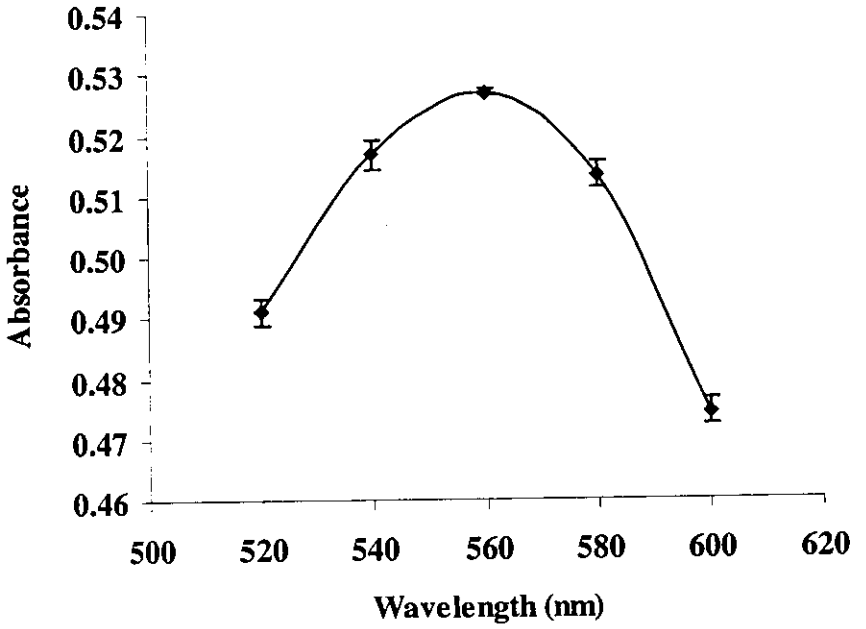


Figure 23 UV-Vis spectrum of 5 μg mL^{-1}, Furazolidone (FZ) at various wavelengths

Table 20 Absorbance of 5 μg mL^{-1}, Nitrofurazone - Nitrofurantoin (NZ-NF) at various wavelengths

Wavelength (nm)	Absorbance*
480	0.67
500	0.81
520	0.88
540	0.82
560	0.65

* 5 replications, RSD < 4%

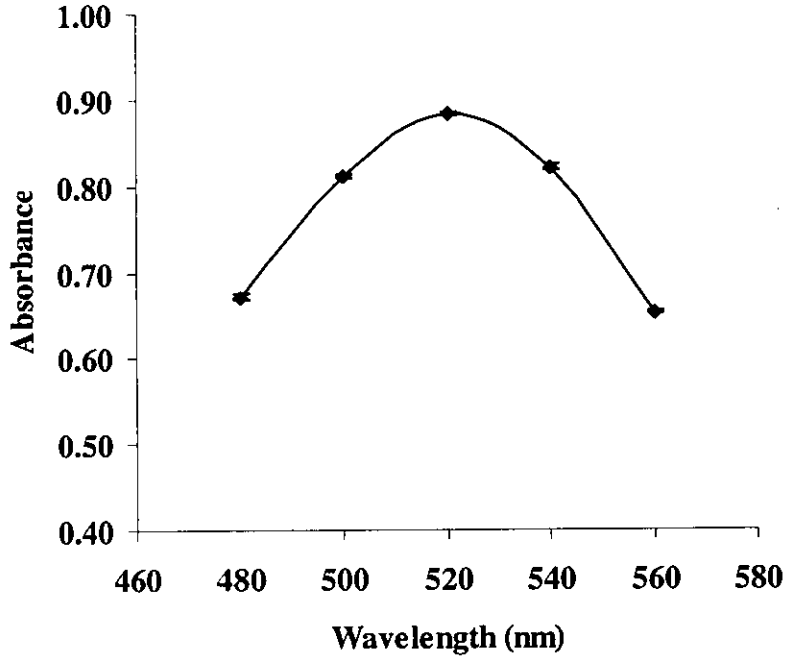


Figure 24 UV-Vis spectrum of 5 μg mL^{-1}, Nitrofurazone- Nitrofurantoin (NZ-NF) at various wavelengths

Table 21 Absorbance of 5 μg mL^{-1}, Nitrofurazone-Furazolidone (NZ-FZ) at various wavelengths

Wavelength (nm)	Absorbance
495	0.91
515	1.08
535	1.14
555	1.06
575	0.91

*5 replications, RSD < 4%

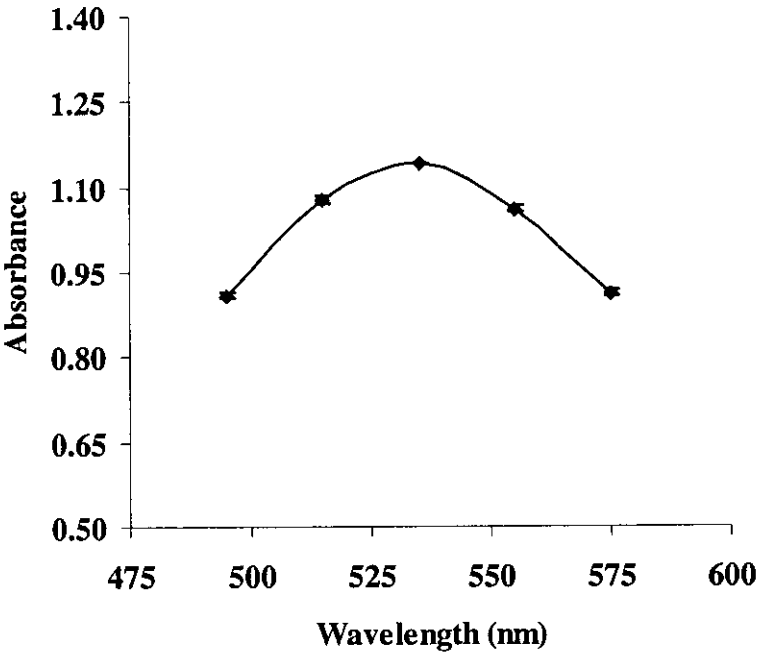


Figure 25 UV- Vis spectrum of 5 μg mL^{-1}, Nitrofurazone - Furazolidone (NZ-FZ) at various wavelengths

Table 22 Absorbance of 5 μg mL^{-1}, Nitrofurantoin-Furazolidone (NF-FZ) at various wavelengths

Wavelength (nm)	Absorbance
475	0.58
495	0.65
515	0.69
535	0.66
555	0.62

* 5 replications, RSD < 4%

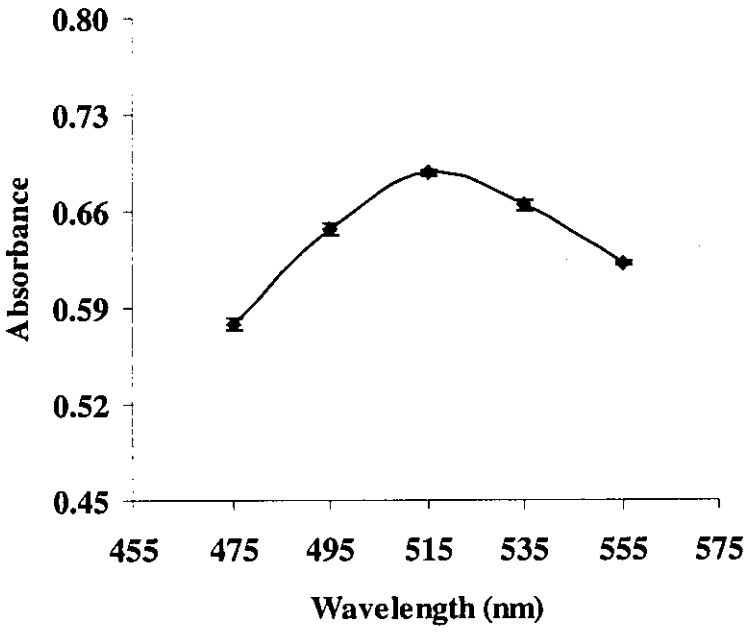


Figure 26 UV-Vis spectrum of 5 μg mL^{-1}, Nitrofurantoin- Furazolidone (NF-FZ) at various wavelengths

Table 23 Absorbance of 5 μg mL^{-1}, Nitrofurazone-Nitrofuratoin-Furazolidone (NZ-NF-FZ) at various wavelengths

Wavelength (nm)	Absorbance
475	0.93
500	1.16
525	1.26
550	1.15
575	0.91

* 5 replications, RSD < 4%

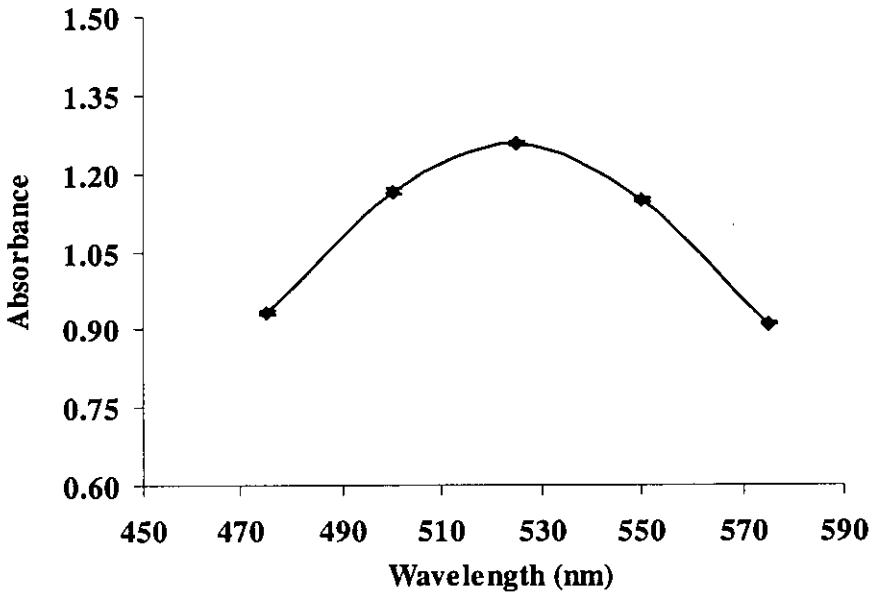


Figure 27 UV-Vis spectrum of 5 μg mL^{-1}, Nitrofurazone-Nitrofuratoin-Furazolidone (NZ-NF-FZ) at various wavelengths

Table 24 Summary the maximum absorption wavelength of nitrofurans

Compounds	Maximum wavelength (nm)
Nitrofurazone (NZ)	530
Nitrofurantoin (NF)	430
Furazolidone (FZ)	560
Nitrofurazone- Nitrofurantoin (NZ-NF)	520
Nitrofurazone-Furazolidone (NZ-FZ)	535
Nitrofurantoin-Furazolidone (NF-FZ)	515
Nitrofurazone-Nitrofuratoin-Furazolidone (NZ-NF-FZ)	525

(3.) Optimum concentration of potassium hydroxide

The effect on the absorbance due to the concentration of potassium hydroxide solution, 0.5-2.0 mol L^{-1}, is shown Tables 25-31 and UV-Vis spectrum in Figures 28-34. The highest absorbance is at 1.5 mol L^{-1}.

Table 32 summarizes the optimum conditions of UV-Vis spectrophotometric analysis.

Table 25 Absorbance of 3 μg mL^{-1}, Nitrofurazone (NZ) at various potassium hydroxide concentrations, λ_{max} = 530 nm

Concentration of KOH (M)	Absorbance
0.5	0.49
1.0	0.49
1.5	0.51
2.0	0.50

*5 replications, RSD < 4%

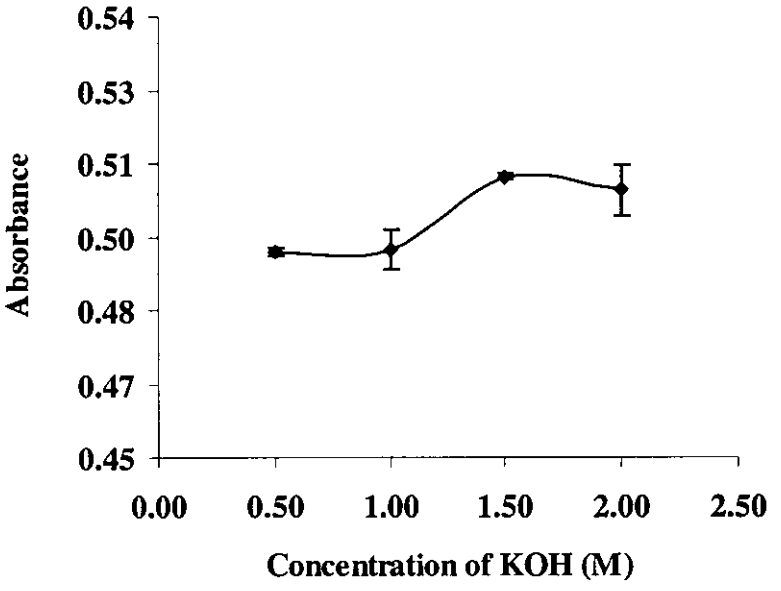


Figure 28 Effect of potassium hydroxide concentration for 3 μg mL^{-1} of Nitrofurazone (NZ) at λ_{max} = 530 nm

Table 26 Absorbance of 3 μg mL^{-1}, Nitrofurantoin (NF) at various potassium hydroxide concentrations, λ_{max} = 430 nm

Concentration of KOH (M)	Absorbance
0.5	0.12
1.0	0.20
1.5	0.26
2.0	0.16

*5 replications, RSD < 4%

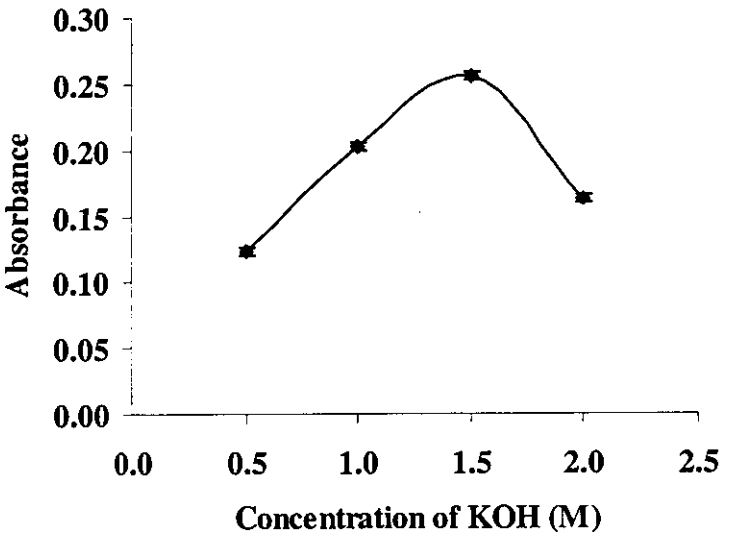


Figure 29 Effect of potassium hydroxide concentration for 3μg mL^{-1} of Nitrofurantoin (NF) at λ_{max} = 430 nm

Table 27 Absorbance of 3 μg mL^{-1}, Furazolidone (FZ) at various potassium hydroxide concentrations, λ_{max} = 560 nm

Concentration of KOH (M)	Absorbance
0.5	0.28
1.0	0.29
1.5	0.31
2.0	0.30

* 5 replications, RSD < 4%

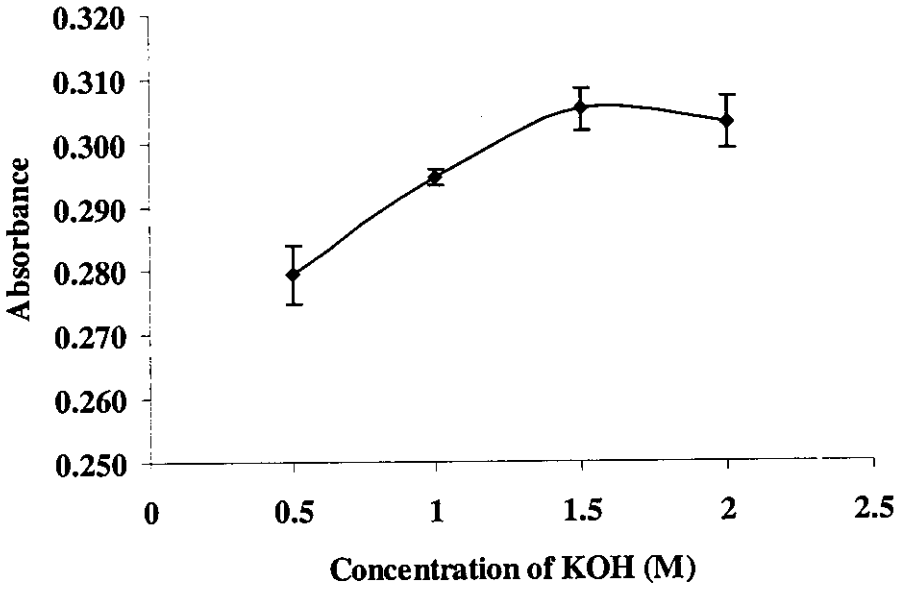


Figure 30 Effect of potassium hydroxide concentration for 3μg mL^{-1} of Furazolidone (FZ) at λ_{max} = 560 nm

Table 28 Absorbance of 3 μg mL^{-1}, Nitrofurazone- Nitrofurantoin (NZ-NF) at various potassium hydroxide concentrations, λ_{max} = 520 nm

Concentration of KOH (M)	Absorbance
0.5	0.26
1.0	0.28
1.5	0.29
2.0	0.29

* 5 replications, RSD < 4%

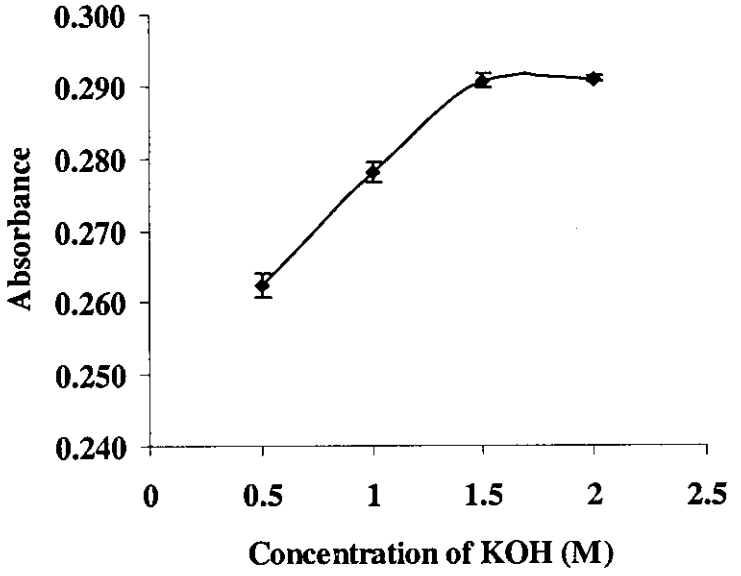


Figure 31 Effect of potassium hydroxide concentration for 3μg mL^{-1} of Nitrofurazone- Nitrofurantoin (NZ-NF) at λ_{max} = 520 nm

Table 29 Absorbance of 3 μg mL^{-1}, Nitrofurazone-Furazolidone (NZ-FZ) at various potassium hydroxide concentrations, λ_{max} = 535 nm

Concentration of KOH (M)	Absorbance
0.5	0.39
1.0	0.39
1.5	0.40
2.0	0.38

*5 replications, RSD < 4%

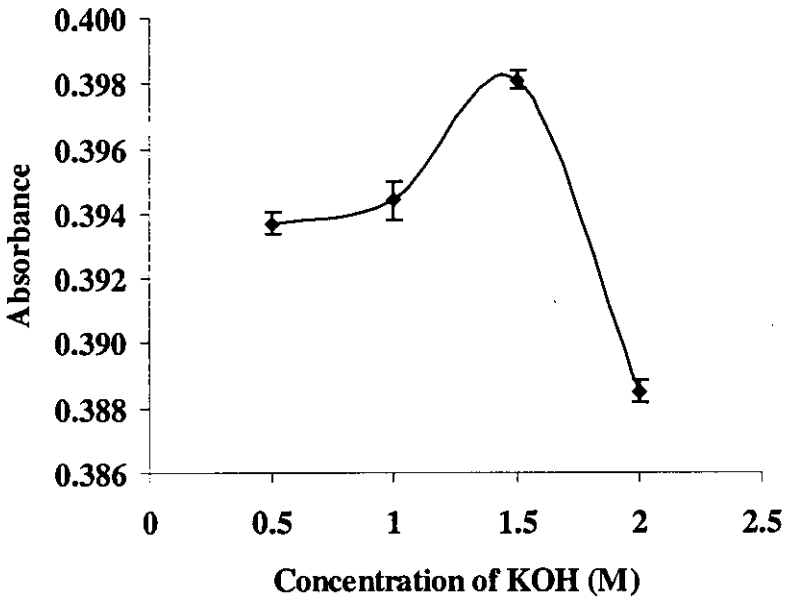


Figure 32 Effect of potassium hydroxide concentration for 3 μg mL^{-1} of Nitrofurazone-Furazolidone (NZ-FZ) at λ_{max} = 535 nm

Table 30 Absorbance of 3 μg mL^{-1}, Nitrofurantoin-Furazolidone (NF-FZ) at various potassium hydroxide concentrations, λ_{max} = 515 nm

Concentration of KOH (M)	Absorbance
0.5	0.16
1.0	0.18
1.5	0.20
2.0	0.19

* 5 replications, RSD < 4%

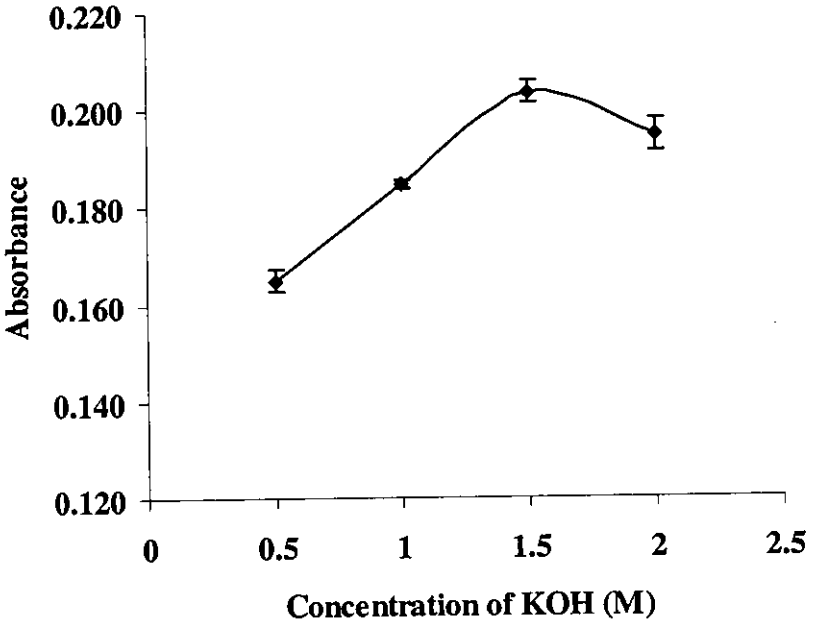


Figure 33 Effect of potassium hydroxide concentration for 3 μg mL^{-1} of Nitrofurantoin-Furazolidone (NF-FZ) at λ_{max} = 515 nm

Table 31 Absorbance of 3 μg mL^{-1}, Nitrofurazone-Nitrofurantoin-Furazolidone (NZ-NF-FZ) at various potassium hydroxide concentrations, λ_{max} = 525 nm

Concentration of KOH (M)	Absorbance
0.5	0.27
1.0	0.28
1.5	0.29
2.0	0.29

* 5 replications, RSD < 4%

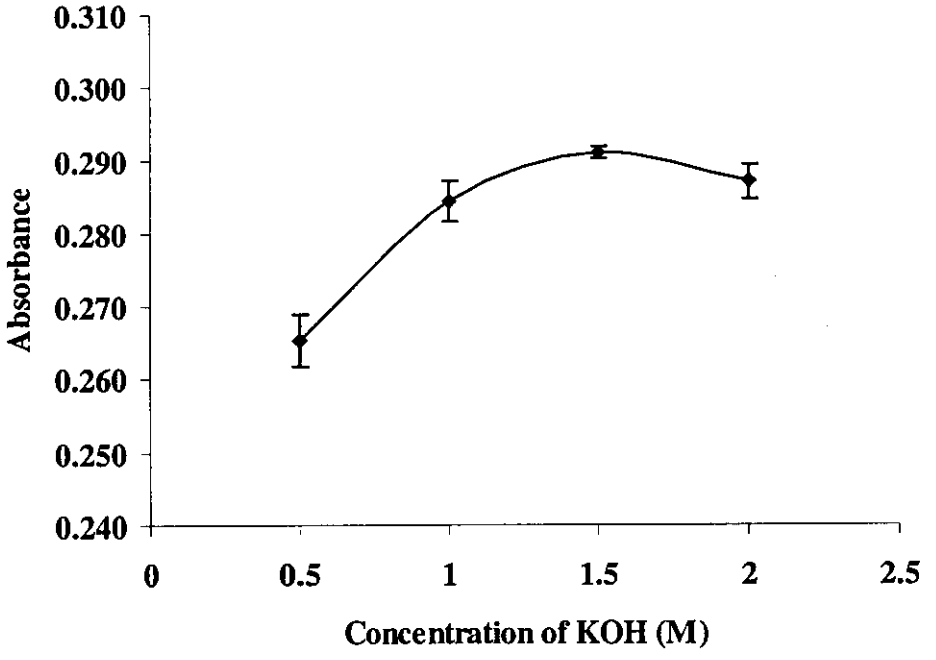


Figure 34 Effect of potassium hydroxide concentration for 3 μg mL^{-1} of Nitrofurazone-Nitrofurantoin-Furazolidone (NZ-NF-FZ) at λ_{max} = 515 nm

Table 32 The optimum conditions of UV-Vis spectrophotometric analysis

Parameters	Conditions
Time of color forming	15 seconds
Maximum wavelength (nm)	Table 24
Methanolic potassium hydroxide concentration	1.5 mol L^{-1}

(4.) Concentration ratio of nitrofurans

The Beer-Lambert law (or Beer's law) describes the relationship between absorbance and concentration of an absorbing species and is usually written as:

$$A_\lambda = \varepsilon_\lambda \cdot b \cdot c \qquad (6)$$

Where A_λ is the measured absorbance at specific wavelength

b is the path length

c is analyte concentration in mol L^{-1}

ε_λ is the molar absorptivity for a specific wavelength (L mol^{-1} cm^{-1})

The molar absorptivity is a quantity characteristic of the substance which depends on a particular wavelength (nm). According to the above equation the values of A and ε are wavelength dependent. Since the absorbance is directly proportional to the analyte concentration. So a high concentration may cause the wavelength to change (Perkampus, 1992). In this work, the mixtures of nitrofurazone (NZ), nitrofurantoin (NF) and furazolidone (FZ) were prepared to study the change of maximum absorption wavelength at various concentration ratios using optimum conditions in Table 32. Figures 35-37 show the maximum wavelength at various concentration ratios.

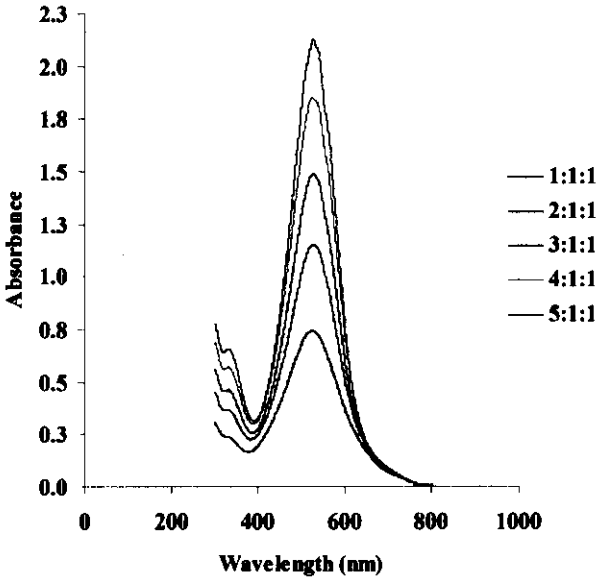


Figure 35 The maximum wavelength at various concentration ratios of nitrofurazone (NZ) in the mixture (NZ : NF : FZ)

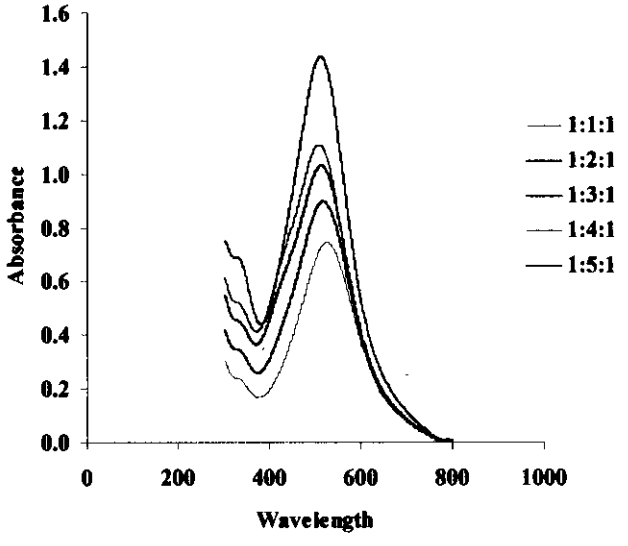


Figure 36 The maximum wavelength at various concentration ratios of nitrofurantoin (NF) in the mixture (NZ : NF : FZ)

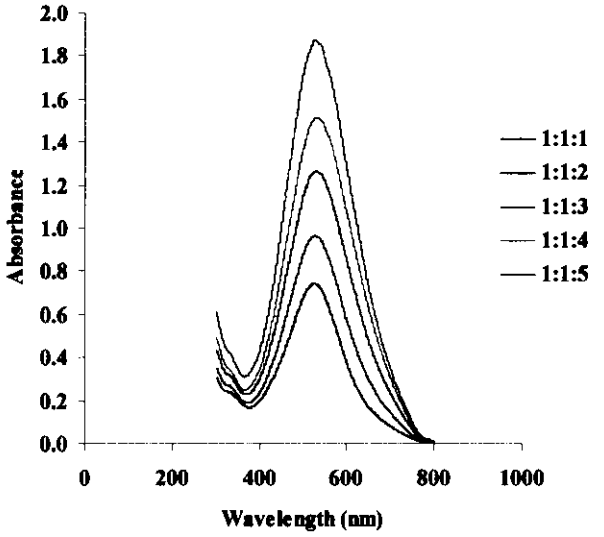


Figure 37 The maximum wavelength at various concentration ratios of furazolidone (FZ) in the mixture (NZ : NF : FZ)

The results showed that the maximum wavelengths of various concentration ratios of nitrofurazone and furazolidone were not different. For nitrofurantoin concentration ratios were different from 1:1:1 ratio less than 3 nanometer. Thus, the concentration ratio at 1 : 1 : 1 was applied for the analysis.

3.2.2 UV-Vis spectrophotometric method validation

(1.) Limit of Detection (LOD)

In this work, the concentrations of nitrofurans where S/N is more than 3 are shown in Table 33. The LOD and sensitivity (slope) of furazolidone in this work are better than those reported by Díaz *et al.* (1994) (Table 34).

Table 33 Detection limit of nitrofurans (S/N>3)

Compounds	Detection limit (mg L^{-1})
Nitrofurazone (NZ)	0.15
Nitrofurantoin (NF)	0.50
Furazolidone (FZ)	0.15
Nitrofurazone- Nitrofurantoin (NZ-NF)	0.20
Nitrofurazone-Furazolidone (NZ-FZ)	0.20
Nitrofurantoin-Furazolidone (NF-FZ)	0.50
Nitrofurazone-Nitrofuratoin-Furazolidone (NZ-NF-FZ)	0.25

Table 34 Comparison of LOD of furazolidone

Authors	Calibration line	Detection limit
Díaz *et al.* (1994)	$y = 0.049x + 0.001$	0.19 $\mu g\ mL^{-1}$
This work	$y = 0.106x + 0.006$	0.15 $\mu g\ mL^{-1}$

(2.) Linearity

A plot of absorbance versus concentration must be linear for Beer's law. At high concentration this may be deviated from the Beer's law (Figure 38).

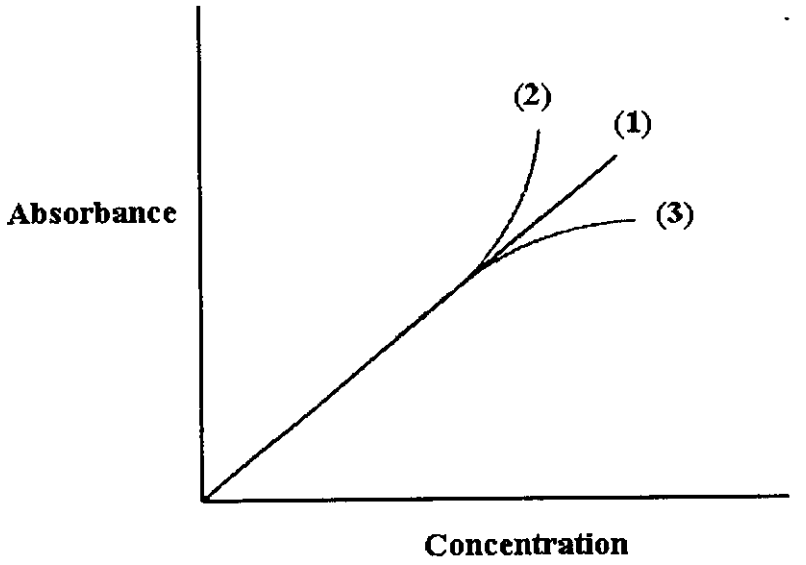


Figure 38 Plot of absorbance vs concentration (1) obeyed with Beer's law (2), (3) deviated from Beer's law.

Tables 35-41 and Figures 39-52 show the concentration ranges which were used to evaluate the linearity of nitrofurans. At higher concentrations the plots become non linear and deviated from Beer's law and are not appropriate for the quantitative analysis. The linear concentration ranges of nitrofurans which obeyed Beer's law are shown in Figures 40, 42, 44, 46, 48, 50 and 52 and are summarized in Table 42.

Table 35 The absorbance of nitrofurazone (NZ) at various concentrations, λ_{max} = 530 nm

Concentration of NZ (mg L^{-1})	Absorbance
0.1	0.01
0.3	0.04
0.5	0.07
0.7	0.10
1	0.14
5	0.69
10	1.39
13	1.75
15	1.97
20	2.39

* 5 replications, RSD < 4%

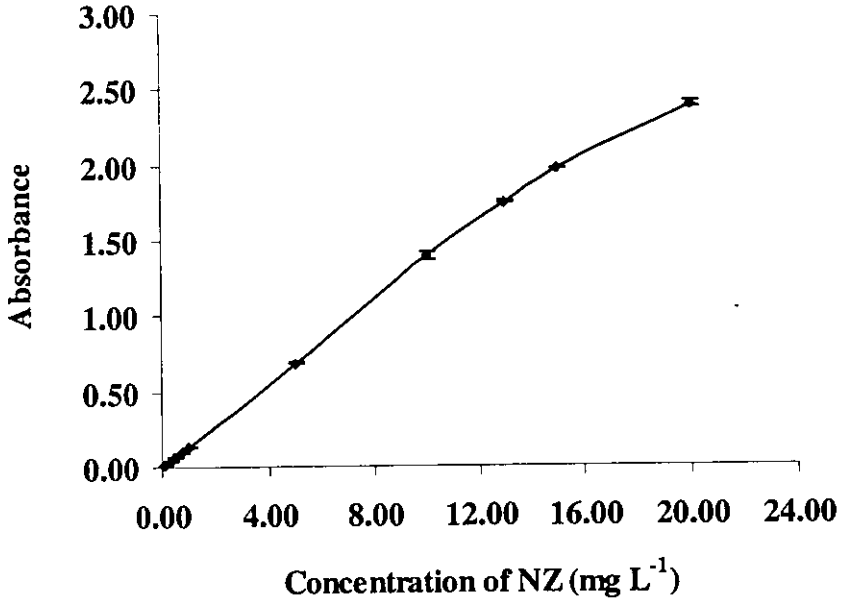


Figure 39 Relationship between absorbance and concentration of nitrofurazone (NZ), λ_{max} = 530 nm

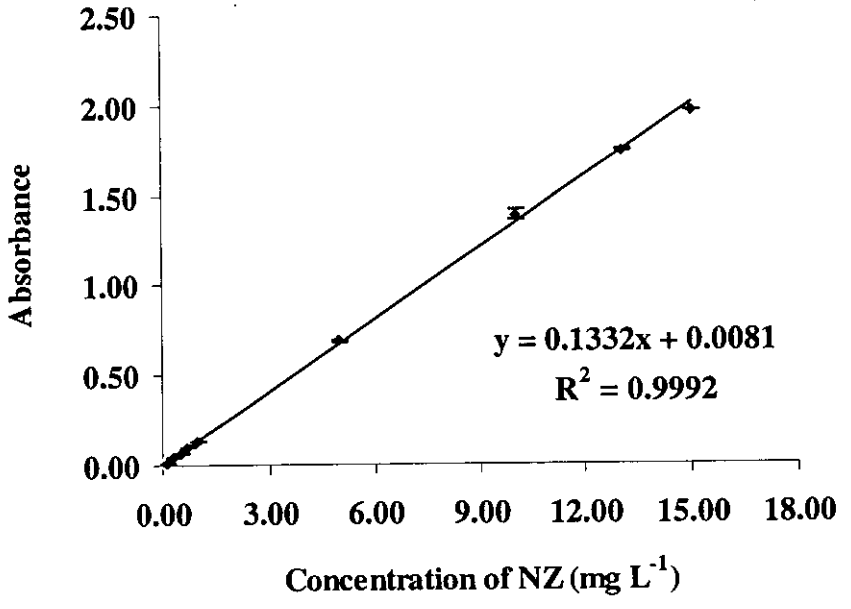


Figure 40 Linearity of nitrofurazone (NZ) (λ_{max} = 530 nm) which obeyed Beer's law

Table 36 The absorbance of nitrofurantoin (NF) at various concentrations, λ_{max} = 430 nm

Concentration of NF (mg L^{-1})	Absorbance
0.3	0.01
0.5	0.02
0.7	0.03
1	0.05
5	0.25
10	0.49
20	0.97
25	1.25
30	1.65

* 5 replications, RSD < 4%

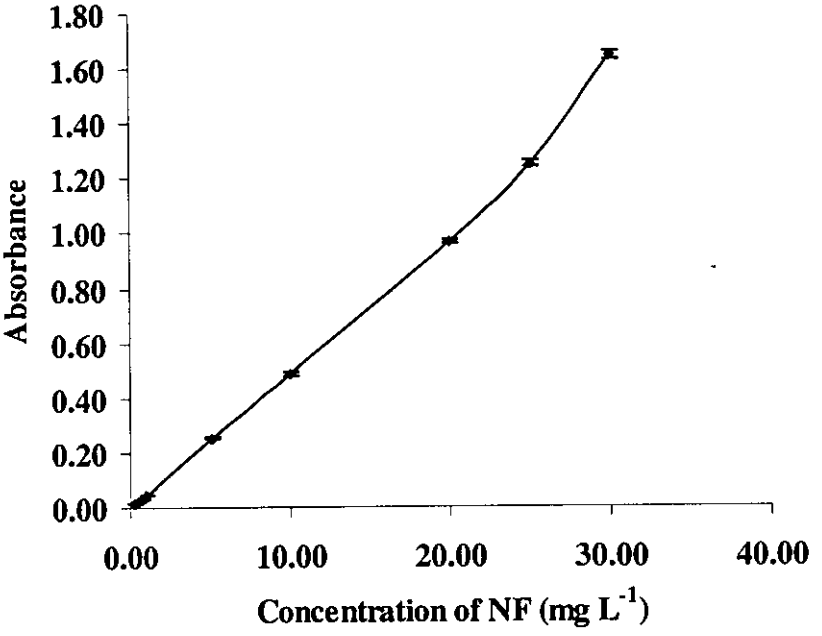


Figure 41 Relationship between absorbance and concentration of nitrofurantoin (NF), λ_{max} = 430 nm

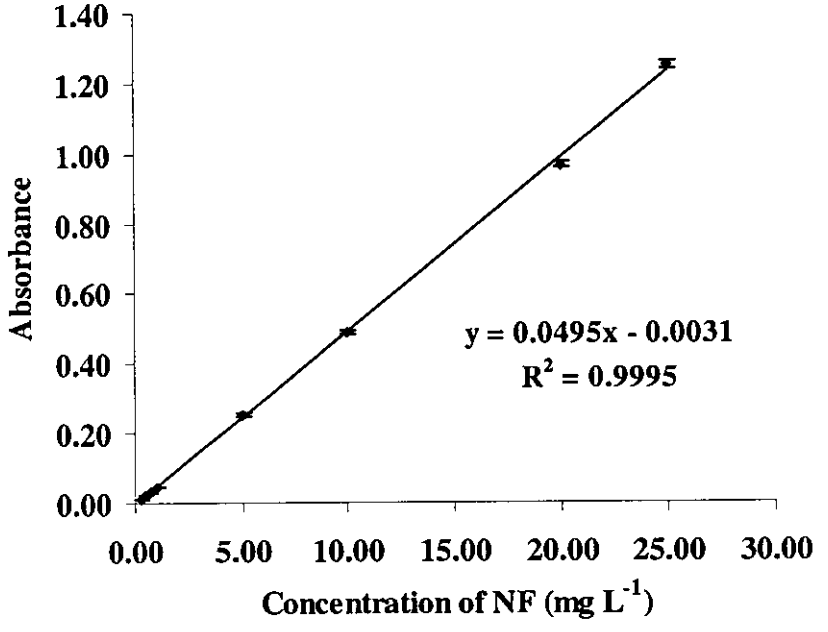


Figure 42 Linearity of nitrofurantoin (NF) (λ_{max} = 430 nm) which obeyed Beer's law

Table 37 The absorbance of furazolidone (FZ) at various concentrations, λ_{max} = 560 nm

Concentration of FZ (mg L^{-1})	Absorbance
0.1	0.01
0.3	0.03
0.5	0.06
0.7	0.08
1	0.10
5	0.54
10	1.08
15	1.60
20	2.10
25	2.41

* 5 replications, RSD < 4%

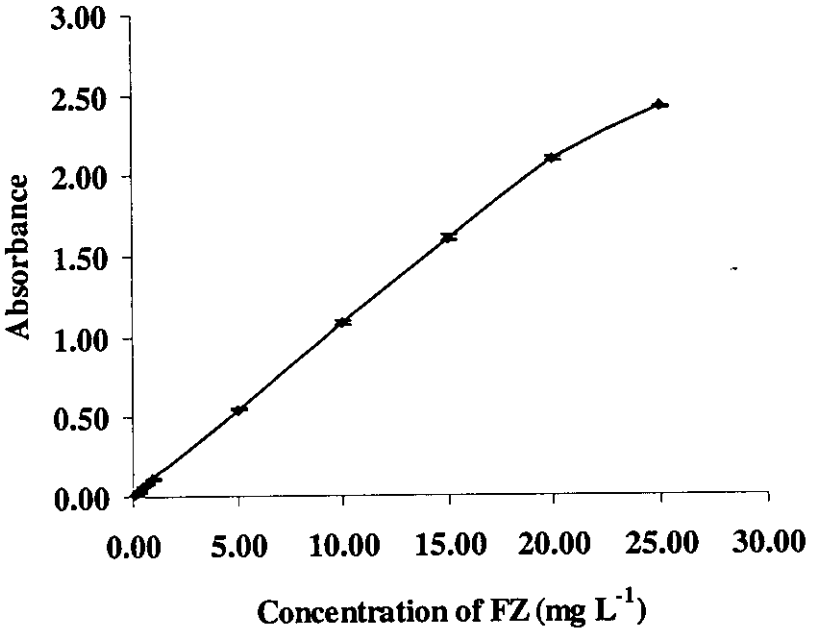


Figure 43 Relationship between absorbance and concentration of furazolidone (FZ), λ_{max} = 560 nm

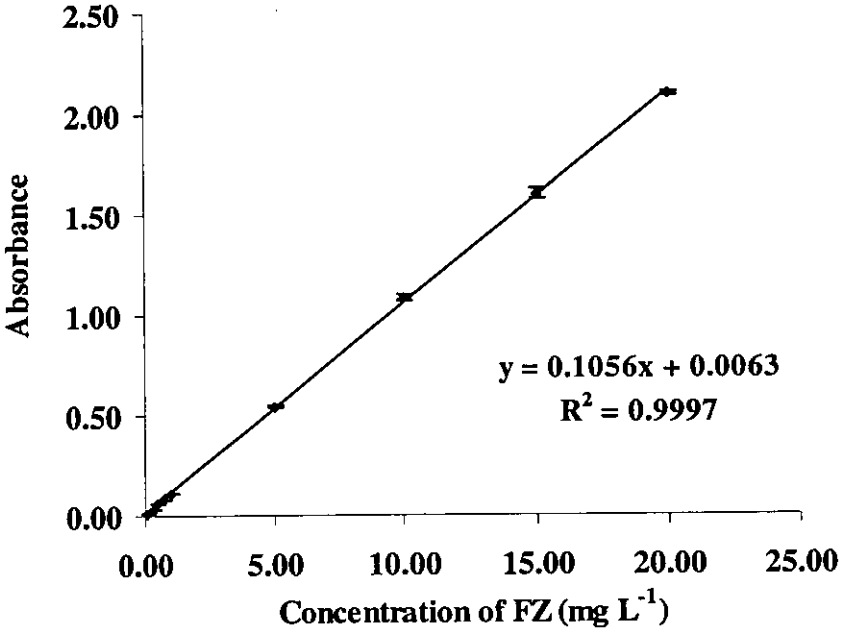


Figure 44 Linearity of Furazolidone (FZ) (λ_{max} = 560 nm) which obeyed Beer's law

Table 38 The absorbance of nitrofurazone-nitrofurantoin (NZ-NF) at various concentrations, λ_{max} = 520 nm

Concentration of NZ-NF (mg L^{-1})	Absorbance
0.1	0.02
0.3	0.05
0.5	0.08
5	0.86
10	1.77
15	2.56
20	2.93

*5 replications, RSD < 4%

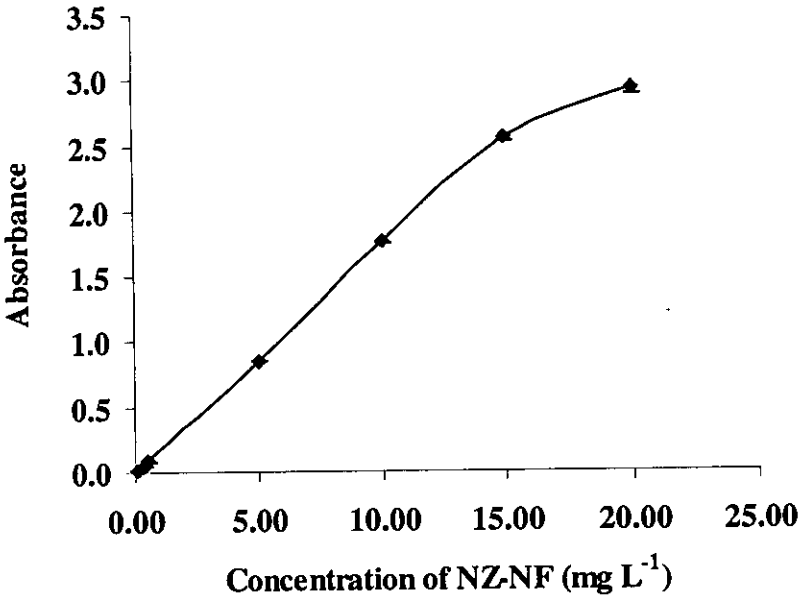


Figure 45 Relationship between absorbance and concentration of nitrofurazone-nitrofurantoin (NZ-NF), λ_{max} = 520 nm

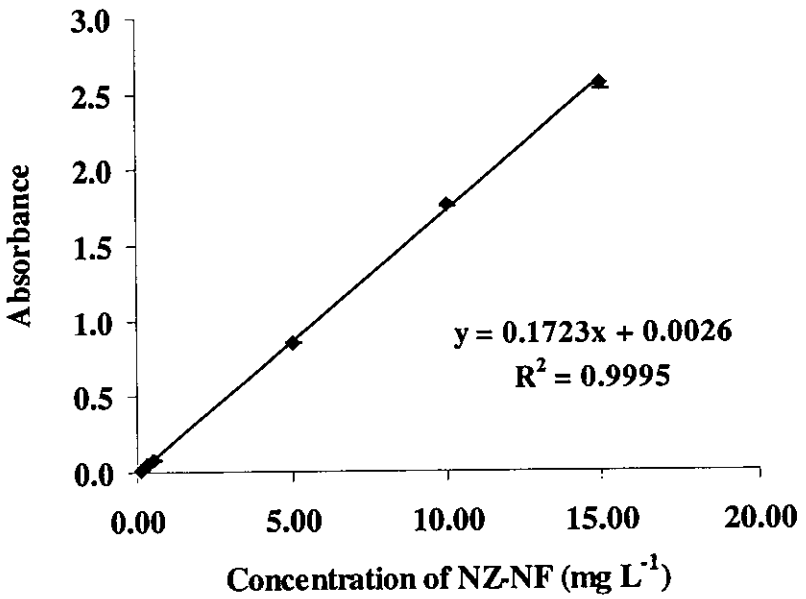


Figure 46 Linearity of nitrofurazone-nitrofurantoin (NZ-NF) (λ_{max} = 520 nm) which obeyed Beer's law

Table 39 The absorbance of nitrofurazone-furazolidone (NZ-FZ) at various concentrations, λ_{max} = 535 nm

Concentration of NZ-FZ (mg L^{-1})	Absorbance
0.1	0.02
0.3	0.07
0.5	0.12
1	0.24
5	1.17
10	2.33
13	2.85

* 5 replications, RSD < 4%

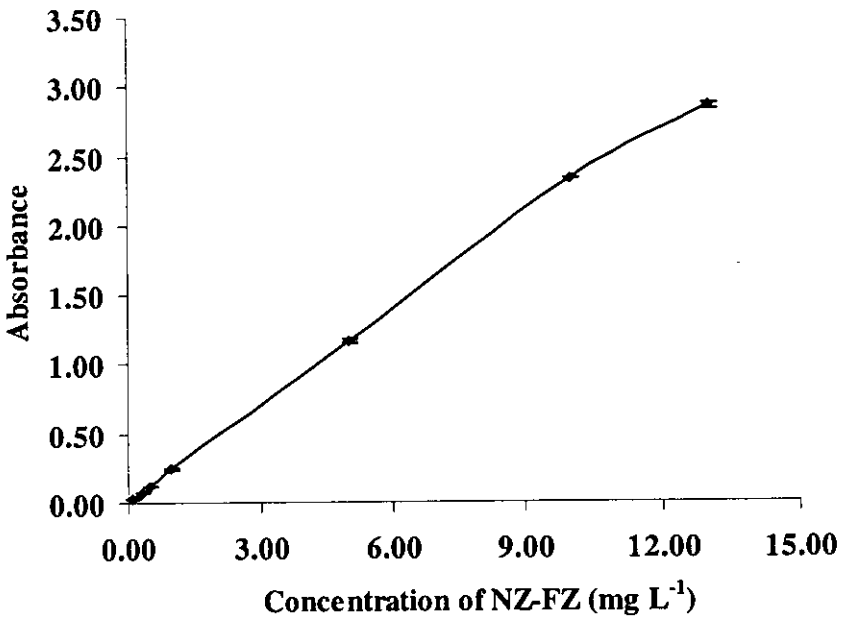


Figure 47 Relationship between absorbance and concentration of nitrofurazone - furazolidone (NZ-FZ), λ_{max} = 535 nm

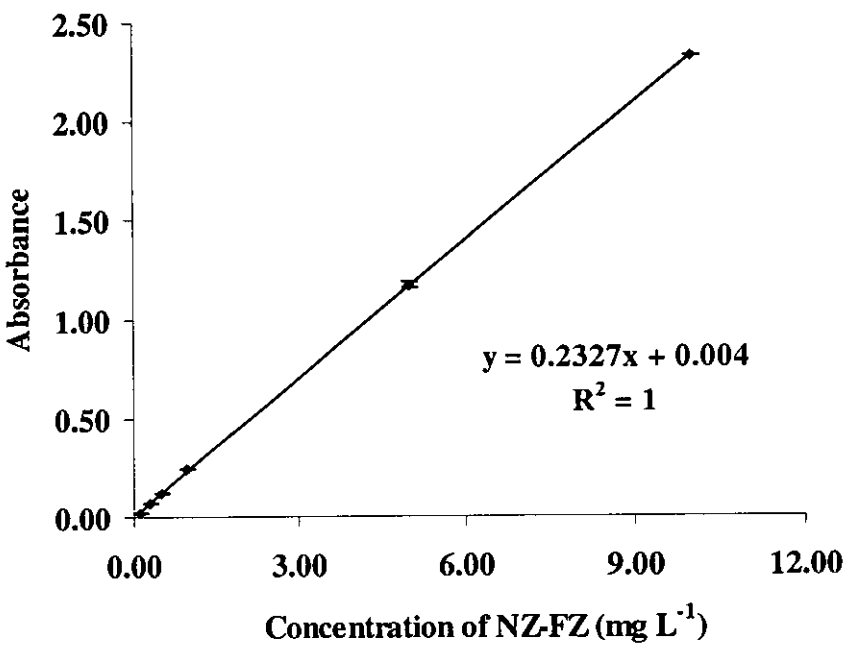


Figure 48 Linearity of nitrofurazone-furazolidone (NZ-FZ) (λ_{max} = 535 nm) which obeyed Beer's law

Table 40 The Absorbance of nitrofurantoin-furazolidone (NF-FZ) at various concentrations, λ_{max} = 515 nm

Concentration of NF-FZ (mg L^{-1})	**Absorbance**
0.1	0.02
0.3	0.04
0.5	0.07
1	1.44
5	0.76
10	1.51
15	2.21
20	2.98
25	3.36

* 5 replications, RSD < 4%

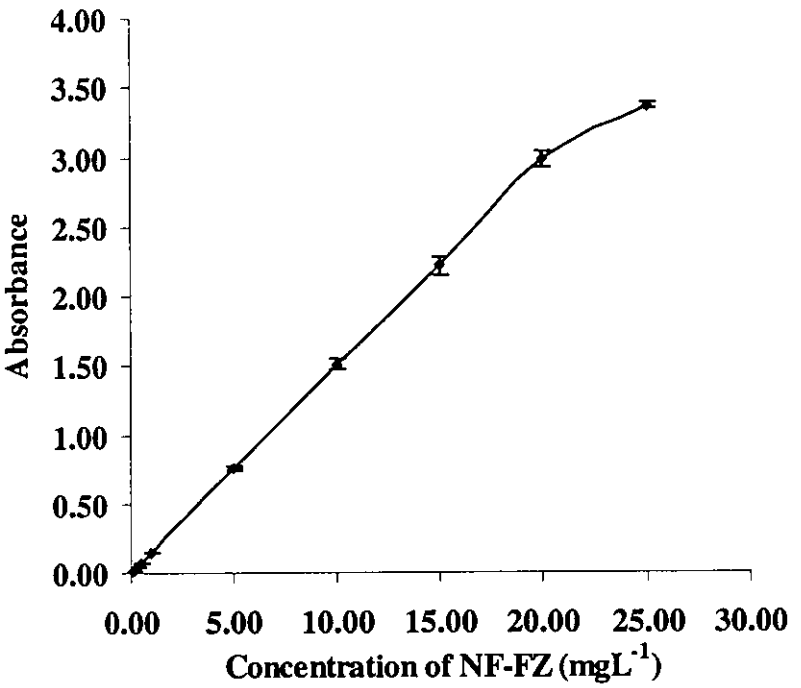


Figure 49 Relationship between absorbance and concentration of nitrofurantoin-furazolidone (NF-FZ), λ_{max} = 515 nm

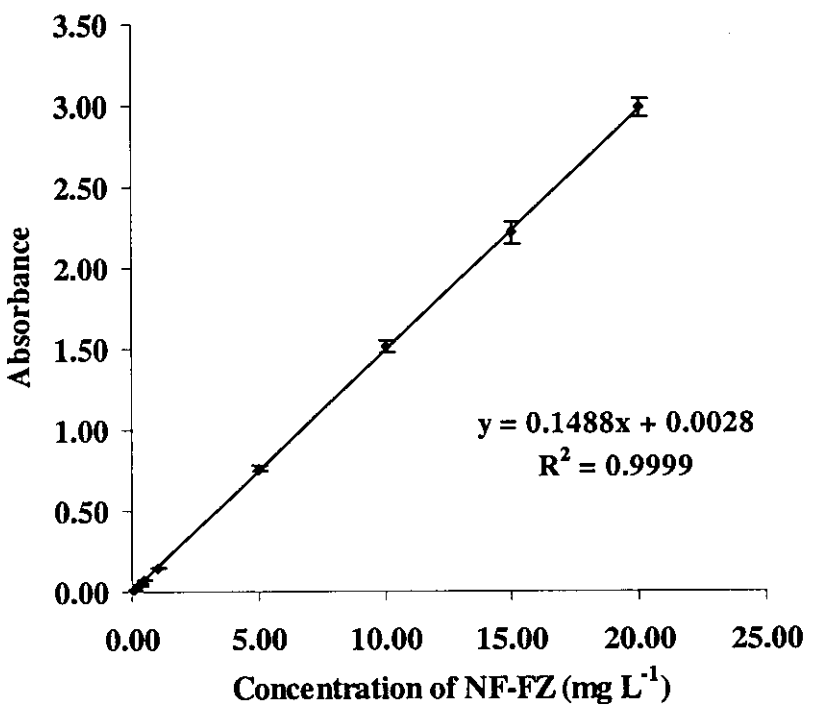


Figure 50 Linearity of nitrofurantoin-furazolidone (NF-FZ) (λ_{max} = 515 nm) which obeyed Beer's law

Table 41 The Absorbance of nitrofurazone-nitrofurantoin-furazolidone (NZ-NF-FZ) at various concentrations, λ_{max} = 525 nm

Concentration of NZ-NF-FZ (mg L^{-1})	Absorbance
0.1	0.03
0.3	0.08
0.5	0.13
1	0.25
5	1.25
10	2.53
13	3.23
15	3.43

* 5 replications, RSD < 4%

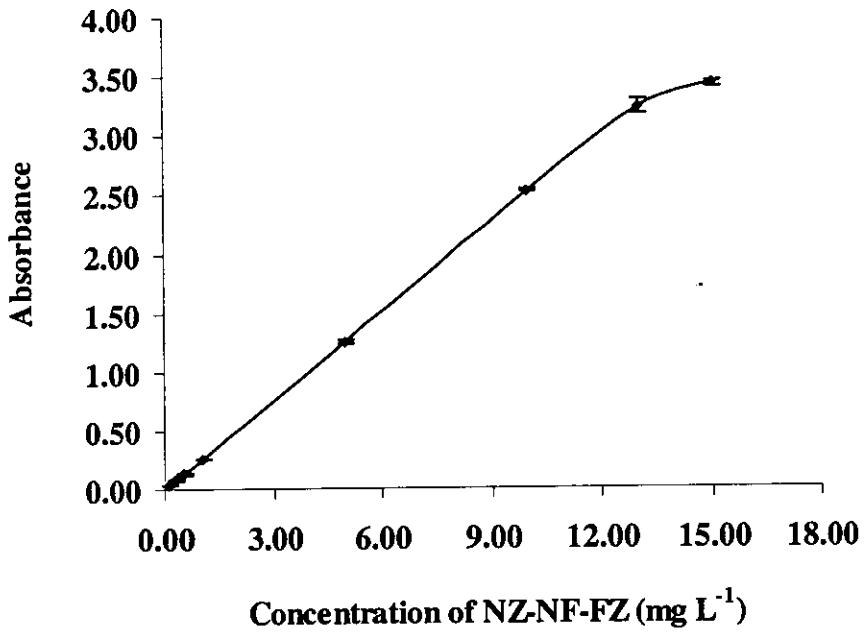


Figure 51 Relationship between absorbance and concentration of nitrofurazone-nitrofurantoin-furazolidone (NZ-NF-FZ), λ_{max} = 525 nm

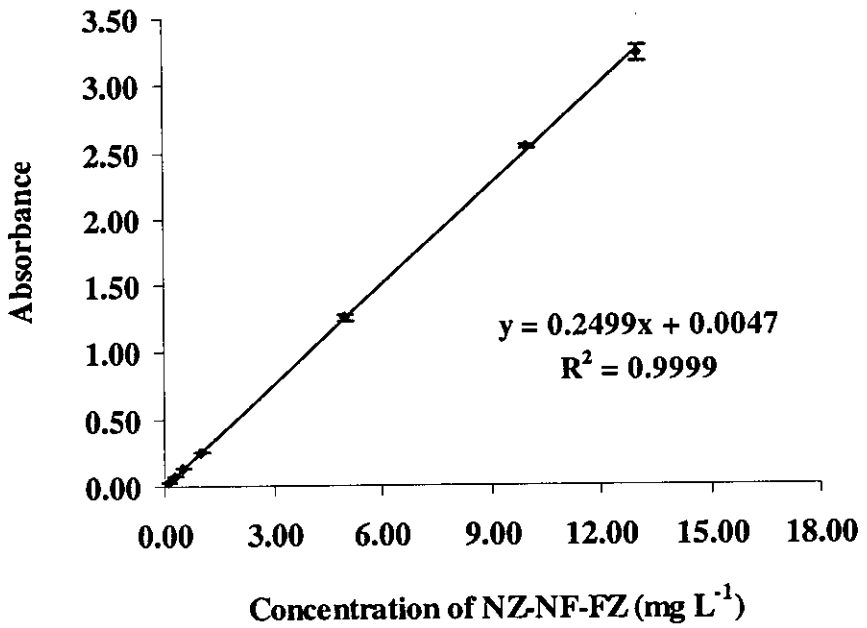


Figure 52 Linearity of nitrofurazone-nitrofurantoin-furazolidone (NZ-NF-FZ) (λ_{max} = 525 nm) which obeyed Beer's law

Table 42 Summary the range of nitrofurans linearity that obeyed Beer's law

Compounds	Linearity (mg L^{-1})
Nitrofurazone (NZ)	0.1-15
Nitrofurantoin (NF)	0.3-25
Furazolidone (FZ)	0.1-20
Nitrofurazone- Nitrofurantoin (NZ-NF)	0.1-15
Nitrofurazone-Furazolidone (NZ-FZ)	0.1-10
Nitrofurantoin-Furazolidone (NF-FZ)	0.1-20
Nitrofurazone-Nitrofuratoin-Furazolidone (NZ-NF-FZ)	0.1-13

3.3 Sample preparation

3.3.1 Extraction time

The extraction time was one of the factors affecting the efficiency of nitrofurans extraction. The extraction time was varied from 1 – 15 hours. The results are shown in Table 43 and Figure 53. Nitrofurazone, nitrofurantoin and furazolidone gave the highest response at 15, 1 and 3 hours, respectively. But the response of nitrofurazone for the extraction time between 1 and 15 hours, and FZ between 1 and 3 hours were not significantly different as calculated by statistical test (P <0.05). So, one hour was selected as the optimum extraction time. This extraction time was shorter than obtained by Lin and Jeng (2001) where the analysis was done by overnight solvent extraction.

Table 43 Responses of nitrofurans at various extraction times

Extraction time (hr)	Response x 10^4* (AU×s)		
	Nitrofurazone (NZ)	Nitrofurantoin (NF)	Furazolidone (FZ)
1	8.69	5.02	9.36
3	8.69	4.89	9.58
5	8.39	4.46	8.85
10	8.37	4.68	8.80
15	8.87	4.63	9.43

*5 replications, RSD < 10%

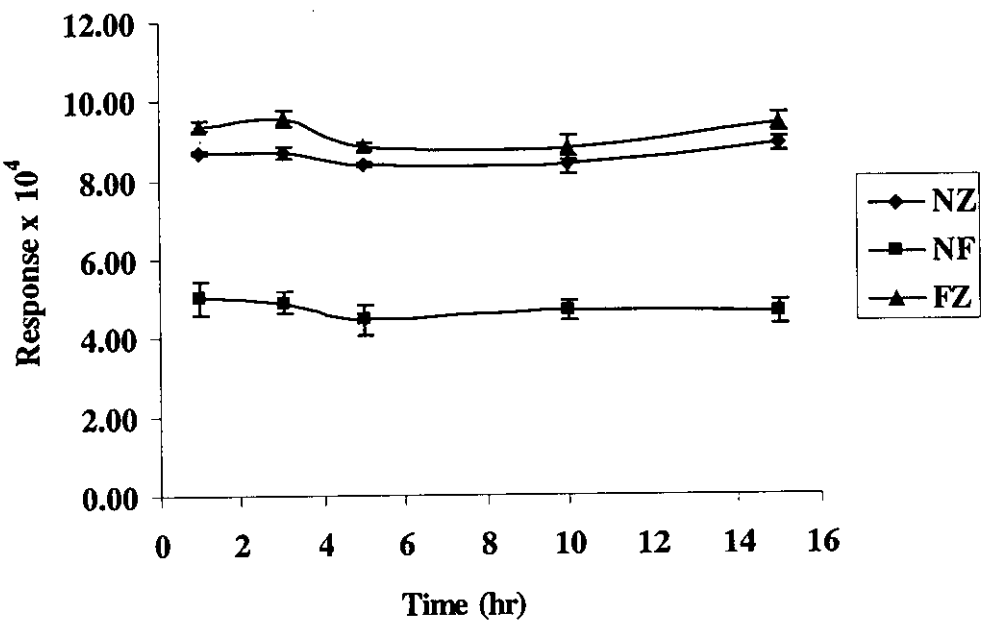


Figure 53 Responses of nitrofurans at various extraction times

3.3.2 Type of solvent

Solvents were used for the extraction of nitrofuran compounds. The extraction solvents were selected from literature, *i.e.*, N, N- dimethylformamide (Díaz *et al.*, 1994, and Lin and Jeng, 2001) and acetonitrile (McCracken and Kennedy, 1997). The results are shown in Table 44 and Figure 54.

Table 44 Response of nitrofurans at various solvent extraction

Type of solvent extraction	Response × 10^4* (AU×s)		
	Nitrofurazone (NZ)	Nitrofurantoin (NF)	Furazolidone (FZ)
N,N-Dimethylformamide	2.42	8.93	8.78
Acetonitrile	0.60	6.97	7.75

* 5 replications, RSD < 10%

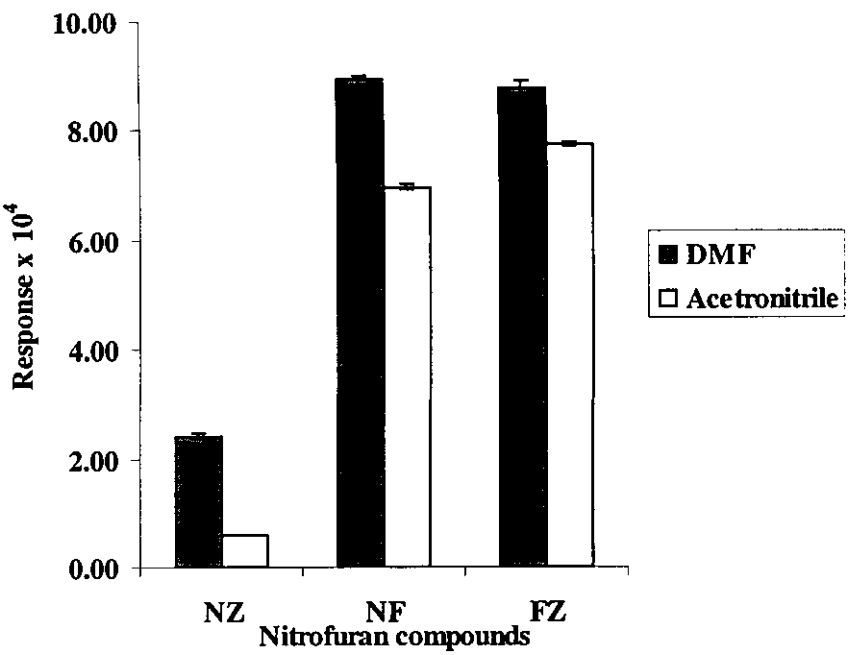


Figure 54 Response of nitrofurans at various solvent extraction

The results show that nitrofurans extracted by N, N-dimethylformamide gave better response than acetonitrile. This is because nitrofurans are slightly polar and the polarity index of N, N- dimethylformamide (6.4) is higher than acetonitrile (5.8) (Sadek, 1996). Thus, N, N-dimethylformamide could extract nitrofurans better than acetonitrile.

3.3.3 Shaking time

Shaking time was another parameter affecting the efficiency of nitrofurans extraction. The shaking was necessary to homogenize the sample and extraction solvent and the shaking time was varied from 0.25-3 minutes. The results (Table 45 and Figure 55) show that 0.25 minute was the appropriate shaking time since it provided the highest response. The time of shaking in this work was less than the work of McCracken and Kennedy (1997), where 30 minute was used in the analysis of nitrofuran in animal feeds. This may be because in this work the sample was ground by a blender, and passed through a 120 mesh sieve, therefore, it was more homogeneous. From the experiment, longer time reduces response of the drugs. Because of nitrofuran's property which decompose under the light. The chemical document from purchasing defined storage for nitrofurantoin and furazolidone at room temperature and at 4 °C for nitrofurazone (Moffat *et al.*, 1986). Therefore, the heating up during shaking caused the decomposition, then response decreased.

Table 45 Response at various shaking time of nitrofuran extraction

Time of shaking (minute)	Response x 10^5* (AU×s)		
	Nitrofurazone (NZ)	Nitrofurantoin (NF)	Furazolidone (FZ)
0.25	7.87	1.76	7.98
3.0	7.62	1.58	6.91
5.0	7.16	1.38	6.33

* 5 replications, RSD < 10%

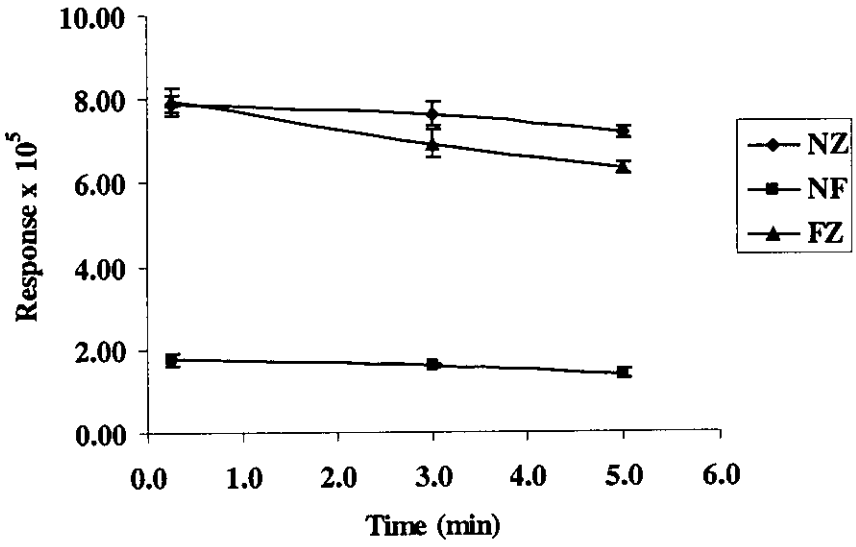


Figure 55 Response using various shaking time of nitrofuran extraction

3.3.4 Type of washing solvent

This work compared between washed and unwashed extractant. Washing was the process of extracting analyte from clean up material by solvent. An unwashed process does not use a solvent to remove the analyte from the clean up material. In this work, alumina was packed into a column. It was a sorbent that was used to remove fats and some other interfering substances in the sample. Then, a sample was introduced onto the top of column. In an unwashed process the extractant passing through the column was analyzed. For a washed process an additional step was performed by passing an appropriate solvent through the column to extract nitrofuran residues down the column. From the comparison between the washed and unwashed method, Table 46 and Figure 56 show the results obtained.

Table 46 Comparison of response between washed and unwashed method

Method	Response x 10^5* (AUxs)		
	Nitrofurazone (NZ)	Nitrofurantoin (NF)	Furazolidone (FZ)
unwashed	1.44	1.02	1.43
washed	1.73	1.23	1.61

* 5 replications, RSD < 10%

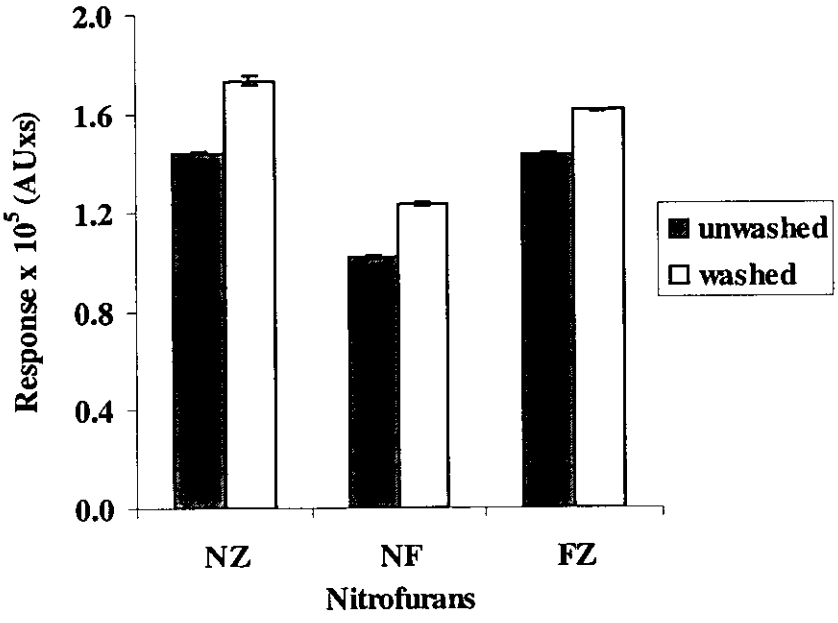


Figure 56 Comparison of response between washed and unwashed method

From the results, the washed method gave a higher response, thus, the washed method was applied. Appropriate washing solvent was then studied. From the results (Table 47 and Figure 57) nitrofurans washed with N, N-dimethylformamide gave higher response than methanol because the solubility of these compounds in N, N- dimethylformamide (> 50 g liter^{-1}) is considerably higher than in methanol (0.22 g liter^{-1}) (Díaz *et al.*, 1994). For this reason, N, N-dimethylformamide was chosen as the washing solvent for nitrofuran analysis.

Table 47 Response of nitrofurans using two washing solvents

Type of solvent extraction	Response x 10^4* (AUxs)		
	Nitrofurazone (NZ)	Nitrofurantoin (NF)	Furazolidone (FZ)
N,N-Dimethylformamide	4.27	3.60	3.86
Methanol	4.01	3.30	3.77

*5 replications, RSD < 10%

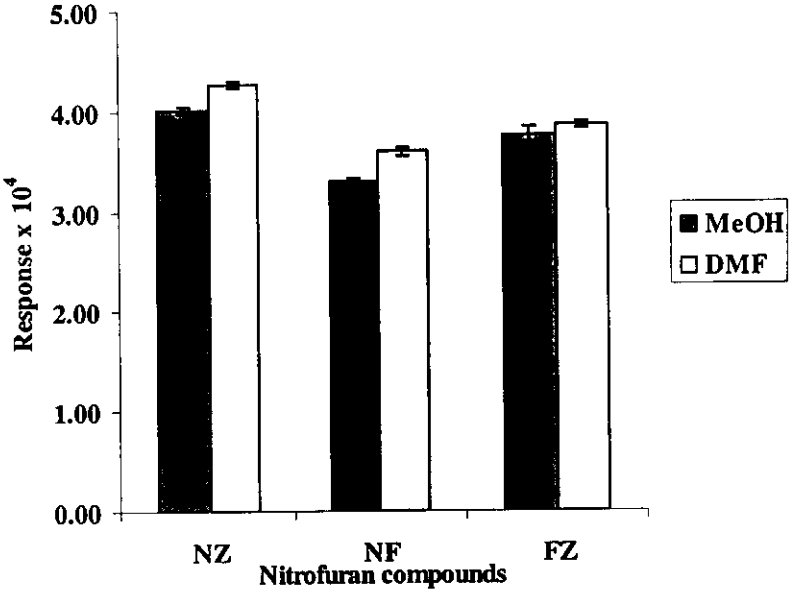


Figure 57 Response of nitrofurans using two washing solvents

3.3.4 Recovery

High recovery of the analyte from the sample is a desirable outcome of sample preparation, and is, therefore, an important characteristic of the extraction procedure . The percentage of recoveries for nitrofurans in the samples are shown in Table 48 and Figure 58. The obtained percentage of recoveries were acceptable follow AOAC method, 80-115% (AOAC, 2002).

Table 48 Percentage of recoveries of the nitrofurans in sample

Nitrofurans	% Recovery ± SD
Nitrofurazone	93.7±1.1
Nitrofurantoin	97.3±1.0
Furazolidone	89.6±1.2

*5 replications, RSD ≤ 10%

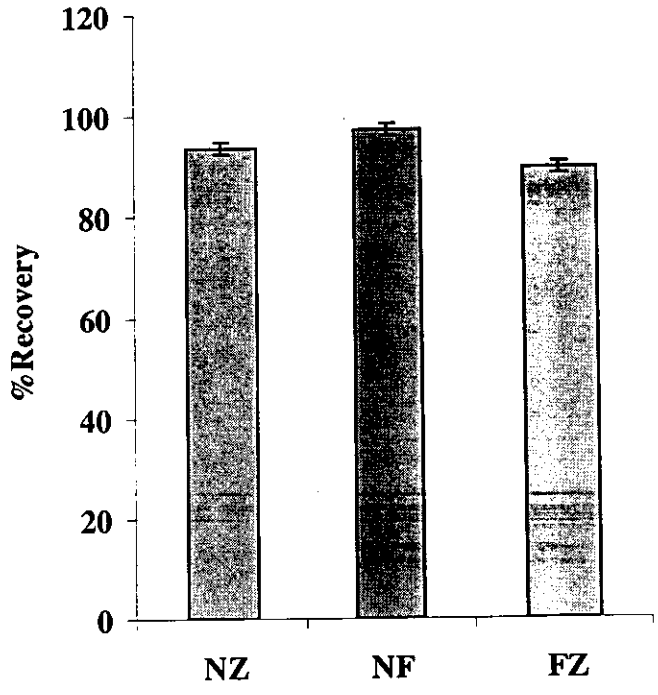


Figure 58 Percentage of recoveries of the nitrofurans in sample

The optimum conditions of sample preparation procedure for the analysis of nitrofurans in animal feed are summarized in Table 49. The chromatograms obtained by these sample preparation conditions (Figure 59) gave high extraction efficiency and baseline resolution.

Table 49 Summary of the optimum conditions of sample preparation

Parameters	Optimum values
Extraction time	1 hour
Type of solvent extraction	N,N- dimethylformamide
Shaking time	15 seconds
Type of washing solvent	N,N- dimethylformamide

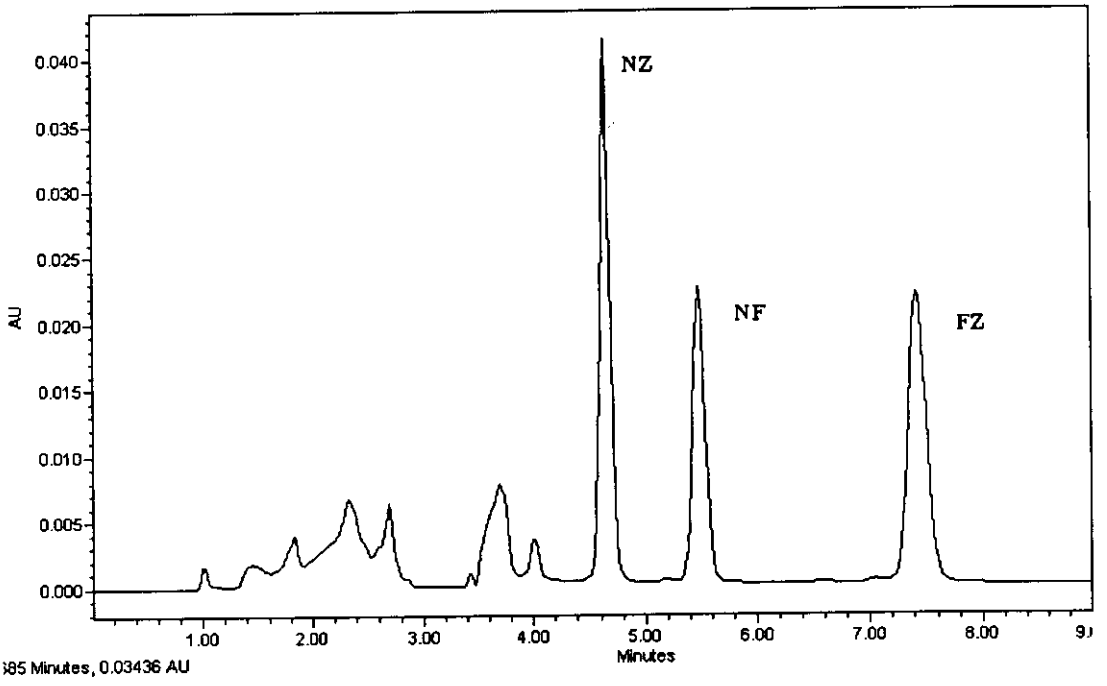


Figure 59 HPLC-UV Chromatograms of nitrofurans from chicken feed sample optimum conditions of sample preparation

3.3.6 Matrix interference

Matrix effect is an influence of one or more undetected components from the sample on the measurement of the analyte concentration. It occur when the physical characteristics of the sample and standard differ considerably. The response of certain analyses may be affected by the presence of co-extractives from the sample (matrix) (Sanco guideline, 2003). To test the matrix effect, aliquots of a standard are added to portions of the sample, thereby allowing any interfere present in the sample to also affect the standard similarly. If no interference were present in this sample, a slope obtained from plotting of measured response versus the concentration of added standard would not be different to the standard calibration. (Eurachem guide, 1998 and Perkin-Elmer Corporation, 1996).

Three brands of animal feed samples in the market were used to study the interference in samples. The standard addition method was used for this study. Known standards *i.e.* 2, 4, 6 and 8 $\mu g\ mL^{-1}$ were added into the samples. The optimum conditions were set for the analysis. Tables 50-53 and Figures 60-63 showed the comparison of the calibration curves between nitrofuran standard and standard addition in samples. The matrix effect was considered from difference between standard calibration and standard addition slopes.

To evaluate the difference of slopes between standard and standard addition (matrix) curves, a statistical test known as a significance test can be employed. Significant tests are widely used to test the truth of a hypothesis which is known as a null hypothesis, denoted by H_0. The null hypothesis is claim that is established for purpose of testing. This claim is either rejected or not rejected. If the evidence is sufficient to reject the null hypothesis, then the alternate hypothesis, denoted by H_1, is accepted. For this work, the null and alternate hypotheses are stated as follows:

H_0 : Slope of regression line between response and concentration in standard and matrix curves are not different.

H_1 : Slope of regression line between response and concentration in standard and matrix curves are different.

In this work, two-way analysis of variance (two-way ANOVA) by R software is used to test the hypothesis. Quantities calculated from the samples information are shown in Table 54-57 (The R Development Core Team, 2006). If P value was less than α (level of significance) then the null hypothesis was rejected at that significant level.

Table 50 Comparison between nitrofurazone (NZ) standard and standard addition curves in animal feed samples by HPLC-UV

Concentration of NZ (mg L^{-1})	Response x 10^{4*} (AUxs)			
	Standard NZ	Chicken feed	Pig feed	Shrimp feed
2	10.15	8.51	8.61	8.62
4	19.94	17.16	17.87	17.68
6	29.85	25.00	27.17	26.20
8	41.07	35.14	35.96	35.59

* 5 replications, RSD < 10%

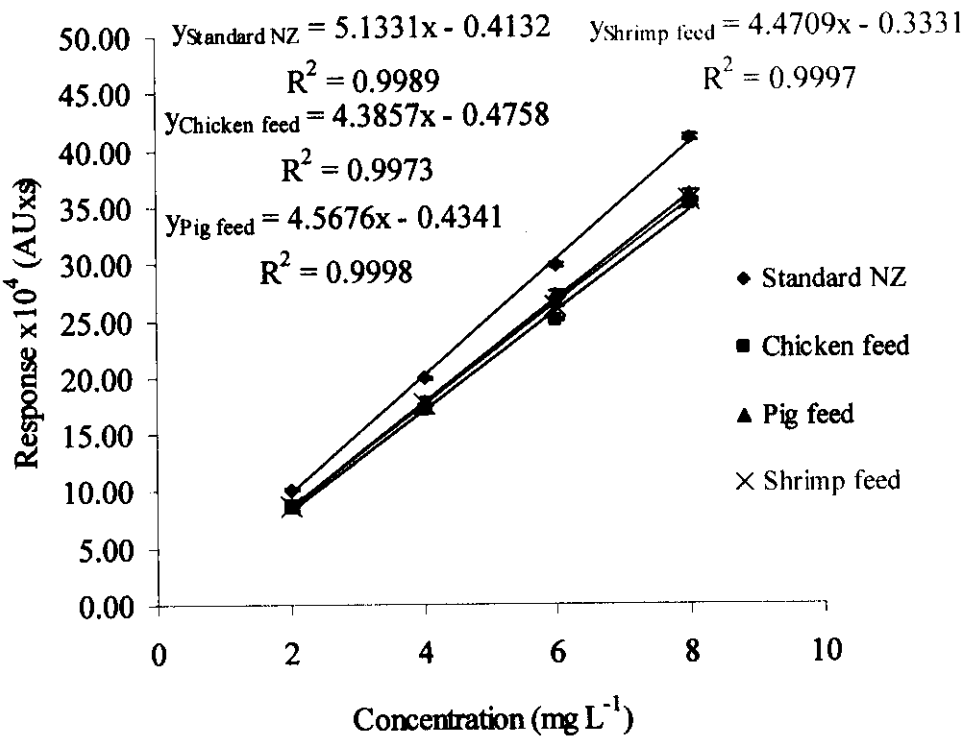


Figure 60 Comparison between nitrofurazone (NZ) standard and standard addition curves in animal feed samples by HPLC-UV

Table 51 Comparison between nitrofurantoin (NF) standard and standard addition curves in animal feed samples by HPLC-UV

Concentration of NF (mg L^{-1})	Response x 10^{4*} (AUxs)			
	Standard NF	Chicken feed	Pig feed	Shrimp feed
2	9.77	8.00	8.41	8.58
4	18.94	16.61	17.89	17.68
6	28.78	24.42	26.84	26.26
8	38.89	34.36	35.93	35.62

* 5 replications, RSD < 10%

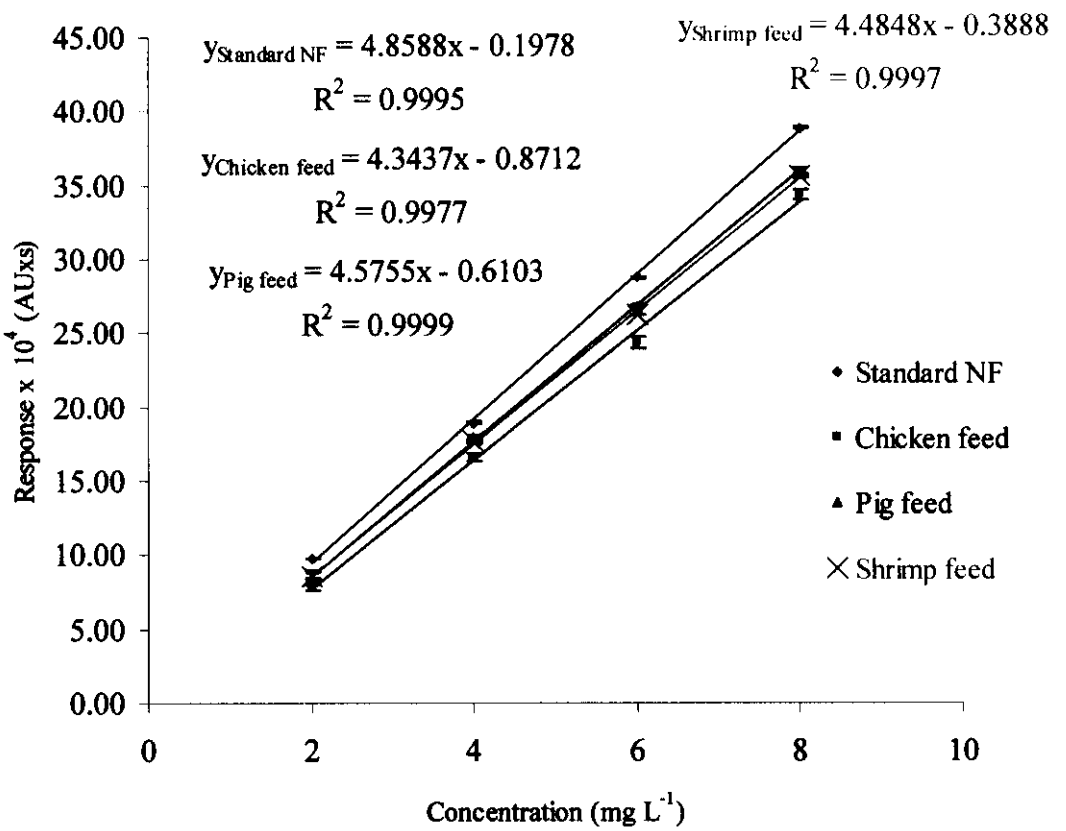


Figure 61 Comparison between nitrofurantoin (NF) standard and standard addition curves in animal feed samples by HPLC-UV

Table 52 Comparison between furazolidone (FZ) standard and standard addition curves in animal feed samples by HPLC-UV

Concentration of FZ (mg L^{-1})	Response x 10^{4*} (AU×s)			
	Standard FZ	Chicken feed	Pig feed	Shrimp feed
2	9.58	8.20	8.33	8.56
4	18.89	17.01	17.65	17.67
6	28.45	25.00	26.86	26.13
8	37.02	34.80	35.26	35.34

* 5 replications, RSD < 10%

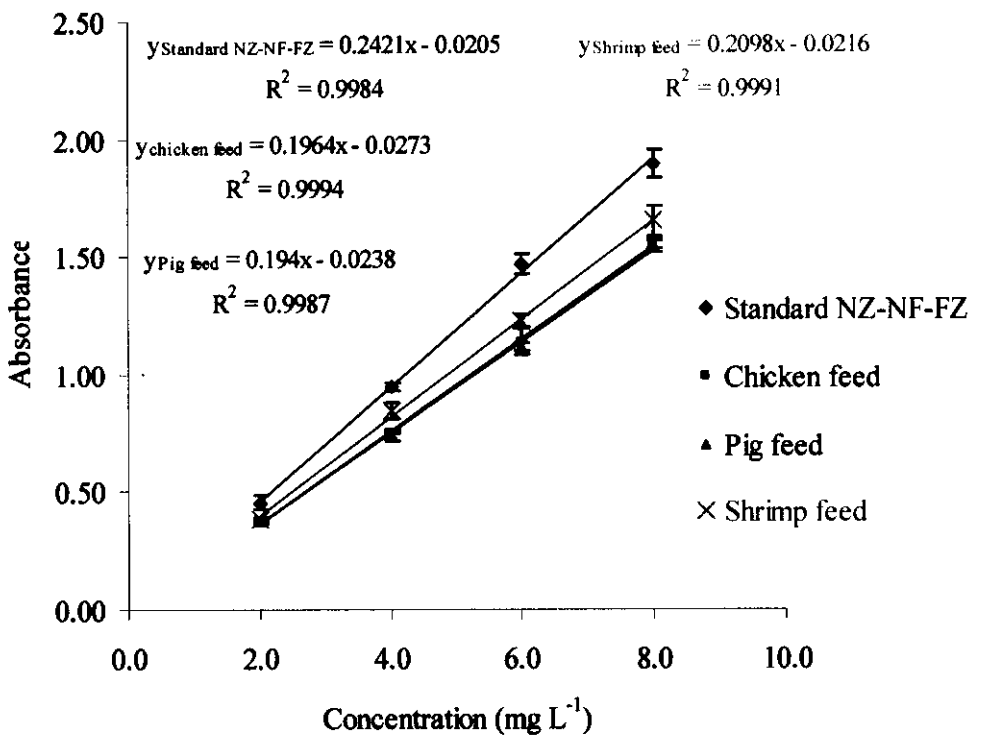


Figure 62 Comparison between furazolidone (FZ) standard and standard addition curves in animal feed samples by HPLC-UV

Table 53 Comparison between NZ-NF-FZ standard and standard addition curves in animal feed samples by spectrophotometric technique

Concentration of NZ-NF-FZ ($mg\ L^{-1}$)	Absorbance*			
	Standard NZ-NF-FZ	Chicken feed	Pig feed	Shrimp feed
2	0.45	0.37	0.38	0.38
4	0.94	0.75	0.74	0.84
6	1.47	1.14	1.12	1.23
8	1.89	1.56	1.55	1.65

* 5 replications, RSD < 10%

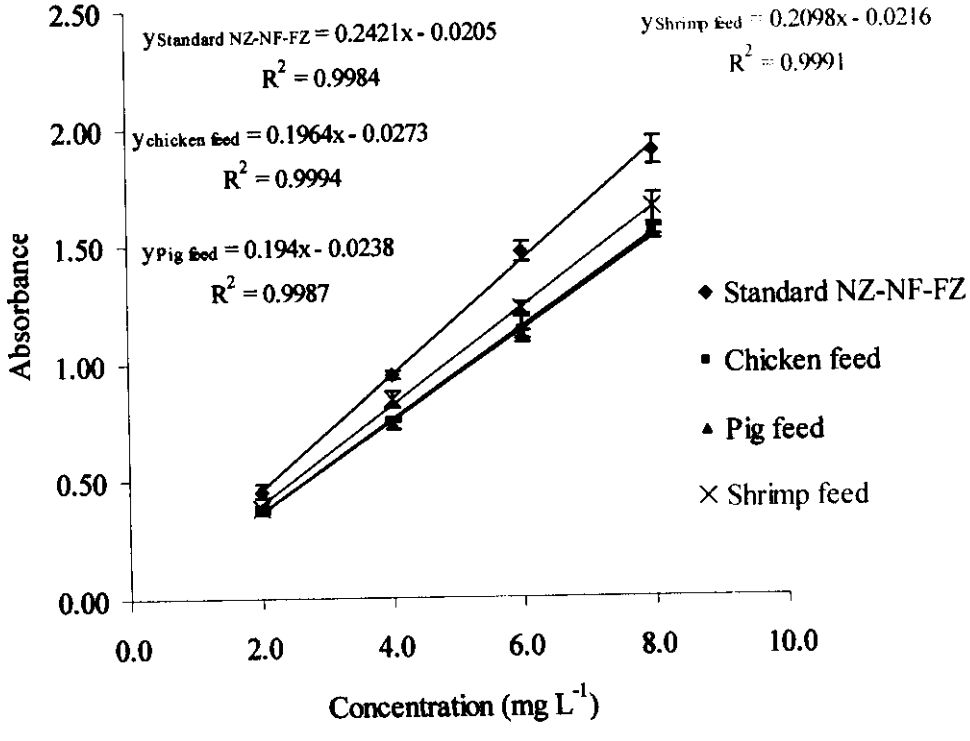


Figure 63 Comparison between NZ-NF-FZ standard and standard addition curves in animal feed samples by spectrophotometric technique

Table 54 Comparison between nitrofurazone (NZ) standard and standard addition curves in animal feed samples by HPLC-UV

Types of animal feed	***Df***	**Sum Sq**	**Mean Sq**	***F***	***P***
Chicken feed	3	$2.8411e^{+09}$	$9.4704e^{+08}$	299.77	$2.2e^{-16}$ ***
Pig feed	3	$1.8593e^{+09}$	$6.1975e^{+08}$	138.79	$2.2e^{-16}$ ***
Shrimp feed	3	$2.2691e^{+09}$	$7.5635e^{+08}$	646.49	$2.2e^{-16}$ ***

Significant codes: '***' ($\alpha = 0.001$)

Where *Df* : Degrees of freedom refer to the number of independent deviation (*Df* = n-1, n : sample size = number of concentration = 4)

Sum Sq : Sum of square refers to an interim quantity used in the calculation of an estimate of the population variance

Mean Sq : Mean of square involves a sum of squared term divided by the number of degrees of freedom

F : F value is the ratio of the two sample variances, *i.e.,* the ratio of the square of the standard deviations, s_1^2 / s_2^2

P : probability

Table 55 Comparison between nitrofurantoin (NF) standard and standard addition curves in animal feed samples by HPLC-UV

Types of animal feed	***Df***	**Sum Sq**	**Mean Sq**	***F***	***P***
Chicken feed	3	$1.4740e^{+09}$	$4.9133e^{+08}$	91.41	$8.865e^{-16}$ ***
Pig feed	3	$5.2660e^{+09}$	$1.7553e^{+08}$	61.132	$2.412e^{-16}$ ***
Shrimp feed	3	$7.6469e^{+09}$	$2.5490e^{+08}$	105.83	$2.2e^{-16}$ ***

Significant codes: '***' ($\alpha = 0.001$)

Where *Df* : Degrees of freedom refer to the number of independent deviation (*Df* = n-1, n : sample size = number of concentration = 4)

Sum Sq : Sum of square refers to an interim quantity used in the calculation of an estimate of the population variance

Mean Sq : Mean of square involves a sum of squared term divided by the number of degrees of freedom

F : F value is the ratio of the two sample variances, *i.e.*, the ratio of the square of the standard deviations, s_1^2 / s_2^2

P : probability

Table 56 Comparison between furazolidone (FZ) standard and standard addition curves in animal feed samples by HPLC-UV

Types of animal feed	***Df***	**Sum Sq**	**Mean Sq**	***F***	***P***
Chicken feed	3	$5.8503e^{+08}$	$1.9501e^{+08}$	23.721	$2.855e^{-8}$ ***
Pig feed	3	$4.9360e^{+07}$	$1.6453e^{+07}$	3.6965	0.02164 *
Shrimp feed	3	$2.5012e^{+08}$	$8.3373e^{+07}$	77.662	$8.894e^{-15}$ ***

Significant codes: '*'($\alpha = 0.1$) '***' ($\alpha = 0.001$)

Where *Df* : Degrees of freedom refer to the number of independent deviation (*Df* = n-1, n : sample size = number of concentration = 4)

Sum Sq : Sum of square refers to an interim quantity used in the calculation of an estimate of the population variance

Mean Sq : Mean of square involves a sum of squared term divided by the number of degrees of freedom

F : F value is the ratio of the two sample variances, *i.e.*, the ratio of the square of the standard deviations, s_1^2 / s_2^2

P : probability

Table 56 Comparison between furazolidone (FZ) standard and standard addition curves in animal feed samples by HPLC-UV

Types of animal feed	*Df*	Sum Sq	Mean Sq	*F*	*P*
Chicken feed	3	$5.8503e^{+08}$	$1.9501e^{+08}$	23.721	$2.855e^{-8}$ ***
Pig feed	3	$4.9360e^{+07}$	$1.6453e^{+07}$	3.6965	0.02164 *
Shrimp feed	3	$2.5012e^{+08}$	$8.3373e^{+07}$	77.662	$8.894e^{-15}$ ***

Significant codes: '*'($\alpha = 0.1$) '***' ($\alpha = 0.001$)

Where *Df* : Degrees of freedom refer to the number of independent deviation (*Df* = n-1, n : sample size = number of concentration = 4)

Sum Sq : Sum of square refers to an interim quantity used in the calculation of an estimate of the population variance

Mean Sq : Mean of square involves a sum of squared term divided by the number of degrees of freedom

F : F value is the ratio of the two sample variances, *i.e.*, the ratio of the square of the standard deviations, s_1^2 / s_2^2

P : probability

Table 57 Comparison between nitrofurazone-nitrofurantoin-furazolidone (NZ-NF-FZ) standard and standard addition curves in animal feed samples by UV-Vis spectrophotometry

Types of animal feed	***Df***	**Sum Sq**	**Mean Sq**	***F***	***P***
Chicken feed	3	0.1144	0.0381	45.819	$1.095e^{-11}$ ***
Pig feed	3	0.1284	0.0428	93.165	$6.754\ e^{-16}$ *
Shrimp feed	3	0.0596	0.0199	25.182	$1.479e^{-18}$ ***

Significant codes: '*'($\alpha = 0.1$) '***' ($\alpha = 0.001$)

Where *Df* : Degrees of freedom refer to the number of independent deviation (*Df* = n-1, n : sample size = number of concentration = 4)

Sum Sq : Sum of square refers to an interim quantity used in the calculation of an estimate of the population variance

Mean Sq : Mean of square involves a sum of squared term divided by the number of degrees of freedom

F : F value is the ratio of the two sample variances, *i.e.,* the ratio of the square of the standard deviations, s_1^2/s_2^2

P : probability

The results found that the null hypothesis is rejected at $P<0.05$ for furazolidone standard curve and matrix (pig feed) curve, and $P<0.001$ for the others. Thus slopes of both curves were statistically different (Miller and Miller, 2000). Therefore, the standard addition method was used for determination nitrofurans to eliminate the presence of a matrix interference in real samples.

3.4 Qualitative and quantitative analysis of animal feed sample

3.4.1 Qualitative analysis

3.4.1.1 Qualitative analysis of the UV-Vis spectrophotometric technique for nitrofuran analysis

The optimum conditions of spectrophotometric technique were used to analyze nitrofurans in animal feed samples. For qualitative analysis, the maximum wavelengths, λ_{max} were used. The maximum of each nitrofurans were;

Nitrofurazone (NZ) 530 nm, nitrofurantoin (NF) 430 nm, furazolidone (FZ) 560 nm, nitrofurazone-nitrofurantoin (NZ-NF) 520 nm, nitrofurazone-furazolidone (NZ-FZ) 535 nm, nitrofurantoin-furazolidone (NF-FZ) 515 nm and nitrofurazone-nitrofuramntoin-furazolidone (NZ-NF-FZ) 525 nm.

3.4.1.2 Qualitative analysis of the HPLC-UV technique for nitrofuran analysis

For HPLC-UV technique, the retention time, t_R were used to qualitative analysis. The average t_R of nitrofurazone, nitrofurantoin and furazolidone were 4.68, 5.88 and 7.73 minutes respectively.

3.4.2 Quantitative analysis

Real samples, chicken feed, pig feed and shrimp feed, were collected from the local animal feed stores. All samples were extracted by solvent extraction, followed by a clean up step and analyzed by HPLC-UV and spectrophotometric technique using standard addition method at the optimum conditions. This is a useful technique that often can make it possible to work in the presence of a matrix interference without eliminating the interference itself, and still make an accurate determination of analyte concentration. The results are shown in Tables 58-61 and Figures 64-67. From the results, no nitrofurans in animal feed samples were detected. This is probably because the Ministry of agriculture issued a proclamation in 2003 which prohibited the import of nitrofurans to be used in animal feed. From this reason, the animal feed producer annul the use of this compound.

Table 58 The results of standard addition method of nitrofurazone (NZ) in each animal feed samples by HPLC-UV

Concentration of NZ (mg L^{-1})	Response x 10^{4*} (AUxs)		
	Chicken feed	Pig feed	Shrimp feed
2	8.51	8.61	8.62
4	17.16	17.87	17.68
6	25.00	27.17	26.20
8	35.14	35.96	35.59

* 5 replications, RSD < 10%

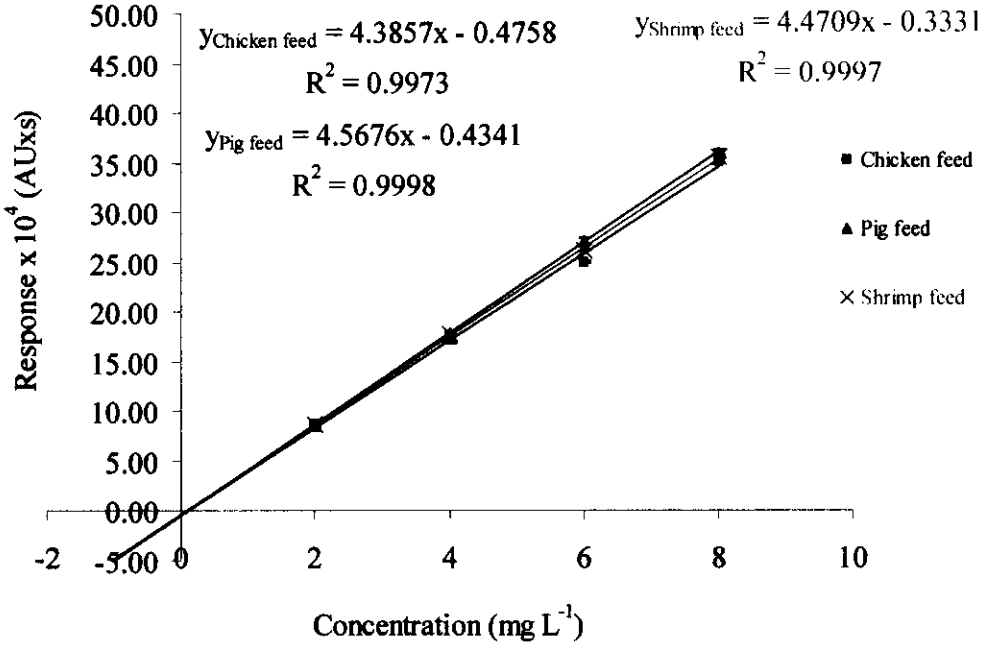


Figure 64 The results of standard addition method of nitrofurazone (NZ) in each animal feed samples by HPLC-UV

Table 59 The results of standard addition method of nitrofurantoin (NF) in each animal feed samples by HPLC-UV

Concentration of NF (mg L^{-1})	Response x 10^{4*} (AU×s)		
	Chicken feed	Pig feed	Shrimp feed
2	8.00	8.41	8.58
4	16.61	17.89	17.68
6	24.42	26.84	26.26
8	34.36	35.93	35.62

* 5 replications, RSD < 10%

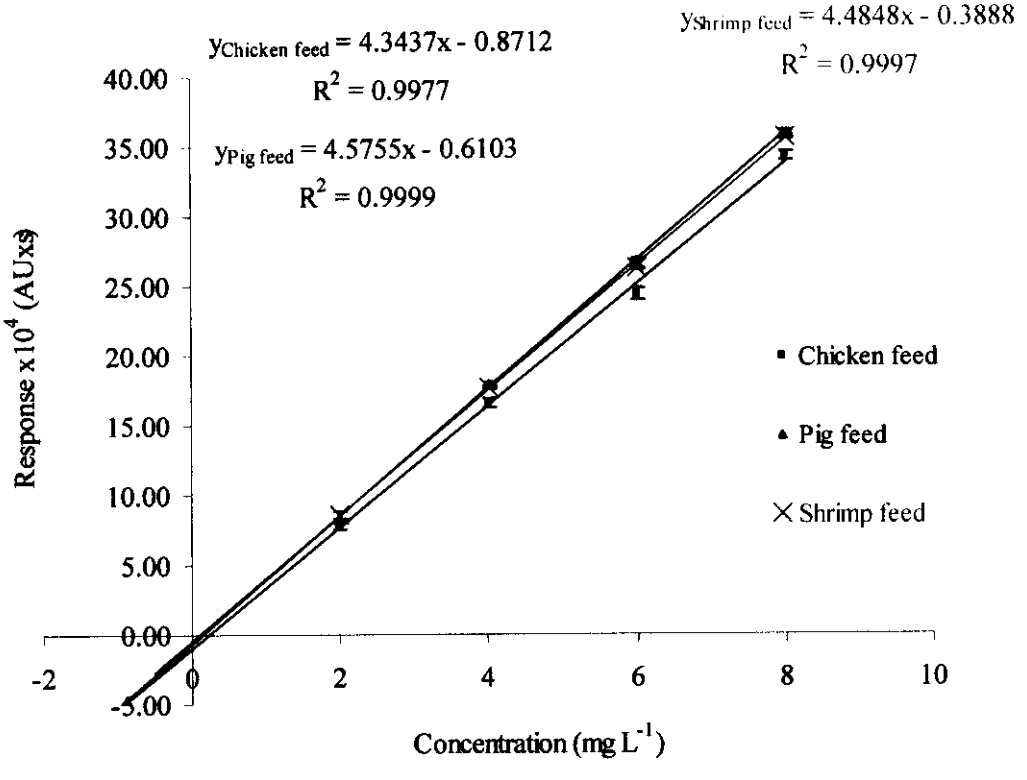


Figure 65 The results of standard addition method of nitrofurantoin (NF) in each animal feed samples by HPLC-UV

Table 60 The results of standard addition method of furazolidone (FZ) in each animal feed samples by HPLC-UV

Concentration of FZ (mg L^{-1})	Response x 10^{4*} (AU×s)		
	Chicken feed	Pig feed	Shrimp feed
2	8.20	8.33	8.56
4	17.01	17.65	17.67
6	25.00	26.86	26.13
8	34.80	35.26	35.34

* 5 replications, RSD < 10%

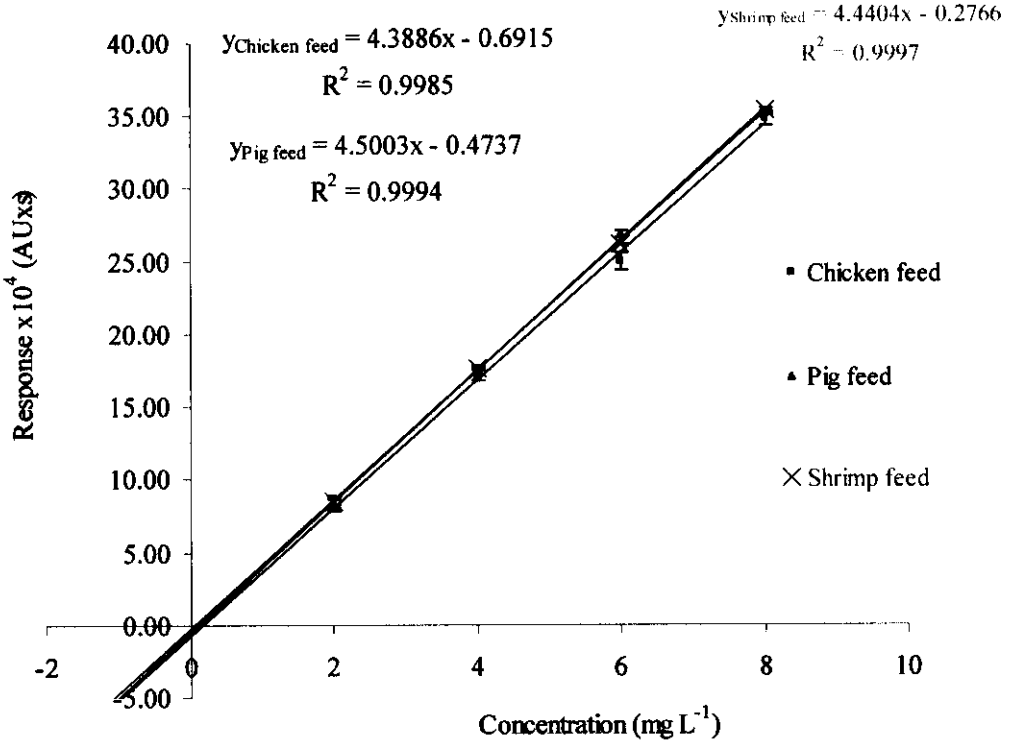


Figure 66 The results of standard addition method of furazolidone (FZ) in each animal feed samples by HPLC-UV

Table 61 The results of standard addition method of NZ-NF-FZ in each animal feed samples by spectrophotometric technique

Concentration of NZ-NF-FZ (mg L^{-1})	Absorbance*		
	Chicken feed	Pig feed	Shrimp feed
2	0.37	0.38	0.38
4	0.75	0.74	0.84
6	1.14	1.12	1.23
8	1.56	1.55	1.65

* 5 replications, RSD < 10%

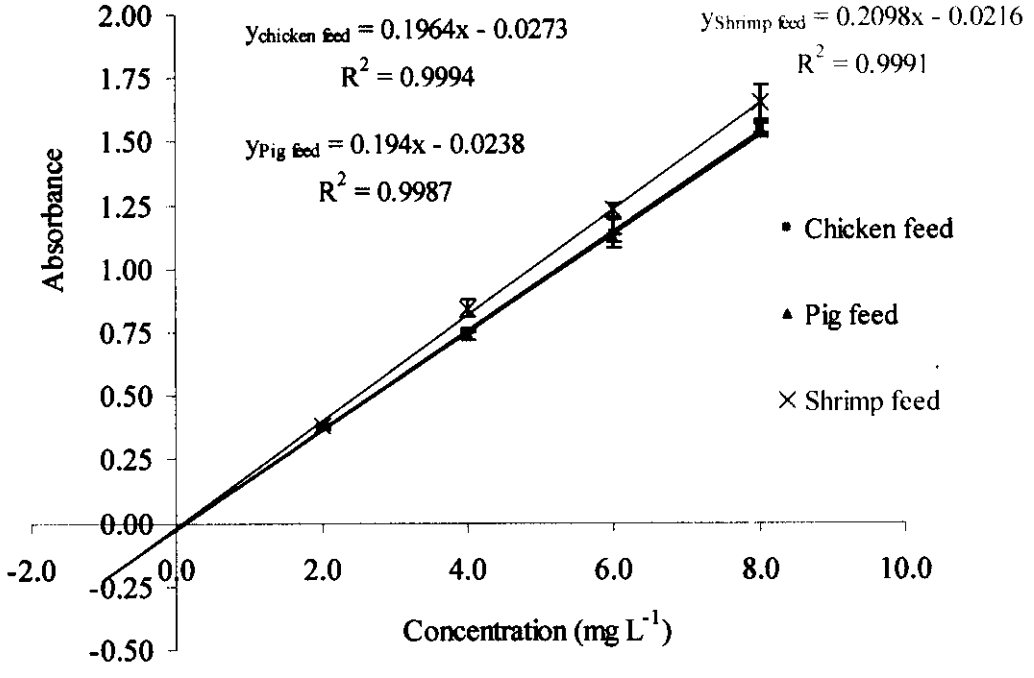


Figure 67 The results of standard addition method of NZ-NF-FZ in each animal feed samples by spectrophotometric technique