CHAPTER 3

RESULTS AND DISCUSSION

This research focused on the determination of salbutamol in lean meat samples with fluorescence detection technique. Two systems were optimized, *i.e.*, spectrofluorometry and ion-pair chromatography with fluorescence detection (IPC-FLD). Sample preparation was done by combining matrix solid phase dispersion (MSPD) with solid phase extraction (SPE).

3.1 Determination of salbutamol by spectrofluorometer

The analysis method for salbutamol in lean meat must be a sensitive and selective method, due to its trace concentration and interference matrices. Fluorescence detection is particularly suitable for salbutamol analysis owing to the intrinsic fluorescence of aromatic phenolic structure. It is a sensitive and selective technique with a possibility of obtaining up to three orders of magnitude, and is more sensitive than absorbance detection, *i.e.*, a low background technique. The selectivity of fluorescence as opp.osed to absorbance detection are (1) due to most organic molecule will absorb UV/visible light but not all will fluorescence and (2) fluorescence utilizes two distinct wavelengths that can decrease the chance that interfering peaks will be detected (Parriott, 1993).

3.1.1 Excitation and emission wavelength

Setting emission wavelength at 309 nm and scanned the excitation spectrum, three excitation wavelengths, *i.e.*, 227, 277 and 310 nm, were obtained (Figure 10 (a)) where 227 nm was chosen as excitation wavelength because when tested by scanning the emission spectrum using blank solution, no interference signal was observed.

Setting 227 nm as excitation wavelength, and scanned for emission wavelength, the emission at 310 nm had the highest intensity (Figure 10 (b)). The excitation and emission wavelengths of salbutamol by this spectrofluorometer were 227 and 310 nm, respectively. These were similarly to those reported by Huclová *et al.* (2003) and Barnett *et al.* (1999), *i.e.*, 230 and 309 nm.
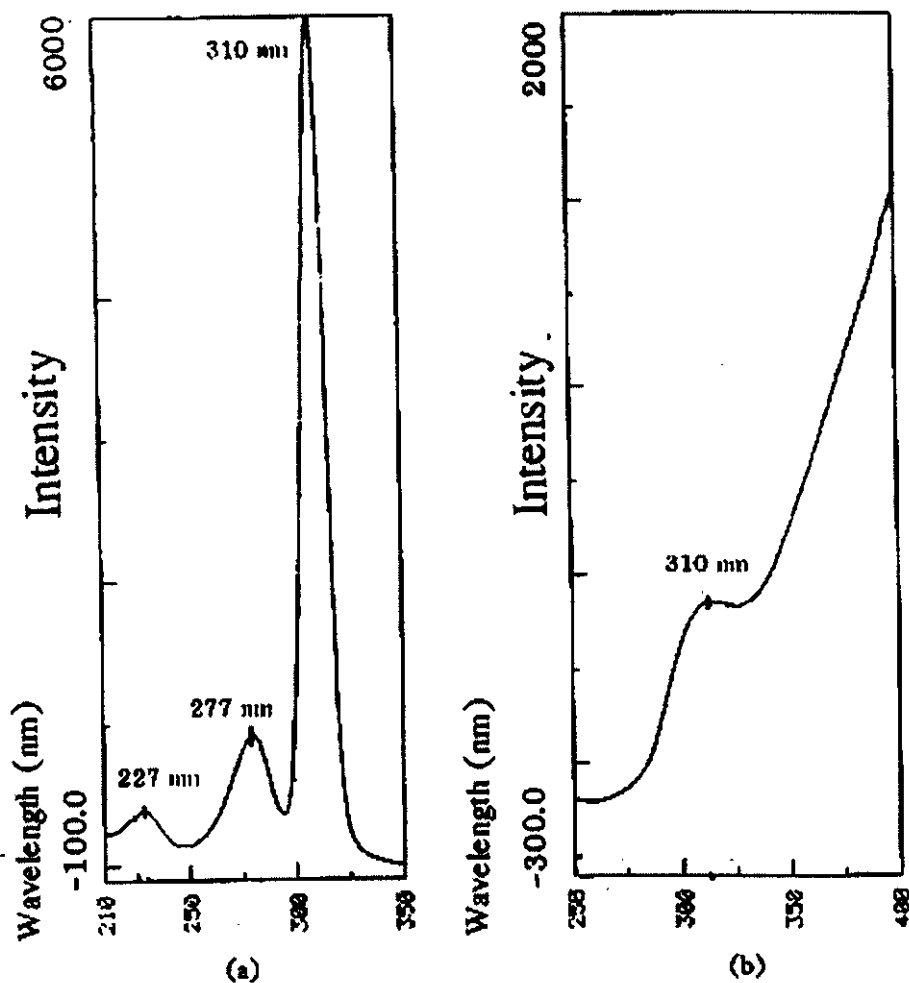

Figure 10 Spectrum of salbutamol standard 0.5 µg mL^{-1} from spectrofluorometer:

      (a) Excitation spectrum by setting emission wavelength at 309 nm.

      (b) Emission spectrum by setting excitation wavelength at 227 nm.

3.1.2 Limit of detection

Limit of detection (LOD) is defined as the smallest quantity of analyte that can be 'reliably' detected. The limit of detection (S/N≥3) (Figure 11) by

spectrofluorometric method was 100 ng mL^{-1} and this was not sensitive enough for trace analysis of salbuatamol in lean meat, (*i.e.*, $\leq$ 1 ng g^{-1} of β-agonists) set by Thai FDA as the acceptable performance of the analysis method (Thai FDA, 2003).

3.1.3 Linear range

The linear range (Figure 11) is defined as the range for which the analytical signal is directly proportional to the amount of analyte present. The linear range of the spectrofluorometric system, where the detector signal is directly proportional to the concentration of the solute, is important in the quantitative analysis. Table 4 and Figure 12 (a), (b) show the linear range of the response (intensity) from the spectrofluorometer to the salbutamol standard solution at various concentrations. This technique showed the linearity from 0.1 to 10 μg mL^{-1} with the the coefficient of determination (R^2) >0.99.
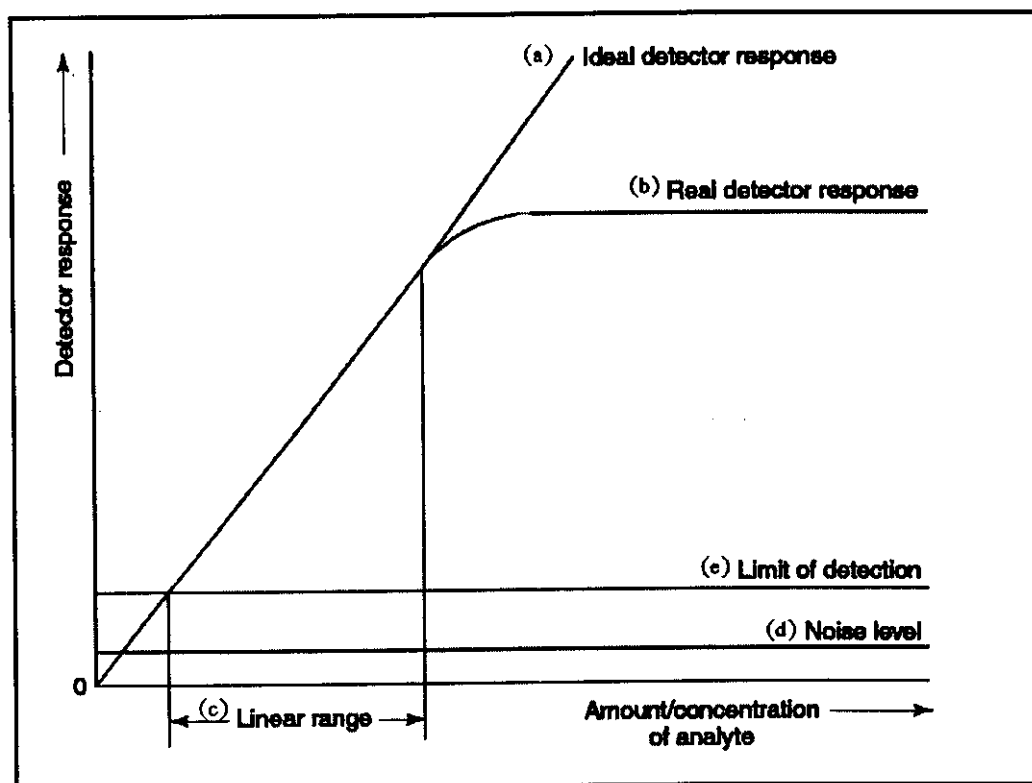

Figure 11 Detector response curve showing (a) 'ideal' behaviour, (b) 'real'behaviour, (c) its linear range, (d) the noise level and (e) the limit of detection at three times the noise level (Ardrey, 2003).
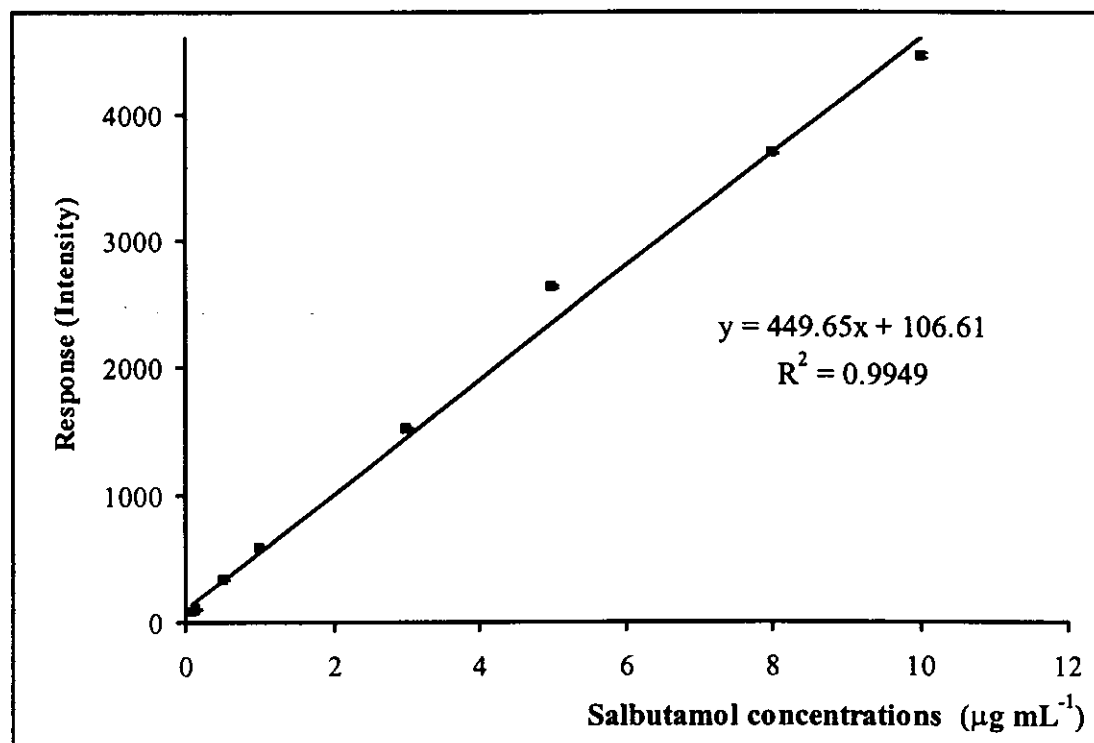
Table 4 Response of salbutamol by spectrofluorometer at various concentrations.

Concentration of salbutamol (μg mL^{-1})	Response * (intensity) ± SD
0.10	75.8 ± 2.0
0.13	91.1 ± 2.4
0.15	107.6 ± 2.5
0.50	335.7 ± 3.6
1.00	587.5 ± 2.8
3.00	1524 ± 11
5.00	2627 ± 12
8.00	3688 ± 7
10.00	4458 ± 17
20.00	7288 ± 12

* 5 replications, RSD < 4%

(a)

$y = 449.65x + 106.61$

$R^2 = 0.9949$
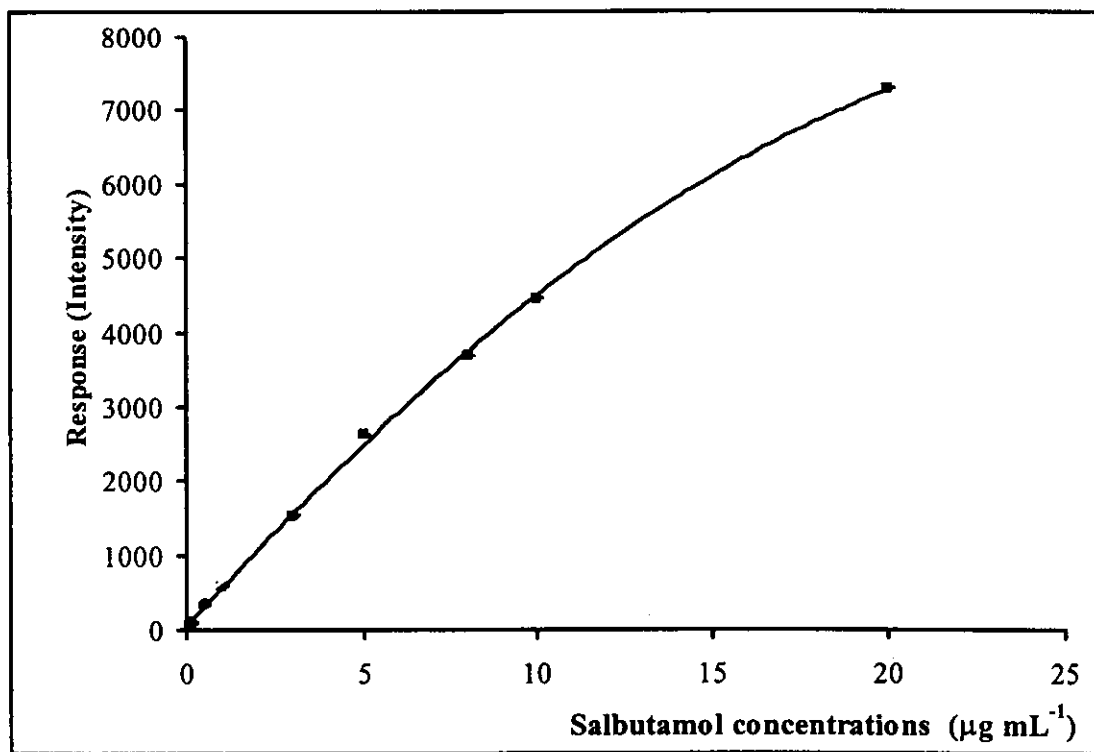
(b)

Figure 12 Response of salbutamol by spectrofluorometry (a) from 0.10 to 20 µg mL^{-1}

(b) linear range from 0.10 to 10 µg mL^{-1}.

From the limit of detection, 0.1 µg mL^{-1}, and the linear range, 0.1-10 µg mL^{-1}, this system may be suitable for the determination of salbutamol in pharmaceutical preparations sample, *e.g.*, tablets, syrups and aerosol that contain sabutamol in high level.

3.2 Determination of salbutamol by HPLC-FLD

Unlike gas chromatography, in which the mobile phase, *i.e.*, the carrier gas, plays no part in the separation mechanism, in HPLC it is the relative interaction of an analyte with both the mobile and stationary phases that determines its retention characteristics (Ardrey, 2003). The separation of any two bands in the chromatogram can be varied systematically by changing experimental conditions. Three parameters in resolution (R_s) equation, *i.e.*, retention factor (k), separation factor(α) and theoretical plate number (N) of column were directly related to experimental conditions.

The parameters k and α were determined by conditions that affect retention or the equilibrium distribution of the sample between the mobile phase and the column packing, *i.e.*, composition of mobile phase, composition of the stationary phase (column) and temperature. Changes in the mobile phase or stationary phases will generally effect both k and α but will have less effect on N. N value is primarily dependent on column quality and can be varied by changing column conditions, *i.e.*, flow rate, column length, particle size. A change in these conditions will not affect k and α as long as the mobile phase and stationary phase types are not changed (Snyder, 1997).

In this part, ion-pair chromatography with fluorescence detection (IPC-FLD) was used to determine salbutamol. Parameters affecting resolution, detection and analysis time of IPC-FLD system were optimized. In IPC-FLD, hexanesulfonate solution (containing acetic acid) and methanol was used as mobile phase and high purity silica base C$_{18}$ (150×4.6 mm with particle size 3 µm) as the stationary phase. Excitation and emission wavelengths of salbutamol and bamethan (IS) were optimized.

3.2.1 Excitation and emission wavelengths

The optimum excitation for IPC-FLD system was obtained by fixing the emission wavelength at 310 nm, the optimum emission wavelength from spectrofluorometry system, and scanned the excitation spectrum. Maximum response was obtained at 220 nm (Figure 13(a)). The excitation wavelength was then fixed at 220 nm and scanned the emission spectrum (Figure 13 (b) and 14 (b)). The maximum response was obtained at 316 nm. The excitation wavelength was scanned again by fixing the emission wavelength at 316 nm and scanned the excitation spectrum (Figure 13 (a) and 14 (a)) and 220 nm provided the maximum response. Thus, the optimum excitation and emission wavelengths of IPC-FLD system were 220 and 316 nm, respectively. Both emission and excitation wavelengths, were closed to many reports (Table 3, Chapter 1).
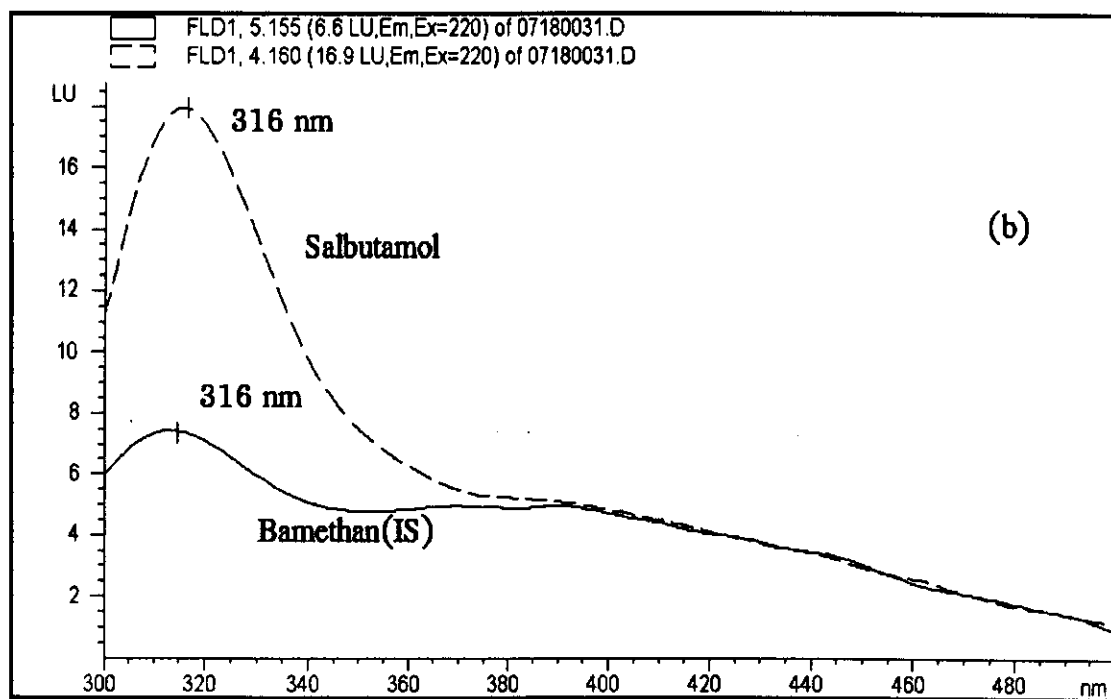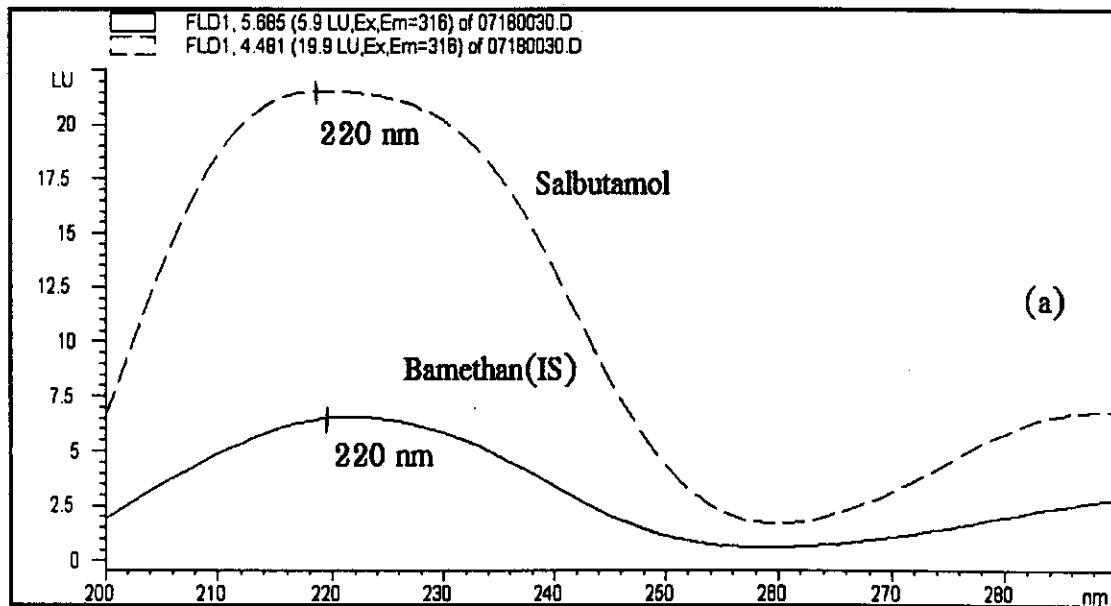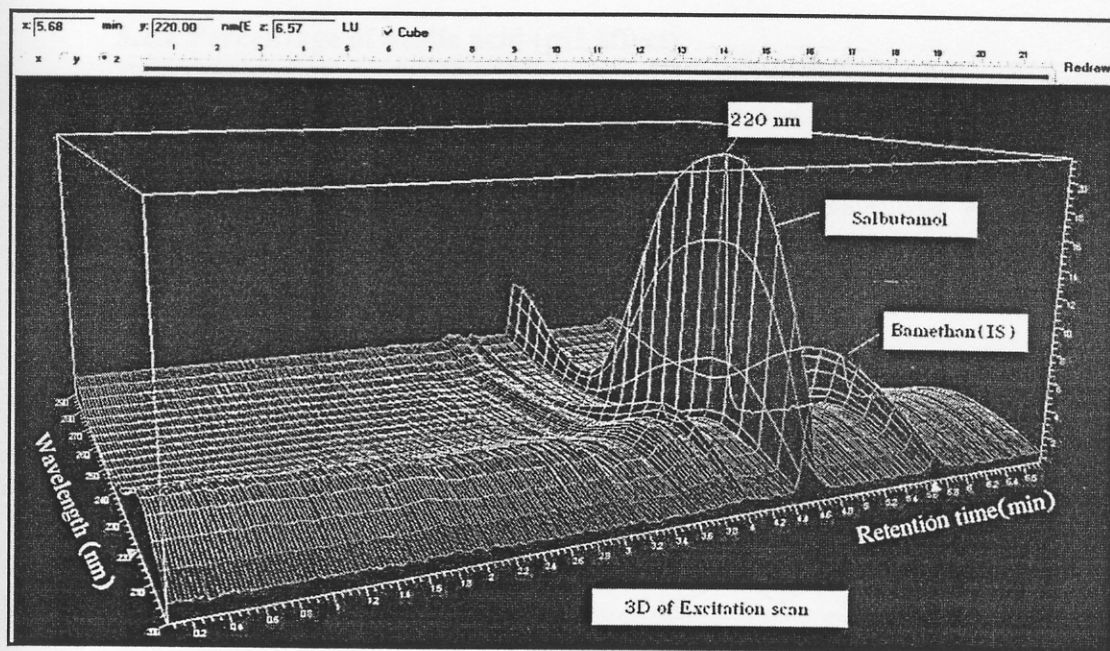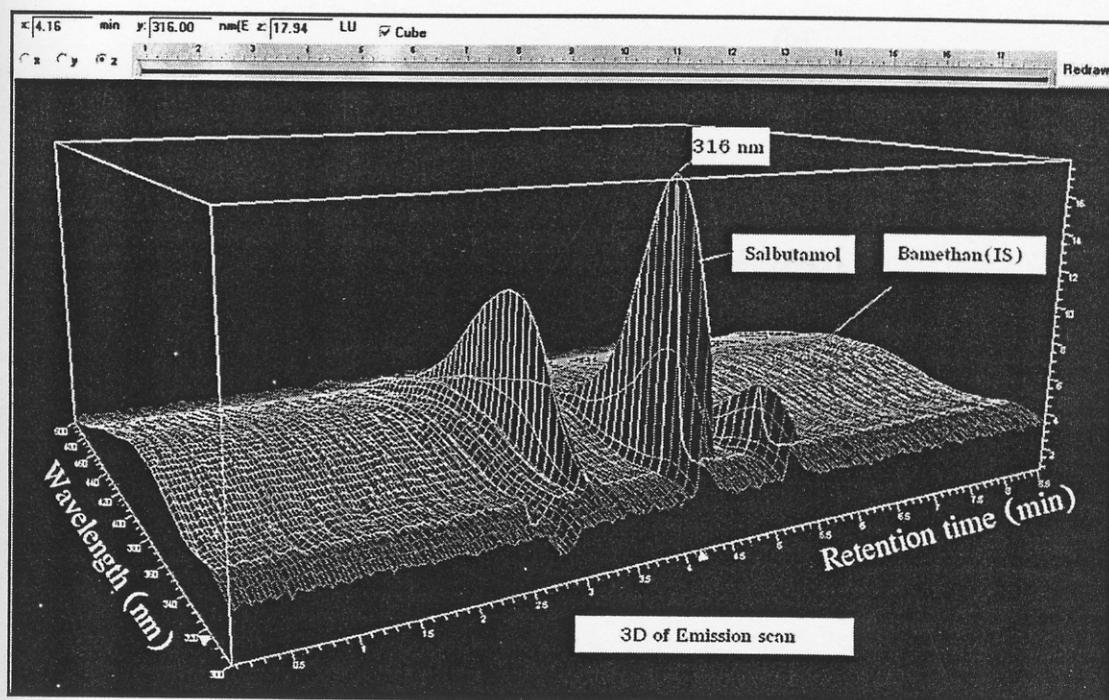
Figure 13 Spectrum of mixed standard salbutamol and bamethan (IS) 20 ng mL^{-1} by

IPC-FLD system (a) Excitation spectrum of salbutamol and bamethan (IS).

(b) Emission spectrum of salbutamol and bamethan (IS).

(a)

(b)

Figure 14 3D plot of mixed standard salbutamol and bamethan (IS) 20 ng mL^{-1} by HP Chemstation program in IPC-FLD system: (a) Excitation scan by setting emission wavelength at 316 nm and (b) Emission scan by setting excitation wavelength at 220 nm.

3.2.2 Percentage of acetic acid (pH effect)

Acetic acid was added to mobile phase to ensure that salbutamol was completely in protonated form (maximum concentration of the ionic form) (Figure 15) while being separated in the IPC-FLD system. The responses at different percentage of acetic acid are shown in Table 5 and Figure 16 and 1.5 % was obtained as the optimum value.
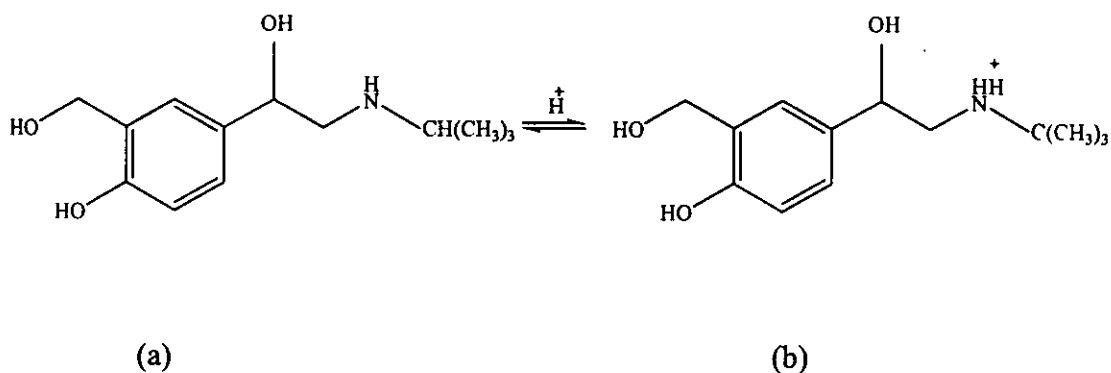

(a)   (b)

Figure 15 Chemical structures of (a) salbutamol and (b) cationic salbutamol under acidic condition (Ouyang *et al.*, 2005).

Table 5 Response of salbutamol and bamethan (IS) at different percentage of acetic acid.

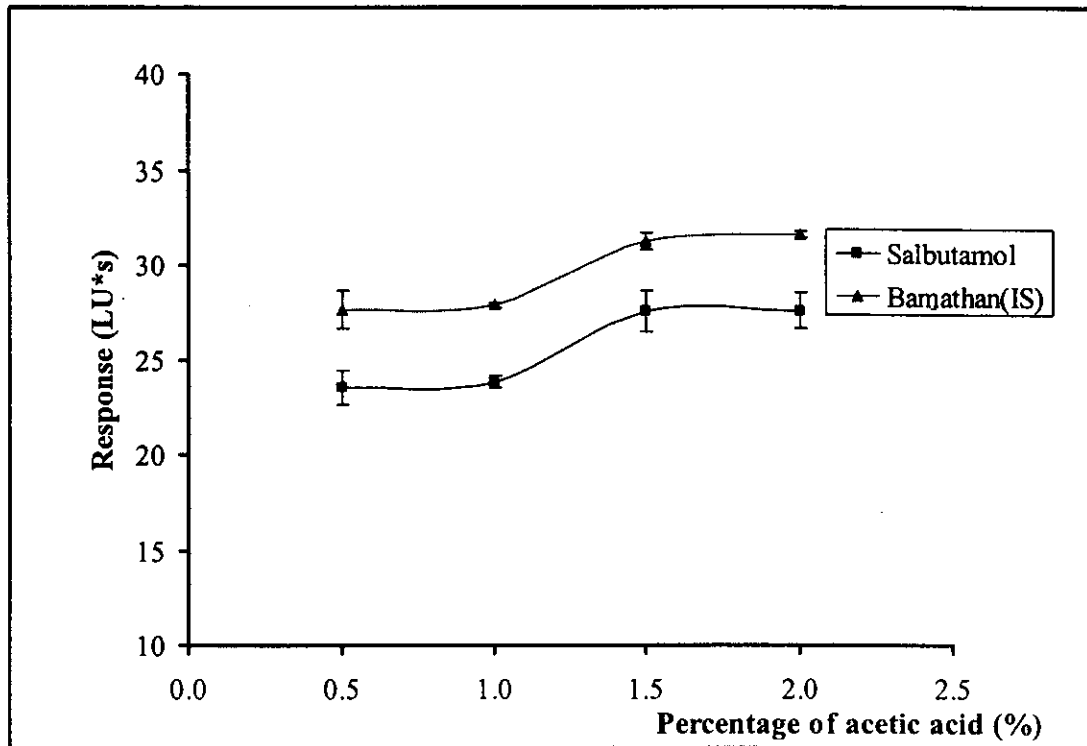| Percentage of acetic acid (%)	Response* (LU*s) ± SD	
	Salbutamol	Bamethan (IS)
0.5	23.5 ± 0.9	27.7 ± 1.0
1.0	23.8 ± 0.3	27.9 ± 0.2
1.5	27.6 ± 1.1	31.2 ± 0.5
2.0	27.6 ± 0.9	31.6 ± 0.2

* 5 replications, RSD < 4%

Figure 16 Response of salbutamol and bamethan (IS) at different percentage of acetic acid.

3.2.3 Concentration of ion-pair reagent

Concentration of ion-pair reagent, *i.e.*, hexanesulfanate solution, in the mobile phase was optimized (experiment 2.6.4.3). The optimum concentration of hexanesulfonate was chosen as a compromise between good separation (consider from retention factor (k) and separation factor (α)), good detection (consider from the response of IPC-FLD at each concentration) and short analysis time. Table 6 shows the retention factor, response and analysis time of salbutamol and bamethan (IS) at different concentration of hexanesulfonate solution. All hexanesulfonate concentration gave separation factor (α) more than 1.05 (not show in the Table). Figure 17 shows the effect of the concentrations of hexanesulfonate solution to the retention of salbutamol and bamethan (IS) on stationary phase of IPC system. At high concentration of ion-pair reagent, salbutamol and bamethan (IS) can retained on stationary phase longer than at low concentration because the stationary phase was saturated with the ion-pair reagent, thus, the ion-exchange and/or reversed-phase processes can occur more and affect the retention of analyte (Snyder *et al.*, 1997). Figure 18 shows the response of salbutamol and bamethan (IS) at difference concentration of hexanesulfonate. By considering the separation, response and analysis time, 3mM hexanesulfonate solution was chosen.

Table 6 Response, retention factor and analysis time of salbutamol and bamethan (IS) at various concentration of hexanesulfonate.

| Concentration of hexanesul-fonate (mM)	Retention factor *(k)±SD		Response *(LU*s) ±SD		Analysis time (min)
	Salbutamol	Bamethan (IS)	Salbutamol	Bamethan (IS)	
0.5	0.538±0.003	0.716±0.002	29.0±0.1	37.7±0.4	5.0
1.0	0.543±0.028	0.759±0.029	31.9±0.4	36.4±0.5	5.2
2.0	0.563±0.028	0.909±0.034	31.8±0.2	32.0±0.2	5.6
3.0	0.633±0.025	1.080±0.038	34.3±0.2	32.8±0.4	5.9
4.0	0.640±0.004	1.156±0.005	32.7±0.3	31.9±0.0	6.1
5.0	0.713±0.011	1.284±0.015	33.2±0.6	31.9±0.2	6.5

* 5 replications, RSD < 4%
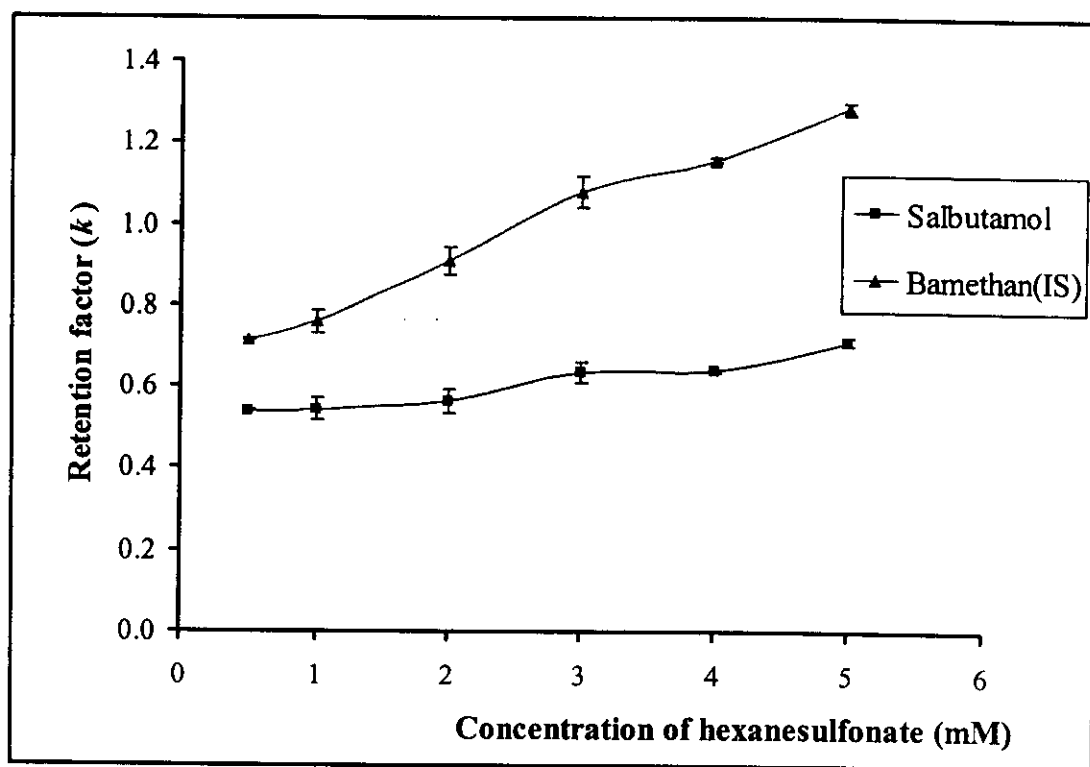
Figure 17 Retention factor of salbutamol and bamethan (IS) at various concentration of ion-pair reagent.
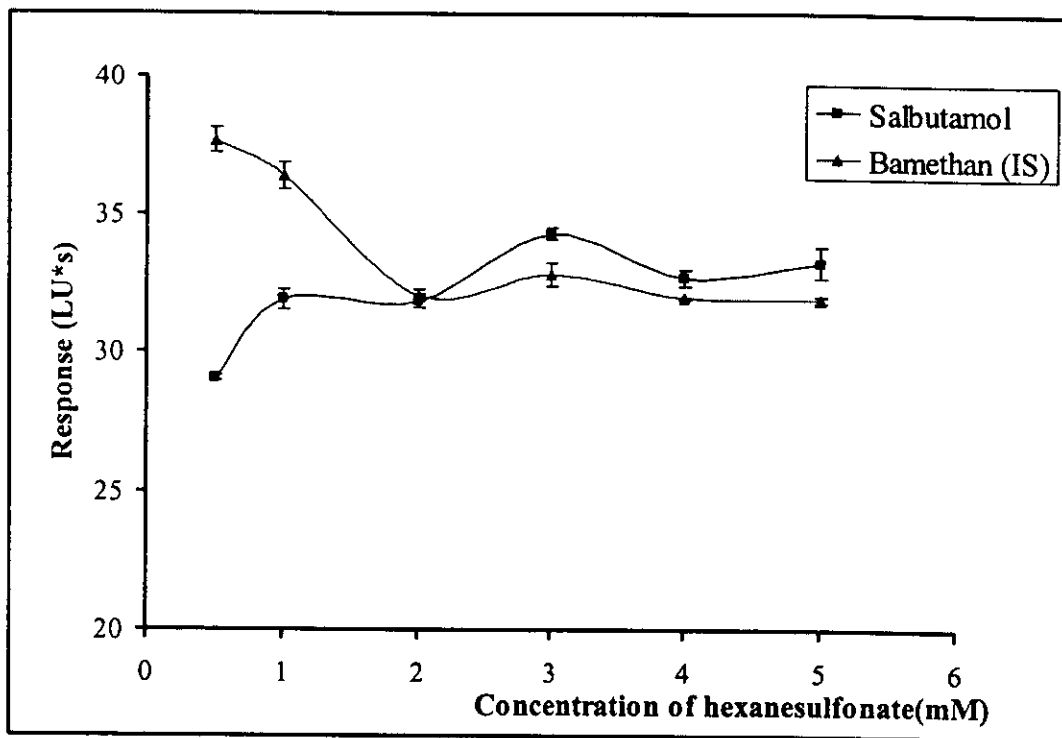
Figure 18 Response of salbutamol and bamethan (IS) at various concentration of ion-pair reagent.

3.2.4 Percentage of methanol (Solvent-strength effect)

The retention factor, separation factor and analysis time of salbutamol and bamethan (IS) at different percentage of methanol are shown in Table 7. Separation factor (α) for all percentages of methanol were more than 1.05. Figure 19 shows the retention factor (k) of salbutamol and bamethan (IS) at each percentage of methanol. At high percentage of methanol, the retention factor of both compounds decrease, moreover, it also caused the decrease of the selectivity factor (Figure 20). An increase in methanol percentage leads to the reduction of relative retention for protonated salbutamol due to the reduction of adsorption of ion-pair reagent to the stationary phase (Snyder *et al.*, 1997). Figure 21 shows the response of salbutamol and bamethan (IS) at various percentage of methanol. When compromised the separation (α between 1.05 and 10, k >1), response and analysis time, 33 % was chosen. However, when this percentage was used to determine fortified sample extractant, salbutamol peak was overlapped with matrix peak as shown in Figure 22 (b), Thus, 30% methanol was then chosen.

Table 7 Response, capacity factor (k), separation factor (α) and analysis time of salbutamol and bamethan (IS) at various percentage of methanol.

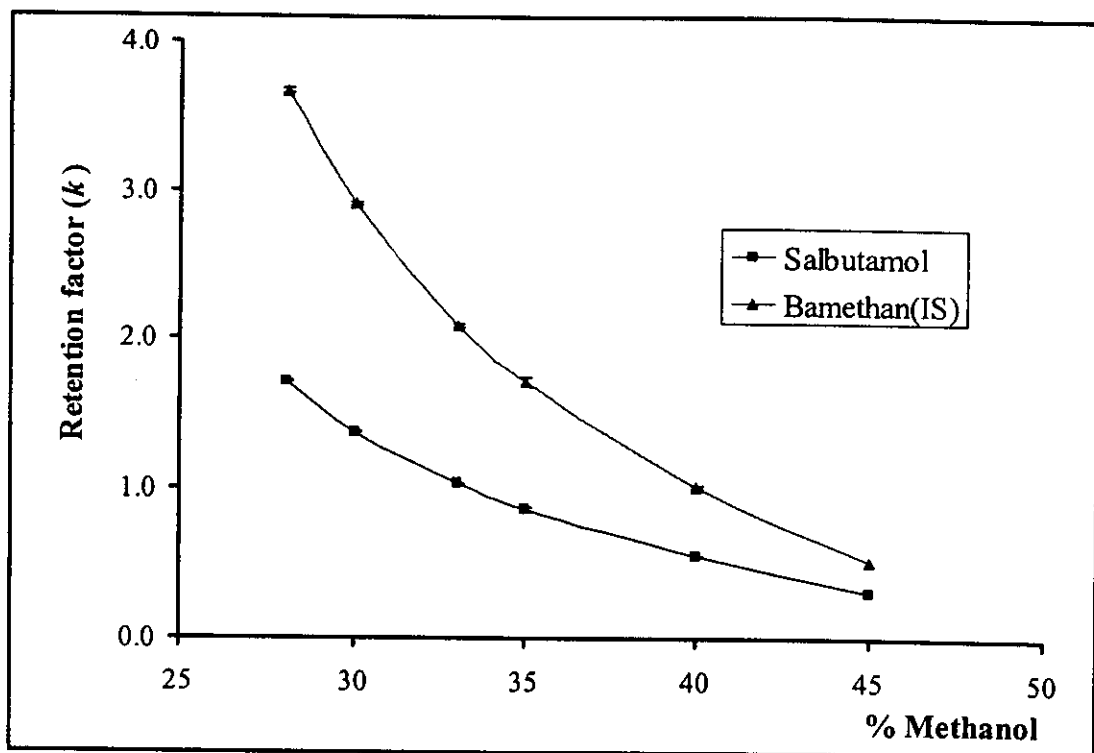| Percentage of methanol (%)	Retention factor* (k)±SD		Separation factor (α)	Response* (LU*s) ±SD		Analysis time (min)
	Salbutamol	Bamethan (IS)		Salbutamol	Bamethan (IS)	
28	1.706±0.004	3.658±0.017	2.144±0.008	41.8±1.2	47.1±0.6	26.0
30	1.365±0.004	2.906±0.011	2.129±0.012	46.2±1.2	50.6±1.1	21.5
33	1.026±0.002	2.065±0.030	2.023±0.011	46.2±1.0	46.9±1.5	20.0
35	0.814±0.026	1.666±0.005	1.996±0.021	47.1±0.6	45.4±0.7	14.5
40	0.547±0.003	1.005±0.008	1.838±0.011	42.1±0.8	48.2±0.9	11.0
45	0.248±0.006	0.567±0.005	1.665±0.034	42.9±0.6	49.5±0.2	8.5

* 5 replications, RSD < 4%

Figure 19 Retention factor of salbutamol and bamethan (IS) at various percentage of methanol.

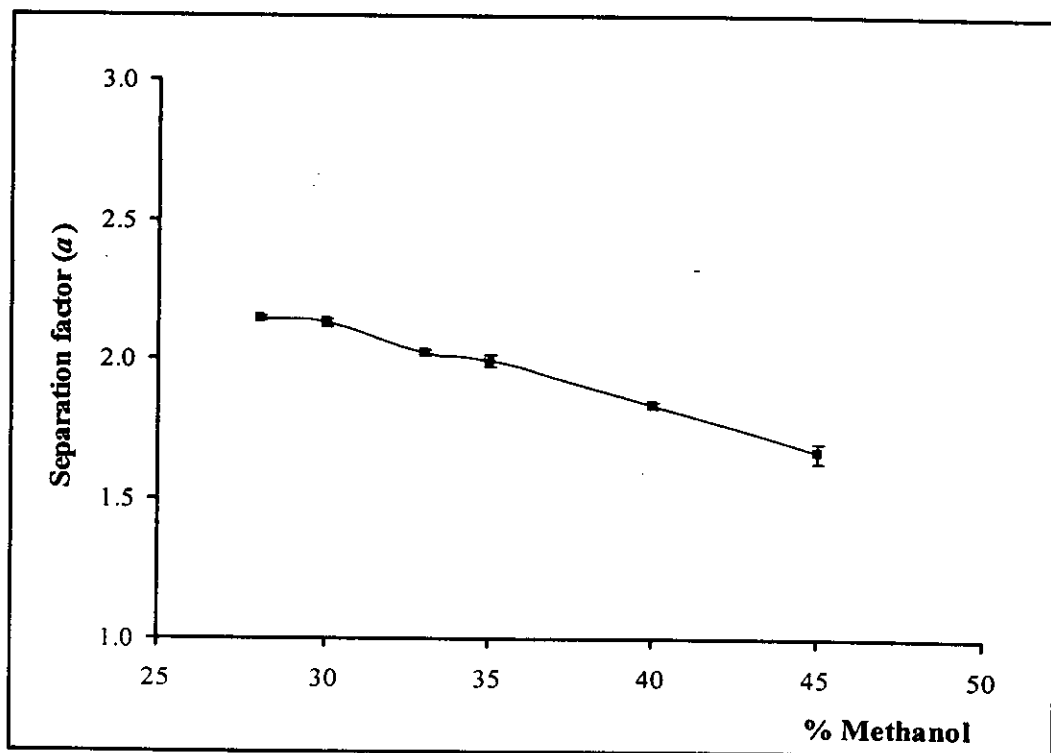**Figure 20** Separation factor (or selectivity) of salbutamol and bamethan (IS) at various percentage of methanol.
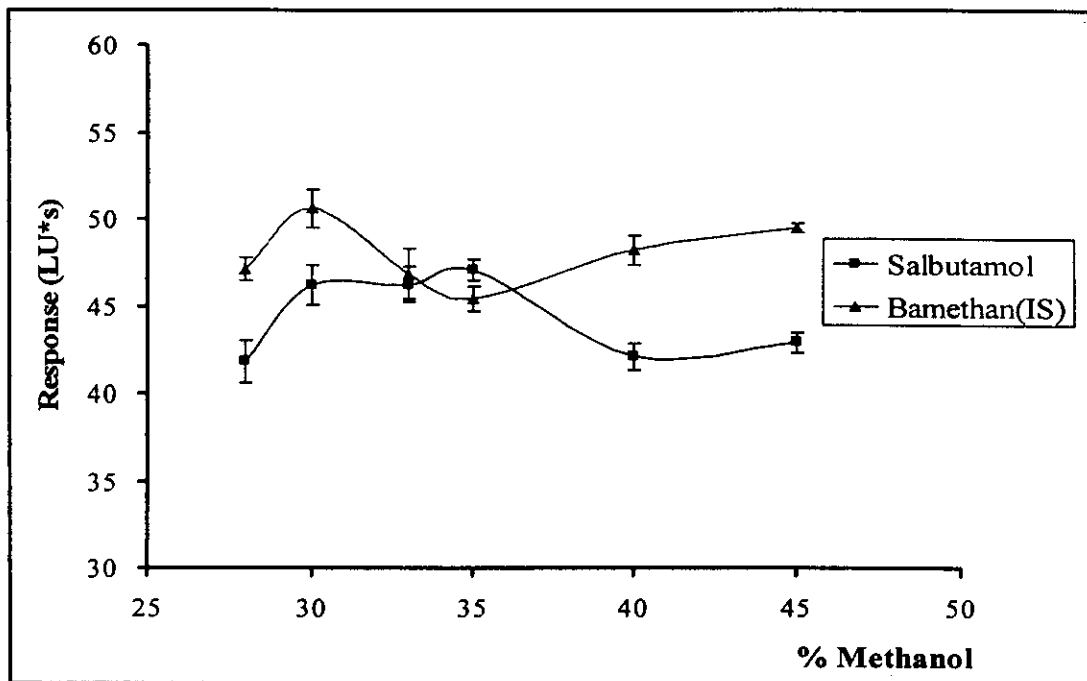
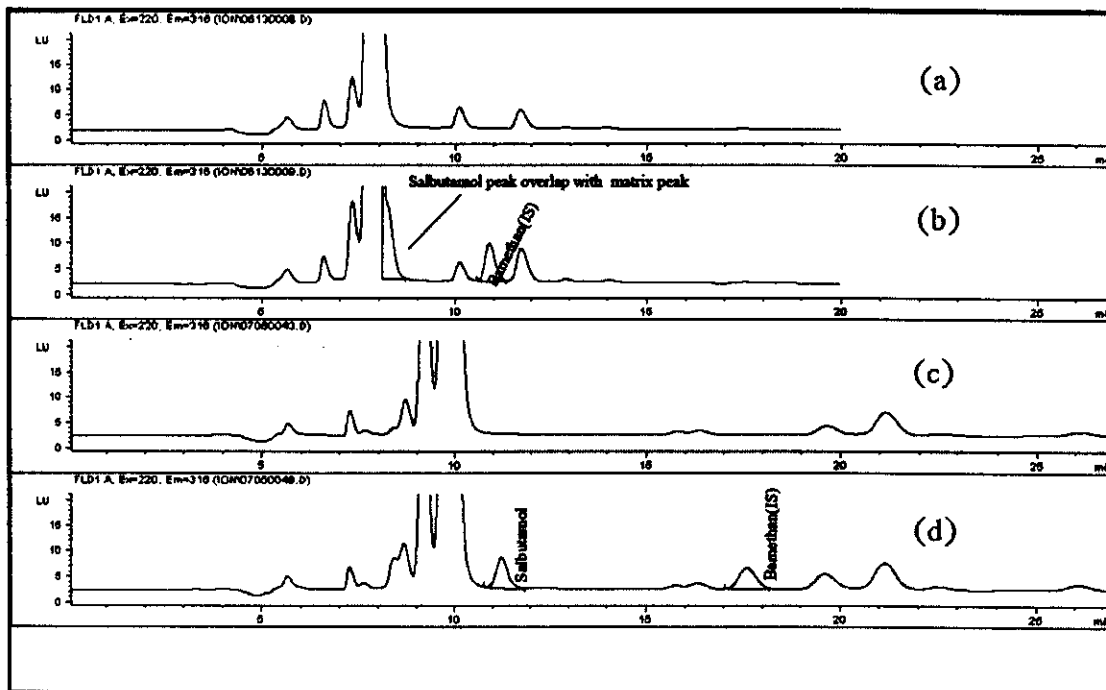**Figure 21** Response of salbutamol and bamethan (IS) at various percentage of methanol.

Figure 22 Chromatogram of (a) blank sample extractant at 33% methanol, (b) fortified sample extractant at 33%, (c) blank sample extractant at 30% methanol and (d) fortified sample extractant fortified sample at 30% methanol.

3.2.5 Temperature

In ion-pair chromatography (IPC), several equilibrium processes are often involved such as sample retention by ion-exchange and/or reversed-phase processes, ionization of sample and sorption of the ion-pair reagent. For reproducible separations by ion-pair HPLC, it is important to thermostat the column (Snyder *et al.*, 1997). The optimum temperature was investigated and the results are shown in Table 8 and Figure 23. The increase in temperature will reduce the retention factor (k) (Figure 23). The decrease of retention factor (k) is the result of a decrease in solvent viscosity as temperature increases. This enhances the mass-transfer rate between the mobile and stationary phase (Neue, 1997). Since the stationary phase of Alltima HP C_{18} column is a monomeric stationary phase, separation factor (α) is not much affected by temperature changes (Neue, 1997) as shown in Table 8. In addition, this IPC-FLD system was operated at a low pH mobile phase, the column packing stability should also be taken into consideration together with the temperature used since at low pH, the high temperature of mobile phase will decrease column life (Snyder *et al.*, 1997). When compromised between the separation, response, analysis time and column packing stability, the optimum temperature of IPC-FLD system was chosen at 35 °C.

Table 8 Response, capacity factor (k), separation factor (α) and analysis time of salbutamol and bamethan (IS) at various column temperature.

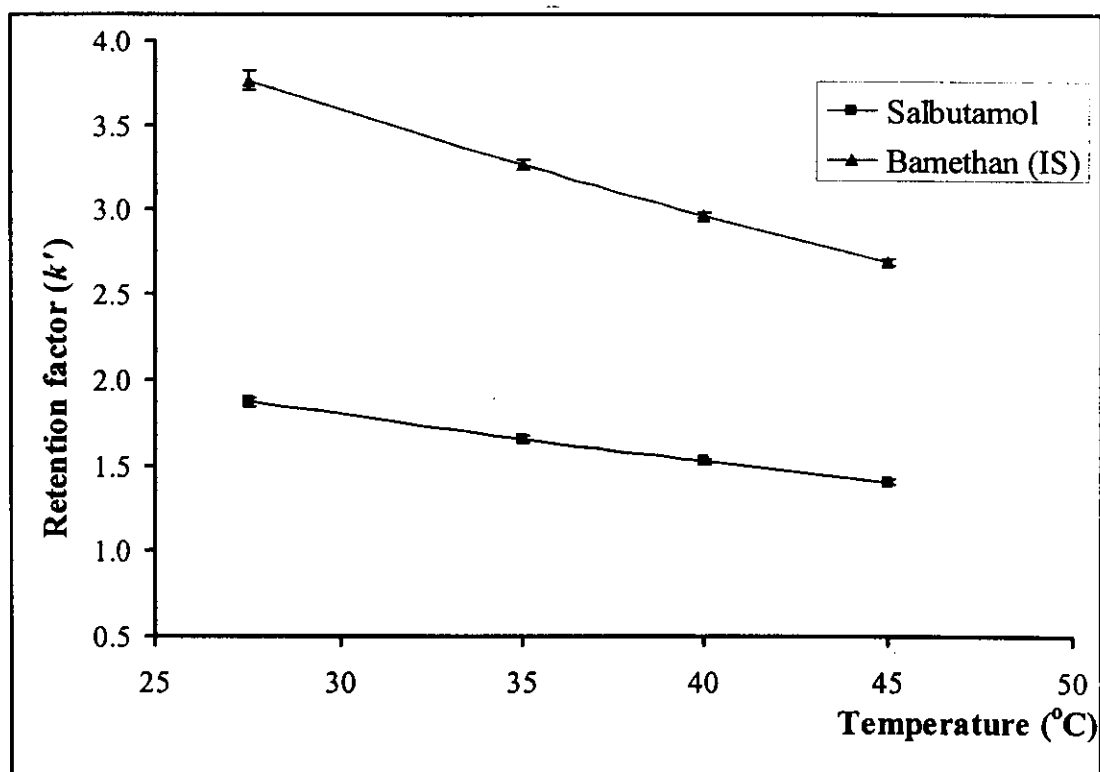| Temperature (°C)	Retention factor* (k)±SD		Separation factor (α)	Response* (LU*s) ±SD		Analysis time (min)
	Salbutamol	Bamethan (IS)		Salbutamol	Bamethan (IS)	
Room temp. 27.5	1.868±0.025	3.763±0.054	2.015±0.011	48±1	48±1	26.0
35	1.652±0.018	3.265±0.034	1.977±0.015	46.7±0.8	49.6±0.1	21.5
40	1.522±0.012	2.966±0.013	1.948±0.014	46.4±0.6	49±2	11.0
45	1.405±0.015	2.687±0.019	1.913±0.027	47.3±0.2	47.9±0.8	8.5

* 5 replications, RSD < 4%

Figure 23 Capacity factor of salbutamol and bamethan (IS) at various column temperatures.

3.2.6 Flow rate

The column efficiency was expressed in term of the theoretical plate number (N) of column and can be calculated by using the equation (3) of Chapter 2:

$$N = 5.54(\frac{t_R}{w_{0.5}})^2$$

where t_R is the retention time of the solute and $w_{0.5}$ is the peak width at half height. For a given set of operating conditions (a particular column and mobile phase, with fixed mobile phase velocity) the quantity N is approximately constant for different band in the chromatogram. In comparing column efficiencies a more useful parameter is the *height equivalent to a theoretical plate* or *plate height* (*HETP* or *H*) which can be expressed as:

$$H = \frac{L}{N} \tag{5}$$

where L is the length of column and H measures the efficiency of the column per unit length. Small H values (large N values), therefore, mean more efficient columns (Hamilton and Swell, 1982). Since the band width increase as the band process down the column, *i.e.*, as t_R increase, N is a measure of the relative band broadening with distance down the column (Hamilton and Swell, 1982). When a sample band migrates through the column, there are five main contributions to band broadening as listed in Table 9. The overall plate height can be represented as the sum of these contributions.

Table 9 Contribution of different band-broadening processes to column plate height H.

Process		
(i) Eddy diffusion (H_F)	$C_e d_p$	
(ii) Moving mobile-phase mass transfer (H_D)	$C_m d_p u/D_m$	$= Au^{0.33}$
(iii) Longitudinal diffusion (H_L)	$C_d D_m/u$	$= B/u$
(iv) Stagnant mobile-phase mass transfer (H_{SM})	$C_{sm} d_p^2 u/D_m$	$= Cu$
(v) Stationary-phase mass transfer (H_S)	$C_s d_f^2 u/D_s$	$= Du$

Note: C_d, C_e, C_m, C_s, C_{sm} = plate height coefficients; d_p = diameter of packing particle; d_f = thickness of stationary phase layer; d_p = particle diameter; D_m = sample diffusion coefficient in the mobile phase; D_s = sample diffusion coefficient in the stationary phase; u = mobile phase velocity; A, B, C, D = constants for a given column (Snyder *et al.*, 1979).

When add these various contributions to H, the equation is:

$$H = Au^{0.33} + \frac{B}{u} + Cu + Du \qquad (6)$$

Term A is the combining of Eddy diffusion and moving mobile phase mass transfer. Eddy diffusion is a function of the size and distribution of the inter particle channels and other nonuniformities in the packed bed. For moving mobile phase mass transfer, molecules in the same flow path will not all move with the same speed due to those molecules close to the particle walls will not move more slowly than those in mid stream, a flow profile will develop across the channel and band broadening will increase.

Term B, longitudinal molecular diffusion, is inversely proportional to the linear velocity; it describes the molecular diffusion in the axial direction.

The C term is stagnant mobile phase mass transfer, when porous stationary phased are used, the intra particle void volume is filled with mobile phase. Analyte molecules must diffuse through this stagnant mobile phase in order to reach the stationary phase. Molecules that diffuse only a short distance into he pore will rapidly back to the mainstream, whereas molecules that diffuse further and spend more time in the pore will be left behind the mainstream, resulting in broadening of the chromatographic band (Hamilton and Swell, 1982). C term can eliminate by avoiding porous particles that is achieved by the use of surface-coated or *pellicular* packings (Snyder *et al.*, 1979).

The last term, D, was stationary phase mass transfer, this term depend a mainly on diffusion for liquid stationary phases and on adsorption-desorption kinetics for solid stationary phases. Hence, to reduce band broadening from this term, liquid layers should be as thin as possible and solid surfaces should be of a homogeneous nature (Hamilton and Swell, 1982).

In liquid chromatography because the solute diffusion coefficient in the mobile phase, D_m, is small and B/u term may often be ignored, *i.e.*, diffusion rate of liquid phase in liquid chromatography was lower than gas phase in gas chromatography. In a well-packed column of porous particles, where stationary phase thickness d_f is small and $D \approx 0$. Then equation (6) reduces to

$$H \approx Au^{0.33} + Cu \qquad (7)$$

To minimize H, it is necessary to use a column packed with small diameter particles, a slow-moving solvent of low viscosity and high separation temperatures, to increase diffusion rates. The comparison of mobile phase on plate height for liquid chromatography and gas chromatography is shown in Figure 24.
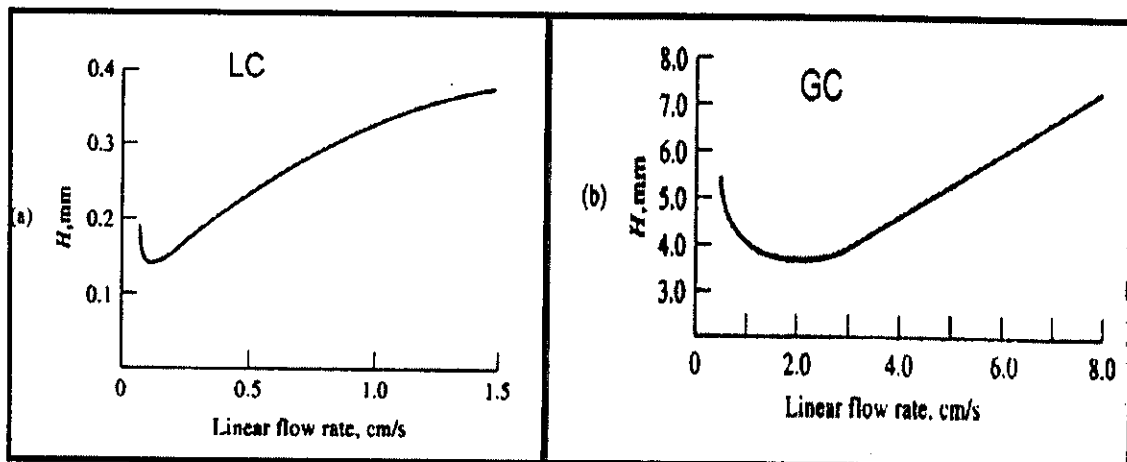

Figure 24 Effect of mobile-phase flow rate on plate height for (a) liquid chromatography and (b) gas chromatography (Hamilton and Swell, 1982).

In this research, a high purity silica base C_{18} (Alltima HP C_{18}), 150×4.6 mm I.D. with particle size 3 μm was used in the analysis and the experimental results are shown in Table 10. The van Deemter plot showed a decrease in efficiency with increasing linear velocity (Figure 25). The low *HETP*s, *i.e.*, high values of plate count at 0.2 mL min^{-1} indicate that the column retained salbutamol and bamethan (IS) for a long period of time. However, the flow rate at 0.4 mL min^{-1} was selected because it showed good separation, using less analysis time with relatively high value of plate count.

Table 10 Plate counts (N) and plate height ($HETP$) of salbutamol and bamethan (IS) at various mobile phase flow rate.

Flow rate (mL min^{-1})	t_o (sec)	Linear velocity[a] (cm sec^{-1})	Plate counts, N		$HETP \times 10^{-3}$(cm)±SD*	
			Salbutamol	Bamethan (IS)	Salbutamol	Bamethan (IS)
0.2	603.180	0.025	11485	9848	1.306±0.016	1.523±0.017
0.3	386.880	0.039	9524	8298	1.575±0.009	1.808±0.026
0.4	289.140	0.052	8640	7331	1.736±0.007	2.046±0.028
0.5	233.040	0.064	7539	6486	1.990±0.044	2.313±0.046
0.6	196.860	0.076	6382	5522	2.351±0.031	2.717±0.034

* 5 replications, RSD < 4%

[a]Linear velocity was calculated from L/t_o.

L was referred to the length of column (15 cm).

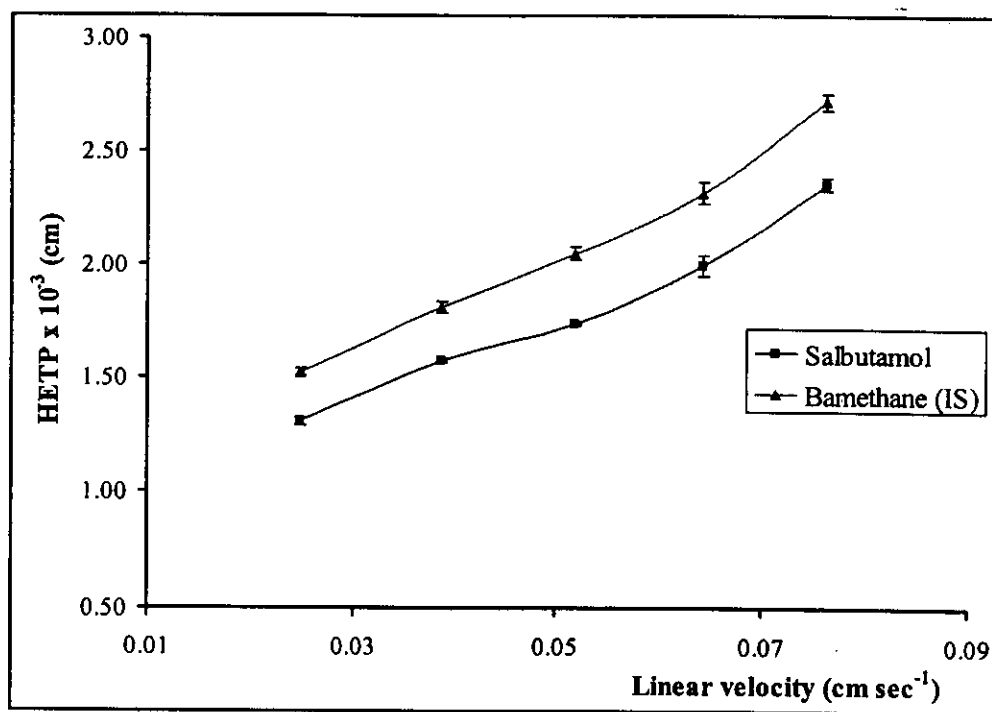t_o was referred to the retention time of an unretained components.

Figure 25 van Deemter plot of salbuamol and bamethan (IS).

All optimized parameters are summarized in Table 11 and Figure 26 shows the chromatogram of mixed standard salbutamol and bamethan (IS) at 20 ng mL^{-1}.

Table 11 Optimum conditions of chromatographic conditions.

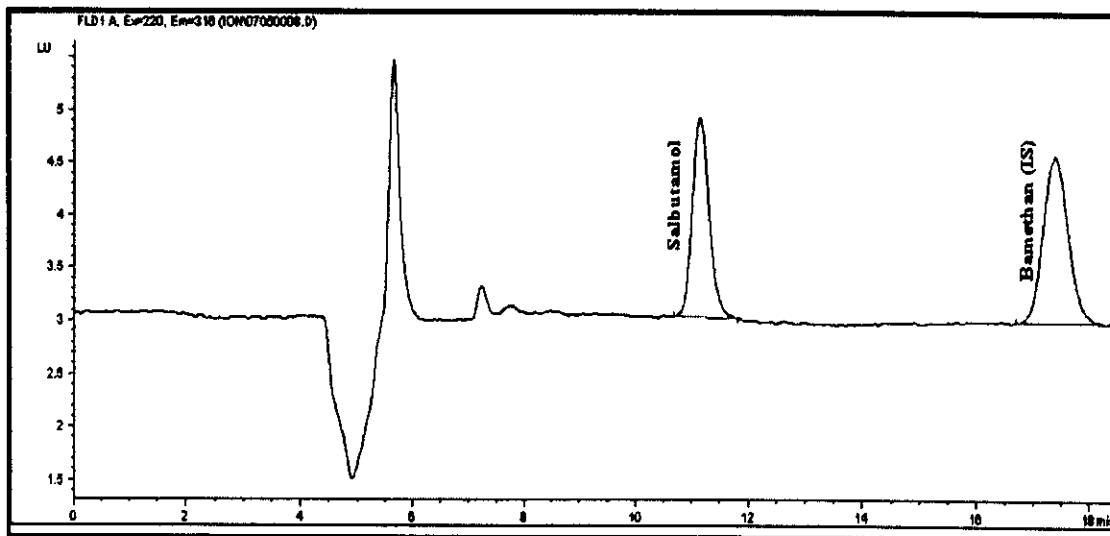| Parameters	Studied values	Optimum values
Excitation/Emission wavelengths (nm)	Scanning for optimum wavelengths	220/316
Percentage of acetic acid (%)	0.5, 1.0, 1.5, 2.0	1.5
Concentration of hexanesulfonate (mM)	0.5, 1.0, 2.0, 3.0, 4.0, 5.0	3.0
Percentage of methanol (%)	28, 30, 33, 35, 40, 45	30
Temperature (°C)	27.5, 35.0, 40.0, 45.0	35.0
Flow rate (mL min^{-1})	0.2, 0.3, 0.4, 0.5, 0.6	0.4

Figure 26 Chromatogram of mixed standard salbutamol and bamethan (IS) at 20 ng mL^{-1} under optimum IPC-FLD conditions.

3.3 System performance of IPC-FLD

3.3.1 Repeatability

Prior to the analysis of samples, the HPLC system must be tested to ensure that it can generate results of acceptable accuracy and precision. The %RSD of retention time and peak area of five repetition injections of standard solution is normally accepted as one of the standard criteria (Snyder *et al.*, 1997). In this work, mixed solution of 20 ng mL^{-1} salbutamol standard and bamethan (IS) was injected to the IPC-FLD system under optimum conditions. The results are shown in Table12. The %RSD of retention time (0.23 and 0.46) and peak area (2.6 and 1.7) were acceptable, *i.e.*, 1% for retention time and 4% for peak area (Snyder *et al.*, 1997).

Table 12 %RSD of retention time and peak area of five repetition injections of mixed solution 20 ng mL^{-1} of salbutamol standard and bamethan (IS).

No.	Retention time (min)		Peak area (LU*s)	
	Salbutamol	Bamethan (IS)	Salbutamol	Bamethan (IS)
1	10.98	17.08	37.90	56.22
2	10.94	17.17	40.12	58.25
3	10.96	17.29	39.29	56.98
4	10.96	17.17	38.88	56.58
5	10.91	17.22	40.51	58.35
Average	10.95	17.19	39.34	57.28
SD	0.025	0.079	1.0	0.97
%RSD	0.23	0.46	2.5	1.7

3.3.2 Limit of detection (LOD)

The limit of detection (LOD) of salbutamol (S/N ≥3) was found to be 0.5 ng mL^{-1}. This LOD is better than that obtained by Kaewklapanyachareon (2001), 3 ng mL^{-1}, Using reverse phase HPLC-FLD with TSK gel-ODS 80Ts (150×4.6 mm) and 4 % acetonitrile containing 0.3% acetic acid as mobile phase.

3.3.3 Linear range

The linear range of IPC-FLD system is the range for which the analytical signal is directly proportional to the amount of analyte present (as Figure 11). In practice, the linear range was demonstrated experimentally by running a series of concentrations as shown in Table 13 and Figure 27. The system showed a wide linear dynamic range, *i.e.*, 0.5 ng mL^{-1} to 12 μg mL^{-1}(the concentration of salbutamol over 12 μg mL^{-1} show bad peak shape), with a good coefficient of determination (R^2) > 0.99.

Table 13 Response at various concentrations of salbutamol.

Salbutamol concentration (ng mL^{-1})	Response (LU*s)* ± SD
0.5**	1.06±0.17
1	2.03±0.05
3	5.89±0.40
5	9.33±0.29
10	19.40±0.76
20	39.34±1.04
50	99.80±3.86
100	225.6± 2.2
500	1047±11
1000	2043.8±8.8
5000	11153±23
10000	21099±56
12000	25150±31
15000	31023±70

* 5 replications, RSD < 4%;
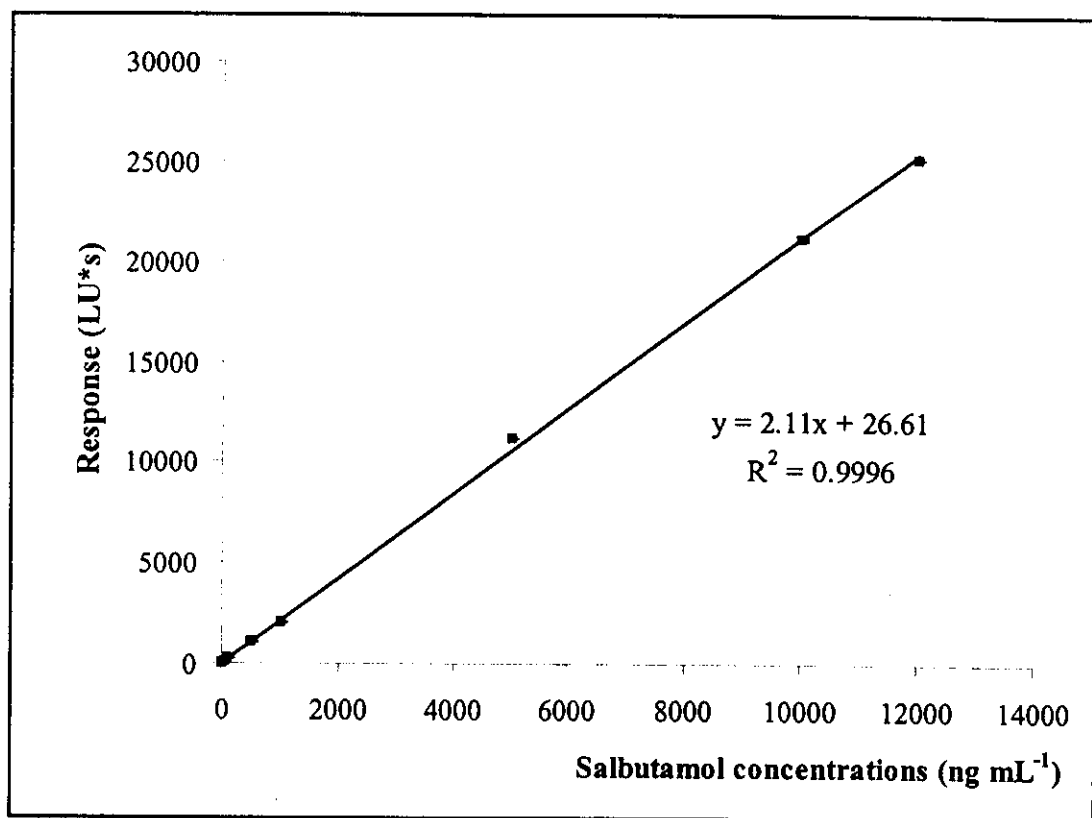
**5 replications, RSD < 15%

Figure 27 Linear range of salbutamol by IPC-FLD system.

3.3.4 Internal standard curve

Bamethan was use as an internal standard (IS) since it met the requirement of IS, *i.e.,* well resolved in the separation. The internal standard is designed to overcome this major source of inaccuracy and also to improve precision. One of the main reasons for using internal standard is for sample requiring significant pretreatment or preparation. Often, sample preparation procedures for muscle sample include many steps, *i.e.*, extraction, centrifugation and clean-up, resulting in sample losses. When add prior to sample preparation, a properly chosen internal standard can be used to correct for these sample losses. The internal standard should be chosen to mimic the behavior of the sample compound in these pretreatment steps (Snyder *et al.*, 1997).

With the internal standard method, a calibration plot was obtained by preparing and analyzing calibration solutions containing various concentrations of salbutamol with a fixed concentration of bamethan (IS), 20 ng mL^{-1}. The peak area

ratio of salbutamol to bamethan (IS) is determined for each calibration solution prepared (Table14), and this ratio was plotted *versus* the salbutamol concentration in Figure 28.

The concentration can be calculated by determining the response factor (*RF*) for the internal standard plot if the latter is linear with the intercept

$$RF = \frac{A_{SB} / A_{BT}}{M} \qquad (8)$$

Where A_{SB} / A_{BT} is the area ratio of the salbutamol-bamethan (IS) in the calibration standard solution and M is the salbutamol concentration in the calibration standard solutions. In this case, RF is the slope of the line (Snyder *et al.*, 1997) in Figure 27, in this case is 0.30 mL ng^{-1}. The concentration of salbutamol in sample (C_s) can be obtained by:

$$C_s = \frac{A_{SB} / A_{BT}}{RF} \qquad (9)$$

Table 14 Relationship between the concentration of salbutamol and the ratio of peak area of salbutamol to bamethan (IS).

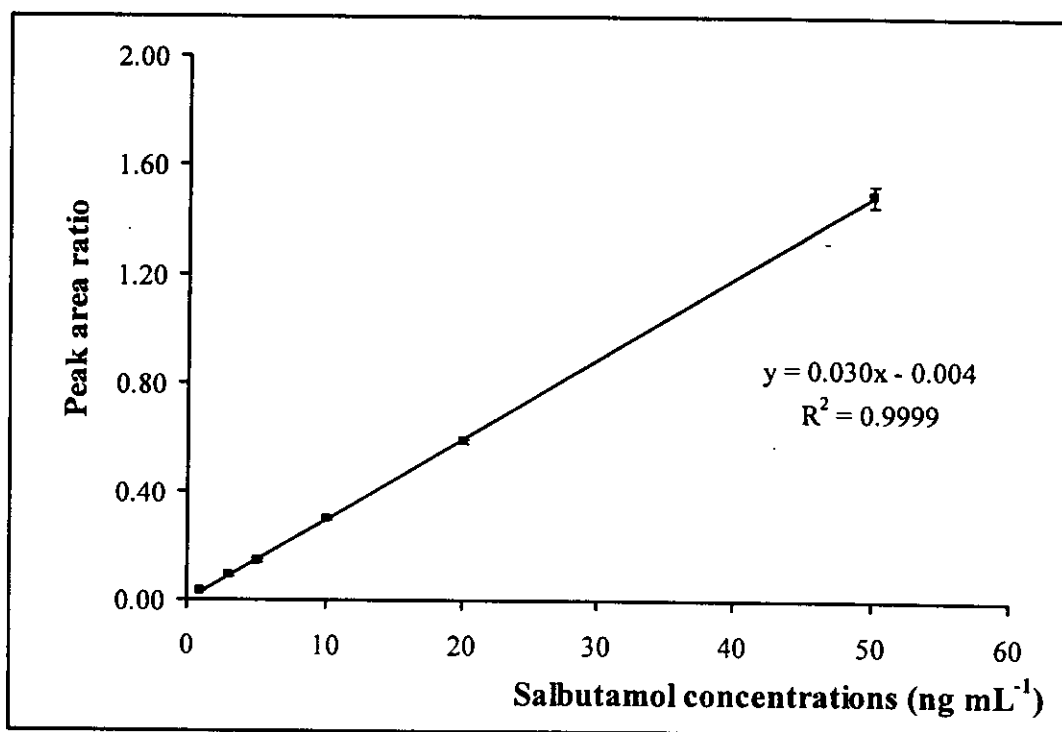| Salbutamol concentration (ng mL^{-1})	Peak area ratio* ±SD
1	0.031 ± 0.001
3	0.088±0.006
5	0.141±0.004
10	0.298±0.007
20	0.585±0.008
50	1.49±0.04

* 5 replications, RSD < 4%

Figure 28 Internal standard curve: salbutamol/bamethan (IS) peak area ratio *versus* salbutamol concentrations.

3.4 Sample preparation

Salbutamol in lean meat samples were extracted and purified by combining matrix solid phase dispersion (MSPD) and subsequent solid phase extraction (SPE). The parameters affecting extraction efficiency of salbutamol were optimized. The MSPD process incorporates the classical methods of the use of abrasive to disrupt sample architecture and cellular membranes and components, with the solvent being bound to the abrasive solid support. In this manner the tissue can be completely disrupted and distributed over the surface of solid support so as to maximize the interactions of the solid support and bounded liquid phase chemistry and interactions with the individual cellular components and, thus, their subsequent interactions with one another (Crouch and Barker, 1997).

3.4.1 Matrix solid phase dispersion (MSPD)

MSPD involves homogenization of a small amount of sample tissue with the sorbent in a glass pestle and mortar. The mechanical shearing forces produced by the grinding process disrupt the structure of the tissue, dispersing the sample over the surface area and, simultaneously, lipid materials and internal membrane lipids associate with the sorbent material by hydrophilic and hydrophobic interactions. The process caused the mixture to become semi-dry and a homogeneous blend of sample and sorbent is the result (Boyd et al., 1995). The selectivity of an MSPD procedure depends on the sorbent/solvent combination used.

3.4.1.1 Type of sorbent

Salbutamol molecule contains polar and non-polar groups as well as an ionizable amine function in its chemical structure which is able to interact with different solid phase (Bergés, 1999). Optimization of sorbent type involved, polar, *i.e.*, aluminum oxide acid, aluminum oxide neutral, Florisil and Silica gel 60, and non-polar, *i.e.*, C_{18} (Supelclean ENVI-18, Supelclean LC-18) sorbent. Both C_{18} sorbents show higher response than polar sorbent (Table 15, Figure 29), because they can interact with salbutamol *via* bonded phase (hydrophobic) and solid support that is

silica base (hydrophilic) (Barker[a], 2000). In addition, Supelclean ENVI-18 show higher response than Supelclean LC-18 due to the different in physical properties of the sorbent that affect to extraction efficiency , *i.e.*, particle size, surface area, pore size, and %carbon load. In this study, Supelclean ENVI-18 was chosen as sorbent in MSPD procedure. This sorbent, C_{18}, was usually used to extract drug residues include β-agonist compound (Barker[a], 2000).

Table 15 Response of salbutamol at each type of sorbent.

Type of sorbent	Response* (LU*s)±SD
Supelclean ENVI-18	86±12
Supelclean LC-18	82±12
Aluminum oxide acid	71.9±7.9
Aluminum oxide neutral	61.5±9.0
Florisil	71±11
Silica gel 60	65.2±9.1

* 5 replications, RSD < 15 %

silica base (hydrophilic) (Barker[a], 2000). In addition, Supelclean ENVI-18 show higher response than Supelclean LC-18 due to the different in physical properties of the sorbent that affect to extraction efficiency , *i.e.*, particle size, surface area, pore size, and %carbon load. In this study, Supelclean ENVI-18 was chosen as sorbent in MSPD procedure. This sorbent, C_{18}, was usually used to extract drug residues include β-agonist compound (Barker[a], 2000).

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Florisil	71±11
Silica gel 60	65.2±9.1

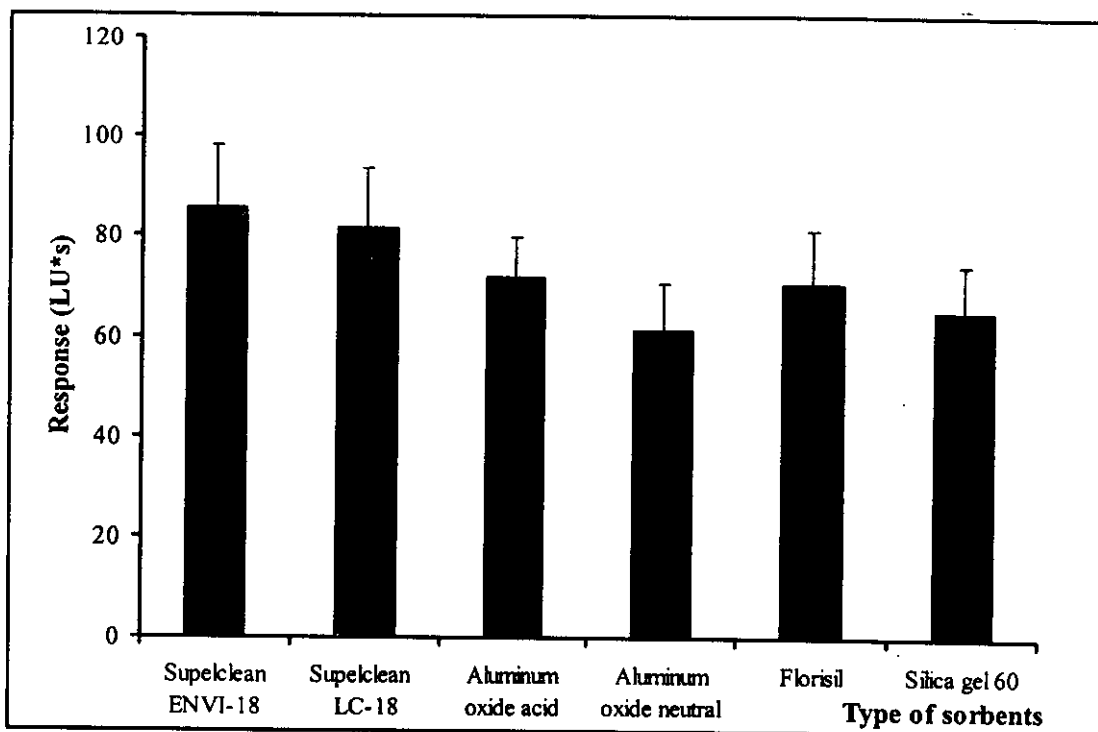* 5 replications, RSD < 15 %

Figure 29 Response of salbutamol at each type of sorbent.

3.4.1.2 Type of washing solvent

The purposed of MSPD cartridge washing is to selectively remove undesired compounds from the sorbent without eluting the salbutamol. The solvent was chosen so that it does not displace the component of interest, but weakly bound components are effectively displaced. In addition, the washing step removes unwanted material from the pores and interstices of the packed bed (Simpson, 2000). MSPD column was washed with various type of washing solvent (experiment 2.7.4.2) and the results are shown in Table 16 and Figure 30. Washing MSPD column with hexane and followed with water gave low response due to hydrophilic nature of salbutamol. It was considered that the water wash is partially removing salbutamol from the sorbent in washing step. The highest response was obtained when MSPD column was washed with the mixture of hexane and diethyl ether (60:40), therefore this washing solvent was chosen. This washing solvent was also used in the work of Boyd and coworker (1995).The relative standard deviation of five replications, <20%, is acceptable (Commission Decision 2002/657/EC, 2002).

Table 16 Response of salbutamol at various type of washing solvent.

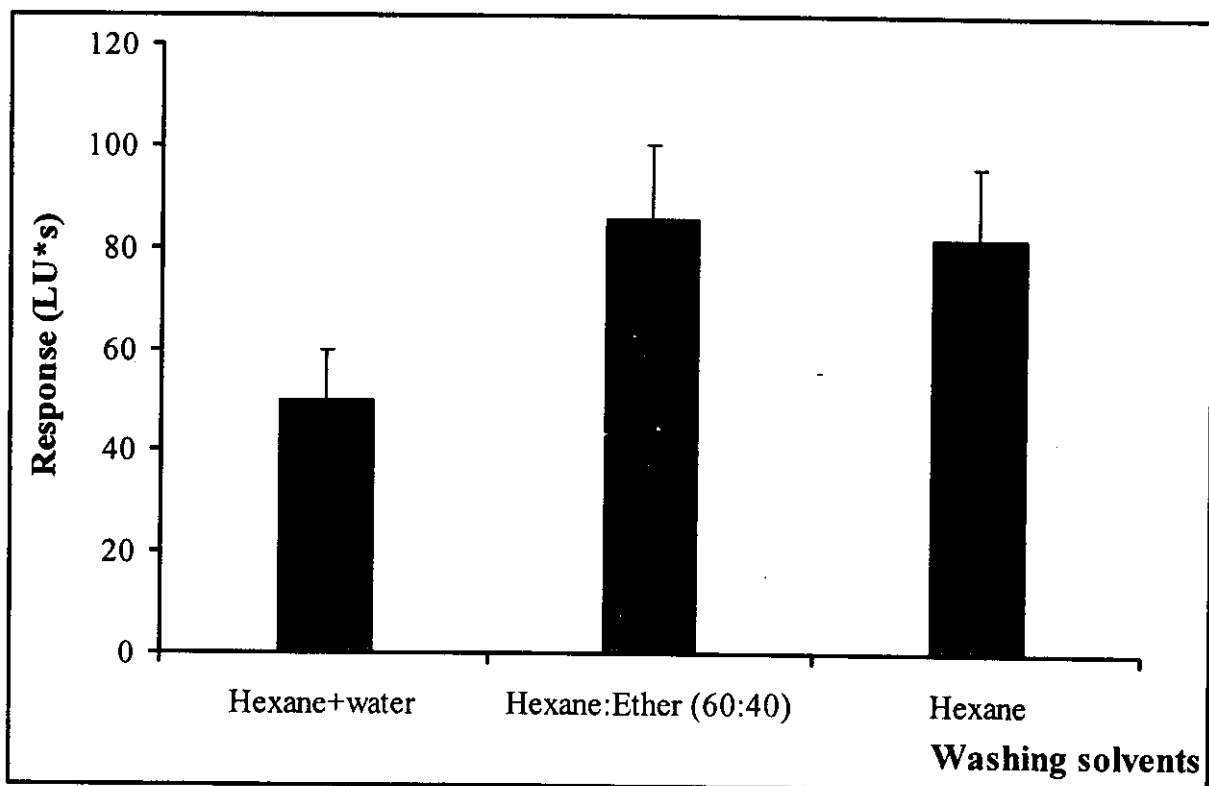| Type of washing solvent	Response* (LU*s)±SD
Hexane followed with water	50±10
Mixture of hexane and ether (60:40)	86±14
Hexane	82±14

* 5 replications, RSD < 20 %

Figure 30 Response of salbutamol at various type of washing solvent.

3.4.1.3 Volume of washing solvent

The mixture of hexane:diethyl ether (60:40) has the washing power on polar compound more than hexane, *i.e.*, hexane and diethyl ether have the elutropic value (E_0) at 0 and 0.38 (Simpson, 2000), respectively. Excessive volume of this washing solvent can elute some salbutamol from the MSPD column as shown in Table 17 and Figure 31. Hexane:diethyl ether (60:40) 8 mL provided the highest response, therefore, 8mL was chosen as the optimum washing volume of MSPD column.

Table 17 Response of salbutamol at each washing solvent volume.

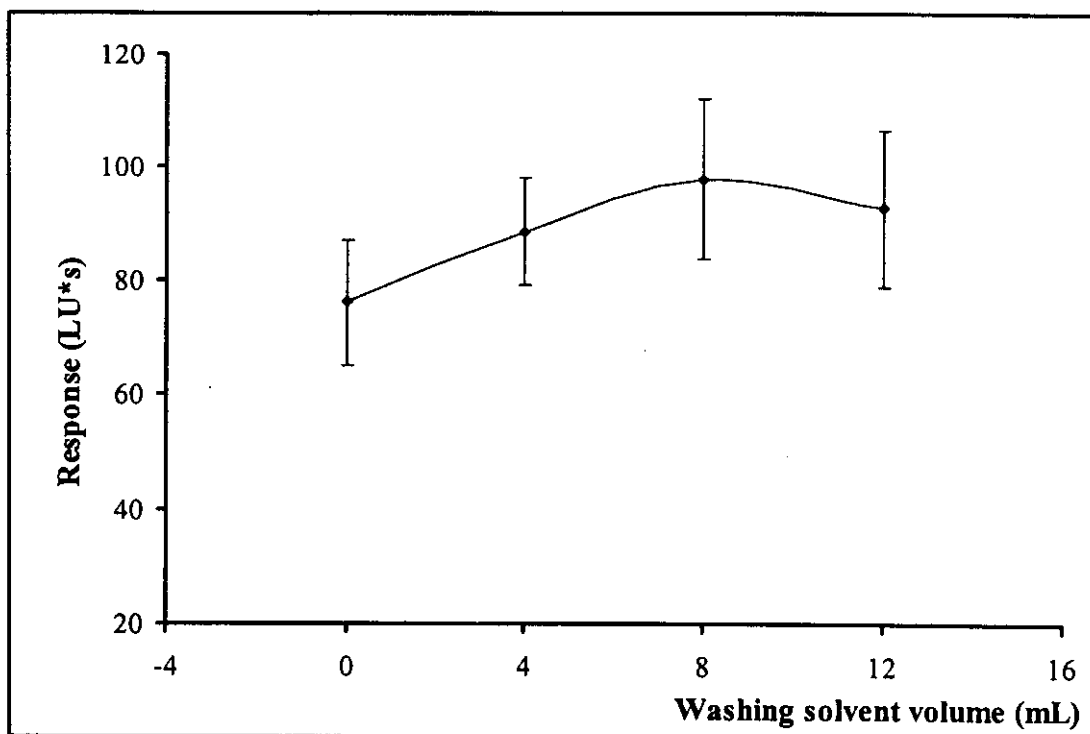| Volume of hexane:diethyl ether (60:40) (mL)	Response* (LU*s)±SD
0	76±11
4	88.6±9.5
8	98±14
12	93±14

*5 replications, %RSD < 15%

Figure 31 Response of salbutamol at each volume of hexane:diethyl ether
(60:40).

3.4.1.4 Type of eluting solvent

The relative polarity of eluting solvent to that of the packing material played a significant role in determining what remains on the column and what is eluted (Barker[b], 2000). The eluting solvent should be one that the analyte is soluble, and often overcome primary and secondary retention mechanism. Other factors to consider include volatility of the solvent if further concentration required. Table 18 and Figure 32 show that methanol gave the highest response when used to eluted salbutamol, therefore methanol was then selected as eluting solvent of MSPD column. The relative standard deviation (%RSD) of five replications, <20%, is acceptable (Commission Decision 2002/657/EC, 2002).

Table 18 Response of salbutamol at each type of eluting solvent.

Type of eluting solvent	Response* (LU*s)±SD
Ethanol	31.5±6.1
Methanol	89±12
Acetonitrile	48.3±3.5
90 % methanol	78±14

*5 replications, %RSD < 20%
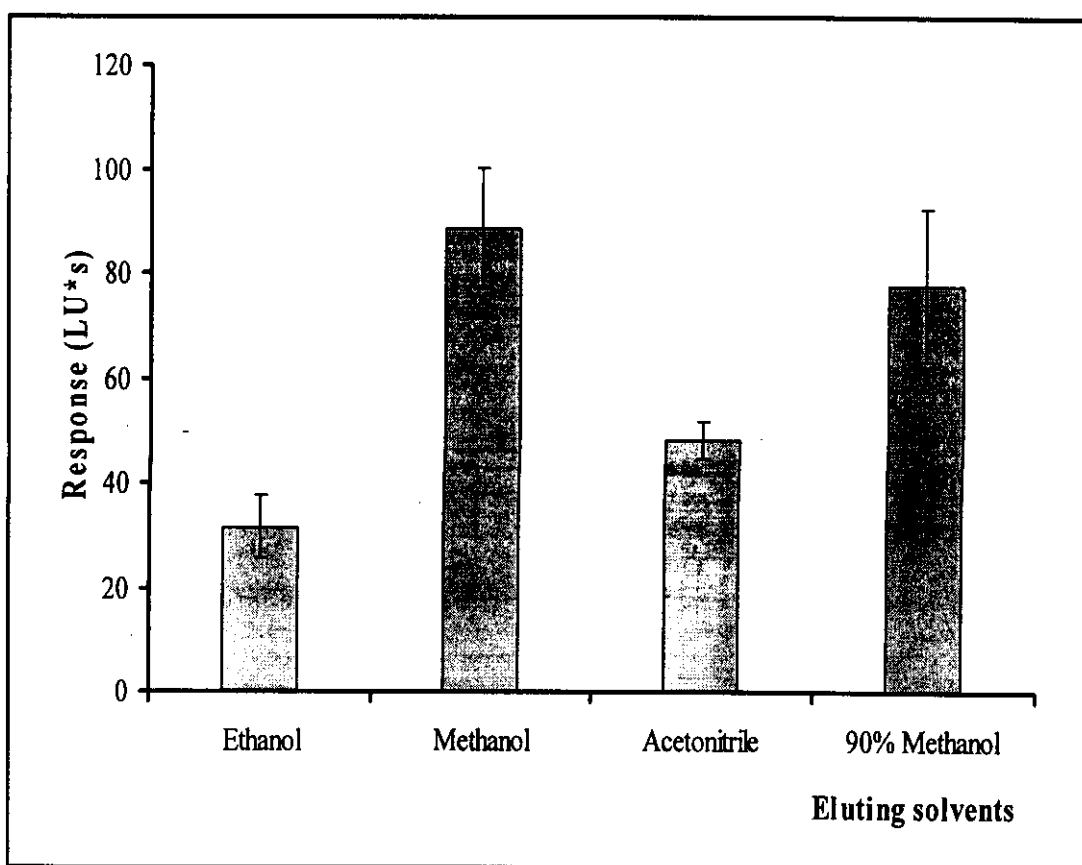
Table 32 Response of salbutamol at each type of eluting solvent.

3.4.1.5 Eluting solvent flow rate

The rate at which solvents flow through the column is also significant in the effectiveness of a separation. Since the time that the analyte to be separated remains on the column is directly proportional to the extent of equilibration between stationary and moving phase. Thus, the eluent flow rate should not be too rapid. Table 19 and Figure 33 show the responses at various flow rates. The low flow rate, 0.15 and 0.3 mL min^{-1} showed higher response than other flow rate. In this study 0.3 mL min^{-1} was chosen as optimum eluting solvent flow rate because it used shorter analysis time.

Table 19 Response of salbutamol at each various eluent flow rate.

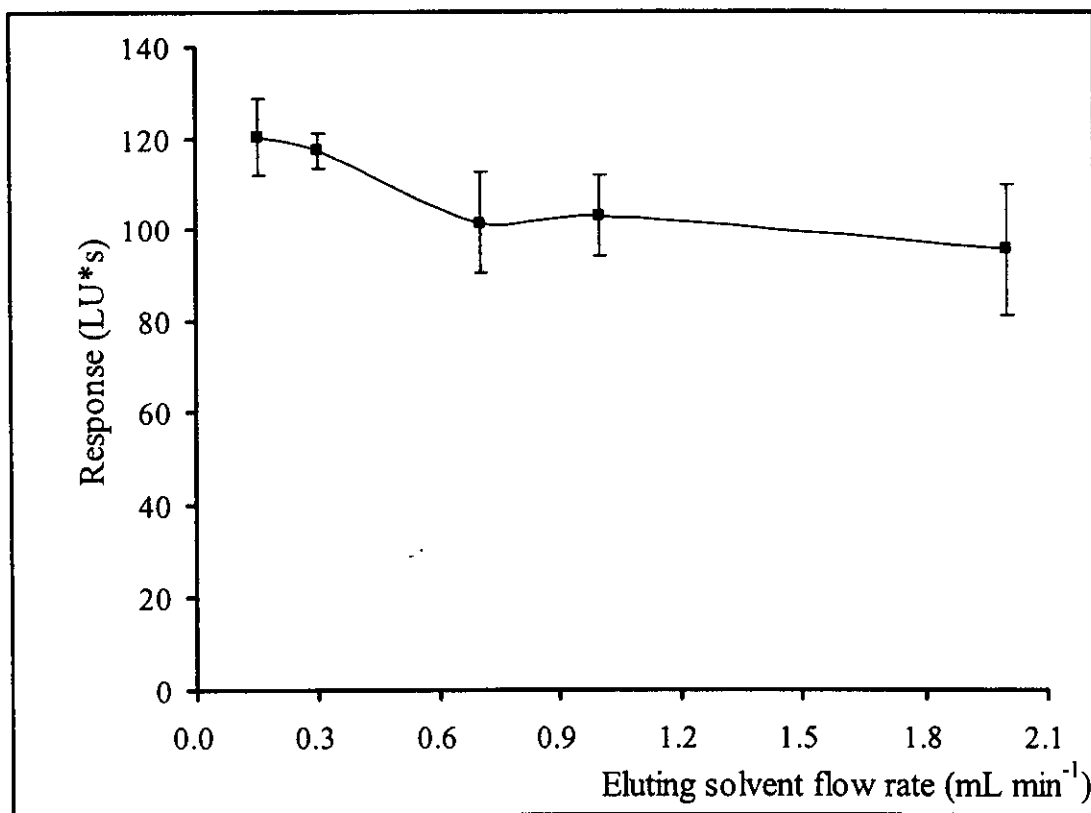| Flow rate (mL min^{-1})	Response* (LU*s)±SD
0.15	120.3±8.4
0.3	117.2±4.0
0.7	101±11
1.0	102.8±8.8
2.0	95±14

* 5 replications, %RSD < 15 %

Figure 33 Response of salbutamol at various eluent flow rate.

3.4.1.6 Volume of eluting solvent

The volume of solvent used in extraction processes was also an important parameter because it would affect the extraction efficiency. If the volume is too small it may not be sufficient to elute all the analyte on the C_{18} sorbent.. Experiment 2.7.4.6 varied the volume of methanol and the results are in Table 20 and Figure 34. The results showed 8 mL and 12 mL of eluting solvent (methanol) gave the same response, thus, to minimize the amount of methanol and analysis time, 8 mL was chosen to elute salbutamol from C_{18} sorbent. This volume was less than the report of Boyd *et al.*(1995), 12 mL.

Table 20 Response of salbutamol at various volume of eluting solvent.

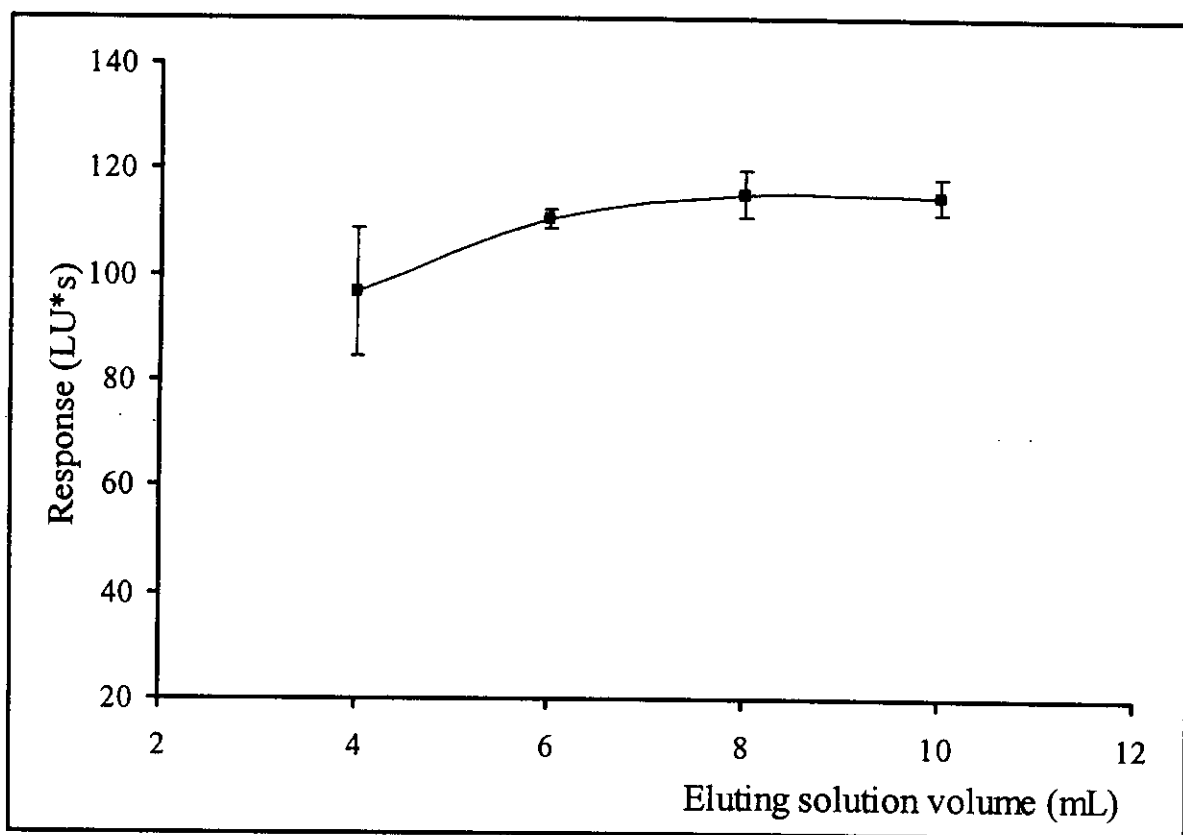| Volume of eluting solvent (mL)	Response * (LU*s)±SD
4	97±12
6	110.6±1.8
8	115.3±4.4
10	114.9±3.4

* 5 replications, RSD < 15 %

Table 34 Response of salbutamol at various volume of eluting solvent.

3.4.2 Solid phase extraction (SPE), clean up

Due to the very complex nature of the lean meat sample matrix, it is necessary to perform a separate clean-up of the eluate. For this study, Strata X SPE cartridge was used in the sample clean-up step. Strata X is the patent polymeric sample preparation sorbent for reverse phase extraction of acidic, basic and neutral compounds (Phenomenex, 2005).

3.4.2.1 Flow rate of sample solution

Separation efficiency varies with flow rate and sample load. If the sample solution flow rate is too high, components may not be interacting sufficiently with the sorbent. The result is loss of resolution, analyte breakthrough, and poor recovery. The optimum flow rate of sample solution was studied in experiment 2.7.5.1. Table 21 and Figure 35 show the response at different sample solution flow rates. As the resulting peak areas were not much difference, a negative impact of the high velocity on the extraction efficiency may be excluding. Therefore, high flow rate of sample solution, 4 mL min^{-1} was chosen, due to its use short analysis time.

Table 21 Response of salbutamol at various flow rate of sample solution.

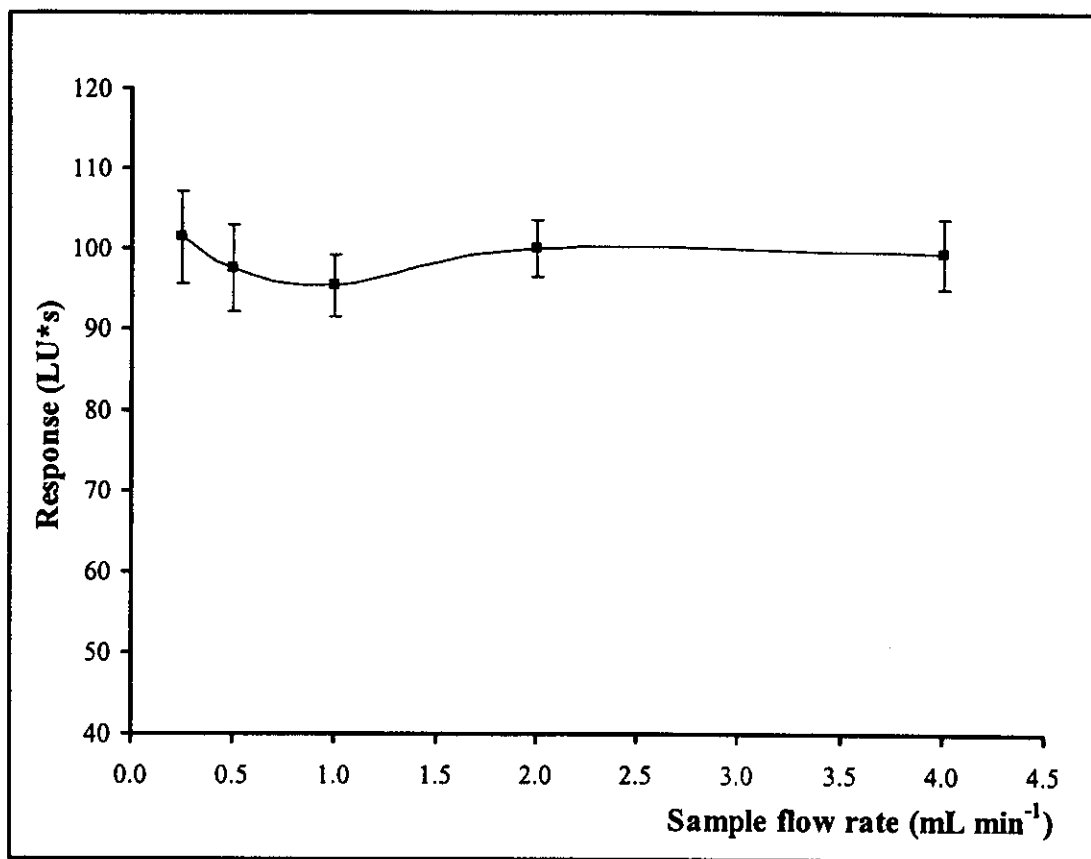| Flow rate (ml min^{-1})	Response* (LU*s)±SD
0.25	101.5±5.7
0.5	97.5±5.3
0.7	95.4±3.8
1.0	100.1±5.7
2.0	99.4±3.5
4.0	101.5±4.4

* 5 replications, RSD < 15 %

Figure 35 Response of salbutamol at various flow rate of sample solution.
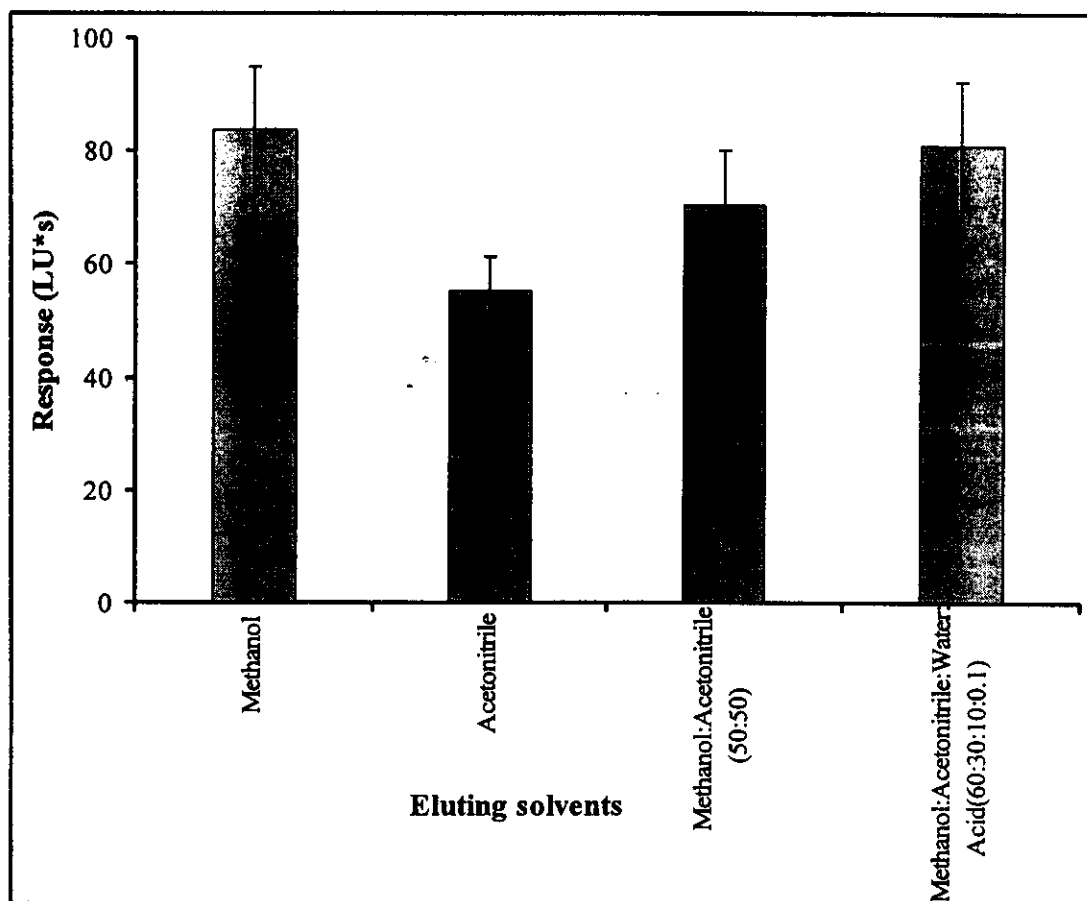
3.4.2.2 Type of eluting solvent

A strong solvent displaces salbutamol (components of interest) in the washing step. Solvent molecule takes the place of adsorbed analytes (salbutamol) on the surface. The nature and volume of elution solvent must be enough to replace and no proportion of the component of interest remains on the surface or in the pore or interstitial volumes (Simpson, 2000). Various type of eluting solvent was used to elute salbutamol from SPE cartridge (experiment 2.7.5.2). The results (Table 22 and Figure 36) showed that methanol and the mixture of methanol: acetonitrile: water with 0.1% acetic acid (60:30:10) provided the highest response. To avoid the preparation a mixed solvents; methanol: acetonitrile: water with 0.1% acetic acid (60:30:10), methanol was selected as eluting solvent.

Table 22 Response of salbutamol at various type of eluting solvent.

Type of eluting solvent	Response* (LU*s)±SD
Methanol	84±11
Acetonitrile	55.3±5.8
Mixture of methanol and acetonitrile (50:50)	70.5±9.6
Mixture of methanol: acetonitrile: water with 0.1% acetic acid (60:30:10)	80±11

* 5 replications, RSD < 15 %

Table 36 Response of salbutamol at various type of eluting solvent.

3.4.2.3 Flow rate of eluting solvent

The effect of eluting solvent flow rates are shown in Table 23 and Figure 37. The resulting peak areas of every flow rate did not show any difference, like the sample solution flow rate, a negative impact of the high velocity on the extraction efficiency may be excluding. Therefore to minimize the analysis time, a high flow rate, 2 mL min^{-1}, of eluting solvent was used.

Table 23 Response of salbutamol at various flow rate of eluting solvent.

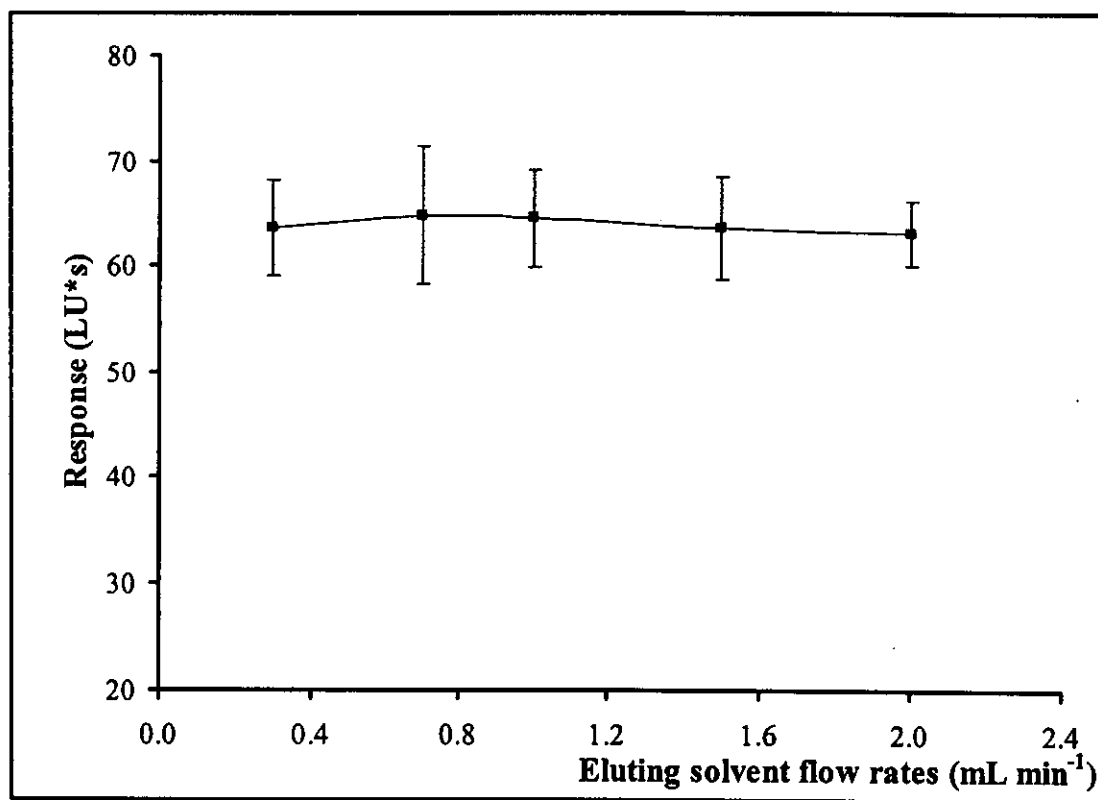| Flow rate (ml min^{-1})	Response* (LU*s)±SD
0.3	63.6±4.5
0.7	64.8±6.6
1.0	64.5±4.6
1.5	63.6±4.9
2.0	63.1±3.1

* 5 replications, RSD < 10 %

Figure 37 Response of salbutamol at various flow rate of eluting solvent.

3.4.2.4 Volume of eluting solvent

The volume of eluting solvent in solid phase extraction (SPE) technique was an important parameter because it would affect the extraction efficiency. If the volume is too small it may not be sufficient to elute all the analyte from the cartridge but if too large it will increase waste. Therefore, it was necessary to optimize the volume of eluting solvent in order to minimize use and at the same time maintained the high response. In experiment 2.7.5.4, fractions of 0.1 mL of the eluting solution were collected. Table 24 and Figure 38 show that after 5 fractions (0.5 mL). There was no salbutamol response. Therefore, to ensure that all salbutamol was eluted, excessive methanol 0.7 mL was chosen as the volume of eluting solvent. This volume was less than the recommend value, 1 mL in "Strata X SPE user's guide".

All optimum conditions in sample preparation procedure by MSPD linked to SPE were summarized in Table 25

Table 24 Response of salbutamol at each collected fractions (0.1 mL per fraction).

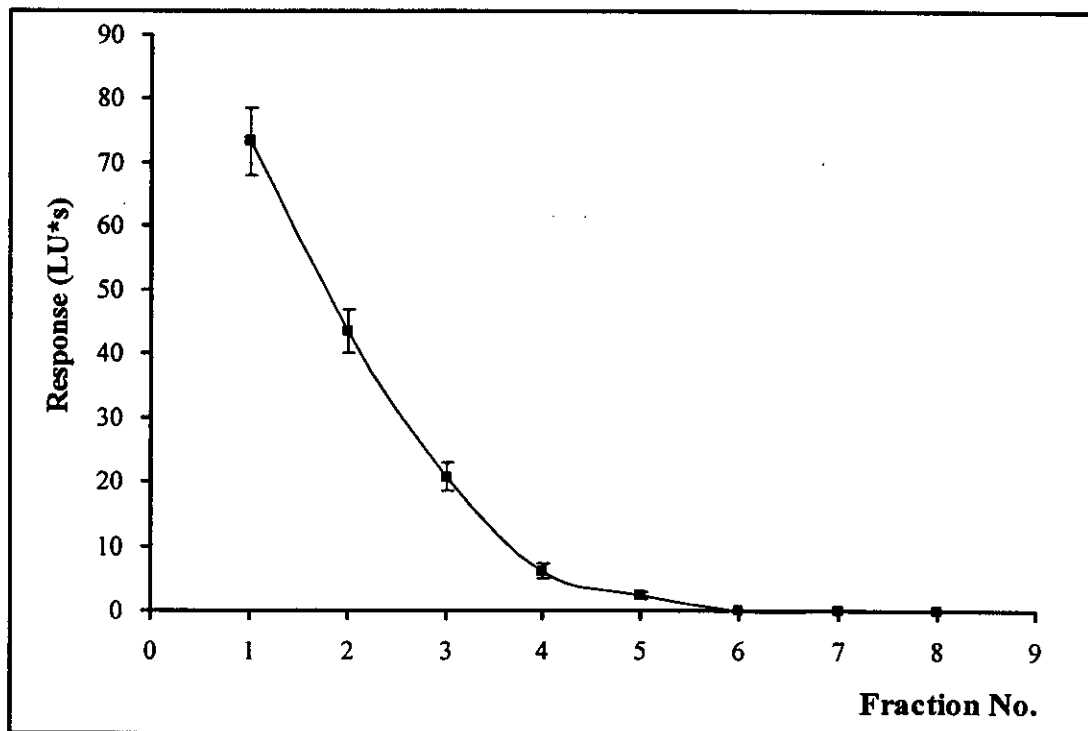| Fraction No.	Response* (LU*s)±SD
1	73.3±5.4
2	43.4±3.3
3	20.7±2.2
4	6.1±1.2
5	2.4±0.5
6	0.0±0.0
7	0.0±0.0
8	0.0±0.0

* 5 replications, RSD < 20 %

Figure 38 Eluting profile of salbutamol from Strata X SPE cartridge.

Table 25 Optimum conditions of sample preparation procedure.

Parameters	Studied values	Optimum values
Matrix solid phase dispersion (MSPD)		
-Type of sorbent	Supelclean ENVI-18, Supelclean LC-18, silica gel 60, aluminum oxide acid, alumnium oxide neutral, florisil	Supelclean ENVI-18
-Type of washing solution	hexane, hexane followed with water, mixture of hexane and diethyl ether (60:40)	mixture of hexane and diethyl ether (60:40)
-Volume of washing solution (mL)	0, 4, 8, 10	8
-Type of eluting solvent	methanol, ethanol, acetonitrile, 90 % methanol	methanol
-Flow rate of eluting solvent (mL min^{-1})	0.15, 0.3, 0.7, 1.0, 2.0	0.3
-Volume of eluting solvent (mL)	4, 6, 8, 12	8
Solid phase extraction (SPE)		
-Flow rate of sample solution (mL min^{-1})	0.25, 0.5, 1.0, 2.0, 4.0	4.0
-Type of eluting solvent	methanol, acetonitrile, mixture of methanol and acetonitrile (50:50), mixture of methanol: acetonitrile: water with 0.1% acetic acid (60:30:10)	methanol
-Flow rate of eluting solvent (mL min^{-1})	0.3, 0.7, 1.0, 1.5, 2.0	2.0
- Volume of eluting solvent (mL)	Elution profile of 1.5 mL	0.7

3.4.3 Matrices interferences

In food analysis, the various compositions in food could interfere with the interest analyte. If the analysts are not aware of these interferences, these interferences can lead to a number of the effect. They may have the effect of apparently enhancing the concentration of the analyte. Interference would usually affect the slope of the calibration curve, so that it will be differently from the slope of the analyte of interest and the slope of the calibration curve in the method of additions may affect the linearity. This effect potentially indicated the possible present of hidden interferences (Eurachem guide, 1998). In this study, porcine and bovine lean meat sample were used for the interferences in samples study. Several amounts of knew standard were added into the samples (2.7.6). The optimum conditions were set for determining the addition sample and standard salbutamol. The results from matrix interference studies in porcine and bovine lean meat samples are shown in Tables 26-27 and Figures 39-40.

In this experiment the use of different concentrations is a controlled factor since the concentration are chosen by experimenter. The curve on which the experiment is performed introduces uncontrolled variation or random factor. The slopes of external standard curve and matrix curve were tested using two-way analysis of variance (two-way ANOVA) by taking the null hypothesis (H_0) that the interaction of both slope is not significant and alternative hypothesis (H_1) that the interaction of slope is significant. If P value is less than α (level of significance) then the null hypothesis was rejected at that significant level.

Table 26 Response of standard salbutamol and fortified porcine lean meat samples at various salbutamol concentrations.

Salbutamol concentrations (ng mL^{-1})	Response (LU*s) ± SD	
	Standard*	Fortified Porcine **
1	2.03± 0.05	1.65± 0.27
3	5.89± 0.24	5.25± 0.62
5	9.33± 0.29	9.1± 1.3
10	19.4± 0.8	13.9± 0.2
20	39.4± 1.0	33.4± 2.8
50	99.8± 3.9	75.3± 3.3

* 5 replications, RSD < 4 %;   ** 5 replications, RSD < 15 %
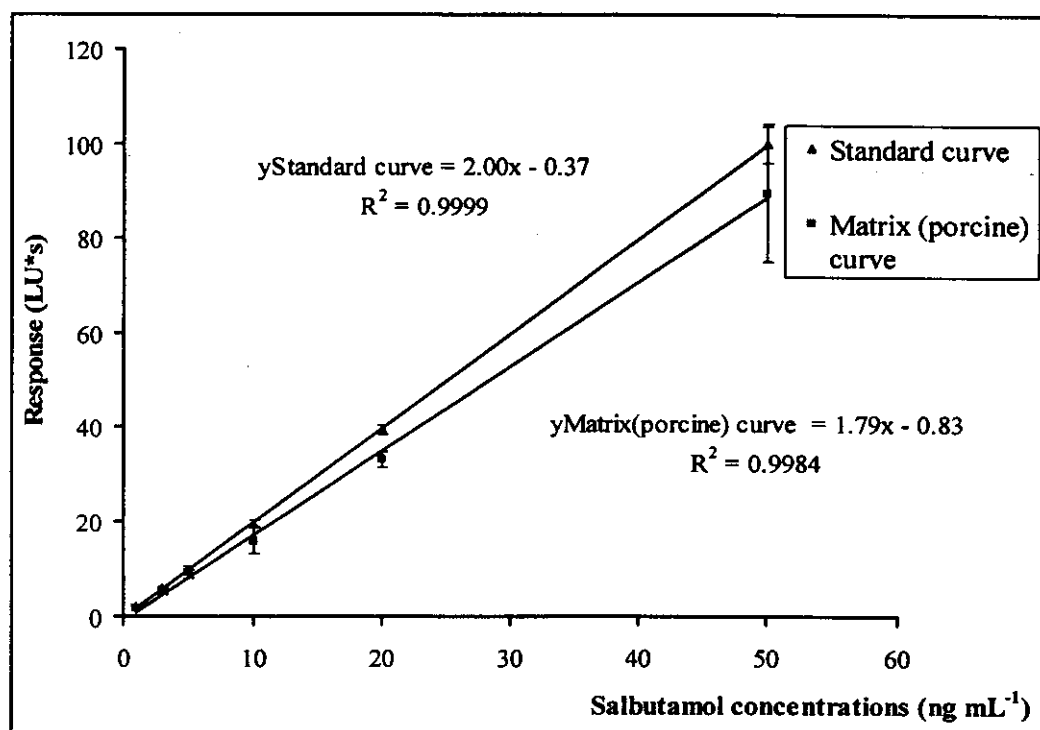
Figure 39 Matrix (porcine) curve compare with standard curve.

Table 27 Response of standard salbutamol and fortified bovine lean meat samples at various salbutamol concentrations.

Salbutamol concentrations (ng mL^{-1})	Response* (LU*s) ± SD	
	Standard*	**Fortified Porcine ** **
1	2.03± 0.05	1.67± 0.11
3	5.6± 0.2	5.45± 0.20
5	9.3± 0.3	9.18± 0.80
10	19.4± 0.8	15.5± 1.9
20	39± 1	35.4± 3.4
50	100± 4	86.5± 2.7

* 5 replications, RSD < 4 %;   ** 5 replications, RSD < 15 %
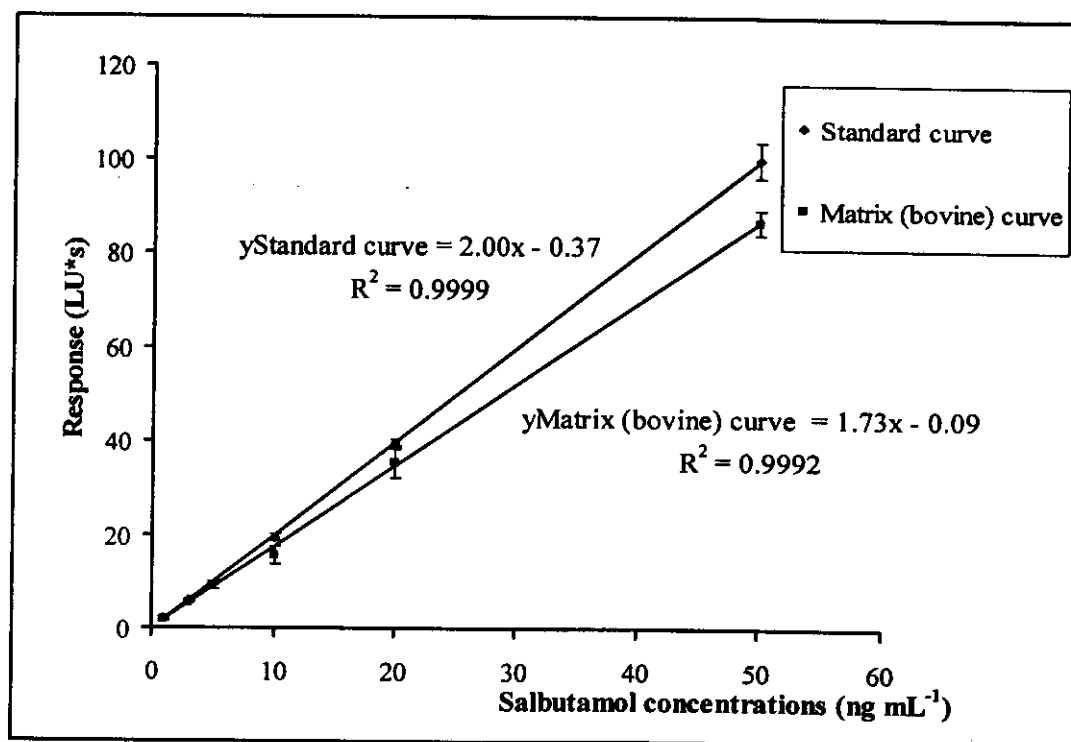
Figure 40 Matrix (bovine) curve compare with standard curve.

Table 28 Result of statistical test using two-way ANOVA by R solfware.

Matrix	*Df*	Sum Sq	Mean Sq	*F*	*P*
Porcine	5	213	43	2.1146	0.079727*
Bovine	5	314	63	15.609	4.173×10^{-3}***

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

where: *Df* is the degree of freedom, it refers to the number of independent deviations

= n-1 (n is the number of concentrations = 6)

Sum Sq is the sum of squares, it refers to an interim quantity used in the calculation of an estimate of the population variance

Mean Sq is mean square is a sum of squared terms divided by the number of degree of freedom

F 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 is probability (Miller and Miller, 2000) .

Table 28 shows the result of two-way ANOVA for slope of regression line to study the interaction between the response and concentration in standard group and matrix group. The slope of regression line in each group was significant difference, *P*<0.1 for standard curve and matrix (porcine) curve, and *P*<0.001 for standard curve and matrix (bovine) curve. Therefore quantitative analysis of salbutamol should be determined from matrix-based calibration curve, *i.e.,* the technique generally use in biological matrix analysis (US-FDA, 2001; Commission Decision 2002/657/EC, 2002).

3.5 Method validation

3.5.1 Selectivity

Selectivity is the ability of analytical method to differentiate and quantify the analyte in the presence of other compounds in the sample (US-FDA, 2001). The selectivity of the method could be demonstrated by IPC-FLD analysis of six blank porcine lean meat samples from difference sources. No interferences of the analyte were observed because of the high selectivity of fluorescence technique. Figure 41 shows an HPLC chromatogram for six blanks porcine muscle samples indicating no interference peak at the retention time of salbutamol and bamethan (IS).
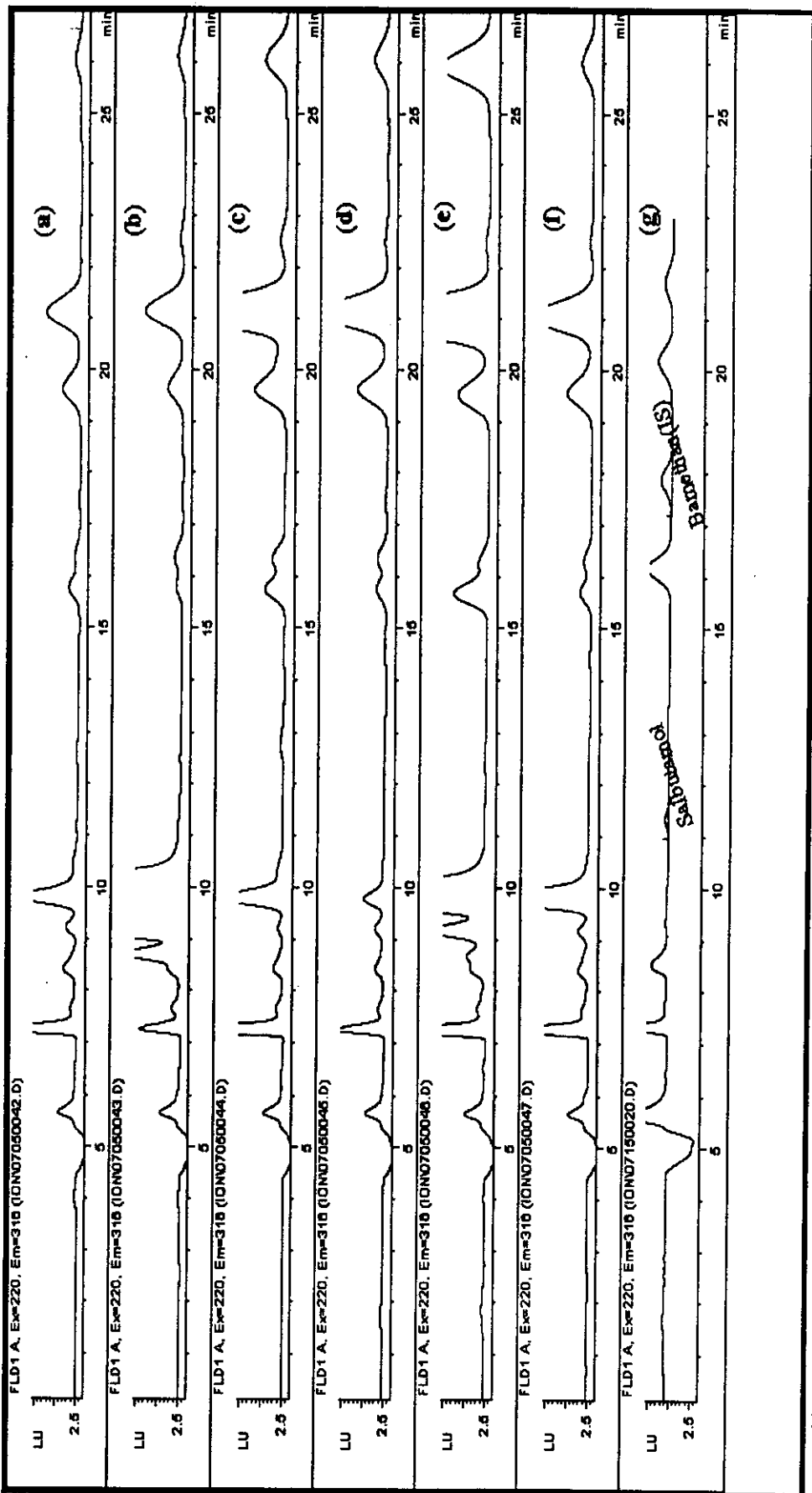
Figure 41 HPLC chromatogram of six blank porcine lean meat (a-f) and fortified porcine sample with salbutamol 5 ng g^{-1} and bamethan (IS) 20 ng g^{-1} (g).

3.5.2 Accuracy, precision and recovery

Accuracy, in terms of mean recovery, and precision, in term of %RSD, were assessed by replicated analyses of samples (fortified at 3 different concentrations within the calibration range). The recoveries and %RSD for the fortified bovine and porcine lean meat samples are indicated in Table 28. The recovery ranged from 82 to 88 %. The RSDs ranged from 4 to 13 %. The accuracy and precision data demonstrated that the method meets US-FDA regulatory method requirement of 80-110 recovery and a RSD at ±15 for the fortified muscle sample (US-FDA, 2001). Table 29 shows the recovery of salbutamol from fortified lean meat (n=5) at different concentrations.

Table 29 The recovery of salbutamol at three concentrations.

Sample Matrix	Concentration (ng g^{-1})					
	3		25		40	
	Recovery±SD	%RSD	Recovery±SD	%RSD	Recovery±SD	%RSD
Porcine	82±10	12	85±8	9	86±7	8
Bovine	88±11	13	85±10	12	82±3	4

3.5.3 Calibration/Standard curve

Calibration is best prepared in the sample matrix (matrix-base calibration curve), to ensure quantitative accuracy. Trace analysis sample are often prepare in the matrix, so that the sample preparation step is an integral part of the calibration procedure (Snyder *et al.*, 1997). Moreover, a proper internal standard must be used to compensate the non-reproducibility in the extraction procedure. In this study, matrix-base calibration curve were established by employing fortified porcine and bovine muscle samples (with salbutamol in the concentration range 1-50 ng g^{-1} and bamethan (IS) 20 ng g^{-1}) under optimum conditions as describe in the proceeding section. As in the case of standards solution, the peak area ratios of

salbutamol and bamethan (IS) from extracted fortified sample were plotted *versus* the concentrations of salbutamol and the detector response was found to be linear for salbutamol in the range of concentration studied as shown in Tables 30-31 and Figures 42-43. Both coefficient of determination (R^2) of the curve were more than 0.99. For the determination of salbutamol in real sample, the signal intensity was converted to concentration. The concentration of the analyte can then be calculated using equation (9).

Figures 44 and 45 show the chromatogram of fortified porcine and bovine at 20 ng g^{-1} in matrix-based calibration curve, comparing with standard and matrix blank chromatograms.

Table 30 Relationship between the fortified concentrations of salbutamol in porcine lean meat and the ratio of peak area of salbutamol to bamethan (IS).

Fortified concentration (ng g^{-1})	Peak area ratio*± SD
1	0.031±0.004
3	0.103±0.009
5	0.185±0.021
10	0.306±0.045
20	0.767±0.041
50	1.42±0.26

* 5 replications, RSD < 15%

$$y = 0.034x - 0.006$$
$$R^2 = 0.9995$$
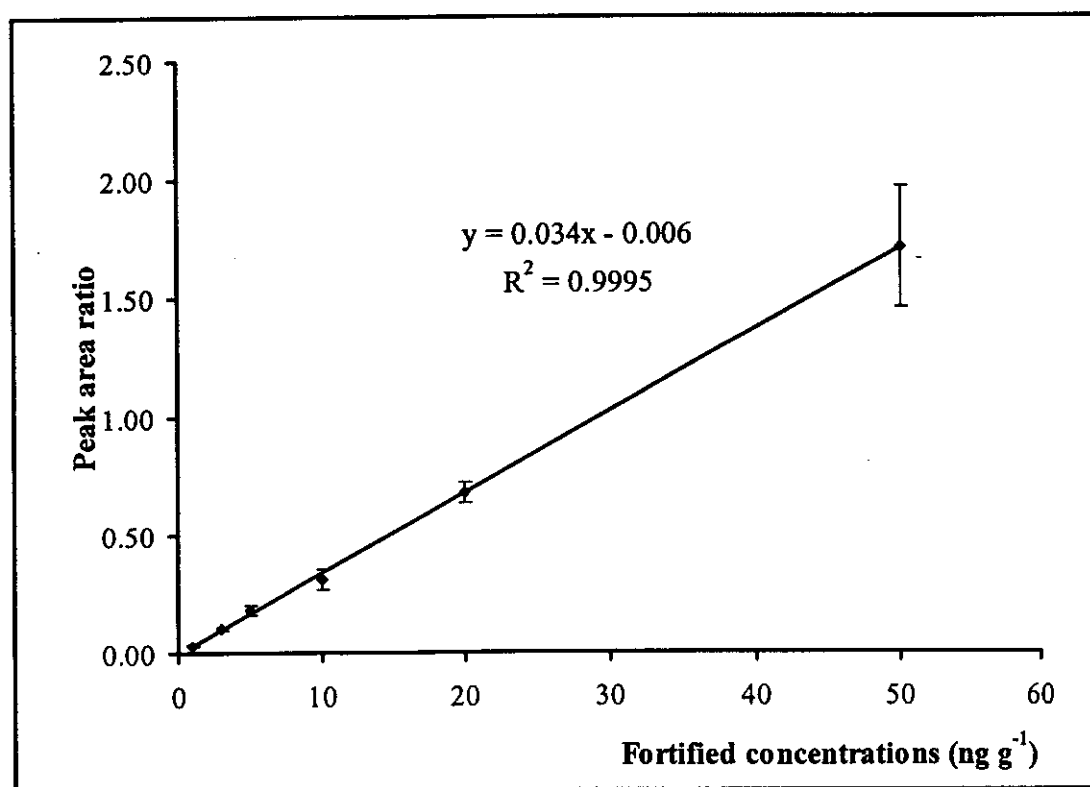
Figure 42 Matrix-based calibration from fortified porcine lean meat.

Table 31 Relationship between the fortified concentrations of salbutamol in bovine lean meat and the ratio of peak area of salbutamol to bamethan (IS).

Fortified concentration (ng g^{-1})	Peak area ratio*± SD
1	0.031 ± 0.003
3	0.107 ± 0.009
5	0.169 ± 0.007
10	0.296 ± 0.024
20	0.694 ± 0.049
50	1.65 ± 0.17

* 5 replications, RSD < 15%
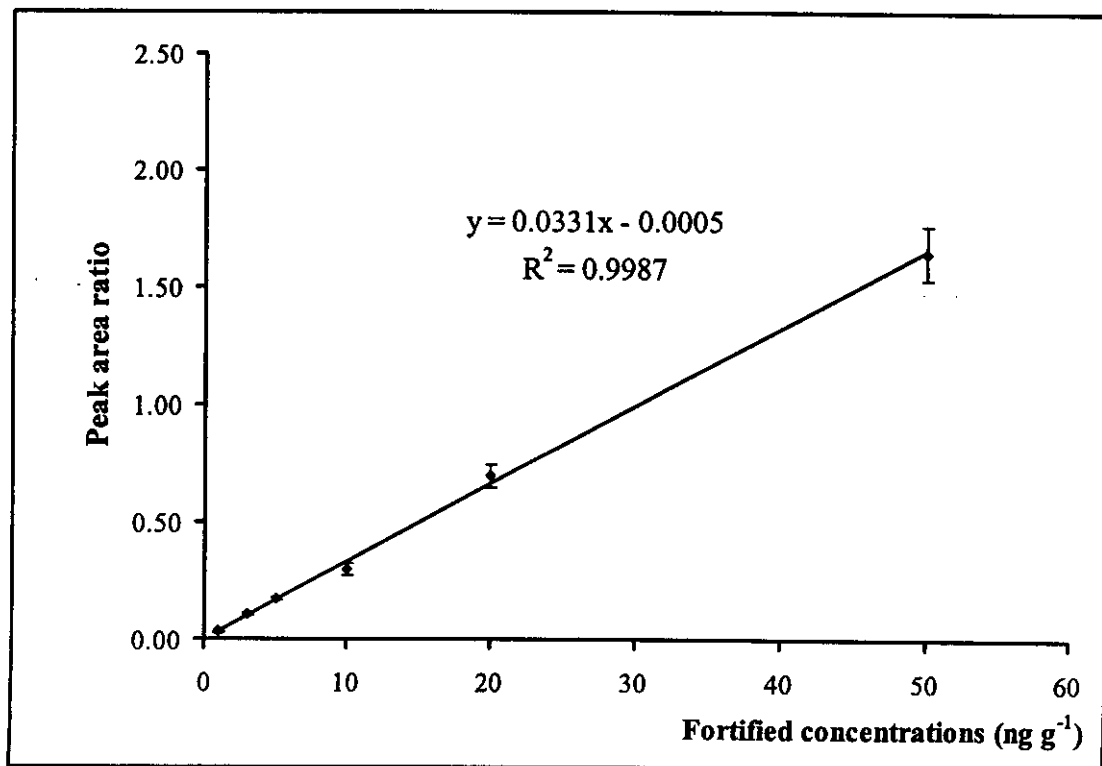
Figure 43 Matrix-based calibration from fortified bovine lean meat.
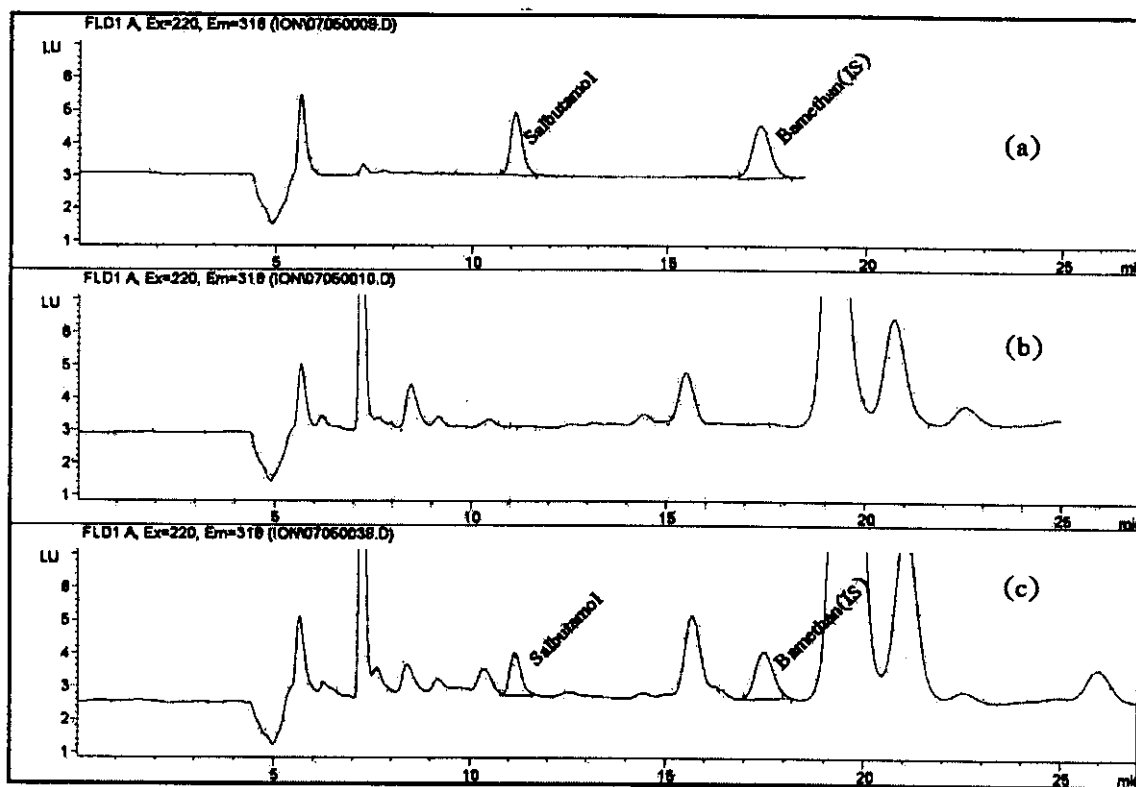
Figure 44 The HPLC chromatogram of; (a) mixed standard solution salbutamol and bamethan (IS) 20 ng mL^{-1}; (b) blank porcine sample and (c) fortified porcine sample with salbutamol and bamethan at 20 ng g^{-1}.
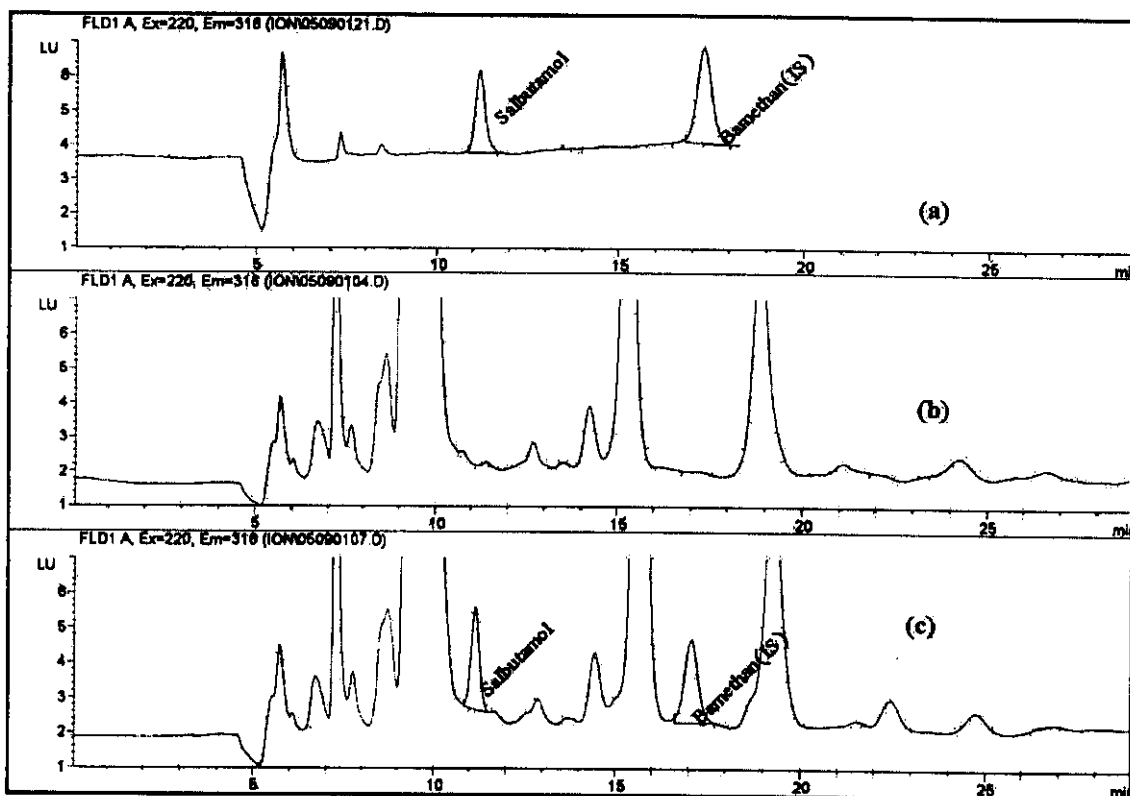
Figure 45 The HPLC chromatogram of (a) mixed standard solution salbutamol and bamethan (IS) 20 ng mL^{-1} ;(b) blank porcine sample; and (c) fortified bovine sample with salbutamol and bamethan at 20 ng g^{-1}.

3.5.4 Limit of Quantification (LOQ)

The method LOQ was taken to be the lowest level that could be quantitated with recoveries of 80-120% and a RSD not exceeding ±20%. The LOQs were 1 ng g^{-1} for porcine and bovine lean meat sample that meet the acceptable value for analysis method performance setting by Thai FDA.

3.6 Qualitative and quantitative analysis of lean meat sample

Porcine and bovine lean meat samples, six samples each, were purchased from local fresh markets and local supermarkets. All samples were prepared and analyzed at the optimum conditions.

3.6.1 Qualitative analysis

The optimum conditions of IPC-FLD were used to analyse salbutamol in lean meat samples. For qualitative analysis, the retention time, t_R, of the sample chromatogram was compared with retention time of the standard chromatogram to identify the salbutamol peak. The average t_R of salbutamol was 11.20 minute.

3.6.2 Quantitative analysis

Quantitative analysis of salbutamol was done by comparing the peak area ratio, salbutamol to bamethan (IS) of sample to the matrix-based calibration curve. First the matrix-based calibration curve was used for the determining of salbutamol in lean meat, salbutamol was not detected. Therefore, the standard addition method was applied with the same sample. The results and standard addition calibration curve of each sample are shown in Tables 32-43 and Figures 46-57. Table 44 shows salbutamol concentration of the samples analyzed by standard addition methods. The standard addition method confirmed that the concentration of salbutamol in all real samples were lower than the limit of quantification (LOQ), 1 ng g^{-1}. The presence of salbutamol in lean meat indicated that there are still abuses of this drug in animal farm even though it was banned by Thai government.

Table 32 Result of standard addition calibration curve of salbutamol in porcine sample 1.

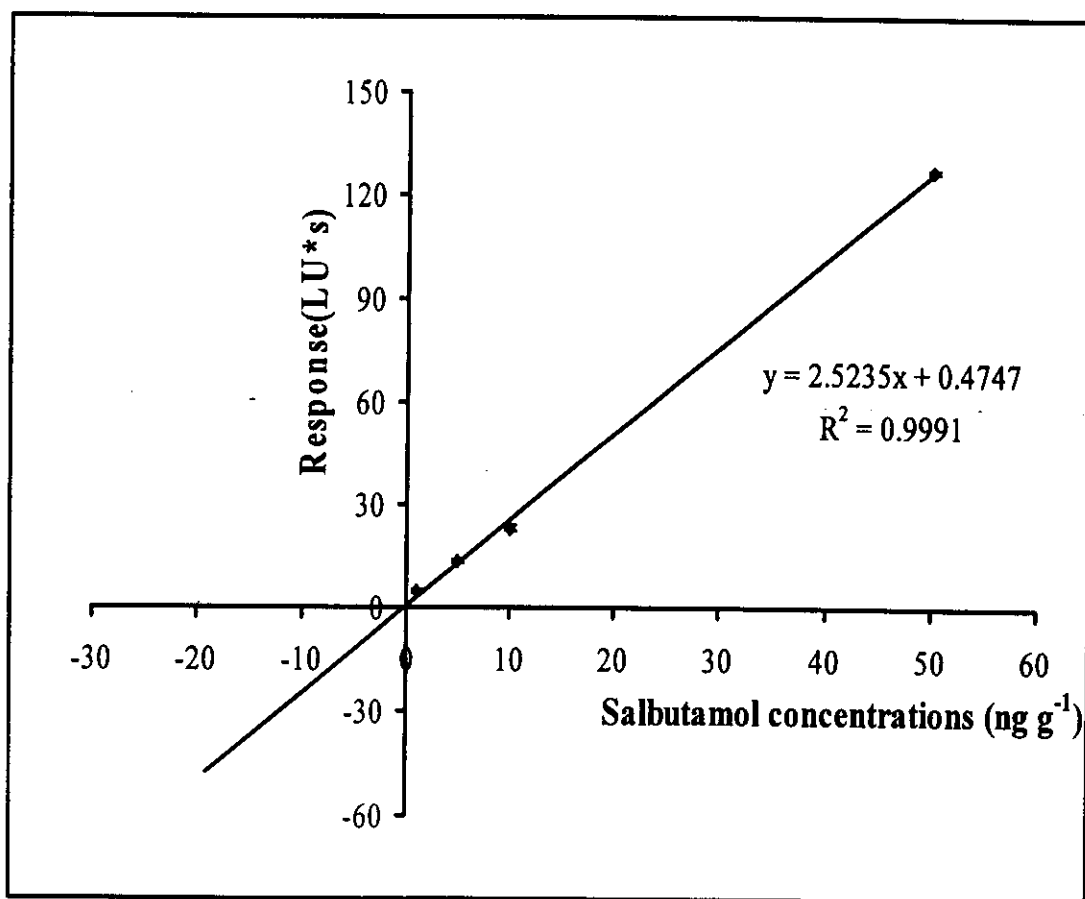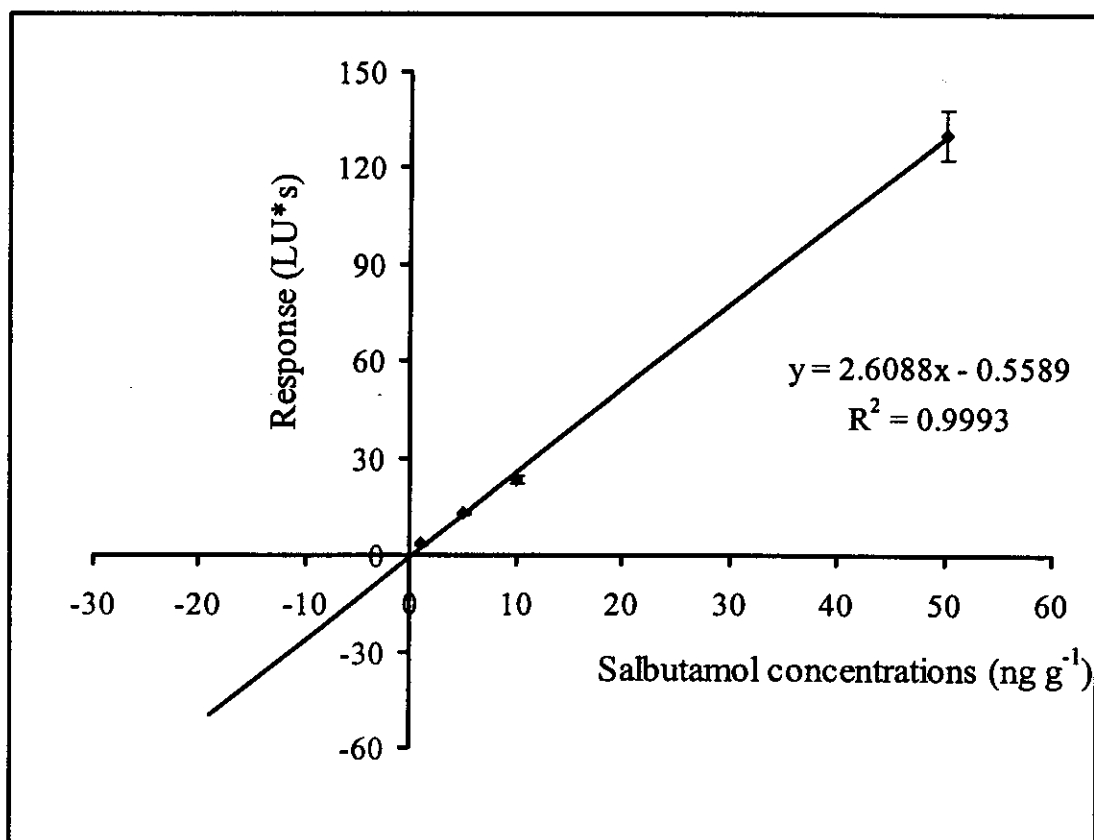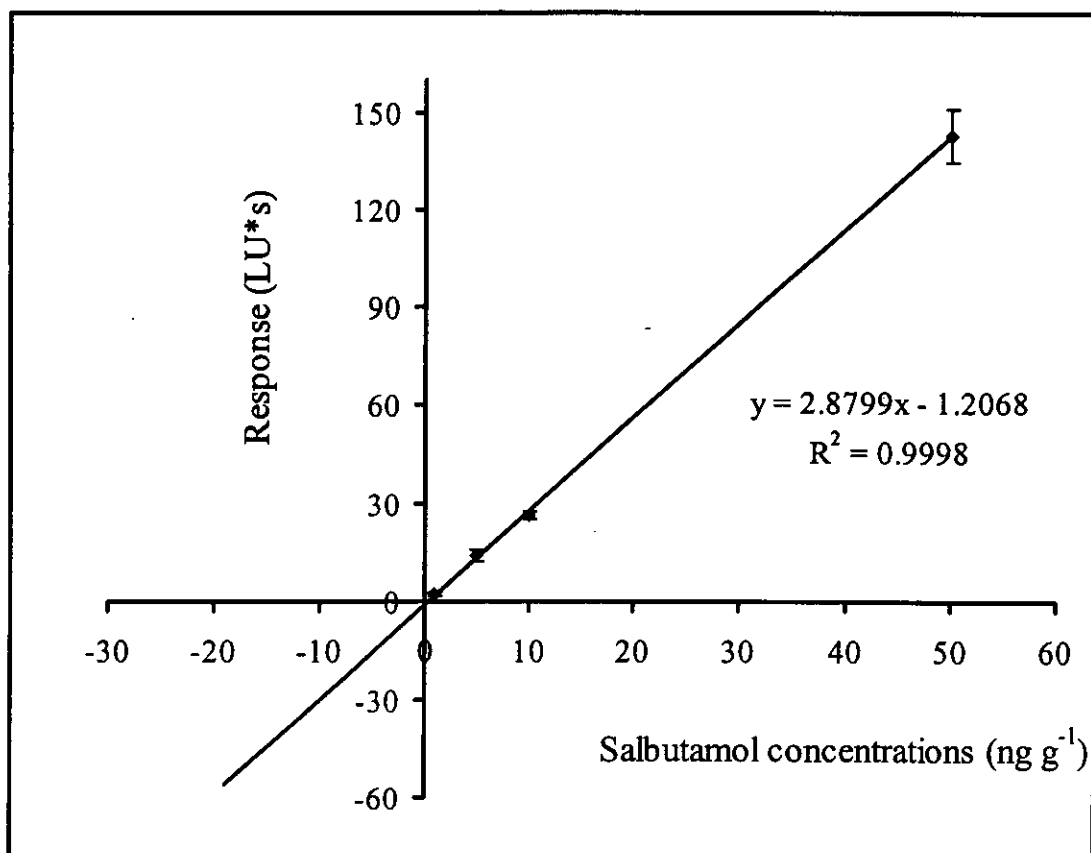| Concentration of salbutamol (ng g^{-1})	Response* (LU*s) ±SD
1	4.6±0.5
5	13.6±0.4
10	23.2±0.7
50	127.1±0.7

*3 replications, RSD < 15%

Figure 46 Standard addition calibration curve of salbutamol in porcine sample 1.

Table 33 Result of standard addition calibration curve of salbutamol in porcine sample 2.

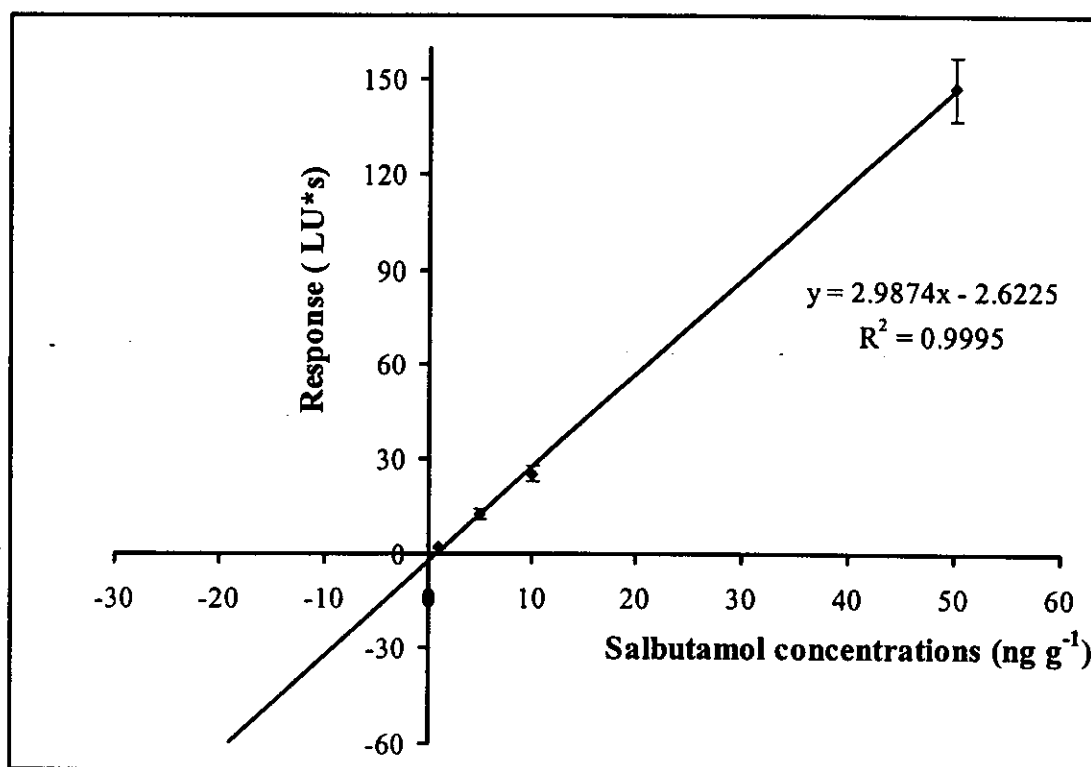| Concentration of salbutamol (ng g^{-1})	Response* (LU*s) ±SD
1	3.4±0.1
5	12.8±0.3
10	23.4±1
50	130±8

*3 replications, RSD < 15%

Figure 47 Standard addition calibration curve of salbutamol in porcine sample 2.

Table 34 Result of standard addition calibration curve of salbutamol in porcine
sample 3.

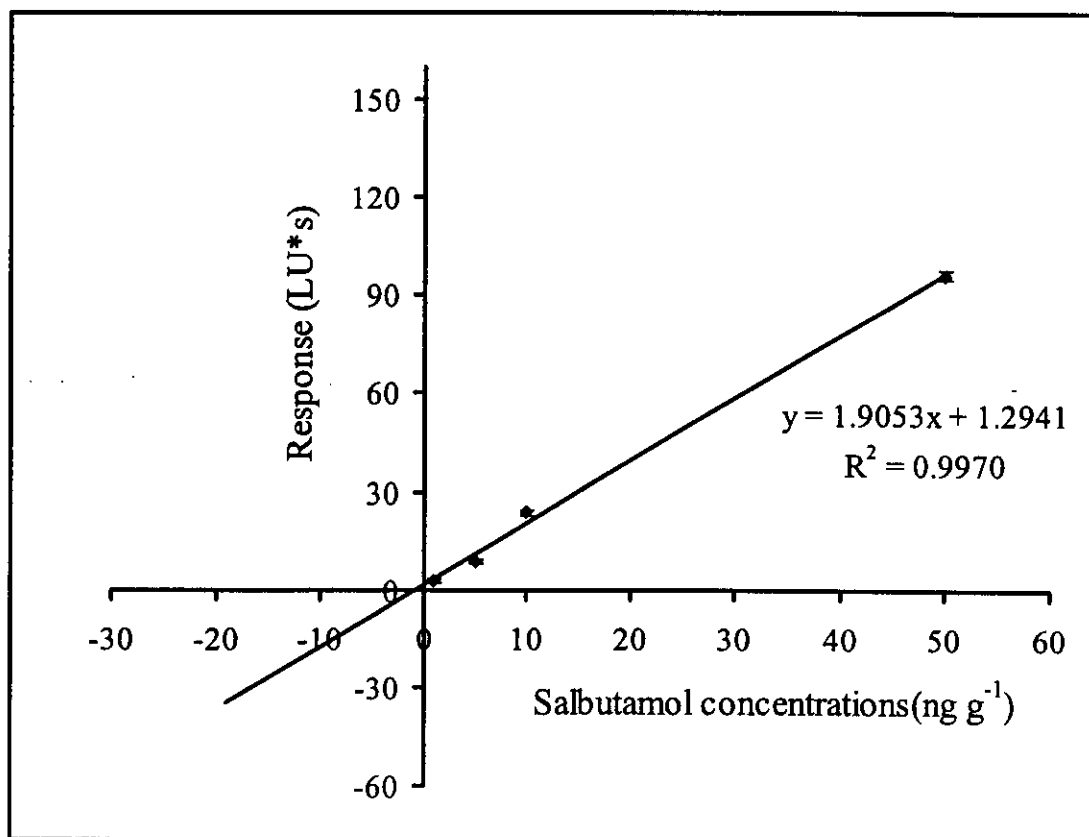| Concentration of salbutamol (ng g^{-1})	Response* (LU*s) ±SD
1	2.2±0.4
5	13.8±1.7
10	26.3±1.2
50	143.0±8.0

*3 replications, RSD < 15%

Figure 48 Standard addition calibration curve of salbutamol in porcine sample 3.

Table 35 Result of standard addition calibration curve of salbutamol in porcine sample 4.

Concentration of salbutamol (ng g^{-1})	Response* (LU*s) ±SD
1	1.9±0.3
5	12.4±1.7
10	25.2±2.5
50	147.1±10.4

*3 replications, RSD < 15%

Figure 49 Standard addition calibration curve of salbutamol in porcine sample 4.

Table 36 Result of standard addition calibration curve of salbutamol in porcine sample 5.

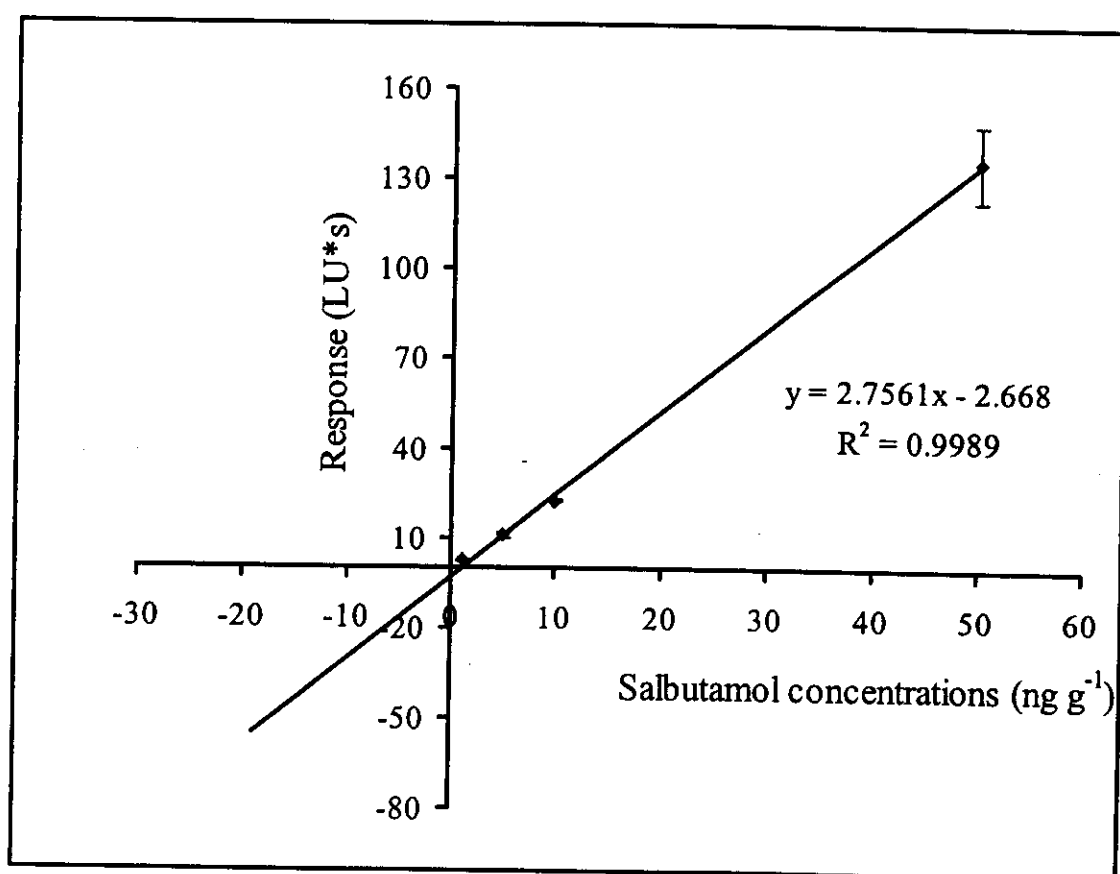| Concentration of salbutamol (ng g^{-1})	Response* (LU*s) ±SD
1	2.7±0.6
5	8.5±0.5
10	23.6±0.9
50	96.1±1.4

* 3 replications, RSD < 15%

Figure 50 Standard addition calibration curve of salbutamol in porcine sample 5.

Table 37 Result of standard addition calibration curve of salbutamol in porcine sample 6.

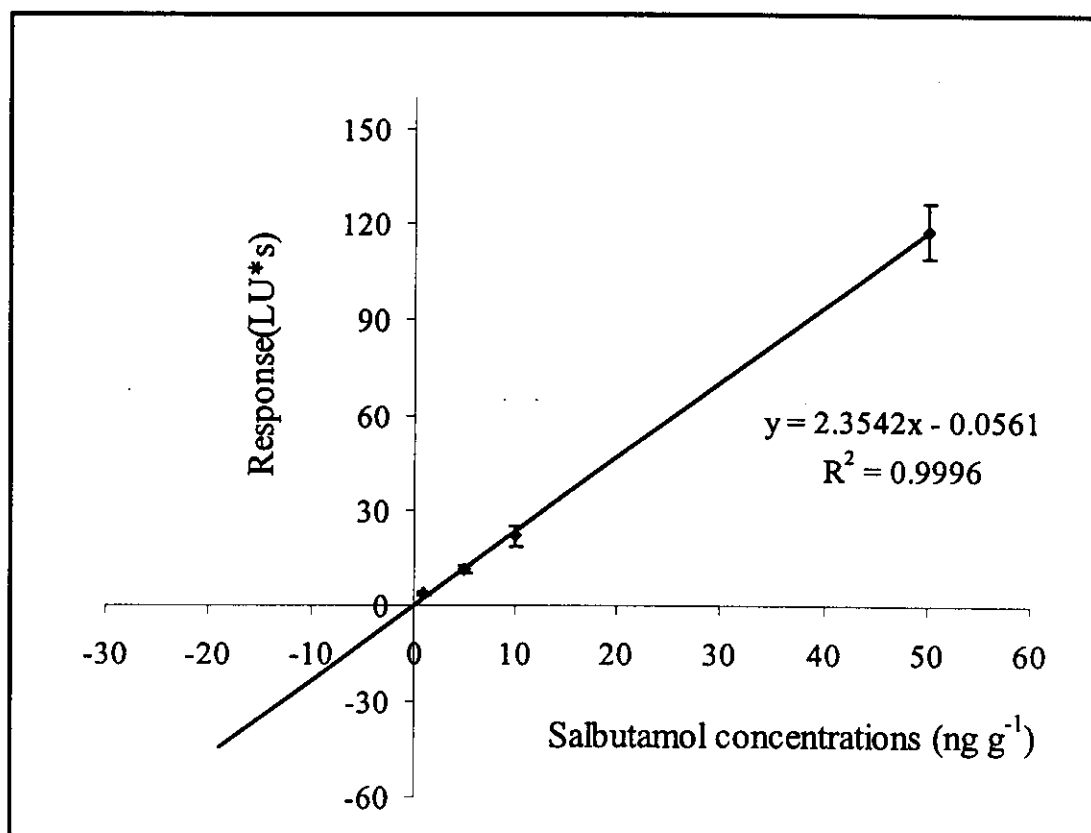| Concentration of salbutamol (ng g^{-1})	Response* (LU*s) ±SD
1	2.4±0.2
5	11.0±0.9
10	22.2±0.3
50	135.6±12.8

* 3 replications, RSD < 15%

Figure 51 Standard addition calibration curve of salbutamol in porcine sample 6.

Table 38 Result of standard addition calibration curve of salbutamol in bovine sample1.

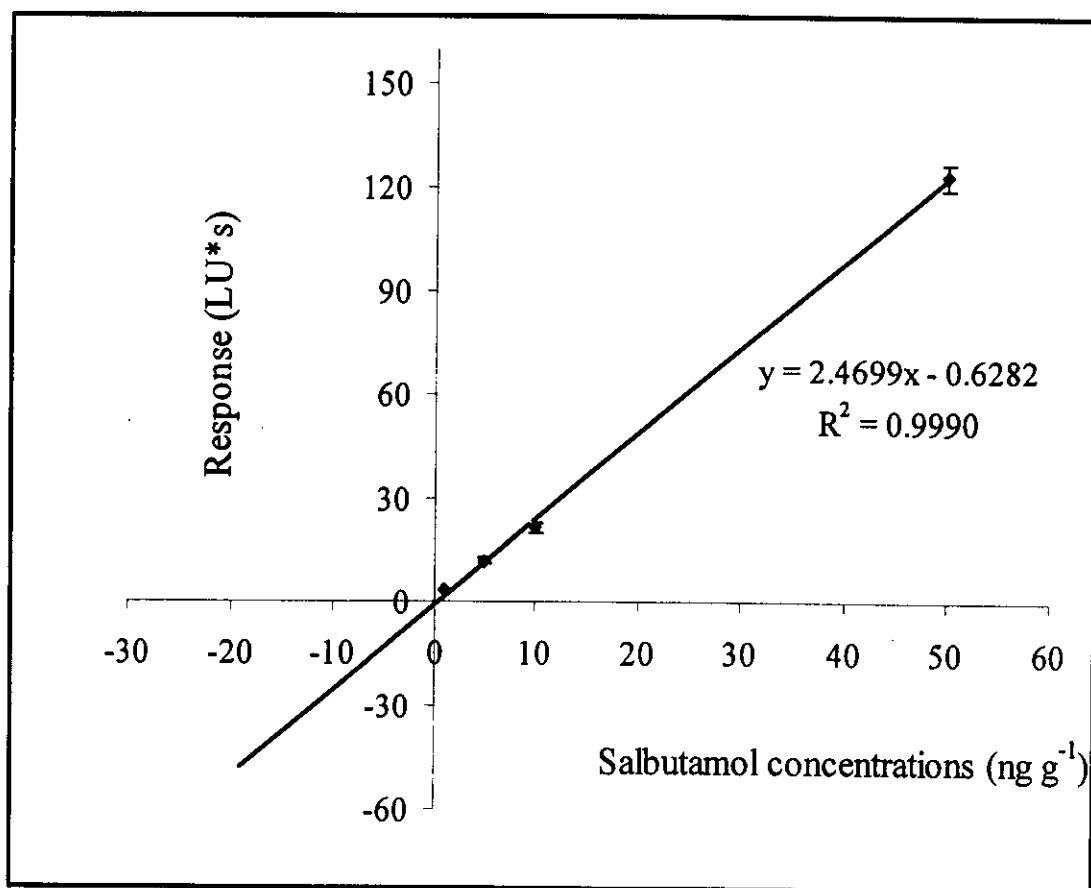| Concentration of salbutamol (ng g^{-1})	Response* (LU*s) ±SD
1	3.6±0.6
5	11.5±1.3
10	22.1±3.2
50	117.9±8.6

* 3 replications, RSD < 15%

Figure 52 Standard addition calibration curve of salbutamol in bovine sample 1.

Table 39 Result of standard addition calibration curve of salbutamol in bovine sample 2.

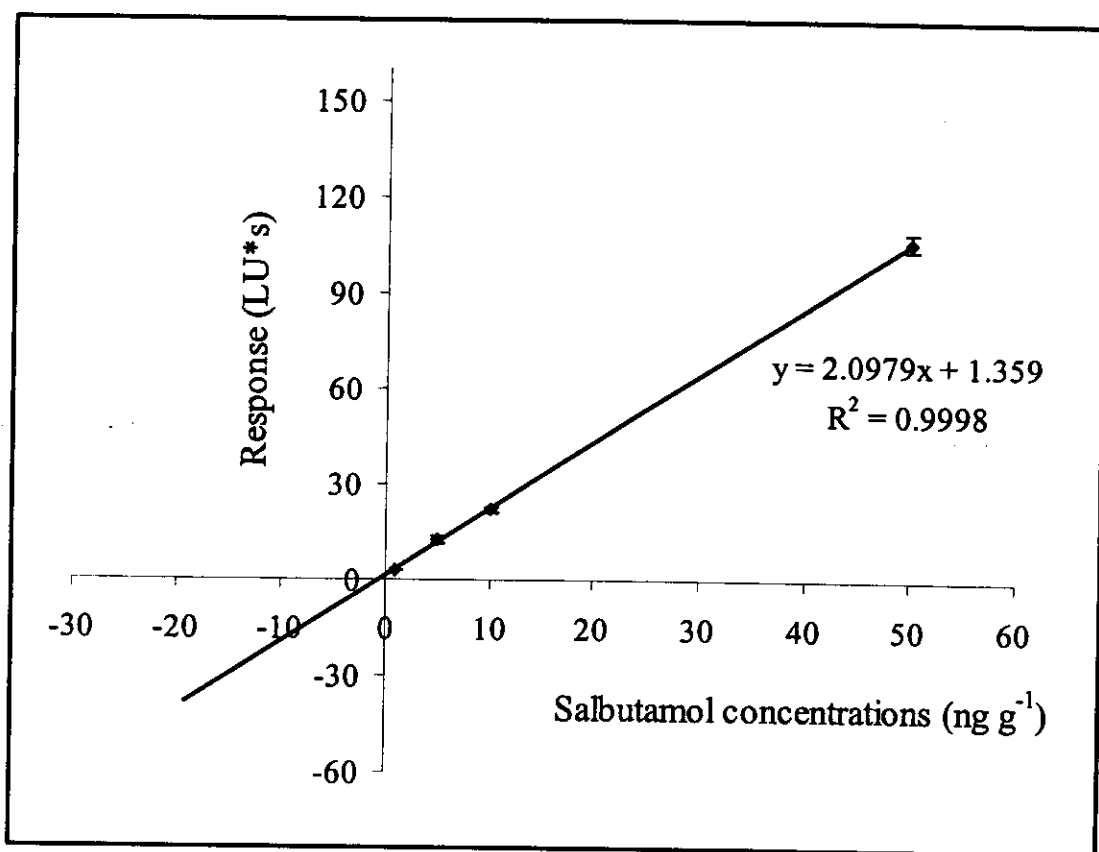| Concentration of salbutamol (ng g^{-1})	Response* (LU*s) ±SD
1	3.6±0.2
5	12.0±0.8
10	21.6±1.4
50	123.3±3.8

* 3 replications, RSD < 15%

Figure 53 Standard addition calibration curve of salbutamol in bovine sample 2.

Table 40 Result of standard addition calibration curve of salbutamol in bovine sample 3.

Concentration of salbutamol (ng g^{-1})	Response* (LU*s) ±SD
1	2.99±0.05
5	12.8±1.2
10	21.8±0.6
50	106.3±2.7

* 3 replications, RSD < 15%

Figure 54 Standard addition calibration curve of salbutamol in bovine sample 3.

Table 41 Result of standard addition calibration curve of salbutamol in bovine sample 4.

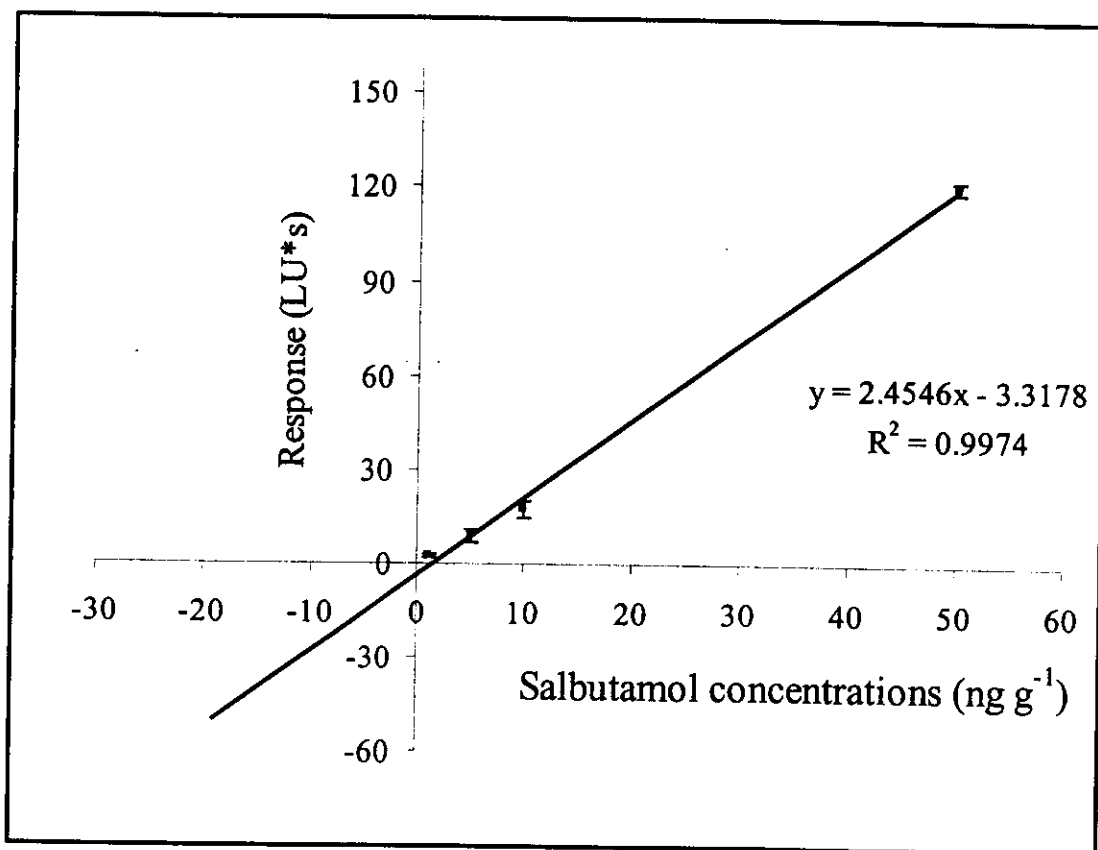| Concentration of salbutamol (ng g^{-1})	Response* (LU*s) ±SD
1	2.2±0.5
5	8.9±2.0
10	17.5±2.8
50	120.1±1.7

* 3 replications, RSD < 15%

Figure 55 Standard addition calibration curve of salbutamol in bovine sample 4.

Table 42 Result of standard addition calibration curve of salbutamol in bovine sample 5.

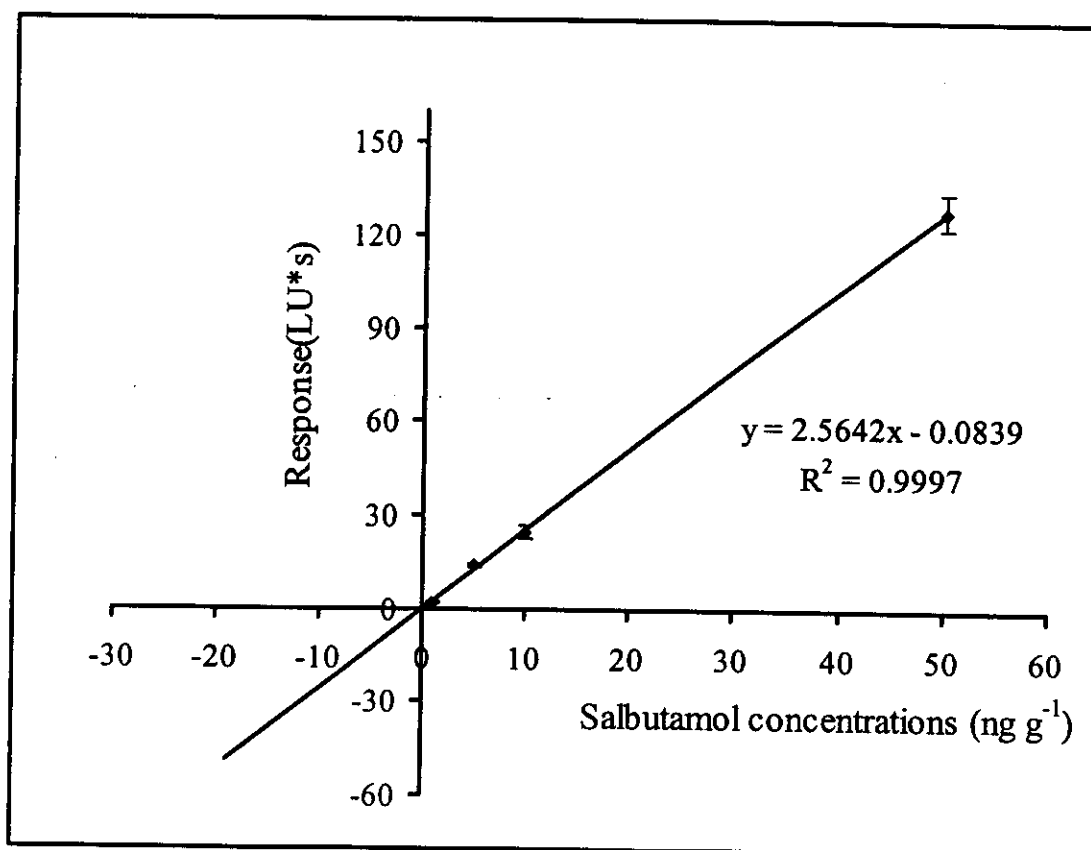| Concentration of salbutamol (ng g^{-1})	Response* (LU*s) ±SD
1	1.9±0.4
5	14.2±0.6
10	24.7±2.1
50	128.2±5.9

* 3 replications, RSD < 15%

Figure 56 Standard addition calibration curve of salbutamol in bovine sample 5.

Table 43 Result of standard addition calibration curve of salbutamol in bovine sample 6.

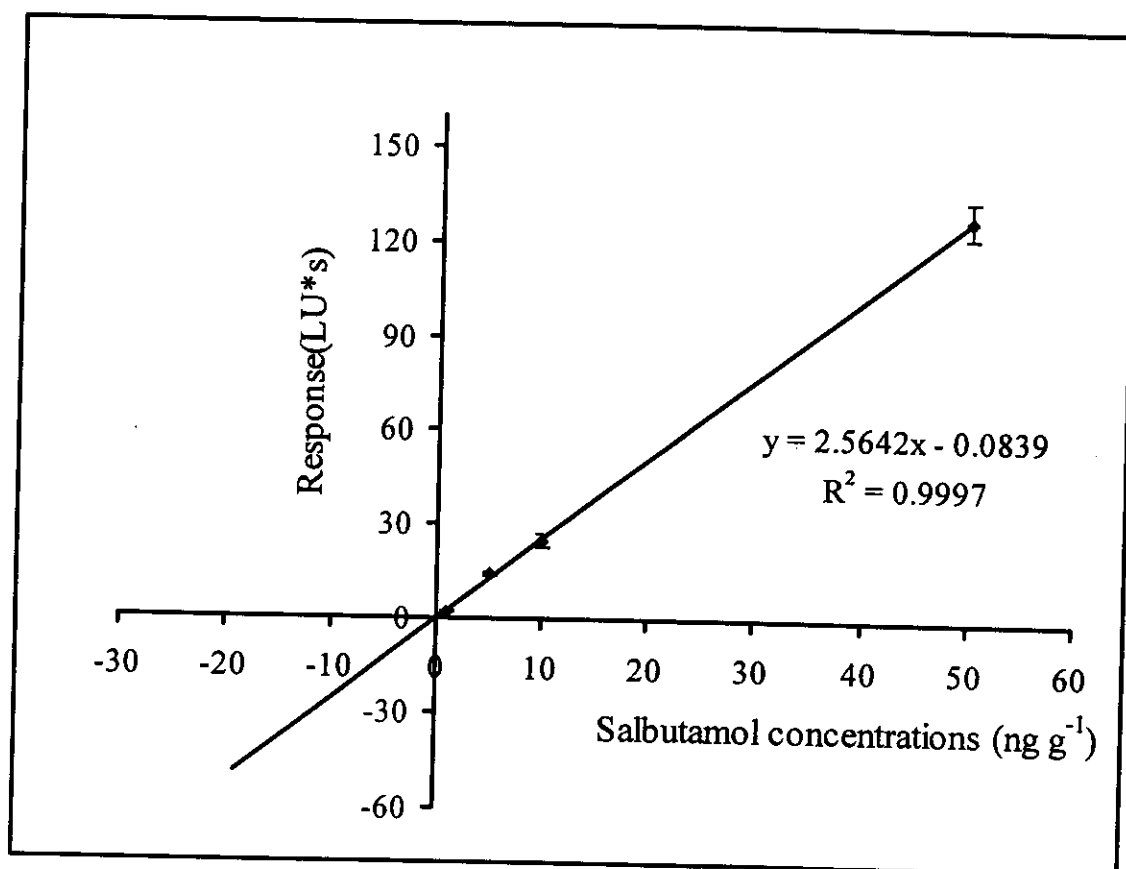| Concentration of salbutamol (ng g^{-1})	Response* (LU*s) ±SD
1	1.6±0.2
5	11.4±0.6
10	23.5±1.9
50	159.5±4.1

* 3 replications, RSD < 15%

Figure 57 Standard addition calibration curve of salbutamol in bovine sample 6.

Table 44 The salbutamol concentrations in porcine and bovine samples by standard addition.

Sample No.	Salbutamol concentration (ng g^{-1}) ±SD	
	Porcine	Bovine
1	0.2±0.1	ND
2	ND	ND
3	ND	0.6±0.2
4	ND	ND
5	0.7±0.1	ND
6	ND	ND

ND= Not Detectable