CHAPTER 2

EXPERIMENTAL

2.1 Chemicals and materials

2.1.1 Standard chemicals

- Salbutamol (99.0 %, $C_{13}H_{21}NO_3$, Sigma, USA)
- Bamethan hemisulfate salt (> 99%, $C_{12}H_{19}NO_2 \cdot 0.5H_2O_4S$, Sigma, USA)

2.1.2 Ion pair reagent

- 1-Hexanesulfonic acid sodium salt ($C_6H_{13}NaO_3S$, HPLC grade: BDH, England)

2.1.3 Other chemicals

- Supelclean ENVI-18 (Supelco, USA)
- Dichloromethane (CH_2Cl_2, AR grade: Merck, Germany)
- n-Hexane ($CH_3(CH_2)_4CH_3$, AR grade: Merck, Germany)
- Diethyl ether ($(C_2H_5)_2O$, AR grade: LAB-SCAN, Thailand)
- Acetic acid, Glacial (CH_3COOH, AR grade: J.T. Baker, USA)
- Methanol (CH_3OH, AR grade: LAB-SCAN, Thailand)
- Acetonitrile (CH_3CN, AR grade: LAB-SCAN, Thailand)
- Phosphoric acid (H_3PO_4 , AR Grade : Carlo Erba, France)
- Ultra pure water (H_2O, water was de-ionized with reverse osmosis system and purified with a Maxima ultrapure water instrument to obtain the resisitivity of 18.2 MΩ, ELGA, England)

2.1.4 Solid Phase Extraction (SPE)

- Strata X cartridges (Polymeric, 60 mg, 3 mL: Phenomenex, USA)
- 12-port SPE vaccuum manifold (Alltech, USA.)
- Vacuum pump (Gast, USA)

2.2 Instruments and apparatus

2.2.1 Spectrofluorometer

- Spectrofluorometer FP 777 with chart recorder PLT 396S (Jasco, Japan)
- Fluorescence rectangular cell 10 mm path (Jasco, Japan)

2.2.2 High Performance Liquid Chromatograph- Fluorescence Detector (HPLC-FLD)

- High Performance Liquid Chromatograph 1100 series equipped with a binary pump, micro vacuum degasser, autosampler, thermostatted column compartment, fluorescence detector and linked to a HP ChemStation (Agilent Technologies, Germany)
- Alltima HP C18 column: 150 × 4.6 mm, 3μm (Alltech, USA)
- Alltima HP C18 guard column: 7.5× 4.6 mm, 5μm (Alltech, USA)
- Computer system Compaq EVO (Compaq Computer, Thailand)

2.2.3 Apparatus

- Blender Moulinette S (Molinex, Mexico)
- Analytical Balance AB204-S (Mettler Toledo, Switzerland)
- Centrifuge GLC 2(Sorvall, USA)
- Ultrasonic bath AS7240AT (Automatic Science, Japan)
- Universal Meter with pH probe (WTW, Germany)
- Evaporating rotator (EYELA, Japan)
- Nitrogen gas (TIG, Thailand)
- Water bath (Elma, Germany)
- 12-port Drying attachments (Alltech, USA)

- Vortex Genie-2 (Sciencetific Industries, USA)
- Solvent filtration system (Alltech, USA)
- Microliter pipette 10 μL, 100 μL, 200 μL, 1000 μL (Eppendorf, Germany)
- Amber vial 2 mL with polypropylene screw cap and red rubber septa (Agilent Technologies, USA)
- Centrifuge tube 15 mL (CLP, USA)
- Filter membrane Supor® 0.2 μm 47 mm (Pall, USA)
- Syringe filter Polyvinylidene fluoride (PVDF) 0.2 μm 13 mm (Chromtech, USA)
- Glass microfiber filter GF/F (Whatman, England)
- General glassware such as mortar, pestle, volumetric flask, round bottle flask, cylinder

2.3 Analysis system

Salbutamol is an intrinsic fluorescence compound owing to its aromatic phenolic structure (Boyd *et al.*, 1996). Two fluorescence detection systems were studied in this work. In the preliminary study, spectrofluorometer was used to study the fluorescence property of salbutamol and the system performance of spectrofluorometer was investigated. Then the ion-pair chromatography with fluorescence detection system (IPC-FLD) was used to enhance the selectivity and sensitivity of the analysis method.

Figure 5 shows the block diagram of IPC-FLD system used in this work. Degassed methanol and hexanesulfonate solution were pumped through the C_{18} column by two dual piston pumps. The ion-pair reagent, because of its hydrophobic alkyl group and the charge carried by the reagent (C_6-SO_3^-), was attracted to the stationary phase (C_{18}). This negative charge on the stationary phase is balance by positive ions (Na^+) from the reagent. A protonated salbutamol can now exchanged with Na^+ ion, resulting in the retention of the sample ion by an ion-exchange process, as shown in Figure 6 (Snyder *et al.*, 1997). The eluting compound passed through the

flow cell and absorbed radiation from xenon flash lamp and then fluorescence, emitting light of a longer wavelength. The intensity of light emitted is proportional to the intensity of the excitation source and the quantum efficiency of the process. The emitted radiation is measured by photomultiplier tube and calculated to luminescence unit (LU) by HP ChemStation program.

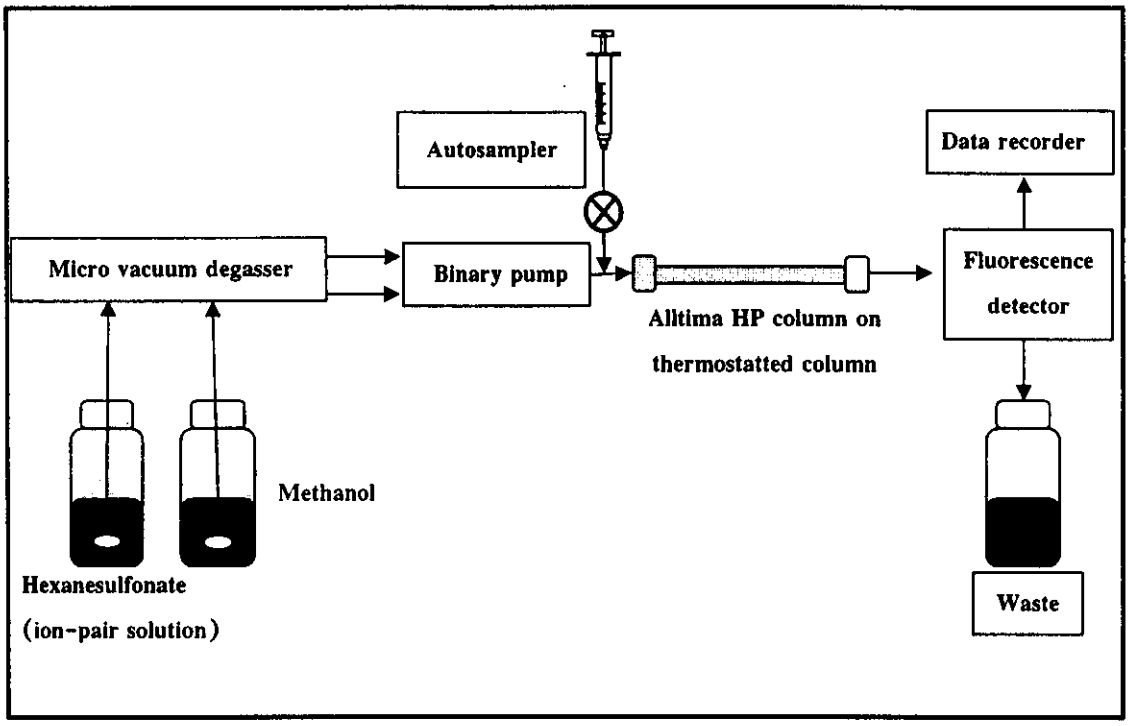

Figure 5 Block diagram showing the components of IPC-FLD.

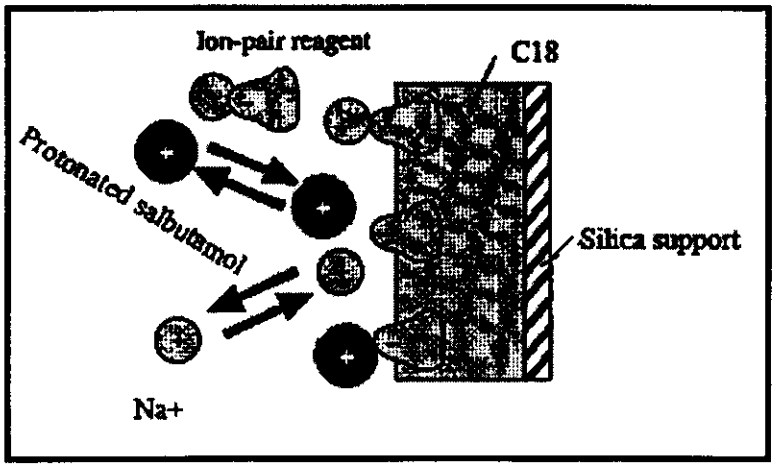

Figure 6 Retention of protonated salbutamol during IPC (Snyder *et al.*, 1997).

2.4 Preparation of salbutamol standard solutions

Standard stock solution, 1000 μg mL^{-1}, was prepared by placing 10.00 mg of salbutamol in a 10 mL volumetric flask and made up the volume with ethanol. Standard solutions were prepared by diluting the standard stock solution to the concentration of 100, 10 and 1 μg mL^{-1} in ethanol. Each standard solution was then transferred into a glass bottle with PTFE-lined screw cap. All bottles were wrapped with aluminum foil and stored at 4 ^{0}C.

2.5 Determination of salbutamol by spectrofluorometer

This technique was used to study the excitation and emission wavelength of salbutamol. The system performance, *i.e.*, the limit of detection (LOD) and linear dynamic range, were also tested. Some chromatographic conditions from the work of Miller *et al.* (1986), that is, mobile phase, 8% acetonitrile (pH = 2.5), and emission wavelength at 309 nm were used as the diluting solution and the first setting emission wavelength in this study.

2.5.1 Diluting solution, 8% (v/v) acetonitrile, pH 2.5

Eight milliliters of acetonitrile (99.7 %) was mixed with distilled water, adjusted to pH 2.5 with phosphoric acid and made up to 100 mL with distilled water.

2.5.2 Salbutamol standard working solution

Salbutamol stock standard solution, 100 μg mL^{-1}, was diluted by the diluting solution to 0.5 μg mL^{-1} . This solution was used to study the excitation and emission wavelengths of spectrofluorometer method.

For linear study, salbutamol standard solution in the range of 0.1-10 μg mL^{-1} was used. These standard solutions were obtained from diluting salbutamol standard solution 100 μg mL^{-1} with the diluting solution.

2.5.3 Excitation (λ_{ex}) and emission (λ_{em}) wavelengths

In fluorescence detection technique, the emitted light is directly proportional to the intensity of the excitation light and using these two wavelengths decreased the chance that interfering (co-eluting) peaks will be detected (Parriott, 1993). Thus, the optimum excitation (λ_{ex}) and emission (λ_{em}) wavelengths of salbutamol were studied using salbutamol standard solution, 0.5 μg mL^{-1}, and blank, dilution solution. Using a spectrofluorometer, this was done by first setting the emission wavelength at 309 nm (Miller *et al.,* 1986) and scanned the excitation spectrum to obtain the excitation wavelengths that gave the responses. Each of these excitation wavelengths was then tested by scanning the emission spectrum using blank solution. The chosen excitation wavelength was the one that did not give any signal at 309 nm (the emission wavelength found by Miller *et al.* (1986)). The chosen excitation wavelength was then set and scanned to obtain the emission wavelength of this system using salbutamol standard solution.

2.5.4 Limit of detection (LOD)

The limit of detection (LOD) was the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value (US-FDA, 1999).The LOD of spectrofluorometry technique was investigated at optimum excitation and emission wavelengths. The LOD was the minimum concentration of salbutamol that had the signal with the signal to noise ratio at more than three (S/N ≥3) (US-FDA, 1999).

2.5.5 Linear range

Salbutamol was diluted to the concentrations from 0.10 to 10 μg mL^{-1} and measured with spectrofluorometer at the optimum wavelengths. Five replications were tested for all analysis. The intensity was plotted *versus* the concentration of salbutamol to obtain the linear dynamic range. Linearity was determined by considering the coefficient of determination (R^2).

2.6 Determination of salbutamol by ion-pair chromatography with fluorescence detection (IPC- FLD)

Salbutamol in protonated form can be separated by ion-pair chromatography and determined by fluorescence detection. Hexanesulfonate solution was used as the ion pair reagent in the mobile phase. Bamethan standard solution was used as internal standard (IS), for reliable quantitative determination and compensation for non-reproducibility in the extraction procedure. Chromatographic conditions of IPC-FLD affecting the detection and separation of salbutamol and bamethan (IS) were studied, *i.e.,* excitation (λ_{ex}) and emission (λ_{em}) wavelengths, percentage of acetic acid (pH effect), concentration of ion-pair reagent, percentage of methanol (solvent-strength effect), temperature and flow rate. After optimization, system performance was investigated.

2.6.1 Bamethan internal standard (IS) stock solution

Internal standard was prepared by placing 12.30 mg of bamethan hemisulfate salt in a 10 mL volumetric flask and made up the volume with ethanol to give a final concentration of 1000 μg mL^{-1}. The intermediate solutions were prepared by diluting this standard stock solution, 1000 ng μL^{-1}, with ethanol to the concentrations of 100, 10 and 1 ng μL^{-1}. Each standard solution was then transferred into a glass bottle with PTFE-lined screw cap. All bottles were wrapped with aluminum foil and stored at 4 ^{0}C.

2.6.2 Salbutamol standard working solution

Salbutamol standard working solutions were prepared by diluting 1 μg mL^{-1} salbutamol standard solution with 2% acetic acid to concentrations in the range of 1-50 ng mL^{-1}.

2.6.3 Degassing of mobile phase

Degassing of mobile phase was an important step to reduce the possibility of bubbles forming in the HPLC system and to eliminate dissolved gases in mobile phase such as oxygen that can react with the mobile/ stationary phase during

the analysis (Hamilton and Swell, 1982). The mobile phase of IPC-FLD system, methanol and hexanesulfonate solution, were filtered through the 0.2 μm 47 mm filter membrane Supor® by solvent filtration system and degassed by ultrasonic vibrations.

2.6.4 Optimization of IPC-FLD conditions

In the optimization process of chromatographic analysis, resolution and elution time was the most important (Rouessac and Rouessac, 2000). In addition, fluorescence signal and optimum wavelengths for excitation and emission can be strongly dependent on separation conditions, *e.g.*, solvent polarity, pH and temperature (Snyder *et al.*, 1997). Thus, the final separation conditions may require a compromise between good resolution, good detection and short analysis time.

Figure 7 shows the measurement of resolution for two analytes. The time taken for the two analytes to elute from a chromatographic column with a particular mobile phase are their retention times, t_A and t_B. Since retention time will vary with column length and mobile phase flow rate, it is more useful to use the *capacity factor* or *retention factor*, *k*. This relates the retention time of an analyte to the time taken by an unretained compound, *i.e.*, one which passes through the column without interacting with the stationary phase, to elute from the column under identical conditions (t_0). The retention factor of analyte A can be represented mathematically by the following equation:

$$k_A = \frac{t_A - t_0}{t_0} \qquad (1)$$

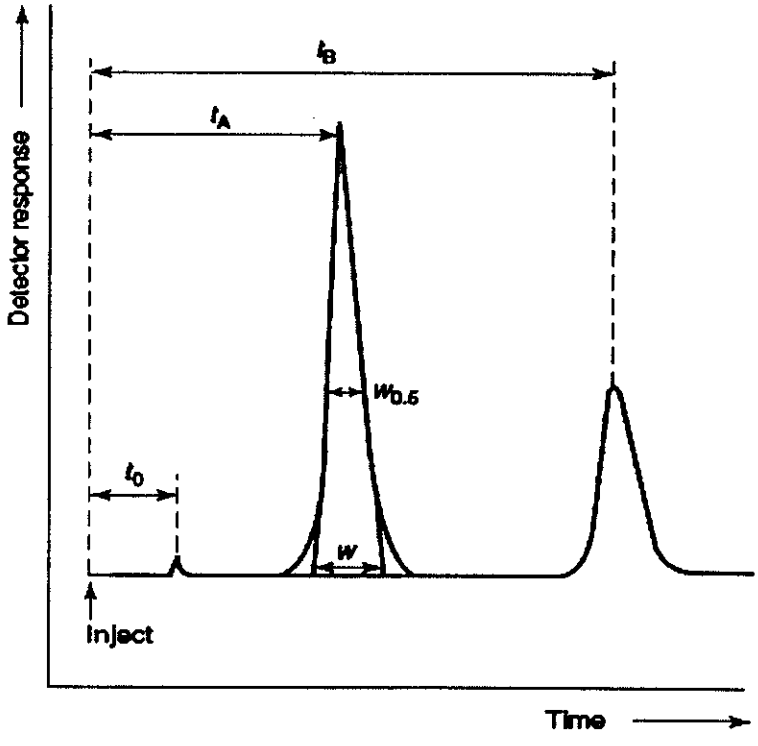

Figure 7 Illustration of HPLC parameters: t_0, retention time of non-retained component; t_A and t_B, retention time of analytes A and B; w, width of peak at base; $w_{0.5}$, width at half-height (Ardrey, 2003).

The separation of two components, A and B, was termed the *selectivity* or *separation factor* (α) and relates to the retention behavior the two analytes (Ardrey, 2003):

$$\alpha = \frac{k_B}{k_A} = \frac{t_B - t_o}{t_A - t_o} \tag{2}$$

The parameters k and α were determined by conditions that affect retention or the equilibrium distribution of the sample between the mobile phase and column packing, *i.e.*, composition of mobile phase, composition of the stationary phase and temperature. The number of theoretical plates or plate number (N) was dependent on column quality and can be varied by changing column condition, *i.e.*, flow rate of mobile phase. The mathematical relationships between the number of plates, the

retention time of analyte and the width of the response is shown in the following equations:

$$N = 16\left(\frac{t_A}{w_A}\right)^2 = 5.54\left(\frac{t_A}{w_{0.5}}\right)^2 \quad (3)$$

Resolution, R_s, can be expressed in terms of three parameters, *i.e.*, retention factor (k), separation factor (α) and the column plate number (N) as following equation.

$$R_s = \frac{1}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_B}{1 + k_B}\right)\sqrt{N} \quad (4)$$

where k_B is the retention factor of the second of the two components and N is the number of theoretical plates measure for that component (Ardrey, 2003).

In optimization process the conditions that affecting k and α values, *e.g.*, concentration of ion-pair reagent, percentage of methanol and temperature, were first varied, then varied the column conditions that affecting N value, *e.g.*, flow rate (Snyder *et al.*, 1997). Optimization was done by changing one parameter and keeping other parameters constant. When an optimum was obtained it was used to optimize the next parameter. Mixed standard of salbutamol, 20 ng mL^{-1}, and bamethan (IS) solution, 20 ng mL^{-1}, in 2 % acetic acid was used for the chromatographic conditions investigation.

2.6.4.1 Excitation (λ_{ex}) and emission (λ_{em}) wavelengths

For the same reason as in spectroflurometry, optimization of excitation (λ_{ex}) and emission (λ_{em}) wavelengths of HPLC-FLD was done by modifying some parameters, *i.e.*, flow rate and the ratio of hexanesulfonate to methanol in mobile phase, from the application note of HPLC method for albuterol (salbutamol) in serum (Waters, 2003) to be used with the Alltima HP column. The modified parameters were the composition of the mobile phase that consisted of 5 mM hexanesulfonate with 1 % acetic acid and methanol (65:35) at a flow rate of 0.6 mL min^{-1} and injection volume of 100 µL. Using IPC-FLD, this was done by setting

the emission wavelength at the optimum obtained from 2.5.3. (spectrofluorometer method) the excitation wavelengths of salbutamol and bamethan (IS) were investigated by scanning the excitation spectrum. Then chose the the wavelength that gave the highest response as excitation wavelengths and these were used to find the optimum emission wavelengths of salbutamol and bamethan (IS) by scanning the emission spectrum. The wavelengths that gave the highest response (LU) were chosen as the optimum emission wavelengths.

2.6.4.2 Percentage of acetic acid (pH effect)

Acetic acid was added to hexanesulfonate solution to convert salbutamol into protonated form. Various percentages (v/v) of acetic acid, 0.5, 1.0, 1.5 and 2.0, were added to the 5 mM hexanesulfonate solution. Each percentage was done in five replications. The mobile phase consisted of 5 mM hexanesulfonate (with acetic acid) and methanol (55:45) flowed at a rate 0.6 mL min^{-1}. Standard salbutamol and bamethan (IS) was injected to the IPC-FLD setting at the optimum excitation and emission wavelengths. Response (peak area) was plotted *versus* percentage of acetic acid. Optimum percentage of acetic acid was the one that gave the highest response.

2.6.4.3 Concentration of ion-pair reagent

To obtain the optimum concentration of ion-pair reagent, hexanesulfonate was prepared at concentrations, 0.5, 1, 2, 3, 4 and 5 mM, with 1.5% v/v acetic acid (the optimum percentage from 2.6.4.2). Each concentration was done in five replications. The mobile phase was hexanesulfonate (with acetic acid) and methanol (55:45) with a flow rate of 0.4 mL min^{-1}. Standard salbutamol and bamethan (IS) was injected to the HPLC-FLD at the optimum excitation and emission wavelengths. Retention factor (k) and separation factor (α) of salbutamol and bamethan (IS) of each concentration were calculated. Optimum concentration of hexanesulfonate was the concentration that gave $1 < k < 10$ (Snyder *et al.*, 1997) and α between 1.05 and 10 (Hamilton and Swell, 1982) with good response and short analysis time.

2.6.4.4 Percentage of methanol (Solvent-strength effect)

The mobile phase consisted of 3 mM hexanesulfonate with 1.5% acetic acid (the optimum values from 2.6.4.2 and 2.6.4.3) and methanol at a flow rate of 0.4 mL min^{-1}. The percentage of methanol in the mobile phase was studied at 28, 30, 33, 35, 40 and 45. Standard salbutamol and bamethan (IS) was injected to the HPLC-FLD at the optimum excitation and emission wavelengths. Optimum percentage of methanol was the percentage that gave $1< k < 10$ (Snyder *et al.*, 1997) and α between 1.05 and 10 (Hamilton and Swell, 1982) with good response and short analysis time.

2.6.4.5 Temperature

The optimum temperature of the ion-pair chromatography (IPC) system was studied by setting the column temperature at room temperature (27.5), 35, 40 and 45 °C. The mobile phase was 3 mM hexanesulfonate with 1.5% acetic acid and methanol (70:30) at a flow rate of 0.4 ml min^{-1} (optimum values from 2.6.4.2 to 2.6.4.4). Standard of salbutamol and bamethan (IS) was injected to the HPLC-FLD at the optimum excitation and emission wavelengths. Optimum temperature of HPLC system was the temperature that gave $1< k < 10$ (Snyder *et al.*, 1997) and α between 1.05 and 10 (Hamilton and Swell, 1982) with good response and short analysis time.

2.6.4.6 Flow rate

In this investigation the flow rate of the mobile phase consisted of 3 mM hexanesulfonate (with 1.5 % acetic acid) and methanol (70:30) (optimum values from 2.6.4.2 to 2.6.4.5) was varied at 0.2, 0.3, 0.4, 0.5 and 0.6 mL min^{-1}. Five replications were done for each flow rate. The retention time and peak width at half height were determined from the chromatogram to calculate the height equivalent to a theoretical plate (HETP). A van Deemter graph was plotted to determine the optimum flow rate.

2.6.5 System performance of IPC-FLD

2.6.5.1 Repeatability

The repeatability of the chromatographic method was determined by performing the analysis of mixed solution of salbutamol standard and bamethan (IS) at 20 ng mL^{-1}.The solution was injected 5 times with an automatic injector and relative standard deviation (RSD) was obtained for the retention time and peak area. The RSD should be less than 1% and 4% for retention time and peak area respectively (Snyder *et al.*, 1997).

2.6.5.2 Limit of detection (LOD)

Salbutamol standard solution, 100 ng mL^{-1}, was diluted with 2 % acetic acid (Shishani *et al.*, 2003) to obtain the concentration in the range of 0.25-5.0 ng mL^{-1} and was analysed with optimum IPC-FLD conditions. The minimum concentration of salbutamol standard that gave the signal to noise ratio more than three ($S/N \geq 3$) was the limit of detection for this system (US-FDA, 1999).

2.6.5.3 Linear range

Salbutamol standard solution 100, 10 and 1 μg mL^{-1} were diluted with 2 % acetic acid to the concentrations from 0.5 ng mL^{-1} to 12 μg mL^{-1}. Each solution was injected into the IPC-FLD system at optimum conditions. Five replications were prepared for all analysis. Peak area was plotted *versus* concentration of salbutamol to obtain the linear dynamic range. Linearity was determined by considering the coefficient of determination (R^2).

2.6.5.4 Internal standard curve

Salbutamol standard solution 1 μg mL^{-1} was diluted with 2 % acetic acid to the concentration from 0.5 to 50 ng mL^{-1}. Each concentration contained 20 ng mL^{-1} of bamethan (IS). All standard solutions were injected into the IPC-FLD system at optimum conditions. Five replications were prepared for all analysis. The internal standard curve was obtained by plotting the relative response of salbutamol (salbutamol response devided by bamethan response) *versus* its concentration.

Linearity was determined by considering the considering the coefficient of determination (R^2).

2.7 Sample preparation

Sample preparation was done by using a combination of matrix solid-phase dispersion (MSPD) and solid phase extraction (SPE) techniques followed those previously described by Boyd *et al.* (1995). Some modified conditions were applied, *i.e.*, volume of MSPD eluent and clean up sample solution with polymeric sorbent, strata X SPE cartridge, before determination by ion-pair chromatography with fluorescence detection (IPC-FLD). Fortified lean porcine sample with 50 ng g^{-1} salbutamol and bamethan (IS) was used to study the optimum sample preparation conditions.

2.7.1 Preparation of salbutamol standard fortification solution

Salbutamol standard solution, 10 μg mL^{-1}, was diluted using ethanol to cover the range of 50 ng mL^{-1} to 2.5 μg mL^{-1} .These solutions were used to fortified blank samples to validate the method.

2.7.2 Fortified sample

Fortified sample, a sample enriched with a known amount of salbutamol and bamethan (IS), was used to study the optimum sample preparation conditions and method validation. Fortification was carried out by pipetting 10 μL of salbutamol and bamethan onto 0.50 g of blank sample tissue and left for 10 minute prior to extraction. Salbutamol standard in the range of 50 ng mL^{-1} to 2.5 μg mL^{-1} and 2.5 μg mL^{-1} of bamethan (IS) were used to fortify in porcine and bovine lean meat in matrix-based calibration curve. Fortified lean porcine sample at 50 ng g^{-1} salbutamol and bamethan (IS) was used to study the optimum sample preparation conditions.

2.7.3 Pretreatment of octadecyl (C_{18}) packing material

C_{18} packing material was pre-washed by placing 15 g of the packing in a 20 mL glass syringe barrel (glass microfiber filter GF/F was used as frit) and washed sequentially with 30 mL each of hexane, dichloromethane and methanol. The

packing was dried by vacuum aspirator. C_{18} packing material may cause irritation to skin, eyes, and respiratory tract. It must be handled with appropriate safety precaution including the use of protective gloves, goggles and lab coat.

2.7.4 Optimization of matrix solid-phase dispersion (MSPD) extraction

Figure 8 shows a schematic diagram of sample extraction by matrix solid phase dispersion (MSPD) technique. Visible fat from the sample was first removed before cut into small pieces and homogenized in a blender. A 0.50 g of the sample was placed into a glass or coated mortar (external diameter 80 mm) then 2.00 g of packing material was added and gently ground with the tissue using a pestle to obtain a homogeneous material. The mixture was transferred to a 10 mL syringe barrel pre-plugged with a glass micro fiber filter GF/F disc. Another filter paper disc was placed at the head of the column. The mixture was compressed to a volume of 4.5 ml with a syringe plunger. Then the barrel was placed on a vacuum manifold. The column was washed with washing solvent and after the solvent had flowed through the column, a positive pressure was applied to remove all solvent from the column. The pestle and mortar was rinsed twice with an eluting solvent and transferred the rinsing solution to the column. The eluate was centrifuged at 3,000 rpm for 10 minute, transferred the supernatant to a clean round bottle flask and evaporated to dryness by a rotary evaporator at 50 °C. The MSPD parameters affecting the extraction efficiency were investigated as follows. Five replications were done for each experiment. The one that provided the highest response was then select.

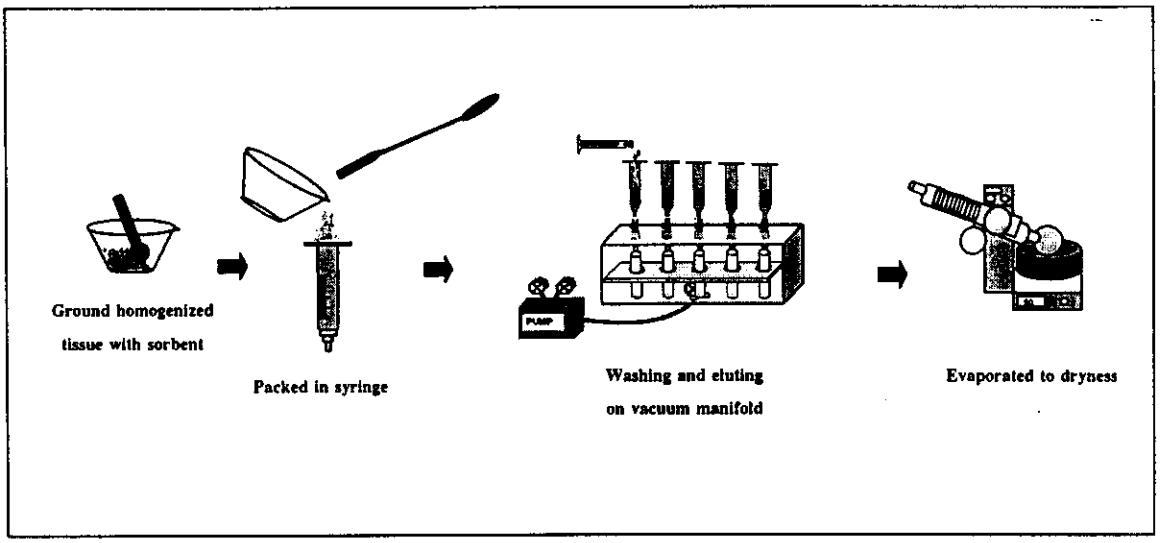

Figure 8 Sample extraction by matrix solid phase dispersion.

2.7.4.1 Type of sorbent

In this work a sorbent was used for grinding and assisting in the sample extraction process by solubilizing lipids, unfolding and disrupting cell membrane and internal structure of tissue sample (Simpson, 2000). Six types of both polar and non polar sorbent were investigated. C_{18}, Supelclean ENVI-18 and Supelclean LC-18, (Boyd *et al.*, 1995) was studied for the non polar sorbent. The polar sorbent, Silica gel 60 (Ramos *et al.*, 1999), aluminum oxide acid (Shishani *et al.*, 2003), alumnium oxide neutral and florisil was used in this study. Each of these was ground with the fortified sample then carried through the sample preparation procedure and analyzed by IPC-FLD at optimum conditions.

2.7.4.2 Type of washing solvent

Washing solvent for MSPD packing must remove the fat or undesired compounds in lean meat sample without eluting salbutamol. Tested washing solvents were hexane (Boyd *et al.*, 1994), hexane followed with water (Boyd *et al.*, 1994) and mixture of hexane and diethyl ether (6:4) (Boyd *et al.*, 1995). The extractant was analyzed by IPC-FLD at optimum conditions.

2.7.4.3 Volume of washing solvent

To minimize the washing solvent used and still maintained the high response, optimum washing solvent from experiment 2.7.4.2, the mixture of hexane and diethyl ether (6:4), was studied at various volumes (0, 4, 8 and 10 mL). The optimum value was the smallest volume that gave the highest response.

2.7.4.4 Type of eluting solvent

The eluting solvent was studied base on the solvent relative polarity. Four solvents, methanol (Boyd *et al.*, 1995) and solvents that have polarity index closed to methanol, *e.g.*, ethanol, acetonitrile and 90 % methanol were studied.

2.7.4.5 Flow rate of eluting solvent

The flow rate of eluting solvent through the column is also significant for the effectiveness of a separation. In addition, the flow control is important to ensure the reproducibility. The flow rate was varied at 0.15, 0.3, 0.7, 1.0 and 2.0 mL min^{-1}. The flow rate that gave the highest response with short analysis time was then selected.

2.7.4.6 Volume of eluting solvent

Methanol was selected as eluent from experiment 2.7.4.4 to elute salbutamol from C_{18} sorbent. To minimize the eluent used and at the same time maintained the highest response, the volume of methanol was varied at 4, 6, 8 and 10 mL. The optimum value was the smallest volume that gave the highest response.

2.7.5 Optimization of Solid phase extraction (SPE), clean up

For the clean up step by SPE, strata X cartridges were used. They were first conditioned with 1 mL of methanol and equilibrated with 1 mL of distilled water. The dry extracts (methanol extracts from 2.7.4.6 was dried by rotary evaporator) were reconstituted in 3 mL of distilled water and transferred to the cartridge. The cartridge was then washed with 1 mL of 5 % methanol and dried under vacuum for 30 s. Salbutamol was eluted with eluting solvent and dried under nitrogen at 60 °C to dryness. Before analysed by optimum IPC-FLD system, the dried substance were

reconstituted with 0.5 mL of 2 % acetic acid and filtered through a 0.2 μm Polyvinylidene fluoride (PVDF) disposable syringe filter. A 100 μL volume of the filtrate was injected. Optimization of SPE parameters were done as follows. Five replications were done for each experiment. The optimum value was the one that provided the highest response.

2.7.5.1 Flow rate of sample solution

The flow rate of sample solution through the SPE cartridge may affect the separation efficiency. The flow rate was studied at 0.25, 0.50, 1.0, 2.0 and 4.0 mL min^{-1}. The flow rate that gave the highest response with short analysis time was then selected.

2.7.5.2 Type of eluting solvent

The eluting solvent used with the SPE cartridge should be able to dissolve the analyte and can overcome the SPE retention mechanism. Four eluting solvents were studied, methanol (Boyd *et al.*, 1995), acetonitrile (polarity index is close to methanol). The recommended eluting solvent from Strata X user's guide, mixture of methanol and acetonitrile (50:50) and the mixture of methanol: acetonitrile: water at 60:30:10 with 0.1% acetic acid were also tested.

2.7.5.3 Flow rate of eluting solvent

For the same reason as in MSPD column, the flow rate of eluting solvent through the SPE cartridge played a significant role in the effectiveness of a separation. The flow rate was varied at 0.3, 0.7, 1.0, 1.5 and 2.0 mL min^{-1}.

2.7.5.4 Volume of eluting solvent

To minimize the eluent used and at the same time maintained the highest response, the volume of eluting solvent was studied as an elution profile. This was done by collecting 0.1 mL of eluting fraction continuously for 15 fractions, *i.e.*, a total of 1.5 mL. The eluent was dried and reconstituted with 2 % acetic acid, filtered and analyzed by HPLC-FLD at optimum conditions. The optimum volume of eluting

solvent was the volume that did not show the analyte signal. Figure 9 summarizes the sample preparation procedure.

2.7.6 Matrices interferences

Matrix effects play an important role in accuracy and precision of measurement (Mitra, 2003). Fortified samples with known quantity of salbutamol and bamethan (IS) were used to determine the matrix effect on the sample preparation and analysis. Salbutamol standards, 50, 150, 250, 500, 1000 and 2500 ng mL^{-1} 10 μL, and bamethan (IS), 1000 ng mL^{-1} 10 μL, were added to 0.5 g porcine lean meat and bovine lean meat. The fortified samples were extracted by MSPD-SPE and analyzed by HPLC-FLD at optimum conditions. The responses were plotted against the known concentration of salbutamol. The slope of the standard curve and matrix-based curve were compared to evaluate the matrix effects using two-way analysis of variance (ANOVA) by R program (R Development Core Team, 2006).

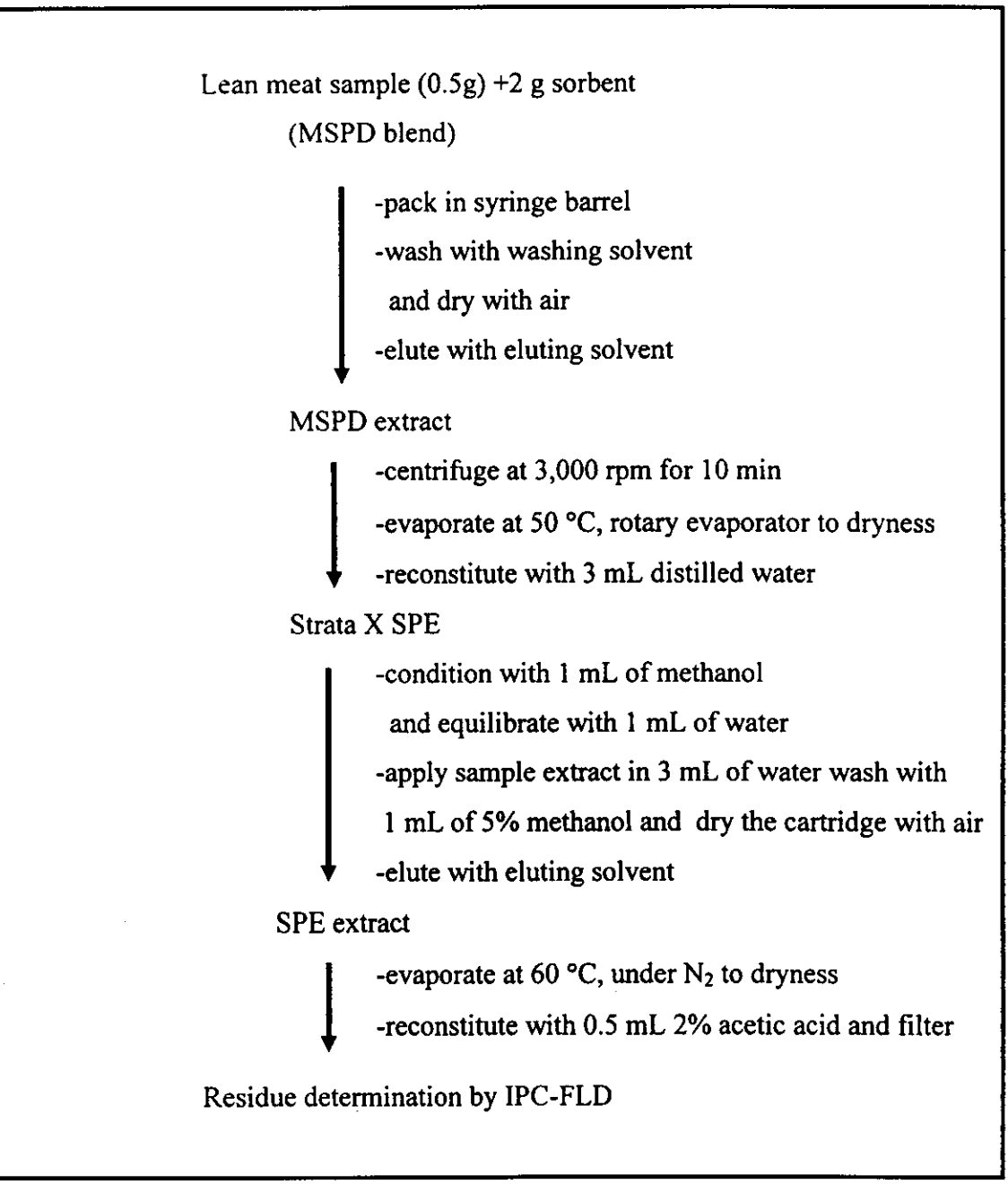

Figure 9 Summary of the sample preparation procedure.

2.8 Method validation

The validation parameters were validated according to US-FDA bioanalytical method validation guidance and the Commission Decision 2002/657/EC as follows.

2.8.1 Selectivity

Blank porcine sample was porcine lean meat with no salbutamol and was purchased from fresh market at Faculty of Natural Resources, Prince of Songkla University, Hatyai, since it was not treated with salbutamol. The method's selectivity was investigated by analyzing 6 different samples, purchased on different days. Each blank sample was tested for interference using proposed extraction procedures and optimum chromatographic conditions.

2.8.2 Accuracy, Precision and Recovery

Accuracy and precision of method were studied at the same time by analzing five fortified porcine and bovine lean meat samples with salbutamol at 3, 25 and 40 ng g^{-1} and bamethan (IS) at 20 ng g^{-1}.

The acceptable criteria for accuracy was evaluated by the percentage of recovery that should be in the range of 70-110% for fortified sample at 3 ng mL^{-1} and 80-110% for 25 and 40 ng mL^{-1}(Comission Decision 2002/657/EC, 2002).

The precision of method was evaluated by the coefficient of variation (CV). The CV for repeated analysis of fortified sample should not exceed the level calculated by the Horwitz equation.

$$CV = 2^{(1-0.5 \log C)}$$

Where C is the mass fraction expressed as a power (exponent) of 10, *e.g.*, 1 mg $g^{-1} = 10^{-3}$ (Commission Decision 2002/657/EC, 2002).

For mass fractions lower than 100 ng g^{-1} the application of the Howitz equation gave unacceptable high values. Therefore, the CV for the concentrations lower than 100 ng g^{-1} should be as low as possible. In this case 23% was taken as a guideline for the coefficient of variation (calculate from Horwitz equation; CV at 100 ng g^{-1} =23%).

2.8.3 Calibration curve (Matrix-based calibration curve)

A calibration curve is the relationship between instrument response and known concentration of the analyte. The method calibration curve was investigated in the fortified sample, both bovine and porcine, with salbutamol in the concentration range 1.0-50 ng g^{-1} and bamethan (IS) at 20 ng g^{-1}. The peak area ratio of salbutamol to bamethan, *versus* the concentration of salbutamol was plotted. Matrix-based calibration curve of at least six point (non-zero standard) were used. Blank (processed without internal standard) and zero samples (processed with internal standard) were also analysed to confirm absence of interferences, these two samples were not use to construct the calibration curve. The linearity was determined by considering the coefficient of determination (R^2).

2.8.4 Limit of Quantification (LOQ)

The limit of quantification of a method was consider as the lower limit of quantification (LLOQ), the lowest standard concentration on the calibration curve, if the following criteria were met:

- The analyte response at the LLOQ should be at least 5 times the response compare to blank response (US-FDA, 2001).
- Analyte peak (response) identifiable, discrete and reproducible with a precision 20 % and accuracy of 80-120 % (US-FDA, 2001).

2.9 Qualitative and quantitative analysis of lean meat sample

Two types of real samples, porcine and bovine lean meat, from fresh market and supermarket were studied quantitatively and qualitatively.

2.9.1 Qualitative analysis of IPC-FLD technique for salbutamol analysis

The retention time of the sample chromatogram was compared with retention time of the standard chromatogram to identify the salbutamol peak.

2.9.2 Quantitative analysis

Bamethan standard solution 1 μg mL^{-1} 10 μL was fortified on porcine and bovine lean meat sample and extracted with combined MSPD-SPE before determined by IPC-FLD. The quantitative analysis was carried out by comparing the peak area ratio, salbutamol to bamethan, of sample to the matrix-matched calibration curve. The non detectable (ND) or lower than LOQ samples was confirm by using standard addition method.

2.9.3 Standard addition

The standard addition was performed in the sample that showed non detectable response (ND) or lower than the LOQ of salbutamol. The extracted sample solution was divided up into 5 equal portions, salbutamol was added to all portions by increasing levels of standard concentration from 0, 1, 5, 10 to 50 ng mL^{-1}. The samples were analyzed and peak area response *versus* the final concentration is plotted. The original concentration is then determined by extrapolation to the x-axis (Harris, 1995).