CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

Veterinary drugs are widely used in food-producing animal for both treatment and prevention of diseases. Drugs are also added to animal feeds to improve the efficiency of their conversion into edible tissue (Shepherd, 1991). The misuse of these veterinary drugs may lead to drug residues in food animal tissue and this may cause allergic reaction and has direct toxic effect on human including environmental and economic problems. Many countries, including Thailand, the use of some drugs in animal farm to minimize the exposure of drug residues to humans and safeguard public health. Thai Food and Drug Administration (FDA) has banned some veterinary drugs in animal tissues including the β-agonist compounds, especially salbutamol. However, this compound is still being found in porcine tissue, e.g., muscle, liver and kidney (Food and Drug Administration, 2002 and 2003 and Department of Medical Sciences, 1998 and 2004).

In 2004, Thai government has established a policy on food safety to provide food quality and safety assurance, and salbutamol is used as one of the indicators to evaluate the achievement of this policy. Thai FDA has set the acceptable performance of the analysis method for β-agonist compounds and its metabolite to ≤ 1 $\mu g\ kg^{-1}$ (Thai Food and Drug Administration, 2003). Routine laboratory analysis in Thailand relies on commercially test kits base on enzyme immunoassay (EIA), e.g., β-agonist EIA of Euro- Diagnostica, but false positive or negative result can occurred (Boyd et al., 1996). An alternative method based on the combination of high performance liquid chromatography with fluorescence detection (selective detector for salbutamol), HPLC-FLD, has been developed for routine analysis of salbutamol in porcine tissue (Kaewklapanyachareon, 2001), however, the detection limit was still

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high, 10 µg kg^{-1}. Thus, the objective of this work is to develop an analysis method to enhance the performance of HPLC-FLD method to determine salbutamol at the required level, *i.e.*, ≤ 1 µg kg^{-1} level.

1.2 Review of Literatures

1.2.1 β-agonists

β-agonists are synthetic derivatives of naturally occurring catecholamines (adrenaline, nordrenaline and dopamine). There are two main classes of β-agonists, the more polar substituted phenol type (salbutamol, terbutaline) and the less polar substituted aniline type (clenbuterol, malbuterol). These compounds have a common β-hydroxyamine group at the side chain, but are differentiated from each other by the different substitutents on the aryl moiety as shown in Figure 1 (O'Keeffe *et al.*, 1999).
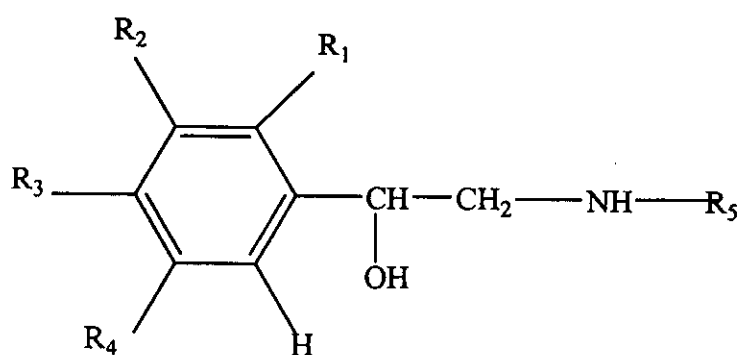
	R1	R2	R3	R4	R5
Salbutamol	H	CH_2OH	OH	H	$C(CH_3)_3$
Terbutaline	H	OH	H	OH	$C(CH_3)_3$
Orciprenaline	H	OH	H	OH	$CH(CH_3)_2$
Ractopamine	H	H	OH	H	$CH(CH_3)CH_2PhOH$
Fenoterol	H	OH	H	OH	$CH(CH_3)(CH_2)_2PhOH$
Clenbuterol	H	Cl	NH_2	Cl	$C(CH_3)_3$
Clenpenterol	H	Cl	NH_2	Cl	$C(CH_3)_2CH_2CH_3$
Clenproperol	H	Cl	NH_2	Cl	$CH(CH_3)_2$
Cimbuterol	H	CN	NH_2	H	$C(CH_3)_3$
Cimaterol	H	CN	NH_2	H	$CH(CH_3)_2$
Bromobutero	H	Br	NH_2	Br	$C(CH_3)_3$
NA 1141	H	Cl	NH_2	Cl	$C(CH_3)_2CH_2OH$
Mabuterol	H	Cl	NH_2	CF_3	$C(CH_3)_3$
Mapenterol	H	Cl	NH_2	CF_3	$C(CH_3)_2CH_2CH_3$

Figure 1 General structure of β-agonists (Ramos, 2000).

β-agonists act through binding to β_2-receptor located on various cell type including adipose tissue, muscle tissue, endocrine system and blood flow (Moloney *et al.*, 1991) and produce direct observable physiological effects, *i.e.*, increase in heart rate, dilation of coronary vessels, relaxation of bronchial and uterine muscle tone, stimulation of glygenolysis and insulin release (Boyd *et al.*, 1996). These lead to decrease lipogenesis, fat synthesis and storage, and increased lipolysis, fat mobilisation and hydrolysis that can increase lean/fat ratio (Moloney *et al.*, 1991). The rate of fat accumulation in adipocytes or growth of the adipose tissue mass slows, particularly in ruminants, resulting in a leaner animal. The magnitude of these changes is influenced by dose and duration of treatment with the β-agonists (Dunshea *et al.*, 2005). The unwanted side effects of β-agonists are predictable extensions of their pharmacological action: tachycardia, muscle tremor, palpitations, gastrointestinal symptoms, cephalalgia, vertigo, weakness, nervousness and confusion (Brambilla *et al.*, 1997).

In the 1990s there were eight reports on the acute food poisoning from β-agonist drug (clenbuterol) in European and Asian countries (Table 1). The main symptoms found were tremors, palpitation and tachycardia, nervousness and general malaise. The risks associated with the presence of residues of β-agonists in meat have led to ban on the use of these compounds as the growth promoter in European Union (Council Directive 96/22/EC, 1996). Moreover, β_2-selective adrenoreceptors are drugs that stimulate the central nervous system and potentially produce a certain amount of anabolic-like effects obtained when higher doses of these compounds are administered (Martineau, 1992 cited in Ventura *et al.*, 2000). For this reason, the International Olympic Committee (IOC) restricts the use of salbutamol in athletes only by inhalation and, even then, it must be declared in writing to the relevant medical authority prior to the competition (Felix *et al.*, 2006).

Table 1 Food poisoning from β-agonist compounds.

Year	Country	Cases	Source	Concentration ($\mu g\ kg^{-1}$)	References
1990	Spain	135	bovine liver	160-291	Martínez-navaro, 1990
1990	France	22	veal liver	-	Pulce *et al.*, 1991 cited in Salleras *et al.*, 1995
1991	Spain	59	cinnamon (accidental contaminant)	-	Salleras *et al.*, 1995
1992	Spain	232	veal liver, veal tongue, cannelloni	19-5395	Salleras *et al.*, 1995
1995	Italy	16	fillet, rump steak	500	Maistro *et al.*, 1995
1996	Spain	15	veal liver	500	Bilbao-Garey *et al.*, 1997
1996	Italy	62	beef	4500	Brambilla *et al.*, 1997
1997	Hong Kong	17	pig lung, pig liver	-	Chan, 1998

1.2.2 Salbutamol

Salbutamol or albuterol is the phenolic substituted β-agonists developed in 1960's. It is direct acting sympathomimetric with β_2-adrenergic activity, employed as bronchodilator for the treatment of asthma and chronic obstructive pulmonary disease. It is also used to arrest premature labor in pregnancy (Dol and Knochen, 2004). It is administered clinically as a racemic mixture even though the *R* (-) enantiomer is carrying most of the therapeutically bronchodilating effects (Bergés *et al.*, 1999). Similarly, these compounds are employed in veterinary medicine as bronchorelaxant and tocolytic agents. Moreover, in animal, β-agonists promote an

increase of the tissue muscle/fat ratio and, for this reason, salbutamol has been misused in animal farm as a growth promoter (Ventura *et al.*, 2000) by adding salbutamol 3 mg to 1 kg feed (Kuptapan, 2003). In Thailand, the used of salbutamol in meat producing animals is now banned due to its health effects. However, illegal uses still exist. Some details of salbutamol are shown below, in Figure 2 and Table 2.

Salbutamol

CAS registry: 18559-94-9 (free base); 51022-70-9 (sulfate); 50293-90-8 (hydrochloride);

Systematic name: α^1-[[(1, 1-Dimethylethyl)-amino] methyl]-4-hydroxy-1,3-benzene-dimethanol; α^1-[(*tert*-butylamino)methyl]-4-hydroxy-*m*-xylene-α,α'-diol; 2-(*tert*-butylamino)-1-(4-hydroxy-3-hydroxy-methyl-phenyl) ethanol; 4-hydroxy-3-hydroxymethyl-α-[(*tert*-butylamino) methyl] benzyl alcohol; albuterol

Trade names: Aerolin; Airet; Albuterol; Asmaven; Bronchovaleas; Cobutolin; Ecovent; Loftan; Proventil; Salbutard; Salbumol; Salbuvent; Sultanol; Venetlin; Ventodisks; Ventolin; Ventide; Volmax.

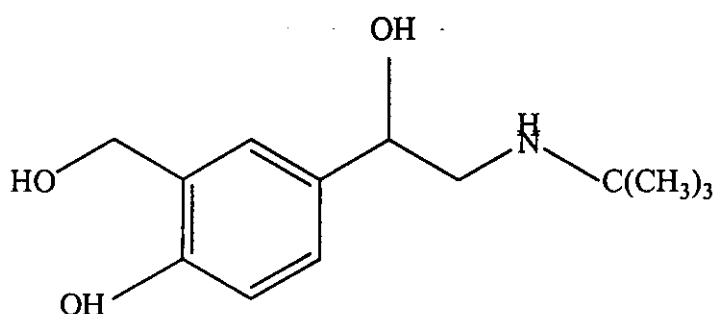Molecular formula: $C_{13}H_{21}NO_3$

Molecular weight: 239.31

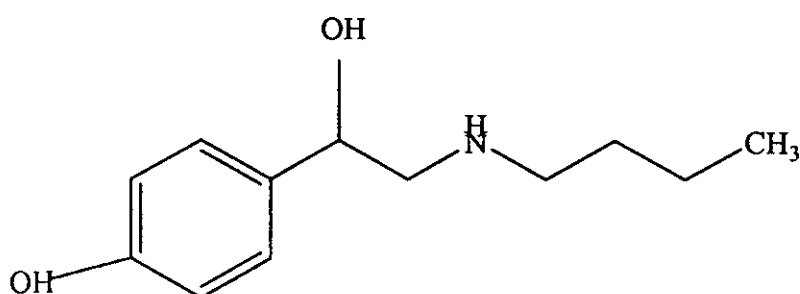
Figure 2 Structure of salbutamol

Figure 3 Structure of bamethan (IS)

Table 2 Physical properties of salbutamol and bamethan (IS)

Chemicals	Molecular weight	Color	Physical state	Melting point (°C)	Solubility	Dissociation Constant (pKa)	Uses
Salbutamol	239.31	Yellow or white	Crystalline powder	157-158 °C	Water; Ethanol	9.3, 10.3	Bronchodilator and tocolytic
Bamethan	209.28	-	Crystals	123.5-125 °C	Water	9.0, 10.2	Vasodilator (peripheral)

Sources: O'Neil *et al.*, 2001; Mills III and Roberson, 1987; Moffat *et al.*, 1986; Plumb, 1999.

In this work, an internal standard was used to control variations throughout the extraction and determine process. Bamethan (Figure 3) was chosen as an internal standard followed Miller and Greenblatt (1986), Bland *et al.* (1990), Loss *et al.* (2000) and Waters Corporation (2003). Its physical properties are shown in Table 2, other name and properties are also listed below. Other internal standards that have been used with HPLC-FLD were propanolol (Koh *et al.*, 2003) and terbutaline (Ouyang *et al.*, 2005).

Bamethan (Internal standard, IS)

CAS registry: 3703-79-5(free base); 5716-20-1(sulfate)

Systematic name: α-[(Butylamino)methyl]-4-hydroxybenzenemethanol; α-[(butyl- amino) methyl]-p-hydroxy-benzyl alcohol; 1-(p-hydroxyphenyl) -2-butylaminoethanol;1-(4-hydroxyphenyl)-1-hydroxy-2-butyl- aminoethane; 2-butylamino-1-p-hydroxyphenyl ethanol.

Trade names: Vasculat; Vasculit; Bupatol; Garmian; Butedrin; Rotesar; Vascunicol

Molecular formula: $C_{12}H_{19}NO_2$

Molecular weight: 209.28

1.2.3 Measurement of salbutamol residue

The measurement of drug residue in biological samples, *e.g.*, serum, blood and urine has focused on drug level after therapeutic dosing (pharmacokinetic studies) or after illegal use (Boyd *et al.*, 1996). The illegal uses of salbutamol were doping in sports or growth-enhancing in animal. Plasma and serum samples were frequently used for pharmacokinetic studies while urine sample was still used to analyse sample material in doping control. The World Anti-Doping Agency (WADA) prescribes that when a concentration of salbutamol (free plus glucuronide) is greater than 1000 ng mL^{-1} it is considered as an adverse analytical finding of anabolic agent (World Anti-Doping Agency, 2004). To monitor the abuse as growth-enhancing in animal, many sample materials were used for different reasons. The samples were collected from farmhouses, urine, faeces, hair and feed. Sampling of faeces is much easier and faster than urine, and the residue levels of β-agonists are comparable. At slaughter, edible tissues (liver, kidney and muscle) can be sampled next to body fluids (plasma, urine and bile) and eye samples (Euro-Diagnostica, 2003). In Thailand, edible tissues (liver, kidney and muscle) were sampled for analysis, in compliance with Food Safety policy of the Thai government.

1.2.4 Sample pretreatment

Before sample extraction and clean-up procedures, the sample must be prepared for the reliable and accurate results. This is particularly relevant to complexity nature of samples. For liquid sample, urine, plasma and serum suspended matter could be removed by centrifugation or filtration. Solid samples, such as liver and muscle, require a more intensive sample pretreatment to ensure that almost all analytes are exposed to the extracting solvent. One important pretreatment step in urine and liver samples is deconjugating its metabolite by enzyme or acid digestion. The mincer or homogenizer was frequently used for grinding and mechanical dispersion of the sample before extraction and clean-up procedure (Boyd et al., 1996).

1.2.5 Sample extraction and clean-up

1.2.5.1 Liquid-liquid extraction (LLE)

Liquid-liquid extraction (LLE) and solid phase extraction (SPE) were generally used to extract salbutamol from biological matrices. Many works reported the use of liquid-liquid extraction (LLE) for salbutamol from serum, plasma, liver and muscle samples. Di (2-ethylhexyl) phosphate (DEHP) has been use as ion pair reagent for extraction into chloroform in serum (Loss et al., 2000) or plasma (Hutchings., 1983; Miller and Greenblatt., 1986). Salbutamol was then back extracted into diluted acid prior to the injection to HPLC. Tan and Soldin (1984) used C_{18} SPE cartridge to clean-up serum before ion pair extraction using DEHP and heptanesulfonic acid, recovery of 79% was reported. Sagar and coworkers (1992) reported on the use of sodium dodecylsulfate (ion-pair reagent) to extract salbutamol from plasma and obtained a detection limit of 1 ng mL^{-1} and recovery of > 85% with 5.4% relative standard deviation (RSD).

The use of LLE for muscle, liver and kidney of porcine samples was described by Kaewklapanyachareon (2001). Minced sample was homogenized with 5% meta-phosphoric acid and methylene chloride where the methylene chloride layer was discarded. Meta-phosphoric layer was adjusted to pH 11-12 and extracted with tert-butyl alcohol:ethyl acetate (3:7). The extractant was dried and cleaned-up by extracted with ethyl acetate again before being analysed by HPLC-FLD. The reported

limit of quantification (LOQ) was 10 ng g^{-1} and recovery was 73.5-86.0 with 0.8-6.3 % RSD. This technique can improve the extraction selectivity of target analyte by the solvent selection or a combination of solvent. However, LLE presents some disadvantages, *i.e.*, high amount used of high price solvent, foaming or emulsion formation, complicated or variety of extraction and clean-up step, the deterioration of laboratory hygiene and work safety conditions, due to the manipulation of organic solvents and environmental contamination (Ramos, 2000).

1.2.5.2 Solid phase extraction (SPE)

Solid phase extraction (SPE) is one of the most popular techniques used for sample extraction/purification (Ramos, 2000). In analysis of salbutamol, SPE can be use as the only method in both extraction and clean up step, or can be used together with other procedure, such as LLE, matrix solid phase dispersion (MSPD) and supercritical fluid extraction (SFE). Salbutamol structure contains polar and non-polar groups as well as an ionizable amine that are able to interact with different solid phases. Four types of SPE have been used, *i.e.*, diatomateous earth, reverse-phase (C_8, C_{18} and Oasis HLB), mixed phase with ion-exchange and lipophilic properties (Bond-Elut Certify, XtractT and Clean Screen DAU) and normal phase (silica, alumina, diol and amine). The extraction mechanisms by adsorption, polar, non-polar, interactions with a polymeric sorbent with dual retention capability to retain polar and non-polar compounds and interactions with a combined phase involving hydrophobic and cation-exchange bonding have been described. The high recovery of salbutamol, about 80%, was obtained with the C_{18} cartridge (non-polar mechanism), however mixed-phase sorbent (reverse phase and cation exchange) showed better result in the simultaneous extraction of multi β-agonists (Bergés *et al.*, 1999; Ramos *et al.*, 1999). For liquid sample (urine, plasma and serum) extraction, this technique offers the advantages over LLE including, high recovery, less solvent usage, no foaming or emulsion problems, shorter sample-preparation, easier operation and incorporation into an automated process (Kataoka, 2003). However, this technique cannot be directly applied to solid sample (muscle, liver and kidney), other extraction technique must be performed before solid phase extraction (Simpson, 2000).

1.2.5.3 Matrix solid phase dispersion (MSPD)

In 1989, Barker and coworkers have adapted SPE, *i.e.*, matrix solid phase dispersion (MSPD), to extract solid sample. The matrix solid phase dispersion (MSPD) was an alternative residue extraction and clean-up technique. This technique has been most frequently applied to the isolation of drugs, herbicides, pesticides and other pollutants from animal tissue, fruit and vegetables. The method involves the dispersion of the sample over a solid support, followed by a preliminary purification and the subsequent elution of the analytes with a relatively small volume of solvent (Barker[b], 2000). A method to extract five β-agonists from liver tissue using MSPD was developed by Boyd *et al.* (1996), liver sample was blended manually with octadecylsilane (C_{18}) sorbent using morta and pestle. The resulting mixture was transferred to a column constructed from a syringe barrel, then washed with hexane-diethyl ether (6:4) and eluted the analyte with methanol. The extractant was digested with glucuronidase/sulfatase enzyme (Helix pomatia juice) solution and cleaned-up with C_{18} SPE cartridge and analysed by radioimmunoassay (RIA). This method is suitable for screening samples at approximately 0.5 ng g^{-1} levels. MSPD technique can directly extract solid sample of SPE in solid sample extraction, reduce analytical time and organic solvent consumption in solid sample (muscle, liver and kidney) analysis.

1.2.5.4 Supercritical fluid extraction (SFE)

Supercritical fluid extraction (SFE) technique has been used by O'Keeffe *et al.* (1999) to extract salbutamol and clenbuterol from bovine liver. The homogenised liver sample was digested with glucuronidase/sulfatase enzyme (Helix pomatia juice) over night and lyophilized for 72 h. The moisture was removed, ground to reduce particle size, then mixed with hydromatrix before packed into an SFE vessel. The SFE conditions reported were, SF-CO_2, modified with 1.5 mL methanol; temperature, 100 °C; pressure, 690 bar (10,000 lb in^{-2}; density, 1.042 g mL^{-1}) ; flow rate, 2 L min^{-1} for 10 min (20 L of depressurized CO_2 in total). The obtained extractant was cleaned-up with neutral alumina SPE column. Salbutamol and clenbuterol were determined with enzyme immunoassay (EIA). The recovery of salbutamol was 85±7 %. This technique used less organic solvent and a large number

of samples can extract at the same time. However, fresh tissue sample cannot directly be applied to this technique. Moisture and sample particle sizes were shown to affect salbutamol extractability. Sample pretreatment by drying and grinding was generally needed.

1.2.6 Detection of salbutamol

After a suitable extraction and clean-up procedure has been carried out, salbutamol could be detected by spectrophotometric, electrochemical or mass spectrometric techniques, normally following chromatographic separation, or by immunoassay (Boyd *et al.*, 1996).

1.2.6.1 Spectrophotometric detection

There were several reports on the use of spectrophotometric detection in a flow injection analysis (FIA) system for the determination of salbutamol in pharmaceutical and biological samples. Since, salbutamol has a low UV extinction coefficient (Shepherd, 1991), direct spectrophotometric measurement at the maximum wavelengths of either 276 or 225 nm can be carried out only in the absence of other UV-absorbing substances. Dol and Knochen (2004) reported the additive interferences of UV-detection due to the fairly low absorptivity and the existence of co-extractant. The forming of colour complexes was usually used to improve salbutamol absorbtivity. Šatínský and coworkers (2002) proposed an FIA system to determine salbutamol with Folin-Ciocalteau reagent (FC). The FC, based on the reduction of reagent by phenolic group of salbutamol, was a complexing agent for salbutamol and the absorbance was measured at 750 nm. However, this method is rather non-selective in the presence of other substances containing redox group. Therefore, SPE microcolumn (filled with 10-30 mg of silica gel chemically modified with carboxylic acid) was used to adsorb salbutamol before eluted to form complex with FC. In this case salbutamol is adsorbed and other compounds are not and this can help to overcome the problem. This system can determine 50 salbutamol tablet samples per hour with an average content of salbutamol in a tablet = 2.04±0.02 mg (declare content 2 mg of salbutamol in one tablet) and RSD (n=10) was 0.82%. In

urine analysis, frequency was 30 samples per hour and detection limit was 1 $\mu g\ mL^{-1}$ with RSD 1.7% (Šatínský *et al.*, 2002).

In 2004, Dol and Knochen reported the increase in selectivity by the formation of a complex with 4-aminoantipyrine to form chromogen and detected at 500 nm. In real sample analysis, salbutamol tablet and solution, the contents found by this system agreed with the HPLC method (established in the United States Pharmacopeia), *i.e.*, relative difference of contents less than 1.6%, with precision similar to the HPLC method (1-2%RSD).

In another development, Huclová and coworkers (2003) proposed the use of fluorescence and chemiluminescence to provide a selective and sensitive FIA system to determine salbutamol in urine and serum samples. Salbutamol showed a fluorescence signal in acidic medium with excitation and emission wavelength of 230 and 309 nm, and underwent the chemiluminescence reaction with acidic potassium permanganate. The fluorescence detection enabled the linear range of 0.05-100 $\mu g\ mL^{-1}$ (RSD 2.69%) with the detection limit of 0.2 $\mu g\ mL^{-1}$ and sample throughput of 24 samples per hour. For chemiluminescence, salbutamol was determined in the linear range 0.05-10 $\mu g\ mL^{-1}$ (RSD 1.53%) with detection limit 0.03 $\mu g\ mL^{-1}$ and sample throughput of 42 samples per hour. However, when real sample (urine and serum) analysis was performed with this system fluorescence detection showed better recovery.

Determination of salbutamol in pharmaceutical formulations and biological liquids by spectrophotometric detection (in FIA system) exhibits similarly or slightly lower sensitivity as conventional methods (HPLC, CE) but more rapid (Šatínský *et al.*, 2002). However, the sensitivity of spectrophotometric detection is not enough to determine trace salbutamol in lean meat tissue.

1.2.6.2 Gas chromatography

Gas chromatography (GC) is not suitable for direct analysis of salbutamol since the compound has polar groups (-OH, -NH$_2$). So, it needs to be converted to derivative (Zhang *et al*, 2006). However, GC has been widely used for the separation of salbutamol when combined with a sensitive and specific mass spectrometer. Mass spectrometric (MS) detection provides clear identification of

residues. Interfacing GC with MS may be *via* electron impact (EI) or chemical ionization (CI). In the EI mode the derivatized salbutamol molecule is volatilized and bombarded by electron to produce a protonated molecular ion (M^+). In the CI mode, a reactant gas, *e.g.*, ammonia, is bombarded with electrons to become ionized and collides with salbutamol molecules, there by ionizing it. The spectrum is then scanned for the molecular and fragment ions. For quantitative analysis, the MS instrument may be set to monitor the most abundant ions (SIM) (Boyd *et al.*, 1996).

Derivatization of salbuatmol before determining with GC-MS is not only to improve the poor gas chromatographic performance of this compound, but also to modify its fragmentation under electron impact (EI) (Polettini, 1996). Derivatizing reagents for salbuamol derivatives were boronic acid, *e.g.*, methylboronic acid and butylboronic acid, and silylating reagent, *e.g.*, *N*-methyl-*N*-trimethylsilyl trifluoroacetamide (MSTFA) and Bis(trimethylsilyl)trifluoroacetamide (BSTFA). When derivatized with boronic reagents salbutamol forms cyclic boronate and this has many advantages such as the high abundances of the most specific ions when compared to the corresponding EI silyl derivatives (Ramos *et al.*, 1998), the possibility to use shorter chromatographic columns, reducing time and saving helium (Reig *et al.*, 2005). The main disadvantage of derivatized with boronic reagents is its unsuitability to a multi β-agonists analysis since the formation of cyclic boronate does not occur in the β-agonists compounds with additional hydroxyl group in the *meta* position, *e.g*, fenoterol and terbutaline (Damasceno *et al.*, 2000). Ramos and coworkers (1998) also described simultaneous methyl- and butylboronic derivatization of eight β-agonists in urine analysis. This provide a detection limit of salbutamol at 2.6 ng L^{-1} and 22.8% recovery with %RSD (n=5) of 13.8-36.6.

Although cyclic boronate derivatives can be used as a comprehensive derivatization method for salbutamol analysis since it gave highly selective mass spectra but not all β-agonist compounds could be derivatized (Damasceno *et al.*, 2000). Because of this, the stability of boronic derivatives (Figure 4) has also been studied by stored it at either room temperature or -20 °C (Ramos *et al.*, 1998; Reig *et al.*, 2005). The result showed the decrease of salbutamol derivative right after the derivatization at room temperature and a good stability was restricted to

only the first day when stored at -20 °C. Base on these studies, salbutamol derivative with boronic reagents should be kept for a minimum period of time.
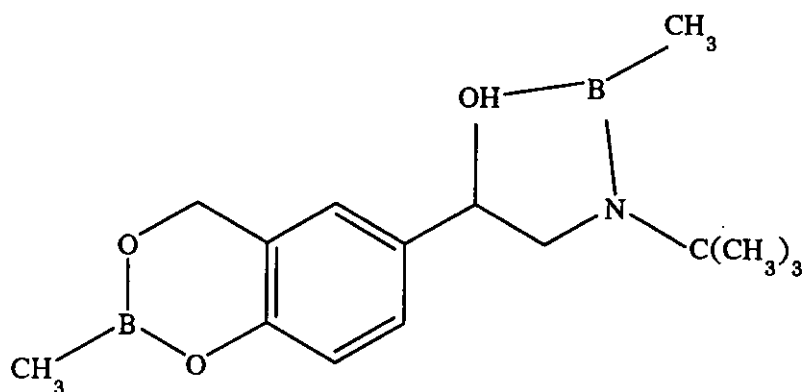
Figure 4 Structure of the bis-MBA derivative (Polettini, 1996).

There are also several reports that used silylating reagents for salbutamol derivatives, *i.e.*, N, O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and *N*-methyl-*N*-trimethylsilyl trifluoroacetamide (MSTFA). Either MSTFA or BSTFA alone is recommended for screening purposes owing to the formation of suitable derivatives for most of β-agonist compounds (Damasceno *et al.*, 2000) , *i.e.*, the hydroxy group of the β-ethanolamine chain in β-agonist structure was converted into trimethylsilylating (TMS) derivative. In this case Tris-*O*- trimethylsilyllation derivative (Tris-*O*-TMS) was formed for salbutamol. In EI mode, Tris-*O*-TMS showed intense fragment ion at *m/z* 369 corresponding to the protonated fragments complementary to m/z 86. The limit of detection (in urine analysis) was 0.5 ng mL^{-1}.

Ventura *et al.* (2000) carried out a multi β-agonist analysis in urine based on enzyme hydrolysis followed by solid phase extraction and derivatized with MSTFA. Salbutamol was determined in selected ion monitoring (SIM) acquisition mode. Detection limit was 0.5 ng mL^{-1} and recovery was 74.3 % with %RSD 9.1. Hernández-Carrasquilla (2000) reported the extraction of several β-agonists from bovine retina by liquid-liquid extraction (LLE) and Bond Elut Certify SPE column before derivatized with MSTFA at 60 °C for 15 min, the recovery of salbutamol (n=17) was 27.0±2.9. Saleh *et al.* (2000) claimed to achieve quantification

of salbutamol in serum at 2 ng mL^{-1} and a recovery of 60-65% by derivatizing the extractant with MSTFA at 60 °C for 30 min.

In 1996, Couper and Drummer derivatized three β-agonists from postmortem blood with Bis (trimethylsilyl) trifluoroacetamide with 1% trimethyl- chlorosilane (BSTFA-1%TMCS) for 15 min at 70 °C. The sample was sonicated and cleaned up with C$_{18}$ Sep-Pak SPE cartridge, the method gave a recovery of salbutamol at 85 % with % RSD 22.0-13.0. The report has pointed out that the MSTFA is a better alternative than BSTFA since it can be injected directly to the GC-MS with out fouling of the mass selective detector (MSD) ion source. Additionally, derivative stability is increased probably because MSTFA prevents loss of TMS groups (Hernández-Carrasquilla, 2000).

In 2000, Damasceno and coworkers evaluated different derivatizing agents, *i.e.*, silylative reagent, acylative reagent and boronic reagent, in the derivatization procedure of seven β-agonists. In silylation procedure, only hydroxyl groups are involved in the derivatization and the main products of salbutamol is Tris-*O*-Trimethylsilyl (Tris-*O*-TMS) derivative. In the EI mass spectra of the salbutamol-TMS derivatives, the base peak corresponds to a fragment at m/z 369. For acylation procedure, pentafluoropropionic anhydride was used, salbutamol derivative was not formed. However in combined silyllation and acylation procedure, salbutamol-*N*-TFA-tris-*O*-TMS derivative was the main product with mass spectrum *m/z* 369. The formation of cyclic methylboronates, using boronic reagent, an additional ring was formed with substituents of the aromatic ring. The methylboronate mass spectra of salbutamol are characterized by the abundant ions [M-15]$^{+}$ and the loss of [OBCH$_3$]$^{+}$ from the [M-15]$^{+}$, m/z 230.

The GC-MS approach has advantages of high sensitivity and selectivity for salbutamol analysis, but derivatization was required prior to analysis (Boyd *et al.*, 1996).

1.2.6.3 High performance liquid chromatography

High performance liquid chromatography (HPLC) was developed for salbutamol determination in biological sample and to study the pharmacokinetics in plasma (Ouyang *et al.*, 2005). Normal phase HPLC has been less

popular with only a few reports. Normal phase HPLC with fluorescence detection has been use to determine salbutamol in human plasma on silica column, with the detection limit of 0.5 ng mL^{-1} and 97.1-106.3 % recovery with % RSD less than 12.6 (Bland *et al.*, 1990). In 2003 Dimova also studied the behavior of salbutamol on normal phase HPLC by separated salbutamol on diol column with UV detector at 279 nm. The retention of salbutamol on diol column occurred through formation of H-bonds between its phenolic OH-groups and the silanol and diol OH-groups of the stationary phase.

A more popular approach is reverse phase HPLC owing to the hydrophobic interaction of the molecules with C_{18} or C_8 stationary phase. Alternatively, salbutamol could be ionized in aqueous solution at relative low pH value. The cation exchange column and diluted nitric acid was used as mobile phase (Ouyang *et al.*, 2005). Ion pair conditions have been used in conjunction with reverse phase chromatography to determine β-agonist. Ion pair reagents used were pentanesulfonic acid (Shishani *et al.*, 2003), octanesulfonic acid (Koole *et al.*, 1999), heptanesulfonic acid (Tan and Soldin, 1984) and hexanesulfonic acid (Waters, 2003).

The UV-Vis spectrophotometric detection, HPLC coupled with UV or diode array detector (DAD), was less used to determine salbutamol in biological matrix since UV light is not sufficiently absorb at low concentration. Thus, detection limit of the method was higher than its presented concentration in the sample. The use of HPLC-DAD was reported by Chin-En and Fusao (1994) for the determination of clenbuterol and salbutamol in swine serum and muscle. A photodiode array was set at 196 nm for the determination of salbutamol. System performance for salbutamol analysis showed detection limit at 100 μg L^{-1}, recovery of fortified serum was 86.7% and 62.9% for fortified muscle.

Selectivity and sensitivity of HPLC method can be improved by using electrochemical detector, *i.e.*, amperometric and coulommetric detections. These detectors can be used to determine salbutamol due to the presence of oxidizable phenolic hydroxyl group on the aromatic part of the molecule (Boyd *et al.*, 1996) which demonstrate the reaction on a thin-layer glassy carbon electrode (Tan and Soldin, 1984), porous graphite electrode (Emm *et al.*, 1988; Zhang *et al.*, 2004) and carbon fiber micro-flow cell (Sagar *et al.*, 1992). Electrochemical cell was usually

operated at high positive potential, 500-800 mV and detection limit (for plasma and serum samples) by this detector was usually less than 1 µg L^{-1}. For a large number of samples, coulometric detection is more suitable than amperometric detection since amperometric detector requires frequent maintenance (electrode polishing) due to electro deposition and adsorption on electrode surface (Zhang *et al.*, 2004). Selectivity and sensitivity of this detection could be enhanced by coupling it with ion-pair chromatography (IPC) where a detection limit of 0.4 µg L^{-1} was obtained (Tan and Soldin., 1984). Efficiency of an electrochemical detector could also be enhanced by using larger surface area electrode, *e.g.*, porous graphite electrode. In 1992 Sagar *et al.* developed carbon-fiber based flow electrode that showed some advantages over conventional electrochemical detectors using glassy carbon electrodes, *e.g.*, easy to prepare, flexible to operate and suffers less trouble from air bubbles and leaks. In addition, this kind of detector exhibited a better detection limit (1 µg L^{-1}) to that obtained using a conventional glassy carbon electrode flow detector (3 µg L^{-1}). However, electrochemical detection has some disadvantages that electrode needed intensive care or maintenance. To obtain reproducible results, some care must be taken, for example, the sensitivity of the working electrodes gradually declined, and cleaning of these electrodes was necessary (Zhang *et al.*, 2004).

Fluorescence detection was an alternative detector for salbutamol analysis due to the intrinsic fluorescence of the aromatic structure (Boyd *et al.*, 1996). Typical excitation/emission wavelengths are around 220/309 or 273/310 nm with the lowest detection limit of 0.25 µg L^{-1} in urine sample (Boulton and Fawcett, 1995). Table 3 summarized the determination of salbutamol by HPLC coupled with fluorescence detector.

Determination of salbutamol by HPLC is more suitable than by GC since derivatization is not required and it is more compatible with biological sample.

Table 3 Summary of determination of salbutamol by HPLC-FLD.

Mobile phase	Column	$\lambda_{ex}/\lambda_{em}$ (nm)	Sample matrix	Detection limit	Author
8% v/v acetonitrile containing 0.15% v/v phosphoric	Zorbax ODS 5μm (250×4.6 mm)	230/309	Plasma	1 μg L^{-1}	Hutchings, 1983
Water:acetonitrile (92:8), adjust pH 2.5 with phosphoric acid	C$_{18}$ 5μm (150×4.6 mm)	220/309	Plasma	1 μg L^{-1}	Miller and Greenblatt, 1986
Methanol containing 0.25%, 2M ammonium acetate (pH 7.5)	Spherisorb silica 3 μm (100×4.6 mm)	220/-	Plasma	0.5 μg L^{-1}	Bland et al., 1990
Hexane:dichloromethane:methanol: TFA (243:140:17:1)	Chirex 3022 3 μm (100×4.6 mm)	220/309	Plasma and urine	0.75 μg L^{-1}	Boulton and Fawcett, 1995
Hexane:dichloromethane:methanol: TFA (240:140:20:1)	Chirex 3022 (100×4.6 mm)	230/309	Urine	S (+) 10.8 μg L^{-1} R (-) 10.4 μg L^{-1}	Bergés et al., 1999
7% v/v acetonitrile containing 0.15% v/v phosphoric	Spherisorb ODS 10 μm (250×4.6 mm)	230/306	Serum	1 μg L^{-1}	Loss et al., 2000
4% acetonitrile containing 0.3 % phosphoric acid	TSK gel-ODS 80 Ts (150×4.6 mm)	226/310	Muscle, liver and kidney	10 μg kg^{-1}	Kaewklapanyachareon, 2001
50 mM phosphate buffer containing 1% TEA (pH 2.8):methanol (85:15 v/v)	NovaPak C$_{18}$ 5 μm (150×4.6 mm)	230/320	Plasma	5 μg L^{-1}	Koh et al., 2003
0.01 M ammonium acetate (pH 3.0) containing 6% (v/v)	C$_{18}$ (250×4.6 mm)	273/310	Plasma	-	Ouyang et al., 2005

Note: λ_{ex}, λ_{em} = Excitation wavelength, Emission wavelength; TEA = Triethylamine; TFA= Trifluoroacetic acid.

Determination of salbutamol by liquid chromatography coupling with mass spectrometry (LC-MS or LC-MS-MS) is also more suited than GC-MS because it can analyze salbutamol without derivatization procedure (Boyd *et al.*, 1996). This detection provides more structural information at low analyte levels than any other detection technique. Selectivity is best when full spectral scans can be acquired to compare sample and standard peak (Aerts *et al.*, 1995). Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) interface was used in salbutamol analysis. Salbutamol was analyzed by selected reaction monitoring (SRM) for the fragmentation m/z 240-m/z 222 and m/z 240-m/z 166 (Jacobson *et al.*, 2003), multiple reaction mode (MRM); 240 m/z-148 m/z (Schmeer *et al.*, 1997) and selected ion monitoring (SIM) m/z 166 (Zhang *et al.*, 2006). Analysis of salbutamol with LC-MS or LC-MS-MS generally provides ng g^{-1} or sub - ng g^{-1} detection limits for salbutamol, more or less independent of the biological matrix which is analyzed. However, combined HPLC with MS detection was an expensive technique for routine laboratories and it always used as confirmatory method for residue analysis (Aerts *et al.*, 1995).

1.2.6.4 Capillary electrophoresis (CE)

Capillary electrophoresis (CE) is becoming an important technique for salbutamol analysis in biological matrix (Zhou *et al.*, 2001; Chen *et al.*, 2005) and pharmaceutical formula (Malkki-Laine and Hartikinen, 1996; Felix *et al.*, 2006) due to its high speed, high separation efficiency, and ultra-small sample (Chen *et al.*, 2005). Moreover, CE is known to be one of the most powerful analytical techniques for chiral separations (enantiomeric separation), *i.e.*, S-(+) salbutamol and R-(-) salbutamol analysis, by addition of suitable cyclodextrins (Esquisabel *et al.*, 1997; Servais *et al.*, 2006).

Many detection techniques were coupled to CE to determine salbutamol such as diode array detector (DAD), electrochemical detection (ECD) and mass spectrometry (MS). CE with amperometric detection (CE-AD) was used by Zhou and coworkers (2001) to separate and determine three β-agonists in serum. This method was simple and fast since a serum sample could be directly applied to the CE-AD system without any sample pre-treatment except filtration. Detection limit for

salbutamol was 2.0×10^{-7} mol L^{-1} and average recovery ranged between 92.2 and 99.5% with RSD less than 4.71%. For another electrochemical detector, CE coupled with contactless conductivity detection (CE-C^{4}D) was used by Felix *et al.* (2006) to determine salbutamol in syrups. Just before measurements, syrups were diluted with the running electrolyte and a detection limit at 3.3×10^{-5} mol L^{-1} was obtained.

However, sample loadability and concentration sensitivity in capillary electrophoresis still limits its use as a separation technique prior to detection. Different attempts have been make to enhance sensitivity in CE by employing sample concentration technique prior to the CE separation. Isotachophoresis (ITP) technique was usually used for sample enrichment on line with CE but the time required was longer than 1 h. Thus, electro extraction (EE), *i.e.*, using an electric field in liquid-liquid extraction, was combined with ITP (EE-ITP) to enable the fast extraction of charge salbutamol from large volumes of organic solvents into a 100 μm I.D. fused-silica capillary. Automation could easily be accomplished, as the whole procedure was performed in single capillary. Vlis and coworkers (1995) on lined EE-ITP with CE-MS (EE-ITP-CE-MS) to determine salbutamol down to 2×10^{-9} mol L^{-1}. However, this technique was not applied for the analysis of salbutamol in real samples.

Although the analysis techniques for salbutamol (extraction and determination methods) have been widely studied, there are only a few reports on the determination of salbutamol in lean meat and its detection limit was quite high, *i.e.*, 100 ng g^{-1} (Chin-En and Fusao, 1994), 25 ng g^{-1} (Trakulosot, *et al.*, 2001) and 10 ng g^{-1} (Kaewklapanyacharoen, 2001). Muscle or meat samples contain lower concentration of salbutamol and its matrix complexity making extraction/detection more difficult, require large solvent consumption and long analysis time. This work focused on the methodology for the determination of salbutamol in lean meat as alternative method for routine analysis laboratory. The method consisted of optimization of two fluorescence detection systems, *i.e.*, spectrofluorometry and high performance liquid chromatography with fluorescence detection (HPLC-FLD), and optimization of sample preparation, *i.e.*, combined matrix solid phase dispersion (MSPD) with solid phase extraction (SPE). Moreover, ion pair reagent was added to HPLC-FLD to enhance the selectivity of method.

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1.3 Objectives

The objectives of this research are to optimize spectrofluorometric and ion-pair chromatography with fluorescence detector conditions for qualitative and quantitative analysis of salbutamol and to study the appropriate sample preparation and analysis of salbutamol in lean meat sample.