DETERMINATION OF ENROFLOXACIN AND CIPROFLOXACIN RESIDUES IN CHICKEN BREAST AND EGGS BY HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY AND LIQUID CHROMATOGRAPHY MASS SPECTROMETRY

JERAWAN TANSIRI

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Thesis
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ABSTRACT

Enrofloxacin is an antibiotic widely used in animal husbandry. Foods from animal origin such as pork, beef, chicken, fish, and shrimp, especially chicken and eggs are suspected to be contaminated by enrofloxacin and its metabolite ciprofloxacin. Antibiotic contamination causes deterioration in meat quality and affects human health. Screening tests for enrofloxacin and ciprofloxacin residues in chicken breast and eggs by high performance thin layer chromatography have been developed. Appropriate solvents were used for extracting the residues from samples before analyzing by high performance thin layer chromatography, which could distinguish the analytes from other substances. The detection limit, sensitivity and specificity in chicken breast were 100 ppb, 90% and 100%, respectively. The detection limit, sensitivity and specificity in eggs were 100 ppb, 80% and 100%, respectively. Liquid chromatography mass spectrometry was used as the confirmatory method to analyze the same samples that were used in validation experiments of high performance thin layer chromatography. According to liquid chromatography mass spectrometry, all samples conformed. Chicken breast and egg samples from various sources around Bangkok were sampled and tested by high performance thin layer chromatography. From 116 samples of chicken meat, only one sample exceeded the limit of enrofloxacin residue (100 ppb). All 62 egg samples were within the same limit of enrofloxacin residue (100 ppb).

KEYWORDS: ENROFLOXACIN/CIPROFLOXACIN/RESIDUES/HPTLC/ LC-MS/ CHICKEN/EGG

52 pages

การหาปริมาณเอ็นโรฟลอกส์ซาซินและไซโปรฟลอกส์ซาซินตกค้างในเนื้ออกไก่และไข่โดยวิธีไฮเพอร์ฟอร์มานซ์ทินแลเยอร์โครมาโทกราฟีและลิควิดโครมาโทกราฟีแมสเสปกโทรเมทรี

DETERMINATION OF ENROFLOXACIN AND CIPROFLOXACIN RESIDUES IN CHICKEN BREAST AND EGGS BY HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY AND LIQUID CHROMATOGRAPHY MASS SPECTROMETRY

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บทคัดย่อ

เอ็นโรฟลอกส์ซาซินเป็นยาต้านจุลชีพกลุ่มควิโนโลน ซึ่งใช้กันแพร่หลายในสัตว์ที่เลี้ยงไว้เพื่อเป็นอาหารสำหรับมนุษย์ และอาหารที่มีต้นกำเนิดจากสัตว์ ได้แก่ หมู วัว ไก่ ปลา และกุ้ง โดยเฉพาะอย่างยิ่งในผลิตภัณฑ์จากเนื้อไก่และไข่อาจมีการปนเปื้อนยาต้านจุลชีพเอ็นโรฟลอกส์ซาซินและเมทาบอไลท์ไซโปรฟลอกส์ซาซิน การปนเปื้อนของยาย่อมส่งผลให้อาหารไม่มีคุณภาพและส่งผลต่อสุขภาพของมนุษย์ การตรวจคัดกรองการปนเปื้อนยาเอ็นโรฟลอกซาซินในเนื้อไก่และไข่ที่พัฒนาขึ้นนี้อาศัยหลักการไฮเพอร์ฟอร์มานซ์ทินแลเยอร์โครมาโทกราฟี ทำได้โดยการสกัดสารออกจากตัวอย่างด้วยตัวทำละลายที่เหมาะสมแล้วนำสารสกัดมาตรวจหาสารด้วยไฮเพอร์ฟอร์มานซ์ทินแลเยอร์โครมาโทกราฟี ที่ได้พัฒนาขึ้นทำให้สามารถแยกสารออกจากสารอื่นในตัวอย่างได้ การตรวจประเมินปริมาณเบื้องต้นที่ระดับ 100 พีพีบี ความไวของวิธีเท่ากับร้อยละ 90 และ มีความจำเพาะของวิธีเท่ากับร้อยละ 100 ในตัวอย่างเนื้อไก่ ส่วนตัวอย่างไข่ไก่ พบว่าสามารถตรวจประเมินปริมาณเบื้องต้นที่ระดับ 100 พีพีบี ความไวของวิธีเท่ากับร้อยละ 80 และ มีความจำเพาะของวิธีเท่ากับร้อยละ 100 วิธีลิควิดโครมาโทกราฟีแมสเสปกโทเมทรี ได้ถูกนำมาตรวจสอบตัวอย่างที่ได้ตรวจคัดกรองเบื้องต้นแล้วในขั้นการตรวจสอบความถูกต้องของวิธีไฮเพอร์ฟอร์มานซ์ ทินแลเยอร์โครมาโทกราฟี พบว่าผลการทดสอบจากทั้งสองวิธีมีความสอดคล้องกัน ทำให้วิธีคัดกรองที่พัฒนาขึ้นนี้มีความน่าเชื่อถือมากยิ่งขึ้น จากการประเมินคุณภาพวัตถุดิบจากแหล่งวัตถุดิบภายในเขตกรุงเทพมหานครและปริมณฑล พบว่ามีตัวอย่างเนื้อไก่ที่ไม่ผ่านการทดสอบ คือมีปริมาณยาต้านจุลชีพเอ็นโรฟลอกซาซินตกค้างเกินกว่าระดับการยอมรับ (100 พีพีบี) 1 ตัวอย่าง จากตัวอย่างเนื้อไก่ที่สุ่มตรวจ 116 ตัวอย่าง และไม่พบการปนเปื้อนเกินระดับ 100 พีพีบี จากตัวอย่างไข่ไก่ที่สุ่มตรวจ 62 ตัวอย่าง

52 หน้า

CONTENTS

CONTENTS (cont.)

LIST OF TABLES

LIST OF TABLES (cont.)

LIST OF FIGURES

LIST OF ABBREVIATIONS

AR	analytical grade
cm	centimeter
EU	European Union
g	gram
HPLC	high-performance liquid chromatography
HPTLC	high performance thin layer chromatography
hr	hour
LC-MS	liquid chromatography-mass spectrometry
ID	internal diameter
LOD	limit of detection
LOQ	limit of quantitation
mg	milligram
min	minute
mL	milliliter
mm	millimeter
MRL	maximum residue limit
nm	nanometer
SIM	selected ion measuring
TLC	thin layer chromatography
RSD	relative standard deviation
SD	standard deviation
UV	ultraviolet light
VIS	visible light
r^2	correlation coefficient
µg	microgram
µL	microliter
µm	micrometer

CHAPTER I

INTRODUCTION

Agricultures play an important role in Thailand. Foods from plants and animals are needed for domestic supply and export. Poultry is one kind of goods for both domestic market and export. Moreover, it is now on the top 10-exported goods to European countries. In order to increase the supply for the markets, using of antibiotics in poultry are persuaded. Antibiotic has benefits in the treatment of infectious diseases and use as the growth enhancement. At the same time, antibiotic has disadvantage in reducing of food quality and impact human health. Nowadays, many countries around the world have concerned about this problem. The monitoring programs on the quality of the foods are established. Enrofloxacin is a member of fluoroquinolone antibiotic. It has been widely used in poultry against both Gram positive and Gram negative bacteria. The mechanisms of action are inhibiting topoisomerase II (DNA gyrase) in Gram negative bacteria in the DNA metabolism, and topoisomerase IV is found to be a target in Gram positive bacteria. It is only used in animals, not in human because of its toxicity to human (1). Enrofloxacin provides 2 major metabolites; enrofloxacin glucuronide and ciprofloxacin (2) (Fig. 1-2).

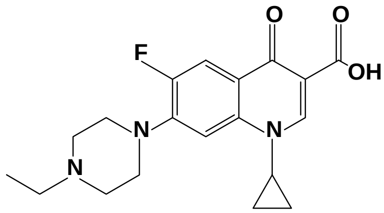


Fig.1 Enrofloxacin

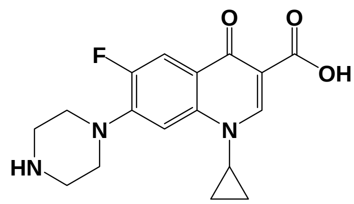


Fig.2 Ciprofloxacin

At present, US FDA has announced the Agency's final decision to no longer allow distribution or use of enrofloxacin for the treatment of bacterial infections in poultry because there were reported that the use of enrofloxacin in poultry caused resistance in *Campylobacter*, a bacteria that causes food-borne illness. Chickens and turkeys normally harbor *Campylobacter* in their digestive tracts without causing poultry to become ill. Enrofloxacin does not completely eliminate *Campylobacter* from the birds' intestinal tracts, and those survive *Campylobacter* bacteria are resistant to fluoroquinolone drugs. These resistant bacteria multiply in the digestive tracts of poultry. They persist and spread through transportation and slaughter, and are found on chicken carcasses in slaughter plants and retail poultry meat. These fluoroquinolone-resistant organisms are transferred to humans and cause the development of fluoroquinolone-resistant Campylobacter in humans, which can be health hazard (3). However, there should be an action to this situation in Thailand. The monitoring program on the quality of the foods from those animals seems to be a part of quality assurance system. Therefore the screening and quantitative analytical methods for enrofloxacin residues and its major metabolites, ciprofloxacin, are necessary. Maximum residue limit (MRL) for the sum of enrofloxacin and ciprofloxacin in chicken muscle, liver and kidney are 100, 200 and 300 ppb, but no established limit for eggs (4).

Various analytical methods were developed for screening of enrofloxacin and ciprofloxacin in chicken. Microbial assay were used in screening of 10 quinolones in chicken muscle which provided the limit of detection around their permitted limits. This method has disadvantage in lacking of selectivity, time consuming and complicated techniques (5). ELISA techniques were used for determining of fluoroquinolones residues in chicken meat and they have been developing to provide better selectivity and separation efficiency by combination with other techniques such as fluorescence tagging. The sensitivities of the methods were 100 – 200 ppm. The limitations of these methods were low sensitivity, short lifetime, and complication in interpretation of the results (6-8). Good screening method should overcome these advantages. High performance thin layer chromatography (HPTLC) is one mode of chromatography. It enables to separate trace impurities or major component from each other. From the information provided for that separation we can identify each component. Moreover, it is a simple, high sample through put, and low cost.

Many quantitative methods were reported using liquid chromatography with different detectors such as ultraviolet detector (single wavelength and diode array detector) (9-10), fluorescence detector (11-14), mass spectrophotometer and tandem mass spectrophotometer (15-20). Capillary electrophoresis with mass spectrophotometer was reported for analysis of quinolones in chicken and fish (21). Most methods use many solvents, much time consuming and complicated techniques make them unsuitable for routine works. The aim of this study is to develop a simple and rapid screening method for enrofloxacin and ciprofloxacin residues in chicken breast and eggs using HPTLC, and quantitative method using LC-MS.

CHAPTER II

LITERATURE REVIEW

1. Enrofloxacin

Enrofloxacin is a member of fluoroquinolones antibiotic which is developed from nalidixic acid. It is used for treatment of bacterial infection caused by gram positive and gram negative bacteria such as *Mycoplasma*, *Mycobacterium*, *Chlamydia Legionella* and *Bruella*. The mechanisms of actions are inhibition of topoisomerase II; DNA gyrase in DNA formation of gram negative bacteria and inhibition of topoisomerase IV in gram positive bacteria. Enrofloxacin is used for veterinary medicine only because it is toxic to human (1). The major metabolites of enrofloxacin are enrofloxacin glucuronide and ciprofloxacin (2).

1.1 Physicochemical properties of enrofloxacin

Enrofloxacin exhibits maximum UV absorption at various wavelengths in different solvents such as the maximum wavelength at 276 and 318 nm in acetate buffer, and at 280 and 322 nm in methanol and ethanol. The pH of the solvent influences its maximum wavelengths. The maximum wavelengths at 276 – 280 and 314 – 322 nm are obtained in the pH about 2.8 to 8.8. Enrofloxacin has fluorescent property. The maximum emission and excitation wavelengths are 439 and 315 nm at pH 6.8 (22).

Enrofloxacin has four forms such as acidic cation, neutral unionized species, intermediate zwitterian ion and basidic ion, which pKa_1 of 5.94 and pKa_2 of 8.70.

The solubility of enrofloxacin depends on the pH of the solution. The maximum solubility is obtained at the pH of 5.02 and the higher concentration of acetate buffer, the greater the solubility.

1.2 Medical uses

Since 1996 enrofloxacin has approved by US FDA, it is used for treatment of bacterial infections such as *Escherichia coli* and *Pasteurella miltoida* in chickens and turkey. This medicine is restricted to use only the some animal diseases because it causes bacterial resistant to the other medicines in poultry (3). The contamination of the medicine can occur from the cages through the slaughters which across from sick animals to other healthy animals. Human are affected with this problem by higher rates in bacterial resistant, higher rates in allergy, and higher mortality rates (24). The maximum residue limits have been established to control the quality of the meats such as the limit of enrofloxacin residue which is shown in Table 1.

Table 1. Maximum residue limits of enrofloxacin in poultry (4)

Medicine	Residue	Animal	MRLs (μg/kg)	Tissue
Enrofloxacin	Enrofloxacin and ciprofloxacin	poultry	100	muscle
			100	skin and fat
			200	liver
			300	kidney

1.3 Analytical methods

Various analytical methods have been developed for determination of enrofloxacin and ciprofloxacin residues in chicken. Microbial assay by agar diffusion method were used in screening of 10 quinolones in chicken muscle which provided the limit of detection around their permitted limits (5). The smaller inhibition zones were observed at low level of standard solution which caused interferences of tissue matrix, and at high level, the differences were not substantial. The test kits for antimicrobial screening in pig and chicken meat, serum, and urine were developed (7). The principle of the tests was to inhibit the growth of bacteria in vitro. The detection limit for enrofloxacin was 8 ppm. These methods were not able to detect the residue at the MRLs level, and they have problem of high cost, lack of specificity, and time consuming.

ELISA techniques were used for determining of fluoroquinolones residues in chicken meat. They have been developed to provide better selectivity and separation efficiency by combination with other techniques (6-8). The sensitivities of the methods were 100 – 200 ppm. The limitations of these methods were low sensitivity, short lifetime, and complication in interpretation of the results.

Many quantitative methods were reported using liquid chromatography with different detectors such as ultraviolet detector (single wavelength and diode array detector) (9-10) fluorescence detector (11-14), mass spectrophotometer and tandem mass spectrophotometer (15-20). Capillary electrophoresis with mass-spectro-photometer was used to analyze of quinolones in chicken and fish (21). Most methods use many solvents, much time consuming and complicated techniques make them unsuitable for routine works (Table 2).

High performance thin layer chromatography (HPTLC) is one mode of chromatography. It enables to separate trace impurities or major component from each other. From the information provided for that separation we can identify each component.

The substances are differently moved on stationary phases which depend on their physicochemical properties, the type of stationary phases and mobile phases. The Rf value is calculated to show the position of the substance on the stationary phases with each system. The Rf value is between 0 to 1 and calculated by the formula

$$Rf = \frac{\text{The length of substance move}}{\text{The length of solvent move}}$$

HPTLC screening method for quinolone residues in pig muscle were reported. Pig samples were extracted then cleaned up with solid phase extraction. The first detection was inspected by UV illumination at 312 nm then turbium chloride was sprayed and inspected by UV illumination at 312 nm again. The detection limit was 15 ppb. This method was reliable with the good sensitivity and specificity. Moreover, it was sensitive, rapid, and low cost (25).

The aim of this study is to develop a simple and rapid screening method for enrofloxacin residues and its major metabolites in chicken breast and eggs using HPTLC. The LC-MS was developed to use as a standard method as well.

Table 2 Analytical techniques for enrofloxacin residues

Analytes	Sample	Extraction procedure			Method of analysis	Reference
		Extraction technique	Reagents	Solid phase extraction		
Enrofloxacin Ciprofloxacin Danofloxacin Difloxacin Flumaquin	Turkey muscle	Liquid extraction	Acetronitrile +phosphoric acid	ENV+Isolute	LC-UV, LC-MS, LC-MS/MS	Clemente M, et al.
Enrofloxacin Ciprofloxacin Sarafloxacin Oxolinic acid Danofloxacin Flumaquin Difloxacin Marbofloxacin	Pig muscle Milk aquaculture products	Liquid extraction	Water extract	Isolute C18	LC-MS/MS	Hoof NV, et al.
Enrofloxacin Sarafloxacin Danofloxacin	Chicken muscle and liver	Liquid extraction	0.1 M phosphate, pH 9 buffer Acetonotrile, Hexane, Ether	-	LC-fluorescence LC-MS/MS	Marilyn J

Table 2 Analytical techniques for enrofloxacin residues (continue)

Analytes	**Sample**	**Extraction procedure**			**Method of analysis**	**Reference**
		Extraction technique	**Reagents**	**Solid phase extraction**		
Enrofloxacin Ciprofloxacin Nalidixic acid Ofloxacin Norfloxacin Danofloxacin Enofloxacin Sarafloxacin Flumaquin	Chicken muscle Egg yolk	Liquid extraction	0.1% TFA in acetronitrile 0.75 M sodium hydroxide in acetronitrile	Merck lichrolute C18	HPLC-UV	Christodoulou EA, et al.
Enrofloxacin Ciprofloxacin Sarafloxacin Oxolinic acid Danofloxacin Flumaquin Difloxacin Marbofloxacin	Chicken muscle	Liquid extraction	Acetonitrile, ammonia	Isolute C18	LC-MS/MS	Schneider MJ, et al.
Enrofloxacin Ciprofloxacin Sarafloxacin Difloxacin	Chicken muscle and liver	Liquid extraction enzyme digestion sonication	10%TCA+ acetronitrile Collagenase+ protease	C18, C8, SDB, NH2, bezenesulfonic acid	LC-fluorescence	Posynak A, et al.

CHAPTER III
MATERIALS AND METHODS

1. Materials

1.1 Instruments and equipments

Name	Source/Supplier
Analytical balance	AL204, Mettler Toledo,USA
Centrifuge	Labofuge200, Heraeus
Densitometer	Camag, Linomat5, TLC scanner
Liquid Chromatography Mass Spectrometer	LC-10ADVB (pump), DGU-12AM (degasser), SCL-10AVP (system controller), LCMS-2010IV (LCMS), Shimadzu, Japan
TLC aluminum sheets Silica gel 60F 254	Merck, Germany
HPTLC aluminum sheets Silica gel 60F 254	Merck, Germany
HPLC column, C18, 3µ, 2.1-mm I.D. x 150 mm	Hypersil GOLD, USA
Nylon filter 0.2 µm, 46 mm in diameter	National Scientific, UK
Sep-Pak C18, 500 mg, 3 mL	Water, USA

1.2 Chemicals

Name	Grade	Source/Supplier
Acetic acid	AR	Lab-Scan Analytical Sciences, Thailand
Acetonitrile	HPLC	Lab-Scan Analytical Sciences, Thailand
Ammonium acetate	LC-MS	Ajax Finechem, Australia

1.2 Chemicals (cont.)

Name	Grade	Source/Supplier
25% Ammonia solution	AR	Carlo erba, Italy
Celite 545	AR	Fluka, Switzerland
Ciprofloxacin hydrochloride	AR	Zhejiang guobang Pharmaceutical co.,Ltd., China
Enrofloxacin	AR	Xinchang, China
Trichloroacetic acid	AR	Riedel-de Haen, Aldrich, Germany
Methanol	AR	Lab-Scan Analytical Sciences, Thailand
Methylene chloride	AR	Lab-Scan Analytical Sciences, Thailand

2. Method

2.1 Sample preparation

2.1.1 Sample collection and sample size

Five hundred grams of chicken breast and twenty eggs were collected from each market in Bangkok and the provinces around the city such as supermarket, market, and temporary market as described in Table 3. They were kept in a freezer (at -20 ^{O}C) and extracted prior to the analysis. Sample sizes are shown in Table 4 and sampling sources are listed in Appendix 1.

Table 3 Classification and definition of the market (27)

Type of market	Description
Supermarket	The market building with regular operation or at least 1 time per week
Market	The market with no structure building and regular operation or at least 1 time per week
Temporary market	The market with no structure building and temporary operation or at particular days

Table 4 Sample size

Sample	Source of sample			Total number of samples
	Supermarket	Market	Temporary market	
Chicken breast	27	32	57	116
Egg	5	40	17	62

2.1.2 Extraction procedure for enrofloxacin in chicken breast and eggs (28)

(1) Five hundred grams of chicken breast were homogenized and 20 grams were extracted with 40 mL of 0.1% trichloroacetic acid in methanol. The mixture was shaken for 30 min and filtered through a Whatman no.1 filter paper. The filtrate was centrifuged at 10000*g* for 10 min, the clear supernatant was collected and added with 2 g of anhydrous sodium sulfate. The tube of mixture was centrifuged again at 10000*g* for 10 minutes. Twenty milliliters of the clear supernatant was evaporated until dry, using water bath at a temperature 40 – 45 ^{O}C and nitrogen stream. The residue was dissolved in 0.1% trifluoroacetic acid before cleaned up with Sep-Pak C18, which were preconditioned with 2 mL of methanol and 2 mL of water then eluted with 1.5 mL of 0.1% trichloroacetic acid in acetonitrile and 0.5 mL of acetonitrile. Before being analyzed by HPTLC, the eluent was evaporated to dryness with nitrogen then 200 μL of 0.1% trichloroacetic acid in methanol was added. The eluent was filtered through 0.2-μm membrane prior to LC-MS.

(2) Twenty whole eggs were prepared in the same manner as

described in (1) except the samples were extracted with 40 mL of 0.75 M sodium hydroxide in acetonitrile.

2.2 Standard preparation

The standard solutions of 100 ppb of enrofloxacin and ciprofloxacin were prepared by diluting the stock solution with methanol. Standard stock solution of enrofloxacin and ciprofloxacin were diluted with 0.1 % trichloroacetic acid in methanol.

2.3 HPTLC screening method

2.3.1 Optimization of HPTLC

HPTLC mobile phases were optimized to give an appropriate separation. Seven mobile phases were tested and the best system was used (29).

(1) Methylene chloride: methanol: 25% ammonium hydroxide: acetronitrile (4:4:2:1)

(2) Methylene chloride : ethanol : 25% ammonium hydroxide (2:2:1)

(3) Methylene chloride : methanol : 25% ammonium hydroxide (150:75:15)

(4) Methanol : water : Methylene chloride: toluene : diethylamine (40:8:40:20:14)

(5) Methylene chloride : methanol : 2-propanol : 25% ammonium hydroxide (3:3:5:2)

(6) Methanol : 2-propanol : 25% ammonium hydroxide (5:5:2)

(7) Acetone : methanol : 25% ammonium hydroxide (5:3:2)

2.3.2 Detection limit

Both enrofloxacin and ciprofloxacin standard solutions at the concentration of 100 ppb were added into the blank extraction. The standard solutions were varyied from 5, 10, 15, 20, 25 and 30 μL, were tested by HPTLC for selectivity and detection limit. The application volume which gave the signal to noise ratio of 3:1 was assigned as the detection limit and used as application volume of the HPTLC test. The samples were extracted and screened for enrofloxacin residues using HPTLC. The HPTLC condition were

HPTLC plate : HPTLC aluminum silica gel 60F 254

Mobile phase : propanol: methanol : 25% ammonium hydroxide 5:5:2

Application volume : 10 μL

Developed length : 75 millimeters

Detection : blue fluorescence at 366 nm

2.3.3 Validation of HPTLC screening method (21)

Twenty of positive samples and twenty of negative samples were prepared and were tested to determine sensitivity, specificity and accuracy.

Positive samples were prepared by adding both standard enrofloxacin and ciprofloxacin solutions into the chicken and egg samples to obtain a concentration of 100 ppb. The samples were extracted before test. Negative samples were samples which contained no analytes.

Sensitivity, specificity and accuracy were evaluated as following

$$\text{Sensitivity} = \frac{\text{True positive} \times 100}{\text{Total number of positive samples}}$$

$$\text{Specificity} = \frac{\text{True negative} \times 100}{\text{Total number of negative samples}}$$

$$\text{Accuracy} = \frac{(\text{True positive} + \text{True negative}) \times 100}{\text{Total number of positive and negative samples}}$$

2.3.4 Interpretation of results

A suspected non compliant sample with the same color and R_f value with the standard solution revealed any spot from the sample preparation more intense than that of the standard solution (100 ppb of enrofloxacin and ciprofloxacin). The result was confirmed by an LC-MS method.

2.4 Quantification and confirmatory method of enrofloxacin by LC-MS

A suspected non compliant sample from screening test by HPTLC method was further confirmed and quantitative analyzed for the residual contaminations by LC-MS. The LC-MS condition was modified from Clemente M, et al (9), were as followed:

LC condition

Column	:	Hypersil GOLD (3μ, 2.1-mm ID x 150 mm)
Mobile phase	:	0.02 M ammonium acetate (pH 2.5): acetonitrile 88:12
Flow rate	:	0.3 ml/min

MS condition

Acquisition mode	:	SIM (selected ion measuring) (+)
Interface temperature	:	250 °C, CDL temperature : 230 °C
Nebulizing Gas Flow	:	0.18 L/min, Heat block : 200 °C
Voltage	:	1.6 kV
Detection	:	m/z Ciprofloxacin = 331.05, Enrofloxacin = 359.00 , and Norfloxacin = 319.10

The developed LC-MS method was validated for the quantitative analysis of enrofloxacin, ciprofloxacin and norfloxacin (internal standard) in chicken and egg extracts. The parameters to be considered in the method validation study was linearity (r^2), accuracy (recoveries), precision (%RSD), limit of detection (LOD) and limit of quantitation (LOQ).

2.4.1 Validation of LC-MS

(1) Linearity and range

Five concentrations of standard solutions at the concentration of 0.72 to 24 ppb of enrofloxacin and ciprofloxacin with norfloxacin as internal standard were prepared. Each solution was injected in triplicates (n = 3). The linear regression of the standard curve was obtained by plotting peak area ratio between both reference standards and internal standard versus concentrations. The linear regression equation and correlation coefficient (r^2) was calculated. A calibration curve was described by the equation:

$$y = ax + b$$

where,

y = peak area ratio between peak area of an analyte and peak area of internal standard;

a = the slope;

x = the concentration of an analyte;

and b = the intercept of a line fit to the data.

The correlation coefficient (r^2) should be more than 0.995.

(2) Accuracy

The accuracy of a measurement is defined as the closeness of the measured value to the true value. Typically, accuracy is presented and determined from recoveries study performed by standard addition method. The standard enrofloxacin and ciprofloxacin were added into samples and analyzed. The percent recoveries were calculated by the following equation:

$$\% \text{ Recovery} = \frac{X_{found} \times 100}{X_{added}}$$

where,

X_{found} = the concentration of standard found in the spiked sample;

and X_{added} = the concentration of the standard added.

(3) Precision

Precision was determined by the percent relative standard deviation (%RSD). Three different concentrations of standard, 9.6, 12 and 24 ppb of enrofloxacin and ciprofloxacin, were analyzed and each concentration was injected triplicates.

The percent relative standard deviations (%RSD), was determined from the following equation:

$$\%RSD = \frac{SD \times 100}{\bar{X}}$$

where,

SD = the standard deviation from the mean value;

and $\bar{X}$ = the mean value.

The value (%RSD) should be less than 2.0 %.

(4) Limit of detection (LOD)

LOD was calculated by the following equation**;**

$$LOD = 3S/N;$$

where,

S/N = Signal to noise.

(5) Limit of quantitation (LOQ)

LOQ was calculated by the following equation**;**

$$LOQ = 10S/N;$$

where,

S/N = Signal to noise.

2.3.5 Screening for enrofloxacin residues in chicken breast and eggs

Samples from various markets were screened for enrofloxacin residues by HPTLC method. The confirmatory method such LC-MS was employed for samples which showed positive results with HPTLC method.

CHAPTER IV
RESULTS AND DISSCUSION

1. Optimization

Seven mobile phase systems were tested for separation of enrofloxacin and ciprofloxacin using TLC silica gel 60 F_{254} and HPTLC silica gel 60 F_{254} as stationary phases. The R_f values are shown in Tables 5 and 6.

Table 5 R_f values of enrofloxacin and ciprofloxacin on TLC silica gel 60 F_{254}

Mobile phase	R_f value	
	Enrofloxacin	Ciprofloxacin
1. Dichloromethane : methanol : 25% ammonium hydroxide : acetonitrile (4:4:2:1)	0.62	0.57
2. Dichloromethane : ethanol : 25% ammonium hydroxide (2:2:1)	0.68	0.49
3. Dichloromethane : methanol : 25% ammonium hydroxide (150:75:15)	0.71	0.36
4. Methanol : water : dichloromethane : toluene : diethylamine (40:8:40:20:14)	0.21	0.09
5. Dichloromethane: methanol : 2-propanol : 25% ammonium hydroxide (3:3:5:2)	0.47	0.37
6. Methanol : 2-propanol : 25% ammonium hydroxide (5:5:2)	0.41	0.27
7. Acetone : methanol : 25% ammonium hydroxide (5:3:2)	0.54	0.36

Table 6 R_f values of enrofloxacin and ciprofloxacin on HPTLC silica gel 60 F_{254}

Mobile phase	R_f value	
	enrofloxacin	ciprofloxacin
1. Dichloromethane : methanol : 25% ammonium hydroxide : acetonitrile (4:4:2:1)	0.67	0.63
2. Dichloromethane : ethanol : 25% ammonium hydroxide (2:2:1)	0.25	0.10
3. Dichloromethane : methanol : 25% ammonium hydroxide (150:75:15)	0.51	0.33
4. Methanol : water : dichloromethane : toluene : diethylamine (40:8:40:20:14)	0.72	0.61
5. Dichloromethane: methanol : 2-propanol : 25% ammonium hydroxide (3:3:5:2)	0.45	0.38
6. Methanol : 2-propanol : 25% ammonium hydroxide (5:5:2)	0.42	0.33
7. Acetone : methanol : 25% ammonium hydroxide (5:3:2)	0.45	0.35

The results from TLC silica gel 60 F254 and HPTLC silica gel 60 F254 showed good separation, the analytes were appeared in the middle of the stationary plates in systems 1-3, and good separations with low R_f values were shown in systems 5-7. The R_f values of ciprofloxacin and enrofloxacin were too low when using solvent system 4. HPTLC silica gel 60 F254 gave more obvious bands of analytes than TLC silica gel 60 F254. Therefore, HPTLC and mobile phase systems 1-3 and 5-7 were chosen to develop a screening method for enrofloxacin residues.

The standard spiked chicken extract was applied to the chosen systems. The interferences bands from the matrix of samples were appeared near the analyte band around the middle of the plate. System 6 gave the better separation because the other interferences from the matrix were move over the analytes. The best HPTLC system for chicken breast samples were as follow:

Stationary phase : HPTLC silicagel 60 F254
Mobile phase : propanol: methanol: 25% ammonia (5:5:2)
Application volume : 10 μl
Developed length : 75 mm
Detection : UV at 366 nm

2. Validation of HPTLC

2.1 Detection limit

Both standard enrofloxacin and ciprofloxacin standard solutions were added into the blank extracts to get standard concentration of 100 ppb. The application volumes of standard solutions were ranged from 5, 10, 15, 20, 25 and 30 μL, which equivalent to 25, 50, 75, 100 and 150 ng of standard per spot on HPTLC, respetively. The detection limit was achieved at the application volume of 10 μL which gave a clear response and good separation between enrofloxacin and ciprofloxacin. The chromatograms are shown in Figure 3.

2.2 Accuracy, sensitivity, specificity

Twenty of positive chicken samples and twenty of chicken negative samples for validations were prepared as described in chapter III to determine sensitivity, specificity and accuracy. Fifteen of positive egg samples and eleven of egg negative samples for validations were prepared as directed in chapter III.

The validation results showed the sensitivities of 90 % and 80 % in chicken breast and eggs, respectively. The specificities were 100 % in both chicken breast and eggs, respectively. The accuracies were 95 and 88 % in chicken breast and eggs, respectively. The validation results are shown in Table 7 and the chromatograms are shown in Figures 4 - 7.

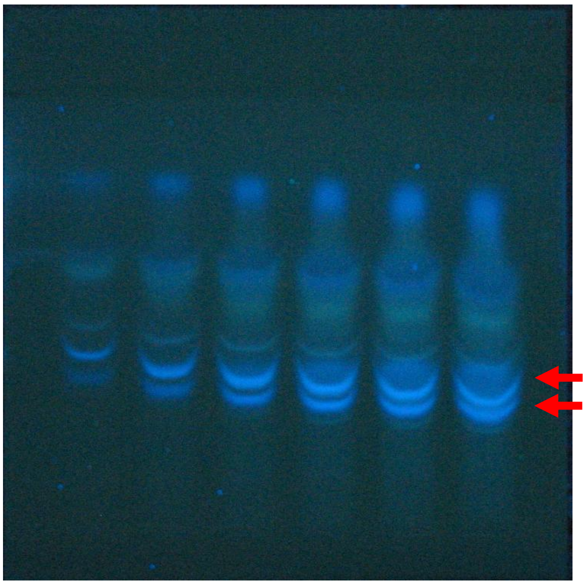
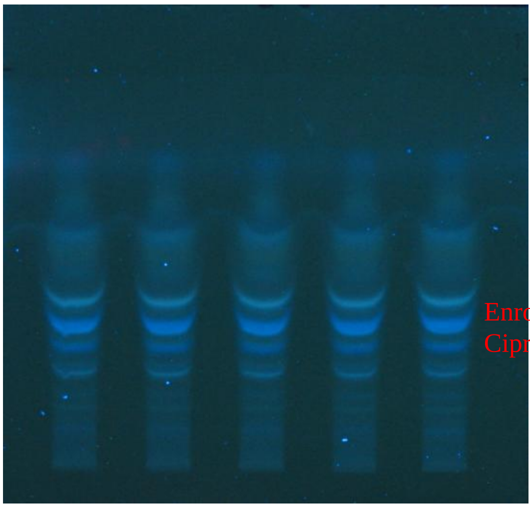


Figure 3 Detection limit**;** chromatograms of standard enrofloxacin and ciprofloxacin solutions (100 ppb) at 5, 10, 15, 20, 25, and 30 µl.

Stationary phase	HPTLC silica gel 60 F254
Mobile phase	Propanol: methanol: 25% ammonia (5:5:2)
Application volume	5, 10, 15, 20, 25, and 30 µl
Developed length	75 mm
Detection	UV at 366 nm

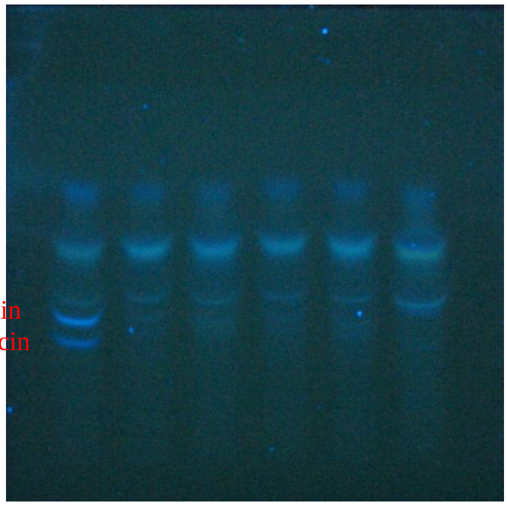
Table 7 Validation for HPTLC screening for enrofloxacin chicken and eggs.

Sample	No. of positive sample			No. of negative sample			Validation parameter		
	True positive	False negative	Total	True negative	False positive	Total	Sensitivity	Specificity	Accuracy
Chicken	18	2	20	20	0	20	90	100	95
Egg	12	3	15	11	0	11	80	100	95



1 2 3 4 5

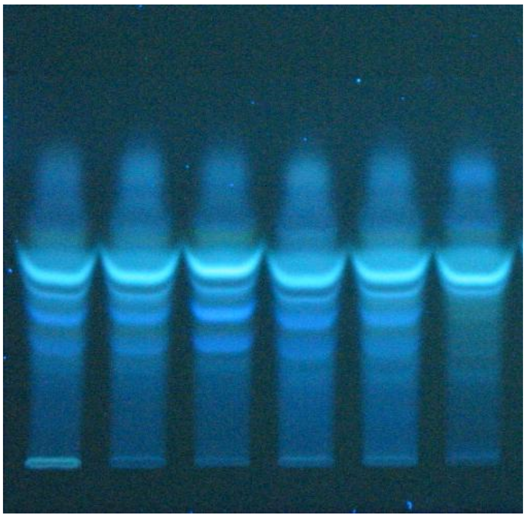
Figure 4 Chromatograms of five positive chicken samples for validation



Standard 1 2 3 4 5

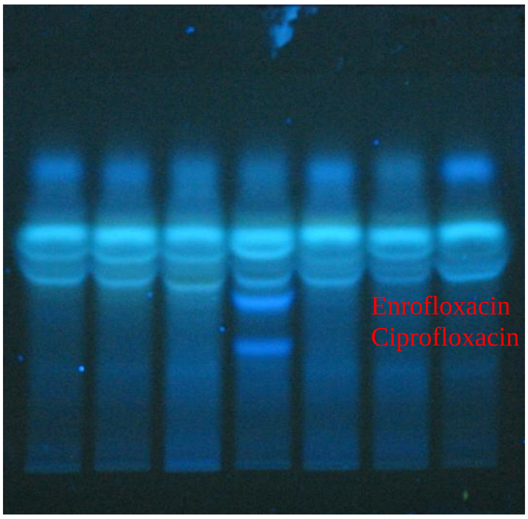
Figure 5 Chromatograms of five negative chicken samples for validation

HPTLC condition	
Stationary phase	HPTLC silicagel 60 F254
Mobile phase	propanol: methanol: 25% ammonia (5:5:2)
Application volume	10 µl
Developed length	75 mm
Detection	UV at 366 nm



1 2 3 4 5 Blank

Figure 6 Chromatograms of five positive egg samples for validation



1 2 3 standard 4 5 6

Figure 7 Chromatograms of six negative egg samples for validation

HPTLC condition	
Stationary phase	HPTLC silicagel 60 F254
Mobile phase	propanol: methanol: 25% ammonia (5:5:2)
Application volume	10 µl
Developed length	75 mm
Detection	UV at 366 nm

3. LC-MS

3.1 Quantification and confirmatory method of enrofloxacin by LC-MS

A suspected noncompliant sample from screening test by HPTLC method was further confirmed and quantitative analyzed for the residual contaminations by LC-MS. The chromatograms of enrofloxacin, ciprofloxacin and norfloxacin (internal standard) were obtained as shown in Figure 8. The LC-MS chromatograms of sample showed the responses of enrofloxacin, ciprofloxacin and norfloxacin as in Figure 9.

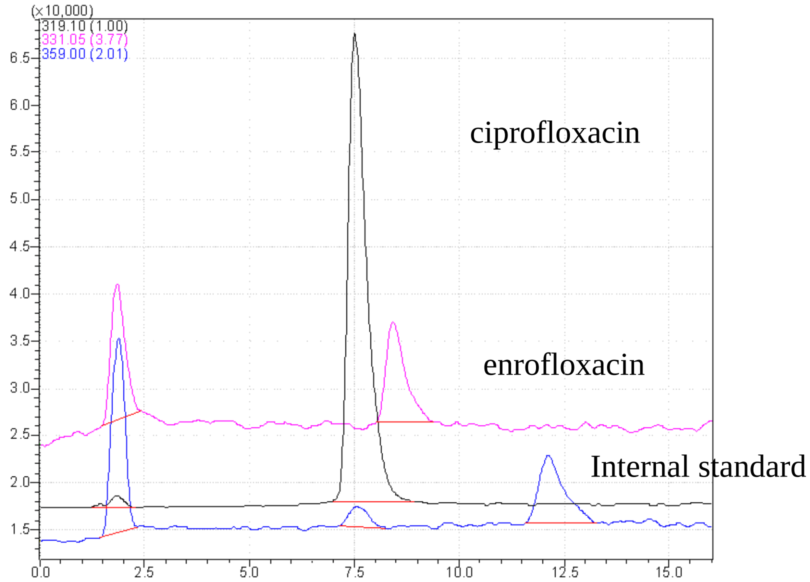


Figure 8 LC-MS chromatograms of enrofloxacin (m/z 359), ciprofloxacin (m/z 331.05), and norfloxacin (internal standard, m/z 319.10).

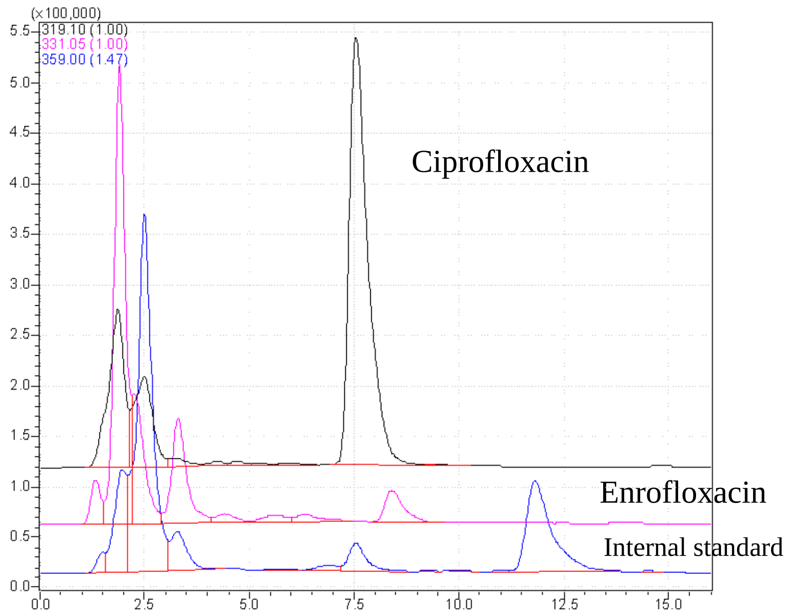


Figure 9 LC-MS chromatograms of sample extraction**;** enrofloxacin (m/z 359), ciprofloxacin (m/z 331.05), and norfloxacin (internal standard, m/z 319.10).

3.2 Method validation of LC-MS

3.2.1 Linearity

The linearity of the method was studied using three replicate injections ($n=3$) of 5 µL of standard enrofloxacin and ciprofloxacin covering the range of 0.72 – 24 ppb. Calibration data calculated from peak area ratio versus concentrations are shown in Tables 8 and 9. The calibration curves of standard enrofloxacin and ciprofloxacin are shown in Figures 10 and 11.

The representative linear regression equation for standard enrofloxacin was: $y = 0.0132x + 0.0812$;

Where;

y = peak area ratio of standard ciprofloxacin and internal standard norfloxacin;

x = concentration of ciprofloxacin standard in ppb.

The representative linear regression equation for standard ciprofloxacin was: $y = 0.0076\,x + 0.0509$;

Where;

Y = peak area ratio of standard ciprofloxacin and internal standard norfloxacin;

x = concentration of ciprofloxacin standard in ppb.

Obviously, the correlation coefficients (r^2) of both curves were within the acceptance criteria (r^2 should be more than 0.995). Thus, this calibration curve could be used to determine of enrofloxacin and ciprofloxacin contents in chicken breast and eggs.

Table 8 Peak area ratio of standard enrofloxacin and internal standard norfloxacin at various concentrations

Injection no.	Concentration (ppb)					
	0.72	2.4	4.8	9.6	12	24
1	0.07436	0.11539	0.12949	0.22664	0.22324	0.38955
2	0.09841	0.11508	0.15099	0.19812	0.23961	0.41129
3	0.09451	0.12066	0.14944	0.24349	0.24365	0.40800
Average	0.08909	0.11704	0.14331	0.22275	0.2355	0.40295
SD	0.01291	0.00314	0.01199	0.02293	0.01081	0.01172
%RSD	14.48	2.68	8.36	10.29	4.57	2.91
			Slope	0.0132		
			y-intercept	0.0812		
			r^2	0.9975		

Table 9 Peak area ratio of standard ciprofloxacin and internal standard norfloxacin at various concentrations

Injection no.	Concentration (ppb)					
	0.72	2.4	4.8	9.6	12	24
1	0.04485	0.07204	0.09159	0.12419	0.14393	0.22798
2	0.05763	0.06459	0.08629	0.12491	0.14264	0.22914
3	0.05906	0.05493	0.09576	0.13555	0.14001	0.24143
Average	0.05385	0.06385	0.09121	0.12822	0.14219	0.23285
SD	0.00638	0.00858	0.00475	0.00636	0.00199	0.00745
%RSD	14.52	13.44	5.20	4.96	1.41	3.20
			Slope	0.0076		
			y-intercept	0.0509		
			r^2	0.9988		

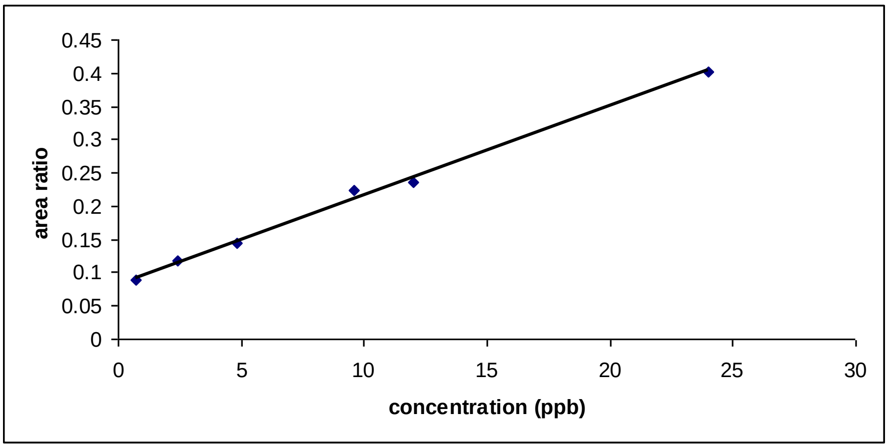


Figure 10 Standard calibration curve of enrofloxacin at the concentration range of 0.72-24 ppb

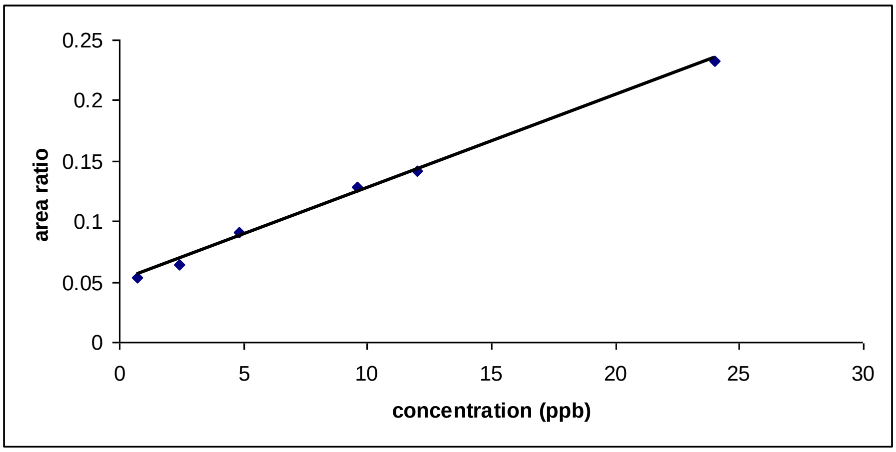


Figure 11 Standard calibration curve of ciprofloxacin at the concentration range of 0.72-24 ppb

3.2.2 Accuracy

The accuracy of method was evaluated using the recovery of enrofloxacin and ciprofloxacin in chicken breast extracts. In the method of standard addition, known amounts of an analyte are spiked into a chicken breast extracts that may contain some quantity of analyte. The amount found was calculated by subtracting total amount from amount originate in sample. Percent recovery of enrofloxacin and ciprofloxacin were shown in Tables 10 and 11, respectively.

Table 10 Recovery of enrofloxacin in chicken breast (n=1)

Amount added (µg)	Total amount (µg)	Amount found (µg)	% Recovery
blank	80.78	-	-
120	216.43	135.65	113.04

Table 11 Recovery of ciprofloxacin in chicken breast (n=1)

Amount added (µg)	Total amount (µg)	Amount found (µg)	% Recovery
blank	42.57	-	-
120	172.75	130.18	108.58

3.2.3 Precision

The precision of the method was expressed as percentage of relative standard deviation (%RSD). The low %RSD value indicating that the method has good precision. Three different concentrations, 9.6, 12 and 24 ppb, of standard enrofloxacin and ciprofloxacin solutions were prepared and analyzed.

Percent relative standard deviations (%RSD) of enrofloxacin and ciprofloxacin are shown in Tables 12 and 13, respectively.

Table 12 Peak area ratio and relative standard deviations (%RSD) of standard enrofloxacin at various concentrations

Injection no.	Concentration (ppb)		
	9.6	12	24
1	0.22664	0.22324	0.38955
2	0.20451	0.23961	0.41129
3	0.22323	0.24365	0.40800
Average	0.21813	0.23550	0.40295
SD	0.01192	0.01081	0.01172
%RSD	5.46	4.57	2.91

Table 13 Peak area ratio and relative standard deviations (%RSD) of standard ciprofloxacin at various concentrations

Injection no.	Concentration (ppb)		
	9.6	12	24
1	0.12419	0.14393	0.22798
2	0.12491	0.14264	0.22914
3	0.13555	0.14001	0.24143
Average	0.12822	0.14219	0.23285
SD	0.00636	0.00199	0.00745
%RSD	4.96	1.41	3.20

3.3.4 Limit of detection

The limit of detection of enrofloxacin and ciprofloxacin were calculated from signal to noise ratio equal to 3:1 which were 0.06 and 0.10 ppb, respectively. The chromatograms of enrofloxacin and ciprofloxacin at the level of the limit of detection are shown in Figure 12.

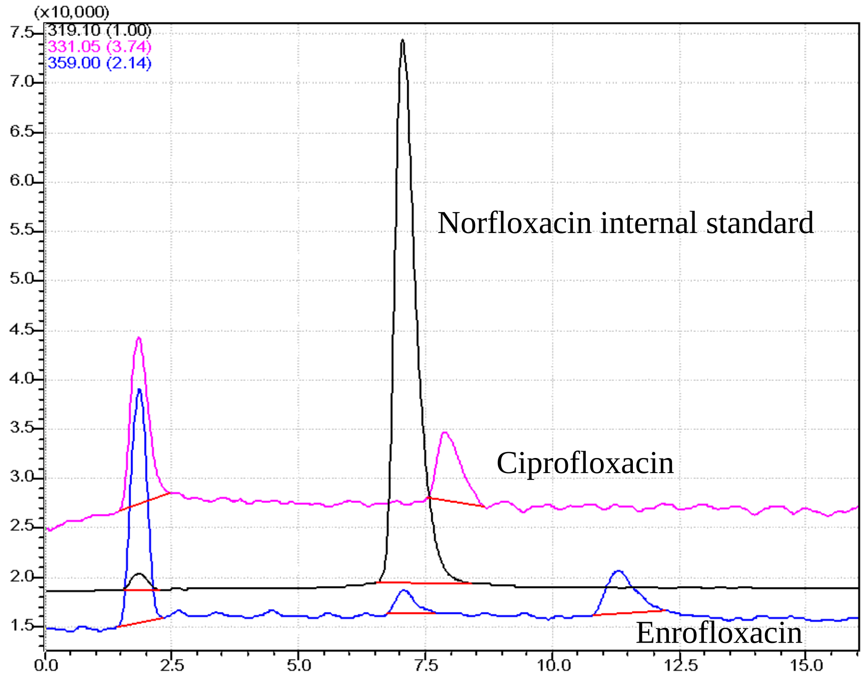


Figure 12 Chromatograms of enrofloxacin and ciprofloxacin at the limit of detection of 0.06 and 0.10 ppb, respectively.

3.2.5 Limit of quantitation (LOQ)

The limit of detection of enrofloxacin and ciprofloxacin were calculated from signal to noise ratio equal to 10:1 which were 0.18 and 0.31 ppb, respectively. The chromatograms of enrofloxacin and ciprofloxacin at the level of the limit of quantitation are shown in Figure 13.

4. Screening for enrofloxacin residues in chicken and eggs samples

The chicken breast and eggs from the various markets were collected for screening of enrofloxacin residues by HPTLC method. The chromatograms are shown in Figure 14. The samples which contained enrofloxacin and ciprofloxacin more than 100 ppb, determined by HPTLC method, was then further investigated by LC-MS. From the HPTLC result only one from 116 chicken samples was positive. The positive sample was re-extracted and analyzed by LC-MS, the LC-MS result was negative. No positive sample was found from HPTLC in egg samples. The screening results are shown in Tables 14 and 15.

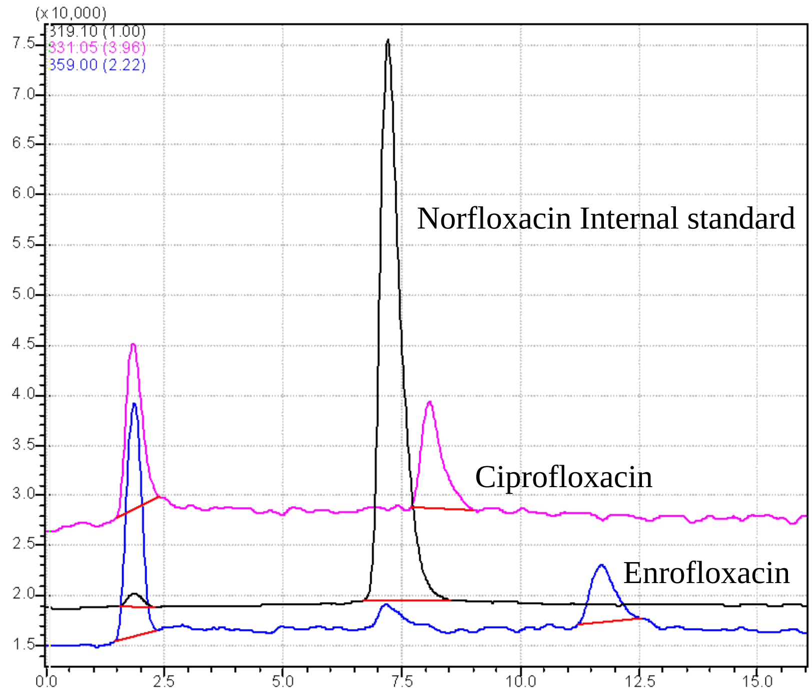


Figure 13 Chromatograms of enrofloxacin and ciprofloxacin at the limit of quantitation 0.18 ppb and 0.31 ppb respectively.

Table 14 Screening results of enrofloxacin in chicken samples from various markets.

Type of market	Total sample	No. of passed sample	No of failed sample
Shopping mall	27	26	1
Market	52	52	0
Temporary market	37	37	0
Total	116	115	1

Table 15 Screening results of enrofloxacin in egg samples from various markets.

Type of markets	Total sample	No. of passed sample	No of failed sample
Shopping mall	5	5	0
Market	40	40	0
Temporary market	17	17	0
Total	62	62	0

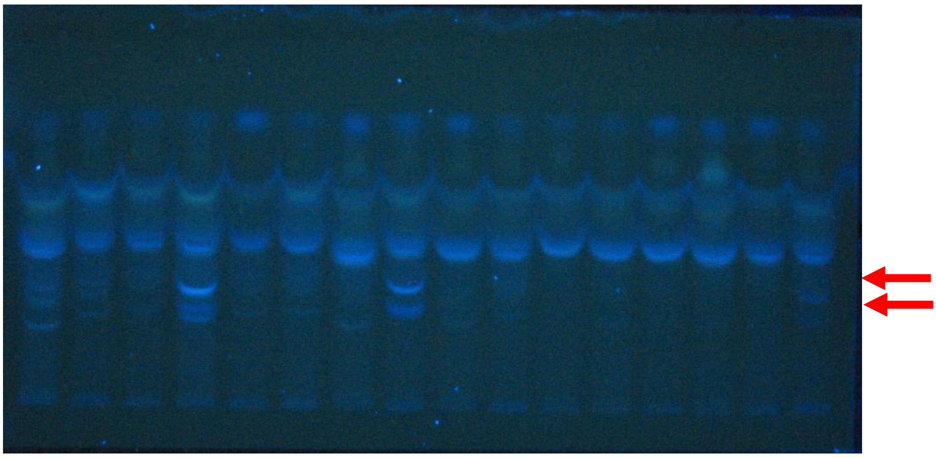


Figure 14 Chromatograms of enrofloxacin by HPTLC in chicken samples

Stationary phase	HPTLC silicagel 60 F254
Mobile phase	propanol: methanol: 25% ammonia 5:5:2
Application volume	10 μl
Developed length	75 mm
Detection	blue fluorescence

5. Quantification and confirmatory method of enrofloxacin by LC-MS

To compare HPTLC method and LC-MS, twenty of positive chicken samples and twenty of chicken negative samples for validations of HPTLC method were analyzed by LC-MS. The results are shown in Tables 16 and 17. Fifteen of positive egg samples and eleven of egg negative samples for validations of HPTLC method were analyzed by LC-MS. The results are shown in Tables 18 and 19.

The analysis of positive chicken and egg samples by HPTLC showed the results conformed to the LC-MS results. The positive samples which showed positive results in HPTLC were positive in LC-MS. The negative samples which showed negative results in HPTLC were negative in LC-MS.

Moreover, the results from analysis of positive chicken and egg samples by LC-MS, the recovery of the enrofloxacin analysis were 45.6 – 84.4% in chicken samples and 36.8 – 70.4 % in egg samples. These errors might come from natural variations of the samples, sample preparation, testing condition, sample storages, etc. The further investigation of these errors should be studied.

Table 16 Results from analysis of negative chicken samples by LC-MS.

Sample	HPTLC result	LC-MS result (ppb)	
		Ciprofloxacin	Enrofloxacin
1 M244	Passed	<0.24*	<0.4*
2 M187	Passed	7.00	30.24
3 M188	Passed	9.68	53.03
4 M230	Passed	12.68	54.03
5 M234	Passed	2.42	58.30
6 M002	Passed	< 0.24*	< 0.4*
7 M041	Passed	<0.24*	<0.4*
8 M044	Passed	<0.24*	<0.4*
9 M049	Passed	<0.24*	<0.4*
10 M052	Passed	<0.24*	<0.4*
11 M056	Passed	<0.24*	<0.4*
12 M074	Passed	<0.24*	<0.4*
13 M076	Passed	<0.24*	<0.4*
14 M149	Passed	<0.24*	<0.4*
15 M150	Passed	<0.24*	<0.4*
16 M174	Passed	<0.24*	<0.4*
17 M178	Passed	<0.24*	<0.4*
18 M181	Passed	<0.24*	<0.4*
19 M240	Passed	<0.24*	<0.4*
20 M200	Passed	<0.24*	<0.4*

Where; * = not more than LOD

Table 17 Results from analysis of positive chicken samples by LC-MS.

Sample	HPTLC result	LC-MS result (ppb)	
		Ciprofloxacin	Enrofloxacin
1 M001	Failed	59.6	78.8
2 M048	Failed	43.2	51.2
3 M104	Failed	55.6	72.8
4 M223	Failed	61.2	70.0
5 M238	Failed	48.4	53.6
6 M205	Failed	59.6	74.4
7 M180	Failed	54.4	69.6
8 M153	Failed	54.4	68.0
9 M186	Failed	42.0	58.0
10 M202	Failed	68.8	79.2
11 M204	Failed	56.0	80.0
12 M216	Failed	51.6	51.2
13 M217	Failed	50.4	60.8
14 M229	Failed	60.0	72.8
15 M242	Failed	60.8	84.4
16 M171	Failed	43.2	57.6
17 M194	Failed	46.4	60.8
18 M152	Failed	5.2	6.8
19 M206	Failed	0.4	2.0
20 M195	Failed	41.6	45.6

Table 18 Results from analysis of negative egg samples by LC-MS.

Sample	HPTLC result	LC-MS result (ppb)	
		Ciprofloxacin	Enrofloxacin
1 E009	Passed	<0.24*	5.6
2 E041	Passed	5.2	<0.4*
3 E020	Passed	4.8	<0.4*
4 E029	Passed	4.8	<0.4*
5 E037	Passed	2.4	<0.4*
6 E047	Passed	4.0	2.8
7 E011	Passed	7.8	0.6
8 E014	Passed	3.2	1.5
9 E023	Passed	0.8	0.5
10 E051	Passed	2.9	2.2
11 E034	Passed	8.0	1.5

Table 19 Results from analysis of positive egg samples by LC-MS.

Sample	HPTLC result	LC-MS result (ppb)	
		Ciprofloxacin	Enrofloxacin
1 (E019)	Failed	47.6	68.8
2 (E030)	Failed	55.2	69.6
3 (E036)	Failed	59.6	70.4
4 (E038)	Failed	54.8	60.8
5 (E049)	Failed	45.2	60.4
6 (E022)	Failed	8.0	39.6
7 (E031)	Failed	<0.40*	36.8
8 (E046)	Failed	9.6	51.2
9 (E043)	Failed	<0.40*	30.8
10 (E017)	Failed	2.4	26.4
11(E001)	Failed	0.8	30.0
12 (E008)	Failed	64.4	292.8
13(E018)	Failed	722.4	812.8
14 (011)	Failed	500.0	562.0
15(E003)	Failed	574.8	530.0

Where * = not more than LOD

Samples 11 – 15 were extracted at 30 days before tested by LC-MS. These results were not included in recovery calculation.

6. Comparison of HPTLC method and LC-MS

HPTLC screening method was developed, validated, and applied to chicken breast and eggs. Even the lower detection limit and accuracy than LC-MS were obtained. The detection limit was reached the MRL limit of enrofloxacin residues and better than the other screening method reviewed in Chapter II. This method overcame the limits of screening method with low cost, rapid, and reliable.

The comparison of HPTLC method and LC-MS is shown in Table 20 and the expenses in analysis by HPTLC are shown in Table 21.

Table 20 Comparison of HPTLC method and LC-MS

Descriptions	HPTLC	LC-MS
Expenses per analysis (baht)	300 -500	3,000-5,000
Time (hr)	3-5	4-6
Detection limit of enrofloxacin (ppb)	100	0.06
Specificity	100	-
Accuracy (% recovery)	45-80 %	113 %
Precision (% RSD)	-	4 %

Table 21 Expenses in HPTLC screening method

Descriptions	Expenses (baht)
Chemicals	150-200
N2 fee	50
Scientific materials	50-100
Utilities	10 % of all expenses
Total	270- 440

CHAPTER V
CONCLUSION

The purposes of this research were to develop a rapid and simple method for screening of enrofloxacin residues in chicken breast and eggs by HPTLC, to develop a confirmatory and quantitative method by LC-MS, and to evaluate the quality of chicken breast and eggs which were sold in the markets in Bangkok and provinces around Bangkok. The HPTLC method was developed to reach the low maximum residues limit of enrofloxacin in chicken breast which was 100 ppb. The detection limit, sensitivity and specificity in chicken breast were 100 ppb, 90% and 100%, respectively. The detection limit, sensitivity and specificity in eggs were 100 ppb, 80% and 100%, respectively. Liquid chromatography mass spectrometry was used as the confirmatory method to analyze the same samples that were used in validation experiments of High Performance Thin layer Chromatography. According to liquid chromatography mass spectrometry results, all samples were conformed. Chicken breast and egg samples from various sources around Bangkok were sampled and tested by High Performance Thin layer Chromatography. From 116 samples of chicken meat, only one sample was out of the limit of enrofloxacin residues (100 ppb). All 62 egg samples were within the same limit of enrofloxacin residues (100 ppb). Enrofloxain residues contaminations reduced the quality of foods and indicated the problems of inappropriate using of enrofloxacin in poultry animals in Thailand. However, this screening method by HPTLC had its limitations in lacking of structural information. The confirmatory methods such as LC-MS, LC-MS/MS were employed to the positive results from screening test.

The errors of this screening method by HPTLC were from the nature and the variations of sample sources, sample preparation techniques such as homogenization and extractions, the conditions of the system, and sample storage was influenced. These factors should be further studied for better solutions.

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APPENDIX

Market list for chicken sampling

No.	Code	Name of market	Sampling date
1	M001	ตลาดสดราชวัตร	2/6/52
2	M002	ตลาดสดราชวัตร 2	2/7/52
3	M003	ตลาดสดราชวัตร 3	2/8/52
4	M004	เตาปูน 1	2/9/52
5	M006	ตลาดบางแค 1	7/6/52
7	M008	พระสมุทรเจดีย์ 2	7/8/52
8	M010	ตลาดบางแค2	7/9/52
9	M011	ตลาด อตก.1	7/10/52
10	M041	ตลาดสดมักกะสัน	14/06/52
11	M042	ตลาดสดมักกะสัน 2	14/06/52
12	M043	ตลาดสดห้วยขวาง	14/06/52
13	M044	ตลาดสดห้วยขวาง	14/06/52
14	M045	ตลาด แฮปปี้แลนด์ 1	14/06/52
15	M047	ตลาดโชคชัย 4 ร้านที่1	14/06/52
16	M048	ตลาดโชคชัย 4 ร้านที่2	14/06/52
17	M049	ตลาดบางกะปิ 1	14/06/52
18	M050	ตลาดบางกะปิ 2	14/06/52
19	M051	ตลาดบางจาก 1	14/06/52
20	M052	ตลาดบางจาก 2	14/06/52
21	M053	ตลาดอ่อนนุช 1	14/06/52
23	M055	ตลาดสดพระโขนง1	14/06/52
24	M056	ตลาดบางเขน 1	16/06/52
25	M068	ตลาดธนบุรี 1	18/06/52
26	M069	ตลาดธนบุรี 2	18/06/52
27	M070	ตลาดบางขุนนนท์1	18/06/52

Market list for chicken sampling (cont.)

No.	Code	Name of market	Sampling date
28	M071	ตลาดบางขุนนนท์2	18/06/52
29	M072	ตลาดชัยพฤกษ์	18/06/53
30	M073	ตลาดบางขุนศรี 2	18/06/52
31	M076	ตลาดสามย่าน 1	19/06/52
32	M081	ตลาดสี่พระยา 3	19/06/52
33	M090	ตลาดเจริญกรุง 3	19/06/52
34	M096	ตลาดสำเหร่ 1	22/06/52
35	M098	ตลาดสวนหลวง 1	24/06/52
36	M099	ตลาดสวนหลวง 2	24/06/52
37	M150	ตลาดแสงดาว 1	7/7/52
38	M151	ตลาดบางบอน 1	7/7/52
39	M152	ตลาดบางบอน 2	7/7/52
40	M159	ตลาดพระปิ่น3 -2	8/7/52
41	M170	ตลาดสมภพ 2	11/7/52
42	M186	ตลาดห้วยขวาง 3	13/07/52
43	M214	ตลาดโพธิ์สามต้น 1	17/07/52
44	M215	ตลาดโพธิ์สามต้น 2	17/07/52
45	M222	ตลาดรัตนโกสินทร์200ปี 1	19/07/52
46	M223	ตลาดรัตนโกสินทร์200ปี 2	19/07/52
47	M227	ตลาดอตก. 2	19/07/52
48	M228	ตลาดอตก. 3	19/07/52
49	M229	ตลาดสะพานขาว 1	19/07/52
50	M233	ตลาดคลองแค 1	20/07/52
51	M244	ตลาดกลางบางใหญ่ 1	22/07/52
52	M246	ตลาดกลางบางใหญ่ 3	22/07/52

Market list for chicken sampling (cont.)

No.	Code	Name of market	Sampling date
53	M005	ลตัสเตาปูน 1	2/6/52
54	M009	Big C พระประแดง 1	7/7/52
55	M022	Emporium 1	11/6/52
56	M023	Siam Paragon 1	11/6/52
57	M024	The Mall ท่าพระ 1	11/6/52
58	M025	โลตัส อ่อนนุช 1	11/6/52
59	M026	โลตัส พระราม 1	11/6/52
60	M074	Macro จรัญสนิทวงศ์	18/06/52
61	M075	Tops บางรัก 1	19/06/52
62	M147	โลตัสเตาปูน 2	7/7/52
63	M148	โลตัสบางแค2	7/7/52
64	M149	The Mallบางแค 2	7/7/52
65	M156	คาร์ฟูร์บางใหญ่	8/7/52
66	M171	Big C อ้อมใหญ่1	11/7/52
67	M178	โลตัสเอกซ์เพรส วัดใหญ่ในคลองบางปลากด	12/7/52
68	M179	โลตัสเอกซ์เพรส วัดแคในคลองบางปลากด	12/7/52
69	M190	Tops มาบุญครอง	13/07/52
70	M195	โลตัสปิ่นเกล้า 14/07/52	14/07/52
71	M197	Save-E	14/07/52
72	M203	คาร์ฟูร์บางบอน	15/07/52
73	M211	โลตัส วัดไชยฉิมพลี	16/07/52
75	M216	โลตัสบางปะกอก	17/07/52
76	M217	Tops นครหลวง	17/07//52
77	M224	คาร์ฟูร์รามอินทรา	19/07/52
78	M225	คาร์ฟูร์ มีนบุรี	19/07/52

Market list for chicken sampling (cont.)

No.	Code	Name of market	Sampling date
79	M226	Tops รังสิต	19/07/52
80	M104	ตลาดนัดวัดแค	24/06/52
81	M174	ตลาดนัดหมู่บ้านหรรษา -1	11/7/52
82	M176	ตลาดนัด แหลมฟ้าผ่า -1	12/7/52
83	M181	ตลาดนัดวัดแค2	12/7/52
84	M183	ตลาดนัดสุขสวัสดิ์	12/7/52
85	M185	ตลาดนัดการเคหะดินแดง2	13/07/52
86	M189	ตลาดนัดรามคำแหงซอย5	13/07/52
87	M193	สี่แยกทศกัณฑ์2	14/07/52
88	M194	ตลาดนัด Safe center	14/07/52
89	M198	ตลาดนัดศาลายา 1	15/07/52
90	M199	ตลาดนัดศาลายา 2	15/07/52
91	M200	ตลาดนัดกระทุ้มล้น	15/07/52
92	M201	ตลาดนัดวัดอุดมรังสี 1	15/07/52
93	M202	ตลาดนัดวัดอุดมรังสี 2	15/07/52
94	M204	ตลาดนัดหมู่บ้านเศรษฐกิจ	15/07/52
95	M205	ตลาดนัดเจ๊ใหญ่ 1	15/07/52
96	M206	ตลาดนัดเจ๊ใหญ่ 2	15/07/52
97	M207	ตลาดนัดหมู่บ้านพระปิ่น 2 -2	16/07/52
98	M208	ตลาดนัดหมู่บ้านพระปิ่น 2 -3	16/07/52
99	M209	ตลาดนัดวัดไชยฉิมพลี 1	16/07/52
100	M210	ตลาดนัดวัดไชยฉิมพลี 2	16/07/52
102	M235	ตลาดนัดสวนผัก	21/07/52
103	M236	ตลาดนัดสวนผัก 2	21/07/52
104	M237	ตลาดนัดอารีย์ ซอย7 -1	21/07/52

Market list for chicken sampling (cont.)

No.	Code	Name of market	Sampling date
105	M238	ตลาดนัดอารีย์ ซอย7 -2	21/07/52
106	M240	ตลาดนัดวัดแก้ว 1	21/07/52
107	M242	ตลาดนัดวัดมะลิ	21/07/52
108	M243	ตลาดนัดวัดท่าพระ	21/07/52
109	M247	ตลาดนัดวัดส้มเกลี้ยง 1	22/07/52
110	M248	ตลาดนัดวัดส้มเกลี้ยง 2	22/07/52
111	M249	ตลาดนัดข้างโพธิสาร	22/07/52
112	M116	ตลาดนัดวัดน้อยใน 2	22/07/52
113	M072	ชัยพฤกษ์1	18/06/52
114	M141	วัดบางแวก	4/7/52
115	M153	ตลาดบางแค	7/7/52
116	M180	ซอยวัดใหญ่	12/7/52
117	M239	ซอยศาสนา	21/07/52

Market list for egg sampling

No.	Code	Name of market	Sampling date
1	E001	โลตัส เตาปูน	2/6/2552
2	E002	บางแค	7/6/2552
3	E003	บางขุนศรี	9/6/2552
4	E004	พระโขนง	14/6/2552
5	E005	พระโขนง	14/6/2552
6	E006	วงเวียนใหญ่	19/6/2552
7	E007	สะพานขาว	27/6/2552
8	E008	พุทธมนฑลสาย 2	29/6/2552
9	E009	ตลาดพระปิ่น 3	8/7/2552
10	E010	เพชรเกษม ซ. 71	11/7/2552
11	E011	รามคำแหง ซ. 5	13/7/2552
12	E012	ตลาดนัดเซฟเซ็นเตอร์	14/7/2552
13	E013	คาร์ฟู บางบอน	15/7/2552
14	E014	ซีพี เฟรชมาร์ค สาขาวัดฉิม	16/7/2552
15	E015	ตลาดสดสะพานขาว	19/7/2552
16	E016	โลตัส สามพราน	20/7/2552
17	E017	ตลาดสดไฟฟ้า นครชัยศรี	20/7/2552
18	E018	ตลาดสดกระทุ่มแบน	20/7/2552
19	E019	Tops สาขามาบุญครอง	6/8/2552
20	E020	ตลาดนัด ธนินธร 1	20/8/2552
21	E021	ตลาดนัด ธนินธร 2	20/8/2552
22	E022	ตลาดนัด ม.ไวท์เฮาส์ 1	20/8/2552
23	E023	ตลาดนัด ม.ไวท์เฮาส์ 2	20/8/2552
24	E024	ตลาดนัดเซียร์รังสิต	20/8/2552
25	E025	ตลาดนัดบ่อนไก่ 1	20/8/2552

Market list for egg sampling (cont.)

No.	Code	Name of market	Sampling date
26	E026	ตลาดนัดบ่อนไก่ 2	20/8/2552
27	E027	ตลาดนัดเมืองเอก 1	20/8/2552
28	E028	ตลาดนัดเมืองเอก 2	20/8/2552
29	E029	ตลาดสดประทานพร 1	20/8/2552
30	E030	ตลาดสดประทานพร 2	20/8/2552
31	E031	ตลาดสดรามอินทรา กม.4	20/8/2552
32	E032	ตลาดนัด (ซ. รามอินทรา 34) 2	20/8/2552
33	E033	ตลาดนัด (ซ. รามอินทรา 34) 1	20/8/2552
34	E034	ตลาดสดลำลูกกา	20/8/2552
35	E035	ตลาดสดปากคลองตลาด 1	20/8/2552
36	E036	ตลาดสดปากคลองตลาด 2	20/8/2552
37	E037	ตลาดสดสี่มุมเมือง	20/8/2552
38	E038	ตลาดสดยิ่งเจริญ	20/8/2552
39	E039	ตลาดนัดท่าพรานนก	20/8/2552
40	E040	ตลาดสดรามอินทรา กม.2	20/8/2552
41	E041	ตลาดสดสำเหร่	21/8/2552
42	E042	ตลาดสดเงินวิจิตร	21/8/2552
43	E043	ตลาดสด สน. พลับพลาไชย	21/8/2552
44	E044	ตลาดสดบางแค	21/8/2552
45	E045	ตลาดสดวงเวียนใหญ่	21/8/2552
46	E046	ตลาดสดคลองเตย	21/8/2552
47	E047	ตลาดสดบางขุนศรี	22/8/2552
48	E048	ตลาดสดสีลม	25/8/2552
49	E049	ตลาดพงษ์เพชร	25/8/2552
50	E050	ตลาดอมรพันธ์	25/8/2552

Market list for egg sampling (cont.)

No.	Code	Name of market	Sampling date
51	E051	ตลาดมิ่งขวัญ	25/8/2552
52	E052	ตลาดสมโชติ	25/8/2552
53	E053	ตลาดท่าน้ำเมืองนนท์	25/8/2552
54	E054	ตลาดประชานิเวศน์ 3	25/8/2552
55	E055	ตลาดลาดพร้าว (อุดมลาภ)	25/8/2552
56	E056	ตลาดไท	25/8/2552
57	E057	ตลาดห้วยขวาง	25/8/2552
58	E058	ตลาดสาธร	25/8/2552
59	E059	ตลาดลาดพร้าว กม.8	27/8/2552
60	E060	ตลาดอุดมสุข	27/8/2552
61	E061	ตลาดโชคชัย 4	27/8/2552
62	E062	ตลาดบางจาก	27/8/2552
63	E063	ตลาดบางกะปิ	27/8/2552

BIOGRAPHY

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