MULTIRESIDUE ANALYSIS OF BANNED SUBSTANCES IN LIVESTOCK DRINKING WATER BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

CHUSAK ARDSOONGNEARN

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entitled

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ABSTRACT

Residues from many classes of antimicrobial drugs have become food safety issue of public concern due to their potential to pose risks to consumers. Antimicrobial drugs such as nitrofurans (nitrofurazone (NFZ), nitrofurantoin (NFT), furazolidone (FZD) and furaltadone (FTD)); nitroimidazoles (metronidazole (MNZ), ronidazole (RNZ) and dimetridazole (DMZ)); and chloramphenicol (CAP) are strictly prohibited in the livestock industry. Nevertheless, misuses of these substances are still present in animal feed and drinking water. This work focused on development of liquid chromatography coupled to an ion trap mass spectrometer (LC-MS ion trap) for the simultaneous separation of these drugs in animal drinking water. The HPLC analysis was performed on a Prodigy ODS-3 column, 2.0×150 mm, 5 µm with a guard cartridge at a flow rate of 0.2 mL/min, column oven temperature 40°C, and injection volume 10 µL. Factors affecting HPLC separation (i.e. buffer pH and concentrations), solid phase extraction (SPE), and ion trap MS using electrospray ionization (ESI) in tandem mass spectrometry mode (MS/MS) were optimized. The eight antimicrobial agents were separated in 22 min using gradient elution of acidified water (pH 5.0) and acetonitrile, nebulizer gas at 35 psi, drying gas and dry temperature at 9 L/min and 325 °C, respectively. The linearity was acceptable (r^2 = 0.979 – 0.999) with the precision and accuracy range from 3.4 – 26.6% and 88.4 – 110.1%, respectively. Limit of detection (LOD) and limit of quantitation (LOQ) for each compound was in a range of 0.002 – 0.06 µg/L and 0.005 - 0.25 µg/L, respectively. The validated method was applied to simultaneous determination of eight antimicrobial drugs in 40 animal drinking water samples collected from standard farms in Thailand. The prohibited drugs were not detected in any samples. This finding may reflected the efficiency of residue control measures in livestock production that have been strictly applied since 2002.

KEY WORDS: NITROFURANS/ NITROIMIDAZOLES/CHLOROAMPHENICOL/ LIVESTOCK DRINKING WATER/ HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY - MASS SPECTROMETRY

99 pages

การวิเคราะห์สารต้องห้ามหลายชนิดในน้ำเลี้ยงปศุสัตว์โดยวิธีไฮเพอร์ฟอร์มานซ์ลิควิดโครมาโทกราฟี-แมสสเปกโทรเมตรี

MULTIRESIDUE ANALYSIS OF BANNED SUBSTANCES IN LIVESTOCK DRINKING WATER BY HIGH - PERFORMANCE LIQUID CHROMATOGRAPHY - MASS SPECTROMETRY

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บทคัดย่อ

สารตกค้างจากยาต้านจุลชีพหลายกลุ่มเป็นปัญหาด้านความปลอดภัยทางอาหารที่อยู่ในความสนใจของสาธารณชนเนื่องจากความเสี่ยงที่อาจก่อให้เกิดอันตรายต่อสุขภาพของผู้บริโภค ยากลุ่มต้านจุลชีพเช่น กลุ่มไนโตรฟูแรนส์ (ไนโตรฟูราโซน (NFZ), ไนโตฟูแรนโตอิน (NFT), ฟูราโซลิโดน (FZD) และฟูรัลตาโดน (FTD)), กลุ่มไนโตรอิมิดาโซล (เมโทนิดาโซล (MNZ), โรนิดาโซล (RNZ) และไดเมตริดาโซล (DMZ)) และ คลอแรมเฟนิคอล (CAP) ถูกห้ามใช้ในการปศุสัตว์ อย่างไรก็ตาม ยังมีการลอบใช้ยาในกลุ่มดังกล่าวโดยผสมในอาหารหรือน้ำเลี้ยงสัตว์ ในการศึกษานี้มุ่งพัฒนาวิธีวิเคราะห์ด้วยเครื่องมือลิควิดโครมาโทกราฟีร่วมกับแมสสเปกโทรเมตรีชนิดกักไอออน (LC-MS ion trap) เพื่อวิเคราะห์สารเหล่านี้ในน้ำเลี้ยงปศุสัตว์ในการวิเคราะห์เดียว การวิเคราะห์ด้วย HPLC ใช้คอลัมน์ Prodigy ODS-3 ขนาดเส้นผ่านศูนย์กลางภายใน 2.0 มม. ยาว 150 มม. ขนาดอนุภาค 5 ไมครอน พร้อม guard cartridge โดยใช้อัตราการไหล 0.2 มล.ต่อนาที อุณหภูมิตู้บรรจุคอลัมน์ 40 องศาเซลเซียส ปริมาตรฉีด 10 ไมโครลิตร ทำการศึกษาปัจจัยต่างๆ ที่ส่งผลต่อการแยกสารของ HPLC (เช่น ความเข้มข้น และค่าความเป็นกรด-เบสของบัฟเฟอร์) การสกัดแยกสารด้วยเฟสของแข็ง (SPE) การปรับแต่งค่าแมสสเปกโทรเมตรีโดยใช้วิธีอิเลคโทรสเปรย์ (ESI) และวิเคราะห์มวลแบบ tandem mass spectrometry (MS/MS) ยาต้านจุลชีพทั้งแปดชนิดสามารถแยกจากกันได้ภายในเวลา 22 นาที โดยใช้เฟสเคลื่อนที่ประกอบด้วยน้ำ pH 5.0 และอะซีโทไนไตรล์โดยเปลี่ยนแปลงสัดส่วนขณะแยกสาร (gradient elution) ใช้เนบิวไรเซอร์ก๊าซ 35 ปอนด์ต่อตารางนิ้ว อัตราเร็วและความดันของก๊าซที่ทำให้แห้งเท่ากับ 9 ลิตรต่อนาที และ 325 °ซ ตามลำดับ ความเป็นเส้นตรงของสมการ (r^2) อยู่ระหว่าง 0.979 – 0.999 ความเที่ยงและความแม่นอยู่ระหว่างร้อยละ 3.4 – 26.6 และ 88.4 – 110.1 ตามลำดับ ระดับต่ำสุดที่สามารถตรวจพบได้ (LOD) และระดับต่ำสุดที่สามารถหาปริมาณได้ (LOQ) อยู่ในช่วง 0.002 – 0.06 และ 0.005 – 0.25 ไมโครกรัมต่อลิตร ตามลำดับ เมื่อใช้วิธีที่ผ่านการทดสอบความใช้ได้ของวิธีนี้ทดสอบตัวอย่างน้ำจากฟาร์มมาตรฐานทั่วประเทศจำนวน 40 ตัวอย่าง ไม่พบการตกค้างของสารต้องห้ามทุกตัวอย่าง แสดงให้เห็นถึงประสิทธิภาพของมาตรการควบคุมสารตกค้างที่ดำเนินการมาตั้งแต่ปี 2002

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LIST OF ABBREVIATIONS

ACN	acetonitrile
AR	analytical grade
AVG	average
B (%B)	refers to the strong solvent in a binary-solvent mobile phase (% v/v)
C_{18}	chain length (octadecyl) of alkyl bonded phase
CI	chemical ionization
CV	coefficient of variation
DAD	diode-array detector
DI H_2O	deionized water
EI	electron ionization
EIC	extracted ion chromatogram
ESI	electrospray ionization (MS)
HLB	hydrophilic-lipophilic balance
HPLC	high-performance liquid chromatography
ID	internal diameter, d_c
IS	internal standard
k	retention factor
L	column length (cm)
LC-MS	liquid chromatography-mass spectrometry
LOD	limit of detection
LOQ	limit of quantitation
M	molarity
MeOH	methanol
mg	milligram
min	minute
mL	millimeter
mM	milimolar

LIST OF ABBREVIATIONS (cont.)

MS	mass spectrometry
MS/MS	tandem mass spectrometry
N	column plate count
pH	negative logarithm of hydrogen ion concentration
pK_a	acidity constant for and acid or protonated base
psi	pound per square inch
r^2	coefficient of determination
RPC	reversed-phase chromatography
R_s	resolution
%RSD	percent relative standard deviation
SD	standard deviation
SPE	solid phase extraction
t_R	retention time (min)
v/v	volume by volume
°C	degree Celsius
µg	microgram

CHAPTER I
INTRODUCTION

Food safety has become an issue of public concern for many years. Occasionally, many substances such as antimicrobial drug, pesticide and microbial organism residues found in food samples exceed the permitted limits. The non-compliance residues may result from contaminations during harvest or production processes, improper withdrawal periods of pesticides and animal drugs uses and abuses of prohibited substances.

In March 2002, meat products, mainly poultry and seafood, exported from Thailand to the EU community were firstly reported to be contaminated with nitrofuran's metabolites and chloramphenicol (1-3). The contaminated consignments were condemned due to genotoxicity and carcinogenicity of these substances (1, 4). Many similar cases were continuously reported as rapid alerts in the year 2002, causing significant economic losses due to the rejection of exported livestock and aquaculture products (2). Thai government responded this crisis immediately and seriously revised the existing legislation and regulations including import restrictions of 16 chemicals that were prohibited according to Annex IV of EU Council regulation (EEC) No 2377/90 (MRL regulation) (5) and to the USFDA list of drugs prohibited from extra-label use in food animals by Import and Export Act B.E. 2522 (1979) (2).

Major classes of antibiotics that are commonly used in livestock include nitrofuran, nitroimidazole and chloramphenicol. Nitrofurans are antibiotics, which were banned from livestock production in the European Union during mid 1990s due to their carcinogenicity and mutagenicity (4). Nitrofurans (e.g. furazolidone (FZD), furaltadone (FTD), nitrofurantoin (NFT) and nitrofurazone (NFZ) (3)) are antibiotics that were widely and effectively used in livestocks as growth promoters and for the prevention and treatment of gastrointestinal infections caused by *Escherichia coli*, *Salmonella* spp., *Mycoplasma* spp., *Coccidia* spp., coliforms and some other protozoa. Nitroimidazoles are veterinary drugs that are used for treatment and prevention of

certain bacterial and protozoal diseases in poultry as well as for swine dysentery, thus they are classified as coccidiostats. The most popular nitroimidazoles used as additives are metronidazole (MNZ), dimetridazole (DMZ), and ronidazole (RNZ). However, these compounds can cause mutagenicity, carcinogenicity and toxicity (6, 7). For this reason, the EU has prohibited their use as additives in feed for food-producing species as described in Annex IV of the Council Regulation(EC) 2377/90 (5). Chloramphenicol (CAP) is considered as a potent antibiotic for treating pneumonic and enteric disorders. CAP has been recommended for prevention of secondary infections associated with chronic respiratory diseases in poultry. However, CAP is, in certain susceptible individuals, associated with serious toxic effects in human such as bone marrow depression, and fatal aplastic anemia (8, 9). Since this condition is dose independent, CAP has been banned for uses in food producing animals in many countries including EU and Thailand.

In order to monitor and control these banned drugs abuse, Thai government had several restricted measures from farm level to slaughter house to ensure the zero tolerance level of these residues. Many analytical methods have been used for the control of these drugs in various matrices (e.g. animal muscles, visceral organs, milk, honey, feed and farm water). Techniques from conventional to highly sophisticated techniques such as tandem mass spectrometry have been used for determinations of these compounds (10-14). An extensive reviews show that a simultaneous multiclass analysis of prohibited drugs in livestock drinking water is not available.

This study was aimed to develop a high performance liquid chromatographic (HPLC) method interfaced with an ion trap mass spectrometer for multiclass analysis of prohibited drugs in livestock drinking water. Target analytes included nitrofurans (NFZ, NFT, FZD and FTD), nitroimidazoles (MNZ, RNZ and DMZ) and CAP. The HPLC analyses were performed on Prodigy ODS (3), 2.0x150 mm, 5µm with guard cartridge using a gradient elution of acetonitrile and water at pH 5.0 at a flow rate of 0.2 mL/min, column oven temperature 40°C, injection volume 10 µL. The eight antimicrobial agents were separated within 22 min using nebulizer gas at 35 psi, drying gas and dry temperature at 9 L/min and 325 °C, respectively. Sample preparation was achieved by solid phase extraction using hydrophilic-

lipophilic balance polymeric packing materials. Among the eight different eluent compositions, 1% acetic acid in acetonitrile : methanol (80 : 20, v/v) provided the satisfied recovery (%R = 60.1 – 106.0) for the target analytes using their corresponding label isotopes as internal standards.

The method was validated in terms of linearity and range, precision (repeatability and intermediate precision), accuracy, limit of detection and limit of quantitation. The linearity shows correlation of determination of 0.979 – 0.999. Precision was less than 26.6% and accuracy was between 88.4 and 110%. The method was sensitive with LOD and LOQ of 0.002 - 0.06 and 0.005 - 0.25 µg/L, respectively. Finally, the developed method was applied for the determination of eight prohibited substances in livestock drinking water collected from standard farms in Thailand. The banned drug residues were not found in all investigated samples.

CHAPTER II
LITERATURE REVIEW

2.1 Nitrofurans

2.1.1 General information

Nitrofurans are broad-spectrum bactericidal drugs (15), containing 5-nitrofuran moiety in their structure (Figure 2.1). These drugs have been widely used in veterinary practice as feed additives in food-producing animals such as poultry, swine, cultured fish, shrimps (3, 16) and bee (17, 18) in order to treat bacterial infection and as growth promoter. They are highly active against *Salmonella* spp., coliforms, *Mycoplasma* spp., *Coccidia* spp., and some other protozoa (15). FZD, FTD and NFZ were allowed by USFDA for the treatment of protozoan and bacterial infection in poultry and swine before withdrawn in 1991 (19).

Normally, nitrofurans were administered to herd or flock of food-producing animal through animal feed or drinking water at the concentration of 8 – 400 mg/kg. However, their metabolites could be detected in trace amounts in animals that were fed with feed containing FZD and FTD as low as 30 µg/kg (3).

Nitrofurans have very short half-lifes in animal and are rapidly metabolized by releasing their side chains. These metabolites can bound to protein and easily extracted under acidic condition. For example, the 3-amino-2-oxazolidinone (AOZ), side chain of furazolidone could be released from protein-bound metabolites in microsomes, hepatocytes and pig livers under mild acidic condition and the condition in human stomach can release AOZ from tissue-bound metabolites (20).

Analytes	Exact mass	pK_a*	R	Metabolites
Nitrofurantoin (NFT)	238.034	7.2		1-Aminohydantoin (AH)
Nitrofurazone (NFZ)	198.039	10.0		Semicarbazide (SEM)
Furazolidone (FZD)	225.039	4.9		3-Amino-2-oxazolidinone (AOZ)
Furaltadone (FTD)	324.107	5.0		3-Amino-5-morpholinomethyl-2-oxazolidinone (AMOZ)

*from Ref. (21)

Figure 2.1 Chemical structure of nitrofurans and their metabolites.

In some country, such as Argentina legislation established the tolerance limit for nitrofurans in animal feeds and water for poultry as 400 μg/kg and 2 μg/L, respectively (22) while some international organization, such as the European Union Commission categorized nitrofurans as prohibited substances and established a zero tolerance for these compound and their metabolites in Annex IV of Regulation 2377/90/EEC (5). Therefore, the substances in this group cannot be used in veterinary medicinal product for food producing animals.

2.1.2 Determination of nitrofurans

High-Performance Liquid Chromatography (HPLC) couples with UV or diode array detector have been used for a long time for determinations of nitrofuran in various samples such as animal feed, water, animal tissues and products of animal origin. The concept of determination of parent compound residues had been superseded by their side chain metabolites after the fact that nitrofurans are rapidly metabolized and their residues can be found as tissue-bound metabolites (23). These protein-bound metabolites are stable and can be extracted to free side chain metabolites under acidic condition before derivatization with suitable chromophore to facilitate detection by UV detector.

Side chain residues of the most popular nitrofurans; nitrofurantoin, nitrofurazone, furazolidone and furaltadone, are 1-Aminohydantoin (AH), Semicarbazide (SEM), 3-Amino-2-oxazolidinone (AOZ) and 3-Amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), respectively (Figure 2.1). These metabolites could be derivatized with derivatizing agent such as 2-nitrobenzaldehyde (2-NBA) (11) to enhance UV absorption enabling to be detected with UV detector. Moreover, these target compounds show strong signal intensity of product ions when tandem mass spectrometry is applied as a detector (24).

Table 2.1 summarizes published literatures related to liquid chromatography of nitrofurans in different food matrices.

Table 2.1 Liquid chromatographic methods for the determination of nitrofurans

Analyte	Method	Matrices	Column	MP	Significant results	Ref.
NFZ, NFT, FZD and FTD	HPLC-DAD	Water (filtration)	100 × 4.6 mm silica-based Chromolith monolith	8/92 (v/v) acetonitrile/50 mM KH_2PO_4 (pH 4.5)	0.21 - 0.27 μg/L	(22)
		Feed (solvent extraction)			2.1 and 2.7 μg/kg	
NFZ, NFT, FZD and FTD	HPLC-DAD	Feed (solvent extraction, Sep-Pack NH_2 cartridge)	Lichrospher 60, RP-select B, 5 μm, 250mm × 4mm, with guard column	14mM ammonium acetate (pH 4.6) (A) and ACN (B), gradient elution	47 – 98 μg/kg (CCα)	(3)

Table 2.1 Liquid chromatographic methods for the determination of nitrofurans (continued)

Analyte	Method	Matrices	Column	MP	Significant results	Ref.
	LC-MS/MS		Zorbax Eclipse XDB–C18, 5 µm, 150mm × 2.1mm, with guard column		7 – 21 µg/kg (CCα)	
AHD, AOZ, SEM and AMOZ	LC-ESI-MS/MS	Milk (solvent extraction)	Luna C18(2) column (150mm×2mm i.d., 3 µm), with guard column	0.5mM acetate ammonium containing 20% methanol (A) and methanol (B), gradient elution	0.12 – 0.29 µg/kg (CCα)	(11)
AHD, AOZ, SEM and AMOZ	LC-ESI-MS/MS	Honey (polymeric SPE)	Symmetry Shield C18 reversed-phase (15 cm × 2.1 mm, 3.5 µm), with guard column	A [water containing concentrated acetic acid 0.025% (v/v) (pH 3.6)] and B (acetonitrile), gradient elution	0.07-0.46 µg/kg (CCα)	(18)

Table 2.1 Liquid chromatographic methods for the determination of nitrofurans (continued)

Analyte	Method	Matrices	Column	MP	Significant results	Ref.
AHD, AOZ, SEM and AMOZ	LC-ESI-MS/MS	Salt (solvent extraction)	C18 column (100mm × 2.1mm, 4μm	A(%) (water, 0.1%acetic acid), B (%) (90%acetonitrile, 10% water and0.1% acetic acid), gradient elution	0.03 - 0.10 μg/kg (CCα)	(25)
AHD, AOZ, SEM and AMOZ	UHPLC–MS/MS	Animal plasma (solvent extraction)	Acquity UHPLC®BEH C18 (100mm×2.1mm, 1.7μm), with guard column	A: ammonium acetate (0.5mM) and B: MeOH, gradient elution	0.054 – 0.071 μg/kg (CCα)	(26)
FZD and AOZ	HPLC-UV	Fish feed (solvent extraction)	C18 column (250 mm × 4.6 mm, 5 μm)	0.1% phosphoric acid in acetonitrile/water (40:60, v/v)	0.4 ng/g	(16)

Table 2.1 Liquid chromatographic methods for the determination of nitrofurans (continued)

Analyte	Method	Matrices	Column	MP	Significant results	Ref.
	LC-ESI-MS/MS		Pinnacle II C18 column (150mm × 2.1 mm, 5 µm)	0.25% formic acid in 0.5 mmol/L ammonium acetate(A) and acetonitrile/water (9:1, v/v) (B), gradient elution	0.05 ng/g	
AHD, AOZ, SEM and AMOZ	LC-ESI-MS/MS	Honey (Oasis HLB SPE)	Inertsil ODS-3 5 µm, 150 × 2.1 mm, with guard column	10 mM NH_4Ac (A) and 100% methanol (B), gradient	0.25 ng/g (LOQ)	(27)
FZD	HPLC-UV	Animal feed (solvent extraction)	µBondapak, 10 µm, C18 reversed-phase column, 300×3.9 mm I.D.	0.01 M sodium acetate pH 6.0 and acetonitrile (8:2)	1 mg/kg	(28)
	LC-MS		LiChrospher RP-18, 125 × 4 mm I.D., 5 µm	0.1 M ammonium acetate-acetonitrile (65:35, v/v)	0.1 mg/kg	

Table 2.1 Liquid chromatographic methods for the determination of nitrofurans (continued)

Analyte	Method	Matrices	Column	MP	Significant results	Ref.
AHD, AOZ, SEM and AMOZ	LC-ESI-MS/MS	chicken, turkey and shrimp muscle	Luna C18 column (150mm×2.0mm, 3μm) with guard column	(A): methanol and ammonium formate (10mM, pH 3.5)(v/v, 9 + 1) (B): methanol and ammonium formate (10mM, pH 3.5) (v/v, 1 + 9), gradient elution	CCα: 0.1 - 0.7 μg/kg, CCβ: 0.1 - 0.9 μg/kg	(23)
AHD, AOZ, SEM and AMOZ	LC-MS/MS ion trap	Shrimp and poultry	Zorbax XDB-C18, 2.1 mm × 150 mm, 3.5 μm	(A): Water + 0.1% acetic acid (B): Acetonitrile + 0.1% acetic acid, gradient elution	LOQ: 0.125 – 0.5 μg/kg	(29)

2.2 Nitroimidazoles

2.2.1 General information

Nitroimidazoles are antiprotozoal and antimicrobial agents that have been used in human and veterinary medicine. They are imidazole heterocyclic compounds containing a NO_2 group on the fifth position of its ring; e.g. metronidazole (MNZ), dimetridazole (DMZ), and ronidazole (RNZ) (7) (Figure 2.2). These substances are added to animal feed for the prevention of several contagious disease such as swine dysentery (6), histominiasis and coccidiosis in poultry, genital tricchoniasis in cattle and hemorrhagic enteritis in swine (30). Moreover, these substances are used as growth promoter and for improvement of feed efficiency (31). However, it has been report that these compounds have carcinogenic and mutagenic properties (6, 7, 30, 32-34); therefore, the European Union has banned the use of nitroimidazoles for food-producing animals and laid down in Annex IV of Regulation 2377/90/EEC (5, 7, 35, 36).

Nitroimidazoles are rapidly metabolized within a few hours after administration. Their major metabolites, 2-hydroxymethyl-1-methyl-5-nitroimidazole (HMMNI) from dimetridazole and ronidazole, 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole (MNZ-OH) from metronidazole, are considered carcinogenic and mutagenic compounds similarly to their parent compounds since they still contain the nitroimidazole ring (31, 32).

Analytes	Exact mass	pK_a*	R_1	R_2
Metronidazole (MNZ)	171.064	2.58	OH	CH_3
Ronidazole (RNZ)	200.055	1.32	CH_3	NH_2 O O
Dimetridazole (DMZ)	141.054	2.81	CH_3	CH_3

*from Ref. (7)

Figure 2.2 Chemical structures of nitroimidazoles.

2.2.2 Determination of nitroimidazoles

Several analytical methods have been reported for the determination of nitroimidazoles and their metabolites such as electrophoresis, gas chromatography, gas chromatography coupled - mass spectrometry, high-performance liquid chromatography and liquid chromatography - mass spectrometry (34). Recently, LC-MS/MS has become the most approved technique for nitroimidazole determination due to its advantage over the GC technique that requires prederivatization procedure. However, sample pretreatment is still the most important part since the target organs and type of sample matrices may contain interferences that lessen the method performances.

Table 2.2 summarizes reports on liquid chromatography of nitroimidazoles.

Table 2.2 Liquid chromatographic methods for the determination of nitroimidazoles

Analyte	Method	Matrices	Column	MP	Significant results	Ref.
MNZ, DMZ, IPZ, RNZ and HMMNI	LC-MS	Water (HLB SPE)	C_{18} (150 × 2.1mm ID, 5µm) with guard column	5.0 mM aqueous ammonium acetate buffer, pH 4.3 (A) and acetonitrile (B), gradient elution	0.2 µg/L	(6)
MNZ, DMZ, IPZ, RNZ, SCZ and TIN	LC-MS	Feed (HLB SPE)	Symmetry C18 column 150 × 2.1 mm ID, 5 µm, with guard column	pH 4.3 ammonium acetate 50 mM (A) and acetonitrile (B), gradient elution	1.2 – 6.1 ng/mL	(35)
MNZ, DMZ, IPZ, RNZ and their metabolites	LC-ESI-MS/MS	Egg (MIP)	Symmetry Shield RP18 (2.1 × 150 mm, 3.5 µm, with guard column	0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B), gradient elution	<0.34 µg/kg (CCα)	(12)

Table 2.2 Liquid chromatographic methods for the determination of nitroimidazoles (continued)

Analyte	Method	Matrices	Column	MP	Significant results	Ref.
MNZ, DMZ, IPZ, RNZ and corresponding hydroxyl metabolites	UPLC-MS/MS	Swine kidney (solvent extraction/Oasis MCX SPE)	Acquity BEH C18 column (50 mm×2.1 mmi.d., 1.7µm)	water (solvent A) and acetonitrile (solvent B), gradient elution	0.05 – 0.5 µg/kg	(31)
MNZ, DMZ, IPZ, RNZ, their corresponding hydroxyl metabolites, carnidazole (CRZ), ornidazole (ORZ) and ternidazole (TRZ)	LC-ESI-MS/MS	Plasma (solvent extraction)	Luna C18 column (100 mm×2 mm), 3µm, with guard column	0.1% acetic acid (A) and acetonitrile acidified with 0.1% acetic acid (B), gradient elution	0.5 – 1.6 ng/mL (CCα)	(36)
MNZ, DMZ, RNZ, IPZ and their metabolites (MNZOH, HMMNI, IPZOH,)	LC-ESI-MS/MS	Poultry muscle, plasma and egg (solvent extraction/SCX SPE)	Gemini C18 column (150 mm×2.0 mm,5 µm), with guard column	A (0.1% formic acid in acetonitrile) and B (0.1% formic acid in water), gradient elution	0.05 – 0.53 µg/kg (CCα)	(32)

Table 2.2 Liquid chromatographic methods for the determination of nitroimidazoles (continued)

Analyte	Method	Matrices	Column	MP	Significant results	Ref.
MNZ, DMZ, IPZ, RNZ, their corresponding hydroxyl metabolites, carnidazole (CRZ), ornidazole (ORZ), tinidazole (TNZ) and ternidazole (TRZ)	LC-ESI-MS/MS	Egg (solvent extraction)	Luna C18 column (100 ×2) mm, 3 µm, with guard column	0.1% acetic acid (A) and acetonitrile acidified with 0.1% acetic acid (B), gradient elution	0.33 - 1.26 µg/kg (CCα)	(30)
MNZ, DMZ, IPZ, RNZ, their two corresponding metabolites, AHD, AOZ, SEM and AMOZ	LC-ESI-MS/MS	Pork (solvent extraction/Oasis HLB SPE)	SunFire C8 column (150 mm×2.1 mm,I.D., 5 µm)	A (water) and B (acetonitrile), gradient elution	0.01 – 0.2 µg/kg	(37)

Table 2.2 Liquid chromatographic methods for the determination of nitroimidazoles (continued)

Analyte	Method	Matrices	Column	MP	Significant results	Ref.
MNZ, DMZ, RNZ tinidazole (TNZ), ornidazole (ONZ),secnidazole (SNZ) and hydroxydimetridazole (DMOHZ)	HPLC-UV	Meat (solvent extraction/SCX SPE)	Diamonsil^{TM} C18 column, (5 µm, 250 mm×4.6 mm i.d.)	deionized water–methanol–acetonitrile, gradient elution	0.2 µg/kg	(34)
DMZ, MNZ, RNZ, hydroxymetronidazole (MNZOH), hydroxyipronidazole (IPZOH), and 2-hydroxymethyl1-methyl-5-nitroimidazole (HMMNI)	LC-ESI-MS/MS	Pork meat (solvent extraction)	Gemini C18 column (5 µm, 150 mm×3 mmI.D.), with guard column	10 mM ammonium formate, titrated to pH 3.5 with formic acid. Eluent A consisted of 90:10 (v/v) of buffer/acetonitrile, eluent B of 10:90 (v/v) buffer/acetonitrile, gradient elution	0.29–0.44 µg/kg	(33)

Table 2.2 Liquid chromatographic methods for the determination of nitroimidazoles (continued)

Analyte	Method	Matrices	Column	MP	Significant results	Ref.
Amoxicillin, metronidazole, cefazolin, sulfamethoxazole and chloramphenicol	HPLC-DAD	Waste water (influent)	RP Purospher RP-18 endcapped column (250 mm×4mm, 5 µm) with guard column	water adjusted with phosphoric acid (pH 2.50 ± 0.02) and ACN, gradient elution	LOD: 3.0 – 14 µg/L	(38)

2.3 Chloramphenicol

2.3.1 General information

Chloramphenicol (CAP) is a broad spectrum bacteriostatic antibiotic originally derived from *Streptomyces venezuelae* (Figure 2.3). It is effective against variety of pathogens, including aerobic and anaerobic gram-negative and gram-positive bacteria, rickettsia, mycoplasma and chlamydia (39). Chloramphenicol can cause non-dose-dependent, irreversible aplastic anemia in susceptible individual (40) that may lead to leukaemia (8). Because of its adverse effect, CAP was prohibited from the use in food-producing animals in EU, and is classified in Annex IV of Regulation 2377/90/EEC (5, 10).

Metabolites of chloramphenicol include nitroso-chloramphenicol, chloramphenicol glucuronide, hydroxymphenicol, dehydro-chloramphenicol, etc. All show cytotoxic and genotoxic properties such as chromosomal aberration and sister chromatid exchange in human (10). In certain conditions, chloramphenicol glucuronide can be deconjugated to the parent form chloramphenicol by bacteria (41).

$C_{11}H_{12}Cl_2N_2O_5$
Average mass = 323.129
Exact mass = 322.012

Figure 2.3 Chemical structure of chloramphenicol.

2.3.2 Determination of chloramphenicol

Various methods have been reported for determinations of chloramphenicol, depending on the purposes of analysis and types of matrices. Residues in products from animal origin or trace analysis in environment may require high sensitivity and rapid detection such as ELISA, immunochromatographic assay (9), capillary electrophoresis, micellar electrokinetic chromatography, and surface plasmon resonance biosensor assay (42) or high confidence method such as GC or LC coupled to MS (8, 40, 42-44). GC-MS/MS was previously the official method in many organizations for the quantitation of chloramphenicol residues at very low concentrations but derivatization (silylation) with derivatization agent such as N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) prior to analysis is a prerequisite (42, 43). Therefore, LC-MS/MS has been introduced as a confirmatory method in place of GC-MS/MS to eliminate tedious step in sample preparation.

Table 2.3 summarizes recent researches on liquid chromatography of chloramphenicol.

Table 2.3 Liquid chromatographic methods for the determination of chloramphenicol

Analyte	Method	Matrices	Column	MP	Significant results	Ref.
chloramphenicol (CAP), thiamphenicol (TAP), florfenicol (FF), and florfenicol amine (FFA)	LC-ESI-MS/MS	Chicken muscle	XTerra C8 column (100 mm×2.1 mm i.d., 5 µm)	(A) acetonitrile and (B) water, gradient elution	0.07 µg/kg for CAP and 0.57 – 3.41 µg/kg for TAP, FF and FFA (CCα)	(45)
CAP	GC/MS-NCI (silylation derivatisation)	Urine Water Honey Milk (MIP)	HP-1MS, length 30 m, I.D. 0.25mm, film thickness 0.25µm,	carrier gas He	0.06 ng/mL (CCα) 0.005 ng/mL (CCα) 0.06 ng/g (CCα) 0.03 ng/mL (CCα)	(43)

Table 2.3 Liquid chromatographic methods for the determination of chloramphenicol (continued)

Analyte	Method	Matrices	Column	MP	Significant results	Ref.
CAP	LC-VWD	Honey (dispersive liquid–liquid microextraction (DLLME))	Varian Pursuit-C18 column (5 µm, 4.6 × 250 mm), with guard column	methanol–water (55:45, v/v)	0.06 µg/kg	(14)
CAP	LC-ESI-MS/MS	Shrimp (solvent extraction)	C18 Unisil HPLC column (150 × 4 mm i.d.), 5 mm	solvents: A (water 90% plus acetonitrile 10%) and solvent B (water 10% plus acetonitrile 90%). Isocratic mobile phase (A:B=40:60, v/v)	0.06 µg/kg (CCα)	(10)
Chloramphenicol, enrofloxacin and 29 pesticides	LC-ESI-MS/MS ion trap	bovine milk	SB-C18 column, 2.1 mm × 150 mm, 5 µm	Methanol : 0.2 %formic acid, gradient elution	CAP: LOD 0.3×10^{-3} mg/kg LOQ 1.5×10^{-3} mg/kg	(44)

Table 2.3 Liquid chromatographic methods for the determination of chloramphenicol (continued)

Analyte	Method	Matrices	Column	MP	Significant results	Ref.
Clenbuterol (CLEN), chloramphenicol (CAP) and diethylstilbestrol (DES)	UPLC–MS/MS	bovine milk	Acquity BEH C18 column (50mm×2.1mm, 1.7μm)	(A):5 mM ammonium acetate (B): acetonitrile, gradient elution.	LOQs were 0.03, 0.02 and 0.13 ng/g for CLEN, CAP and DES, respectively,	(46)
Chloramphenicol	HPLC-UV with Ionic liquid–salt aqueous two-phase flotation (ILATPF)	Lake water, feed water, milk and honey	Eclipse XDB-C18 250mm×4.6mm,5μm.	Water : methanol (55:45)	LOD: 0.1 ng/mL LOQ 0.3 ng/mL.	(47)
Chloramphenicol	HPLC-DAD with Solid phase microextraction—Liquid chromatography (SPME)	Urine, tap and sea water	Luna C18 (150×4.6mm i.d, particle size 5μm)	acetonitrile/ammonium acetate buffer (10mM, pH 4.6) mixture (70:30, v/v)	Estimated LOD and LOQ were 37 and 95 ng/mL (urine), 0.1 and 0.3 ng/mL (tap water), 0.3 and 0.7 ng/mL (sea water)	(41)

Table 2.3 Liquid chromatographic methods for the determination of chloramphenicol (continued)

Analyte	Method	Matrices	Column	MP	Significant results	Ref.
Chloramphenicol, thiamphenicol, tetracycline, oxytetracycline, chlortetracycline, metacycline, doxycycline, cefoperazone, ceftriaxone and cefaclor	UPLC-DAD	Milk	Acquity UPLC BEH C18 column (50 mm × 2.1 mm, 1.7 µm,	(A): acetonitrile (B): 10 mmol L^{-1}oxalic acid, gradient elution	LOD: 0.003 to 0.022 µg/g LOQ: 0.01 to 0.08 µg/g For CAP, LOD: 0.004 µg/g LOQ: 0.02 µg/g	(48)
Chloramphenicol	LC–ESI–MS/MS	Milk powder	Luna C18 column (150mm×2mm i.d., 5 µm)	(A): water (B): acetonitryle (80:20; v/v), gradient elution	CCα 0.09 µg/kg CCβ 0.11µg/kg	(49)
Chloramphenicol	LC-ESI-MS/MS ion trap	Muscle tissue samples from bovine, pork, chicken, fish, turkey and rabbit,	Synergi MAX-RP250×3.0mm, 4 µm	20mM ammonium acetate (pH 4.5) /acetonitrile 60/40 (v/v)	CCα 0.15 ng/g CCβ 0.22 ng/g	(50)

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

Table 3.1 List of instruments

Instruments	Source/Supplier
Analytical balance (AX205DR)	Mettler Toledo (Schwerzenbach, Switzerland)
Deionized water system (Milli-Q)	Millipore (Bedford, MA, USA)
HPLC system (1200 series)	Agilent Technologies (Santa Clara, CA, USA)
Ion trap mass spectrometry (HTC)	Bruker Daltonics (Bremen, Germany)
Nitrogen evaporator (TurboVap®LV)	Zymark (Hopkinton, MA, USA)
Rotary evaporator (Eyela N-1000)	Tokyo Rikakikai (Tokyo, Japan)
Rotary evaporator (Laborota 4000)	Heidolph (Schwabach, Germany)
pH meter (MP230)	Mettler Toledo (Schwerzenbach, Switzerland)
Prodigy ODS (3), 2.0x150 mm, 5µm	Phenomenex (Torrance, CA, USA)
Ultrasonic bath (1875D)	Crest (Trenton, NJ, USA)
Vacuum manifold (Visiprep 24™ DL)	Supelco (Bellefonte, PA, USA)
Vacuum pump	Gast (Benton Harbor, MI, USA)
Vortex mixer (Vertex Genie2)	Scientific Industries (Bohemia, NY, USA)

* These instruments were provided by Bureau of Quality Control of Livestock Products, Department of Livestock Development, Pathumthani, Thailand

Table 3.2 List of chemicals and reagents

Name	Source/Supplier
Acetonitrile (AR grade)	BDH (Leicestershire, UK)
Acetonitrile (HPLC grade)	Labscan Asia (Bangkok, Thailand)
Ammonium acetate (AR grade)	BDH (Leicestershire, UK)
Chloramphenicol	Sigma (St. Louis Missouri, USA)
Chloramphenicol-D_5	Dr.Ehrenstorfer (Augsburg, Germany)
Dimetridazole	Sigma (St. Louis Missouri, USA)
Dimetridazole-D_3	Witega (Berlin, Germany)
Formic acid (GR grade)	Merck (Darmstadt, Germany)
Furaltadone	Sigma (St. Louis Missouri, USA)
Furaltadone-D_5	Witega (Berlin, Germany)
Furazolidone	Sigma (St. Louis Missouri, USA)
Furazolidone-D_4	Witega (Berlin, Germany)
Glacial acetic acid (GR grade)	Merck (Darmstadt, Germany)
Methanol (AR grade)	Scharlau (Barcelona, Spain)
Methanol (HPLC grade)	Labscan Asia (Bangkok, Thailand)
Metronidazole	Sigma (St. Louis Missouri, USA)
Metronidazole-$^{13}C_2$, $^{15}N_2$	Witega (Berlin, Germany)
Nitrofurantoin	Sigma (St. Louis Missouri, USA)
Nitrofurantoin-$^{13}C_3$	Witega (Berlin, Germany)
Nitrofurazone	Sigma (St. Louis Missouri, USA)
Nitrofurazone-^{13}C, $^{15}N_2$	Witega (Berlin, Germany)
Oasis HLB cartridges, 200mg/6mL	Waters (Milford, MA, USA)
Ronidazole	Sigma (St. Louis Missouri, USA)
Ronidazole-D_3	Witega (Berlin, Germany)

3.2 Methods

Experiments scheme comprised of method development, method validation and application (Figure 3.1). Initial experiments aimed to define the optimum condition of HPLC, MS and SPE. HPLC conditions were optimized to acquire the best separation of analyte peaks. The MS optimization was fine tuned using parameter ramping modules incorporated in Esquire Control version 6.1 software. Sample preparations were performed using hydrophilic-lipophilic balanced reversed-phase sorbent (HLB) SPE by varying eluent compositions. Method validation was subsequently evaluated with the proposed conditions before applying to animal drinking water samples.

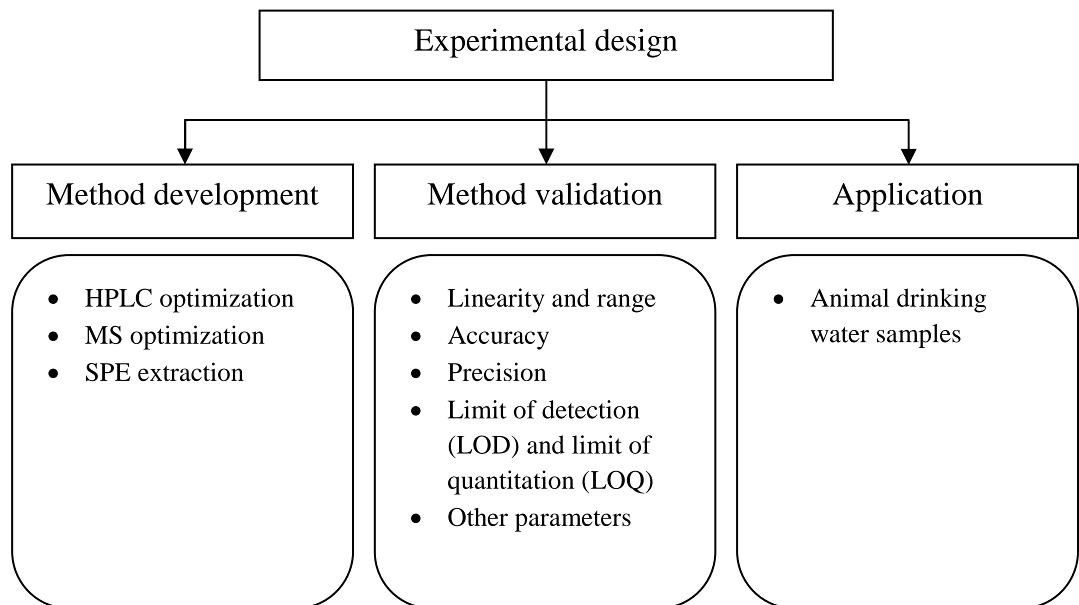


Figure 3.1 Experimental scheme.

3.2.1 Instrumentation

High-performance liquid chromatography tandem mass spectrometry analysis was performed on Agilent 1200 series coupled with Bruker HCT ion trap mass spectrometer. The LC-MS system was controlled by HP workstation XW 6200 using Bruker Daltonic Esquire 6.1 software comprised of Esquire Control version 6.1, DataAnalysis version 3.4, HyStar version 3.2 and QuantAnalysis version 1.8 for

analysis, data manipulation and quantitative analysis. Chromatographic separation was operated in reversed phase mode using octadecylsilane mini or microbore columns with 2.1 or 3.0 mm i.d., 100-150 mm in length and 3-5 μm partical size coupled with guard cartridges. Method development and optimization were studied as described in method development and optimization sections.

3.2.2 Standard preparations

Standard and deuterated isotope labeled internal standards were accurately weighed and diluted with acetonitrile to concentrations of 0.5 and 0.2 mg/mL, respectively. The stock solutions were protected from light and kept in a freezer (-20°C). Intermediate standard solutions were subsequent diluted with acetonitrile to the concentration of 25 μg/mL and kept at 4°C. Working standards were prepared by diluting the stock standard solutions with acetonitrile to appropriate concentrations (e.g. 100 and 200 ng/mL) for linearity and range experiments. The stocks of standard and internal standard solutions were stable at the temperature of 4°C for a month.

For calibration procedure, the internal standard calibration method was applied to this study. NFZ-^{13}C-$^{15}N_2$, NFT-$^{13}C_3$, FZD-D_4, FTD-D_5, MNZ-$^{13}C_2$-$^{15}N_2$, RNZ-D_3, DMZ-D_3 and CAP-D_5 were used as internal standards for NFZ, NFT, FZD, FTD, MNZ, RNZ, DMZ and CAP, respectively. Known amounts of working standard solutions were accurately added into evaporating tubes containing acetonitrile before fortified with fixed amount of working internal standard. Standard mixtures were evaporated under nitrogen stream at 40 °C to dryness, reconstituted with 1 mL 20% acetonitrile and filtered with 0.45 μm nylon syringe filter before analyses. Linearity studies were examined in ranges of 2.5 to 350 μg/L (n = 6).

3.2.3 Method development

3.2.3.1 HPLC optimization

The analyses were performed on an Agilent HPLC 1200 series (Agilent Technologies, Santa Clara, CA, USA) coupled to a Bruker (HTC) ion trap mass spectrometry equipped with ESI (Bruker Daltonics, Bremen, Germany) in alternative polarity switching scan mode MS. The LC-MS column was a Prodigy ODS (3), 2.0x150 mm, 5µ (Phenomenex, Torrance, CA, USA) with a guard cartridge. The mobile phase consist of solvent A (aqueous buffer of ammonium formate and formic acid or ammonium acetate and acetic acid at various concentration and pH) and solvent B (acetonitrile or methanol). Various mobile phase compositions and gradient profiles were studied to obtain the appropriate analytical separation and run time. The flow rate was 0.2 mL/min, and injection volume was set to 10 µL per injection. Ultrahigh pure nitrogen was used for as nebulizer and dry gas at 35 psi and 9 L/min, respectively, with drying temperature of 325 °C.

Optimization of HPLC condition was performed by varying compositions, pH of mobile phase and types of modifiers (Table 3.3). The optimum condition was determined from system suitability parameter such as resolution, plate number and run time.

Table 3.3 List of factors in HPLC optimization

Factor	Range
Buffer concentration (mM)	5 – 40
Type of buffer	ammonium acetate, ammonium formate
pH of buffer	2.0 – 6.0
Type of modifier	formic acid, acetic acid
Amount of organic solvent (%v/v)	Gradient elution profile (5 – 50)
Type of organic solvent	acetonitrile, methanol

Acetonitrile (ACN) and methanol (MeOH) were used as organic solvents, and the aqueous phase were acidified with acetic acid (CH_3COOH) and formic acid (HCOOH) at concentration of 0.05 and 0.1% v/v. The elution profile of each banned substance was studied at the concentration of 1 mg/L, 5-µL injection volume. The parameters used in each mobile phase system were detailed in Table 3.4. The appropriate system was evaluated from resolution parameter.

Table 3.4 Various types of organic solvent and acid modifiers

System	ACN	MeOH	CH_3COOH (v/v)	HCOOH (v/v)	pH of aqueous phase
1	✓	-	-	0.05%	2.9
2	-	✓	-	0.05%	2.9
3	✓	-	-	0.10%	2.7
4	-	✓	-	0.10%	2.5
5	✓	-	0.05%	-	3.2
6	-	✓	0.05%	-	3.3
7	✓	-	0.10%	-	3.2
8	-	✓	0.10%	-	3.2

Isocratic elution using ammonium formate and ammonium acetate buffer at various pH were performed using alternative polarity switching in scan mode MS. The buffer of ammonium formate and formic acid were prepared in 0.3 step of pH value in the range of 2.6 – 5.0 and the buffers of ammonium acetate and acetic acid were also varied in same step in the range of 3.8 – 5.6.

The effects of buffer concentrations were studied using ammonium formate at concentrations of 5, 10, 20 and 40 mM in 0.01% formic acid. The pH of aqueous buffer were ranged from 3.9 - 4.8. The LC-MS was operated in alternative polarity scan mode with mobile phase (ACN : buffer, 15 : 85 (v/v)). The chromatograms were interpreted in term of retention time (t_R).

3.2.3.2 MS optimization

Precursor ions, product ions from MS/MS reactions and fragmentation amplitudes were studied with direct infusion of 1 mg/L of each working standard solution (Table 3.5) into the mass analyzer through electrospray ionization interface (ESI) at a flow rate of 240 μL per hour. Various parameters such as capillary voltage, ion optic (e.g. skimmer, octapole, lens, trap drive voltage), and fragmentation voltage were varied to obtain the highest intensity of precursor and product ions of each compound.

Table 3.5 MS/MS reactions monitored by LC-ESI MS^n

Analyte	MS/MS reactions (m/z)
Positive mode $[M+H]^+$	
MNZ	172.0 > 128.0
MNZ-$^{13}C_2$-$^{15}N_2$	176.0 > 132.0
RNZ	201.0 > 140.0
RNZ-D_3	204.2 > 143.0
DMZ	142.0 > 96.2
DMZ-D_3	145.2 > 99.2
FZD	226.0 > 113.1
FZD-D_4	229.8 > 117.1
FTD	325.2 > 281.1
FTD-D_5	330.2 > 286.2
Negative mode $[M-H]^-$	
NFZ	196.8 > 149.9
NFZ-^{13}C-$^{15}N_2$	199.8 > 152.9
NFT	236.8 > 151.9
NFT-$^{13}C_3$	239.8 >151.9
CAP	321.0 > 257.0
CAP-D_5	326.0 > 262.0

After LC optimization, the analytical run time was divided into segment time and particular MS/MS parameters corresponded to each analyte and internal standard were specified to each segment and calibration curve experiment was performed in MS/MS mode.

3.2.3.3 Sample pretreatment-SPE extraction

Solid phase extraction was employed for sample clean up. Oasis HLB cartridges were connected to a vacuum manifold and 60 mL reservoirs were attached to the top of each SPE cartridge by an adapter. SPE cartridges were conditioned before use by washing with methanol and water prior the applications of samples. After sample loading, the solid phase was washed with 5% methanol in deionized water. Cartridges were then dried with a stream of nitrogen or under vacuum and the analytes were subsequently eluted with optimized eluent and were evaporated to dryness using rotary evaporator before being reconstituted with suitable reagent and filtered through 0.45-µm syringe filters before injection.

Solid phase extraction (SPE) procedure on Oasis HLB cartridges (200 mg / 6 mL) was optimized by varying types of solvents (acetonitrile and methanol), compositions and acidity of eluent (Table 3.6).

The cartridges were conditioned with 5 mL of MeOH, then equilibrate with 5 mL of water before loading of 200 mL of sample. SPE were washed with 5 mL of 5% MeOH and applied with vacuum to dryness before eluting with 4 mL of eluent (2×2). Eluate was dried up using a rotary evaporator, reconstituted with 0.5 mL of 20 mM ammonium acetate : MeOH (80 : 20, v/v), vortexed for 30 sec, sonicated for 15 sec, and filtered through a 0.45-µm syringe nylon filter.

Table 3.6 Types of eluents for SPE optimization

Eluent	Composition
1	Absolute methanol
2	Absolute acetonitrile
3	Acetonitrile : Methanol (50:50, v/v)
4	Acetonitrile : Methanol (80:20, v/v)
5	0.5% acetic acid in acetonitrile : methanol (80 : 20, v/v)
6	1% acetic acid in acetonitrile : methanol (80 : 20, v/v)
7	1.5% acetic acid in acetonitrile : methanol (80 : 20, v/v)
8	2% acetic acid in acetonitrile : methanol (80 : 20, v/v)

Samples used in this experiment were blank animal drinking water. Two hundred milliliters were transferred into each volumetric flask, 1 mL of 2 M sodium thiosulphate was added into the flask, mixed and left for 30 min before spiked with 0.5 mL and 0.2 mL of mixtures of eight IS and working standards, respectively (Table 3.7). Samples were mixed thoroughly and stood for 10 min before loading to previously conditioned SPE. Samples in each eluent were prepared in triplicate (n = 3). Matrix matched standards were prepared in the same fashion of sample with the working standard (Table 3.7) varying spiking volume from 200 – 1400 µL into blank farm water. The concentration of each analyte was shown in Table 3.8.

Table 3.7 Working standard and stable isotope labeled internal standard working solution

	Concentration (mg/L)							
Working standard	MNZ	RNZ	DMZ	NFZ	NFT	FZD	FTD	CAP
	0.375	0.15	0.5	0.375	0.5	0.015	0.025	0.025
Isotope labeled internal working standard	MNZ-$^{13}C_2$-$^{15}N_2$	RNZ-D_3	DMZ-D_3	NFZ-^{13}C-$^{15}N_2$	NFT-$^{13}C_3$	FZD-D_4	FTD-D_5	CAP-D_5
	0.32	0.2	0.56	0.16	0.4	0.016	0.032	0.032

Table 3.8 Matrix matched standard calibration range of concentration in standard preparation

Level	Concentration (µg/L)							
	MNZ	RNZ	DMZ	NFZ	NFT	FZD	FTD	CAP
1	0	0	0	0	0	0	0	0
2	150	60	200	150	200	6	10	10
3	300	120	400	300	400	12	20	20
4	450	180	600	450	600	18	30	30
5	600	240	800	600	800	24	40	40
6	750	300	1000	750	1000	30	50	50
7	900	360	1200	900	1200	36	60	60
8	1050	420	1400	1050	1400	42	70	70
corresponding range in sample	0.375 - 2.625	0.15 - 1.05	0.5 - 3.5	0.375 - 2.625	0.5 - 3.5	0.015 - 0.105	0.025 - 0.175	0.025 - 0.175

Each standard or sample preparation was analyzed using LC-MS ion trap under optimized condition. Eight levels including zero of matrix matched standards were triplicately injected. Linear regression was constructed using Microsoft Excel from concentration ratio of analyte/IS and ratio area for each compound. The obtained results were corrected by enrichment factor of 400.

Figure 3.2 shows the relationship and steps of method development concerning HPLC, MS and SPE. The performance parameters and system suitability were investigated and fine tune adjustment was carried out along the development process until all performance parameters were in acceptable ranges.

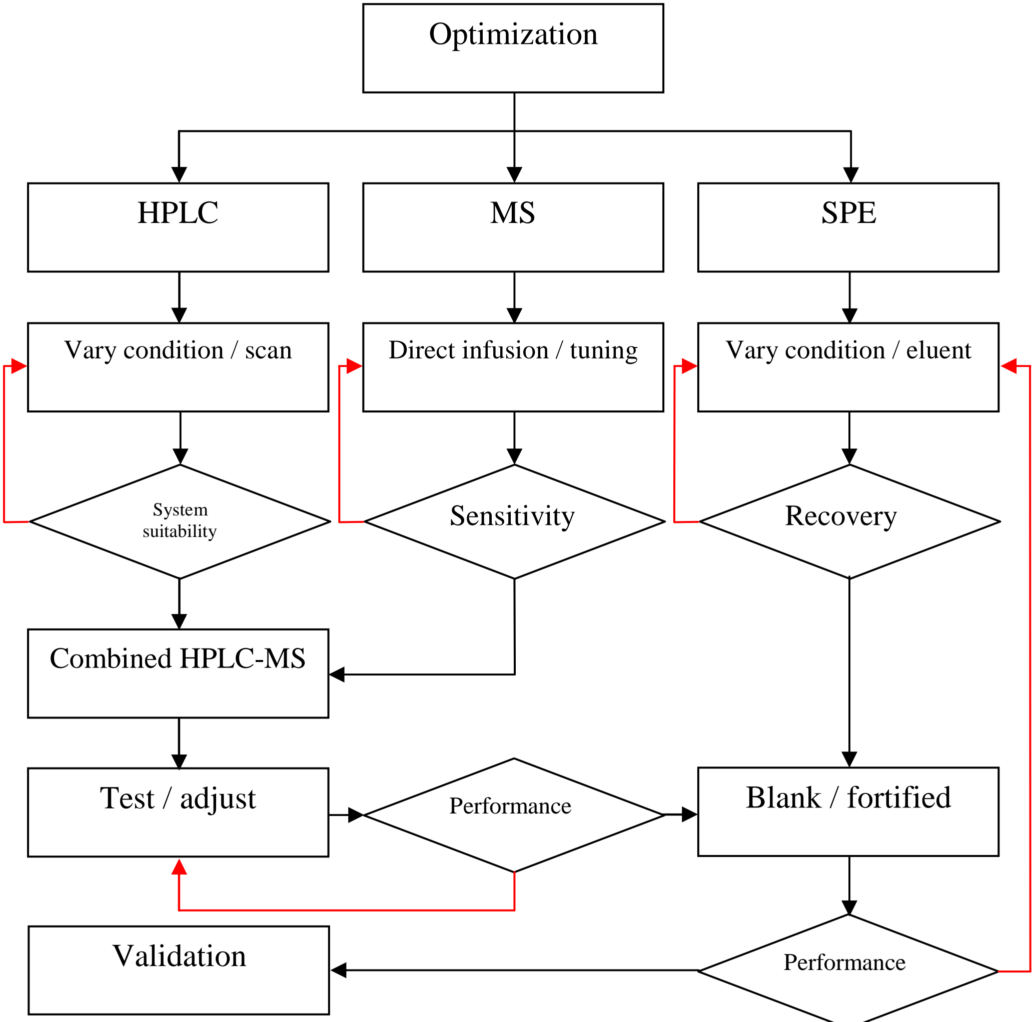


Figure 3.2 Method development scheme.

3.2.4 Method validation

3.2.4.1 Linearity and range

Linearity of the method were determined by analyzing at least six different concentrations of the working standards (n = 3). The regression lines were obtained by plotting concentration ratios of standards and internal standards against ratios of peak area between standards and internal standards. Slopes, intercepts and correlation of determination were calculated using the least-square linear regression.

Matrix matched standards were prepared in the same way as sample pretreatment except working standard solutions were prepared in seven levels. One mL of each level of mixing working standard solution (Table 3.9) was transfered into water blank sample previously added with 1 mL of 2 M sodium thiosulfate. Mixture of stable isotope internal standard solution was spiked for 0.5 mL to each sample (Table 3.10). The ranges of concentration of each compound for method validation were stated in Table 3.11.

Table 3.9 Working standard solution concentration for method validation

Working standard solution	Concentration (μg/L)							
	MNZ	RNZ	DMZ	NFZ	NFT	FZD	FTD	CAP
Level 1	20	6	40	50	40	1	2	5
Level 2	40	12	80	100	80	2	4	10
Level 3	60	18	120	150	120	3	6	15
Level 4	80	24	160	200	160	4	8	20
Level 5	100	30	200	250	200	5	10	25
Level 6	120	36	240	300	240	6	12	30
Level 7	140	42	280	350	280	7	14	35

* One mL of ACN was added to standard blank.

Table 3.10 Stable isotope labeled internal standard concentration for method validation

Concentration (mg/L)							
MNZ-$^{13}C_2$-$^{15}N_2$	RNZ-D_3	DMZ-D_3	NFZ-^{13}C-$^{15}N_2$	NFT-$^{13}C_3$	FZD-D_4	FTD-D_5	CAP-D_5
0.08	0.04	0.2	0.096	0.16	0.0056	0.0128	0.032

Table 3.11 Matrix matched standard calibration ranges for method validation

Level	Concentration (µg/L)							
	MNZ	RNZ	DMZ	NFZ	NFT	FZD	FTD	CAP
1	0	0	0	0	0	0	0	0
2	40	12	80	100	80	2	4	10
3	80	24	160	200	160	4	8	20
4	120	36	240	300	240	6	12	30
5	160	48	320	400	320	8	16	40
6	200	60	400	500	400	10	20	50
7	240	72	480	600	480	12	24	60
8	280	84	560	700	560	14	28	70
corresponding range in sample	0.1 – 0.7	0.03 – 0.21	0.2 – 1.4	0.25 – 1.75	0.2 – 1.4	0.005 – 0.035	0.01 – 0.07	0.025 - 0.175

3.2.4.2 Precision

Method precision was determined by repeatability (within day) and intermediate precisions (between days). The intermediate precisions were studied in three concentration levels on three different days (single laboratory validation) (n = 10). Results were reported as means ± standard deviation (SD) and %relative standard deviation (%RSD). Intermediate precision was analyzed using one way analysis of variance (one-way ANOVA) with least significant difference (LSD) by SPSS®. Statistical probability (*p*-value) less than 0.05 indicated a statistically significant difference between groups.

3.2.4.3 Accuracy

Accuracies were demonstrated in term of recovery, fractions of the analytes were added to tested samples (fortified or spiked samples) at three different concentrations (lower, middle and high of calibration curve, n = 10), and percentage recoveries (% R) were calculated as following:

$$\%R = [(CF-CU)/CA] \times 100$$

Where CF was the concentration of analyte measured in the fortified sample; CU was the concentration of analyte measured in the unfortified sample; CA was the concentration of analyte added (measured value, not determined by method) in fortified sample.

3.2.4.4 Limit of detection (LOD) and Limit of quantitation (LOQ)

Limit of detection was achieved using ten measurements of ten independent sample blanks fortified at lowest acceptable concentration (Table 3.12), measured once each and express LOD as the analyte concentration corresponding to mean sample blank value + 3SD and LOQ of method equal mean sample blank value + 10SD (sample standard deviation; SD).

Further investigation was performed by spiking of each analyte to blank farm samples at the LOD levels obtained in the previous step. Samples were treated as previously described before LC-MS analysis. Each analyte peak was visualize examination and spike concentrations were adjusted before repeat the procedure to confirm LOD.

Table 3.12 Lowest fortified concentration for LOD determination

Concentration (μg/L)							
MNZ	RNZ	DMZ	NFZ	NFT	FZD	FTD	CAP
0.0125	0.005	0.015	0.035	0.025	0.0005	0.0005	0.005

3.2.4.5 Other parameter

Other parameters such as ruggedness and robustness were conducted as appropriate (51, 52) by varying degree of some potential factors (e.g. composition) to the method performance.

For peak identification criteria in LC-MS/MS, one precursor and two daughters must be detected with identification points at least 4 according to Commission Decision 2002/657/EC (53)

The four points are required for Group A substances, which include the current investigated drugs (e.g. MNZ, RNZ, DMZ, NFZ, NFT, FZD, FTD and CAP)

For mass spectrometric detection, the relative intensities of the detected ions, express as a percentage of the intensity of the most intense ion, shall correspond to those of the calibration standard, at comparable concentration within the following tolerances (Table 3.13).

Table 3.13 Maximum permitted tolerances for relative ion intensities using a range of mass spectrometric techniques

Relative intensity (% of base peak)	EI-GC-MS (relative)	CI-GC-MS, $GC\text{-}MS^n$ LC-MS, $LC\text{-}MS^n$ (relative)
> 50 %	± 10 %	± 20 %
> 20 % t o 50 %	± 15 %	± 25 %
> 10 % t o 20 %	± 20 %	± 30 %
≤ 10 %	± 50 %	± 50 %

3.2.5 Application

Animal drinking water was sampled by regional Department of Livestock Development officers. Forty samples of animal drinking water were collected from registered standard farms in various provinces. The samples were analyzed using the proposed method. Six blind samples fortified one or more of target compound in the linearity range were analysed per batch as quality control.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Method development

Optimization of HPLC condition, MS parameters and sample pretreatment procedures were performed to achieve the optimal condition for analysis of MNZ, RNZ, DMZ, NFZ, NFT, FZD, FTD and CAP.

4.1.1 HPLC optimization

Factors affecting HPLC separation included types of organic solvent and acid modifiers, pH of buffer and buffer concentrations were investigated.

4.1.1.1 Organic solvent and acid modifier

Results from organic solvents and acid modifiers effects (Table 3.4, see Chapter III) were shown in Figures 4.1 – 4.5. All analytes were eluted within 35 minutes except CAP. Under these acidic condition (pH 2.5 – 3.4); FTD, MNZ, RNZ and DMZ were closely eluted within 15 min while the other three substances; NFZ, NFT and FZD were eluted between 15 – 35 min. FTD was well eluted under acidic condition (pH < 3 (system 1 - 4, Figures 4.2 – 4.3)) and was more retained at higher pH (system 5 - 8, Figures 4.4 -4.5). The elution order of three nitroimidazoles was almost the same in system 1 – 5 and 7, MNZ was first eluted followed by RNZ and DMZ except in system 6 and 8 (Figures 4.4B and 4.5B). However, when acetonitrile was used, MNZ and RNZ were more separated apart (Figures 4.4A and 4.5A). Three nitrofurans, NFZ, NFT and FZD, were less affected from acidic condition than that of FTD. The elution order of this group was similar in all systems. NFT was firstly eluted followed by NFZ and FZD. The separations among these three substances was satisfied in systems which contained acetonitrile as organic modifier, while overlapping peaks were observed in the mobile phase containing methanol (system 4, 6 and 8; Figure 4.3B, 4.4B and 4.5B). Therefore, acetonitrile was chosen as the organic modifier in the next optimization. Formic acid significantly affected FTD

and DMZ, and slightly affected NFZ and NFT than that of acetic acid at the same concentration. However, more investigation was needed to evaluate the appropiate use of acid. Resolution data in Table 4.1 indicated that system 7 was the most separation system since it provided acceptable resolution for all peaks.

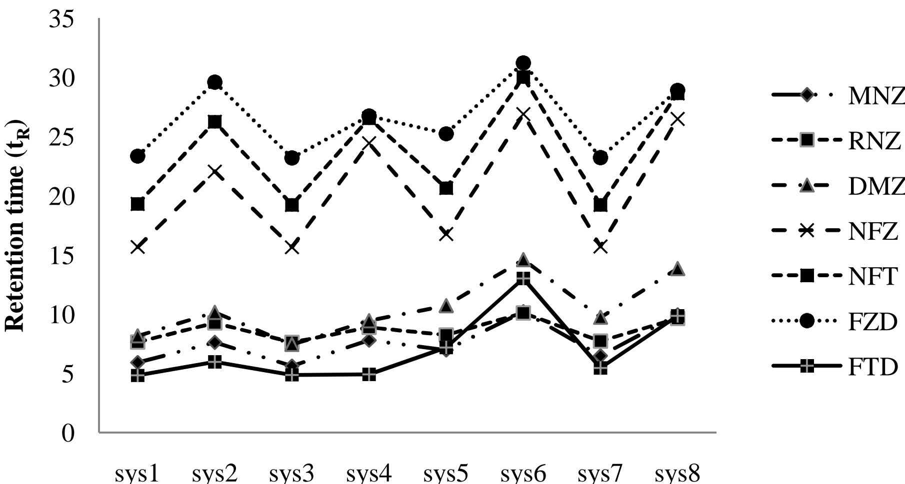


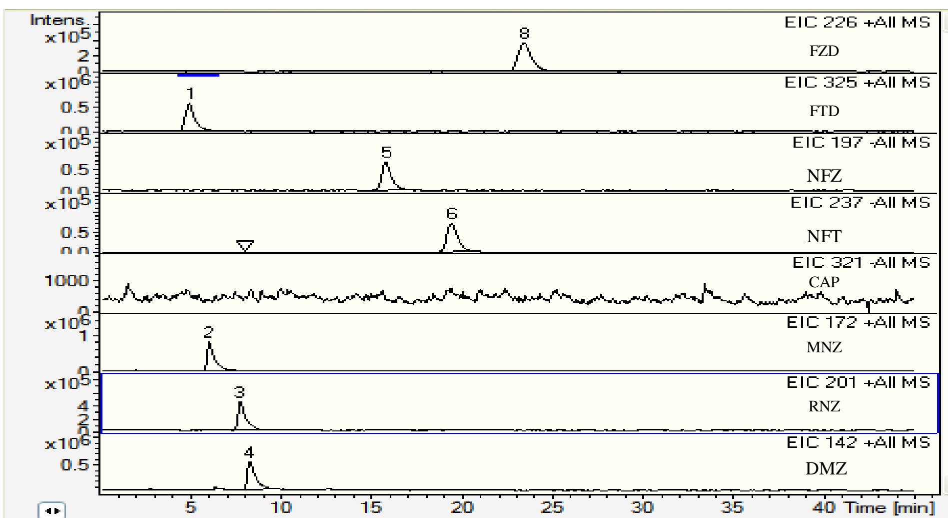
Figure 4.1 Plot of retention time vs various composition of mobile phase modified organic solvent and acid.

Table 4.1 Resolution of each pair of peaks*

Pair of elution order	sys1	sys2	sys3	sys4	sys5	sys6	sys7	sys8
1	1.2	1.8	1.0	2.8	0.3	0.2	1.2	0.2
2	2.0	2.1	2.2	1.2	1.3	2.9	1.9	0.1
3	0.6	1.1	0.2	0.6	3.0	1.6	2.8	4.3
4	7.8	11.0	9.0	11.8	6.0	10.4	6.1	10.5
5	3.2	3.1	3.3	1.4	3.3	2.1	3.2	1.5
6	3.1	2.3	3.1	0.1	3.5	0.9	3.3	0.2

* CAP was not eluted within 40 min

A) System 1



B) System 2

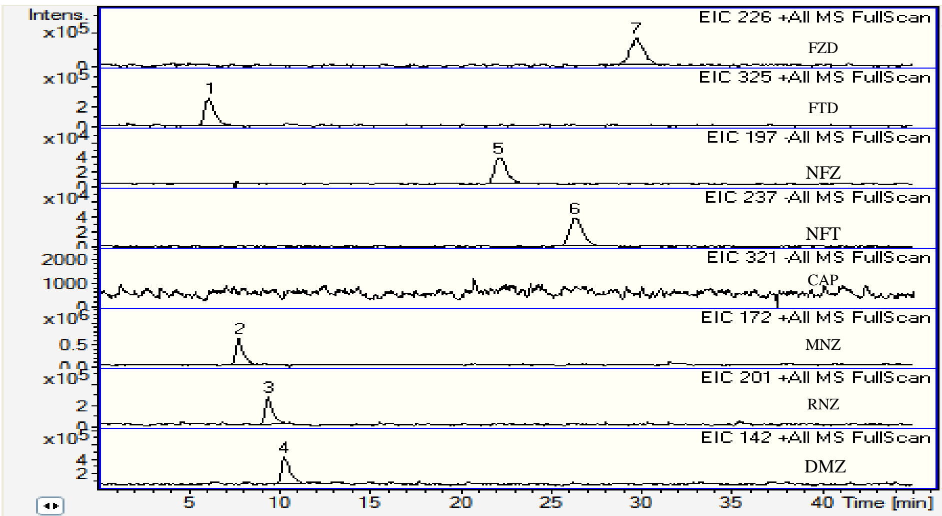
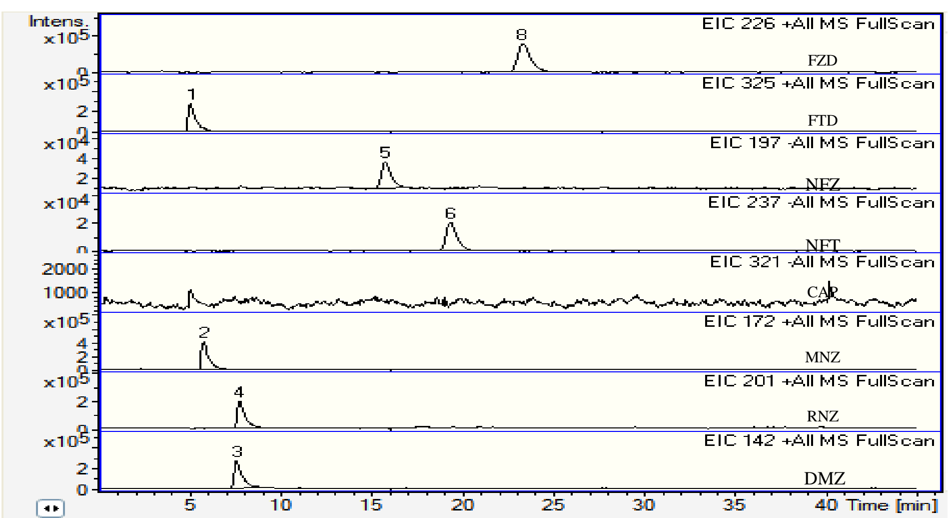


Figure 4.2 Effects of organic solvent and acid modifiers. HPLC condition: standard mixture solution (1 mg/L) in isocratic elution. Condition A) ACN : 0.05% HCOOH (v/v) (10 : 90), B) MeOH : 0.05% HCOOH (v/v) (10 : 90); Prodigy ODS (3), 2.0x150 mm, 5µm with guard cartridge, 40 °C, flow rate: 0.2 mL/min. MS condition: alternative polarity switching, scan mode. Numbers over peaks were elution orders.

A) System 3



B) System 4

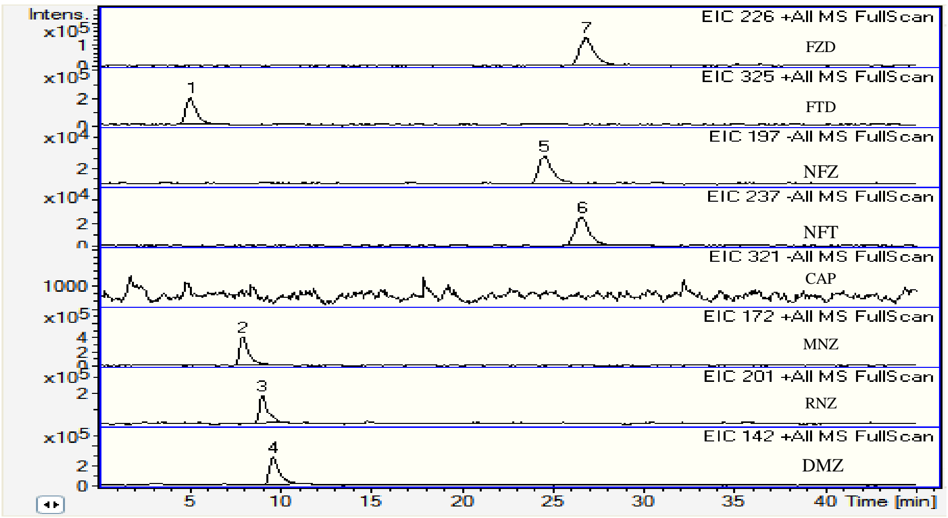
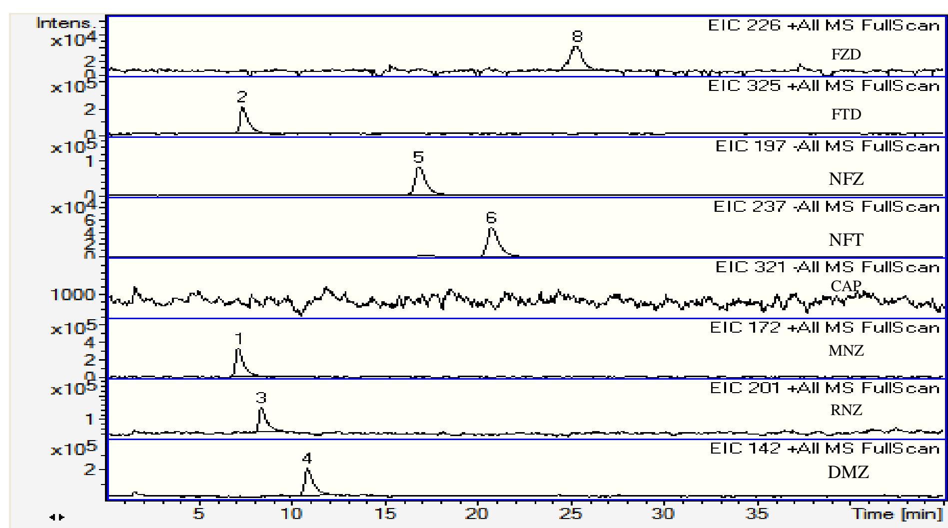


Figure 4.3 Effects of organic solvent and acid modifiers. HPLC condition: standard mixture solution (1 mg/L) in isocratic elution. Condition A) ACN : 0.1% HCOOH (v/v) (10 : 90), B) MeOH : 0.1% HCOOH (v/v) (10 : 90). Other conditions are as in Figure 4.2.

A) System 5



B) System 6

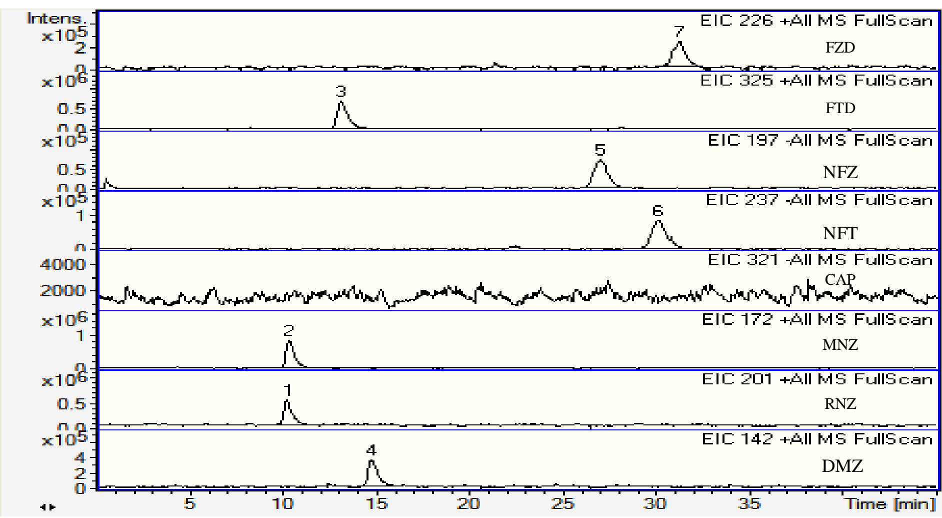
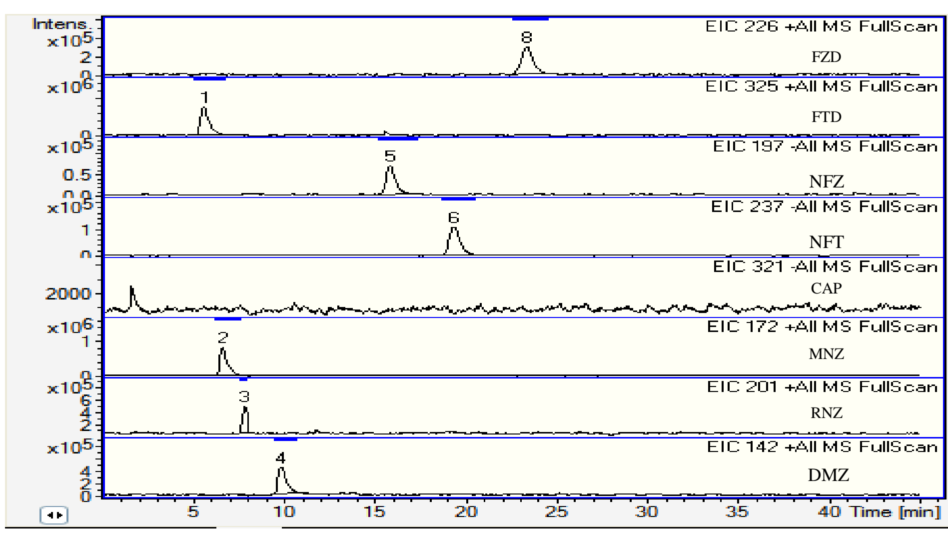


Figure 4.4 Effects of organic solvent and acid modifiers. HPLC condition: standard mixture solution (1 mg/L) in isocratic elution. Condition A) ACN : 0.05% CH_3COOH (v/v) (10 : 90), B) MeOH : 0.05% CH_3COOH (v/v) (10 : 90). Other conditions are as in Figure 4.2.

A) System 7



B) System 8

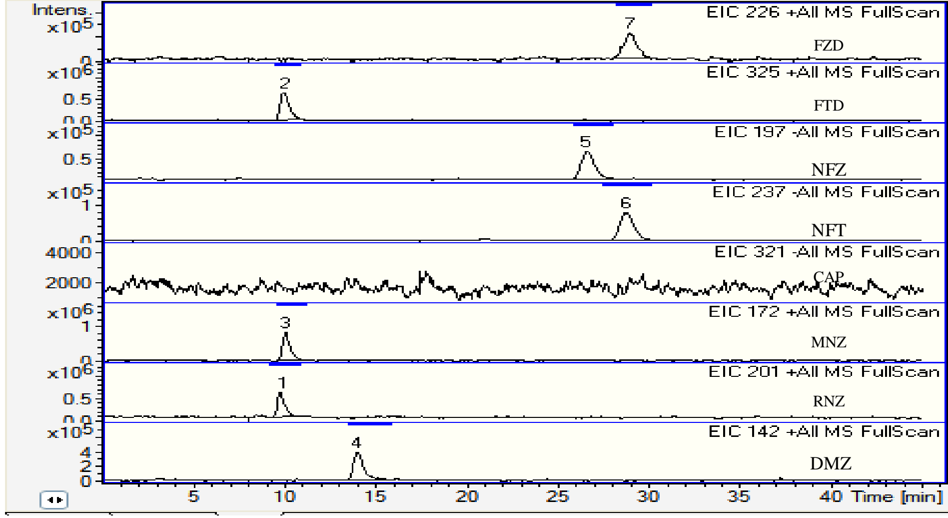


Figure 4.5 Effects of organic solvent and acid modifiers. HPLC condition: standard mixture solution (1 mg/L) in isocratic elution. Condition A) ACN : 0.1% CH_3COOH (v/v) (10 : 90), B) MeOH : 0.1% CH_3COOH (v/v) (10 : 90). Other conditions are as in Figure 4.2.

4.1.1.2 pH of buffer

From Figures 4.6 and 4.7, retention time of FTD significantly decreased with the decrease of buffer pH, whereas retention time of other substances remained constant. Moreover, in both buffer (A and B) FTD peak was not observed at pH above 4.4. In addition, CAP was not eluted in 40 min over the studied pH ranges. Up to this point, isocratic elution could not provide satisfied separation of the analytes, thus gradient elution was required to optimize the separation. The appropriate pH of ammonium formate buffer system was at 3.5 and above 4.4, whereas the proper pH was above 4.4 for ammonium acetate. Therefore, gradient elutions were studied at pH 3.5 and 5.0 using ammonium formate and ammonium acetate, respectively.

Table 4.2 Retention time of the investigated drug under isocratic elution of 20 mM ammonium formate buffer at various pH and ACN (90 : 10)

Sys	pH	Retention time (min)							
		MNZ	RNZ	DMZ	NFZ	NFT	FZD	FTD	CAP
9	2.6	5.3	7.0	7.1	13.7	16.2	19.9	7.3	*
10	2.9	5.9	7.5	8.6	14.7	17.8	21.8	8.3	*
11	3.2	6.6	7.9	10.0	15.6	18.9	23.2	10.2	*
12	3.5	6.7	7.8	10.3	15.2	18.5	22.3	12.1	*
13	3.8	6.6	7.5	10.3	14.8	18.1	22.1	16.2	*
14	4.1	6.6	7.5	10.6	14.9	18.2	22.1	23.3	*
15	4.4	6.7	7.6	10.6	15.1	18.6	22.2	34.1	*
16	4.7	7.0	7.9	11.5	16.4	19.8	24.2	*	*
17	5.0	6.5	7.4	10.5	14.5	17.6	21.6	*	*

* more than 40 min

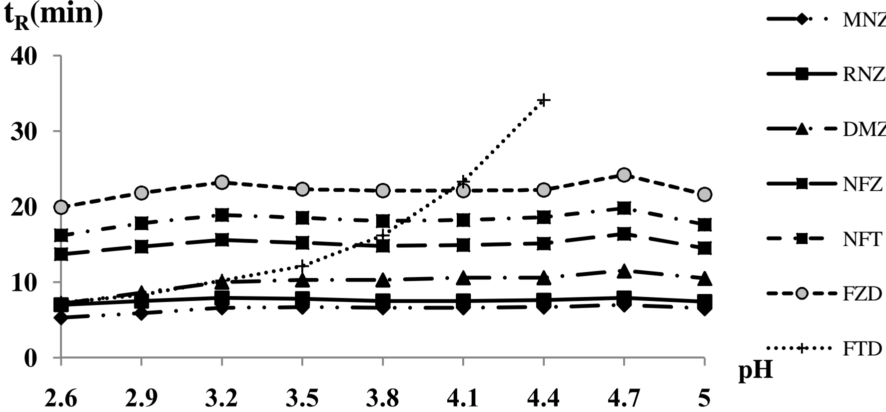


Figure 4.6 Effects of pH of buffer 20 mM ammonium formate : ACN (90 : 10). HPLC condition: Prodigy ODS (3), 2.0 x 150 mm, 5 µm with guard cartridge, 40 °C, flow rate: 0.2 mL/min. MS condition: alternative polarity switching, scan mode.

Table 4.3 Retention time of the investigated drugs under isocratic elution of 20 mM ammonium acetate buffer at various pH and ACN (90 : 10)

Sys	pH	Retention time (min)							
		MNZ	RNZ	DMZ	NFZ	NFT	FZD	FTD	CAP
18	3.8	7.0	7.9	10.9	15.2	17.9	22.6	16.4	*
19	4.1	7.0	8.0	11.4	16.7	20.4	25.0	27.0	*
20	4.4	7.3	8.2	11.5	16.4	20.1	24.8	37.9	*
21	4.7	7.4	8.3	11.6	16.4	20.5	26.1	*	*
22	5.0	7.3	8.2	11.6	16.5	20.3	25.0	*	*
23	5.3	6.9	7.9	11.4	16.3	19.5	24.3	*	*
24	5.6	7.0	7.9	11.2	15.8	19.0	23.8	*	*

* more than 40 min

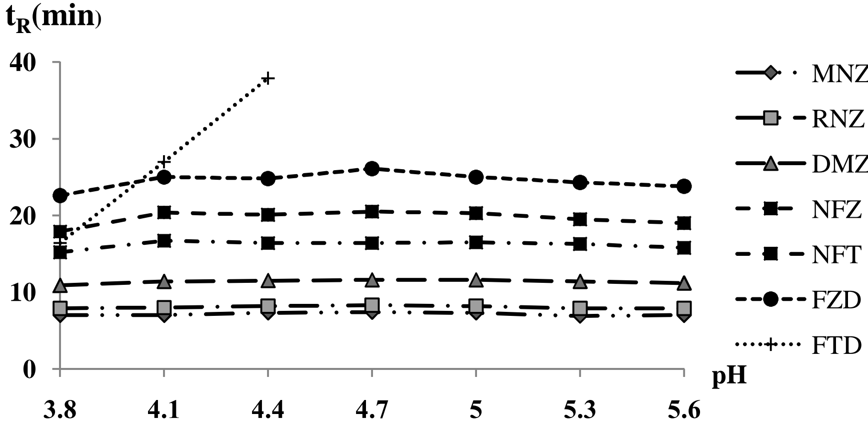


Figure 4.7 Relationship between pH of aqueous buffer and retention time of each analyte; 20 mM ammonium acetate. Other conditions are as in Figure 11.

4.1.1.3 Buffer concentration

The chromatograms obtained in buffer concentration variation were interpreted in term of retention time (t_R) (Table 4.4). Concentrations of formate buffer did not affect separation of all compounds except for FTD and CAP. Results were in agreement with effect of pH in that the retention time of FTD decreased under acidic condition as well as that of CAP (Figure 4.8). At the buffer concentration of 40 mM, accumulation of non-volatile buffer around the orifice on spray shield at the entrance of mass optics occurred after several injections. Therefore, buffer should be used as low as possible to avoid ion suppression effects or interfering to analysis.

Table 4.4 Retention time of target compounds under different concentrations of ammonium formate

Sys	Buffer conc.	Retention time (t_R), min							
		MNZ	RNZ	DMZ	NFZ	NFT	FZD	FTD	CAP
25	5 mM	4.6	5.4	7.3	9	10.8	13.7	10.1	42.4
26	10 mM	4.4	5.1	7	8.4	10.2	12.8	12.8	39.4
27	20 mM	4.7	5.5	7.6	9.2	11.1	14.1	17.7	33.9
28	40 mM	4.7	5.4	7.5	9.1	11	13.9	21.2	43.8

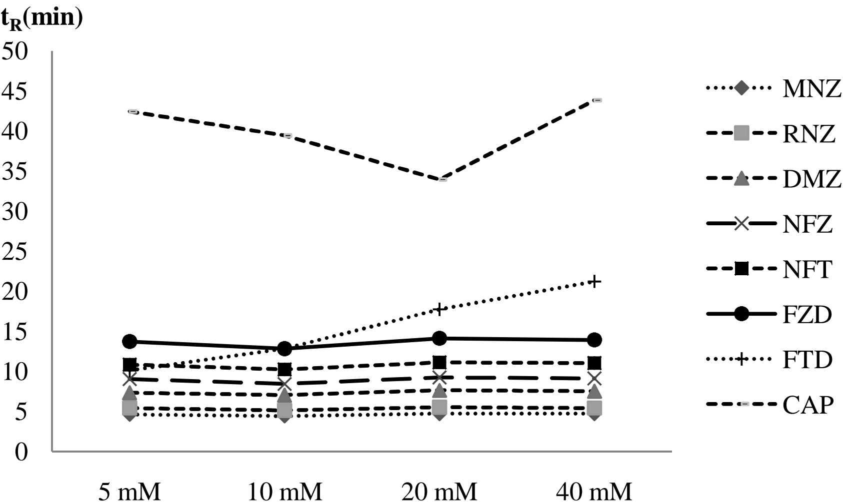


Figure 4.8 Effect of formate buffer concentration on retention time. Other conditions are as Figure 11

4.1.1.4 Final HPLC optimization

Initial HPLC optimization in 1.1.1 to 1.1.3 could not provide satisfactory resolution for all analytes. Final optimization was required and was described in this section by varying several factors including buffer concentrations and gradient elution.

Further investigation was carried on effects of concentrations of buffer at a specific pH value to retention time. Results showed that the concentrations of 5 to 20 mM ammonium formate at pH of 3.5 did not affect the analyte elution (Table 4.5 and Figure 4.9).

Figure 4.10 showed the comparison of retention time under gradient elution. For ammonium formate buffer, the concentrations were 10 and 20 mM and the pH were slightly adjusted from 3.5 to 3.6 to increase separation between DMZ and FTD. All substances were separated but segmentation between DMZ, FTD and NFZ was difficult. This was due to 1) narrow window segment of FTD and 2) retention time shift during long batch run causing incomplete peaks. The separation obtain from condition B and C; 10 and 20 mM ammonium acetate, pH 5.0 gave more satisfactory separation between peak except for the MNZ and RNZ (Table 4.6).

However, their MS parameters could be compromised in the same segment time. Therefore, the mobile phase comprised of ammonium acetate at pH 5.0 and acetonitrile was chosen for further optimization.

Table 4.5 Retention time of analytes under various concentration of ammonium formate buffer (pH 3.5)

Sys		Retention time (t_R, min)							
		MNZ	RNZ	DMZ	FTD	NFZ	NFT	FZD	CAP
29	5 mM	7.9	8.9	11.5	12.6	15.3	17.2	19.3	27.2
30	10 mM	7.5	8.6	11.1	12.5	14.5	16.5	18.8	27.2
31	20 mM	8.2	9.3	11.7	13.3	15.5	17.2	19.4	27.0

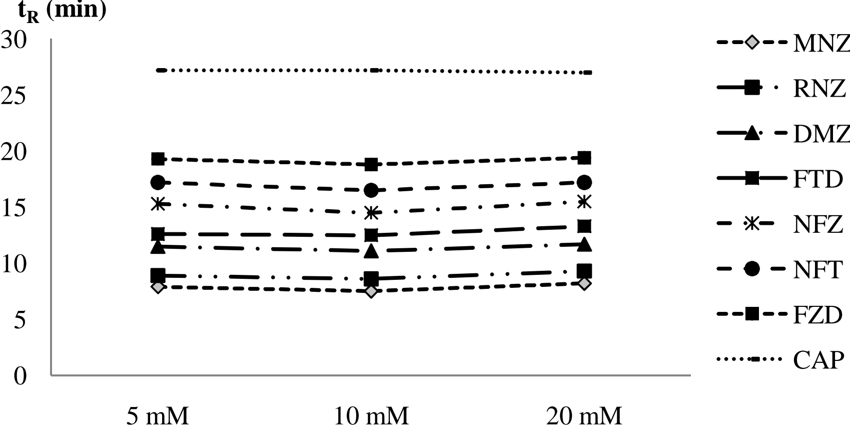


Figure 4.9 Plot of retention time vs various concentration of ammonium formate buffer (pH 3.5). HPLC: in gradient elution of A : B (buffer : ACN, v/v), 0 min B 15%, 15 min B 20%, 20 min B 35%, 26 min B 15%, 30 min B 15%. Other HPLC conditions are as in Figure 11. MS condition; Tandem mass spectrometry (MS/MS)

Table 4.6 Retention time under various types and concentrations of buffer

Sys		Retention time (t_R, min)							
		MNZ	RNZ	DMZ	NFZ	NFT	FZD	FTD	CAP
32	10 mM $HCOONH_4$ pH 3.6	7	7.9	10.4	12.9	14.4	16.2	12.1	25.2
33	20 mM $HCOONH_4$ pH 3.6	6.9	8	10.4	13.1	14.7	16.4	12	24.9
34	10 mM CH_3COONH_4 pH 5.0	7.1	8	11	13.5	14.9	16.8	21.1	25.3
35	10 mM CH_3COONH_4 pH 5.0	7.1	8.1	11	13.5	15.1	17.1	21.1	25.4

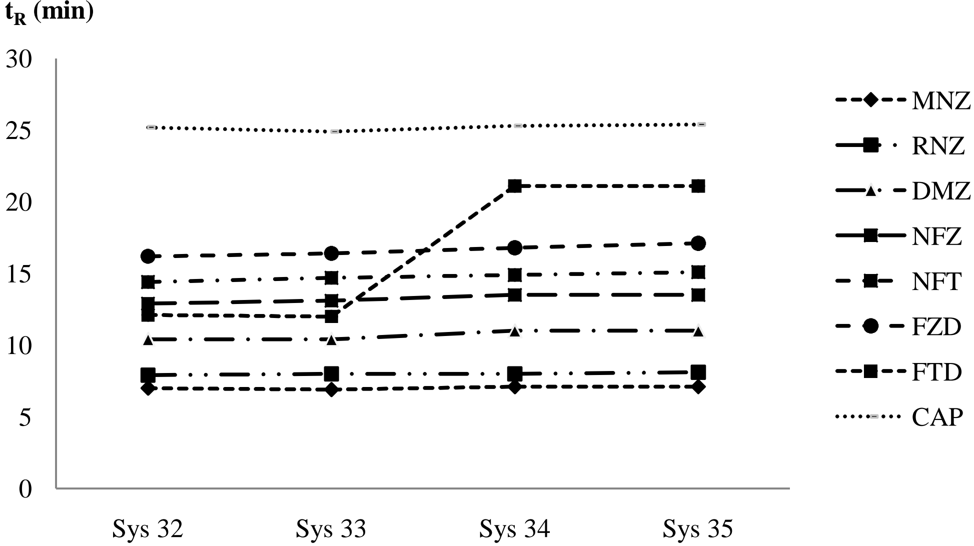


Figure 4.10 Plot of retention time vs various types and concentrations of buffer. HPLC condition as same as in Figure 4.16 in gradient elution of A : B (buffer : ACN, v/v), 0 min B 10%, 15 min B 25%, 25 min B 25%, 26 min B 10%, 35 min B 10%. MS condition; scan mode (positive mode ionization for MNZ, RNZ, DMZ, FTD and FZD; negative mode ionization for NFZ, NFT and CAP).

The next optimization was performed by varying of concentrations of ammonium acetate from 20 mM to 5 mM and without buffer (pH 5.0 water) (Sys 36 = 10 mM; Sys 37 and Sys 38 = 20 mM; Sys 39 and Sys 40 = 5 mM; Sys 41 - 43 = pH 5.0 water (adjusted with diluted acetic acid) and gradient profile were also optimized during variation (Table 4.7).

Table 4.7 Gradient profiles variation

System	Gradient profile
36 and 37	A: acetate buffer, B: 100% ACN; 0 min B 10%, 15 min B 25%, 25 min B 25%, 26 min B 10%, 35 min B 10%
38	A: acetate buffer, B: 100% ACN; 0 min B 15%, 20 min B 30%, 21 min B 15%, 30 min B 15%
39	A: acetate buffer, B: 100% ACN; 0 min B 10%, 15 min B 20%, 20 min B 35%, 21 min B 10%, 30 min B 10%
40	A: acetate buffer, B: 100% ACN; 0 min B 15%, 15 min B 35%, 16 min B 15%, 25 min B 15%
41	A: pH 5.0 water, B 100% ACN; 0 min B 15%, 15 min B 35%, 16 min B 15%, 25 min B 15%
42	A: 10% ACN in pH 5.0 water (v/v), B: 30% ACN in pH 5.0 water (v/v); 0 min B 0%, 14 min B 100%, 15 min B 100%, 16 min B 0%, 25 min B 0%
43	A: 10% ACN in pH 5.0 water (v/v), B: 30% ACN in pH 5.0 water (v/v); 0 min B 0%, 12 min B 100%, 15 min B 100%, 16 min B 0%, 22 min B 0%

Table 4.8 shows that low concentration of acetate buffer (5 mM) and pH 5.0 water gave the same elution profile as that of 10 and 20 mM ammonium acetate buffer (pH 5.0). The pH of aqueous mobile phase plays major role in retention mechanism of target compound in C_{18} column. The separation in each system was satisfied and the mobile phase consisted of pH 5.0 water and ACN (Sys 43) which prepared premixing with weighing was chosen for solid phase

optimization, LOD estimation and method validation due to its convenient in preparation, easy to maintenance equipment and column compatibility. Each compound was eluted within 20 min. The run time was 22 min and MS segment program was set to 7 segments. MNZ and RNZ were in the first segment, DMZ, NFZ, NFT, FZD, FTD and CAP were in specific segments, respectively.

Table 4.8 Retention time under variation of buffer composition and gradient profile

Sys	Retention time (t_R, min)							
	MNZ	RNZ	DMZ	NFZ	NFT	FZD	FTD	CAP
36	7.1	8.0	11.0	13.5	14.9	16.8	21.1	25.3
37	7.1	8.1	11.0	13.5	15.1	17.1	21.1	25.4
38	4.8	5.5	7.5	9.1	10.8	13.0	17.1	22.1
39	7.4	8.2	11.3	14.3	16.0	18.4	24.2	26.5
40	4.8	5.6	7.6	8.9	10.3	12.1	15.2	18.1
41	4.9	5.6	7.6	9.1	10.5	12.4	15.7	18.3
42	6.9	7.9	10.6	12.4	13.5	14.9	17.8	20.2
43	6.0	6.8	9.3	11.1	12.2	13.7	16.1	18.8

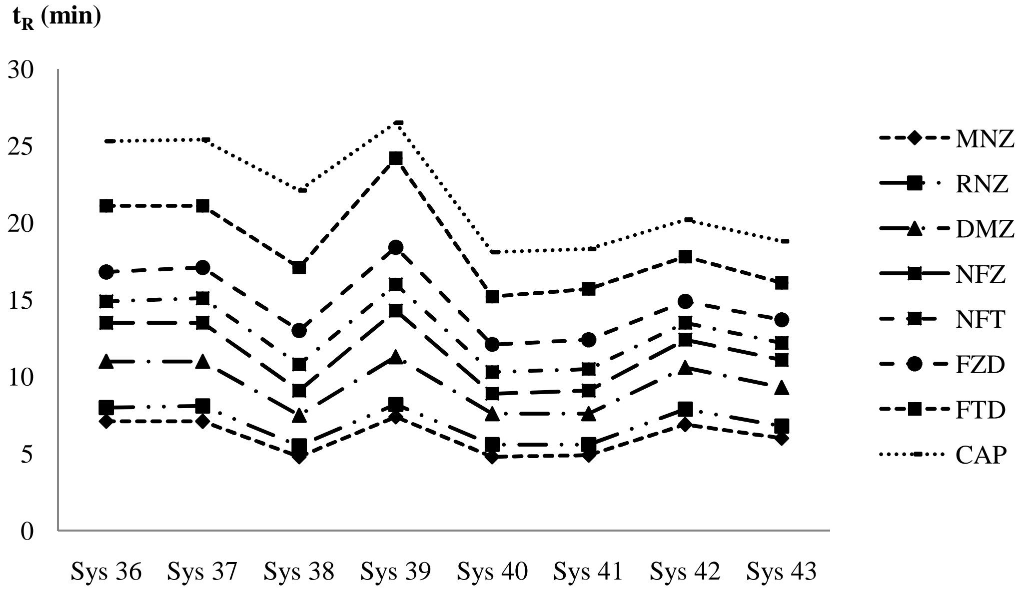


Figure 4.11 Plot of retention time vs various condition of buffer composition and gradient elution profiles.

A mixture of 1 mg/L of reference standards was injected under system 43 (Table 4.7) for peak characteristic evaluation (Figure 4.12). Peak width at half height was calculated using Chemstation for LC system, width at base (W_b), theoretical plate (N) and resolution (R) were calculated using Excel (Table 4.9). Resolution between peaks indicated that all peaks were well separated. However, when MS/MS mode were applied and IS was used for each analyte, the peak became slightly broadened. Therefore, MNZ and RNZ were in the same segment.

Table 4.9 Peak characteristics under system 43 in MS scan mode

	t_R(min)	Width	Width at base (W_b)	N	R_s
MNZ	5.888	0.1466	0.25	8946	
RNZ	6.744	0.187	0.32	7213	3.0
DMZ	9.317	0.2276	0.39	9294	7.3
NFZ	10.957	0.1934	0.33	17801	4.6
NFT	12.115	0.1759	0.30	26309	3.7
FZD	13.695	0.1766	0.30	33352	5.3
FTD	16.434	0.181	0.31	45721	9.0
CAP	18.681	0.1918	0.33	52612	7.1

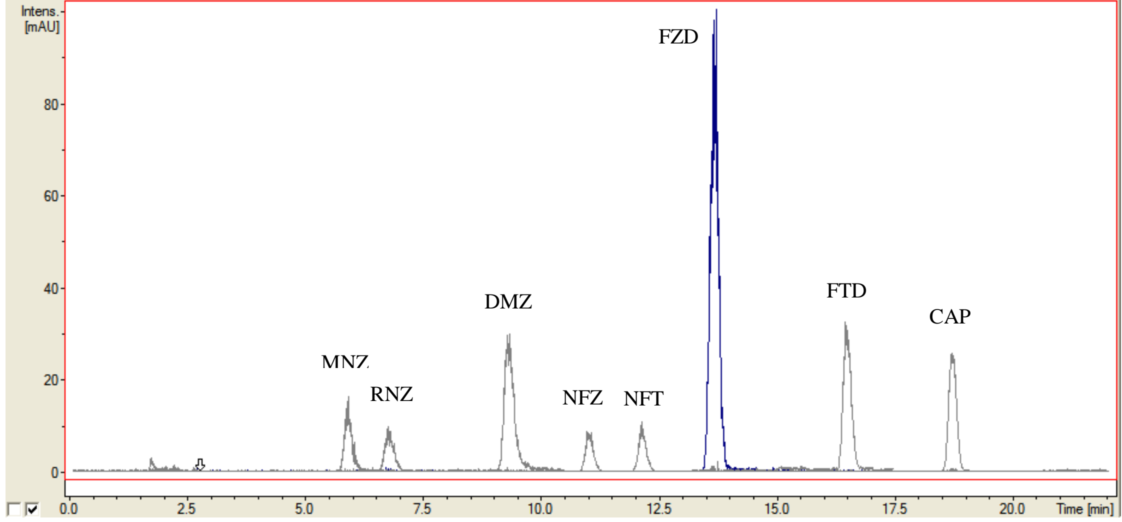


Figure 4.12 Chromatogram of the investigated compounds under system 43. HPLC condition: Prodigy ODS (3), 2.0x150 mm, 5µm with guard cartridge, 40 °C, flow rate: 0.2 mL/min. MS condition: scan mode, segment program.

4.1.2 MS optimization

Tuning of MS parameters by direct infusion of the target compounds and IS were performed using optimization function in Esquire Control version 6.1 (Appendix A). Fine tune MS parameters were obtained in Table 4.10. These parameters were specified to each segment time program. Fine tune of MS parameters (Table 4.10) were used for sample pretreatment optimization, LOD estimation and method validation. The parameters must be confirmed if there are any significant changes of the instrument, after machine calibration and when the performance of analysis is decrease. The MS/MS chromatogram of each compound was accomplished with previously optimized HPLC conditions (Figure 4.13).

The performances of MS parameters were depend upon the instrument condition. The system should be maintenance and obtain calibration from expert periodically. Any defect of the instrument such as MS optical parts, vacuum system also the HPLC parts can result in experimental failure.

Table 4.10 MS optic parameters of each time segment

MS Parameter	**MNZ** (+)	**RNZ** (+)	**DMZ** (+)	**NFZ** (-)	**NFT** (-)	**FZD** (+)	**FTD** (+)	**CAP** (-)
	segment 1		segment 2	segment 3	segment 4	segment 5	segment 6	segment 7
Capillary	-3950		-3950	3775	3075	-4183	-4242	3717
Skimmer	31.7		30.0	-33.3	-26.7	30.0	33.3	-38.3
Cap exit	95.8		95.8	-87.5	-95.8	158.3	137.5	-175.0
Oct 1 DC	8.92		6.58	-7.46	-5.71	7.17	8.92	-9.50
Oct 2 DC	0.68		1.06	-0.39	-0.76	1.24	1.14	-1.33
Trap Drive	35.4		23.3	22.5	23.5	29.2	36.1	35.4
Oct RF	75.0		60.0	70.0	80.0	70.0	95.0	155.0
Len 1	-4.5		-2.1	2.7	1.6	-2.8	-3.6	3.4
Len 2	-62.5		-32.5	49.0	34.0	-37.0	-41.5	62.5
Isolatation Mass (analyte)	172.0	201.0	142.0	196.8	236.8	226.0	325.2	321.0
Fragmentation amplitude	0.75	0.73	0.66	0.80	0.84	0.63	0.67	1.21
Isolatation Mass (IS)	176.0	204.2	145.2	199.8	239.8	229.8	330.2	326.0
Fragmentation amplitude	0.67	0.70	0.74	0.77	0.73	0.63	0.67	1.16
Smart Target	20000		30000	30000	20000	20000	30000	30000
Max Accu Time (ms)	100		50	100	100	100	20	10
Scan (m/z)	70 - 250		80 - 200	110 - 300	110 - 250	100 - 300	150 - 350	150 -350

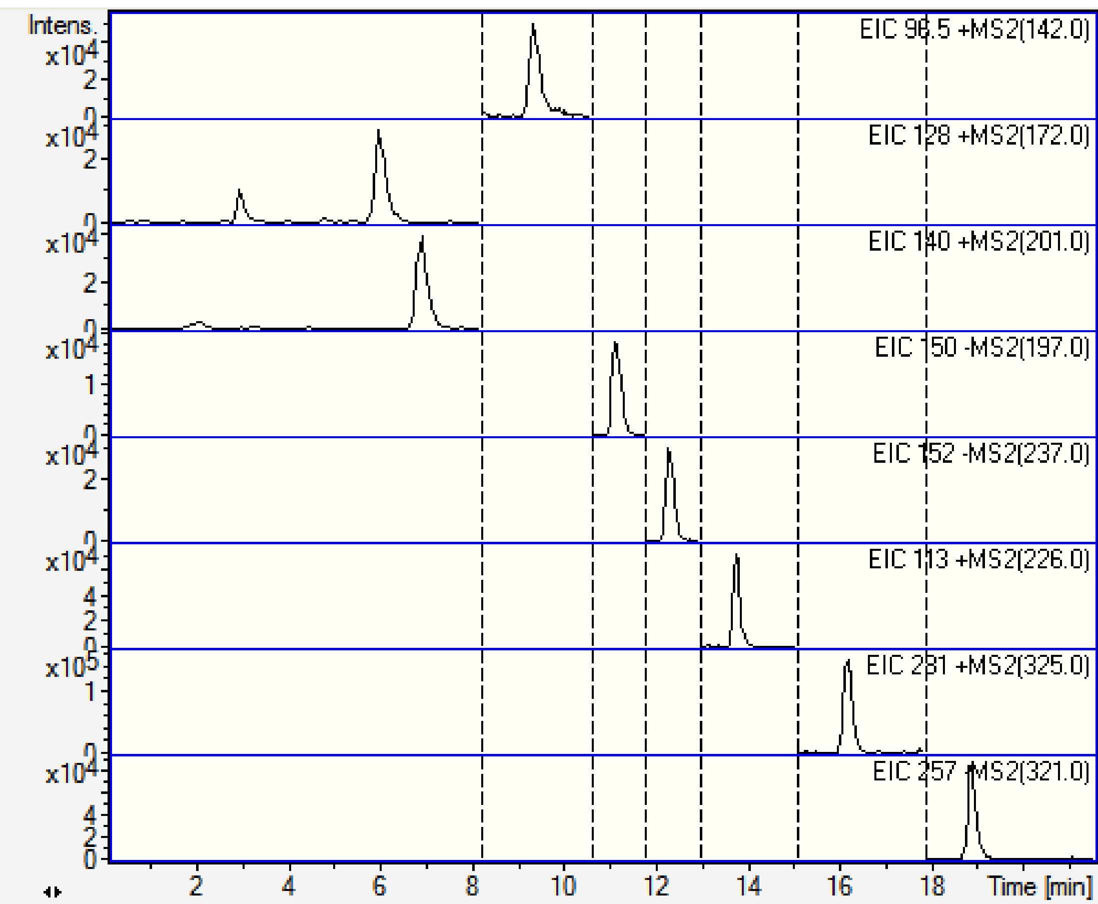


Figure 4.13 Product ions chromatograms obtained by LC-ESI MSn in positive mode using optimized method. Segment 1 (MNZ and RNZ), 2 (DMZ), 5 (FZD) and 6 (FTD), negative mode for segment 3 (NFZ), 4 (NFT) and 7 (CAP).

4.1.3 Sample pretreatment-SPE extraction

The solid phase extraction (SPE) procedure on Oasis HLB cartridges (200 mg / 6 mL) was optimized by varying types of solvents (acetonitrile and methanol), compositions and acidity of eluent as indicated in Table 3.6 (see Chapter III) using previous optimized HPLC and MS method. Each condition was performed in triplicate. The matrix-matched standards were prepared using eluent 6 (Table 3.6, see Chapter III). The results showed good recoveries (%R) of the nitroimidazole groups (i.e. MNZ, RNZ and DMZ) in a range of 60 to 120% were obtained under all conditions (Table 4.11 and Figure 4.14). For DMZ, the recovery rate was higher than 120% in eluent 4 and very high in eluent 7 and 8. The recovery rate of NFZ was quite poor in several conditions except in eluent 2, 6, 7 and 8. It should noted that, the eluent containing MeOH gave poor recovery of NFZ, especially in eluent 1. However, the presence of acid increased elution of NFZ as shown in eluent 6, 7 and 8. NFT was easily eluted in every condition. FZD and FTD were eluted very well in the eluent containing ACN and/or acidic condition, while their recoveries were very poor in eluent 1 (notice that IS of FZD and FTD were not eluted in this condition). CAP was poorly eluted in eluent 1 (lower than 15%) and became better in the presence of ACN. Moreover, recovery rate of CAP was enhanced when eluent was acidic (eluent 6, 7 and 8). Except for CAP, %RSDs of all analytes were between 1.7 and 13.3 using eluent 6, 7 and 8 (Figure 4.15).

The eluent 6 was selected as an optimized eluent for further experiment for LOD determination and method validation. However, eluents 7 and 8 also gave good recovery rate but required more time in evaporation step. Therefore, the optimized eluent composition was 4 mL (2×2 mL) of 1% acetic acid in mixture of ACN : MeOH (80 : 20, v/v).

Table 4.11 HLB SPE eluent optimization

Eluent (n=3)	spike conc. (μg/L)	MNZ	RNZ	DMZ	NFZ	NFT	FZD	FTD	CAP
		0.375	0.15	0.5	0.375	0.5	0.015	0.025	0.025
1	Conc.	0.325	0.122	0.499	0.073	0.527	0.137	0.162	0.005
	%R	86.7	81.5	99.8	19.6	105.4	*	*	19.2
	%RSD	3.6	20.9	69.1	49.8	7.5	33.8	67.2	14.1
2	Conc.	0.409	0.136	0.510	0.231	0.596	0.016	0.025	0.013
	%R	108.9	90.6	102.0	61.5	119.3	106.5	100.2	53.2
	%RSD	9.3	4.7	17.2	9.2	12.2	11.6	4.7	28.9
3	Conc.	0.424	0.137	0.571	0.222	0.538	0.017	0.025	0.013
	%R	112.9	91.4	114.3	59.2	107.7	111.5	101.7	52.4
	%RSD	14.7	7.2	5.1	9.4	12.0	3.3	6.1	10.1
4	Conc.	0.379	0.145	0.612	0.209	0.495	0.016	0.027	0.012
	%R	101.1	96.4	122.4	55.8	99.0	106.4	109.7	47.2
	%RSD	10.6	8.9	10.8	6.0	11.8	10.4	7.0	17.2
5	Conc.	0.377	0.143	0.500	0.214	0.555	0.016	0.025	0.015
	%R	100.4	95.3	100.0	57.0	111.0	104.4	100.9	58.4
	%RSD	12.6	2.9	14.3	7.0	17.1	9.1	10.1	14.4
6	Conc.	0.389	0.155	0.484	0.225	0.498	0.016	0.025	0.017
	%R	103.8	103.6	96.9	60.1	99.6	106.0	101.1	67.5
	%RSD	1.7	9.1	13.3	11.1	11.0	8.6	2.1	35.4
7	Conc.	0.415	0.142	0.587	0.243	0.510	0.016	0.027	0.017
	%R	110.8	95.0	117.4	64.9	102.0	106.7	106.1	66.4
	%RSD	5.3	5.7	6.7	9.4	8.6	3.0	3.1	28.9
8	Conc.	0.422	0.131	0.591	0.226	0.510	0.017	0.027	0.016
	%R	112.4	87.5	118.2	60.4	102.1	110.1	108.2	65.5
	%RSD	4.4	3.7	1.8	3.1	4.6	3.7	7.9	41.8

* could not be determined because IS was not eluted.

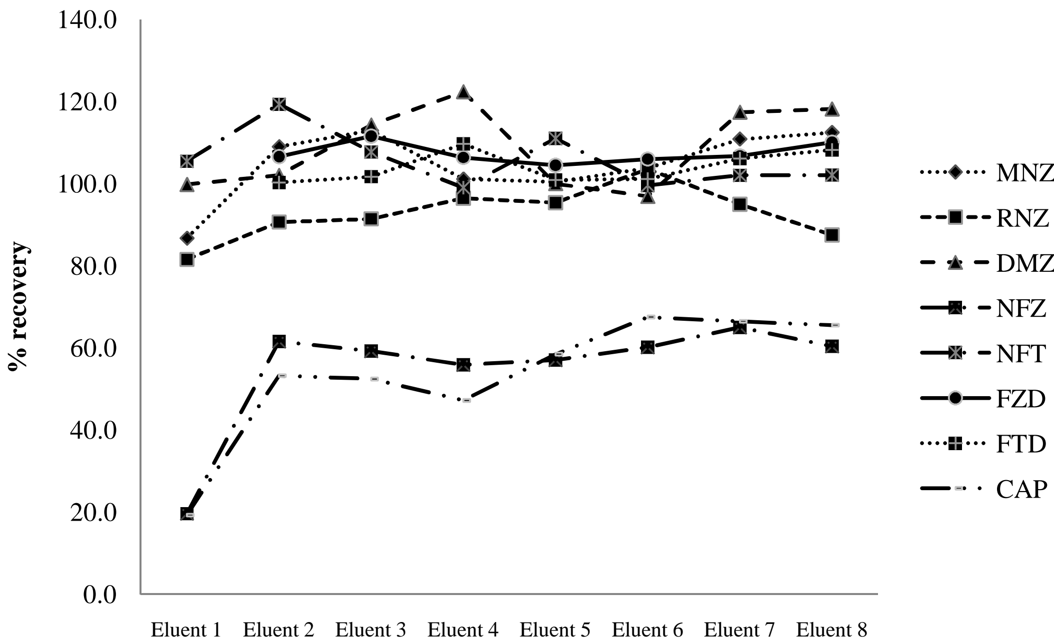


Figure 4.14 %recovery of the variation of eluent composition.

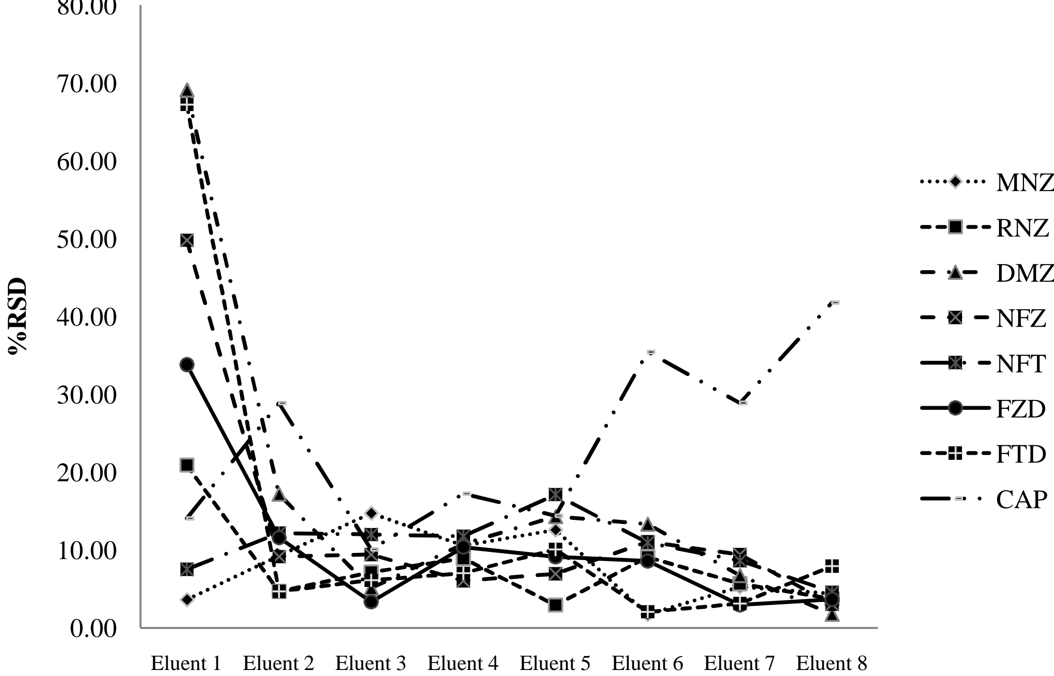
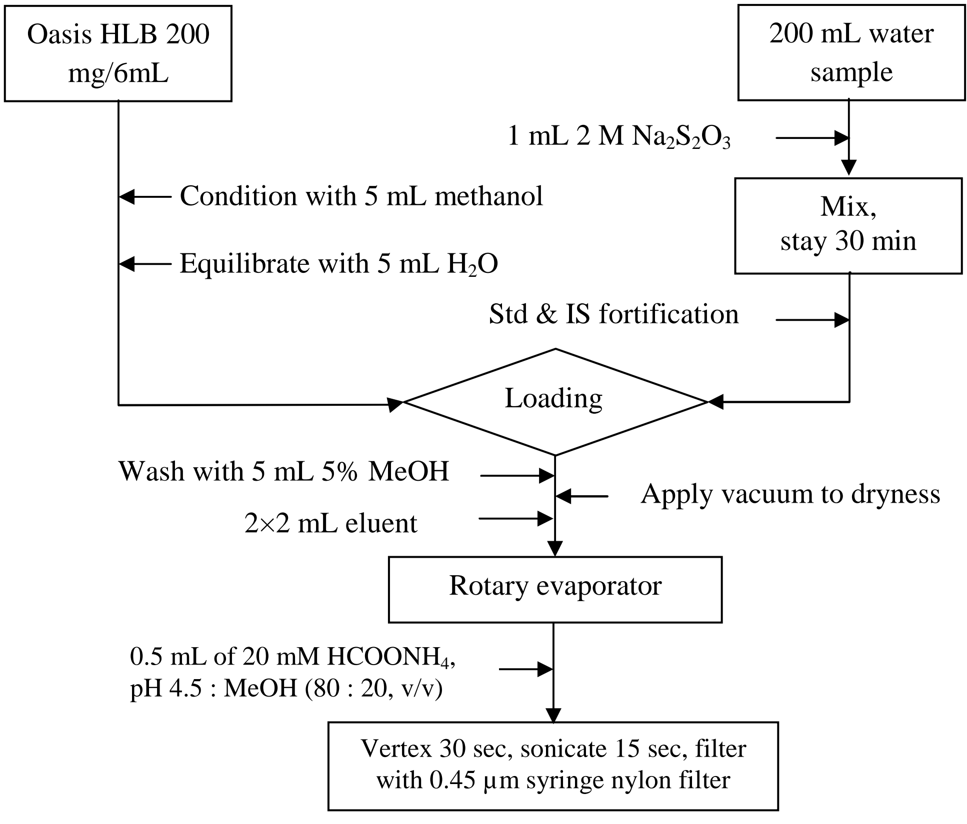


Figure 4.15 %RSD of the variation of eluent composition.

Sample preparation:



HPLC condition:

Prodigy ODS (3), 2.0x150 mm, 5µm with guard cartridge, 40 °C, flow rate: 0.2 mL/min; mobile phase: A: 10% ACN in pH 5.0 water (v/v), B: 30% ACN in pH 5.0 water (v/v); 0 min B 0%, 12 min B 100%, 15 min B 100%, 16 min B 0%, 22 min B 0%; injection volume: 10 µL

MS condition:

MS/MS mode as shown in Table 4.10 using segment time program

Calculation:

$$\text{concentration in sample } (\mu g/L) = \frac{\text{cal conc.from linear equation, } X\ (\mu g/L) \times \text{conc.IS } (\mu g/L)}{\text{enrichment factor } (400)}$$

Figure 4.16 Overall analytical procedure scheme

Figure 4.16 summarizes the overall analytical procedure scheme for the analysis of the investigated drugs in animal drinking water.

4.2 Method validation

4.2.1 Linearity and range

The calibration curve of MNZ, RNZ, DMZ, NFZ, NFT, FZD, FTD and CAP were constructed in triplicate samples of eight different concentrations of the matrix-matched standard solutions for three days. Calibration curves were plots of the area ratios against concentration ratios of target compound and IS. The linearity were range from 0.979 – 0.999 for all analytes in the investigated concentration ranges (Table 4.12 – 4.14).

Calibration curves for determination of matrix effects were also established daily (Table 4.12 – 4.14). Percent differences greater than 10% between matrix-matched standards and instrumental calibration curves indicated presence of matrix effect that are normally occurred in electrospay ionization MS. The result (Table 4.15) showed that matrix effects were randomly occurred in each substance.

Table 4.12 Linearity (day 1)

Analyte	Range (µg/L)	Method linearity		System linearity	
		Linear regression	r^2	Linear regression	r^2
MNZ	(0.1 – 0.7)	y = 0.804x + 0.030	0.991	y = 0.745x + 0.070	0.989
RNZ	(0.03 – 0.21)	y = 0.932x + 0.016	0.995	y = 0.908x + 0.017	0.998
DMZ	(0.2 – 1.4)	y = 0.725x + 0.088	0.998	y = 0.760x + 0.017	0.999
NFZ	(0.25 – 1.75)	y = 0.340x – 0.015	0.996	y = 0.369x – 0.040	0.998
NFT	(0.2 - 1.4)	y = 0.854x – 0.029	0.998	y = 0.856x – 0.027	0.998
FZD	(0.005 – 0.035)	y = 0.986x + 0.017	0.993	y = 0.896x + 0.023	0.996
FTD	(0.01 – 0.07)	y = 1.359x + 0.093	0.979	y = 0.993x – 0.029	0.997
CAP	(0.025 – 0.175)	y = 1.075x – 0.021	0.998	y = 1.050x + 0.021	0.999

Table 4.13 Linearity (day 2)

Analyte	Range (µg/L)	Method linearity		System linearity	
		Linear regression	r^2	Linear regression	r^2
MNZ	(0.1 – 0.7)	y = 0.779x + 0.037	0.999	y = 0.758x +0.025	0.994
RNZ	(0.03 – 0.21)	y = 0.985x + 0.024	0.999	y =0.935 x +0.021	0.997
DMZ	(0.2 – 1.4)	y = 0.845x + 0.035	0.997	y =0.737 x +0.071	0.996
NFZ	(0.25 – 1.75)	y = 0.362x - 0.023	0.996	y = 0.336 x -0.013	0.999
NFT	(0.2- 1.4)	y = 0.996x - 0.130	0.991	y =0.817 x - 0.014	0.999
FZD	(0.005 – 0.035)	y = 0.987x +0.064	0.995	y =1.032 x +0.038	0.998
FTD	(0.01 – 0.07)	y = 1.098x - 0.080	0.992	y =0.814 x +0.007	0.996
CAP	(0.025 – 0.175)	y = 1.277x - 0.016	0.994	y =1.097 x +0.014	0.999

Table 4.14 Linearity (day 3)

Analyte	Range (µg/L)	Method linearity		System linearity	
		Linear regression	r^2	Linear regression	r^2
MNZ	(0.1 – 0.7)	y = 0.755x + 0.058	0.995	y = 0.684x + 0.039	0.995
RNZ	(0.03 – 0.21)	y = 1.062x + 0.000	0.997	y = 0.965x - 0.013	0.997
DMZ	(0.2 – 1.4)	y = 0.764x + 0.067	0.994	y = 0.829x - 0.007	0.993
NFZ	(0.25 – 1.75)	y = 0.375x - 0.046	0.995	y = 0.308x - 0.008	0.998
NFT	(0.2 - 1.4)	y = 0.943x - 0.033	0.997	y = 0.754x + 0.002	0.999
FZD	(0.005 - 0.035)	y = 0.952x + 0.024	0.998	y = 0.882x + 0.046	0.997
FTD	(0.01 – 0.07)	y = 0.987x - 0.011	0.997	y = 1.009x - 0.026	0.996
CAP	(0.025 – 0.175)	y = 0.985x - 0.003	0.996	y = 0.904x - 0.020	0.993

Table 4.15 Matrix effects

Analyte	Day 1				Day 2				Day 3			
	Pure std.	MM std.	D	%D	Pure std.	MM std.	D	%D	Pure std.	MM std.	D	%D
MNZ	0.745	0.804	-0.059	8	0.758	0.779	-0.021	2.7	0.684	0.755	-0.071	10.4
RNZ	0.908	0.932	-0.024	2.6	0.935	0.985	-0.049	5.3	0.965	1.062	-0.096	10
DMZ	0.76	0.725	0.035	4.6	0.737	0.845	-0.108	14.6	0.829	0.764	0.065	7.8
NFZ	0.369	0.34	0.029	7.9	0.336	0.362	-0.026	7.7	0.308	0.375	-0.067	21.7
NFT	0.856	0.854	0.002	0.2	0.817	0.996	-0.178	21.8	0.754	0.943	-0.19	25.1
FZD	0.896	0.986	-0.09	10	1.032	0.987	0.044	4.3	0.882	0.952	-0.07	8
FTD	0.993	1.359	-0.365	36.8	0.814	1.098	-0.284	34.8	1.009	0.987	0.022	2.1
CAP	1.05	1.075	-0.026	2.4	1.097	1.277	-0.18	16.4	0.904	0.985	-0.081	9

4.2.2 Precision

Precision of the optimized method was evaluated from intra-day and inter-day precision. Intra-day precision ranges from 3.4 – 26.6% and inter-day precision were range from 7.2 – 19.6% (Table 4.16 – 4.23).

Table 4.16 Intra-day and inter-day accuracy and precision of MNZ

	Conc.add (µg/L)	MNZ								
		0.1			0.4			0.7		
	No. of sample	day1	day2	day3	day1	day2	day3	day1	day2	day3
	1	0.09	0.08	0.11	0.32	0.41	0.39	0.61	0.64	0.60
	2	0.09	0.12	0.08	0.29	0.40	0.44	0.68	0.66	0.67
	3	0.08	0.07	0.09	0.41	0.41	0.46	0.67	0.57	0.67
	4	0.09	0.11	0.10	0.43	0.37	0.40	0.59	0.59	0.71
	5	0.08	0.09	0.10	0.35	0.42	0.37	0.69	0.61	0.75
	6	0.11	0.11	0.08	0.45	0.39	0.43	0.81	0.65	0.70
	7	0.10	0.11	0.11	0.42	0.44	0.44	0.66	0.66	0.70
	8	0.08	0.12	0.08	0.43	0.36	0.42	0.78	0.67	0.65
	9	0.09	0.11	0.08	0.37	0.41	0.36	0.72	0.64	0.83
	10	0.10	0.10	0.08	0.34	0.42	0.43	0.60	0.65	0.71
Intra-day	average	0.09	0.10	0.09	0.38	0.40	0.41	0.68	0.63	0.70
	%recovery	92.1	101.1	90.1	95.1	100.7	103.6	97.2	90.7	99.8
	SD	0.01	0.02	0.01	0.05	0.03	0.03	0.07	0.03	0.06
	%RSD	9.93	16.12	13.57	13.89	6.36	7.70	10.76	5.30	8.99
Inter-day	average		0.09			0.40			0.67	
	%recovery		94.4			99.8			95.9	
	SD		0.01			0.04			0.06	
	%RSD		14.14			10.00			9.40	
	p-value		0.146			0.159			0.062	

Table 4.17 Intra-day and inter-day accuracy and precision of RNZ

	Conc.add (µg/L)	RNZ								
		0.03			0.12			0.21		
	No. of sample	day1	day2	day3	day1	day2	day3	day1	day2	day3
	1	0.025	0.030	0.036	0.12	0.13	0.11	0.17	0.22	0.21
	2	0.029	0.024	0.031	0.14	0.13	0.11	0.24	0.22	0.20
	3	0.024	0.027	0.033	0.13	0.13	0.14	0.22	0.18	0.23
	4	0.028	0.028	0.033	0.11	0.14	0.12	0.18	0.23	0.19
	5	0.027	0.027	0.029	0.11	0.12	0.12	0.19	0.18	0.20
	6	0.031	0.031	0.027	0.11	0.13	0.13	0.19	0.19	0.20
	7	0.022	0.018	0.029	0.13	0.12	0.10	0.20	0.21	0.19
	8	0.030	0.031	0.026	0.12	0.12	0.11	0.23	0.21	0.22
	9	0.024	0.025	0.029	0.11	0.11	0.11	0.22	0.21	0.19
	10	0.035	0.033	0.034	0.14	0.11	0.11	0.18	0.24	0.22
Intra-day	average	0.03	0.03	0.03	0.12	0.12	0.12	0.20	0.21	0.21
	%recovery	91.9	91.3	102.1	101.9	103.7	96.7	97.2	99.7	97.8
	SD	0.00	0.00	0.00	0.01	0.01	0.01	0.03	0.02	0.02
	%RSD	14.46	16.01	10.91	8.71	7.60	9.29	12.25	8.90	7.83
Inter-day	average		0.03			0.12			0.21	
	%recovery		95.1			100.8			98.2	
	SD		0.00			0.01			0.02	
	%RSD		14.31			8.76			9.54	
	p-value		0.134			0.217			0.832	

Table 4.18 Intra-day and inter-day accuracy and precision of DMZ

	Conc.add (µg/L)	DMZ								
		0.2			0.8			1.4		
	No. of sample	day1	day2	day3	day1	day2	day3	day1	day2	day3
	1	0.145	0.197	0.207	0.86	0.85	0.95	1.24	1.55	1.22
	2	0.196	0.211	0.202	0.74	0.79	0.74	1.34	1.44	1.56
	3	0.171	0.210	0.291	0.94	0.73	0.70	1.32	1.49	1.31
	4	0.191	0.207	0.166	0.73	0.83	0.82	1.13	1.58	1.29
	5	0.216	0.213	0.219	0.88	0.82	0.90	1.26	1.55	1.34
	6	0.270	0.220	0.185	0.80	0.74	0.96	1.33	1.49	1.57
	7	0.214	0.230	0.189	0.81	0.84	0.87	1.40	1.15	1.37
	8	0.178	0.222	0.175	0.82	0.86	0.86	1.36	1.38	1.61
	9	0.216	0.224	0.215	0.63	0.82	0.87	1.59	1.27	1.50
	10	0.179	0.203	0.188	0.87	0.85	0.75	1.46	1.32	1.61
Intra-day	average	0.20	0.21	0.20	0.81	0.81	0.84	1.34	1.42	1.44
	%recovery	98.8	106.8	102.0	101.1	101.7	105.2	96.0	101.6	102.7
	SD	0.03	0.01	0.04	0.09	0.04	0.09	0.12	0.14	0.15
	%RSD	17.18	4.86	17.18	11.02	5.50	10.50	9.23	9.94	10.15
Inter-day	average		0.21			0.82			1.40	
	%recovery		102.5			102.7			100.1	
	SD		0.03			0.08			0.14	
	%RSD		13.94			9.20			9.93	
	p-value		0.464			0.663			0.274	

Table 4.19 Intra-day and inter-day accuracy and precision of NFZ

	Conc.add (µg/L)	NFZ								
		0.25			1			1.75		
	No. of sample	day1	day2	day3	day1	day2	day3	day1	day2	day3
	1	0.243	0.287	0.272	1.13	1.02	0.86	1.88	1.99	1.66
	2	0.263	0.281	0.249	1.01	1.16	1.09	1.86	1.58	1.44
	3	0.243	0.233	0.255	1.16	1.02	1.10	2.06	1.89	2.19
	4	0.247	0.274	0.244	0.91	1.04	1.00	1.69	1.77	1.47
	5	0.286	0.240	0.302	1.02	1.04	1.15	1.77	1.72	1.76
	6	0.240	0.268	0.281	1.02	1.12	1.02	1.75	1.78	1.67
	7	0.287	0.246	0.238	0.80	0.96	1.13	1.45	1.75	1.71
	8	0.285	0.227	0.280	0.89	1.14	0.84	1.72	1.74	1.83
	9	0.287	0.276	0.335	0.99	0.93	0.96	1.90	1.82	1.47
	10	0.262	0.295	0.296	0.92	0.93	0.88	1.71	1.90	1.40
Intra-day	average	0.264	0.263	0.275	0.98	1.04	1.00	1.78	1.79	1.66
	%recovery	105.7	105.1	110.1	98.4	103.7	100.4	101.6	102.5	94.8
	SD	0.02	0.02	0.03	0.11	0.08	0.11	0.16	0.11	0.24
	%RSD	7.75	9.20	11.03	11.23	8.08	11.24	9.06	6.31	14.28
Inter-day	average		0.27			1.01			1.74	
	%recovery		107.0			100.8			99.7	
	SD		0.03			0.10			0.18	
	%RSD		9.39			10.11			10.44	
	p-value		0.489			0.538			0.200	

Table 4.20 Intra-day and inter-day accuracy and precision of NFT

	Conc.add (µg/L)	NFT								
		0.2			0.8			1.4		
	No. of sample	day1	day2	day3	day1	day2	day3	day1	day2	day3
	1	0.201	0.203	0.181	0.73	0.89	0.87	1.18	1.54	1.27
	2	0.214	0.200	0.187	0.80	0.77	0.80	1.42	1.39	1.35
	3	0.168	0.210	0.191	0.70	0.76	0.85	1.40	1.36	1.56
	4	0.209	0.213	0.206	0.98	0.76	0.73	1.41	1.36	1.33
	5	0.188	0.216	0.202	0.97	0.80	0.73	1.63	1.38	1.61
	6	0.231	0.215	0.213	0.93	0.80	0.81	1.50	1.41	1.32
	7	0.225	0.200	0.197	0.91	0.75	0.72	1.50	1.41	1.34
	8	0.224	0.209	0.176	0.85	0.78	0.79	1.45	1.43	1.50
	9	0.228	0.197	0.197	0.79	0.77	0.81	1.51	1.40	1.35
	10	0.210	0.214	0.206	0.83	0.74	0.73	1.53	1.26	1.26
Intra-day	average	0.210	0.208	0.196	0.85	0.78	0.78	1.45	1.40	1.39
	%recovery	105.0	103.9	97.8	106.3	97.6	98.0	103.7	99.7	99.2
	SD	0.02	0.01	0.01	0.10	0.04	0.05	0.12	0.07	0.12
	%RSD	9.38	3.41	5.98	11.33	5.54	6.85	8.09	4.97	8.89
Inter-day	average		0.20			0.81			1.41	
	%recovery		102.2			100.6			100.9	
	SD		0.01			0.07			0.11	
	%RSD		7.23			9.14			7.54	
	p-value		0.063			0.085			0.358	

Table 4.21 Intra-day and inter-day accuracy and precision of FZD

	Conc.add (μg/L)	FZD								
		0.005			0.02			0.035		
	No. of sample	day1	day2	day3	day1	day2	day3	day1	day2	day3
	1	0.0050	0.0044	0.0049	0.018	0.022	0.021	0.032	0.036	0.033
	2	0.0048	0.0046	0.0051	0.019	0.018	0.021	0.034	0.036	0.039
	3	0.0050	0.0053	0.0040	0.017	0.020	0.022	0.036	0.040	0.034
	4	0.0060	0.0046	0.0049	0.020	0.020	0.022	0.037	0.029	0.033
	5	0.0054	0.0043	0.0041	0.019	0.019	0.022	0.034	0.031	0.036
	6	0.0041	0.0044	0.0042	0.021	0.021	0.020	0.038	0.034	0.034
	7	0.0053	0.0047	0.0047	0.019	0.019	0.020	0.035	0.033	0.033
	8	0.0058	0.0044	0.0042	0.022	0.021	0.021	0.035	0.038	0.030
	9	0.0045	0.0048	0.0057	0.022	0.023	0.018	0.032	0.035	0.031
	10	0.0050	0.0053	0.0049	0.021	0.018	0.022	0.033	0.034	0.037
Intra-day	average	0.0051	0.0047	0.0047	0.02	0.02	0.02	0.03	0.03	0.03
	%recovery	102.1	93.6	93.5	99.4	100.3	104.2	99.4	99.3	97.5
	SD	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	%RSD	10.77	7.59	11.62	8.88	7.33	5.78	5.49	9.07	7.85
Inter-day	average		0.005			0.02			0.03	
	%recovery		96.4			101.3			98.7	
	SD		0.001			0.002			0.003	
	%RSD		10.71			7.44			7.40	
	p-value		0.099			0.323			0.828	

Table 4.22 Intra-day and inter-day accuracy and precision of FTD

	Conc.add (μg/L)	FTD								
		0.01			0.04			0.07		
	No. of sample	day1	day2	day3	day1	day2	day3	day1	day2	day3
	1	0.005	0.012	0.010	0.03	0.05	0.04	0.05	0.10	0.07
	2	0.009	0.010	0.011	0.05	0.04	0.04	0.07	0.08	0.07
	3	0.005	0.011	0.009	0.03	0.04	0.03	0.07	0.07	0.06
	4	0.009	0.010	0.010	0.04	0.04	0.04	0.08	0.06	0.06
	5	0.010	0.012	0.007	0.04	0.04	0.04	0.07	0.06	0.06
	6	0.009	0.010	0.007	0.04	0.04	0.04	0.07	0.06	0.06
	7	0.008	0.010	0.009	0.05	0.04	0.03	0.07	0.06	0.06
	8	0.011	0.010	0.009	0.04	0.04	0.04	0.07	0.06	0.06
	9	0.012	0.008	0.007	0.05	0.04	0.03	0.08	0.06	0.05
	10	0.012	0.011	0.010	0.04	0.04	0.03	0.08	0.07	0.07
Intra-day	average	0.01	0.01	0.01	0.04	0.04	0.04	0.07	0.07	0.06
	%recovery	89.9	104.1	88.4	101.6	96.1	88.8	101.7	96.5	88.4
	SD	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.01	0.01
	%RSD	26.61	11.45	16.68	18.51	9.18	10.64	10.61	17.50	10.59
Inter-day	average		0.01			0.04			0.07	
	%recovery		94.1			95.5			95.5	
	SD		0.002			0.01			0.01	
	%RSD		19.61			14.44			14.15	
	p-value		0.109			0.111			0.082	

Table 4.23 Intra-day and inter-day accuracy and precision of CAP

	Conc.add (μg/L)	CAP								
		0.025			0.1			0.175		
	No. of sample	day1	day2	day3	day1	day2	day3	day1	day2	day3
	1	0.024	0.030	0.026	0.094	0.101	0.099	0.191	0.211	0.168
	2	0.024	0.024	0.025	0.103	0.084	0.084	0.164	0.127	0.150
	3	0.024	0.017	0.024	0.114	0.080	0.110	0.187	0.153	0.187
	4	0.024	0.027	0.026	0.094	0.105	0.108	0.151	0.164	0.169
	5	0.025	0.029	0.028	0.097	0.088	0.116	0.168	0.178	0.190
	6	0.027	0.027	0.027	0.087	0.105	0.108	0.188	0.183	0.205
	7	0.024	0.025	0.027	0.097	0.108	0.101	0.187	0.166	0.172
	8	0.025	0.027	0.032	0.116	0.107	0.102	0.180	0.157	0.191
	9	0.032	0.027	0.022	0.110	0.094	0.119	0.164	0.170	0.195
	10	0.027	0.021	0.026	0.083	0.105	0.110	0.156	0.174	0.175
Intra-day	average	0.026	0.025	0.026	0.099	0.098	0.106	0.174	0.168	0.180
	%recovery	102.5	101.6	105.4	99.4	97.7	105.6	99.2	96.1	102.9
	SD	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.02	0.02
	%RSD	10.46	15.12	9.51	11.12	10.66	9.45	8.42	12.92	9.00
Inter-day	average	0.0			0.10			0.17		
	%recovery	103.2			100.9			99.4		
	SD	0.0			0.01			0.02		
	%RSD	11.6			10.60			10.26		
	p-value	0.8			0.227			0.342		

4.2.3 Accuracy

Accuracy in term of recovery was performed by the standard addition method by spiking each compound at the level of low, middle and high concentrations of calibration curves to blank animal drinking water. Percent recoveries of MNZ, RNZ, DMZ, NFZ, NFT, FZD, FTD and CAP were found in ranges of 90.1 – 103.6%, 91.3 – 103.7%, 96.0 – 106.8%, 94.8 – 110.1%, 97.6 – 106.3%, 93.5 – 104.2, 88.4 – 104.1% and 96.1 – 105.6, respectively (Table 4.24).

Table 4.24 Summary of accuracy and precision

	Intra-day		Inter-day	
	%R	%RSD	%R	%RSD
MNZ	90.1 – 103.6	5.3 – 16.1	94.4 – 99.8	9.4 – 14.1
RNZ	91.3 – 103.7	7.6 – 16.0	95.1 – 100.8	8.8 – 14.3
DMZ	96.0 – 106.8	4.9 – 17.2	100.1 – 102.7	9.2 – 13.9
NFZ	94.8 – 110.1	6.3 – 14.3	99.7 – 107.0	9.4 – 10.4
NFT	97.6 – 106.3	3.4 – 11.3	100.6 – 102.2	7.2 – 9.1
FZD	93.5 – 104.2	5.5 – 11.6	96.4 – 101.3	7.4 – 10.7
FTD	88.4 – 104.1	9.2 – 26.6	94.1 – 95.5	14.1 – 19.6
CAP	96.1 – 105.6	8.4 – 15.1	99.4 – 103.2	10.3 – 11.6

4.2.4. Limit of detection (LOD) and Limit of quantitation (LOQ)

LOD and LOQ were calculated from 10 blank samples spiked at lowest acceptable concentrations as shown in Table 3.12. The results (Table 4.25) showed that LOD of each target compound was in the range of 0.001 – 0.035 µg/L. The LODs were confirmed by spiking of the calculated amounts to blank water samples and performed sample pretreatment before evaluated by visualization. The adjusted value for LODs and LOQs for each compound was in a range of 0.002 – 0.06 µg/L and 0.005 - 0.25 µg/L, respectively (Table 4.26). Figure 4.17 and 4.18 showed chromatogram at LOD and LOQ, respectively.

Table 4.25 Calculated LOD and LOQ of each target compound

Analyte	Slope	Intercept	r^2	Concentration (µg/L)		
				Spike conc.	LOD (mean spike + 3sd)	LOQ (mean spike +10sd)
MNZ	0.6859	0.0586	0.990	0.013	0.02	0.04
RNZ	0.8302	0.0123	0.997	0.005	0.01	0.02
DMZ	0.8933	0.0738	0.990	0.015	0.12	0.26
NFZ	0.6832	-0.0496	0.989	0.035	0.06	0.13
NFT	0.9544	-0.0232	0.998	0.025	0.04	0.09
FZD	1.0299	0.0418	0.998	0.001	0.001	0.002
FTD	1.3234	-0.1095	0.998	0.001	0.001	0.003
CAP	0.8171	0.0053	0.991	0.005	0.01	0.03

Table 4.26 Adjusted LOD and LOQ after peak confirmation

Analyte	Concentration (µg/L)				
	Spike conc.	LOD (mean spike + 3sd)	Adjusted LOD	LOQ (mean spike +10sd)	Adjusted LOQ
MNZ	0.013	0.02	0.03	0.04	0.10
RNZ	0.005	0.01	0.01	0.02	0.03
DMZ	0.015	0.12	0.05	0.26	0.20
NFZ	0.035	0.06	0.06	0.13	0.25
NFT	0.025	0.04	0.04	0.09	0.20
FZD	0.001	0.001	0.002	0.002	0.005
FTD	0.001	0.001	0.002	0.003	0.01
CAP	0.005	0.01	0.01	0.03	0.025

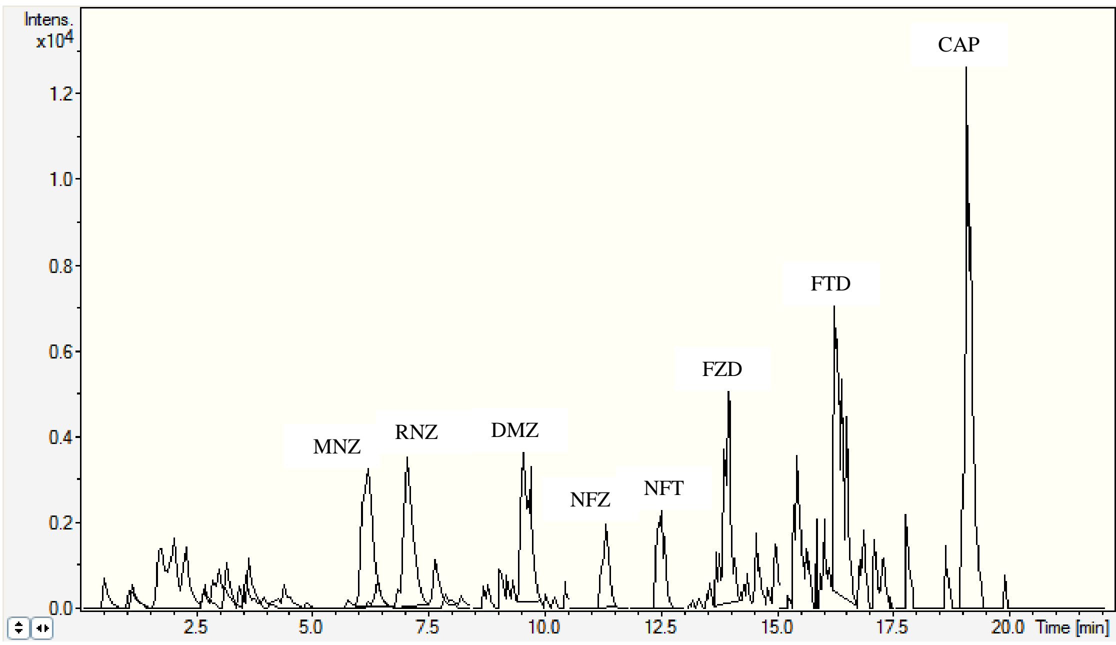


Figure 4.17 Extracted ion chromatogram from MS/MS full scan at LOD level; 1: MNZ, 2: RNZ, 3: DMZ, 4: NFZ, 5: NFT, 6: FZD, 7: FTD and 8: CAP.

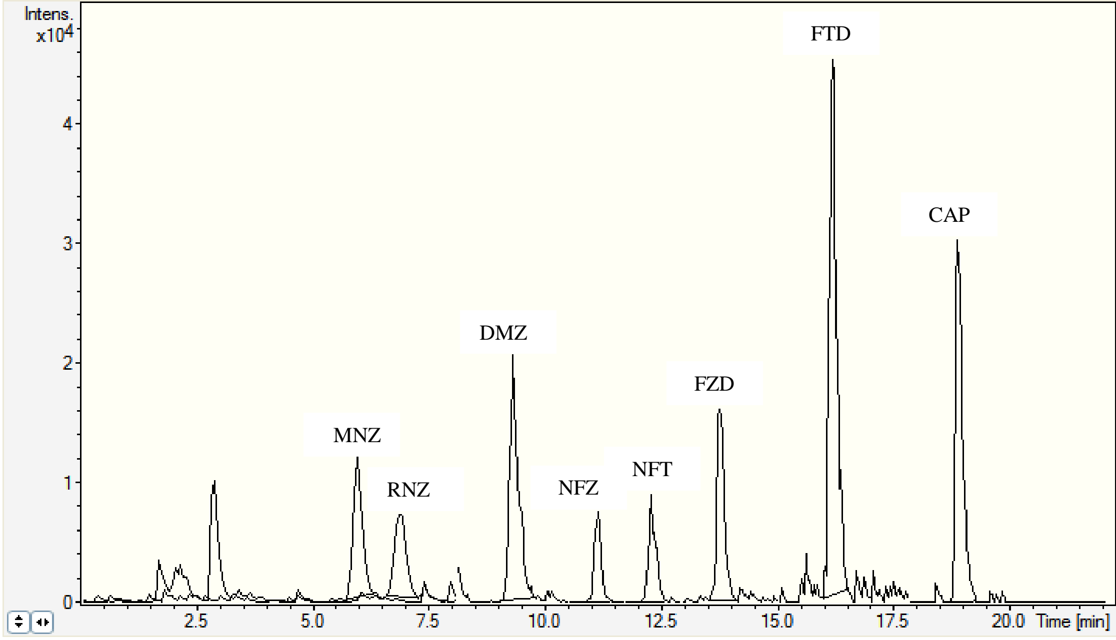


Figure 4.18 Extracted ion chromatogram from MS/MS full scan at LOQ level; 1: MNZ, 2: RNZ, 3: DMZ, 4: NFZ, 5: NFT, 6: FZD, 7: FTD and 8: CAP.

4.2.5 Ruggedness

Ruggedness data were obtained from 1) varying amounts of acids in eluent (Table 3.6). The data showed that %recovery of each analyte at lower than 1 µg/L spiked level was satisfied in the range of 60 – 120% (Table 4.11). 2) varying of pH of water in optimized mobile phase around proposed value of 5.0 (4.5, 5.0 and 5.5). The retention time data showed that t_R were not affected despite of slightly chance of pH around 5.0 except that of FTD (Table 4.27 and Figure 4.19). However, the analysis of each target compound could be performed simultaneously at the pH 4.5 – 5.5.

Table 4.27 Ruggedness data of pH of mobile phase

Analyte	Retention time at various pH of water in mobile phase (t_R, min)					
	pH 4.5	pH 5.0	pH 5.5	mean	sd	%RSD
MNZ	6.1	6.1	6.1	6.1	0.02	0.32
RNZ	7.0	6.9	7.0	6.9	0.07	1.00
DMZ	9.5	9.4	9.4	9.4	0.02	0.20
NFZ	11.1	11.1	11.1	11.1	0.04	0.35
NFT	12.3	12.2	12.0	12.2	0.15	1.25
FZD	13.8	13.7	13.8	13.8	0.06	0.42
FTD	15.8	16.5	17.4	16.6	0.77	4.65
CAP	18.9	18.8	19.1	18.9	0.16	0.87

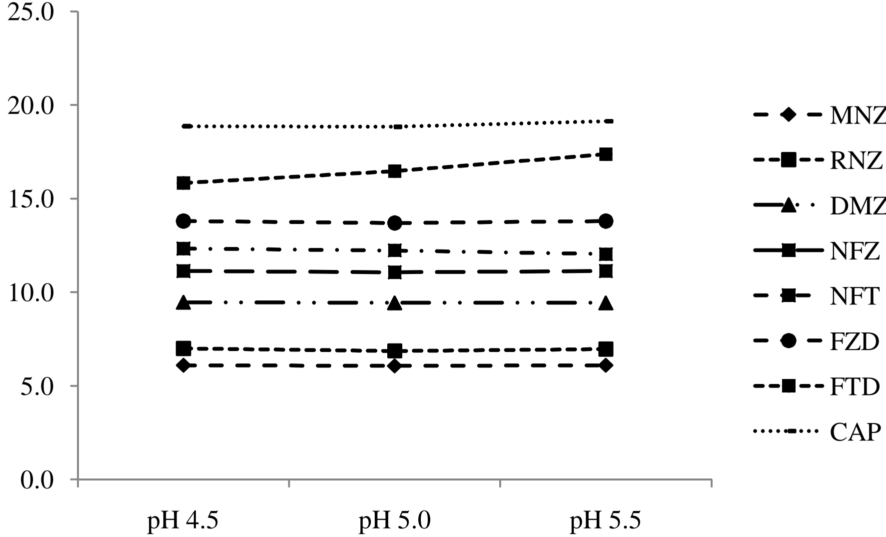


Figure 4.19 Plot of retention time at various pH value around optimized value (pH 5.0), HPLC condition: Prodigy ODS (3), 2.0x150 mm, 5µm with guard cartridge, 40 °C, flow rate: 0.2 mL/min. MS condition: MS/MS mode, segment program. Gradient profile: system 43, run time: 25 min.

4.3 Applications

The developed and validated method was applied for the determination of eight banned substances in livestock drinking water collected from standard farm in various provinces. Two batches (20 samples per batch) were analysed including six blind samples per batch. Recoveries of spiked blind samples were acceptable (60 – 120%) (Table 4.28 – 4.31). Among the investigated samples (40 samples), no drug substances were detected. This finding may reflected the efficiency of banned substances control scheme that has been applied since nitrofurans and chloramphenicol crisis in 2002.

Table 4.28 Target compounds and concentration in blind samples in batch No.1

Blind No.	Concentration (µg/L)							
	MNZ	RNZ	DMZ	NFZ	NFT	FZD	FTD	CAP
1	0	0	0.9	1.25	0	0.035	0.01	0.1
2	0.2	0	1.1	0	0	0	0.035	0
3	0	0.15	0	0	0	0	0	0
4	0.65	0	0.7	0.375	0	0.025	0.07	0.0625
5	0	0	0.9	1.375	0.5	0	0.02	0.125
6	0.5	0.18	0	0.875	1.1	0	0.015	0.025

Table 4.29 Target compounds and concentration found in blind samples in batch No.1

Blind No.	Concentration found (µg/L) / %recovery							
	MNZ	RNZ	DMZ	NFZ	NFT	FZD	FTD	CAP
1	ND	ND	0.99 / 110.1	1.24 / 99.3	ND	0.04 / 108.0	0.01 / 100.9	0.1 / 110.3
2	0.20 / 98.1	ND	1.24 / 112.6	ND	ND	ND	0.04 / 107.5	ND
3	ND	0.17 / 114.0	ND	ND	ND	ND	ND	ND
4	0.68 / 104.0	ND	0.77 / 110.1	0.35 / 92.8	ND	0.03 / 106.5	0.08 / 111.2	0.06 / 95.8
5	ND	ND	1.0 / 111.4	1.34 / 97.5	0.41 / 82.6	ND	0.02 / 105.6	0.11 / 85.2
6	0.53 / 105.2	0.20 /109.6	ND	0.96 / 109.4	0.95 /86.2	ND	0.01 / 99.7	0.02 / 76.4

Table 4.30 Target compounds and concentration in blind samples in batch No.2

Blind No.	Concentration (µg/L)							
	MNZ	RNZ	DMZ	NFZ	NFT	FZD	FTD	CAP
1	0	0	0	1.125	0	0	0.05	0
2	0	0	0.4	0	0	0	0	0
3	0.2	0.09	0.7	0	0	0	0	0.1125
4	0	0	0	0	0	0	0	0.0875
5	0	0.045	0	0	0	0.0175	0.06	0
6	0	0.045	0.4	0	0.5	0	0	0.15

Table 4.31 Target compounds and concentration found in blind samples in batch No.2

Blind No.	Concentration found (ug/L) / %recovery							
	MNZ	RNZ	DMZ	NFZ	NFT	FZD	FTD	CAP
1	ND	ND	ND	1.0 / 90.7	ND	ND	0.05 / 91.5	ND
2	ND	ND	0.41 / 103.6	ND	ND	ND	ND	ND
3	0.19 / 95.0	0.07 / 77.0	0.82 / 117.6	ND	ND	ND	ND	0.12 / 107.1
4	ND	ND	ND	ND	ND	ND	ND	0.097 / 110.8
5	ND	0.03 / 68.1	ND	ND	ND	0.02 / 97.4	0.06 / 101.0	ND
6	ND	0.04 / 91.9	0.45 / 112.0	ND	0.51 / 102.7	ND	ND	0.13 / 88.4

CHAPTER V
CONCLUSION

The HPLC-MS method for the simultaneous determination of MNZ, RNZ, DMZ, NFZ, NFT, FZD, FTD and CAP was established using their corresponding stable isotope labeled compounds as internal standards. Optimization was performed by varying compositions of mobile phase i.e. pH of mobile phase (2.0 – 6.0), types of modifiers (formic acid and acetic acid), types of buffer (ammonium acetate and ammonium formate) and buffer concentrations (5 – 40 mM) and instrumental parameters, i.e. gradient elution profiles and MS parameters. The separation of eight target compounds was achieved in 22 min using gradient elution of A: 10% ACN in acidified water (pH 5.0) (v/v), B: 30% ACN in acidified water (pH 5.0) (v/v); 0 min B 0%, 12 min B 100%, 15 min B 100%, 16 min B 0%, 22 min B 0%, Prodigy ODS (3) column, 2.0 x 150 mm, 5 µm with guard cartridge, 40 °C, flow rate: 0.2 mL/min with the fine tuned MS parameters in MRM mode. Ultrahigh pure nitrogen was used for ESI interface as nebulizer and dry gas at 35 psi and 9 L/min, respectively, with drying temperature of 325 °C. The optimized condition provided a baseline separation of all compounds with the $R_s \geq 3$ and $N > 8000$. Eluent compositions were optimized for SPE and 4 mL (2×2 mL) of 1% acetic acid in mixture of ACN : MeOH (80 : 20, v/v) was selected for method validation. The optimized LC-MS condition and sample preparation were validated in term of linearity and range, precision, accuracy, LOD and LOQ. Calibration curves were constructed in the range of 0.1 – 0.7, 0.03 – 0.21, 0.2 – 1.4, 0.25 – 1.75, 0.2 – 1.4, 0.005 – 0.035, 0.01 – 0.07 and 0.025 – 0.175 µg/L for MNZ, RNZ, DMZ, NFZ, NFT, FZD, FTD and CAP, respectively. The linearity (r^2 = 0.979 – 0.999) were obtained for all analytes. Precision of the method was evaluated from intra-day and inter-day precision. Intra-day precision ranges from 3.4 – 26.6% and inter-day precision were range from 7.2 – 19.6%. Accuracies were performed in term of recovery by spiking of analytes to 200 mL of blank farm water at the level of low, middle and high of calibration curve of each analyte. Percent recoveries of MNZ,

RNZ, DMZ, NFZ, NFT, FZD, FTD and CAP were found in ranges of 90.1 – 103.6%, 91.3 – 103.7%, 96.0 – 106.8%, 94.8 – 110.1%, 97.6 – 106.3%, 93.5 – 104.2, 88.4 – 104.1% and 96.1 – 105.6, respectively. LODs and LOQs for each compound was in a range of 0.002 – 0.06 µg/L and 0.005 - 0.25 µg/L, respectively. Finally, the optimized and validated method was applied for the determination of eight banned substances in 40 samples of animal drinking water collected from standard farms in various provinces in Thailand. The prohibited substances were not found in all studied samples, whereas recoveries of twelve controlled blind samples were within 60 – 120%.

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APPENDICES

APPENDIX A

Optimization function in Esquire Control version 6.1 is automated feature used for tuning of MS optic parameters by ramping values between low and high limits to obtain highest intensity of specified mass of target compounds. The benefits of this feature are its convenience, accuracy and less time consumption.

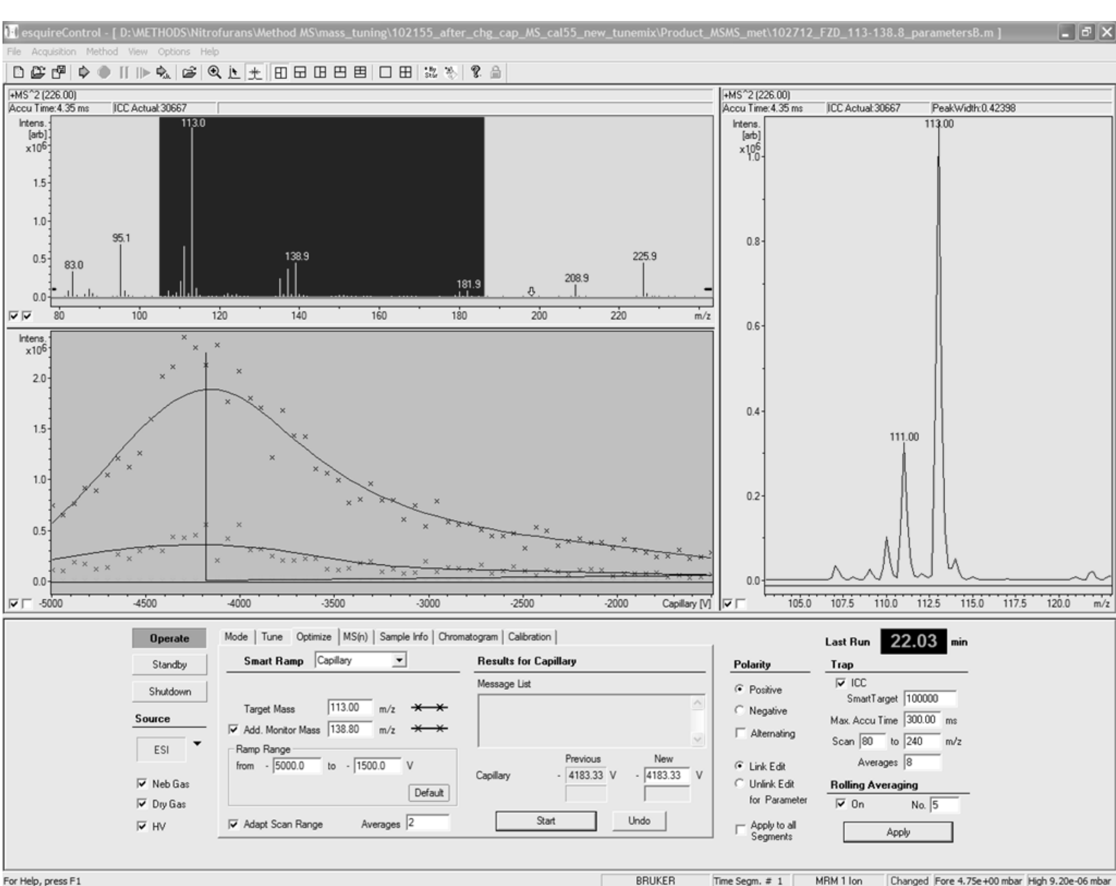

Figure A1 Tuning of MS parameters using Esquire Control.

APPENDIX B

Table B1 Proposed mass fragmentation

Structure	MW (exact mass)	Ionization mode	Product ion 1	Product ion 2
MNZ	171.06	+ MS / MS	128 .04	
RNZ	200.05	+ MS / MS	140.03	
DMZ	141.05	+ MS / MS	92.26	112.18
NFZ	198.04	- MS / MS	149.95	123.99

Table B1 Proposed mass fragmentation (continued)

Structure	MW(exact mass)	Ionization mode	Product ion 1	Product ion 2
NFT	238.03	- MS / MS	151.93	193.90
FZD	225.04	+ MS / MS	113.07	139. 04
FTD	324.11	+ MS / MS	281.12	252.08
CAP	322.01	- MS / MS	256.98	193.93

APPENDIX C

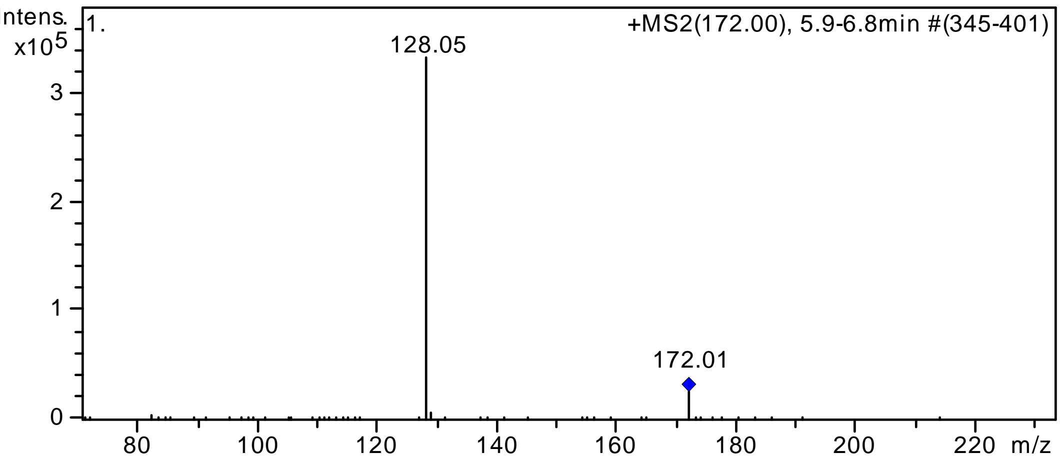

Figure C1 MS/MS mass spectrum of MNZ $[M+H]^+$.

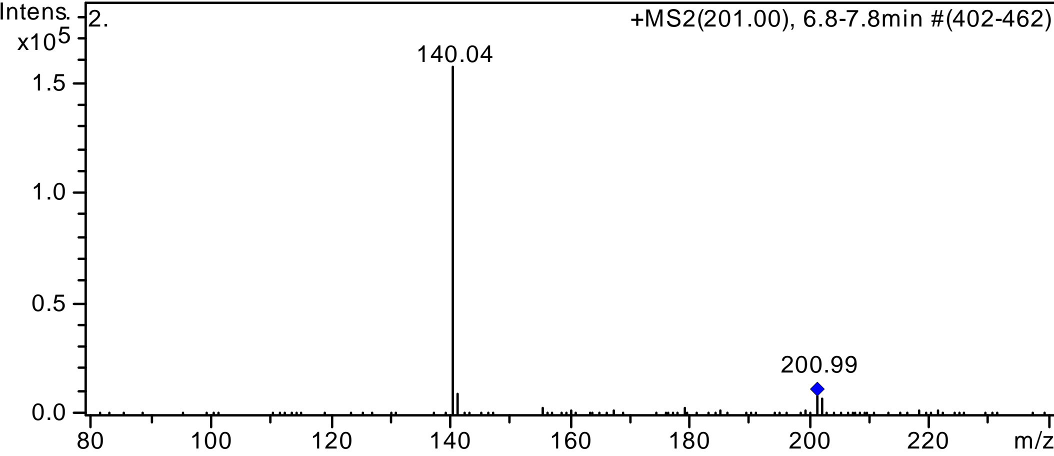

Figure C2 MS/MS mass spectrum of RNZ $[M+H]^+$.

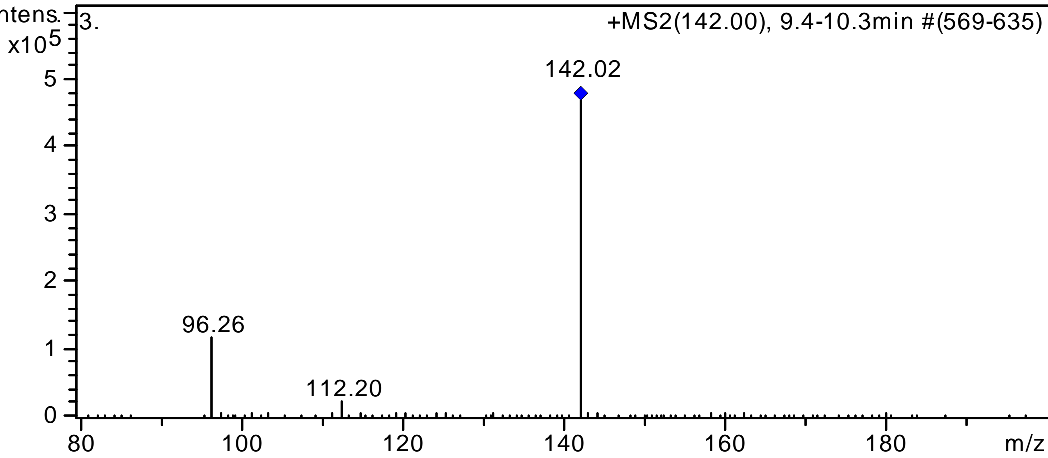

Figure C3 MS/MS mass spectrum of DMZ $[M+H]^+$.

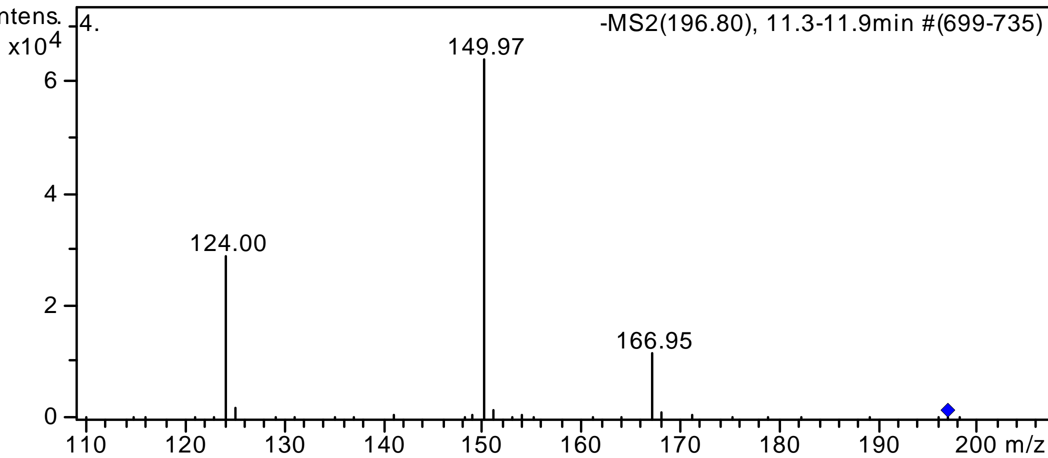

Figure C4 MS/MS mass spectrum of NFZ $[M-H]^-$.

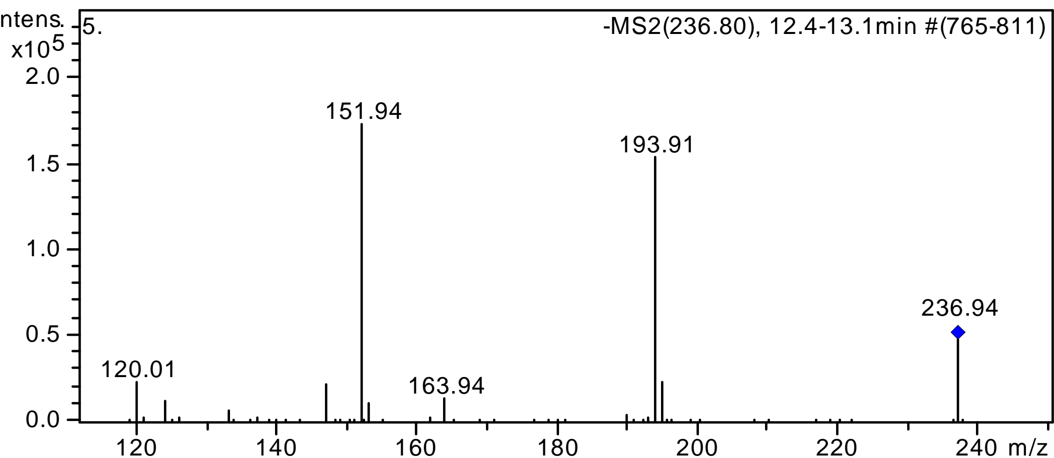

Figure C5 MS/MS mass spectrum of NFT $[M-H]^-$.

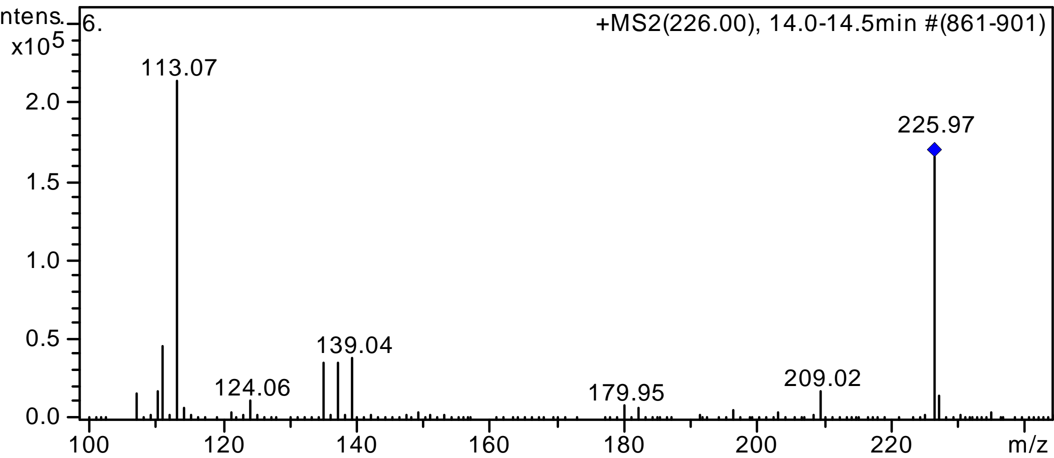

Figure C6 MS/MS mass spectrum of FZD $[M+H]^+$.

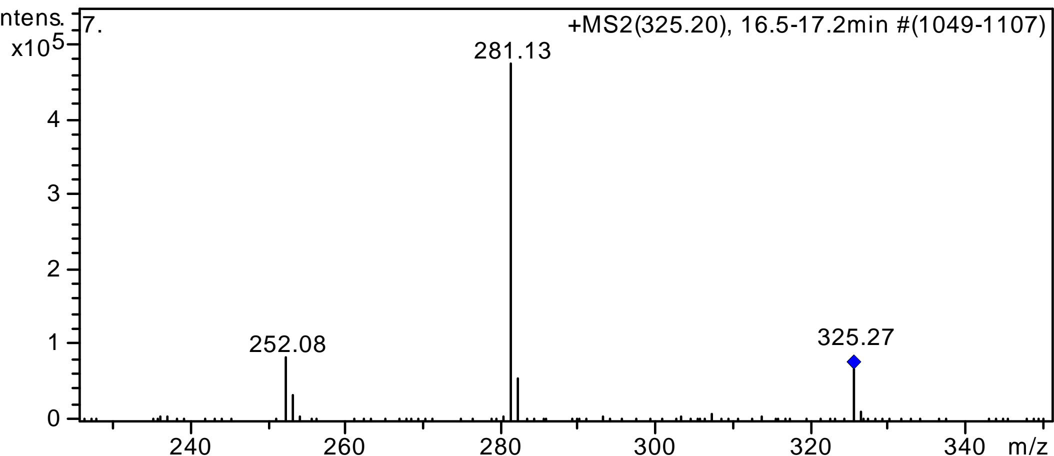

Figure C7 MS/MS mass spectrum of FTD $[M+H]^+$.

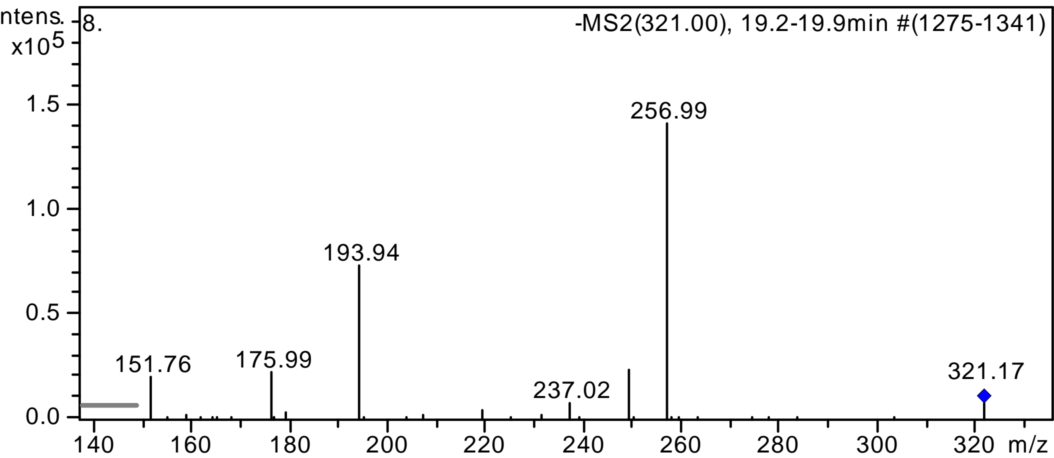

Figure C8 MS/MS mass spectrum of CAP $[M-H]^-$.

APPENDIX D

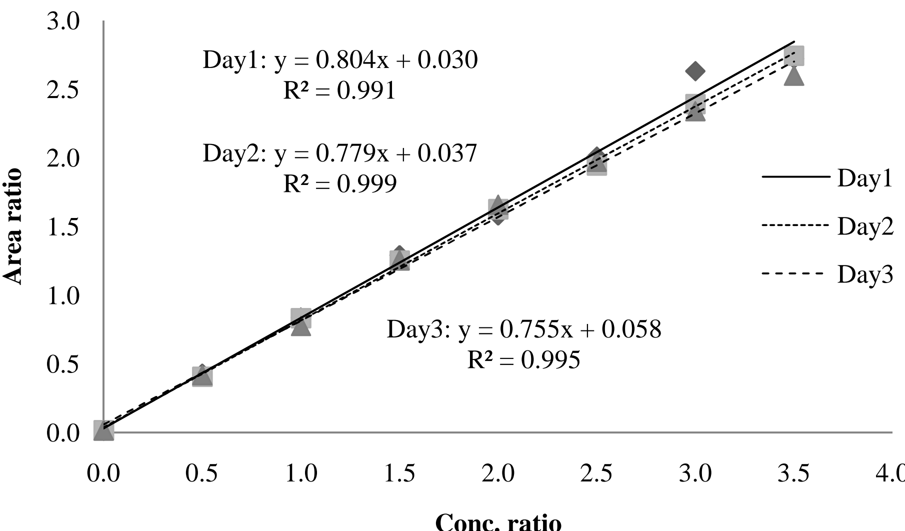

Figure D1 Calibration curve of MNZ on three different days.

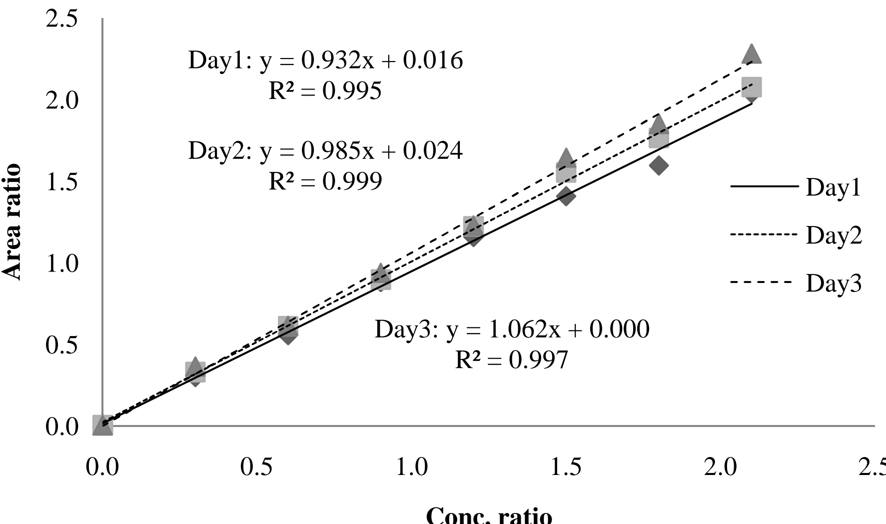

Figure D2 Calibration curve of RNZ on three different days.

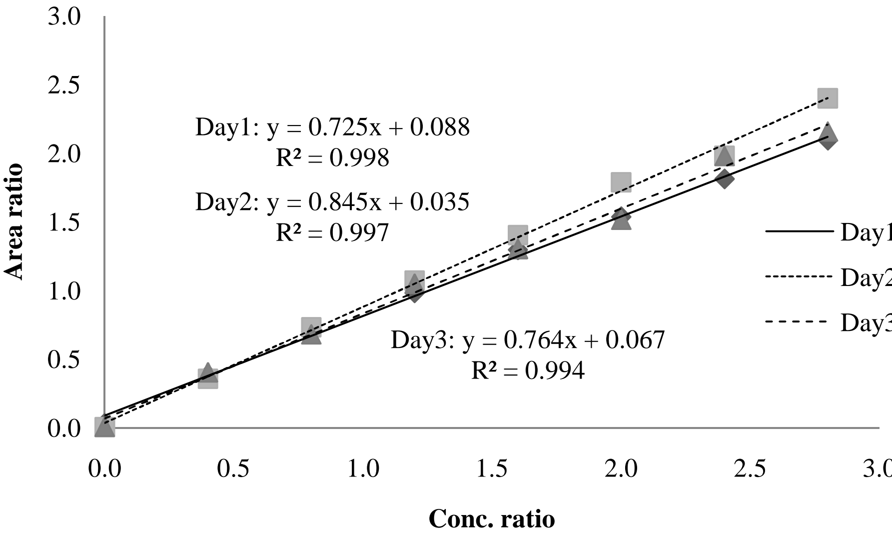

Figure D3 Calibration curve of DMZ on three different days.

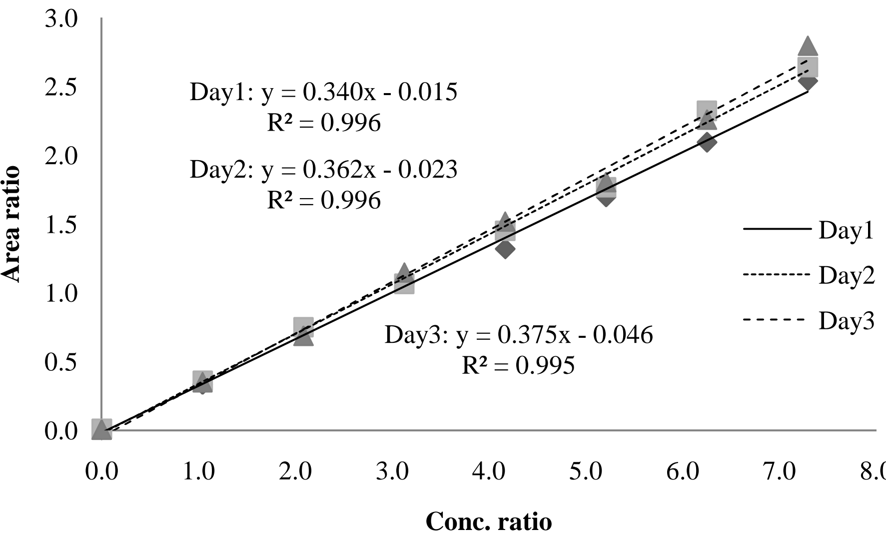

Figure D4 Calibration curve of NFZ on three different days.

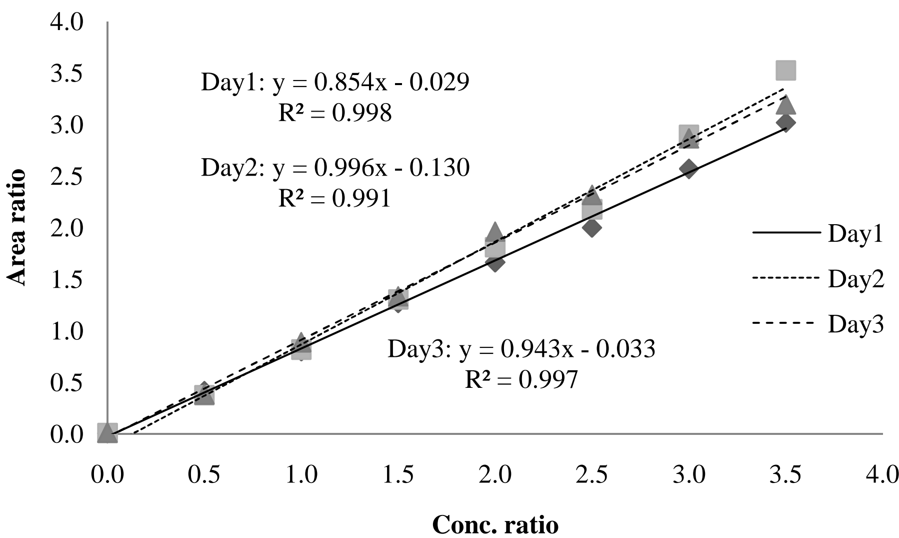

Figure D5 Calibration curve of NFT on three different days.

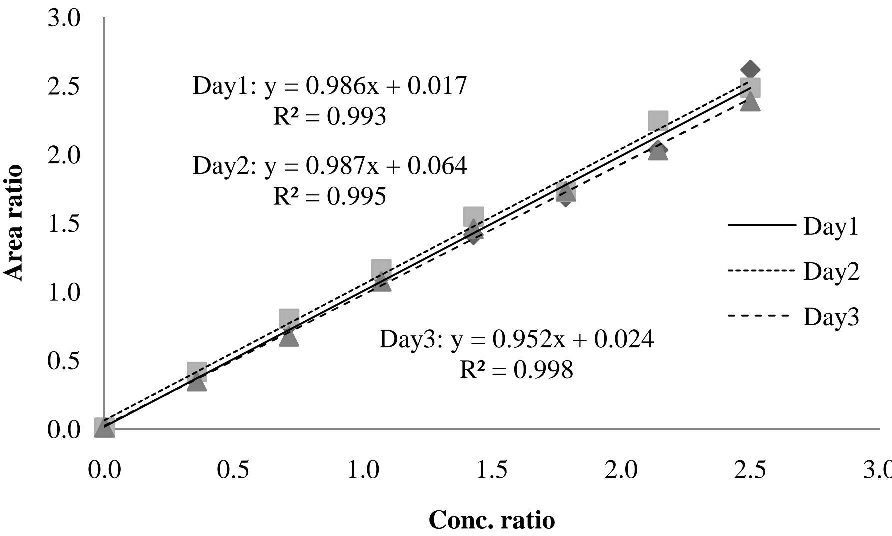

Figure D6 Calibration curve of FZD on three different days.

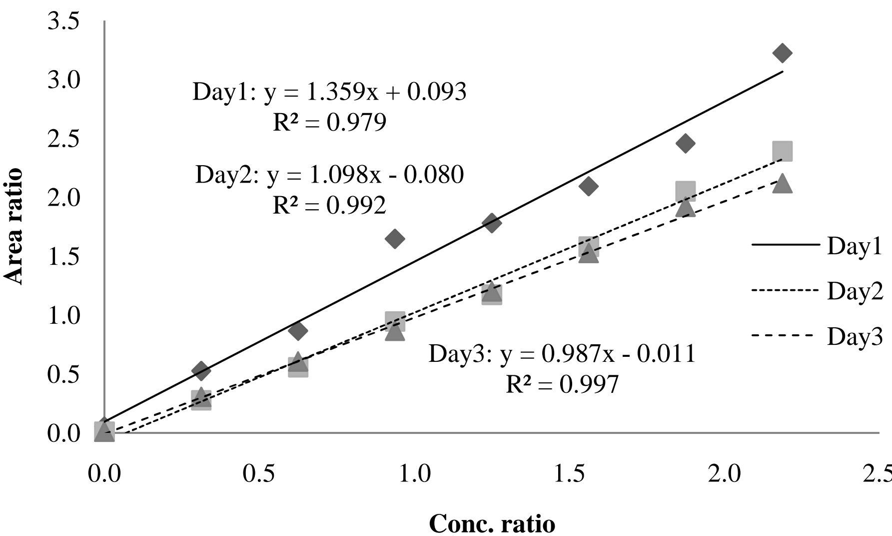

Figure D7 Calibration curve of FTD on three different days.

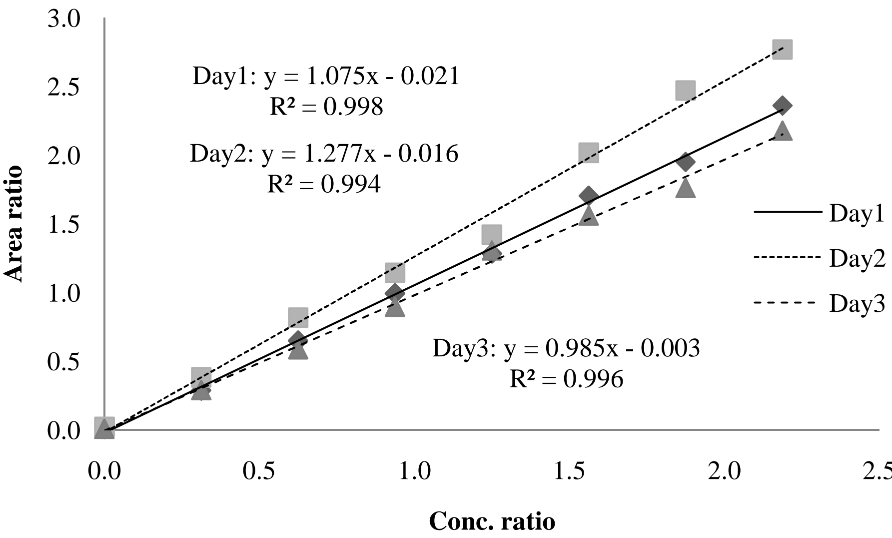

Figure D8 Calibration curve of CAP on three different days.

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