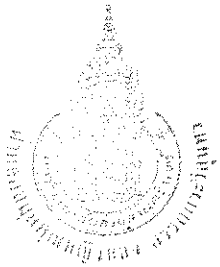


Development of Sample Preparation Technique for Trace Analysis of Oxolinic Acid in Animal Tissues

Opas Bunkoed

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Analytical Chemistry
Prince of Songkla University
2007

Thesis Title	Development of Sample Preparation Technique for Trace Analysis of Oxolinic Acid in Animal Tissues
Author	Mr. Opas Bunkoed
Major Program	Analytical Chemistry

Major Advisor

.....

(Assoc. Prof. Dr. Proespichaya Kanatharana)

Co-advisor

.....

(Assoc. Prof. Dr. Panote Thavarungkul)

Examining Committee:

.....Chairperson

(Assoc. Prof. Dr. Nongporn Towatana)

.....Committee

(Assoc. Prof. Dr. Proespichaya Kanatharana)

.....Committee

(Assoc. Prof. Dr. Panote Thavarungkul)

.....Committee

(Miss Jaruwan Limsajasakul)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Science Degree in Analytical Chemistry

.....

(Assoc. Prof. Dr. Krerkchai Thongnoo)

Dean of Graduate School

ชื่อวิทยานิพนธ์	การพัฒนาวิธีการเตรียมตัวอย่างสำหรับวิเคราะห์กรดออกโซลินิกปริมาณน้อยในเนื้อสัตว์
ผู้เขียน	นายโอภาส บุญเกิด
สาขาวิชา	เคมีวิเคราะห์
ปีการศึกษา	2550

บทคัดย่อ

ใช้เทคนิคลิควิดโครมาโทกราฟีสมรรถนะสูงร่วมกับตัวตรวจวัดฟลูออเรสเซนต์โดยใช้คอลัมน์ Pinnacle II C 18 ความยาว 250 มิลลิเมตร ขนาดเส้นผ่านศูนย์กลางภายใน 4.6 มิลลิเมตร และขนาดอนุภาคที่บรรจุในคอลัมน์ 5 ไมโครเมตร ในการวิเคราะห์กรดออกโซลินิกในเนื้อสัตว์ สภาวะที่เหมาะสมของระบบคือความยาวคลื่น excitation 265 นาโนเมตร และ emission 375 นาโนเมตร ตัวทำละลายเคลื่อนที่ พีเอส 3.5 ประกอบด้วย 45 เปอร์เซ็นต์ อะซิโตไนไตรน์ และ 55 เปอร์เซ็นต์ของสารละลายบัฟเฟอร์กรดออกซาลิก 10 มิลลิโมลาร์ อัตราการไหล 0.9 มิลลิลิตรต่อนาที และอุณหภูมิของคอลัมน์ 25 องศาเซลเซียส ภายใต้สภาวะดังกล่าวสามารถวิเคราะห์กรดออกโซลินิกภายใน 6 นาที การทดสอบความเหมาะสมของระบบให้ผลเป็นที่ยอมรับตาม ICH guideline โดยมีช่วงความเป็นเส้นตรง 1 นาโนกรัมต่อมิลลิลิตร ถึง 8 ไมโครกรัมต่อลิตร ค่าสัมประสิทธิ์ความเป็นเส้นตรง (coefficient of determination: R^2) มากกว่า 0.999 และมีขีดจำกัดของการตรวจวัด 1.0 นาโนกรัมต่อมิลลิลิตร

เตรียมตัวอย่างโดยการสกัดกรดออกโซลินิกในตัวอย่างเนื้อสัตว์ด้วยคลื่นเหนือเสียงและทำความสะอาดตัวอย่างโดยใช้ตัวดูดซับของแข็ง จากปัจจัยต่างๆ ที่มีผลต่อประสิทธิภาพการเตรียมตัวอย่าง พบว่าวิธีนี้มีข้อดีคือ ใช้ได้ง่าย สามารถลดระยะเวลาของการวิเคราะห์ และมีราคาประหยัด

ศึกษาปัจจัยเพื่อพิสูจน์การใช้ได้ของวิธีได้แก่ ความจำเพาะเจาะจง ช่วงความเป็นเส้นตรง ความเที่ยงตรง ความแม่นยำ ร้อยละของการได้กลับคืน ขีดจำกัดการตรวจวัด ขีดจำกัดการตรวจวัดเชิงปริมาณ ความเสถียร และความทนทานของวิธี พบว่าวิธีนี้มีความจำเพาะเจาะจงกับตัวอย่าง กุ้ง ปลา เนื้อไก่ ตับไก่ เนื้อหมู และเนื้อวัว มีช่วงความเป็นเส้นตรงตั้งแต่ 5 ถึง 500 นาโนกรัมต่อกรัม ความผิดพลาดสัมพัทธ์ของการทดลองภายในหนึ่งวันและระหว่างวันน้อยกว่า 10 เปอร์เซ็นต์ ค่าความเบี่ยงเบนมาตรฐานสัมพัทธ์ของการศึกษาภายในหนึ่งวันและระหว่างวันน้อยกว่า 8 และ 9

เปอร์เซ็นต์ตามลำดับ ร้อยละของการได้กลับคืนมากกว่า 85 เปอร์เซ็นต์สำหรับตัวอย่าง กุ้ง ปลา เนื้อไก่ ตับไก่ เนื้อหมู และมากกว่า 74 เปอร์เซ็นต์สำหรับเนื้อวัว ให้ค่าขีดจำกัดของการตรวจวัดและขีดจำกัดของการตรวจวัดเชิงปริมาณต่ำกว่าค่าสูงสุดที่มีการอนุญาตให้มีตกค้างได้ ซึ่งกำหนดโดยองค์การค้าสหภาพยุโรป จากการทดสอบพบว่ากรดออกโซลินิกมีความเสถียรในตัวอย่างทั้งในช่วงระยะเวลาสั้น สามรอบของการแช่แข็งและการละลาย ช่วงระยะเวลายาว และสารมาตรฐานมีความคงสภาพที่อุณหภูมิ 4 องศาเซลเซียส เป็นเวลาสามเดือนโดยมีการเปลี่ยนแปลงของสัญญาณน้อยกว่า 15 เปอร์เซ็นต์ การศึกษาความทนทานของวิธีพบว่าการเปลี่ยนแปลงสภาวะทางโครมาโทรกราฟีเล็กน้อยไม่ทำเกิดการเปลี่ยนแปลงคุณสมบัติทางโครมาโทรกราฟที่สำคัญ

การวิเคราะห์เชิงคุณภาพและเชิงปริมาณไม่พบกรดออกโซลินิกในตัวอย่างที่ซื้อจากตลาดสด วิธีนี้จะเป็นประโยชน์สำหรับตรวจสอบการตกค้างของกรดออกโซลินิกเมื่อมีการเกิดโรคระบาดในสัตว์และมีการใช้กรดออกโซลินิกในการรักษาและเหมาะสมสำหรับการวิเคราะห์กรดออกโซลินิกในเนื้อสัตว์ที่รับประทานได้

Thesis Title	Development of Sample Preparation Technique for Trace Analysis of Oxolinic Acid in Animal Tissues
Author	Mr. Opas Bunkoed
Major Program	Analytical Chemistry
Acadamic Year	2007

Abstract

High sensitive high performance liquid chromatography with fluorescence detection and a Pinnacle II C18, 250×4.6 mm, 5 μm column was used to determine oxolinic acid in animal tissues. Optimum conditions of HPLC system were excitation wavelength 265 nm, emission wavelength 375 nm, the mobile phase was a mixture of acetonitrile and 10 mM oxalic acid buffer (45:55, v/v, pH 3.5), flow rate 0.9 mL min^{-1} and column temperature 25 °C. Under the optimum conditions oxolinic acid was detected in less than 6 minutes. System suitability tests are acceptable within the ICH guideline and this system provided linear dynamic range of 1 ng mL^{-1} to 8 μg mL^{-1} with a coefficient of determination higher than 0.999 and a detection limit of 1.0 ng mL^{-1}.

Oxolinic acid in edible animal tissues was extracted by ultrasonic extraction and cleaned-up with solid-phase extraction (SPE). The influences of several parameters on the efficiency were investigated. The advantages of this method are reduced the extraction time, simple, and cost effective sample preparation.

The method was evaluated for various validation parameter *i.e.* selectivity, linearity, accuracy, precision, recovery, LOD, LOQ, stability and robustness. The method is selective for oxolinic acid in shrimp, fish, chicken meat, chicken liver, pork and beef. The proposed method was found to be linear in the range 5 to 500 ng g^{-1}. The inter-day and intra-day relative errors are less than 10 %. The intra-day relative standard deviations (RSD) were lower than 8% and lower than 9 % for inter-day. Recoveries of oxolinic acid were higher than 85% for shrimp, fish, chicken meat, chicken liver, pork and higher than 74 % for beef. The limits of detection and

quantification obtained are below the maximum residue level (MRL) established by the European Union. Oxolinic acid were stable in extracted sample when tested for short term, three freeze-thaw cycles, long term and standard solution was also stable at 4 °C for three months with <15% variation. Method robustness indicated that minor changes of the operational parameters do not lead to essential changes of the chromatographic.

Qualitative and quantitative analysis of oxolinic acid were not found in real samples that were purchased from fresh markets. This method would be useful for the oxolinic acid residues analysis or monitoring of this compound when there is an epidemic in animals that will require oxolinic acid treatment. In addition, this method was suitable for determination of oxolinic acid in animal tissues.

Acknowledgements

The completion of this thesis would be quite impossible without the help of many people, whom I would like to thank.

I express my sincere thanks to my advisors Associate Professor Dr. Proespichaya Kanatharana and Associate Professor Dr. Panote Thavarungkul for their valuable advice and suggestions throughout the course of this work.

I would also like to thank:

- The examination committee members of this thesis for their valuable time

- The Center for Innovation in Chemistry: Postgraduate Education and Research Program in Chemistry (PERCH-CIC), funded by the Royal Thai Government, for the scholarship and research support

- Staffs of the Chemistry Department for their help in some technical aspects of this thesis

- The Chemistry Department, Faculty of Science and Graduate School, Prince of Songkla University

- My parents, my sisters and my brothers for their love and understanding, encouragement and supports.

Finally, I would like to thank my friends in the Analytical & Environment Chemistry/Trace Analysis Research Unit and Biophysics Research Unit: Biosensors & Biocurrents who helped me in innumerable ways during these years.

Opas Bunkoed

The Relevance of This Research Work

Analysis of oxolinic acid residued in animal tissues is a Master of Science Thesis in Analytical Chemistry. This research provides some new knowledge on the analysis technique relates to food safety. Organizations that can be use the outcome of this work include

- Ministry of Public Health
- Ministry of Environment and Natural Resource
- Ministry of Education

Contents

	Page
CHAPTER 1: Introduction	1
1.1 Background and rationale	1
1.2 Quinolones	3
1.3 Oxolinic acid	8
1.4 Sample preparation	10
1.4.1 Liquid-Liquid extraction (LLE)	11
1.4.2 Microwave-assisted extraction (MAE)	13
1.4.3 Matrix solid phase dispersion (MSPD)	15
1.4.4 Accelerated solvent extraction (ASE)	18
1.4.5 Solid phase extraction (SPE)	19
1.4.6 Ultrasonic extraction	23
1.5 Analytical techniques for oxolinic acid	24
1.5.1 Gas chromatography (GC)	25
1.5.2 Capillary electrophoresis (CE)	25
1.5.3 High performance liquid chromatography (HPLC)	29
1.5.4 Other techniques	32
1.6 Method validation	34
1.6.1 Recovery	34
1.6.2 Limit of detection (LOD) and quantification (LOQ)	35
1.6.3 Calibration	35
1.7 Objectives	36
CHAPTER 2: Experimental	37
2.1 Chemicals and materials	37
2.1.1 Standard chemical	37

Contents (Continued)

	Page
2.1.2 Other chemicals	37
2.1.3 Solid phase extraction (SPE) materials	38
2.1.4 Samples	38
2.2 Instruments and apparatus	38
2.2.1 High performance liquid chromatograph	38
2.2.2 Apparatus	38
2.3 Analysis system	39
2.4 Standard solution	41
2.4.1 Oxolinic acid standard stock solutions	42
2.4.2 Oxolinic acid standard working solutions	42
2.5 Mobile phase preparation	42
2.6 Optimization of HPLC -FLD conditions	42
2.6.1 Excitation and emission wavelengths	43
2.6.2 Composition of mobile phase	44
2.6.2.1 Organic modifier	44
2.6.2.2 Percentage of organic modifier	44
2.6.2.3 pH of mobile phase	44
2.6.2.4 Buffer concentration	45
2.6.3 Mobile phase flow rate	45
2.6.4 Column temperature	45
2.7 System performance of HPLC-FLD	46
2.7.1 System Suitability test	46
2.7.1.1 Capacity factor (k')	47
2.7.1.2 Theoretical plates (N)	47
2.7.1.3 Peak asymmetry and Tailing factor (T)	48
2.7.1.4 Repeatability	49

Contents (Continued)

	Page
2.7.2 Linear dynamic range	49
2.7.3 Limit of detection	50
2.8 Sample preparation	50
2.8.1 Fortification of sample	50
2.8.2 Evaluation of spiking sample	50
2.8.3 Optimization of sample preparation	51
2.8.4 Optimization of ultrasonic extraction (USE)	52
2.8.4.1 Extraction solvent	52
2.8.4.2 Solvent volume	52
2.8.4.3 Extraction time	53
2.8.4.4 Stability of Oxolinic acid under ultrasonic condition	53
2.8.5 Optimization of solid phase extraction (SPE)	53
2.8.5.1 Preparation of solid phase extraction cartridges	54
2.8.5.2 Type of sorbent	55
2.8.5.3 Type of eluting solvent	55
2.8.5.4 Sample flow rate	56
2.8.5.5 Volume of eluting solvent	56
2.8.5.6 Flow rate of eluting solvent	56
2.8.6 Effect of defatting	56
2.9 Matrix mach calibration curve	58
2.10 Sampling	59
2.11 Method validation	60
2.11.1 Selectivity	61
2.11.2 Range and linearity	61
2.11.3 Accuracy	61

Contents (Continued)

	Page
2.11.4 Precision	62
2.11.5 Recovery	63
2.11.6 Limit of detection (LOD) and quantification (LOQ)	63
2.11.7 Stability	64
2.11.8 Robustness studies	66
2.12 Qualitative and quantitative analysis of oxolinic acid in edible animal tissues	67
2.12.1 Qualitative analysis	67
2.12.2 Quantitative analysis	67
2.12.2.1 Standard addition method	67
CHAPTER 3: Results and Discussion	69
3.1 Optimization of HPLC-FLD conditions	69
3.1.1 Excitation (λ_{ex}) and Emission (λ_{em}) wavelengths	69
3.1.2 Composition of mobile phase	71
3.1.2.1 Organic modifier	71
3.1.2.2 Percentage of organic modifier	73
3.1.2.3 pH of mobile phase	75
3.1.2.4 Buffer concentration	76
3.1.3 Flow rate	77
3.1.4 Temperature	82
3.2 System performance of HPCL-FLD	85
3.2.1 System suitability tests	85
3.2.2 Linear dynamic range	87
3.2.3 Limit of detection	89
3.3 Sample preparation	92
3.3.1 Evaluation of spiking of sample	92
3.3.2 Optimization of ultrasonic extraction	93

Contents (Continued)

	Page
3.3.2.1 Extraction solvent	93
3.3.2.2 Solvent volume	97
3.3.2.3 Extraction time	98
3.3.2.4 Stability of oxolinic acid under ultrasonic condition	100
3.3.3 Optimization of Solid phase extraction	101
3.3.3.1 Type of sorbent	101
3.3.3.2 Type of eluting solvent	103
3.3.3.3 Sample flow rate	106
3.3.3.4 Volume of eluting solvent	107
3.3.3.5 Flow rate of eluting solvent	109
3.3.3.6 Effect of Defatting	110
3.3.3.7 Matrix effects	112
3.4 Method validation	117
3.4.1 Selectivity	117
3.4.2 Range and linearity	122
3.4.3 Accuracy	123
3.4.4 Precision	123
3.4.5 Recovery	124
3.4.6 Limit of detection (LOD) and quantification (LOQ)	125
3.4.7 Stability	127
3.4.8 Robustness	130
3.5 Qualitative and quantitative analysis of oxolinic acid in edible animal tissues	132
3.5.1 Qualitative analysis	132
3.5.2 Quantitative analysis	132
CHAPTER 4: Conclusions	140

List of Tables

Table		Page
1.1	List of the structure of quinolone antimicrobials	6
1.2	Maximum residue limits (MRL) of oxolinic acid in various edible animal tissues	9
1.3	Main advantages and disadvantages of HPLC technique	32
1.4	Summary of determination of oxolinic by HPLC technique	33
2.1	HPLC-FLD starting operation conditions	43
2.2	Optimization of HPLC conditions	46
2.3	Peak asymmetry and peak tailing factor relationship	49
2.4	The starting operation conditions for the optimization of sample preparation	51
2.5	Optimization of sample preparation	57
2.6	The ICH, USP and FAD validation parameters	60
2.7	Robustness study	66
3.1	Chromatographic results of oxolinic acid using different organic modifiers	73
3.2	Chromatogrphic results of oxolinic acid at different percentage of acetonitrile	74
3.3	Effect of pH of mobile phase	76
3.4	Effect of oxalic acid buffer concentration	77
3.5	Retention time and plate height (*HETP*) of oxolinic acid at various mobile phase flow rate	80
3.6	Effect of column temperature	83
3.7	Optimum conditions of chromatographic conditions	84
3.8	System suitability tests	86

List of Tables (Continued)

Table		Page
3.9	RSD of retention time and peak area of oxolinic acid standard solution at 50 ng g^{-1}	87
3.10	Response of oxolinic acid at various concentrations	88
3.11	Limit of detection of oxolinic acid based on standard deviation of y-intercepts of regression line	90
3.12	Response of oxolinic acid at various prolong times	92
3.13	Polarity index and solubility in water of studied solvents	94
3.14	Effect of various extraction solvent on the response of oxolinic acid	94
3.15	Extraction efficiency of various solvent volumes on the response of oxolinic acid extraction	97
3.16	Extraction efficiency of various extraction times on the response of oxolinic acid extraction	99
3.17	Stability of oxolinic acid under ultrasonic condition at various sonication time	100
3.18	Response of oxolinic acid at each type of sorbent	102
3.19	SPE sorbents properties	103
3.20	Response of oxolinic acid at various 3% acidic eluting solvents	104
3.21	Response of oxolinic acid at various percentage of trifluoroacetic acid in eluting solvent	105
3.22	Response of oxolinic acid at various sample flow rate	106
3.23	Effect of volume of eluting solvent on the response of oxolinic acid	108
3.24	Effect of flow rate of eluting solvent on the response of oxolinic acid	109

List of Tables (Continued)

Table		Page
3.25	Recovery of oxolinic acid at different defatting procedure	111
3.26	Optimum conditions of sample preparation procedure	112
3.27	Response of standard oxolinic acid and spiked samples at various oxolinic acid concentrations	113
3.28	Results of statistical test using two-way ANOVA by R software	117
3.29	Parameters corresponding to linear regressions obtained from the calibration curves	122
3.30	Precision and accuracy of the analysis of oxolinic acid	124
3.31	Recovery of oxolinic acid from various samples	125
3.32	Limit of detection and quantification of oxolinic acid in various samples	127
3.33	Stability study	129
3.34	Result for robustness test study	131
3.35	The results of standard addition calibration curve of oxolinic acid in shrimp sample	133
3.36	The results of standard addition calibration curve of oxolinic acid in fish sample	134
3.37	The results of standard addition calibration curve of oxolinic acid in chicken meat sample	135
3.38	The results of standard addition calibration curve of oxolinic acid in chicken liver sample	136
3.39	The results of standard addition calibration curve of oxolinic acid in beef meat sample	137

List of Tables (Continued)

Table		Page
3.40	The results of standard addition calibration curve of oxolinic acid in pork sample	138
3.41	Oxolinic acid concentration edible animal tissue by standard addition method	139
4.1	System suitability test values	141
4.2	Optimum conditions of ultrasonic and solid phase extraction	142
4.3	Comparison of the proposed method with other method	145

List of Figures

Figure		Page
1.1	Source and pathways for the occurrence of quinolones in environment and animal	4
1.2	Basic structure of quinolones	5
1.3	Acid-base equilibria for the quinolones	7
1.4	Chemical structure of oxolinic acid	9
1.5	Principle of microwave assisted extraction systems	15
1.6	Step in a typical MSPD	17
1.7	Accelerated solvent extraction system	19
1.8	Solid phase extraction procedure	22
1.9	(A) Formats of solid-phase extraction, disks, cartridges, and syringe barrels (B) Relative difference in particle size between an SPE disk and a conventional SPE cartridge	23
1.10	Capillary electrophoresis systems	28
1.11	Fragmentation pathways of oxolinic acid by ion-trap CID	31
2.1	Sample preparation procedure to determine oxolinic acid consists of (A) extraction by ultrasonic technique and (B) clean-up by solid phase extraction	40
2.2	Schematic diagram of high performance liquid chromatography system	40
2.3	Optical diagram of fluorescence detector (FLD)	41
2.4	HPLC chromatogram for evaluation system suitability	48
2.5	Solid phase extraction system used for clean-up sample consists of pump, cartridges and manifold	53
2.6	Packed SPE cartridges	55
2.7	Analytical procedures for determination of oxolinic acid in edible animal tissues	59

List of Figures (Continued)

Figure		Page
2.8	The y-residuals of a regression line	64
2.9	The method of standard additions	68
3.1	3D plotted of oxolinic acid standard solution, 0.5 mg L^{-1}. (A) Scanning excitation wavelengths. (B) Scanning emission wavelength	70
3.2	Stationary phase of reverse phase high performance liquid chromatography	72
3.3	Retention time of oxolinic acid at various percentage of acetonitrile	74
3.4	A typical van Deemter plot	78
3.5	Contributions to molecular spreading in LC	79
3.6	van Deemter plot of oxolinic acid	81
3.7	Retention time of oxolinic acid at various flow rate	81
3.8	Capacity factor of oxolinic acid at various column temperatures	83
3.9	Chromatogram of oxolinic acid at 50 ng mL^{-1} under the optimum condition	84
3.10	Linear dynamic range of oxolinic acid by HPLC-FLD system	89
3.11	Calibration curve of oxolinic acid for calculation detection limit	91
3.12	Response of oxolinic acid at various waiting time before extraction	93
3.13	Responses of oxolinic acid at various extraction solvent	95
3.14	Chromatogram of oxolinic acid at various extraction solvents for evaluation of selectivity	96

List of Figures (Continued)

Figure		Page
3.15	Response of oxolinic acid at various solvents volume	98
3.16	Response of oxolinic acid at various extraction time	99
3.17	Recovery of oxolinic acid under the optimum condition of ultrasonic extraction at various sonication times	101
3.18	Response of oxolinic acid at each type of sorbent	102
3.19	Response of oxolinic acid at various eluting solvent	104
3.20	Response of oxolinic acid at different % trifluoroacetic acid	105
3.21	Response of oxolinic acid at different sample flow rate	107
3.22	Response of oxolinic acid at various volumes of eluting solvent	108
3.23	Response of oxolinic acid at various eluting solvent flow rates	110
3.24	Recovery of oxolinic acid at different defatting procedure	111
3.25	Matrix match calibration curve of oxolinic acid in shrimp	113
3.26	Matrix match calibration curve of oxolinic acid in fish	114
3.27	Matrix match calibration curve of oxolinic acid in chicken	114
3.28	Matrix match calibration curve of oxolinic acid in chicken liver	115
3.29	Matrix match calibration curve of oxolinic acid in beef	115
3.30	Matrix match calibration curve of oxolinic acid in pork	116
3.31	HPLC chromatogram (A) blank shrimp sample (B) standard oxolinic acid (C) spiked shrimp sample with oxolinic acid 5 ng g^{-1}	118

List of Figures (Continued)

Figure		Page
3.32	HPLC chromatogram (A) blank fish sample (B) standard oxolinic acid (C) spiked fish sample with oxolinic acid 5 ng g^{-1}	119
3.33	HPLC chromatogram (A) blank chicken meat sample (B) standard oxolinic acid (C) spiked chicken meat sample with oxolinic acid 5 ng g^{-1}	119
3.34	HPLC chromatogram (A) blank chicken liver sample (B) standard oxolinic acid (C) spiked chicken liver sample with oxolinic acid 5 ng g^{-1}	120
3.35	HPLC chromatogram (A) blank pork sample (B) standard oxolinic acid (C) spiked pork sample with oxolinic acid 5 ng g^{-1}	120
3.36	HPLC chromatogram (A) blank beef sample (B) standard oxolinic acid (C) spiked beef sample with oxolinic acid 5 ng g^{-1}	121
3.37	HPLC chromatogram (A) blank pig liver sample (B) standard oxolinic acid (C) spiked pig liver sample with oxolinic acid 5 ng g^{-1}	121
3.38	Chromatogram of oxolinic acid standard (A) and spiked shrimp sample (B) at the optimum condition	132
3.39	The standard addition calibration curve of oxolinic acid in shrimp sample	134
3.40	The standard addition calibration curve of oxolinic acid in fish sample	135

List of Figures (Continued)

Figure		Page
3.41	The standard addition calibration curve of oxolinic acid in chicken meat sample	136
3.42	The standard addition calibration curve of oxolinic acid in chicken liver sample	137
3.43	The standard addition calibration curve of oxolinic acid in beef sample	138
3.44	The standard addition calibration curve of oxolinic acid in pork sample	139

CHAPTER 1

Introduction

1.1 Background and Rationale

Antibiotics are widely used for preventing and treating several diseases in human and animals (Teuber, 2001; Nakata *et al.*, 2005), as well as for promoting growth in food-producing animals and in industrial farming (Hubert *et al.*, 2000; Di Corcia and Nazzari, 2002; Turiel *et al.*, 2003; Lee *et al.*, 2007). In 1999 the European Federation of Animal Health (FEDESA) estimates the consumption of approximately 4700 tons in veterinary medicine in the European Union and Switzerland (Martínez-Carballo *et al.*, 2007) and its use was increased to an estimated 6051 tons in 2004 (Kools *et al.*, 2007).

Veterinary drugs are administered to various animal species such as shrimp (Graslund *et al.*, 2003; Reed *et al.*, 2004; Zou *et al.*, 2005; Grobbel *et al.*, 2007), chicken (Yorke and Froc, 2000; Jafari *et al.*, 2007; Marchesini *et al.*, 2007; Schneider *et al.*, 2007), fin fish (Ueno *et al.*, 1999), calves (De Liguoro *et al.*, 2003; Berge *et al.*, 2005), pigs (Blackwell *et al.*, 2004; Toussaint *et al.*, 2005) and cows (Guerin-Faublee *et al.*, 2002; Ramirez *et al.*, 2003). The administration of veterinary drugs to animal have the potential to generate drug residues and accumulated in the animals and animal products (Schneider *et al.*, 2007). These residues represent a toxic/hazard potential for the consumer (Stolker and Brinkman, 2005) such as increased allergies (Toldrá and Reig, 2006). To protect human health the European Union (EU) has established the maximum residue limits (MRL) in edible animal tissues depending on the target tissues (Kowalski *et al.*, 2003; Granelli and Branzell, 2007).

Antibiotics such as β-lactams, tetracyclines, sulfonamides, macrolides, aminoglycosides and quinolones have been used as veterinary medicine (Stolker and Brinkman, 2005; Flammer, 2006; Hernandez *et al.*, 2007; Kemper, 2007). Several quinolones are currently widely used to treat various diseases in animals in many countries in the world (WHO, 1998). Oxolinic acid is one of quinolone antibiotic

agents which frequently used in veterinary drugs due to its broad antibacterial spectrum, high potency against Gram-negative bacteria (Pouliquen and Armand, 2000; Delépée *et al.*, 2004) and effectiveness in treating bacteria infections (Cinquina *et al.*, 2003). However, the use of oxolinic acid as veterinary drug can leave residues at trace levels in animal product which has lead to a significant increase in antimicrobial resistance (Tendencia and de la Peña, 2001; Vila, 2007) and have important effect on public health (van Vyncht *et al.*, 2002; Rigos *et al.*, 2004). Therefore, monitoring of these residues in edible animal tissues is necessary to ensure that human food is entirely free from potentially harmful elements (Roudaut and Yorke, 2002).

Analytical techniques include gas chromatography (GC)(Takatsuki, 1992), capillary electrophoresis (CE) (Hernandez *et al.*, 2000; Barrón *et al.*, 2003; Lara *et al.*, 2006) and high-performance liquid chromatography (HPLC) (Ramos *et al.*, 2003; Hermo *et al.*, 2006; Karbiwnyk *et al.*, 2007) have been reported for determination of oxolinic acid. However, GC coupled with mass spectrometric detector required derivatization step, which makes this technique more tedious and CE provided low sensitivity (Bailac *et al.*, 2006). Thus, HPLC coupled with fluorescence detection was used in this work because of its good sensitivity for oxolinic acid (Bailac *et al.*, 2004).

Analysis of oxolinic acid in tissue that has a complex matrix of naturally occurring compounds, *e.g.* lipids, carbohydrates, proteins and vitamins (Núñez *et al.*, 2005), needs an appropriate sample preparation method to reduce the matrix and achieve the optimum analytical result. Most of the analytical method for determination of oxolinic acid only dealt with one matrix or similar matrices using different extraction methods such as extraction of oxolinic acid from eggs (Hassouan *et al.*, 2007), chicken (Yorke and Froc, 2000) and shrimp (Karbiwnyk *et al.*, 2007) using liquid-liquid extraction (LLE), sediments and soils using microwave-assisted extraction (MAE) (Prat *et al.*, 2006) and animal feeds using accelerated solvent extraction (ASE) (Pecorelli *et al.*, 2003). However, the disadvantages of LLE are the need for large volumes of organic solvents, time consuming and demands several steps (Koesukwiwat *et al.*, 2007; Sanz and Martinez-Castro, 2007). Drawbacks of MAE and ASE are the high cost of equipment. This limitation can be reduced by ultrasonic extraction, which is simple, rapid and inexpensive because of no

specialized laboratory equipment is required (Lambropoulou *et al.*, 2006) and extractions can be done simultaneously (Rezić *et al.*, 2005; Tor *et al.*, 2006)

The aim of this work is to develop a simple, rapid and inexpensive sample preparation using ultrasonic for extraction and solid phase extraction (SPE) for clean-up that can be applied to determine oxolinic acid in various edible animal tissues (shrimp, fish, beef, pork, pig liver, chicken meat and chicken liver). The determination is performed using high sensitive reversed phase liquid chromatography with fluorescence detection. The developed method was also validated followed the guidelines of the US Food and Drug Administration (US-FDA), US phamacopeia (USP) and the International Conference Harmonisation (ICH).

1.2 Quinolones

Quinolones are a group of synthetic antibacterial agents widely used in human and veterinary medicine (Kennedy *et al.*, 1998). In 1998 WHO reported that quinolones production and usage is estimated to be about 50 tonnes for proprietary products (mainly USA, European Union, Japan, South Korea) because of their lower prices (WHO, 1998). For the latter they were used for treatment of pulmonary, urinary and digestive infections (Hoof *et al.*, 2005). Their primary target is the bacterial enzyme DNA gyrase or topoisomerase IV, (Hooper, 1999) which renders the DNA molecule compact and biologically active (Li, 2005). Occurrence potential of quinolones residue in environment and food is show in Figure 1.1. Its can enter the environmental mainly as a result of urinary, fecal excretion and of farming industry disposals (Andreu *et al.*, 2007), as well as of aquaculture treatments and the direct discharge of aquaculture products (Prat *et al.*, 2006). After administration of quinolones to animals, some were absorbed in the animal tissues and excreted to the environment (Hirsch *et al.*, 1999).

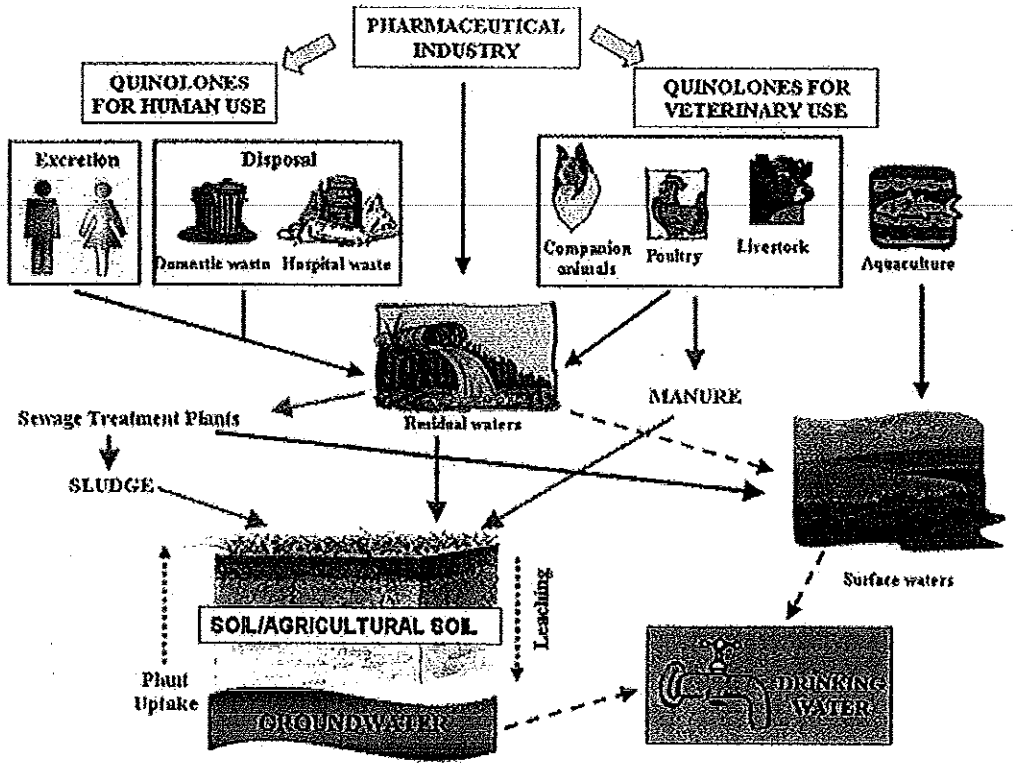


Figure 1.1 Source and pathways for the occurrence of quinolones in environment and animal (Andreu *et al.*, 2007).

Quinolones are nitrogen-containing, eight-membered heterocyclic aromatic compounds with a ketone group at position 4 and a carboxylic group at position 3 (Figure 1.2). A ketone and carboxylic groups at these positions are necessary for DNA gyrase inhibition (Park *et al.*, 2002). The main nucleus usually contains one nitrogen atom (quinolines), but analogues have additional nitrogens at position 2 (cinolines), position 8 (naphthyridines) or positions 6 and 8 (pyridopyrimidines). Several structural modifications (Table 1.1) have enhanced their biological and pharmacological activities. These modifications include the introduction of alkyl or aryl groups at position 1 and fluoro and piperazinyl substitute-ions at positions 6 and 7, respectively. The fluoro group at position 6, which originates fluoroquinolones, widens the spectrum of activity against both gram-negative and gram-positive pathogens (Espinosa-Mansilla *et al.*, 2005). The introduction of a piperazinyl group at position 7 improves activity against *Pseudo-monas aeruginosa* (Joshi, 2002). The

carboxylic group at position 3 makes these compounds acidic (Stolker and Brinkman, 2005). In addition, the 7-piper azinylquinolones include additional amine groups, which are basic. Therefore, in aqueous solution, the 7-piperazinyl quinolones show three different species, which are cationic, zwitterionic and anionic (Stolker and Brinkman, 2005), while the other quinolones can only be neutral or anionic. The existing equilibria for these two types of quinolones in the physiological pH range are shown in Figure 1.3. Only one p*K*a value is referred to as acidic quinolones and two p*Ka* values are called piperazinyl quinolones (Lin *et al.*, 2004). The reported values of p*Ka* for acidic quinolone range from 6.0 to 6.9 (Prat *et al.*, 2006), whereas in the case of 7-piperazinyl quinolones, the proposed values in the ranges 5.5-6.6 and 7.2-8.9 for pKa_1 and pKa_2, respectively (Brighty *et al.*, 2000).

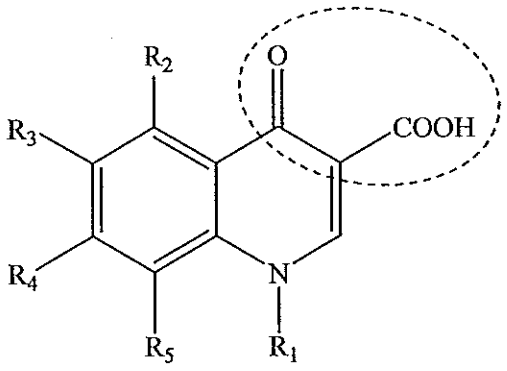


Figure 1.2 Basic structure of quinolone

Table 1.1 List of the structure of quinolone antimicrobials (Belal *et al.*, 1999)

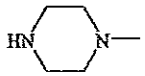
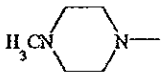
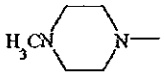
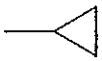
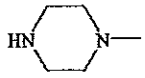
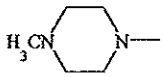
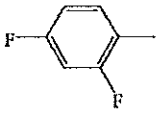
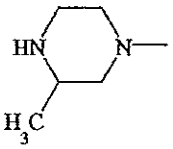
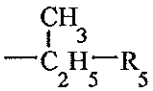
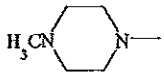
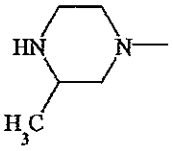
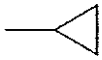
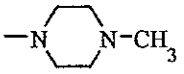
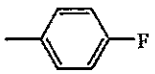
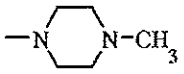
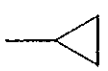
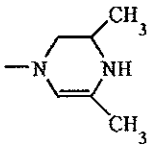
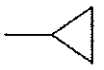
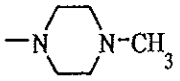
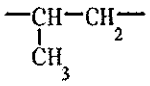
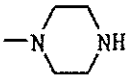
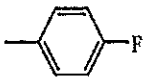
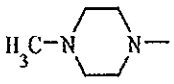
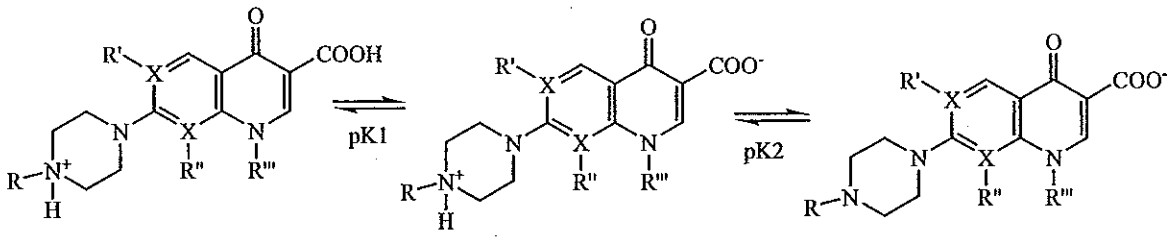
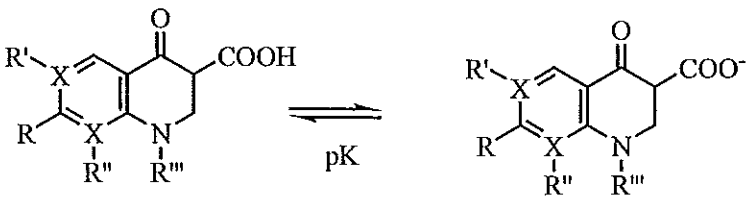
Name	R_1	R_2	R_3	R_4	R_5
Oxolinic acid	$-C_2H_5$	H	-O-	CH_2-O-	H
Norfloxacin	$-C_2H_5$	H	F		H
Pefloxacin	$-C_2H_5$	H	F		H
Amifloxacin	$-NHCH_3$	H	F		H
Ciprofloxacin		H	F		H
Fleroxacin	$-C_2H_5F$	H	F		F
Temafloxacin		H	F		H
Ofloxacin		H	F		O-
Lomefloxacin	$-C_2H_5$	H	F		F
Danofloxacin		H	F		H
Difloxacin		H	F		H
Sparfloxacin		$-NH_2$	F		F

Table 1.1 (Continued)

Name	R_1	R_2	R_3	R_4	R_5
Enrofloxacin		H	F		H
Sarafloxacin		H	F		H
Miloxacin	$-OCH_3$	H	-O-	$-CH_2-O-$	H
Flumequine		H	F	H	$-CH_2-$
Rofloxacin	$-CH_3-CH_2-$	H	F		-S-



7-Piperazinyl quinolone



Acid quinolone

Figure 1.3 Acid-base equilibria for the quinolones

1.3 Oxolinic acid

Chemical formula	: $C_{13}H_{11}NO_5$
Molecular weight	: 261.23
Synonyms	: Dioxacin; Starner; Gramurin; 5, 8-Dihydro-5-ethyl-8-oxo-1, 3-dioxolo [4, 5-g] quinoline-7-carboxylic acid
Classification	: Fluoroquinolones
Physical properties	: white crystalline powder
Melting point	: > 300 °C
Solubility in water	: insoluble

Oxolinic acid (Figure 1.4) is a quinolone antibacterial agents which operates by inhibiting bacterial DNA gyrase activity and producing bacterial death (Saad *et al.*, 2002). It has been widely used as a veterinary drug in food producing animals (Prat *et al.*, 2006), applied in livestock, poultry, shrimp and fish production to prevent or treat infections and, sometimes, as growth promoter. Oxolinic acid is administered by the oral route, in feed, drinking water or as a bolus. The recommended doses are 12 mg kg^{-1} for fin fish (Saad *et al.*, 2002) and 20 mg kg^{-1} for pigs and poultry (EMEA, 1998). It was quickly absorbed after oral administration in all target species and distributed extensively in the tissue (Ramos *et al.*, 2003). It is also reported that residues may persist in fish many days post dosing (US-FDA, 2004). Bioavailability after an oral dose of 10 mg kg^{-1} was approximately 82% in healthy chickens but around 100 % in diseased chickens. Oral bioavailability was also high in pig and calves (EMEA, 1998).

The presence of oxolinic acid residues can be a potential hazard for consumer and increase drug resistant bacteria (Naviner *et al.*, 2007). In human, therapeutic dose levels of oxolinic acid have been reported to induce psychopharmacological effects such as nervous excitation, stereotyped behaviour and insomnia. Oxolinic acid inhibits cytochrome P4501 A2 activity resulting in reduced metabolism of co-administered xenobiotics (Li, 2005). Therefore, several control authorities such as the European Union (EU) has set maximum residue limits (MRL) that allow only trace amounts of oxolinic acid in edible animal tissues (Table 1.2).

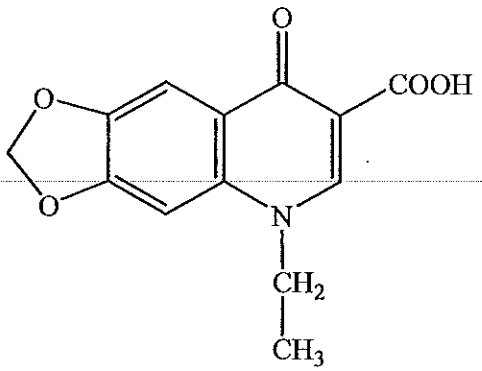


Figure 1.4 Chemical structure of oxolinic acid

Table 1.2 Maximum residue limits (MRL) of oxolinic acid in various edible animal tissues (EMEA, 1998)

Sample	**MRL (μg kg^{-1})**
Chicken	
Meat	100
Fat, skin + fat	50
Eggs	50
Liver, kidney	150
Beef, pork	
Meat	100
Fat, skin + fat	50
Liver, kidney	150
Fish	
Meat	300

1.4 Sample preparation

The basic concept of a sample preparation method is to convert a real matrix into a sample in a format that is suitable for analysis by a separation or other analytical technique (Smith, 2003). Food samples cover a wide range of physical types from dry powders to biological matrices, such as meat, fats and liquids or solutions that makes it hard to isolate and determine analytes of interest (Buldini *et al.*, 2002). Determination of oxolinic acid in complex matrices often requires appropriate sample preparation prior to instrumental analysis (Pouliquen *et al.*, 1997). The step of sample preparation needed depends on the sample matrix and level of analyte to be determined (Smith, 2003). The typical steps within sample preparation include sampling/homogenisation, extraction, clean up and followed by the final analysis (Leitner *et al.*, 2001; Bailac *et al.*, 2004; Huebra *et al.*, 2007). Another step that can be included at several points is derivatisation. This step was used for determination of oxolinic acid when analysis with gas chromatographic technique (Pfenning *et al.*, 1996)

In sample preparation there is often a need to minimise the number of steps of procedure (Berrueta *et al.*, 2001) to reduce time, sources of error, uncertainty and enhance sensitivity (Demeestere *et al.*, 2007). The enhanced sensitivity (less noise) in the detection step and reduction of interfering compounds facilitates identification and confirmation, which are particularly important when investigating the presence or absence of low concentrations of a contaminant in complex sample matrices, such as food (Lõhmus and Kender, 2007).

The selective extractions of analytes are based on the differences in their chemical and physical properties (Li *et al.*, 2002). These typically include molecular weight, charge, solubility (hydrophobicity), polarity, or differences in volatility (Sanz and Martinez-Castro, 2007).

Particle size can also be an important parameter for reproducible results as the extent to which the matrix is broken up can influence the extraction rates. Solid samples can usually be prepared by cutting to small pieces and grind or after drying, followed by solvent or liquid extraction. The analyte is desorbed from the matrix and dissolved into a solvent or fluid. Extraction of an analyte is therefore influenced by

solubility, penetration of the sample by the solvent (mass transfer) and matrix effects. After most of these extraction methods the analytes of interest are obtained in an organic or aqueous solution, which then requires concentration or additional clean-up (Ito *et al.*, 1999; Blackwell *et al.*, 2004; Benito-Pena *et al.*, 2006; Koesukwiwat *et al.*, 2007; O'Connor and Aga, 2007). These extract solutions can then be treated as a liquid samples. However, some samples, such as animal tissue and similarly moist or wet solids, can cause crucial problems. Drying before extraction may not always be practical because of the bulk of the sample, as in meat due to the presence of high proportions of fats (Lõhmus and Kender, 2007), or due to the cells collapsing and hence retaining the analytes. Tissue matrices in particular cause problems, as they tend to clump preventing penetration by the extraction media. However, in some case, in order to obtain the low limits of detection required for trace level analysis, relatively large sample sizes are often required. Larger sample size results in the need for more efficient clean-up techniques and therefore possibly higher costs (Ridgway *et al.*, 2007).

Sample preparation methods include liquid-liquid extraction (LLE), Microwave-assisted extraction (MAE), Matrix solid phase dispersion (MSPD), Accelerated solvent extraction (ASE) and Solid phase extraction (SPE) are employed for oxolinic acid extraction and clean up the samples.

1.4.1 Liquid-liquid extraction (LLE)

Liquid-liquid extraction is based on the relative solubility (polarity) of an analyte in two immiscible phases and is governed by the equilibrium distribution/partition coefficient. LLE is traditionally one of the most common methods of extraction (Hendriks *et al.*, 2007; Hernández-Borges *et al.*, 2007; Palma *et al.*, 2007), particularly for organic compounds from aqueous matrices. LLE can effectively remove proteinaceous components from biological fluids (Ramos *et al.*, 1999). Typically a separatory funnel is used and the two immiscible phases are mixed by shaking and then allowed to separate.

Extraction of oxolinic acid from biological matrices by LLE has been reported by several approaches which include water-immiscible organic solvents,

water miscible organic solvents or acidic and basic solutions (Andreu *et al.*, 2007). Dichloromethane was used as water-immiscible organic solvent to extract oxolinic acid from chicken meat (Bailac *et al.*, 2004) where recovery of 94% was obtained from the spiked sample. The different condition for extraction of oxolinic acid with water-miscible organic solvent (*e.g.* acetonitrile) was also reported (Romero-González *et al.*, 2007). Recoveries were obtained in the range of 66.5-79.5 % for spike samples at 25-100 μg kg^{-1} (Yorke and Froc, 2000; Romero-González *et al.*, 2007).

The major disadvantage of LLE is the need of a large volumes of organic solvents, some of these solvents are hazardous and toxic (Rodriguez *et al.*, 2000). This method is also time-consuming because it requires lengthy solvent-evaporation steps (van der Hoff and van Zoonen, 1999; Ahmed, 2001; Gilar *et al.*, 2001; Ebrahimzadeh *et al.*, 2007; Nagaraju and Huang, 2007) making it difficult to apply the method to large batches of samples. LLE can also be considered as a high risk contamination method, more laborious than other extraction/purification methods, requires expensive glassware (Mitra, 2003) and distillation or evaporation apparatus (Li *et al.*, 2002). It also has limited selectivity, particularly for trace level analysis, there is a need for clean-up or analyte enrichment/concentration steps prior to instrumental analysis (Pyrzynska, 2007).

In many cases, the sample and solvent combination and shaking lead to the formation of emulsions (Xia *et al.*, 2006), which further complicates the efficiency of extraction and adds greatly to the time required for the analyst to complete the protocol. To avoid emulsions, in some cases, salt may be added and centrifugation can be used if necessary. Alternatively matrix solid phase dispersion (MSPD) approach can be used to avoid emulsions (Barker, 2000; Fernandez *et al.*, 2000; Chu *et al.*, 2005). To ensure the complete extraction of an analyte into the required phase, repeat extractions may be necessary (de Fatima Alpendurada, 2000).

1.4.2 Microwave-assisted extraction (MAE)

Microwave-assisted extraction (MAE) is a process of using microwave energy to heat solvents in contact with a sample in order to partition analytes from the sample matrix into the solvent (Sparr Eskilsson and Bjorklund, 2000; Diaz-Cruz and Barcelo, 2007). The partitioning depends on the temperature and the nature of the extractant (Camel, 2000). Microwave-assisted extraction can be used to enhance the extraction efficiency of solvent extractions. MAE has good extraction efficiency, using less solvent and shorter extraction times (Deng *et al.*, 2007; Singh *et al.*, 2007). It was also considered as extraction method because a wide spectrum of compounds can be extracted. However, it is only applied to the thermally stable compounds due to increasing in temperature during extraction. Since the non-polar solvents do not absorb microwave energy, at least some polar solvent, such as water, must be used (Diaz-Cruz and Barcelo, 2007).

The principle of MAE systems is shown in Figure 1.5. When a high dielectric constant solvent is used, the sample is kept in a closed PTFE vessel. The technique resembles pressurized liquid extraction (PFE) because microwaves heat the solvent far above its atmospheric pressure boiling point and the analytes are rapidly extracted from the sample (Sparr Eskilsson and Bjorklund, 2000). In the case of low dielectric constant solvent, the sample can be extracted in an open PTFE vessel with microwaves heating the portion of the sample which has higher dielectric components than the solvent. The advantages of microwave pretreatment are simplicity, low cost of operate, high extraction rate, complete automation and the possibility of simultaneously extracting different samples at the same time without interferences (Rostagno *et al.*, 2007). Integrated microwave extractions allow food to be dried, extracted and concentrated with a single piece of equipment and no sample manipulation.

This technique is widely used in environmental and food analysis (Pena *et al.*, 2006; Barriada-Pereira *et al.*, 2007; Careri *et al.*, 2007; Silvia Díaz-Cruz and Barceló, 2007; Singh *et al.*, 2007). Usually, sample sizes range from 0.5 to 10.0 g and 10 ml of solvent are sufficient for the extraction that may require from less than 1 to 10 min (Cheng *et al.*, 2007). The technique was reported for the determination of oxolinic

acid residues in soils and sediments (Prat *et al.*, 2006). Although, the strong interactions between oxolinic acid and soils or sediments make the analytes difficult to extraction. MAE can improved the speed and efficiency of the extraction process (Labbozzetta *et al.*, 2005). This method provide the absolute recovery rates for the whole process range from 79% to 94% (RSD 3-7%), and detection limits are in the low $\mu g\ kg^{-1}$ level. MAE has been compared to mechanical shaking. The results found that mechanical shaking gave lower absolute recoveries than MAE (Prat *et al.*, 2006)

For the determination of oxolinic acid in food. Hermo and coworker (2005) reported the use of microwave extraction technique for the determination of oxolinic acid residues in pork. The effect of the microwaves power (from 20 to 60%) on the recovery of oxolinic acid was studied there is no clear influence of the microwaves power. They chose a middle power (40%) in order to avoid interferences from the matrix that could be extracted at high energy. LOD and LOQ obtained from MAE were lower than obtained classical extraction (Hermo *et al.*, 2005).

The main drawbacks of MAE are loss of more volatile solutes if the temperature inside the vessel rises rapidly and the vessels need to be cooled to room temperature after extraction and before they can be opened, thus increasing the overall extraction time (Camel, 2000; Ahmed, 2003).

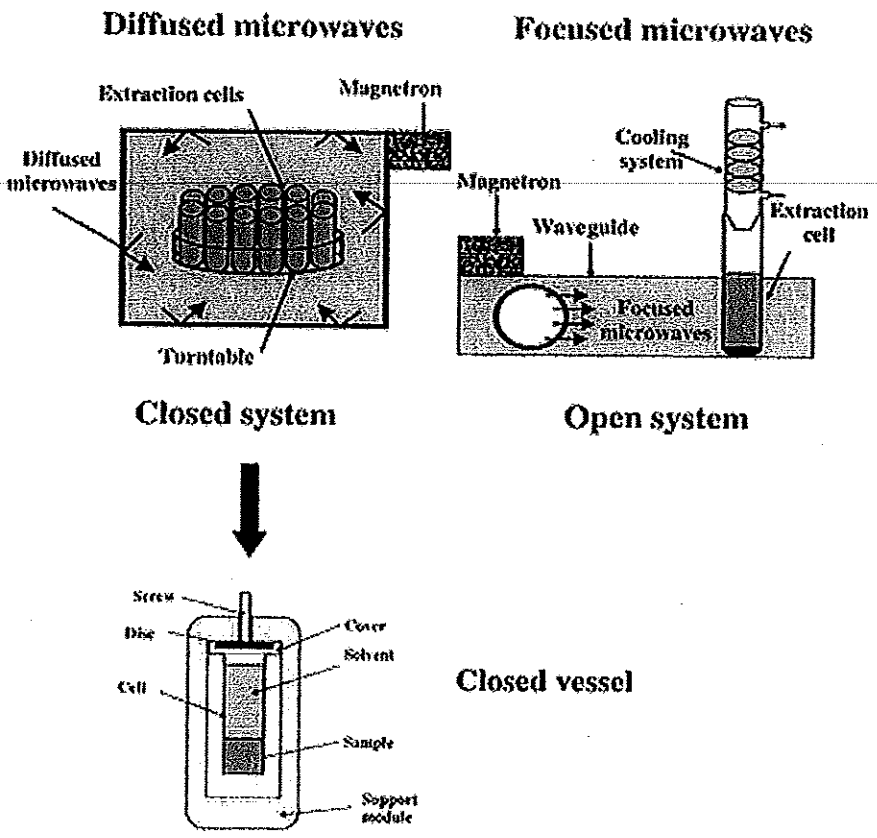


Figure 1.5 Principle of microwave assisted extraction systems (Ahmed, 2003)

1.4.3 Matrix solid phase dispersion (MSPD)

Matrix solid phase dispersion (MSPD) process involves blending a viscous, solid or semi-solid sample with a solid support (Ziakova *et al.*, 2003) such as C18, C8 bonded silica (Elizabeth Horne, 1998; Blasco *et al.*, 2002; Soler *et al.*, 2005; Arribas *et al.*, 2007), graphite carbon black (Shao *et al.*, 2007), Florisil (Bogusz *et al.*, 2004; Hu *et al.*, 2005; Pensado *et al.*, 2005), sodium sulphate (Pensado *et al.*, 2005), aluminium oxide (Kishida and Furusawa, 2001) or silica gel (Zou *et al.*, 2005). The blending used a glass pestle to obtain complete disruption and dispersion of the sample on the solid support. When blending is complete, the sample is then transferred and packed into a suitable column, followed by washing and elution with a small volume of solvent (Kristenson *et al.*, 2006; Dopico-García *et al.*, 2007) (Figure 1.6). MSPD provides both a porous structure to enable the solvent to penetrate the

matrix and extract the analytes, but also has some functionality which can retain the fat/lipids

Several factors that have been examined for their effect in conducting MSPD extractions include

(1) The effect of average particle size diameter. As expected, very small particle sizes (3-10 μm) lead to extended solvent elution times and the need for excessive pressures or vacuum to obtain adequate flow (Barker, 2007).

(2) The character of the bonded-phase or type of solid supports. Depending on the polarity of the phase chosen (Blesa *et al.*, 2003; Lehotay, 2004).

(3) The best ratio of sample to solid support material. The most often applied is 1 to 4 (Xiao *et al.*, 2004), 150mg: 600mg (Teixeira and Costa, 2005), 0.4g: 1.6g (Garcinuno *et al.*, 2004). This ratio is dependent on the application and must be examined as a major variable during method development.

(4) The optimum choice of elution solvents and the sequence of their application to a column. Elution solvent sequence attempts to isolate the analyte or further clean the column of interfering substances with each solvent step. MSPD columns permit isolation of different polarity analytes or entire chemical classes of compounds in a single solvent or in differing polarity solvents passed through the column (Albero *et al.*, 2003; Ramil Criado *et al.*, 2004)

(5) The elution volume. It is very for each application and should be examined to reduce the use of solvent and the unintended co-elution of potential interferences (Barker, 2007; Bogialli and Di Corcia, 2007).

The method has been applied to the isolation of drugs in different food samples (Valenzuela *et al.*, 1999; Barker, 2000; Loveland *et al.*, 2001; Zhang *et al.*, 2005). A porous structure, such as diatomaceous earth (Extrelut) can be used as co-sorbent. A layer of co-sorbent can be packed in the bottom of the column as a further clean-up procedure to enable a more selective extraction (Ferrer *et al.*, 2005). Although some MSPD extracts are clean enough for direct instrumental analysis (Barker, 2007), a further clean-up step is often required, particularly with fatty matrices.

Jarboe and Kleinow (1992) developed MSPD method for detecting oxolinic acid and oxolinic acid-related metabolites in catfish tissue and bile. Mean percent recovery, correlation coefficient, and inter-and intra-assay variabilities were 82.8%, 0.996, 12.5%, and 1.22%, respectively (Jarboe and Kleinow, 1992).

This technique uses less solvent than liquid-liquid extraction and can eliminate the need for multiple extractions (Kristenson *et al.*, 2001). However, the recoveries were sometimes low and variable (Kishida and Furusawa, 2001).

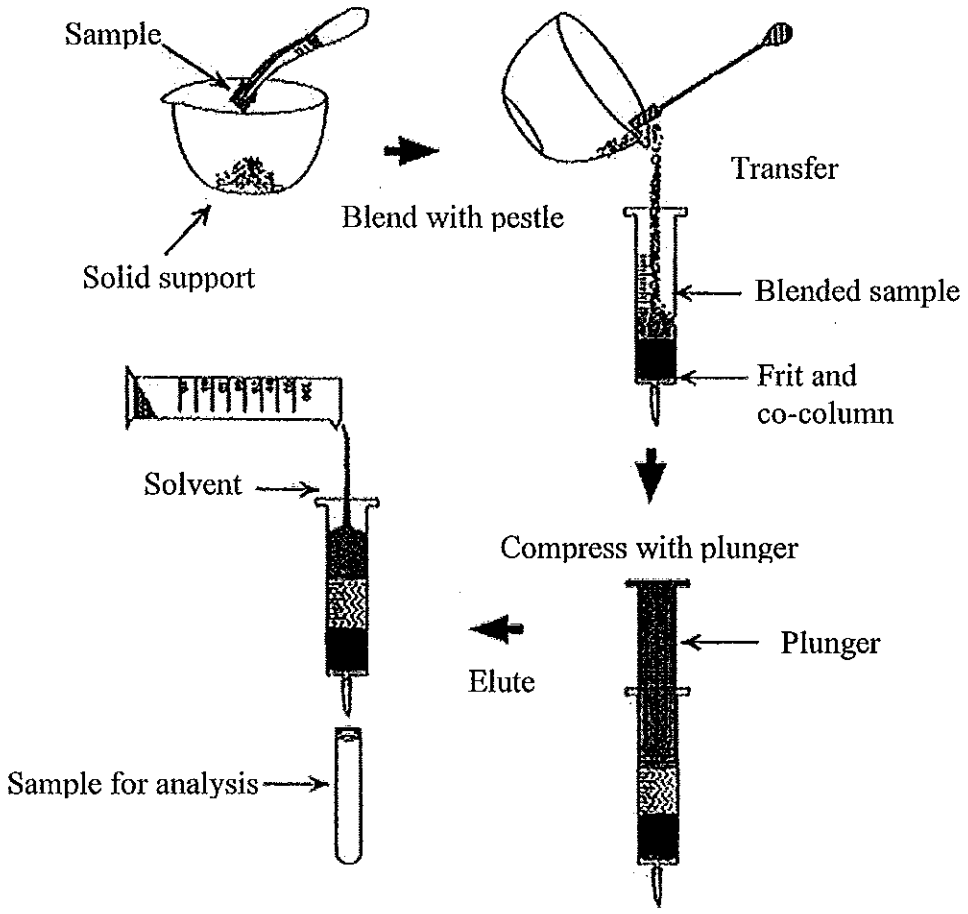


Figure 1.6 Step in a typical MSPD (Barker, 2007)

1.4.4 Accelerated solvent extraction (ASE)

Accelerated solvent extraction (ASE) is also known as pressurized liquid extraction (PLE) (Ahmed, 2003). It was used for environmental and food applications to replace microwave extraction (Hubert *et al.*, 2000; Urraca *et al.*, 2004). It uses liquid solvents at elevated pressures and temperatures (up to 200 bars and 200°C respectively), leading to faster extractions (owing to increased solubilities, better desorptions, and enhanced diffusion) (Camel, 2000; Ahmed, 2003; Li *et al.*, 2003).

The basic experimental set-up utilizing the Dionex ASE 200 instrument is shown in Figure 1.7. The system comprises a stainless-steel extraction cell, where programmed temperature and pressure are controlled by electronic heaters and pumps. By pressuring the sample cell, it is possible to keep the organic solvent in a liquid phase. Extraction steps involve the following sequential steps: loading the sample into the extraction cell; filling the cell with an organic solvent; heating and pressurizing the cell to set-up values (pre-extraction step); transferring the solvent to the collection vial and cleaning the sample with fresh solvent; and, purging the solvent residue from the sample to the collection vial using a suitable gas (Björklund *et al.*, 2000)

Pecorelli and coworker (2003) used ASE method in the determination of oxolinic acid and other quinolones in feeds. Samples were extracted by a metaphosphoric acid/acetonitrile mixture at pH 2.6 and automatically purified onto OASIS HLB cartridges. The procedure was also validated by spiking a feed sample at different levels and linearity, detection limit, quantification limit, accuracy and precision were checked. The automated preparation of the sample permits a very fast analysis which is an important goal for routine purposes (Pecorelli *et al.*, 2003).

Drawbacks of this method include the high cost of equipment, elevated temperatures that may degrade thermolabile analytes and possible blockage through concentration of matrix materials (Ahmed, 2003).

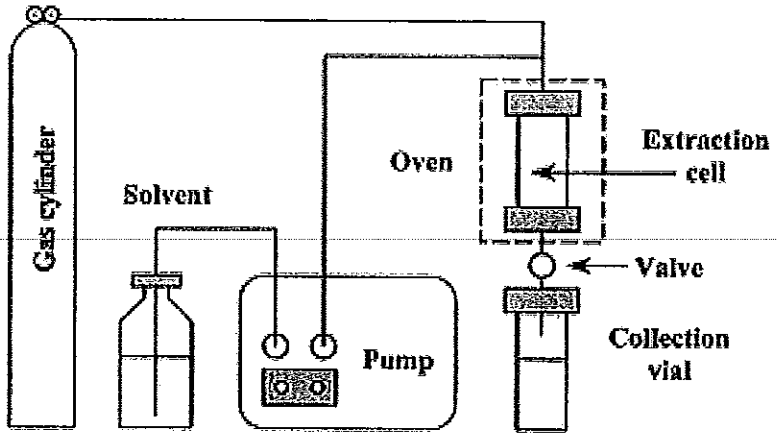


Figure 1.7 Accelerated solvent extraction system (Ahmed, 2003).

1.4.5 Solid phase extraction (SPE)

Solid phase extraction has been developed as an alternative to LLE for the separation, purification, concentration and/or solvent exchange of solutes for solutions (Ahmed, 2001; Sanz and Martinez-Castro, 2007). SPE which involves the use of suitable sorbent to trap analytes and separate them from the bulk of the matrix (Castro *et al.*, 2000; Wells and Yu, 2000). As the sample solution passes through the activated sorbent bed, analytes concentrate on its surface, while the other sample components pass through the bed. The equilibrium between analyte and sorbent is rapidly reached because of the large interface (Boyce, 2006). Many types of sorbent, such as alumina, magnesium silicate and graphitised carbon (Hennion, 2000), various polymeric beds, and ion-exchange media are commercially available (Pico *et al.*, 2000), but the most common material is silica because it is reactive enough to permit its surface to be modified by chemical reaction and yet stable enough to allow its use with a wide range of solutions (Hennion, 1999; Sanz and Martínez-Castro, 2007).

Usually, the particles size of the sorbent is ranged from 50 to 60 μm (Poole, 2003). The materials are similar to that used in liquid chromatography (except for particle size) (Liska, 2000). Sorbents fall into three general classes: non-polar, polar and ion-exchange and their activity is dependent on the properties of the bonded phase (León-González and Pérez-Arribas 2000). The choice of the sorbent is dependent on the food matrix, analytes of interest and their interferences (Ridgway *et al.*, 2007).

Solid phase extraction is performed in four steps (Figure 1.8)

(1) Conditioning; the functional groups of the sorbent bed are solvated in order to activate them for interaction with the sample. Before extraction of analytes, the sorbent bed must be prepared so that it will make intimate and effective surface contact with the liquid sample solution. Most commonly, conditioning is accomplished by passing a small volume of methanol or acetonitrile through the SPE extraction tube or disk (Blasco *et al.*, 2007; Granelli and Branzell, 2007; Horie *et al.*, 2007; Li *et al.*, 2007). Some of this organic solvent is adsorbed on the surface of the sorbent particles, making the surface more hydrophilic and thus more compatible with a primarily aqueous sample solution. Without such treatment the surface of many common sorbents is hydrophobic and is poorly wetted by the hydrophilic sample solution. The polar liquid flows in small channels through the solid phase without making the necessary close surface contact. The conditioning step also serves to elute any adsorbed organic impurities from the SPE bed (Fritz and Macka, 2000).

(2) Retention; the analytes are bound to the bed surface. Typical interactions are hydrophobic (van der Waals forces), polar (hydrogen bonding and dipole-dipole forces) or ion exchange interactions (Masqué *et al.*, 1998; León-González and Pérez-Arribas 2000). The sample may be applied to the SPE system by gravity, pumping, aspirated by vacuum or by an automated system (Camel, 2003).

(3) Rinsing the sorbent bed to remove unwanted materials. (Fritz and Macka, 2000). Undesirable material chemically similar to the analyte may be preferentially removed by washing the sorbent bed with diluted solution of the elution solvent.

(4) Elution of the retained compound; the analytes are desorbed and collected for analysis using a solvent that disrupts the analyte-sorbent interactions.

Method development in SPE is accomplished by investigating pH, ionic strength, polarity and flow rate of the elution solvent and physico-chemical characteristics of the sorbent bed (Pichon, 2000; Camel, 2003). Current SPE is designed in various formats depending on application such disks, cartridges and syring barrels (Hennion, 1999). Figure 1.9 (A) shows the differences types of SPE formats and (B) Relative difference in particle size between an SPE disk and a conventional SPE cartridge. SPE disks differ from SPE cartridges or syringes in that

the disk is a membrane loaded with a solid sorbent, whereas the cartridge or syringe contains the sorbent (Thurman and Snavely, 2000).

Turiel and coworker (2003) evaluated four different SPE sorbent (C18, styrenedivinylbenzene (SDB), C_{18}-cation-exchange and SDB-cation-exchange) for the preconcentration of oxolinic acid in water. The best results were obtained using the C_{18} and SDB-RPS. The recoveries and limits of detection obtained proved the suitability of this procedure for the control of oxolinic acid in surface water (Turiel *et al.*, 2003).

For the study of quinolones (oxolinic acid and other) in chicken samples. Bailac and coworker (2004) chose Oasis HLB, Oasis MAX and SDB-RPS sorbents. Oasis-HLB is a hydrophilic-lipophilic balanced (HLB) copolymer of *N*-vinylpyrrolidone and divinylbenzene, Oasis Max is a mixed-mode polymeric sorbent with strong anion-exchange quaternary amine groups in the surface of the copolymer, and SDB-RPS Empore cartridges are formed by a poly (styrene divinylbenzene) copolymer sorbent that displays slight cation-exchange interactions due to sulphonic groups. Recovery values obtained using any of the three sorbents were high for oxolinic acid. According to these results, the three cartridges could be used to extract oxolinic acid from chicken tissues (Bailac *et al.*, 2004).

Compared to the classic liquid-liquid extraction using a separatory funnel, SPE offers several advantages: practicallity it is much more convenient with easier manipulations; samples can be processed quickly (Fritz and Macka, 2000), reducing laboratory time; there is less of a requirement for the use of expensive, breakable specialty glassware; solvent usage is greatly reduced, no foaming or emulsion problems, decreasing exposure of analysts to these hazardous chemicals and eliminating the problematic disposal of large quantities of organic (Kataoka, 2003; Boyce, 2006) and improved sensitivity and repeatability (Ahmed, 2003).

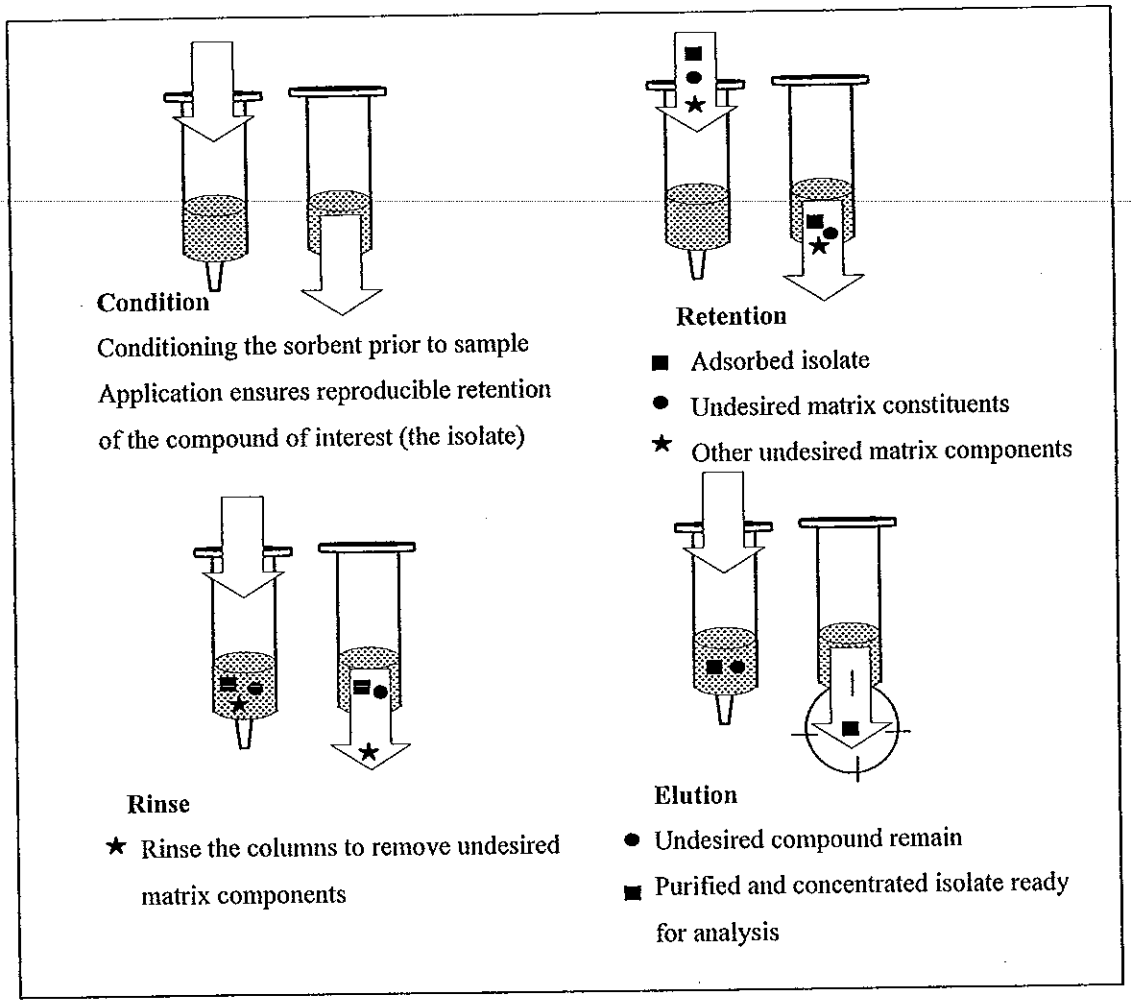


Figure 1.8 Solid phase extraction procedure

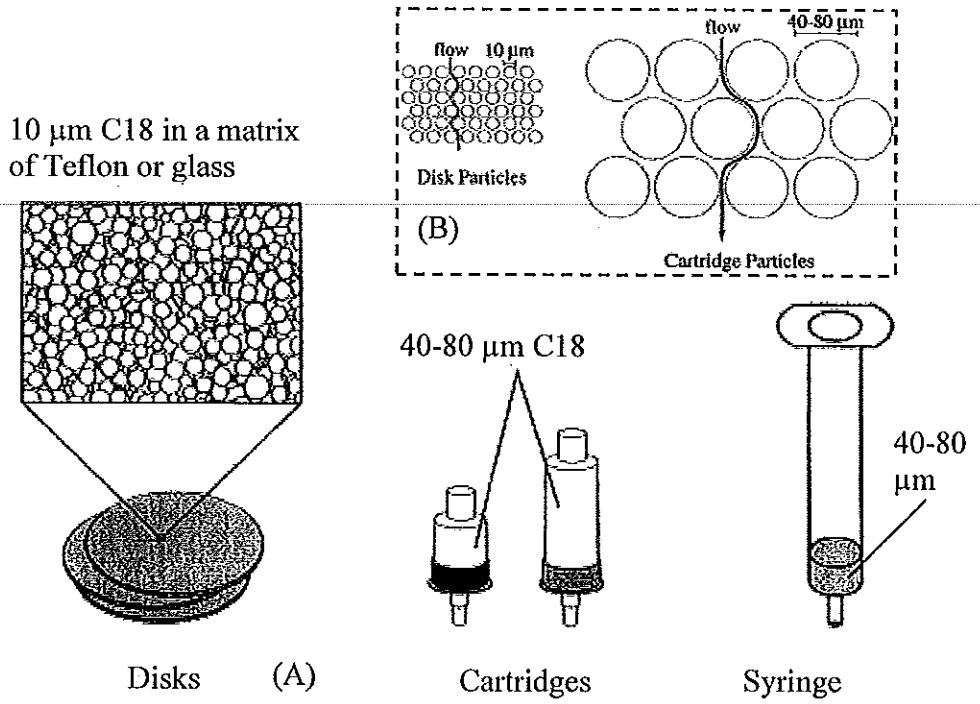


Figure 1.9 (A) Formats of solid-phase extraction, disks, cartridges, and syringe barrels (B) Relative difference in particle size between an SPE disk and a conventional SPE cartridge (Thurman and Snavely, 1999)

1.4.6 Ultrasonic extraction

Ultrasonic extraction uses mechanical energy in the form of a shearing action, which is produced by a low frequency sound wave (<150Hz) (LeBlanc, 2001) to agitate the sample that immersed in organic solvent (Rezić *et al.*, 2005). The mechanical effects of ultrasound induce a greater penetration of solvent into the sample and improve mass transfer. Therefore, an effective mass transfer is leading to the enhancement of extraction with ultrasonic power (Wang *et al.*, 2006).

In ultrasonic extraction, the sample is placed in a suitable glass container and enough solvent added to cover the sample and placed in an ultrasonic bath (Ahmed, 2003). The sample is then sonicated using the sonic bath. The efficiency of extraction depends on the polarity of the solvent, the homogeneity of the matrix and ultrasonication time (Ahmed, 2003; Zuo *et al.*, 2004; Tor *et al.*, 2006; Wang *et al.*, 2006). The mixture of sample and organic solvent is separated by filtration and

washing with the solvent. After extraction, the solvent containing the analyte can be separated by centrifugation or filtration and fresh solvent added (Ramos *et al.*, 2003). The combination of state-of-the-art separation and detection techniques good performance can be obtained, especially by coupling its performance with solid phase extraction (Wu *et al.*, 2006).

Ultrasonic extraction (USE) has been proved to be an expeditious, inexpensive (Blackwell *et al.*, 2004; Goncalves and Alpendurada, 2005) and efficient alternative to conventional extraction such as liquid-liquid and soxhlet extraction (Li *et al.*, 2002) This technique has been successfully applied in both liquid and solid samples for the determination of different group of analytes (Lambropoulou and Albanis, 2003) *i.e.* inorganic and organic compounds (Rezić *et al.*, 2007). USE has the potential to fulfill the requirements of a sensitive and reliable method. It is a very versatile technique due to the possibility of selecting the solvent type or solvent mixture that allows the maximum extraction efficiency and selectivity (Wu *et al.*, 2006; Ridgway *et al.*, 2007). The primary advantage of this method is the fact that several extractions can be done simultaneously and no specialized laboratory equipment is required.

1.5 Analytical techniques for oxolinic acid

Several techniques have been reported for the determination of oxolinic acid in various samples which include gas chromatography, capillary electrophoresis, high-performance liquid chromatography (HPLC) and non-chromatography method such as phosphorimetric sensor.

1.5.1 Gas chromatography (GC)

Since oxolinic acid is quite a polar compound, volatile derivatives must be obtained prior to GC analysis (Hernandez-Arteseros *et al.*, 2002). Gas chromatographic technique for determination of oxolonic acid in fish was described by Takatsumki and coworkers (1991). The method involved extraction of oxolinic acid from the homogenized sample, reduced with $NaBH_4$ and analysed by GC on a fused silica column of DB-S with H_2 as a carrier gas, the concentration range was 0.2–20 ng (Belle *et al.*, 1999). Determination of oxolinic acids in catfish, salmon and shrimps by GC-MS after decarboxylation was also investigated (Pfenning *et al.*, 1996) *i.e.* four diagnostic ions were monitored for oxonilic acid. In all cases, detection was carried out by MS in positive ion mode and signal monitoring was performed in SIM mode (Hernandez-Arteseros *et al.*, 2002).

Although determination of oxolinic acid by GC is possible it involves derivatization of the sample extract making the analytical methods based on this technique more tedious (Ramos *et al.*, 2003), increases the overall analysis time and may lead to errors to analytical technique.

1.5.2 Capillary electrophoresis (CE)

Capillary electrophoresis is a family of related separation techniques that use narrow-bore fused-silica capillaries to separate a complex array of large and small molecules (Fierens *et al.*, 2000; Altria and Elder, 2004; Anastos *et al.*, 2005). The fused silica capillaries are typically 25mm-75 μm I.D. and 50-100 cm in length (Anastos *et al.*, 2005; Perez *et al.*, 2007). High electric field strengths are used to separate molecules based on differences in charge, size and hydrophobicity, resulting in different migration velocities (Anastos *et al.*, 2005; Monton and Terabe, 2006). Sample introduction is accomplished by immersing the end of the capillary into a sample vial and applying pressure, vacuum or voltage. Depending on the types of capillary and electrolytes used, electro-osmotic flow can ensure that both negatively and positively charged species migrate towards the same end of the capillary, where under typical conditions, is towards the cathode end, with neutral species not being

separated and migrating with the electro-osmotic flow (Tagliaro *et al.*, 1996). The CE technique can be classified into several separation techniques include capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF), isotachophoresis (ITP), electrokinetic chromatography (EKC), micellar electrokinetic capillary chromatography (MECC or MEKC), micro emulsion electrokinetic chromatography (MEEKC) and capillary electrochromatography (CEC) (Li, 1993; Petersen *et al.*, 2003; Anastos *et al.*, 2005). A basic schematic of a capillary electrophoresis system is shown in Figure 1.10.

Capillary electrophoresis has been used to analyse food (Kowalski *et al.*, 2003), forensic (Fattorini *et al.*, 2004; Anastos *et al.*, 2005; Cruces-Blanco *et al.*, 2007) pharmaceutical (Wang and Chang, 1998; Faria *et al.*, 2006), environmental (Loos and Niessner, 1999; Chicharro *et al.*, 2005) and biological samples (Hernández *et al.*, 2000) because it offers high separation efficiency, high resolution, high speed, small sample volume requirements and low reagent consumption (Pacáková and Stulík, 1997; Petersen *et al.*, 2003; Ha *et al.*, 2006; Timerbaev *et al.*, 2006; Saavedra and Barbas, 2007; Santalad *et al.*, 2007). However, biological samples contain several interference and proteinaceous components, which can make them difficult to analyse. It is therefore necessary to develop sample-pre-treatment systems to remove interference, proteins and particulate matter (Boone *et al.*, 2001; Simonet *et al.*, 2003; Svec, 2006 ;Puig *et al.*, 2007).

The most widely used detection method in capillary electrophoresis is on-column UV detection because of its simplicity and flexibility (Simonet *et al.*, 2003; Curiel *et al.*, 2007; Injac *et al.*, 2007; Santos *et al.*, 2007), which involves burning off a section of the polyimide coating of the capillary to form an optical window (Tagliaro *et al.*, 1998). However, the short internal diameter of the capillary (detection path length) limits the sensitivity of this detection system, which are 1-2 orders of magnitudes less than that found in HPLC. Several approaches have been adopted to improve the sensitivity in capillary electrophoresis by increasing the path length or inner diameter of the capillary, solute derivatisation, pre-concentration techniques, electrokinetic injection, modified capillary dimensions and use of preconcentrators. Other modes of detection include laser-induced fluorescence (LIF) (Moller *et al.*, 1998; Male and Luong, 2001) , amperometric (Hu *et al.*, 2000; Zhou *et al.*, 2001) and

mass spectrometry (McCourt *et al.*, 2003) are more sensitive detection methods, and can be employed for improvement of the detection limit in CE (Picó *et al.*, 2003; Hernandez-Borges *et al.*, 2007). LIF system was used to determine various quinolones in human-plasma samples and obtained limits of quantitation (LOQ) of 2.5μg L^{-1} (Moller *et al.*, 1998).

A capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MEKC) method for the separation of oxolinic acid and other quinolone in plasma sample was developed by Hernandez and Coworker (2002). A 75 μm × 85 cm uncoated fused-silica capillary was used, with a run buffer consisting of 40 mM sodium tetraborate (pH 8.1) containing 10% (v/v) methanol. Separations were carried out within 13 min, with an applied voltage of 30 kV at 30 °C, with UV detection at 260 nm. A linear relationship was obtained in the concentration range 5-20 mg L^{-1} and detection limits were between 1.1 and 2.4 mg L^{-1}(Hernandez *et al.*, 2000).

Barrón and Coworker (2003) developed capillary electrophoresis system for determine oxolinic acid in chicken tissue, with diode array detection. The analytes were extracted using dichlorometane and pre-concentrated by solid phase extraction (SPE). The recoveries obtained were 94 %. The detection and quantification limits achieved were 15 and 48 μg kg^{-1} respectively. The sensitivity of the method proposed allows the determination of these drugs at a residue level far below their maximum residue limit (MRL) established by the European Union (EU) (Barrón *et al.*, 2003).

Oxolinic acid was also determined in feeds and fish meats samples by CE using UV detection at 254 nm. The buffer solution consisted of 10 mM phosphate at pH 9.00 and methanol (9:1) was found to be the most suitable for separation (Saad *et al.*, 2002).

Lara and coworker (2006) have developed and validated capillary zone electrophoresis-tandem mass spectrometry (CZE-MS/MS) for the identification and simultaneous quantification of oxolinic acid and other quinolones in beef raw milk. Different parameters (*i.e.*, separation buffer composition and electrospray conditions) were optimized in order to obtain both an adequate CE separation and a high sensitivity. The limits of detection and quantification (below 6 and 24 ppb,

respectively) were lower than the maximum residues limits tolerated for these compounds in milk, the recoveries ranging from 81 to 110% (Lara *et al.*, 2006).

The major disadvantage of CE is its low concentration sensitivity (Barrón *et al.*, 2003), usually lower than high performance liquid chromatography (HPLC) (Simonet *et al.*, 2003) and limited for application to the analysis of drug residues in foods (Saad *et al.*, 2002).

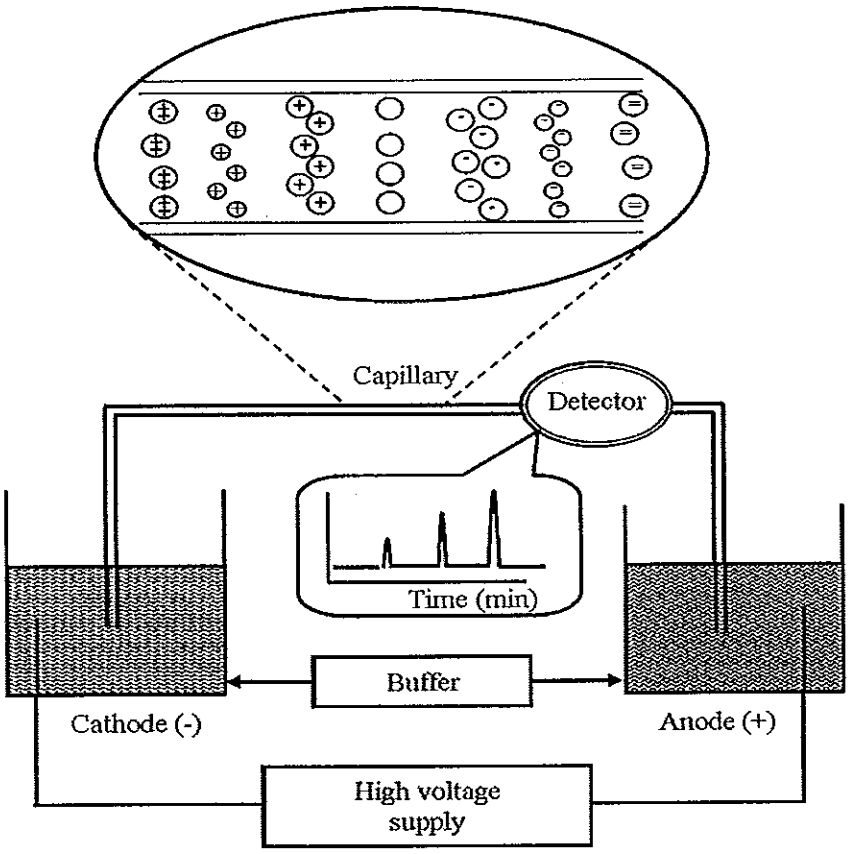


Figure 1.10 Capillary electrophoresis systems

1.5.3 High performance liquid chromatography (HPLC)

HPLC is a separation technique. The separation of oxolinic acid is usually performed with silica-based reversed phased column, mainly C_{18} (Ramos *et al.*, 2003; Turiel *et al.*, 2006; Rubies *et al.*, 2007) or C_8 (Bailac *et al.*, 2006; Hermo *et al.*, 2006; Hassouan *et al.*, 2007; Karbiwnyk *et al.*, 2007) and in some cases C_5 (Pecorelli *et al.*, 2003). Due to the residual silanol groups and metal impurities in column packing materials, conventional reversed-phase column lead to severely tailing peaks (Maraschiello *et al.*, 2001). Therefore, most methods used end capped columns or high purity silica column which are free of trace metals responsible for the strengthening of the acidic properties of silanol groups (Delépée *et al.*, 2002).

Many different HPLC conditions for determination of oxolinic acid are described in the literature, especially concerning the mobile phase, with variations in ionic strength, acidity, or adding modifiers such as citric acid, perchloric acid or tertiary amine salts (Ramos *et al.*, 2003). Mobile phases containing acetonitrile, tetrahydrofuran and methanol are applied almost exclusively to the determination of oxolinic acid, especially when a polymeric column is used. Several tail-reducing agents are added to these mobile phases to improve peak shape. The pH was kept in the range 2-4 in order to reduce silanol ionisation and minimise its interaction with oxolinic acid, which are present as cationic species (Touraki *et al.*, 2001; Delépée *et al.*, 2002; Prat *et al.*, 2006).

Ability of HPLC technique to detect compounds depends on the type of detector used. The choice of the detection system is very important for selectivity and sensitivity (Toldrá and Reig, 2006). Several spectroscopic techniques, such as ultraviolet-visible (UV) absorption (Ueno *et al.*, 1999; Touraki *et al.*, 2001), fluorescence (Pouliquen *et al.*, 2000; Maraschiello *et al.*, 2001; Hassouan *et al.*, 2007) or mass spectrometry (Johnston *et al.*, 2002) are used for oxolinic acid detection. The most used detector of the HPLC determinations is the fluorimetric (Prat *et al.*, 2004). The emission spectrum consists of a wide band centre at 350-400 nm. Fluorescence signal depends strongly on the pH of the medium. Thus, the anionic species do not generally show native fluorescence, whereas the highest fluorescence is obtained at

low pH (from 2.5 to 4.5). At these pH values, neutral species prevail for quinolone (Hernandez-Arteseros *et al.*, 2002).

Determination of oxolinic acid using liquid chromatography coupling with mass spectrometry (LC-MS) was studied and also compared the advantage with ultraviolet (LC-UV) detector (Bailac *et al.*, 2006; Graces *et al.*, 2006). LC-MS offers advantages over other detectors, such as selectivity, identification purposes and possibility of elucidation structures (Chambers *et al.*, 2007). However, The LC-UV method offers lower cost. In both cases, oxolinic acid could be detected at concentration levels below the MRL, although LC-MS allows the determination of a lower concentration of oxolinic acid than does LC-UV, due to the high sensitivity of the technique. Hermo and coworker (2006) also developed method for determination of oxolinic acid in pork and compared between liquid chromatography with ultraviolet detection (LC-UV), liquid chromatography-mass spectrometry (LC–MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). When MS detection was used, the selectivity of the technique allows the multiresidue determination of quinolones in complex matrixes (Toussaint *et al.*, 2002). The LOD and LOQ of all methods are much lower than the MRL fixed by European Union. The use of MS allows the determination of quinolones below of MRL/3 while the use of MS–MS detection allows the determination of residues of the series of quinolones studied below of the MRL/30.

Atmospherically pressure chemical ionization (APCI) has also been introduced for LC-MS and LC-MS/MS with in source collision-induced dissociation (CID). Mass spectrum obtained at low cone CID voltage consists of $[MH]^+$and $[MH+CO_2]^+$, and higher cone voltages, a peak corresponding to $[MH-H_2O]^+$ also appears. APCI-MS/MS was used to determine product ions from the $[MH]^+$ peak following CID in the gas cell. Loss of H_2O was first transition observed followed by loss of $CH_2=CH_2$ (Hernandez-Arteseros *et al.*, 2002). Li and coworker (2006) proposed fragmentation pathways of oxolinic acid by ion-trap CID is shown in Figure 1.11 and obtained the fragmentation as reported by Hernandez-Arteseros and coworker (Li *et al.*, 2006).

One limitation associated with LC-MS analysis is its susceptibility to matrix effect (Buhrman *et al.*, 1996; Fu *et al.*, 1998; King *et al.*, 2000; Dams *et al.*, 2003). Matrix effect is defined as the effect of co-eluting residual matrix components on the ionization of the target analyte. Typically, suppression or enhancement of analyte response is accompanied by diminished precision and accuracy of subsequent measurements (Mei *et al.*, 2002). In the trace level analysis, matrix effects can be highly variable and can be difficult to control or predict (Petrovic *et al.*, 2006).

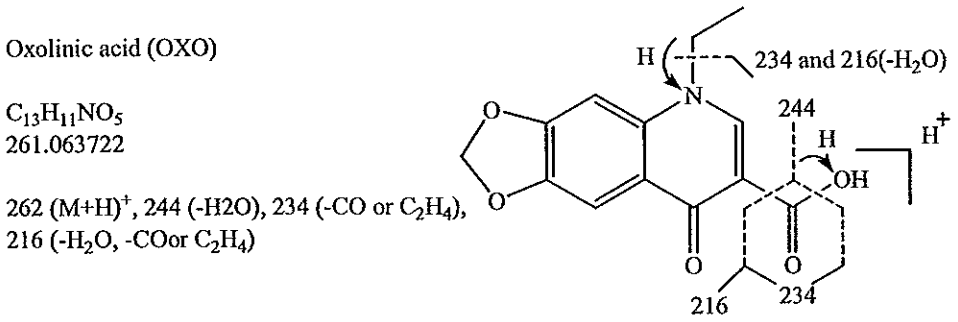


Figure 1.11 Fragmentation pathways of oxolinic acid by ion-trap CID (Le *et al.*, 2006)

HPLC is getting expanded use in control laboratories due to the possibility to analyse simultaneously multiple residues in a sample in relatively short time. Developments of high speed HPLC can reduce sample treatment and analysis time. In addition, this technology is fully automated (injection, elution, washing of column, detection) and computer-controlled. The main advantages and disadvantages are compiled in Table 1.3 (Toldrá and Reig, 2006) and Table 1.4 summarized the determination of oxolinic acid by HPLC technique with various detector.

Table 1.3 Main advantages and disadvantages of HPLC technique

Advantages	Disadvantages
• Short time (few min/sample) to obtain the results	• Expertise required
• Sensitive	• Cost of column
• Specificity depending on detector	• High initial investment (equipment)
• Automatisation leading to higher productivity	• Need of sample preparation (extraction and filtration, addition of internal standard, *etc.*)
• Possibility to find more information from spectra when using diode array detector	

1.5.4 Other techniques

Other non-chromatographic methods for the determination of oxolinic acid are based on phosphorimetric sensor. Capitán-Vallvey and coworkers (2003) proposed a single-use phosphorimetric sensor for determination of oxolinic acid in cow milk and human urine. It was formed by used a rectangular strip of Mylar type polyester as solid support that contained a circular sensing zone, formed by PVC plasticized with tributylphosphate adhered to its surface. When the strip was introduced for 1 hour into a sample solution, the analyte was retained in the sensing zone, making it possible to directly measure the phosphrorescence intensity emitted by the oxolinic acid in the solid phase, at λ excitation 330 nm and λ emission 449 nm. The method's detection limit was 0.01 mg L^{-1}. This method was simple and inexpensive but still has disadvantage relatively long response time when the oxolinic acid concentration is low (Capitán-Vallvey *et al.*, 2003).

Table 1.4 Summary of determination of oxolinic acid in various samples by HPLC technique

Mobile phase	Column	Detector	Sample	Sample treatment	Recovery (%)	Detection Limit (ppb)	Author
Acetonitrile : 0.02 M orthophosphoric acid	LiChroSpher 100 RP-18E (4.6×125 mm)	UV (262 nm)	Shell	(1) Ex. 0.013 M Methanolic oxalic acid (pH 1.4)	72.9	12	Pouliquen *et al.*, 1997
Acetonitrile : 0.02 M phosphoric acid (pH 2.2)	PLRP-S (4.6×150)	FLD	Fish	(1) Ex. Acetonitrile basic solution	67.7-71.0	5	Roudaut and York., 2002
Acetonitrile : citric buffer (pH4.5)	Zorbax Eclipse XDB-C8 (4.6×150 mm)	UV-DAD (250 nm)	Chicken tissues	(1) Ex. DCM (2) Ex. NaOH (3) SPE	87	10	Bailac *et al.*, 2004
Acetonitrile : 0.01 M citric acid (pH 4.5)	Zorbax Eclipse XDB-C8 (4.6×150 mm)	UV-DAD (250 nm)	Pork	(1) Ex. Microwave (2) SPE	85	6	Hermo *et al.*, 2005
Acetonitrile : Oxalic acid buffer (pH 4); (35:65)	Inertsil C8 (4.6×250 mm)	FLD λex 263 nm λem 370 nm	Sediments and soils	(1) Ex. Microwave (2) Ex. NaOH	82-89	3	Prat *et al.*, 2006
0.02 M ammonium acetate: MeCN (86:14,v/v) adjusted to pH 2.5 with formic acid	Zorbax Eclipse XDB-C8 (4.6×150 mm)	MS	Pig plasma	(1) SPE	101.7	8	Garces *et al.*, 2006

1.6 Method validation

The parameters for method validation have been defined in different working groups of national and international committee such as US Food and Drug Administration (US-FDA), United States Pharmacopeia (USP) and the International Conference on Harmonization (ICH). Validation of analytical methods for determination of oxolinic acid in various sample were reported only with some parameters. Normally includes recovery, limit of detection and quantification and calibration.

1.6.1 Recovery

Recovery studies are essential to evaluate the accuracy of analytical methologies (Shabir, 2003; Nadarassan *et al.*, 2007). The best approach for determining recoveries is the analysis of reference material for the matrices and analytes concerned but no reference material for oxolinic acid in edible tissues is currently available. Therefore, recoveries are estimated by mean of surrogates that are assumed to match the behaviour of native analyte in the extraction procedure. However, there is a risk that spiked samples lead to an overestimation of the extraction efficiency (Hernández-Arteseros *et al.*, 2002).

Many of the literature reported the used of spiked samples for evaluated recovery, but information about spiking procedures and spiking levels is either too brief or non-existent (Toussaint *et al.*, 2005; Garcés *et al.*, 2006). Typical spiking volumes range from 10 to 500 µL (Johnston *et al.*, 2002; Barrón *et al.*, 2003; Turiel *et al.*, 2006). Some literature reported information about the equilibration step, which is carried out by leaving the sample to stand for time ranging from several minutes to overnight (Prat *et al.*, 2006). In some case, agitation of sample in contact with the spike for 30 min at room temperature is usually performed prior to leaving the sample overnight at 4 °C (Rubies *et al.*, 2007).

Some studies on the effect of storage of spiked samples on recoveries are reported. Oxolinic acid has been found to be stable in spiked crops and oyster stored at -20 °C over several months (Pouliquen *et al.*, 1994). Oxolinic acid is stable in

salmon meat stored at -20 °C at least for a week. However, when it is stored at 4 °C, recovery decreases after only 24 h (Hernández-Arteseros *et al.*, 2000). The most advisable option of spiking at two or more levels has been often reported and no differences in extraction recoveries were observed at different levels. Because of the great variety of extraction and clean-up procedures, it is not easy to conclusions about recoveries. In general, recoveries are over 60% for most of the analytes and matrices (Yorke and Froc, 2000; Bailac *et al.*, 2004; Beltrán *et al.*, 2004)

1.6.2 Limit of detection (LOD) and quantification (LOQ)

Almost all the published methods report the limit of detection (LOD) and the limit of quantification (LOQ). However, these limits are not calculated in the same way and often authors do not report how LOD was determined. According to the EU Commission Decision 93/256/EEC, LOD must be calculated as the concentration corresponding to three times the peak-to-peak noise. This approach is followed by many authors (Bailac *et al.*, 2004; Hermo *et al.*, 2006).

Some authors report LOQ instead of LOD. It usually corresponds to the lower limit of the dynamic range. For most of the proposed methods, LOD are in the low ng g^{-1} range, which are suitable for residue analysis (Ramos *et al.*, 2003; Karbiwnyk *et al.*, 2007; Hassouan *et al.*, 2007).

1.6.3 Calibration

For quantitative analysis consideration must also be given to the most appropriate preparation of calibration standards. Calibration is a fundamental step in any analytical method. It is usually carried out by means of external standards (Cuadros-Rodríguez *et al.*, 2003), which is the most common approach in LC-based methodologies. Standards usually consist of solutions of pure analytes in the mobile phase or in an appropriate solvent, which are injected into the chromatographic system. In some cases the method of standard additions or matrix matched standards (MMS) (Schneider *et al.*, 2007) may be necessary. MMS is a calibration procedure where an analyte-free matrix is spiked with known amounts of the standard

(Fernández-Fígares *et al.*, 2004). After extraction of the spiked samples, the solutions coming from standards are similar to those coming from samples and, therefore any influence of matrix on the response would be the same for both. Moreover, if the analyte added to the quinolone-free matrices (standards) be haves in the same way as the incurred, this approach allows for the correction of any loss of analyte.

Internal standards (IS) are also used for calibration in some of the methods (Hermo *et al.*, 2005).The use of a suitable internal standard should also be considered (Poole, 1984). It is always another quinolone such as flumequine and cinoxacin was considered as IS for determination of oxolinic acid (Pouliquen *et al.*, 2000; Touraki *et al.*, 2001; Fierens *et al.*, 2002). IS was added to the samples at the beginning of the sample treatment in order to compensate for any uncontrolled effect (Hassouan *et al.*, 2007). However, the sample treatments are in general quite complicated, in some of methods that use an IS as a surrogate (Johnston *et al.*, 2002).

1.7 Objectives

The objectives of this work are to study the appropriate sample preparation procedure for analysis of oxolinic acid in edible animal tissues (shrimp, fish, beef, pork, chicken meat and chicken liver) using ultrasonic for extraction and solid phase extraction for clean up. The analysis is performed by highly sensitive liquid chromatography with fluorescence detector. The developed method was also fully validated to ensure its acceptability as an alternative analysis method followed the guidelines of the US Food and Drug Administration (US-FDA), US phamacoepia (USP) and the International Conference Harmonisation (ICH).

CHAPTER 2

Experimental

2.1 Chemicals and materials

2.1.1 Standard chemical

- Oxolinic acid ($C_{13}H_{11}NO_5$, purity 98%, Sigma, Mo, USA)

2.1.2 Other chemicals

All chemicals and reagents were analytical grade.

- Acetone (CH_3COOCH_3: Merck, Germany)
- Dichloromethane (CH_2Cl_2: Merck, Germany)
- Citric acid ($C_6H_8O_7$: Fluka, Buchs, Switzeland)
- Ethyl acetate ($CHCOOC_2H_5$: LAB-SCAN, Thailand)
- Formic acid (CH_2O_2: Baker, Dagenham, England)
- n-Hexane ($CH_3(CH_2)_4CH_3$: Merck, Germany)
- Acetic acid (CH_3COOH: J.T. Baker, USA)
- Methanol (CH_3OH: LAB-SCAN, Thailand)
- Acetonitrile (CH_3CN: LAB-SCAN, Thailand)
- Oxalic acid ($H_2C_2O_4$: Fisher Scientific Ltd., Leicestershire,UK)
- Phosphoric acid (H_3PO_4: Carlo Erba, France)
- Sodium hydroxide (NaOH: Merck, Darmstadt, Germany)
- Sodium oxalate ($COONa_2$: Carlo Erba)
- Sodium sulphate anhydrous (Na_2SO_4: Merck, Germany)
- Trifluoroacetic acid ($C_2H_1O_2F_3$: Fluka, Buchs, Switzeland)
- Ultra pure water (H_2O, water was de-ionized with reverse osmosis system and purified with a Maxima ultrapure water instrument to obtain the resistivity of 18.2 MΩ, ELGA, England)

2.1.3 Solid phase extraction (SPE) materials

- Polypropylene cartridge, 3 mL
- Oasis HLB cartridge (water, Mildford, MA, USA)
- Supelclean LC-18 sorbent (Supelco, Bellefonte, PA, USA)
- Supelclean EN-18 sorbent (Supelco, Bellefonte, PA, USA)
- Sepra C18-E sorbent (Phenomenex, USA)

2.1.4 Samples

Shrimp, pork, beef, fish, chicken meat and chicken liver were purchased from local markets and Department Stores in Songkhla province.

2.2 Instruments and apparatus

2.2.1 High performance liquid chromatograph

- High performance liquid chromatograph 1100 series equipped with a binary pump, micro vacuum degasser, autosampler, thermostatted column compartment, fluorescence detector and linked to a HP ChemStation
  (Agilent Technologies, Geramany)
- Pinnacle II C18 column: 250×4.6 mm, 5 μm (Restek, Bellefonte, PA, USA)
- Pinnacle II C18 guard column (Restek, Bellefonte, PA, USA)
- Computer system Compaq EVO (Compaq computer, Thailand)

2.2.2 Apparatus

- Analytical Balance AB204-S (Metter Toledo, Switzerland)
- Ultrasonic bath AS7240AT (Automatic Science, Japan)

- Evaporating rotator (EYELA, Japan)
- Vortex Genie-2 (Sciencetific Industries, USA)
- Solvent filtration system (Alltec, USA)
- Microliter pipette 10 µL, 100 µL, 200 µL, 1000 µL, 5000 µL (Eppendorf, Germany)
- Amber vial 2 mL with polypropylene screw cap and red rubber septa (Agilent Technologies, USA)
- Filter membrane Supor® 0.2 µm 47 mm (Pall, USA)
- Syringe filter PVDF 0.2 µm 13 mm (Chromtech, USA)
- Glass microfiber filter GF/F (Whatman, England)
- SPE vacuum manifold (Alltec, USA)
- Vacuum pump (Gast manufacturing, Inc., USA)
- General glassware such as volumetric flask, round bottle flask, Cylinder

2.3 Analysis system

Oxolinic acid in edible animal tissue was analyzed by high performance liquid chromatography with fluorescence detection (HPLC-FLD). The analysis system consists of sample preparation and analysis steps. The sample preparation procedure is summarized as shown in Figure 2.1. Oxolinic acid was extracted by ultrasonic and cleaned-up with solid phase extraction.

Figures 2.2 and 2.3 shows block diagrams of the HPLC system and the optical fluorescence detector. The mobile phase was degassed and pumped through the Pinnacle II column by dual piston pumps. The sample, oxolinic acid, was injected into the C18 column using autosampler and separation based on reversed phase mode. Then, the eluting compound (oxolinic acid) passed through the flow cell and absorbed radiation from xenon flash lamp following emit radiation to photocell. The emitted radiation is measured by photomutiplier tube and calculated to luminescence unit (LU) by HP ChemStation program (Agilent Technologies, 2002).

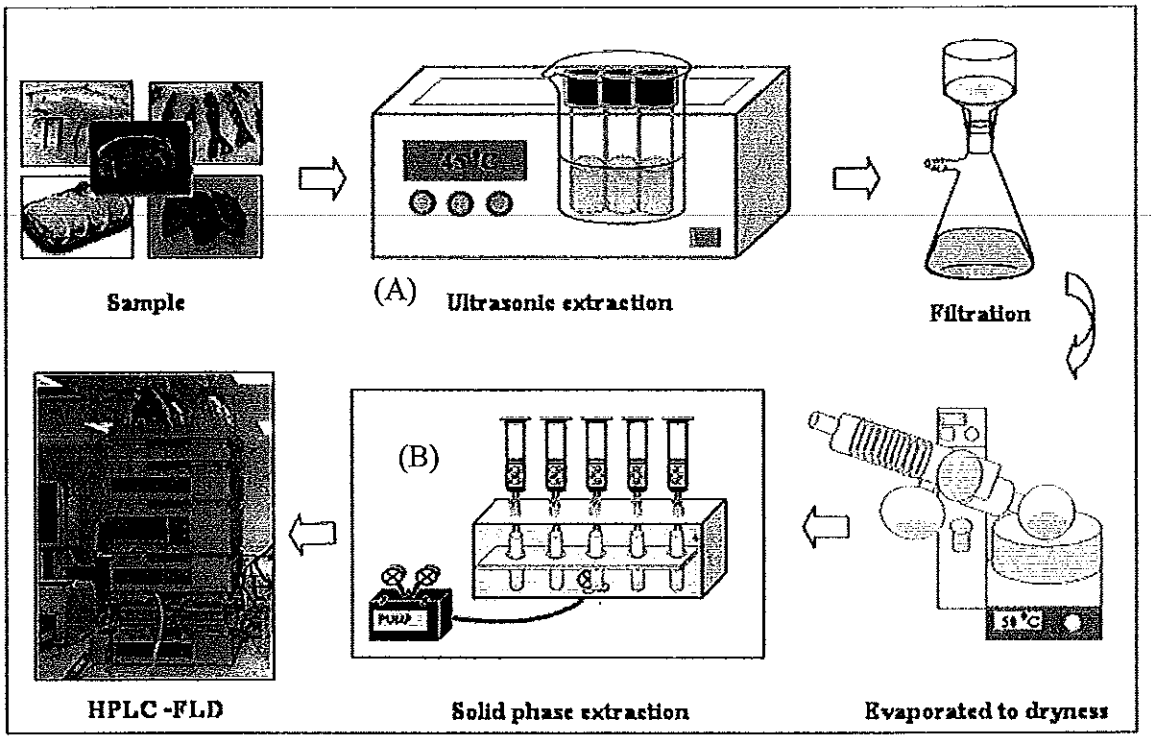


Figure 2.1 Sample preparation procedure to determine oxolinic acid consists of (A) extraction by ultrasonic technique and (B) clean-up by solid phase extraction

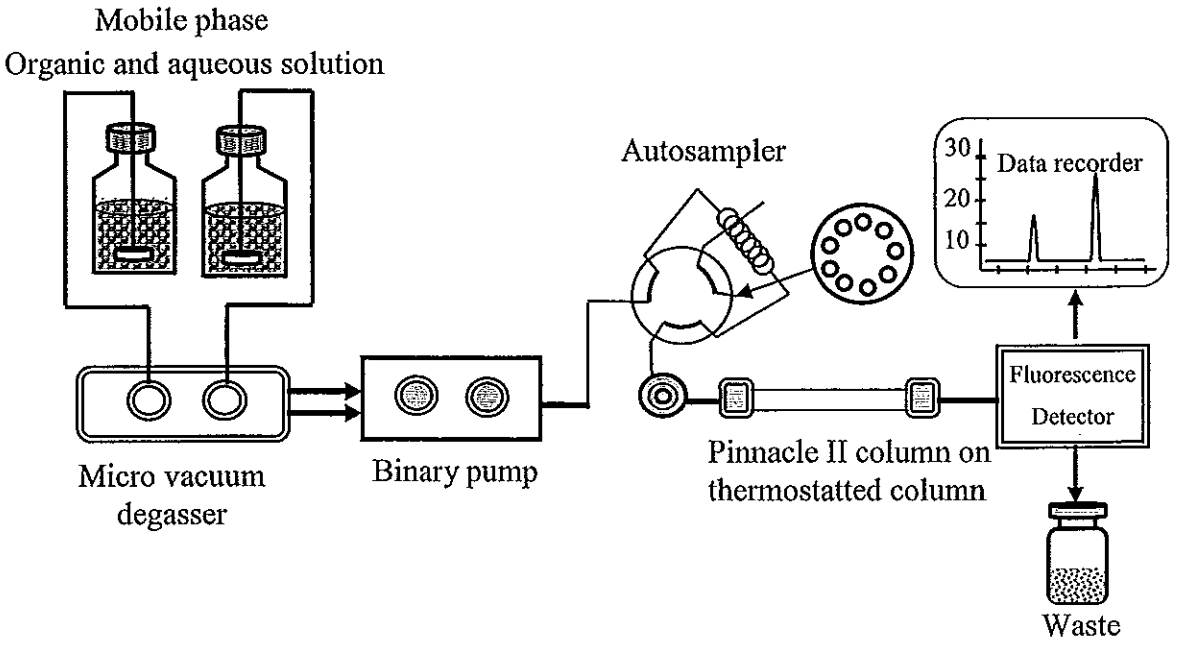


Figure 2.2 Schematic diagram of high performance liquid chromatography system

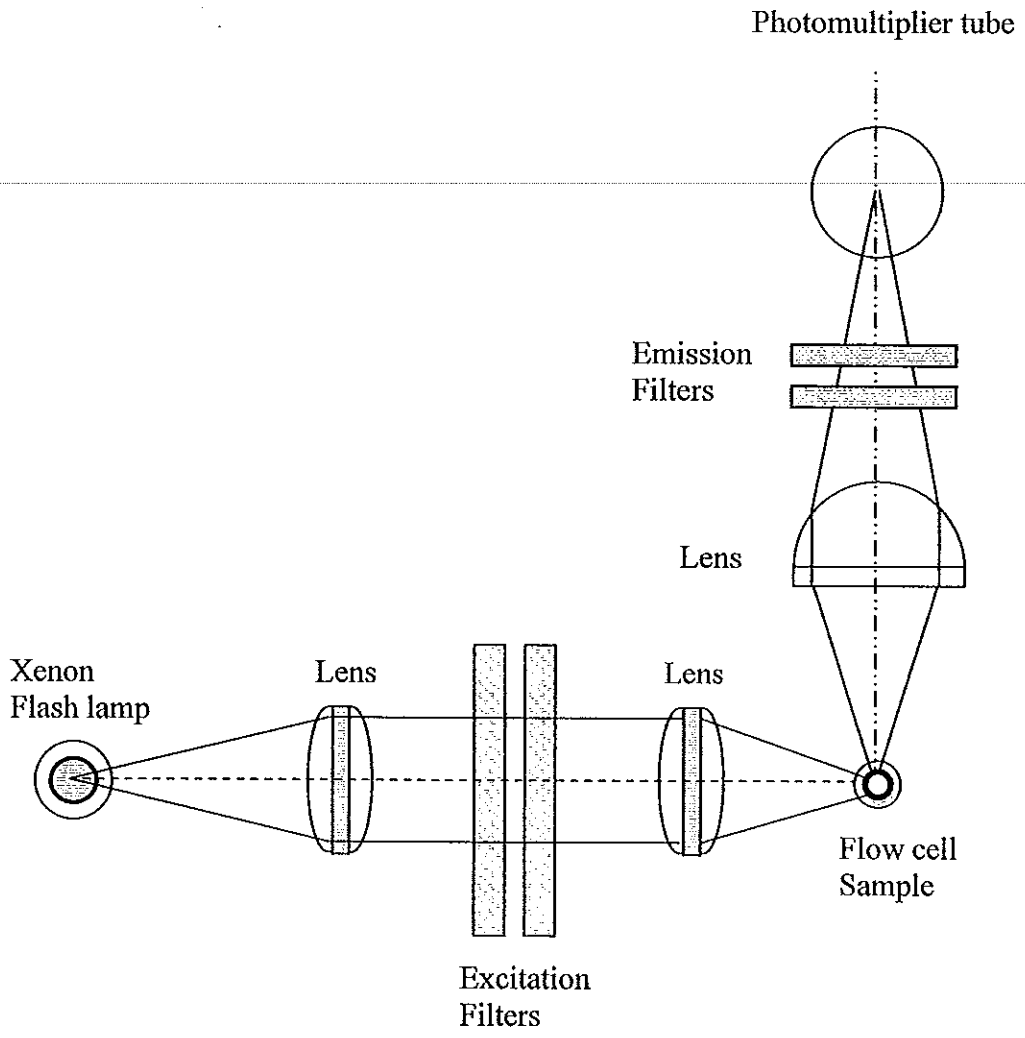


Figure 2.3 Optical diagram of fluorescence detector (FLD) (Snyder and Kirkland, 1979)

2.4 Standard solution

Standard solution is a chemical term which describes a solution of known concentration, prepared using standard substances in one of several ways (IUPAC, 1997). The standard stock solution and working solution of oxolinic acid were prepared and stored in dark bottles to protect them from light.

2.4.1 Oxolinic acid standard stock solutions

The primary stock solution of oxolinic acid was prepared at a concentration of 1000 mg L^{-1} in 0.02 M sodium hydroxide. This was diluted with the mobile phase to a secondary standard stock solution of 2 mg L^{-1} and stored at 4 °C in dark bottles.

2.4.2 Oxolinic acid standard working solutions

Working standard solutions were prepared by diluting the secondary standard stock solution with mobile phase to obtain concentrations in the range of 5-500 ng mL^{-1}.

2.5 Mobile phase preparation

Mobile phase of HPLC system (acetonitrile and oxalic acid buffer solution) was filtered through a 0.2 μm membrane filter. The mobile phase was degassed by ultrasonic vibration for 20 min. The same procedure was also carried out with water which was used to remove buffer solution after analysis.

2.6 Optimization of HPLC -FLD conditions

HPLC conditions were optimized to obtain the highest response with good chromatogram peak shape and short analysis time. The optimized parameters were excitation and emission wavelengths, mobile phase composition, pH of mobile phase, buffer concentration, flow rate and column temperature. In these studies, 20 μL of oxolinic acid working standard solution, 0.5 mg L^{-1}, was injected to HPLC system for optimization. Five replications were performed for each tested value. The starting operating conditions of HPLC system are shown in Table 2.1. Optimization was done by changing one parameter and kept others parameters constant. When an optimum was obtained it was used to optimize the next parameter. After optimization, system's performance was investigated.

Table 2.1 HPLC-FLD starting operation conditions

Parameter	Value	Reference
Excitation wavelength (nm) Emission wavelength (nm)	230-340 300-500	Pouliquen and Armand, 2000
Mobile phase	15 mM oxalic buffer (pH 2.5) : Acetonitrile	Prat *et al.*, 2006
Percentage of organic modifier (%)	40	Roudaut and York, 2002
Mobile phase pH	3.0	Touraki *et al.*, 2000
Mobile phase flow rate (mL min^{-1})	1.0	Ramos *et al.*, 2003
Column temperature (°C)	27	Grushka and Grinberg, 2006

2.6.1 Excitation and emission wavelengths

The most important parameters to be optimized in fluorescence detection are the excitation and emission wavelengths (Agilent Technologies, 2002). To achieve greatest sensitivity, the excitation wavelength of oxolinic acid was investigated by scanning the wavelength from 230 to 340 nm while the emission wavelength was scanned from 300 to 500 nm. The wavelength that gave the highest response was used as the excitation wavelength to optimize the emission wavelength of oxolinic acid by scanning the emission wavelength from 300 to 500 nm. The wavelength that gave the highest response was chosen as the optimum emission wavelength.

2.6.2 Composition of mobile phase

Composition of mobile phase is investigated for the separation and the retention behavior of oxolinic acid on a reversed phase column. Since solvent type and solvent strength of the mobile phase are the common factors used to control retention in isocratic reversed-phase liquid chromatography (Kiridena *et al.*, 2004), the following parameters were optimized *i.e.* organic modifier, percentage of organic modifier, pH of mobile phase and concentration of buffer in mobile phase.

2.6.2.1 Organic modifier

Since, methanol and acetonitrile are the most popular solvent used as organic modifiers in the mobile phase for reversed phase related to lipophilicity estimations (Sun *et al.*, 2006), thus, methanol, acetonitrile and the mixture (1:1) of the two solvents were investigated as possible organic modifier in the mobile phase for separating oxolinic acid at the optimum excitation (265 nm) and emission wavelengths (375 nm) obtained from 2.6.2. The organic modifier that gave a short analysis time and good peak shape was chosen.

2.6.2.2 Percentage of organic modifier

The mobile phase consists of oxalic acid buffer solution and acetonitrile (from 2.6.2.1). The percentage of acetonitrile in the mobile phase was varied *i.e.* 30, 35, 40, 45 and 50 %. Optimum percentage of acetonitrile was the percentage that provided the highest response with $k' > 0.5$ and short analysis time.

2.6.2.3 pH of mobile phase

pH of the mobile phase, consisted of oxalic acid buffer solution and acetonitrile (55:45) (from 2.6.2.2), was prepared at 2.5, 3.0, 3.5, 4.0 and 4.5. Optimum pH of mobile phase was the one that gave the highest response and good

peak shape.

2.6.2.4 Buffer concentration

To obtain the optimum concentration of buffer solution, oxalic acid solution was prepared at 5, 10, 15 and 20 mM. Optimum concentration of oxalic acid buffer solution was the concentration that gave the highest response and good peak shape.

2.6.3 Mobile phase flow rate

The flow rate of mobile phase (oxalic acid buffer solution and acetonitrile (55:45)) was varied at 0.7, 0.8, 0.9, 1.0, 1.1, and 1.2 mL min^{-1}. The retention time and peak width at half height were determined from the chromatogram to calculate the number of theoretical plate (*N*) and height equivalent to a theoretical plate (*HETP*). It was calculated as follows (Snyder *et al.*, 1997).

$$HETP = \frac{L}{N}, N = 5.54\left(\frac{t_R}{W_{1/2}}\right)^2$$

Where L is column length

N is plate number

$W_{1/2}$ is peak width at half-height

t_R is retention time of peak (min).

A van Deemter graph was plotted between *HETP* and flow rate to determine the optimum flow rate. The flow rate that gave the lowest *HETP* and short analysis time were selected.

2.6.4 Column temperature

The optimum column temperature was studied by varied the temperature at 25, 27, 30, 35 and 40 °C. The mobile phase was oxolinic acid buffer solution and acetonitrile (55:45) at the flow rate of 0.9 mL min^{-1} (from 2.6.3). Optimum temperature of HPLC system was the temperature that gave the highest response

and short analysis time. The summary of the optimization of HPLC conditions is shown in Table 2.2.

Table 2.2 Optimization of HPLC conditions

Parameter	Value tested
Excitation wavelength (nm) Emission wavelength (nm)	230-340 300-500
Organic modifier	Methanol, Acetonitrile and Methanol+Acetonitrile (1:1 v/v)
Percentage of organic modifier (%)	30, 35, 40, 45 and 50
Mobile phase pH	2.5, 3.0, 3.5, 4.0 and 4.5
Buffer concentration (mM)	5, 10, 15 and 20
Mobile phase flow rate (ml L^{-1})	0.7, 0.8, 0.9, 1.0, 1.1 and 1.2
Column temperature (°C)	25, 27, 30, 35 and 40

2.7 System performance of HPLC-FLD

A system suitability test provides assurance that a system's performance is appropriate for the analysis (Shabir *et al.*, 2003), 50 ng mL^{-1} of oxolinic acid standard solution was used to studies the system suitability. Then, the linear dynamic range and detection limit of the system were evaluated before analysis of real sample.

2.7.1 System Suitability test

System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns. Parameters such as capacity factor, plate count, tailing factors, and repeatability (RSD retention time and area for five repetitions) are determined and compared against the specifications set for the

method (Shabir, 2003).

. 2.7.1.1 Capacity factor (k')

Capacity factor (k') is a measurement of the retention time of a sample molecule relative to column dead volume. Capacity factor was investigated by injecting a standard solution of oxolinic acid with the automatic injector. The measurement of parameter, t_R and t_0 for k' calculation is illustrated in Figure 2.4. and equation as the following (Snyder *et al.*, 1997)

$$k' = \frac{t_R - t_0}{t_0}$$

Where t_R is retention time of peak

t_0 is void time.

2.7.1.2 Theoretical plates (N)

The theoretical plate is a useful measure of column efficiency. It is the relative ability of given column to provide narrow band and improved separation. The measurement is illustrated in Figure 2.4. It was calculated as follows (Snyder *et al.*, 1997)

$$N = 5.54\left(\frac{t_R}{W_{1/2}}\right)^2$$

Where $W_{1/2}$ is peak width at half-height

t_R is retention time of peak (min).

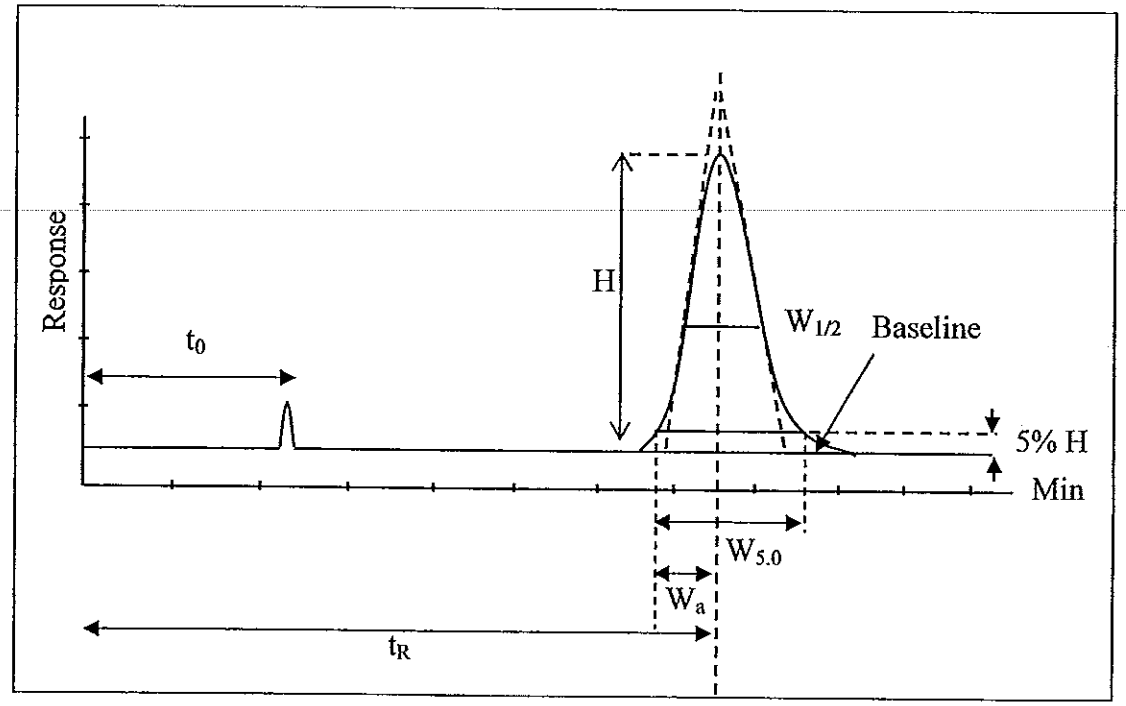


Figure 2.4 HPLC chromatogram for evaluation system suitability; t_R= retention time, t_0= void time, $W_{1/2}$= peak width at haft-height, $W_{5.0}$= peak width at 5%, peak front edge width at 5% height, H= peak height

2.7.1.3 Peak asymmetry and Tailing factor (*T*)

A useful and practical measurement of peak shape is the peak asymmetry factor or tailing factor. Peak asymmetry is measured at 10% of full peak height. Peak tailing factor is measured at 5% of full peak height while the peak asymmetry and the peak tailing factors are interconverted as shown in Table 2.3. The tailing factor (*T*) was used to evaluate peak shape and is recommended to be $T<2$ (US-FDA, 1994). The measurement is illustrated in Figure 2.4. The tailing factor is calculated by the following equation (Snyder *et al.*, 1997)

$$T = \frac{W_{5.0}}{2W_a}$$

Where $W_{5.0}$ is the peak width at 5% height from baseline

W_a is the peak front edge width at 5% height from baseline.

Table 2.3 Peak asymmetry and peak tailing factor relationship (Snyder *et al.*, 1997)

Peak Asymmetry factor (at 10%)	Peak Tailing factor (at 5%)
1.0	1.0
1.3	1.2
1.6	1.4
1.9	1.6
2.0	1.8
2.5	2.0

2.7.1.4 Repeatability

The repeatability of the chromatographic system was determined by injected 10 times of oxolinic acid standard solution with automatic injector and percentage of relative standard deviation (%RSD) was obtained for the retention time and peak area. The %RSD should be less than 1% and 4% for retention time and peak area respectively (Snyder *et al.*, 1997).

2.7.2 Linear dynamic range

Oxolinic acid standard solution was prepared by diluting stock solution with mobile phase (oxalic acid buffer: acetonitrile; 55:45 v/v) to the concentration from 5 ng mL^{-1} to 20 μg mL^{-1}. Each concentration was injected into the HPLC system at optimum conditions obtained previously. Peak area was plotted versus concentration of oxolinic acid to obtain the linear dynamic range. Linearity was determined by considering the coefficient of determination (R^2) (>0.99) (US-FDA, 2000) and peak shape (T<2) (US-FDA, 1994).

2.7.3 Limit of detection

The limit of detection (LOD) of the chromatographic system was determined based on the standard deviation of *y*-intercepts of regression lines. For the preparation of the standard calibration curve, the standard solution of oxolinic acid was prepared by diluting stock solution at the concentration 2 mg L^{-1} with mobile phase. The standard solution of oxolinic acid was prepared in the range of 0.5-50 ng mL^{-1} and was analyzed under the optimum conditions of HPLC system.

2.8 Sample preparation

Ultrasonic solvent extraction (USE) and solid phase extraction (SPE) methods were used in the sample preparation procedures for determination of oxolinic acid in edible animal tissues. Fortified shrimp sample with 50 ng g^{-1} oxolinic acid was used to study the optimum sample preparation conditions.

2.8.1 Fortification of sample

Fortified sample, a sample enriched with a known amount of oxolinic acid, was used to study the optimum sample preparation conditions and method validation. Two grams of blank sample was thawed at room temperature (26 ± 1°C) before placing in a 50 mL glass tube. A 100 μL of oxolinic acid standard solution was then added to the surface and homogenized by vortex for 1 min. The sample was allowed to stand at room temperature for approximately 30 min prior to further processing while covered with aluminum foil to avoid light because it permitted the interaction between the oxolinic acid and the sample.

2.8.2 Evaluation of spiking sample

The waiting time of spiking sample was evaluated to ensure that all analyses were adsorbed in the sample at the chosen waiting time (30 min) before extraction.

Spiked samples at a concentration of 50 ng g^{-1} were stored in the dark for different lengths of time *i.e.* 30 min, 1, 3, 5, 12 and 24 h (five replicates). After these times, the samples were analysed and the responses were compared.

2.8.3 Optimization of sample preparation

Extraction parameters of oxolinic acid from shrimp sample were optimized and each parameter was repeated five times. Five replications of each extractant were analysed by HPLC-FLD system at optimum conditions. The starting operation conditions for optimization of sample preparations are show in Table 2.4. When the optimum value of one parameter was obtained it was used in the optimization of the next parameter.

Table 2.4 The starting operation conditions for the optimization of sample preparation

Parameters	Start operating values
Ultrasonic extraction	
Extraction solvent	Ethyl acetate (Hernández-Arteseros *et al.*, 2002)
Extraction volume	15 mL
Extraction time	30 min
Solid phase extraction	
Type of sorbent	Supelclean C18
Type of eluting solvent	Acetonitrile (Bailac *et al.*, 2006)
Sample flow rate	1 mL min^{-1}
Volume of eluting solvent	10 mL
Flow rate of eluting solvent	1 mL min^{-1}

2.8.4 Optimization of ultrasonic extraction (USE)

Since the presence of water in USE generally decreases extraction efficiency (Wu *et al.*, 2006; Ridgway *et al.*, 2007) therefore, the fortified sample was mixed with 5 grams of anhydrous sodium sulfate to achieve a dried sample. The sample was sonicated for 30 min at room temperature. The extractant was passed through a Whatman 40 filter, the filtrate was transferred into a 100 mL round bottom flask. The extraction was repeated twice. Organic extractants were evaporated on a rotary evaporator at 50 °C to dryness and the residue was dissolved in 2 mL of mobile phase. The concentrated extractant was transferred onto a SPE cartridge for the clean up. The aim of the optimization procedure was to improve the extraction efficiency with minimum solvent and time consumption. Parameters investigated were extraction solvent, solvent volume and extraction time.

2.8.4.1 Extraction solvent

In the extraction process extraction solvent is the most important parameter (Lambropoulou *et al.*, 2006). Therefore, extraction efficiency of various medium polarity organic solvents (ethyl acetate, acetonitrile, dichloromethane, acetone, and methanol) was compared. The solvent that provide the highest response was used as extraction solvent.

2.8.4.2 Solvent volume

To investigate the optimum volume of solvent 2 g of spiked shrimp samples were sonicated for 30 min with various solvent volumes, 5, 8, 10, 15, 20 and 30 mL of ethyl acetate. The least amount of solvent that provided the highest response was selected as extraction volume.

2.8.4.3 Extraction time

Optimum extraction time was determined using 2 g of spiked shrimp samples and sonicated for 5, 10, 15, 20, 30, 40 and 50 min with 10 mL of ethyl acetate. The extraction time which provide the highest response and shortest time was selected.

2.8.4.4 Stability of Oxolinic acid under ultrasonic condition

After the optimum conditions of ultrasonic extraction were obtained the stability of oxolinic acid under these conditions was investigated. The method was as follows: 1 mL standard solution (50 ng mL^{-1} of oxolinic acid) was diluted 10 times by ethylacetate, and then sonicated for 0, 5, 10, 15, 20, 30 and 60 min at 45 °C. Then, ethyl acetate was evaporated on a rotary evaporator at 50 °C to dryness and the residues were dissolved in mobile phase for HPLC analysis.

2.8.5 Optimization of solid phase extraction (SPE)

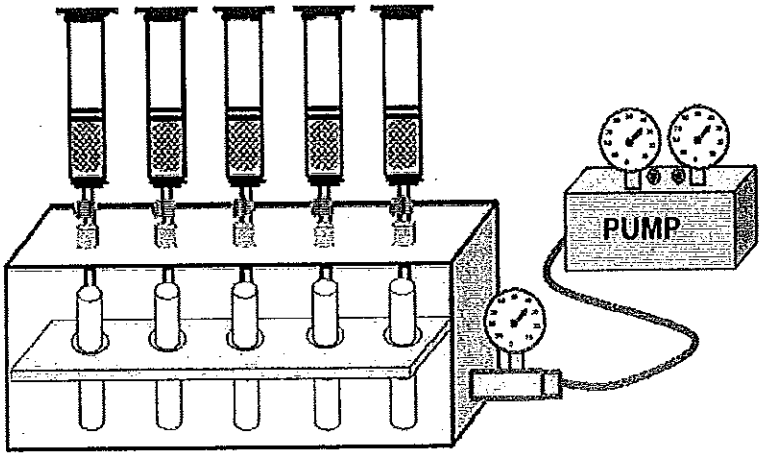


Figure 2.5 Solid phase extraction system used for clean-up sample consists of pump, cartridges and manifold

Figure 2.5 shows the solid phase extraction system which involves the use of sorbents packed in the cartridge to trap analytes and separate them from the bulk of the matrix (Castro *et al.*, 2000; Wells and Yu, 2000). The four steps of solid phase extraction include conditioning, retention, rinsing and elution. In this study, the sorbents were conditioned with 3 mL of methanol and equilibrated with 3 mL of water (Johnston *et al.*, 2002) to eliminate contaminations and to ensure that the sample solution would properly wet the surface of the sorbent particle. After conditioning, 500 µL of extractant was passed through the cartridges where oxolinic acid would be retained. They were then rinsed with 3 ml of hexane to defat followed by 1 mL of water to remove interference. Oxolinic acid was then eluted with eluting solvent. The eluent was evaporated to dryness at 50 °C. The residue was reconstituted with the mobile phase and filtered with a 0.2 µm PVDF syringes filter. Twenty microliters was injected into HPLC system at the optimum conditions. The optimization of solid phase extraction for analysis of oxolinic acid were type of sorbent, type of eluting solvent, sample flow rate, volume of eluting solvent and flow rate of eluting solvent.

2.8.5.1 Preparation of solid phase extraction cartridges

Each cartridge was prepared by first plugged a GF/F glass microfiber filter into the bottom of a 3 ml plastic syringe using a syringe plunger followed by 0.3 g of sorbent then a GF/F glass microfiber was plugged on the top as frit to retain the sorbent. The packed SPE cartridge is shown in Figure 2.6.

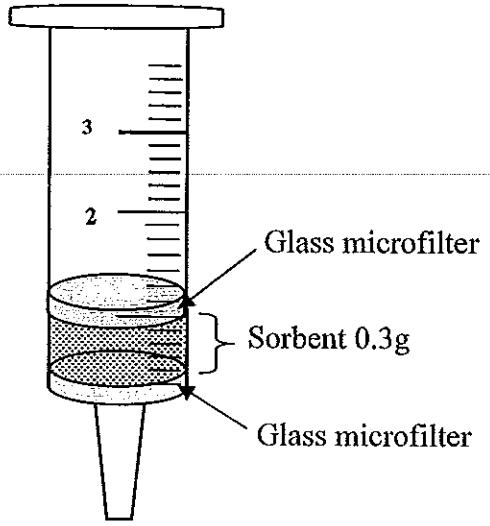


Figure 2.6 Packed SPE cartridges

2.8.5.2 Type of sorbent

Different types of SPE sorbents have been used to separate the oxolinic acid from samples of high matrix (edible animal tissues). Supelclean LC-18, Supelclean EN-18, Sepra C18-E sorbents and Waters Oasis HLB cartridges were compared and the sorbent that gave the highest efficiency (high response and low interference) was chosen.

2.8.5.3 Type of eluting solvent

The eluting solvent should be strong with respected to analytes for high selectivity. Acetonitrile (75%) combined with 25% of difference acidic solutions (Hermo *et al.*, 2006) (phosphoric acid, citric acid, formic acid, acetic acid and trifluoroacetic acid) were tested. The eluting solvents that provide highest response were selected as eluting solvent. Different concentration of trifluoroacetic acid (0.2, 0.4, 1.0, 2.0, 3.0, 4.0 and 5.0) which is the optimum eluting solvent was also studies. The lowest concentration that provided highest response was chosen.

2.8.5.4 Sample flow rate

The sample flow rate was optimized for the maximum efficiency. The flow rate was varied at 0.2, 0.6, 1, 2, 3, and 4 mL min^{-1}. The flow rate that gave the highest response was then selected.

2.8.5.5 Volume of eluting solvent

The volume of eluting solvent should be enough to elute all analyte in order to obtain the highest recovery. The volume of eluting solvent was investigated by varying at 1, 2, 3, 4, 5, and 10 mL. The least solvent volume that provided the highest response was then selected.

2.8.5.6 Flow rate of eluting solvent

Oxolinic acid was eluted from the SPE sorbent using the mixture of trifluoroacetic acid and acetonitrile (25:75, v/v). In order to minimize eluting solvent and short analysis time, the flow rate of eluting solvent was varied at 1, 2, 3 and 4 mL min^{-1}. The flow rate that gave the highest response was then selected.

2.8.6 Effect of defatting

Analysis of oxolinic acid in complex food samples requires defatting step. Hexane which is a non-polar solvent was used in this step (Hermo *et al.*, 2005). The defatting step was investigated in both liquid-liquid extraction and the addition of hexane to the SPE cartridge. The obtained results were also compared with non-defatting. The summary of the optimization for sample preparation is shown in the Table 2.5.

Table 2.5 Optimization of sample preparation

Parameters	**Optimization values**
Ultrasonic extraction	
Extraction solvent	Ethyl acetate, acetonitrile, dichloromethane, Acetone, and methanol
Extraction volume	5, 8, 10, 15, 20 and 30 mL
Extraction time	5, 10, 15, 20, 30, 40 and 50 min
Stability of oxolinic acid	0, 5, 10, 15, 20, 30 and 60 min
Solid phase extraction	
Type of sorbent	Supelclean LC-18, Supelclean EN-18, Ssepra C18-E sorbents and Waters Oasis HLB cartridges
Type of eluting solvent	Various acidic solution (phosphoric acid, citric acid, formic acid, acetic acid and trifluoroacetic acid) in acetonitrile, (25:75, v/v)
Trifluoroacetic acid	0.2, 0.4, 1.0, 2.0, 3.0, 4.0 and 5.0 (%)
Sample flow rate	0.2, 0.6, 1, 2, 3, and 4 mL min^{-1}
Volume of eluting solvent	1, 2, 3, 4, 5 and 10 mL
Flow rate of eluting solvent	1, 2, 3 and 4 mL min^{-1}
Defatting effect	Non defatting, in LLE and SPE procedure

2.9 Matrix match calibration curve

Matrix match calibration curve was carried out by spiking standard oxolinic acid into extracted blank sample (matrix standard; corresponding to blank samples fortified at the end of sample preparation). Oxolinic acid standard solution was added to extracted blank sample by increasing levels of standard concentration in the sample from 5, 10, 20, 50 to100 ng mL^{-1}. Oxolinic acid standard solutions at the same concentration were also injected in the parallel with matrix standard. The experiments were done in five replicates. The matrix match calibration curve was obtained by plotting the peak area versus concentration of oxolinic acid. The response from the chromatogram per unit of concentration of oxolinic acid in the unknown samples is then calculated mathematically from the matrix match calibration curve.

2.10 Sampling

Samples were purchased from fresh markets and department stores in Hat Yai city. Before analysis, the sample was thawed at room temperature for about 1 h then performed as the procedure is shown in Figure 2.7.

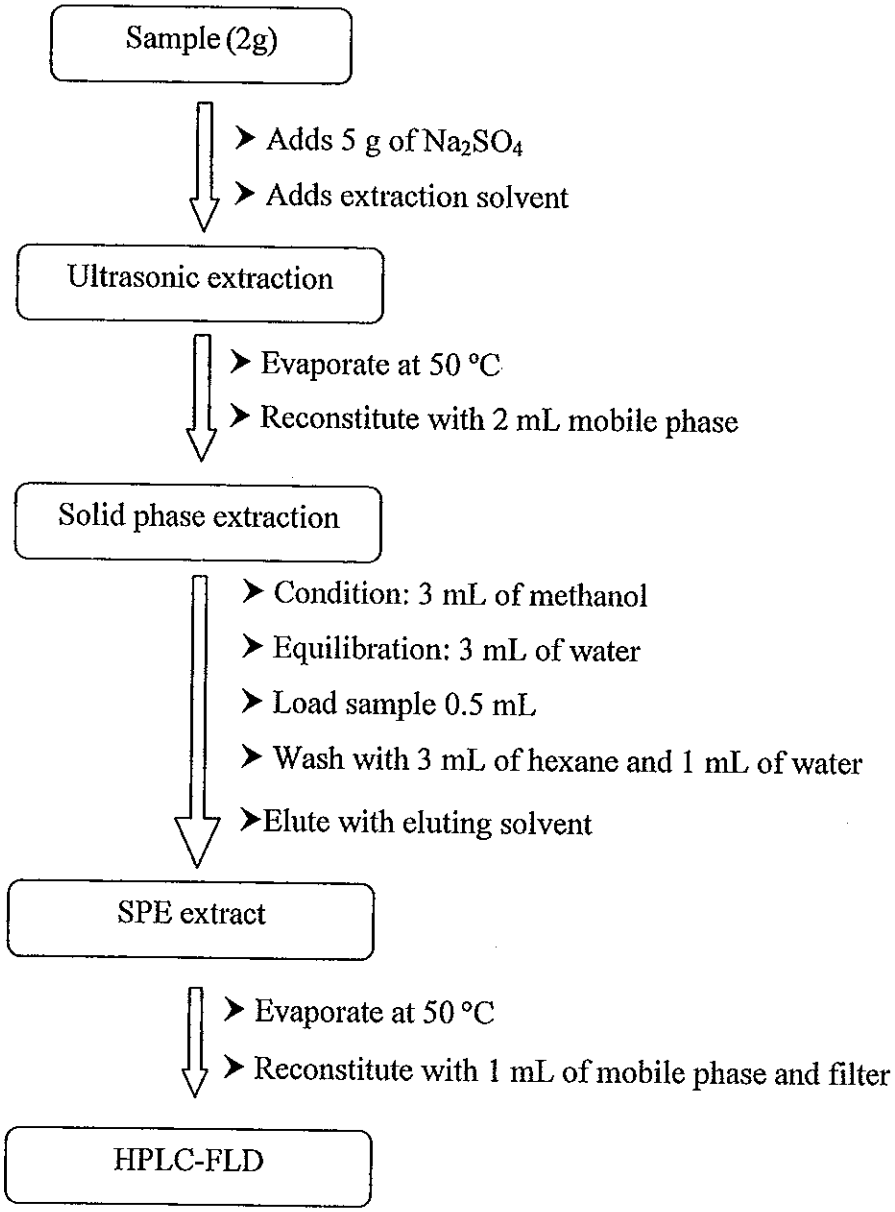


Figure 2.7 Analytical procedures for determination of oxolinic acid in edible animal tissues

2.11 Method validation

Analytical test method validation is carried out to ensure that an analytical methodology is accurate, specific, reproducible and robust over the specified range that an analyte will be analyzed (Feinberg, 2007). Analysis of drug using HPLC were generally validated followed the guidelines of the US-FDA (1994), US Pharmacopeia (USP) and International Conference on Harmonization (ICH) (Table 2.6). The proposed method was then validated to cover those specified by the US Food and Drug Administration (US-FDA), US Pharmacopeia and the International Conference Harmonisation (ICH) guideline for bioanalytical method validation. Parameters include selectivity, linearity, recovery, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ), stability, robustness and system suitability were established.

Table 2.6 The ICH, USP and FAD validation parameters

ICH/USP validation parameters	**US-FDA validation requirements**	**US-FDA GMP (legal) requirements**
Specificity	Sensitivity	Accuracy
Accuracy	Recovery	Sensitivity
Precision	Reproducibility	Specificity
-Repeatability	Robustness	Reproducibility
-Intermediate precision	Sample solution stability	
-Reproducibility	System suitability	
Limit of detection		
Limit of Quantification		
Linearity		
Range		
Ruggedness		
Robustness		

2.11.1 Selectivity

The selectivity of an analytical method is the ability of the method to measure the analyte response in the presence of other components of the sample (Clemente *et al.*, 2006). The selectivity of oxolinic acid was evaluated by comparing six different sources of blank sample and spike sample follow guideline for industry bioanalytical method validation (US-FDA, 2001). The samples were purchased from different markets or on different days in the area of Hat Yai. The method will be selectivity if not presence of other compound in the chromatogram at the same retention time of oxolinic acid.

2.11.2 Range and linearity

Linearity was determined with seven concentration levels (5 -500 ng g^{-1}). Each concentration was performed five times. Linear relationship was obtained between the peak area and the corresponding concentrations. The equation of linear regression was performed using least-squares method. The coefficient of determination was considered and requited more than 0.99 (US-FDA, 2000).

2.11.3 Accuracy

The accuracy of the analytical procedure is the extent to which the test results generated by the procedure and the true value agree (Grushka and Grinberg, 2006). The accuracy of the method was evaluated by spiking blank sample at three concentrations (50, 100 and 200 ng g^{-1}). The accuracy is expressed as the relative error of measurement (RE, %) and should be less than 20% (US-FDA, 2001)

$$\text{RE (\%)} = \frac{(\text{Mean calculated concentration} - \text{true concentration})}{\text{True concentration}} \times 100$$

2.11.4 Precision

Precision demonstrates the ability of an analytical method to produce consistent results. Precision is the degree of reproducibility among a set of individual results, including any internal variations inherent in an analytical method. Precision can be divided into three categories: repeatability, intermediate precision and reproducibility (ICH, 1996; Grushka and Grinberg, 2006).

Repeatability (intra-day precision) demonstrates the precision of the instrument itself and is obtained when the analysis is performed using the same instrumental operating conditions over a short period of time. Intermediate precision (inter-day precision) evaluates the data generated over an extended period of time. Intermediate precision ensures that an analytical method is precise despite random events affecting operating conditions within the same laboratory (Srinubabu *et al.*, 2007). Reproducibility analyzes the performance of an analytical method in more than one laboratory (Ermer and Ploss, 2005).

The precision of method was evaluated include the standard deviation, relative standard deviation, coefficient of variation and confidence interval. Relative standard deviation is the parameter of choice for expressing precision in analytical science, which may also be expressed as a percentage, using the equation:

$$RSD = 100s/ x_m$$

Where $s = [\sum (x_i - x_m)^2 / n-1]^{1/2}, x_m = \sum x_i / n$

n is the total number of measurements

x_i is number of the individual measurements.

The precision was calculated as intra-day precision and inter-day precision, expressed by means of the percentage relative standard deviation. In this work, intra-day-precision was evaluated in one set, of fifteen samples, at three concentration levels (50, 100 and 200 ng g^{-1}). The procedure was repeated on three different days to determine inter-day-precision.

2.11.5 Recovery

The recovery was quantified by calculate the ratio of the slopes of the calibration curves of extracted spike samples with standard solution that prepared at the same concentration. Five replicates of spiked samples at five different concentration levels (5, 10, 20, 50, 100 ng g^{-1}) of oxolinic acid were extracted following the proposed method.

2.11.6 Limit of detection (LOD) and quantification (LOQ)

The LOD and LOQ were established using the calibration curve methodology ($S_{y/x}$ and S_a) with spiked samples at different concentration levels (5, 10, 20, 50 and100 ng g^{-1}). The calculation of these methods according to equation (1) and (2) for LOD and LOQ, respectively.

$$LOD = 3.3 \times \frac{S_{y/x}}{b} \text{ and } LOD = 3.3 \times \frac{S_a}{b} \quad \text{.... (1)}$$

$$LOQ = 10 \times \frac{S_{y/x}}{b} \text{ and } LOQ = 10 \times \frac{S_a}{b} \quad \text{.... (2)}$$

Equation (3) was used to calculate $S_{y/x}$ which estimates the random errors in the y-direction. This equation utilized the y-residual, $y_i - \hat{y}_i$, where $\hat{y}_i$ values are the points on the calculated regression line corresponding to the individual x-values, was described in Figure 2.8. The standard deviation of y-intercept (S_a) was calculated using equation (4).

$$S_{y/x} = \sqrt{\frac{\sum_i \left(y_i - \hat{y}\right)^2}{n-2}} \quad \text{.... (3)}$$

$$S_a = S_{y/x} \sqrt{\frac{\sum_i x_i^2}{n\sum_i \left(x_i - \bar{x}\right)^2}} \quad \text{.... (4)}$$

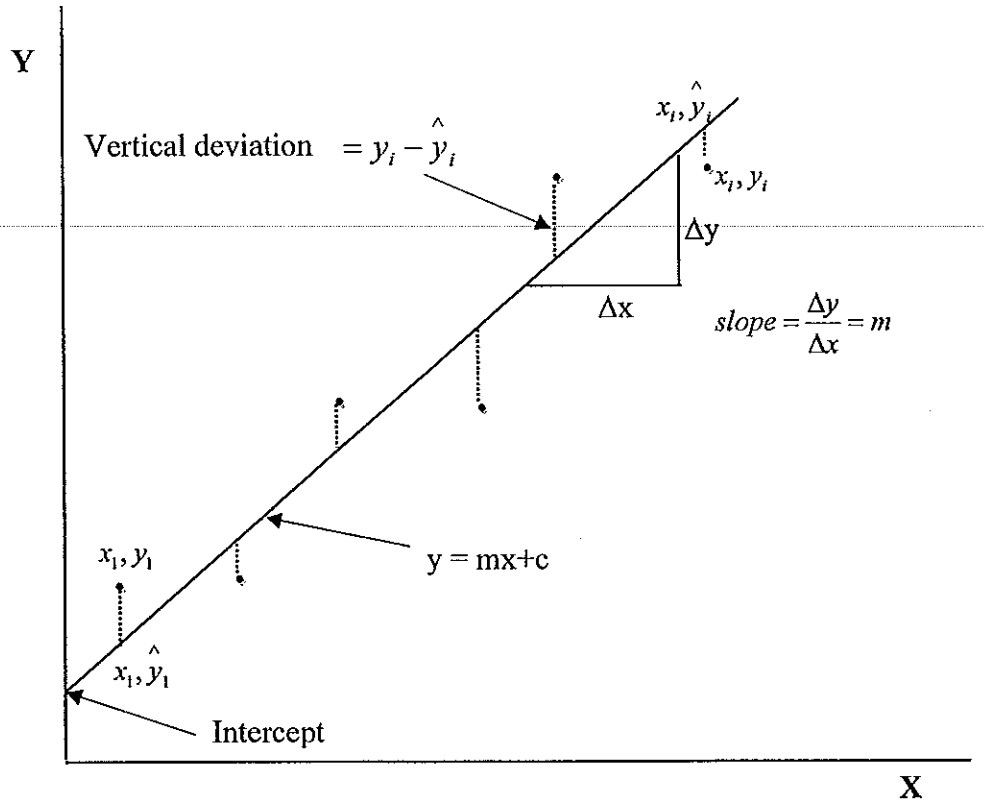


Figure 2.8 The y-residuals of a regression line (King *et al.*, 2000)

2.11.7 Stability

Since the analysis of biological samples for drug testing is not usually performed immediately after sample collection such as instrument breakdown or overnight analyses using autosampler. Instability of the analyte in the sample matrix during storage before analysis may give rise to significant deviations of the analysis result (Hartmann *et al.*, 1998). It is therefore, very important to know the optimal storage conditions to keep drugs stable at least during the storage time. Sample and standards should be tested over at least a 24 h period (depends on need). Stability procedures should evaluate the stability of the analytes during sample collection and handling, after long-term (frozen at the intended storage temperature) and short-term (room temperature) storage, and after going through freeze-and-thaw cycles (Peters *et al.*, 2007). All these studies were evaluated by HPLC-FLD which modified from guidance for industry bioanalytical method validation (US-FDA, 2001).

2.11.7.1 Short-term stability

Five samples at a concentration of 10 ng g^{-1} (low level) and 100 ng g^{-1} (high level) were processed and the extracts dissolved in mobile phase were stored at room temperature and kept at this temperature for 48 h (based on the expected duration that samples will be maintained at room temperature in the intended study in the autosampler). They were analysed at the beginning and after 2, 4, 8, 12, 24, 36 and 48 h to ensure that samples are stable at room temperature for at least 2 days. This study was done by dissolve the samples with the mobile phases. The samples went through the extraction method before being analysed. The accuracy values and standard deviations between the concentration found and initial concentration was used for the stability evaluation.

2.11.7.2 Freeze-and-thaw stability

Five samples at a concentration of 10 ng g^{-1} (low level) and 100 ng g^{-1} (high level) were processed, and the evaporated extracts were dissolved in mobile phase were subjected to three freeze-and-thaw cycles. The samples were stored at -20 °C for 24 h and were then thawed at room temperature. Afterwards, the samples were refrozen for 24 h under the same conditions. This cycle was repeated two more times. The samples was analysed at the beginning of the process and after each freeze/thaw cycle. The concentrations of oxolinic acid found in each determination were compared to ensure that there is no variation during these processes. The accuracy values and standard deviations between the concentration found and initial concentration was used for the stability evaluation.

2.11.7.3 Long-term stability

Five samples at a concentration of 10 ng g^{-1} (low level) and 100 ng g^{-1} (high level) were stored at -20 °C for 3 months. Then, these samples were analysed and compared with three fresh samples at a concentration of 10 ng g^{-1} and five fresh

samples at a concentration of 100 ng g^{-1}. The accuracy values and standard deviations between the concentration found and initial concentration was used for the stability evaluation.

2.11.7.4 Standard aqueous solutions

The stability of aqueous standard solutions was evaluated by kept at 4 °C. The standard solution were analysed every two weeks and were compared with freshly prepared standards.

2.11.8 Robustness studies

The robustness of a method is its ability to remain unaffected by small deliberate variations in method parameters (Kulikov and Zinchenko, 2007). The robustness was evaluated by deliberate variations of the method parameters. The factors selected to examine were variation in mobile phase flow rate, column temperature, mobile phase pH and mobile phase composition (percentage of acetonitrile) (ICH, 1996) .The positive and negative variations from optimum values are shows in Table 2.7. Only one of these parameters was changed on one occasion.

Table 2.7 Robustness study

Variation parameters	Optimum conditions	Variation values
Mobile phase flow rate	0.9 mL min^{-1}	0.8, 1.0 mL min^{-1}
Column temperature	25 °C	20, 25 °C
Mobile phase pH	3.5	3.2, 3.8
Percentage of acetonitrile	45 %	42, 48 %

2.12 Qualitative and quantitative analysis of oxolinic acid in edible animal tissues

2.12.1 Qualitative analysis

The retention time data was applied for the qualitative analysis. The retention time of the sample chromatogram was compared with retention time of the standard chromatogram to identify the oxolinic acid peak.

2.12.2 Quantitative analysis

Quantitative analysis of oxolinic acid in edible animal tissues was based on the response of chromatographic peak that was proportional to the amount of analyte. Peak area of oxolinic acid was compared to standard calibration curve. When it was non detectable (ND) or lower than LOQ the standard addition method was implemented for quantitative analysis.

2.12.2.1 Standard addition method

The standard addition was performed in the sample that showed non detectable response or lower than the LOQ of oxolinic acid. This method was carried out by spiking the oxolinic acid into 2.00 grams of various samples. Oxolinic acid standard solution was added into the sample by increasing levels of standard concentration in the sample from 5, 10, 20, 50 to100 ng g^{-1}. The spiking was done before the sample preparation step. Then the spiked and un-spiked samples were analysed by HPLC under the optimum conditions. The experiments were done in five replicates. The response from the chromatogram per unit of concentration of oxolinic acid in the unknown samples is then calculated mathematically from the calibration curve. The results plotted as shown in Figure 2.9 (Miller and Miller, 2000).

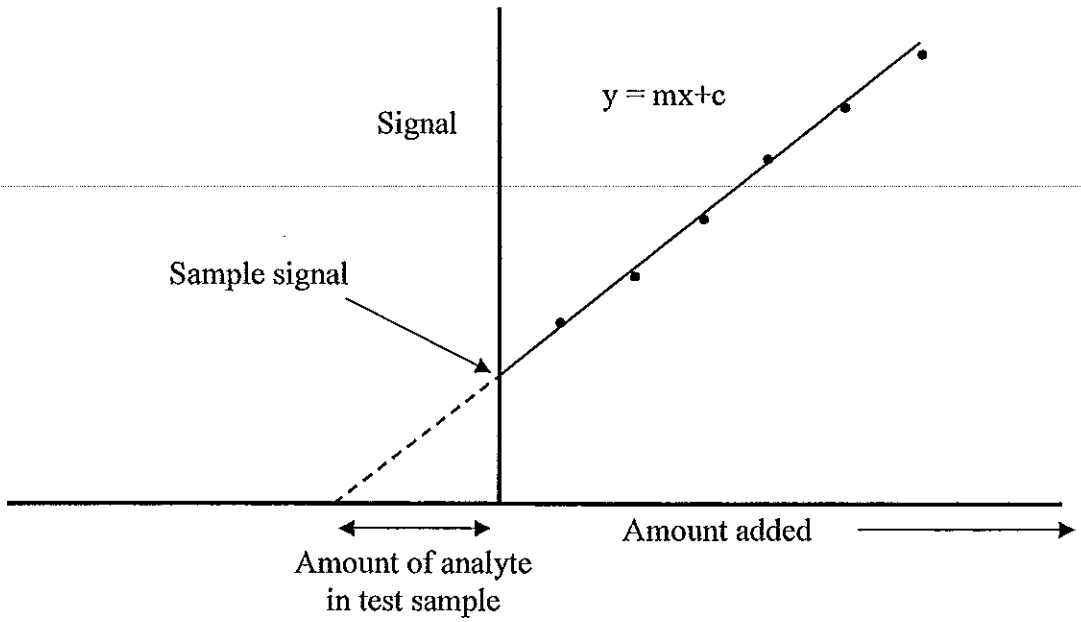


Figure 2.9 The method of standard additions.

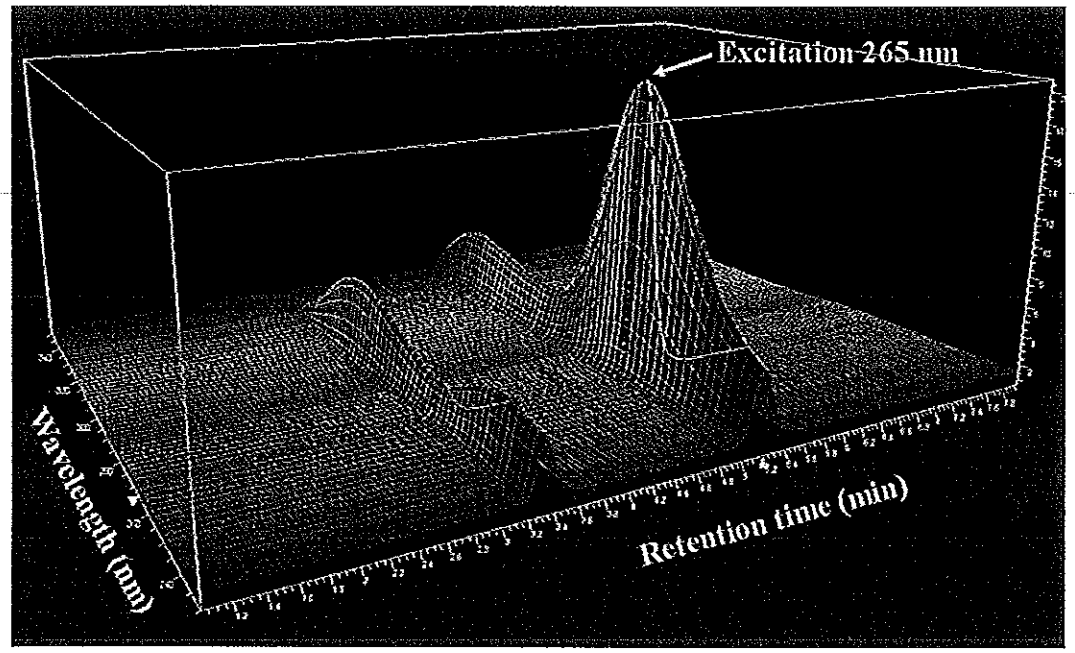
CHAPTER 3

Results and Discussion

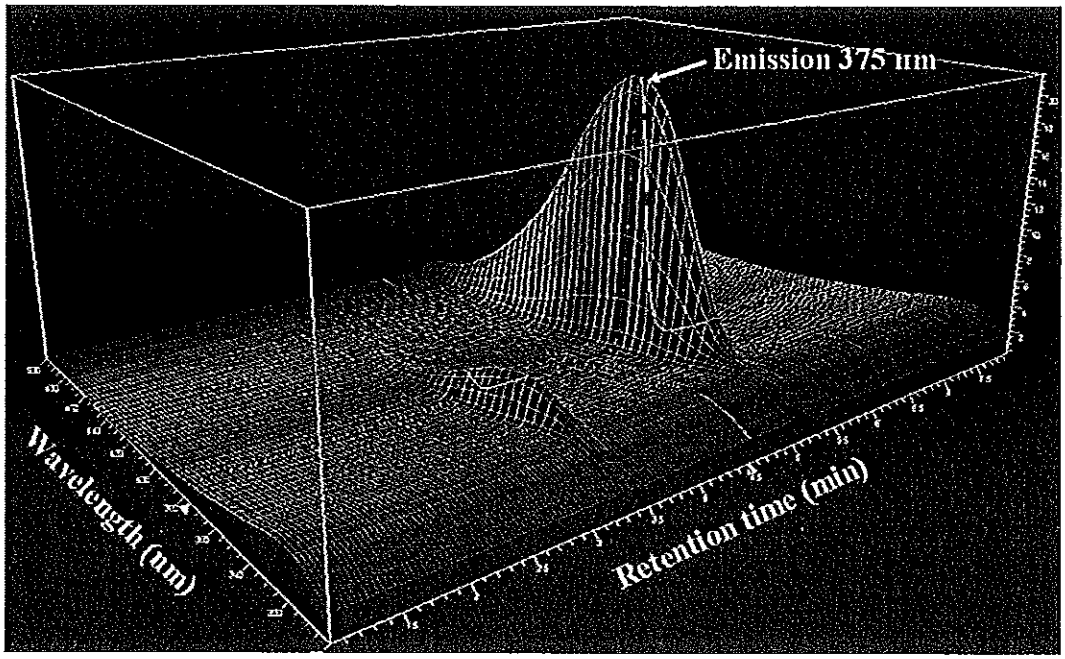
3.1 Optimization of HPLC-FLD conditions

3.1.1 Excitation (λ_{ex}) and Emission (λ_{em}) wavelengths

Determination of oxolinic acid was performed by reversed-phase liquid chromatography (RPLC) with fluorimetric detection (FLD) because it is mainly used for trace analysis (Prat *et al.*, 2004). Oxolinic acid is polar compound and highly fluorescent (Pouliquen and Armand, 2000). In order to obtain the highest fluorescence signals, the excitation and emission wavelength were optimized. Maximum excitation wavelength and emission wavelength were obtained at 265 nm (Figure 3.1(A)) and 375 nm (Figure 3.1(B)), respectively.



(A)



(B)

Figure 3.1 3D plotted of oxolinic acid standard solution, 0.5 mg L^{-1}. (A) Scanning excitation wavelengths. (B) Scanning emission wavelength.

3.1.2 Composition of mobile phase

A general problem for HPLC analysis on basic and polar substances are the severe peak broadening and tailing on reversed-phase columns, due to specific interactions of the bases with the support (Grushka and Grinberg, 2006). The retention of amphoteric compounds (oxolinic acid) is directly dependent on the organic modifier in the mobile phase and mobile phase pH (Ho *et al.*, 2004). The optimizations of the mobile phase composition were organic solvent and aqueous solution.

3.1.2.1 Organic modifier

The influence of organic modifier on the separation of oxolinic acid was studied on C18 column based on reversed phase high performance liquid chromatography (RP-HPLC), where the stationary phase is less polar than the mobile phase. C18 is packing materials with the alkane chains form a molecular "fur" on the silica gel granule (Figure 3.2). The separation based on reversed phase with a purely aqueous mobile phase is not the best results (Grushak and Grinberg, 2006). The alkane "fur" (the alkane groups; C18) needs amount of organic solvent in the mobile phase to help raise the alkane chains from a flat position and allow the free circulation of the solvent molecules. Methanol and acetonitrile are the most popular solvent used as organic modifiers in the mobile phase for reversed phase related to lipophilicity estimations (Grushak and Grinberg., 2006; Sun *et al.*, 2006). Oxolinic acid has both lipophilic and hydrophilic parts in the molecule that can interact with both chromatographic phase. Oxolinic acid interact with organic modifier aggregates and species that exist in the mobile phase with the lipophilic part positioned towards the stationary phase and the hydrophilic groups towards the mobile. In this study, the chromatographics parameters were obtained as shown in Table 3.1. The results confirmed that the organic modifier plays an important role in the retention of oxolinic acid, resulting in different retention time, peak shapes and a little effect on response. Comparisons of three organic modifiers

(Methanol, Methanol+Acetonitrile, Acetonitrile) indicated that peaks were more symmetrical by using acetonitrile than methanol and acetonitrile mixed with methanol. The reason for this was the acetonitrile has a weak hydrogen bond acceptor and usually has a greater impact on the solute salvation than methanol. Besides that acetonitrile also has a high dipole moment that can participate in selective dipole-dipole interaction with certain solute.

In hydro-organic mixtures, both methanol and acetonitrile can self-associate or associate with water molecules to form clusters but in different extent. Methanol is able to form hydrogen bonds (HB) by accepting or donating protons whereas acetonitrile is barely able to form hydrogen bonds. When used as mobile phases, this difference in hydrogen bonding capability influences the sorption of the modifier into the stationary phase as well solute partitioning (Sun *et al.*, 2006).

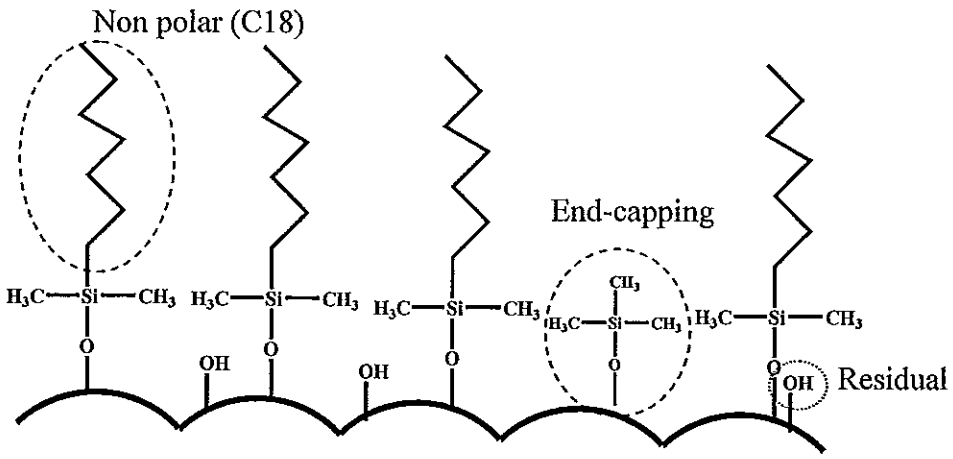


Figure 3.2 Stationary phase of reverse phase high performance liquid chromatography

Table 3.1 Chromatographic results of oxolinic acid using different organic modifiers

Mobile phase	Response (LU*s)	Retention time (min)	Symmetry
Methanol	170 ± 3	9.89 ± 0.04	0.22 ± 0.04
Methanol : Acetonitrile (1:1 v/v)	176± 2	7.46 ± 0.02	0.29 ± 0.01
Acetonitrile	179 ± 3	5.19 ± 0.02	0.54 ± 0.01

3.1.2.2 Percentage of organic modifier (Acetonitrile)

The amount of organic modifier in the mobile phase will have an influence on the retention of analytes that adsorped onto the stationary phase. The retention times of oxolinic acid at different percentages of acetonitrile are shown in Table 3.2 and Figure 3.3. At high percentage of acetonitrile, the retention time and the capacity factor decreased. The best result (high response, short analysis time and k'>0.5) was obtained at 45% of acetonitrile in the mobile phase. Although 50 % acetonitrile provided acceptable values it was not chosen since its capacity factor was very close to the limitation (0.5). Its retention time was also quite short and the peak would appear near the interference substance in real sample.

Table 3.2 Chromatogrphic results of oxolinic acid at different percentage of acetonitrile

Percentage	Response (LU*s)	Retention time (min)	Capacity factor (k')
30	149±4	10.520±0.005	1.640±0.005
35	175±4	7.680±0.004	1.200±0.003
40	178±2	6.330±0.005	0.970±0.007
45	183±2	5.200±0.006	0.800±0.004
50	179±2	4.590±0.008	0.590±0.005

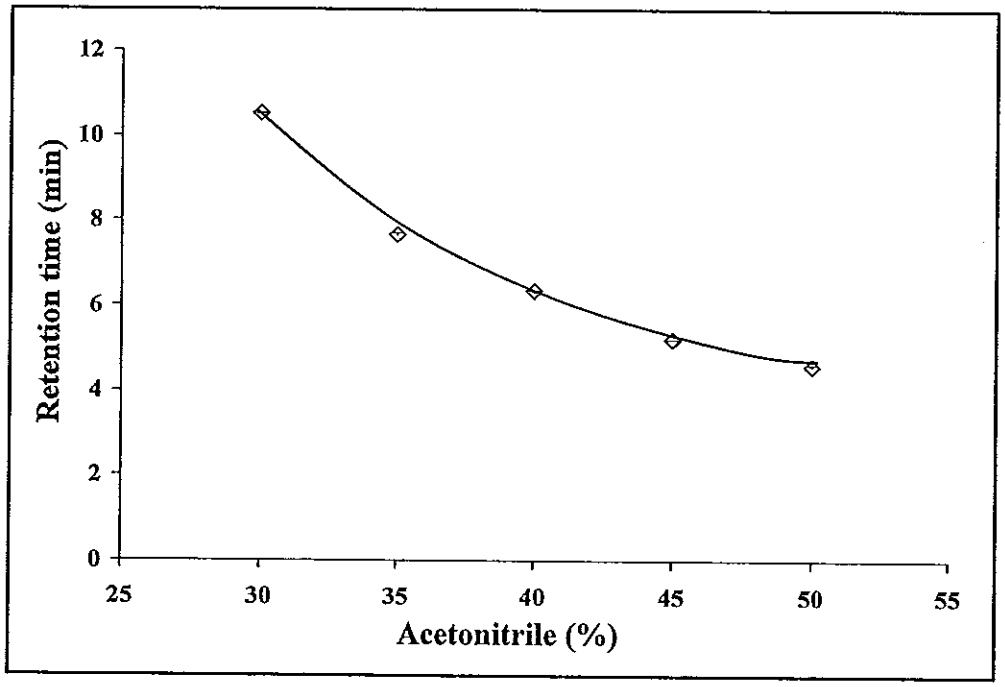


Figure 3.3 Retention time of oxolinic acid at various percentage of acetonitrile

3.1.2.3 pH of mobile phase

The severe peak broadening and tailing on reversed phase column are the general problem for HPLC analysis of basis and polar substances due to specific interactions of the bases with support. Oxolinc acid is ampholytic compound that could give a tailing peak in reversed-phase chromatography. The tailing in reversed phase liquid chromatography is caused by the residue silanol groups and metal impurities in traditional phase column materials. This makes it impossible to achieve the chromatographic separation suitable for residue analysis that can fulfil the requirement of short retention times and good peak shapes. This behavior can be reduced by mean of mobile phase with high acidity and ionic strength (Ramos *et al.*, 2003). Since, pH is one of the most powerful tools for reduced tailing. Therefore pH of mobile phase was optimized for the best peak shape.

A change in mobile phase pH primarily affects the ionization of acidic or basic solutes, with predictable changes in retention based on the Henderson-Hasselbach equation (Wilson *et al.*, 2002). In this work, pH of mobile phase (2.5-4.5) was evaluated and the results are shown in Table 3.3 where increasing pH caused tailing factor to increase. In view of this low pH should be used however, low pH can also cause the loss of organic ligands from a silica surface by solvolysis and subsequent breaking the Si-O-Si bond is the main cause of column deterioration (Claessens and van Straten, 2004). Therefore, pH 3.5 was chosen as the optimum pH of mobile phase to prolong column life.

Table 3.3 Effect of pH of mobile phase

pH	Response (LU*s)	Tailing factor (T) ± SD
2.5	179 ±2	1.28 ± 0.02
3.0	184 ±1	1.36 ± 0.04
3.5	181 ±2	1.41 ± 0.04
4.0	165±2	1.58 ± 0.04
4.5	161±2	1.77 ± 0.09

3.1.2.4 Buffer concentration

As a consequence of the presence of acidic and basic functional groups, oxolinic acid is prone to chemical tailing due to interactions with free silanols in stationary phase (Maraschiello, 2001). Since oxolinic acid shows strong fluorescence in aqueous acid solutions (Delépée and Pouliquen, 2002) aqueous oxalic acid was used to increase the specificity and sensitivity of fluorescence detection that will help to improve the peak shape by masking the residual silanol groups of the stationary phase. Oxalic acid buffer concentration in the mobile phase was tested. Table 3.4 shows the response and tailing factor at difference concentration of oxalic acid. The optimum concentration was chosen as a compromise between high response, good peak shape and also less amount of chemical. The results showed that 10 mM oxalic buffer solution can improve the peak shapes and suppress the tailing phenomena of chromatographic peaks of oxolinic acid.

Table 3.4 Effect of oxalic acid buffer concentration

Oxalic buffer concentration (mM)	Response (LU*s)	Tailing factor (T) ± SD
5	167.4±0.8	1.60±0.06
10	180.7±0.6	1.41±0.04
15	182.3±0.5	1.40±0.03
20	192.5±0.3	1.42±0.05

3.1.3 Flow rate

The column plate number (N) is primarily dependent on column quality but it can be varied by changing flow rate (Snyder *et al.*, 1997). The quantity N is proportional to column length L and can be expressed as $N = L/HETP$, $HETP$ is height equivalent of a theoretical plate. The optimum flow rate (highest column efficiencies) is a maximum plate number and minimum plate height (H) or $HETP$. The theory of the hydrodynamic of chromatography deals with the relationship of the $HETP$ with the flow dynamics and the properties of the stationary phase (Neue, 1997). When plot the $HETP$ against a linear velocity (Figure 3.4), can be described by van Deemter equation

$$HETP = A + \frac{B}{u} + C_m u + C_s u$$

Where $HETP$ is the height equivalent to a theoretical plate, u is the mean velocity of the mobile phase (in fact the interstitial velocity in packed column)

The A term represents the dispersive contribution from the flow profile (Eddy diffusion). The mobile phase moves through the column which is packed with stationary phase. Solute molecules will take different paths through the stationary phase at random (Figure 3.5a). This cause broadening of the solute band, because

different paths are of different lengths, B term arises from axial molecular diffusion; the concentration of analyte is less at the edges of the band than at the center. Analyse diffuses out from the center to the edges (Figure 3.5b). This causes band broadening. If the velocity of the mobile phase is high then the analyte spends less time on the column, which decreases the effects of longitudinal diffusion and C terms are mass transfer terms in mobile phase (C_m) and stationary phase (C_s). The analyte takes a certain amount of time to equilibrate between the stationary and mobile phase (Figure 3.5c-e). If the velocity of the mobile phase is high, and the analyte has a strong affinity for the stationary phase, then the analyte in the mobile phase will move ahead of the analyte in the stationary phase. The band of analyte is broadened. The higher the velocity of mobile phase, the worse the broadening becomes.

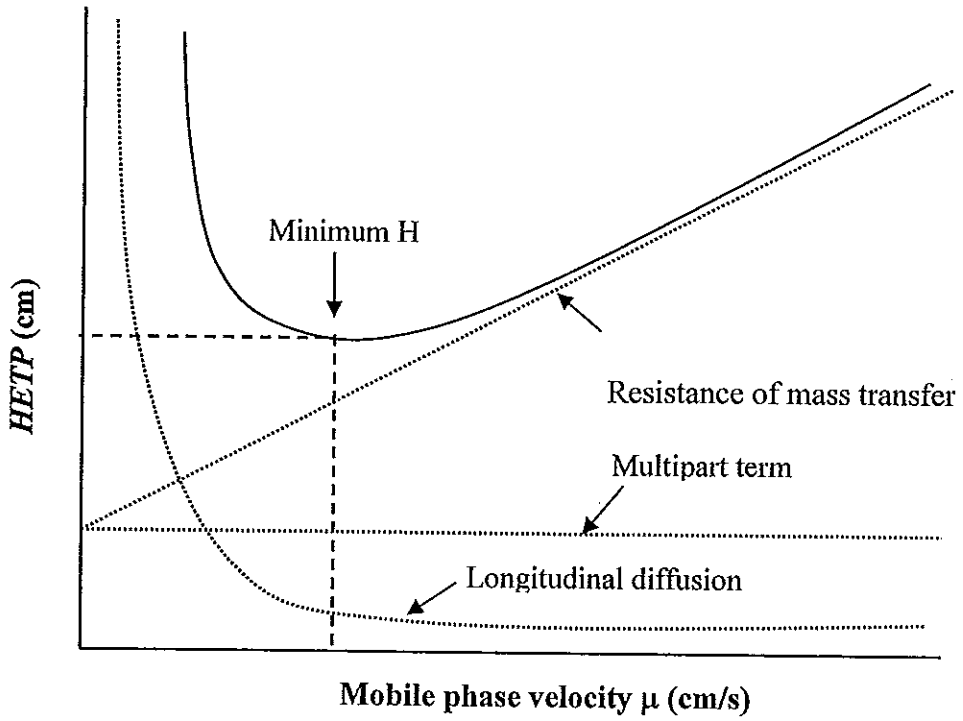


Figure 3.4 A typical van Deemter plot

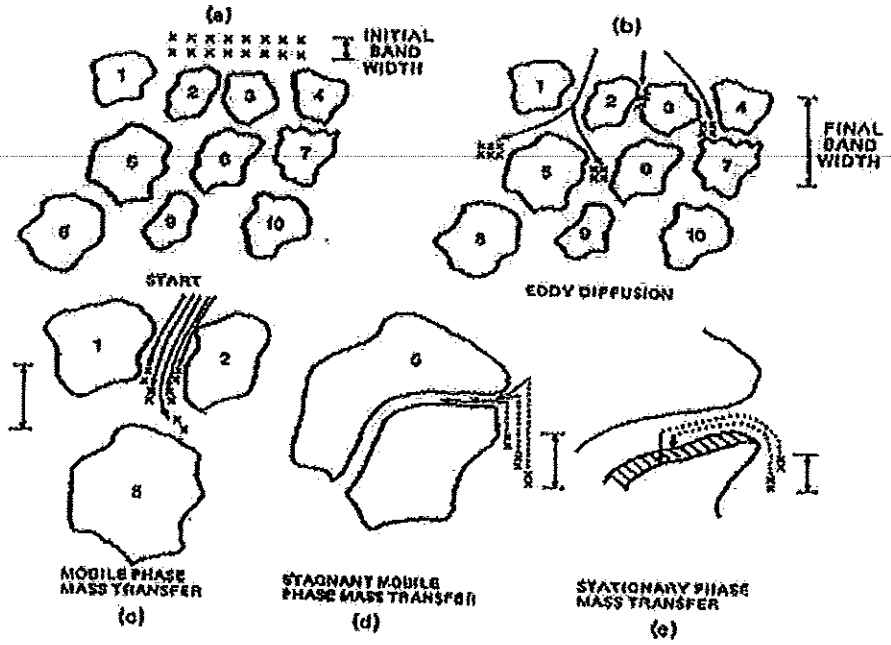


Figure 3.5 Contributions to molecular spreading in LC (Snyder and Kirkland, 1979)

In this work, a silica base C18 (Pinnacle II C18), 250× 4.6 mm I.D. with particle size 5 μm was used in analysis and the results are shown in Table 3.5. The lowest *HETP* is the flow rate 0.7 mL min^{-1} and columns efficiency decreases when flow rate increases (Figure 3.6). However, the retention time also decreased (Figure 3.7). In the general HPLC columns are operated at flow rates higher than the optimum, because higher flow rates allow shorter run times (Snyder *et al.*, 1997). Considering efficiency and analysis time, 0.9 mL min^{-1} was selected as optimal.

Table 3.5 Retention time and plate height (*HETP*) of oxolinic acid at various mobile phase flow rate

Flow rate (mL min^{-1})	**Retention time (min)**	***HETP*×10^{-3} (cm)**
0.7	6.57 ± 0.03	2.96
0.8	5.72 ± 0.01	3.06
0.9	5.11 ± 0.01	3.22
1.0	4.57 ± 0.01	3.40
1.1	4.16 ± 0.01	3.70
1.2	3.81 ± 0.01	4.02

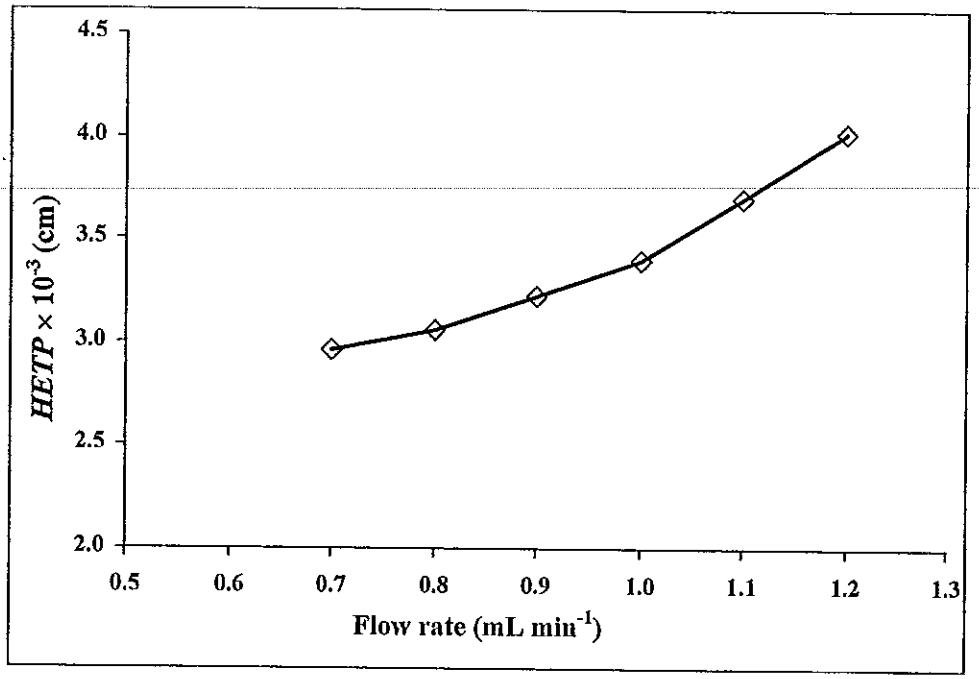


Figure 3.6 van Deemter plot of oxolinic acid

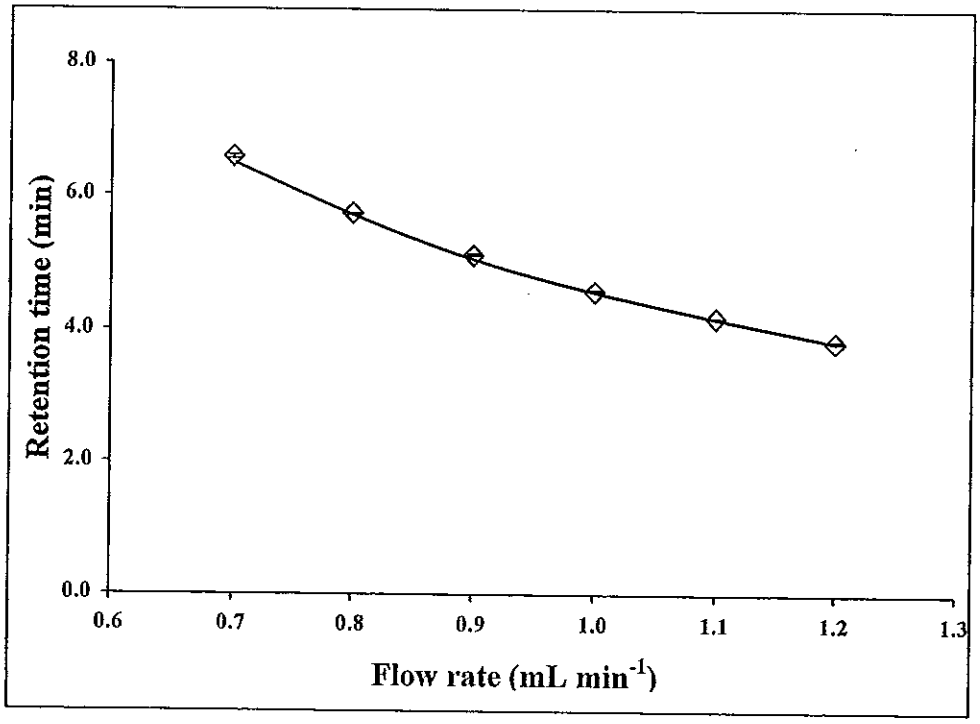


Figure 3.7 Retention time of oxolinic acid at various flow rates

3.1.4 Temperature

Temperature is a factor used to control retention in isocratic reversed-phase liquid chromatography (Kiridena *et al.*, 2004). The contribution of temperature to the retention is mainly given by the enthalpy term of the van't Hoff equation for the retention factor (Greibrokk and Andersen, 2003; Vanhoenacker and Sandra, 2005),

$$\ln k = -\Delta H / RT + \Delta S / R + \ln \beta$$

where ΔH is the enthalpy change associated with the transfer of the solute between phases, ΔS is the corresponding entropy change, R is the molar gas constant, T is the absolute temperature and β is the phase ratio of the column.

Therefore, optimization of column temperature has been considered since it is related to retention and the results are shown in Table 3.6. The increase in temperature reduced the capacity factor and retention time (Figure 3.8) that are the results of a decrease in solvent viscosity, increase in diffusion coefficient and sorption-desorption kinetics as temperature increase (Greibrokk and Andersen, 2003). This enhances the mass-transfer rate between the mobile phase and stationary phase (Neue, 1997). Elevated temperatures usually improve plate heights and the reduction in viscosity allows the increase in flow rates to speed the analysis (Castells *et al.*, 2004). However, the major drawback of the column high temperature is the risk of stationary phase degradation because bonded silicas are largely thermal unstable (Castells *et al.*, 2004). In this work the optimum temperature was chosen at 25 °C as a compromise between the response, analysis time and capacity factor. Small changes in column temperature did not effect the elution, but higher increments of 5 °C caused shortening of the eluting times.

Table 3.6 Effect of column temperature

Temperature (°C)	Response (LU*s)	Retention time (min)	Capacity factor (k')
25	178 ± 1	5.11 ± 0.01	0.826 ± 0.005
27	162 ± 3	5.08 ± 0.02	0.814 ± 0.007
30	157 ± 3	5.04 ± 0.01	0.799 ± 0.004
35	147 ± 5	4.94 ± 0.01	0.765 ± 0.003
40	140 ± 5	4.48 ± 0.01	0.736 ± 0.003

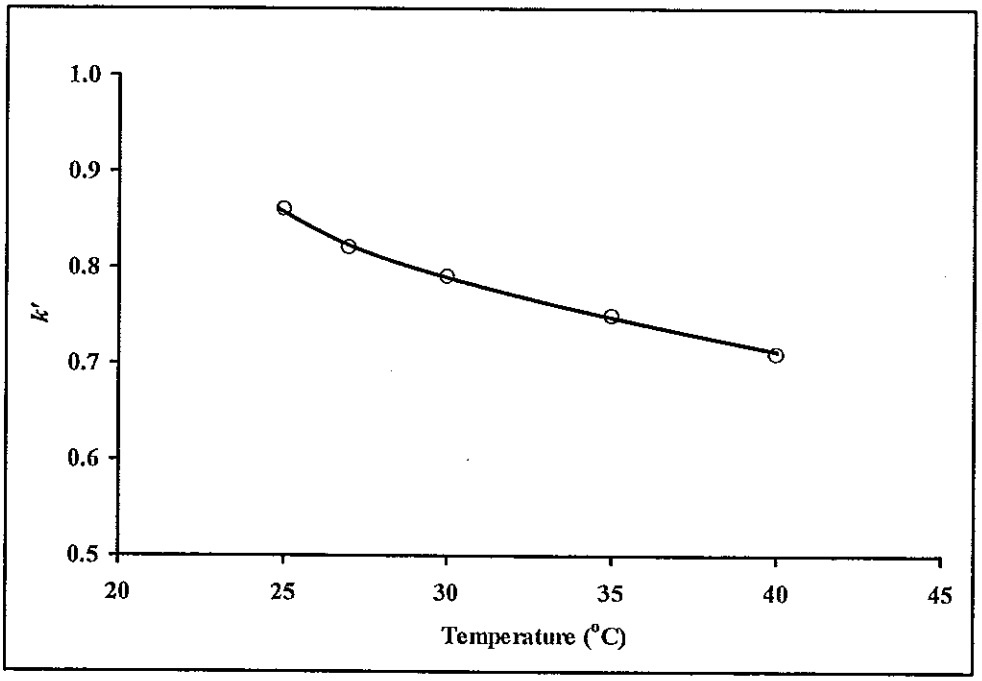


Figure 3.8 Capacity factor of oxolinic acid at various column temperatures

All parameters are summarized in Table 3.7 and Figure 3.9 shows the chromatogram of oxolinic acid standard at 50 ng mL^{-1}

Table 3.7 Optimum conditions of chromatographic conditions

Factor	Optimum value
Detection wavelength	Excitation : 265 nm Emission : 375 nm
Mobile phase A	Acetonitrile (ACN)
Mobile phase B	Oxalic buffer 10 mM
Ratio A:B	45:55
pH of mobile phase	3.5
Flow rate	0.9 mL min^{-1}
Temperature	25 °C

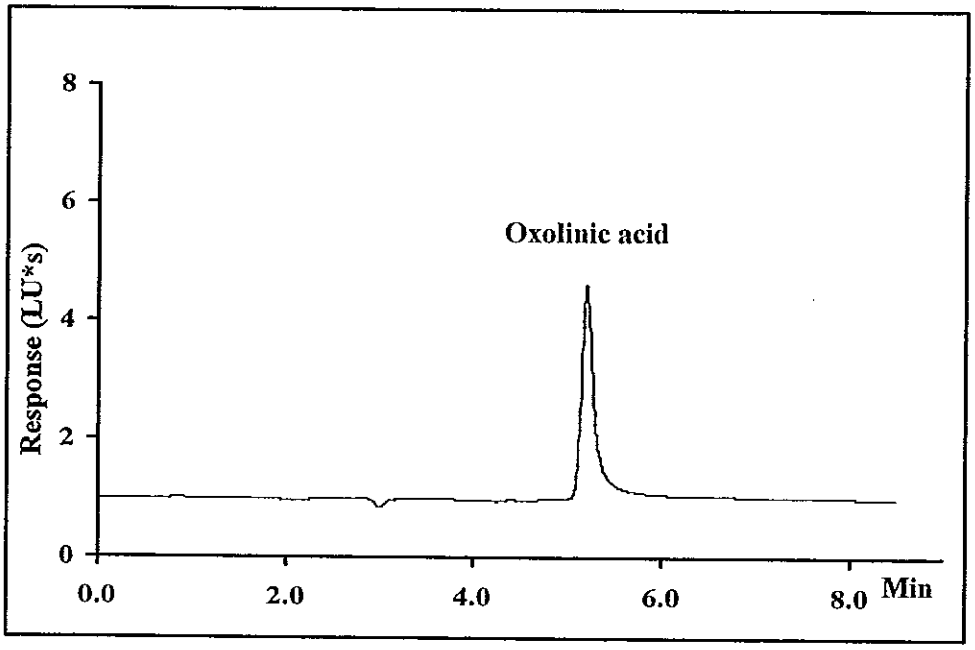


Figure 3.9 Chromatogram of oxolinic acid at 50 ng mL^{-1} under the optimum condition

3.2 System performance of HPCL-FLD

3.2.1 System suitability tests

Prior to the analysis of sample, the HPLC system must be tested to ensure that it can generate results of acceptable accuracy and precision. The results of system suitability testing are shown in Table 3.8.

The capacity factor obtained is within the accepted values, more than 0.5 (Snyder *et al.*, 1997).

The column efficiency was evaluated by considering the plate number. The obtained plate number is more than the recommend value of ICH.

Peak asymmetry is also important for precise peak integration and thus for quantitative information. In this work the tailing factor was within the limits established by ICH guidelines.

The % RSD of retention time and peak area of ten replicate injections of standard solution is normally accepted as one of standard criteria (Snyder *et al.*, 1997). The standard solution of oxolinic acid (50 ng g^{-1}) was injected to the HPLC system under the optimum conditions. The %RSD of the retention time was less than 1% (0.39%) and 4% (1.1%) for peak area (Table 3.9) and these are within the acceptable value of precision (Snyder *et al.*, 1997).

Therefore, the system under optimum conditions can be applied to its intended purpose.

Table 3.8 System suitability tests

Parameter	Recommendation	This work
Capacity factor (k')	$0.5 < k' < 20$	0.912 ± 0.004
Plate (N)	> 2000	7522 ± 113
Tailing factor (T)	≤ 2	1.39 ± 0.03
RSD (Peak area)	<4	1.1
RSD (Retention time)	<1	0.39

Table 3.9 RSD of retention time and peak area of oxolinic acid standard solution at 50 ng g^{-1}

No.	Retention time (min)	Peak area (LU*s)
1	5.16	17.6
2	5.17	17.6
3	5.15	17.5
4	5.15	17.5
5	5.14	17.6
6	5.14	17.9
7	5.12	17.6
8	5.14	17.8
9	5.14	17.9
10	5.12	17.9
Average	5.14	17.7
SD	0.02	0.2
RSD (%)	0.39	1.1

3.2.2 Linear dynamic range

The linear range of HPLC-FID system is the range for which the analytical signal is directly proportional to the amount of analyte present. The system showed a wide linear dynamic range (Table 3.10), 1 ng mL^{-1} to 8 μg mL^{-1} with a coefficient of determination higher than 0.999 (Figure 3.10). Concentration higher than 8 μg mL^{-1} showed bad peak shape.

Table 3.10 Response of oxolinic acid at various concentrations

Concentration (ng mL^{-1})	Response (LU*s) ± SD
1	0.31 ± 0.01
2	0.54 ± 0.04
5	1.41 ± 0.06
10	2.8 ± 0.2
20	6.41 ± 0.02
50	15.2 ± 0.2
100	34.3 ± 0.2
200	66.6 ± 0.6
500	160.4 ± 0.7
1000	336 ± 1
2000	687.6 ± 0.6
5000	1725 ± 2
8000	2820 ± 11

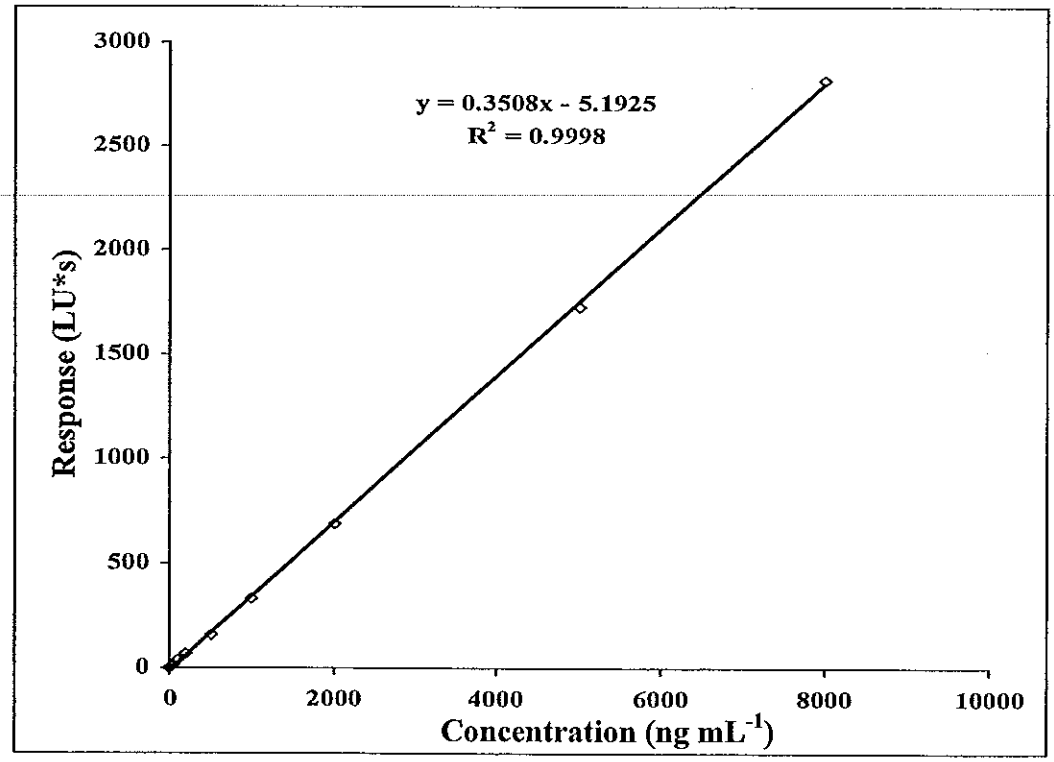


Figure 3.10 Linear dynamic range of oxolinic acid by HPLC-FLD system

3.2.3 Limit of detection

The limit of detection of the HPLC-FLD system was evaluated based on the standard deviation of y-intercepts of regression lines (Ribani *et al.*, 2007) and was found to be 1.03 ng mL^{-1} (Table 3.11, Figure 3.11)

Table 3.11 Limit of detection of oxolinic acid based on standard deviation of y-intercepts of regression line.

Concentration (ng mL^{-1})	**Response (LU*s) ± SD**
1	0.31 ± 0.01
2	0.54 ± 0.04
5	1.41 ± 0.06
10	3.0 ± 0.2
20	6.41 ± 0.02
50	15.2 ± 0.2
Equation	y = 0.3064x-0.0219
R^2	0.999
S_{a*}	0.0954
$S_{y/x**}$	0.177
LOD	1.03 ng mL^{-1}

* Standard deviation of the y intercept of regression line

** Residual standard deviation of the regression line

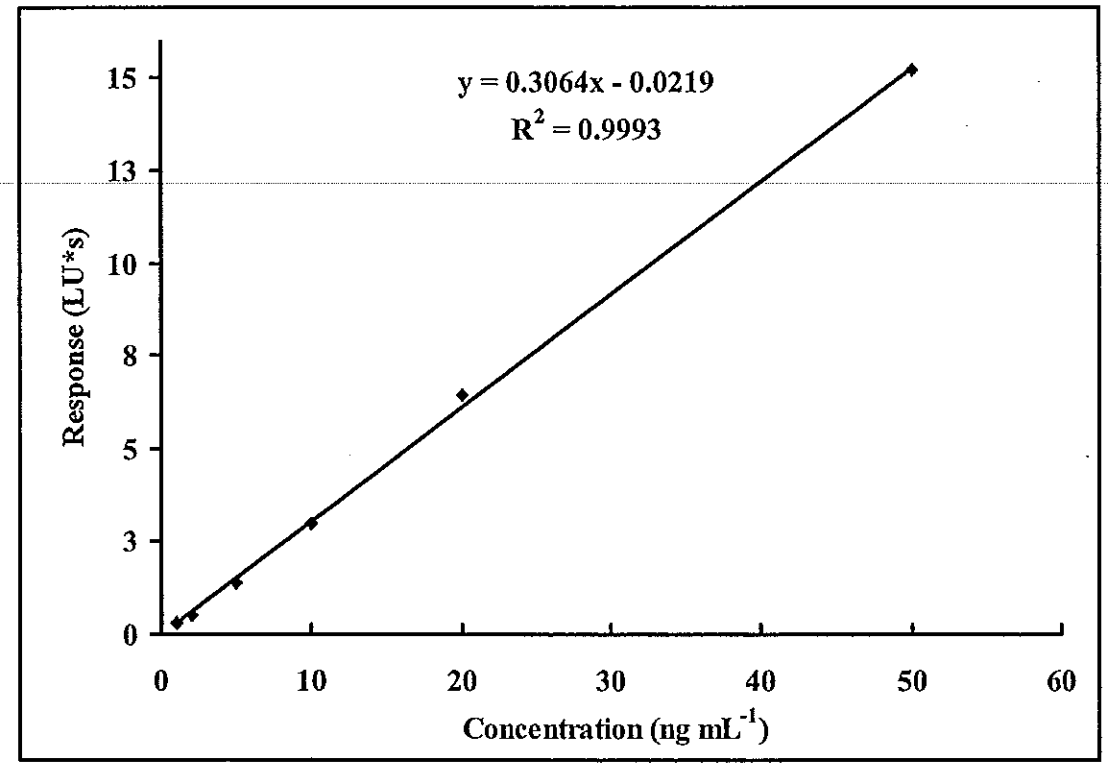


Figure 3.11 Calibration curve of oxolinic acid for calculation detection limit

3.3 Sample preparation

3.3.1 Evaluation of spiking of sample

The spiking sample was evaluated to ensure that all oxolinic acid were adsorbed in the sample at a suitable prolong time before extraction. The responses obtained from oxolinic acid at various prolong time are not significant different (Table 3.12 and Figure 3.12). Thus, 30 min was fixed as the time to wait before extraction samples. Shorter prolong time (<30 min) was not considered because might be some of oxolinic acid are not adsorbed in the sample which caused effect to the accuracy of the method.

Table 3.12 Response of oxolinic acid at various prolong times

Spike sample prolong time (h)	Response (LU*s) ±SD
0.5	8.7 ± 0.5
1	9.0 ± 0.4
3	8.9 ± 0.4
5	8.8 ± 0.5
12	8.8 ± 0.8
24	8.9 ± 0.3

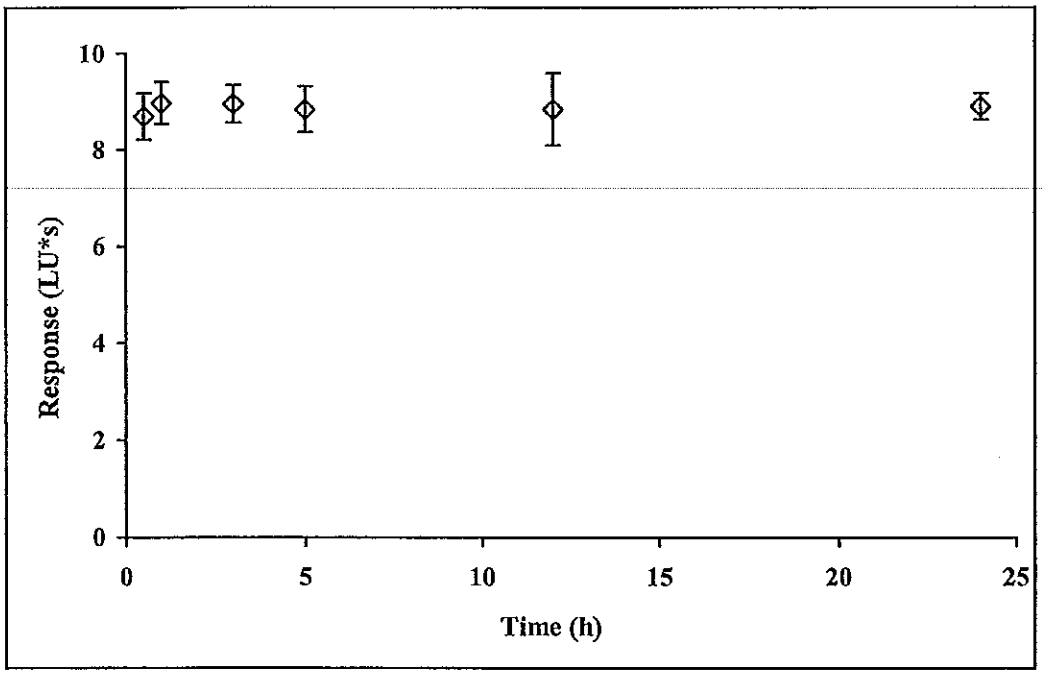


Figure 3.12 Response of oxolinic acid at various waiting time before extraction

3.3.2 Optimization of ultrasonic extraction

3.3.2.1 Extraction solvent

Ultrasonic extraction was used to enhance extraction due to the possibility of selecting the solvent type to obtain maximum extraction efficiency and selectivity (Ridgway *et al.,* 2007). Since, quinolones (oxolinic acid) is soluble in polar organic solvents (Hernández-Arteseros *et al.*, 2002) and is also a lipophilic molecule (Loussouarn *et al.*, 1997), thus in the optimization of extraction solvent, different medium polarity solvents include ethyl acetate, acetonitrile, dichloromethane, acetone and methanol were used for extraction of oxolinic acid. The polarity index and solubility in the water of studied solvents are shown in Table 3.13 and the results are shown in Table 3.14 and Figure 3.13. Results indicated that ethyl acetate is a better solvent for extracting oxolinic acid followed by acetone, acetonitrile, dichloromethane and methanol. Ethyl acetate also has less interference from the matrix (Figure 3.14).

Table 3.13 Polarity index and solubility in water of studied solvents

Solvent	Polarity index	Solubility in water (%w/w)
Dichloromethane	3.1	1.6
Ethyl acetate	4.4	8.7
Acetone	5.1	100
Methanol	5.1	100
Acetonitrile	5.8	100

Table 3.14 Effect of various extraction solvent on the response of oxolinic acid

Solvent	Response (LU*s) ± SD
Ethyl acetate	8.5 ± 0.7
Acetonitrile	6.2 ± 0.4
Dichloromethane	5.7 ± 0.6
Acetone	6.2 ± 0.5
Methanol	5.6 ± 0.6

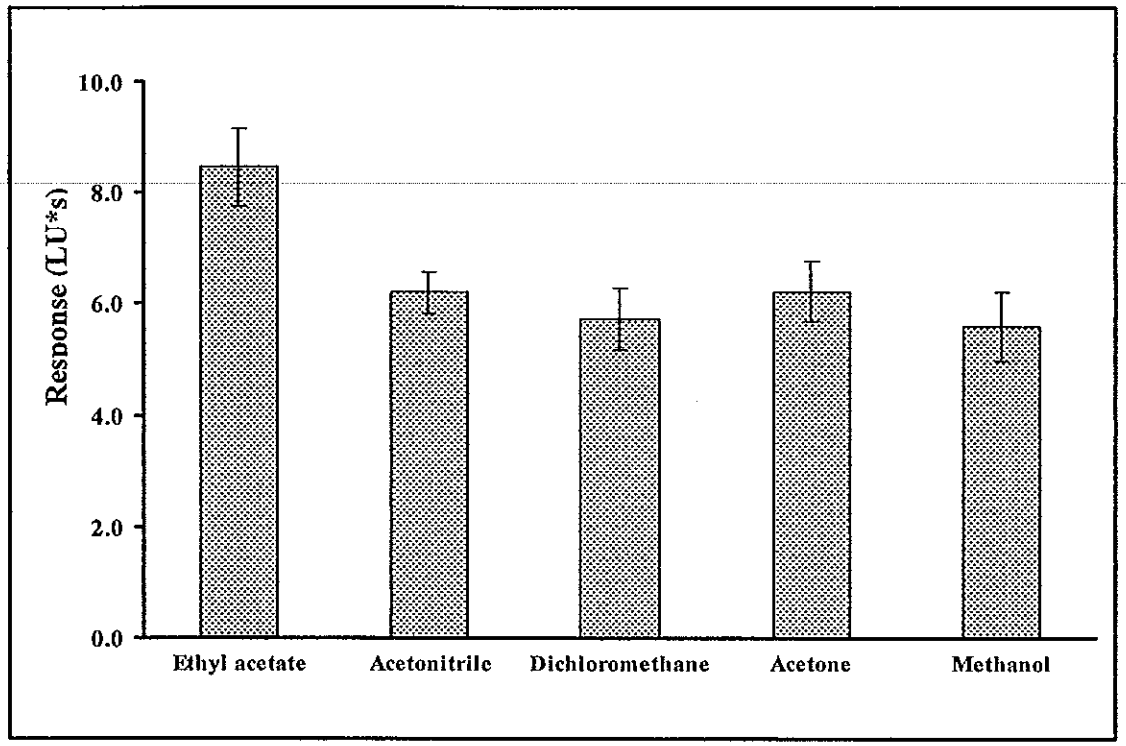


Figure 3.13 Responses of oxolinic acid at various extraction solvent

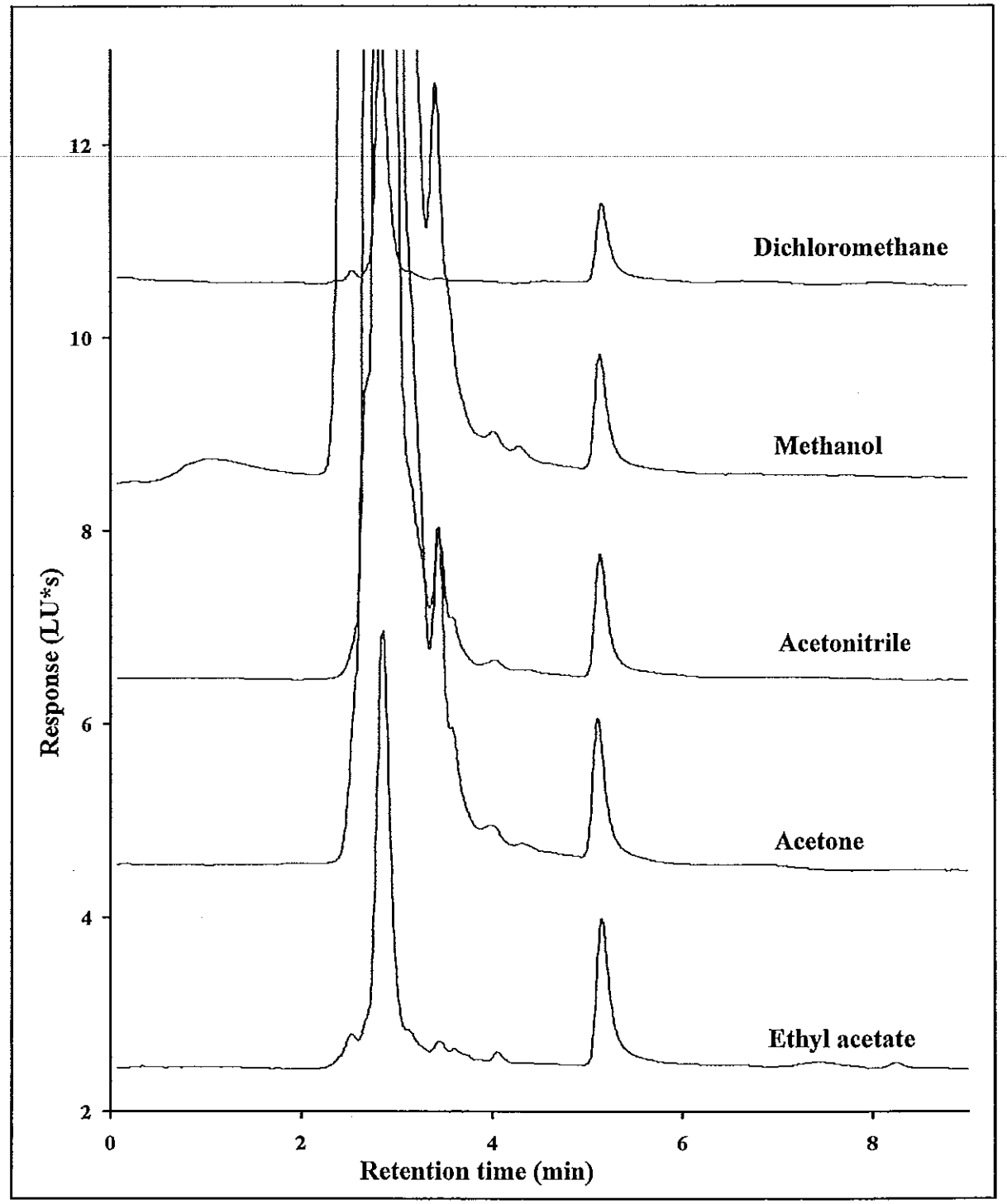


Figure 3.14 Chromatogram of oxolinic acid at various extraction solvents for evaluation of selectivity

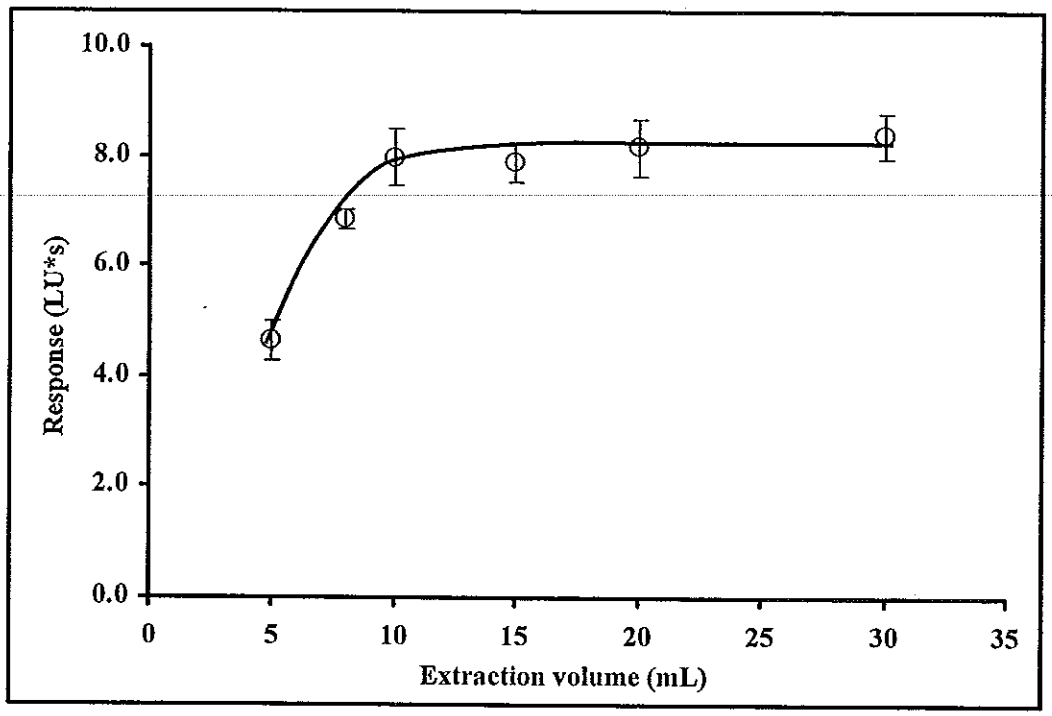


Figure 3.15 Response of oxolinic acid at various solvents volume

3.3.2.3 Extraction time

The optimization of extraction time was to improve the extraction efficiency with minimum time consumption. The results (Table 3.16 and Figure 3.16) showed that the response increased with the extraction time to 15 min and started to become constant. Therefore, 15 min was selected because it was the least time that gave the highest extraction efficiency.

Table 3.16 Extraction efficiency of various extraction times on the response of oxolinic acid extraction

Extraction time (min)	Response (LU*s)
5	5.8 ± 0.2
10	6.8 ± 0.2
15	7.8 ± 0.2
20	8.0 ± 0.5
30	7.9 ± 0.4
40	7.9 ± 0.5
50	8.0 ± 0.6

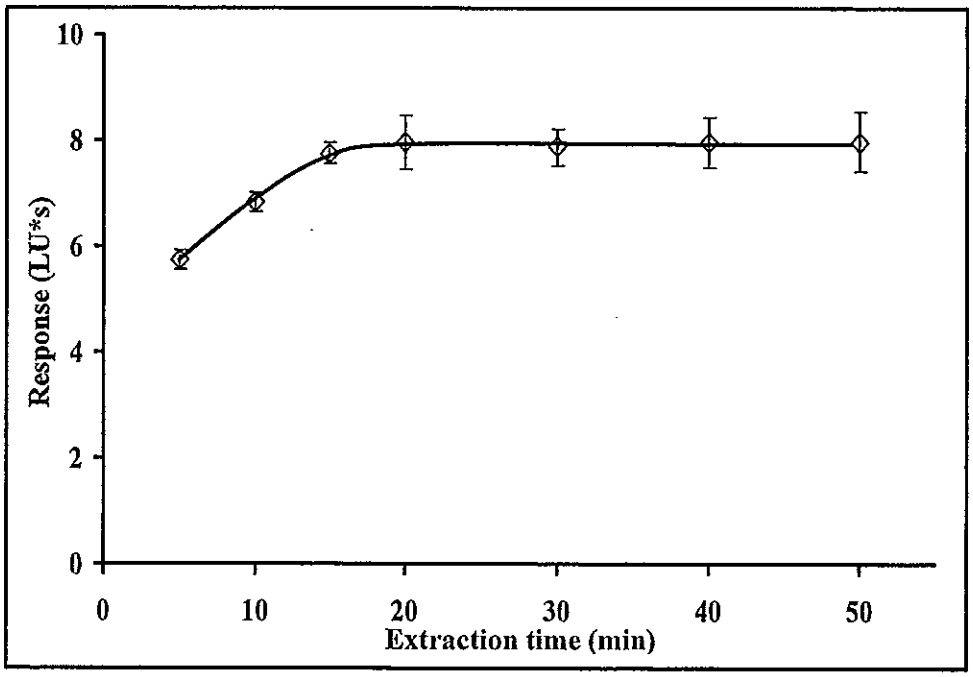


Figure 3.16 Response of oxolinic acid at various extraction time

3.3.2.4 Stability of oxolinic acid under ultrasonic condition

After the optimum conditions of ultrasonic extraction were obtained. The stability of oxolinic acid under this condition was investigated to ensure that oxolinic acid is stable under ultrasonic extraction. The sonication time was varied from 5 min to 60 min at 45 °C. The results (Table 3.17 and Figure 3.17) indicated that no obvious loss was observed within 5-60 min of sonication. Therefore, oxolinic acids were stable under the optimum condition of ultrasonic extraction procedure.

Table 3.17 Stability of oxolinic acid under ultrasonic condition at various sonication times

Time	Recovery ± SD
5	98.2 ± 0.7
10	99.8 ± 0.8
15	97 ± 1
20	101 ± 1
30	99.6 ± 0.5
60	98.0 ± 0.5

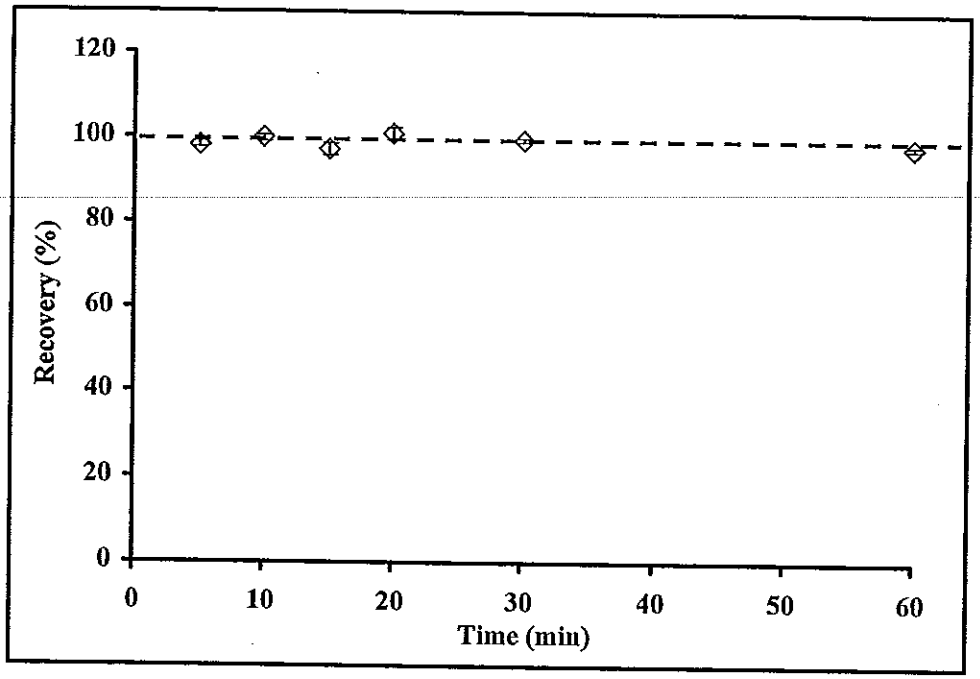


Figure 3.17 Recovery of oxolinic acid under the optimum condition of ultrasonic extraction at various sonication times

3.3.3 Optimization of solid phase extraction

3.3.3.1 Type of sorbent

A comparative study of four sorbents were carried out to achieve the maximum response of oxolinic acid and optimal clean up efficiency, which would provided the methods with suitable limits of detection. The results indicated that Supelclean LC-18 provided the highest response (Table 3.18 and Figure 3.18), due to the different in physical properties of the sorbent that affect the extraction efficiency, *i.e.,* particle size, pore size and percent carbon load (Table 3.19).

Table 3.18 Response of oxolinic acid at each type of sorbent

Type of sorbent	Response (LU*s) ±SD
Supelclean LC-18	12 ± 1
Supelclean EN-18	10 ± 1
Sepra C18-E	10.7 ± 0.7
Oasis HLB	8.3 ± 0.5

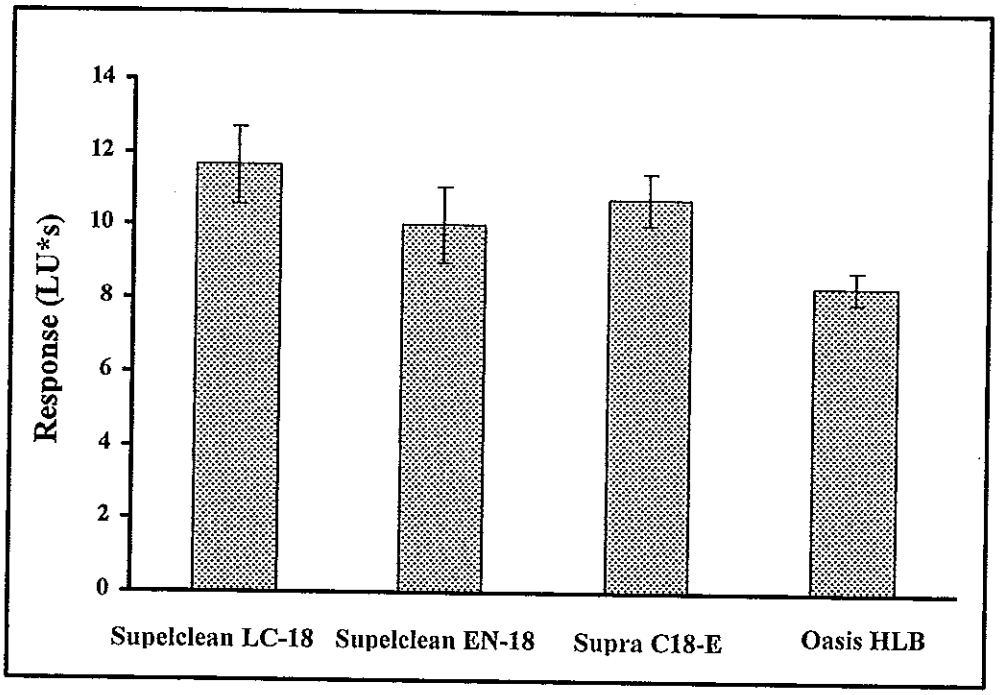


Figure 3.18 Response of oxolinic acid at each type of sorbent

Table 3.19 SPE sorbents properties

Sorbent	%C	Particle size (μm)	Pore size (nm)	End-capped	Solid phase
Supelclean LC-18	10	40-45	6	Yes	C18 (monofunctional silane)
Supelclean EN-18	17	45	6	Yes	C18 bonded to silica
Sepra C18-E	17	50	6.5	Yes	C18 bonded to silica
Oasis HLB	-	30	8	-	Poly (divinylbenzene)-co-N-vinyl pyrrollidone copolymer

3.3.3.2 Type of eluting solvent

Since non-polar sorbents retain the uncharged quinolones when dissolved in a polar solvent (Hernandez-Arteseros *et al.*, 2002) Supelclean LC18 was used as the SPE sorbent to retain oxolinic acid. Oxolinic acid was then eluted with a suitable eluting solvent. Literature refers to SPE reverse phase but amphoteric compounds bind strongly to free silanol groups, and they can not be elute complete with organic solvent (Babić *et al.*, 2006). To overcome this problem acetonitrile combined with various acid solutions (25:75 %) were evaluated. The results in Table 3.20 and Figure 3.19 indicated that trifluoroacetic acid combined with acetonitrile provided the highest response. In order to use minimum solvent consumption percentage of trifluoroacetic acid was also investigated and the results in Table 3.21 and Figure 3.20 indicated that 3% trifluoroacetic acid is suitable.

Table 3.20 Response of oxolinic acid at various 3% acidic eluting solvents

Type of acidic solution (3%)	Response (LU*s) ± SD
Phosphoric acid	9 ± 1
Citric acid	8.4 ± 0.7
Formic acid	15 ± 1
Acetic acid	12 ± 2
Trifluoroacetic acid	17 ± 1

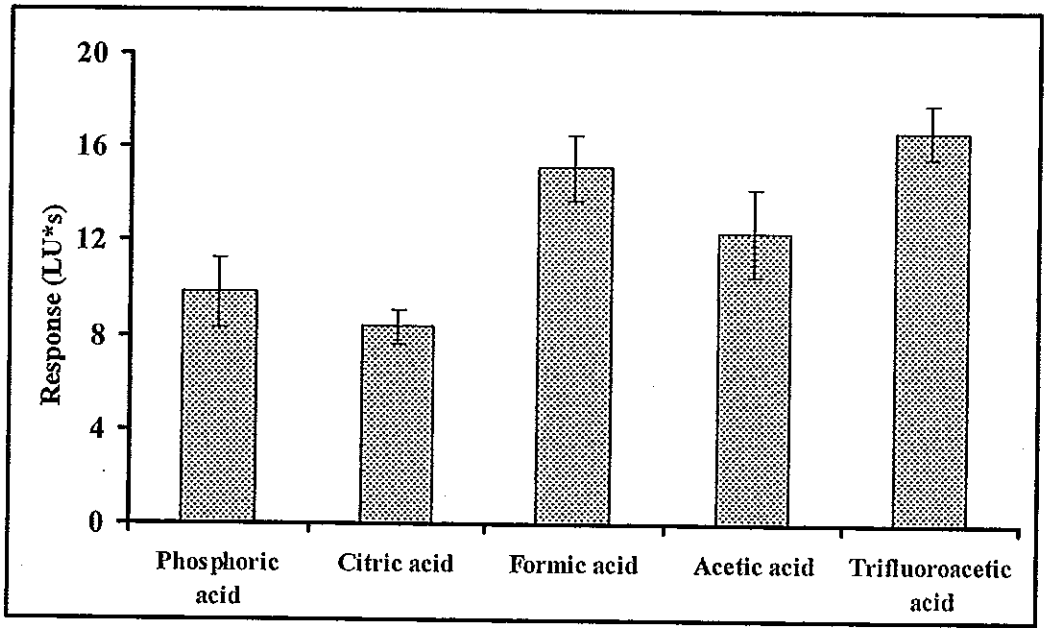


Figure 3.19 Response of oxolinic acid at various eluting solvent

Table 3.21 Response of oxolinic acid at various percentage of trifluoroacetic acid in eluting solvent

Trifluoroacetic acid (%)	Response (LU*s) ± SD
0.2	8.8 ± 0.5
0.4	11 ± 1
1.0	14 ± 1
2.0	17 ± 1
3.0	17.1 ± 0.9
4.0	17.0 ± 0.6
5.0	17.0 ± 0.7

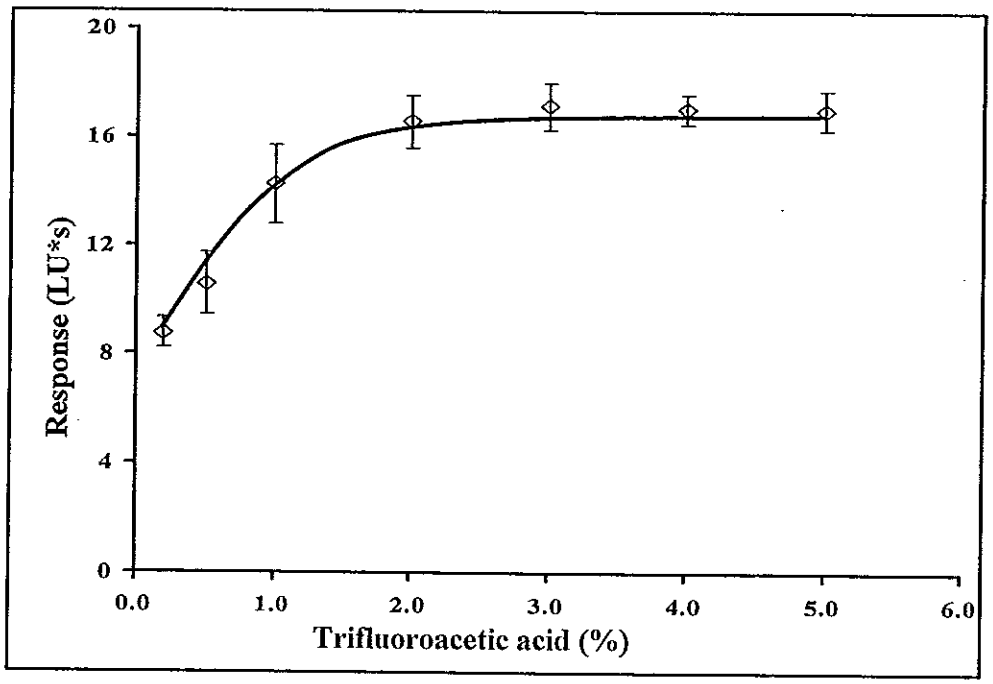


Figure 3.20 Response of oxolinic acid at different % trifluoroacetic acid

3.3.3.3 Sample flow rate

In the solid-phase extraction system, the flow rate of the sample solution is one of the most important parameters which affects the recoveries of analytes and also controls the time of analysis. It was found that flow rates up to 4 mL min^{-1} (the maximum flow rate of vacuum pump) for sample loading on the cartridge had no effect on the recoveries as shown in (Table 3.22) and (Figure 3.21). Therefore, 4 mL min^{-1} was adopted as the flow rate of sample solutions.

Table 3.22 Response of oxolinic acid at various sample flow rate

Flow rate (mL min^{-1})	**Response (LU*s)± SD**
0.2	12.5 ± 0.4
0.6	13 ± 1
1.0	13 ± 2
2.0	14 ± 1
3.0	13.1 ± 0.9
4.0	13 ± 1

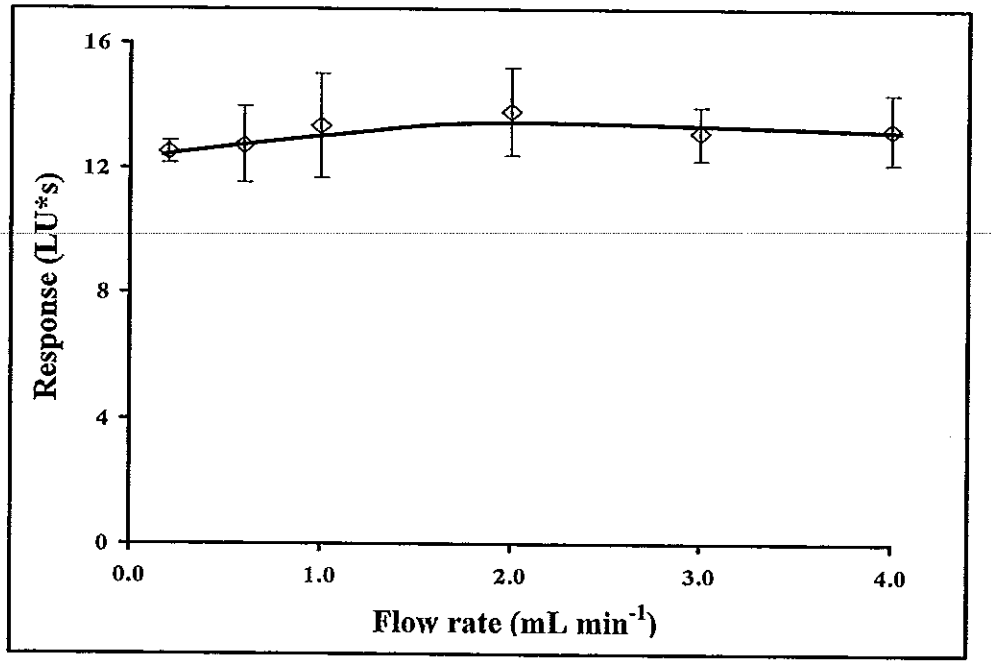


Figure 3.21 Response of oxolinic acid at different sample flow rate

3.3.3.5 Volume of eluting solvent

The volume of eluting solvent in solid phase extraction (SPE) technique is also an important parameter because it will affect the extraction efficiency. If the volume is too small it may not be sufficient to elute all the analyte from the sorbent but if it is too large it will increase waste and cost. Therefore, it was necessary to optimize the volume of eluting solvent in order to minimize cost and waste and at the same time maintained the highest response. In this study, Supelclean LC-18 sorbent was used to retain oxolinic acid for clean-up. Then, oxolinic acid was eluted with acetonitrile-3% trifluoroacetic acid (75:25, v/v) using various volume. The results are shown in Table 3.23 and Figure 3.22, 3 mL gave the highest response and was selected as the appropriate solvent volume to elute oxolinic acid from the sorbent.

Table 3.23 Effect of volume of eluting solvent on the response of oxolinic acid

Eluting solvent volume (mL)	Response (LU*s) ± SD
1.0	13.6 ± 0.8
2.0	15.2 ± 0.4
3.0	16.1 ± 0.4
4.0	16.0 ± 0.2
5.0	16.2 ± 0.4

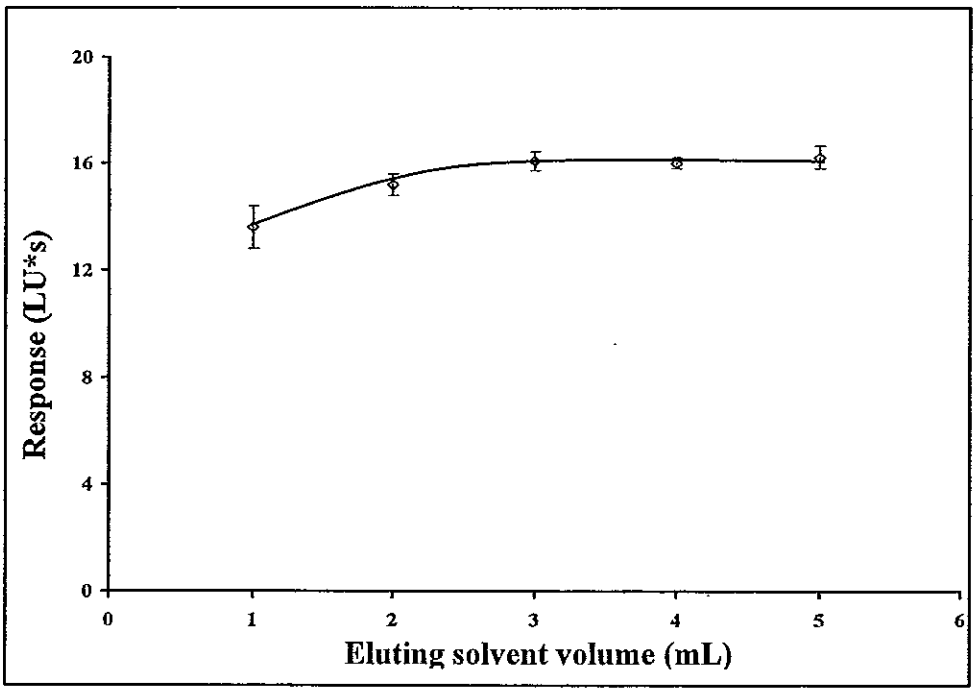


Figure 3.22 Response of oxolinic acid at various volumes of eluting solvent

3.3.3.6 Flow rate of eluting solvent

The effect of eluting flow rates are shown in Table 3.24 and Figure 3.23. The result indicated that 1.0 mL min^{-1} provided the highest response but did not deffer from 2.0 mL min^{-1}. Considering the reproducibility and minimization of the analysis time, 2.0 mL min^{-1} was selected as the optimum flow rate.

Table 3.24 Effect of flow rate of eluting solvent on the response of oxolinic acid

Eluting flow rate (mL min^{-1})	**Response (LU*s) ± SD**
0.5	14 ± 2
1.0	16 ± 2
2.0	16.0 ± 0.9
3.0	15.9 ± 0.9
4.0	15.9 ± 0.8

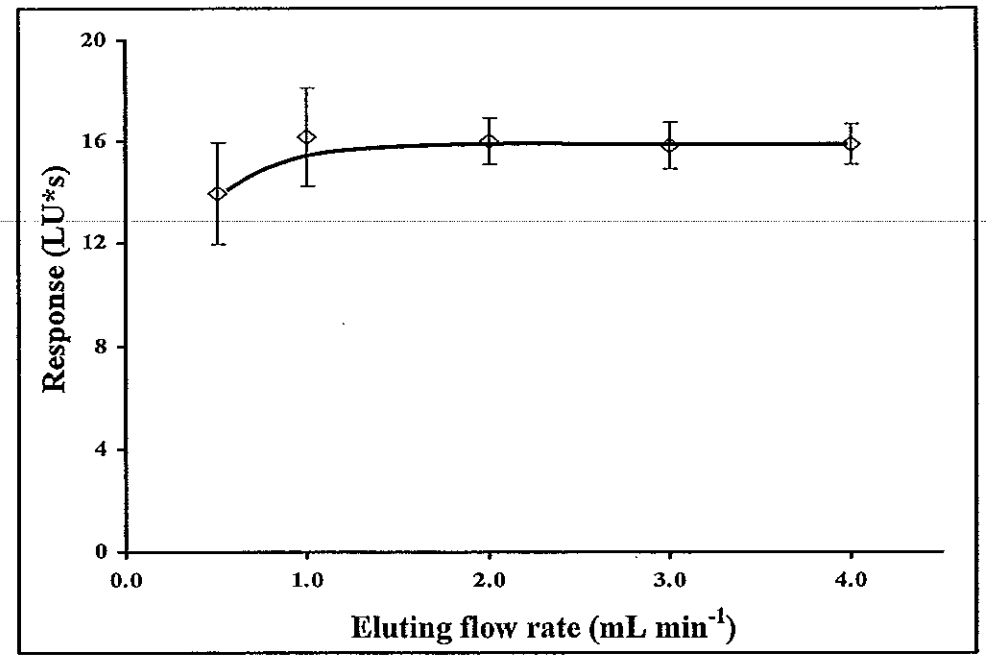


Figure 3.23 Response of oxolinic acid at various eluting solvent flow rates

3.3.3.7 Effect of Defatting

In order to defat the sample, a step using hexane was necessary. Defatting was investigated after extraction of the oxolinic acid in shrimp sample with ultrasonic extraction and compared the result with no defat sample. The defatting procedure was investigated in both liquid-liquid extraction and the SPE cartridge by adding hexane. The results in Table 3.25 and Figure 3.24 indicated that recoveries were better when hexane was added to the SPE cartridge. The lack of interferences in the separation suggests a high specificity of the chromatographic method and a good selectivity of the extraction procedure. Defatting also improved the life time of the guard column.

All optimum conditions of sample preparation procedure are summarized in Table 3.26.

Table 3.25 Recovery of oxolinic acid at different defatting procedure

Defatting procedure	Recovery (%) ± SD
Liquid-liquid extraction	81 ± 4
Addition of hexane in SPE cartridge	85 ± 1
No defatting	79 ± 4

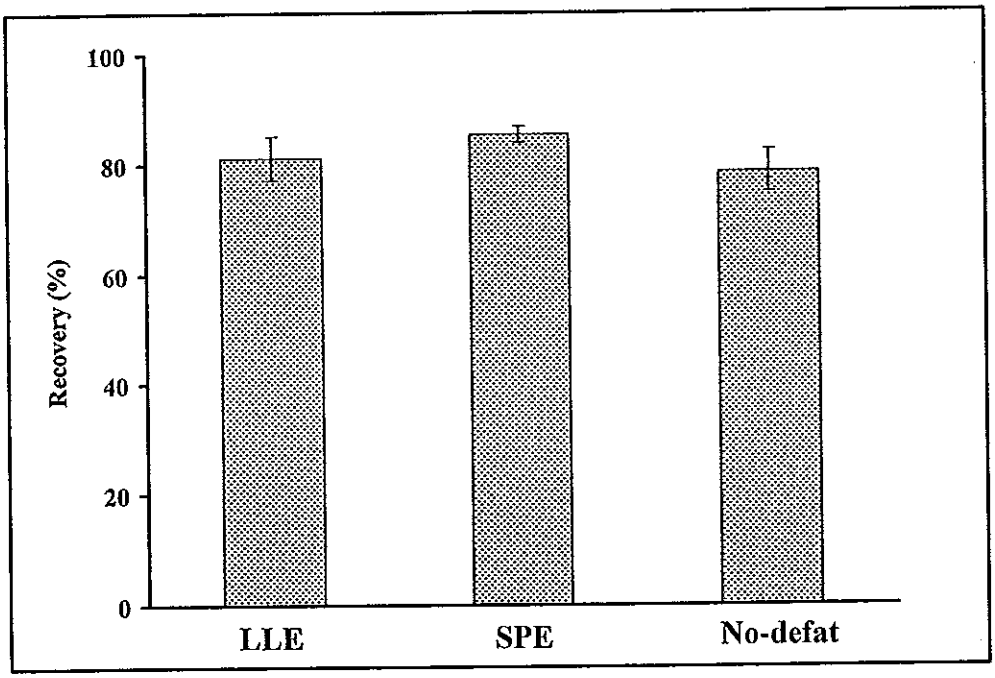


Figure 3.24 Recovery of oxolinic acid at different defatting procedure

Table 3.26 Optimum conditions of sample preparation procedure

Parameters	**Optimum value**
Ultrasonic extraction	
Extraction solvent	Ethyl acetate
Extraction volume (mL)	10
Extraction time (min)	15
Stability of oxolinic acid (min)	60
Solid phase extraction	
Type of sorbent	Supelclean LC-18
Type of eluting solvent	3% trifluoroacetic acid: Acetonitrile (25:75, v/v)
Sample flow rate (mL min^{-1})	4
Volume of eluting solvent (mL)	3
Flow rate of eluting solvent (mL min^{-1})	2
Defatting effect	SPE procedure

3.3.3.8 Matrix effects

For biological analysis the various compositions in the sample could interfere with the interest analyte. If the analytes were not isolated from these interferences, it could lead to a number of effects. They may have the effect of apparent enhancing the concentration of analyte. Interference usually affect the slope of the calibration curve, so the slope of the analyte of interest and the slope of the calibration curve are different in the method of additions that may affect the linearity. The interferences in edible animal were evaluated and the results are shown in Table 3.27 and Figure 3.25-3.30.

Table 3.27 Response of standard oxolinic acid and spiked samples at various oxolinic acid concentrations

Concentration ($ng\ L^{-1}$)	Response (LU*s)± SD						
	Standard	**Shrimp**	**Fish**	**Chicken Meat**	**Chicken liver**	**Beef**	**Pork**
5	1.65 ± 0.03	1.79 ± 0.04	1.82 ± 0.08	1.80 ± 0.04	1.90 ± 0.04	1.60 ± 0.02	1.70 ± 0.04
10	3.4 ± 0.2	3.34 ± 0.06	3.20 ± 0.03	3.09 ± 0.06	3.71 ± 0.06	3.53 ± 0.04	3.48 ± 0.08
20	7.1 ± 0.3	7.4 ± 0.5	6.83 ± 0.06	6.64 ± 0.04	7.01± 0.04	7.0 ± 0.2	6.85 ± 0.04
50	17.8 ± 0.7	19.6 ± 0.6	18.4 ± 0.6	17.9 ± 0.2	17.1 ± 0.2	17.8 ± 0.6	17.9 ± 0.6
100	35.8 ± 0.5	36.8 ± 0.2	36.2 ± 0.4	35.6 ± 0.1	35.7 ± 0.1	36.6 ± 0.7	37.4 ± 0.8

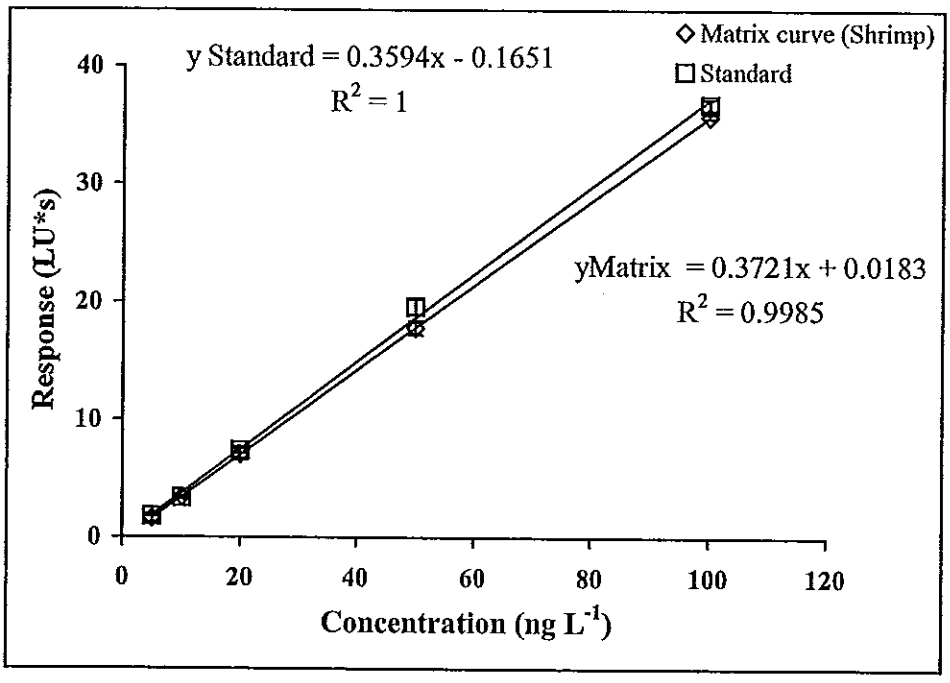


Figure 3.25 Matrix match calibration curve of oxolinic acid in shrimp

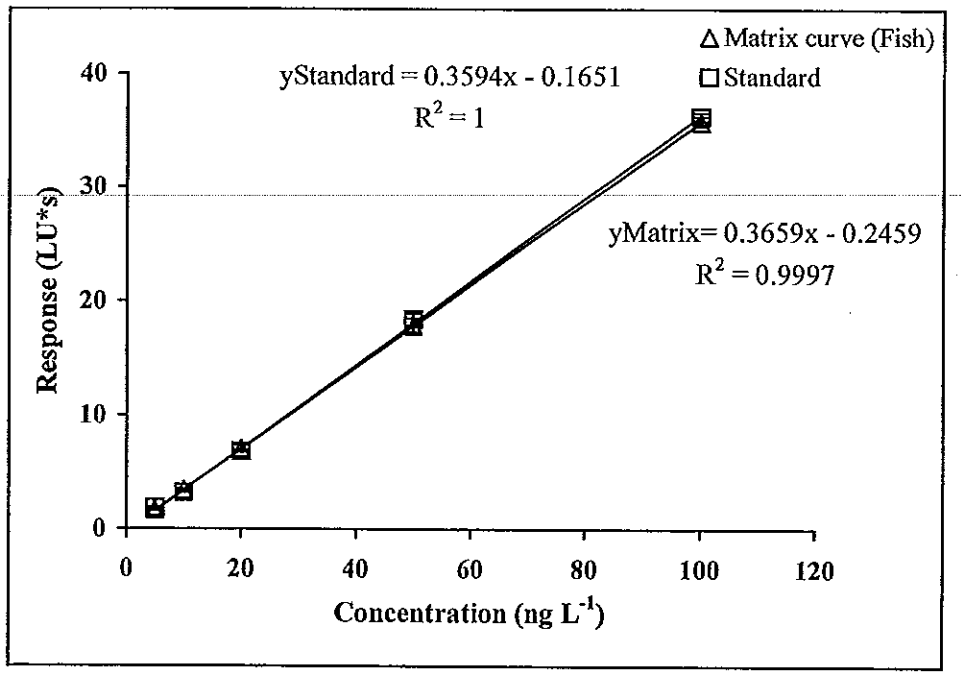


Figure 3.26 Matrix match calibration curve of oxolinic acid in fish

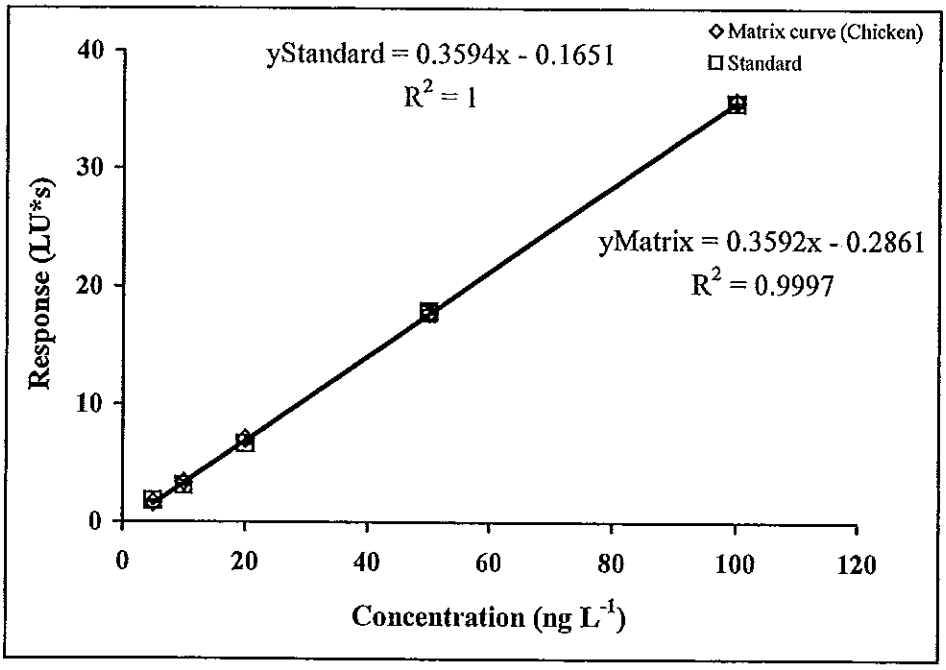


Figure 3.27 Matrix match calibration curve of oxolinic acid in chicken

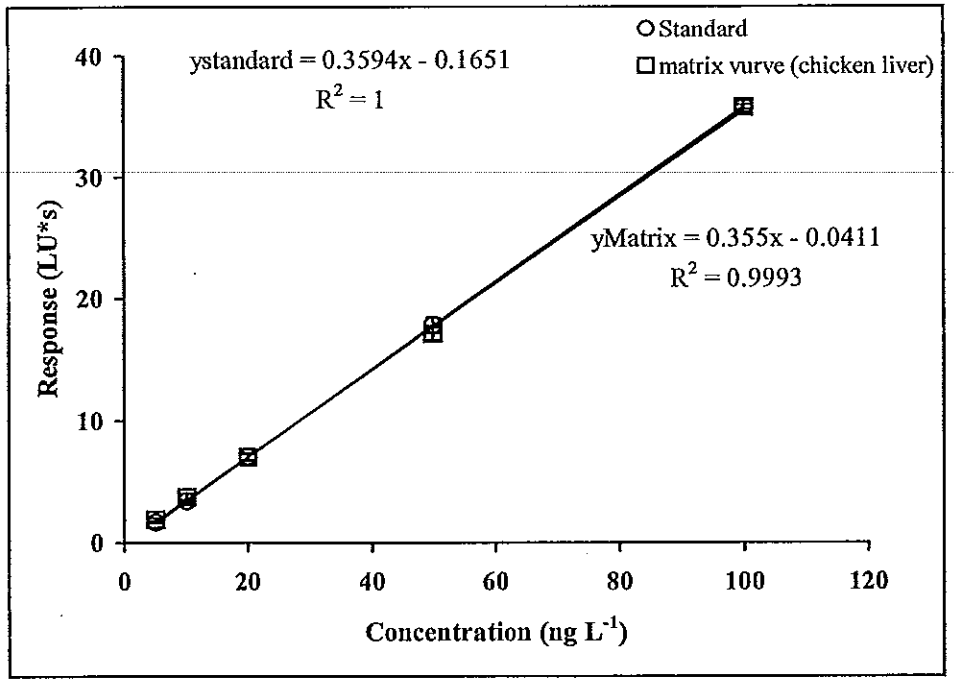


Figure 3.28 Matrix match calibration curve of oxolinic acid in chicken liver

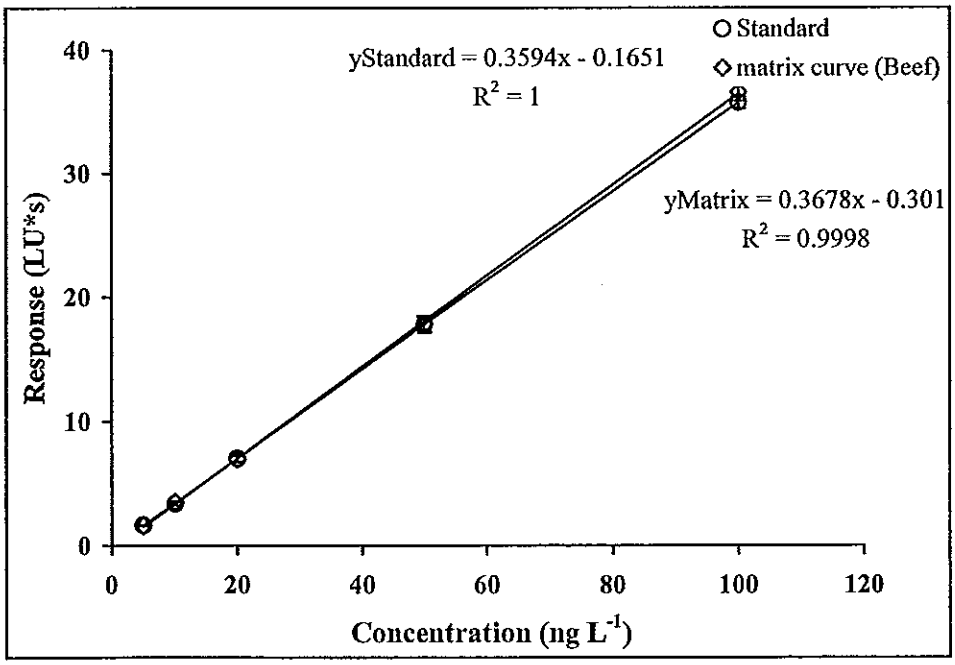


Figure 3.29 Matrix match calibration curve of oxolinic acid in beef

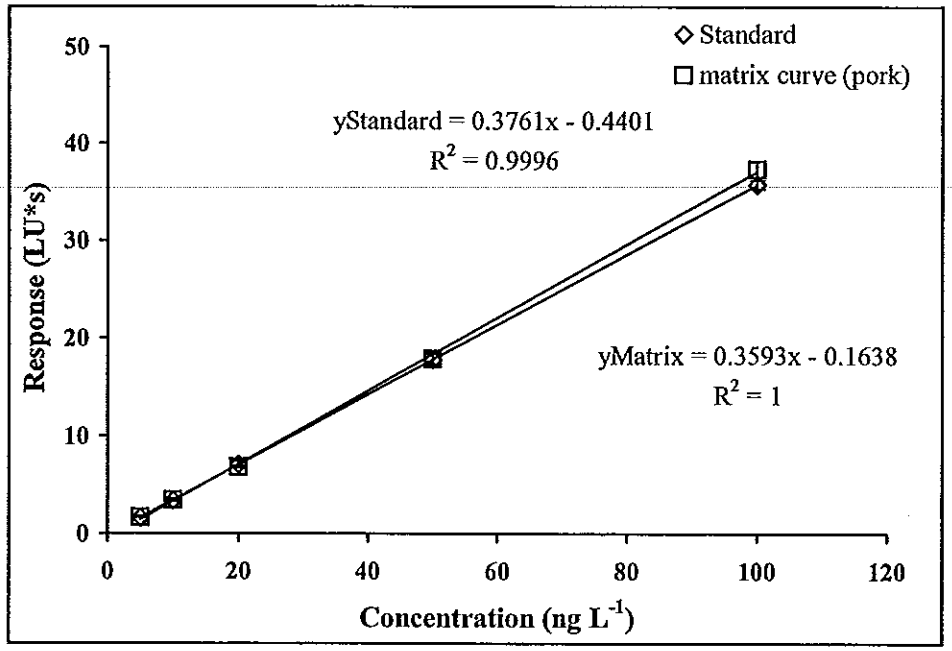


Figure 3.30 Matrix match calibration curve of oxolinic acid in pork

The slopes of standard curve and matrix match calibration curve were test using two-way ANOVA by taking the null hypothesis (H_0) that the interaction of both slopes is not significant and alternative hypothesis (H_1) that the interaction of slope is significant. If P value is less than α (level of significance) then null hypothesis was rejected at that significant level. The results from significant test (Table 3.28) found that the slope of regression line of standard curve and matrix match calibration curve were not significant for fish, chicken, chicken liver and beef, quantitative analysis can be determined using standard curve or matrix match calibration curve. The results were significantly different, $P<(0.01)$ for shrimp and pork. It's possible that some compositions were still in the extracted sample and still have little effect on the response of oxolinic acid. Therefore, quantitative analysis of oxolinic acid in these two types of samples should be determined from matrix match calibration curve.

Table 3.28 Results of statistical test using two-way ANOVA by R software

Matrix	Degree of freedom	Sum of squares	Mean squares	*F*	*P*
Shrimp	4	3.5	0.9	6.210	0.0020380**
Fish	4	0.8	0.2	1.6840	0.1931
Chicken meat	4	0.4	0.1	1.1772	0.3508
Chicken liver	4	1.0	0.2	1.9165	0.1470
Beef	4	0.9	0.2	1.0396	0.4115
Pork	4	3.2	0.8	4.5711	0.008744**

Significant codes; "**" (α=0.01)

Where; *F* is the ratio of the two sample variances

P is probability

3.4 Method validation

3.4.1 Selectivity

Selectivity is the ability of the bioanalytical method to measure and differentiate the analytes in the presence of components that may be present as impurities, degradedness or matrix components. In the evaluation of the selectivity, six blanks of different source of samples and spike samples were analyzed at the level of 50 ng g^{-1}. The selectivity of the method was checked by analyzing different types of blank samples (shrimp, fish, chicken meat, chicken liver, pork, pig liver pork) and compared the results with spike samples and oxolinic acid standard. The results showed no interference substance at the same retention time of oxolinic acid for shrimp (Figure 3.31), fish (Figure 3.32), chicken meat (Figure 3.33), chicken liver (Figure 3.34), pork (Figure 3.35) and beef (Figure 3.36). Only pig liver have a interference substance at the retention time of oxolinic acid (Figure 3.37). It is

possible that the interference might be from feed or some compositions in the liver which are difficult to remove. However, if the detection of oxolinic acid in pig liver is needed it is possible to adjust the chromatographic parameters (*e.g.* mobile phase composition) to separate this interference substance from oxolinic acid. Therefore, the proposed method is selective to shrimp, fish, chicken meat, chicken liver, pork and beef.

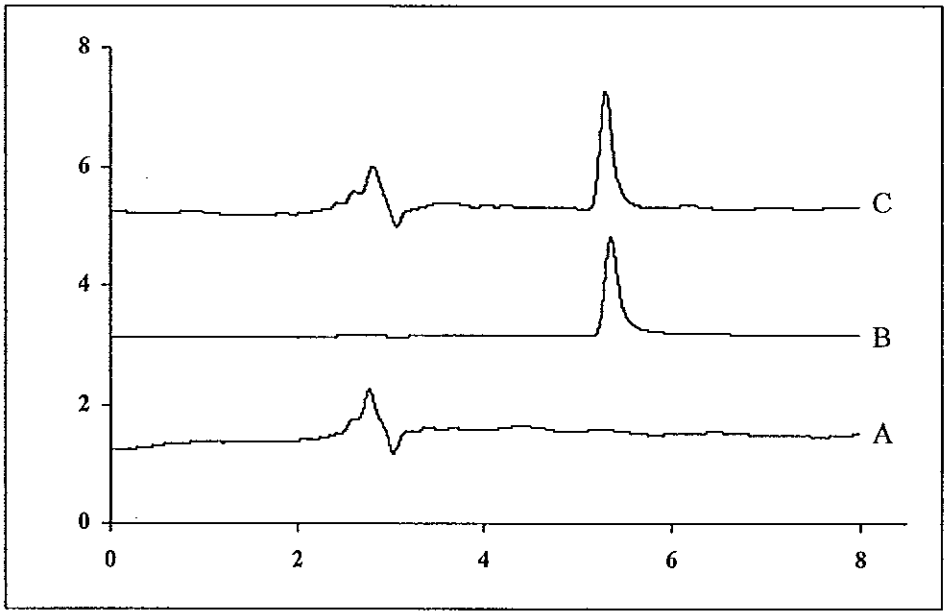


Figure 3.31 HPLC chromatogram (A) blank shrimp sample (B) standard oxolinic acid (C) spiked shrimp sample with oxolinic acid 5 ng g^{-1}

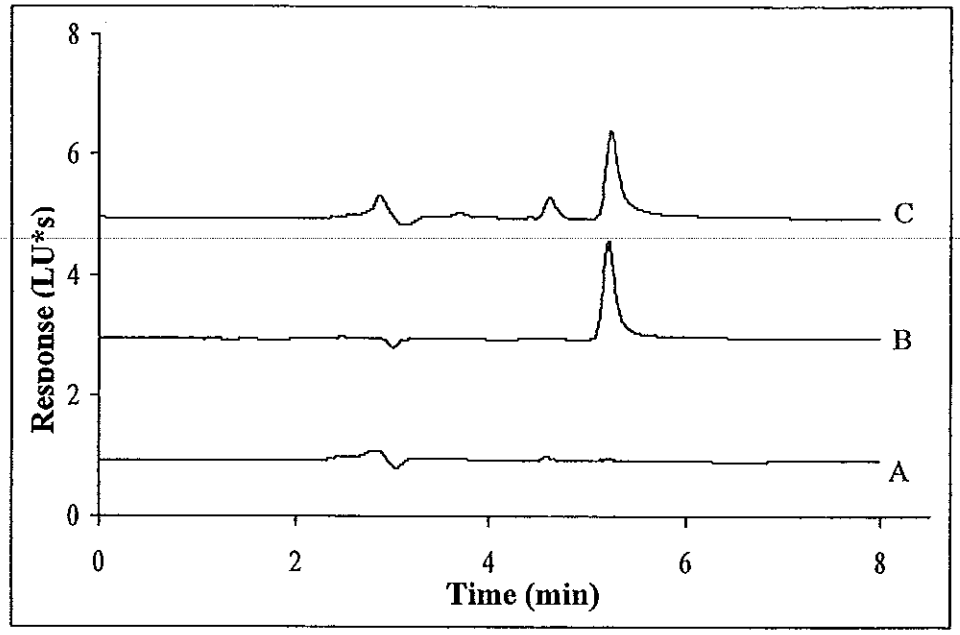


Figure 3.32 HPLC chromatogram (A) blank fish sample (B) standard oxolinic acid (C) spiked fish sample with oxolinic acid 5 ng g^{-1}

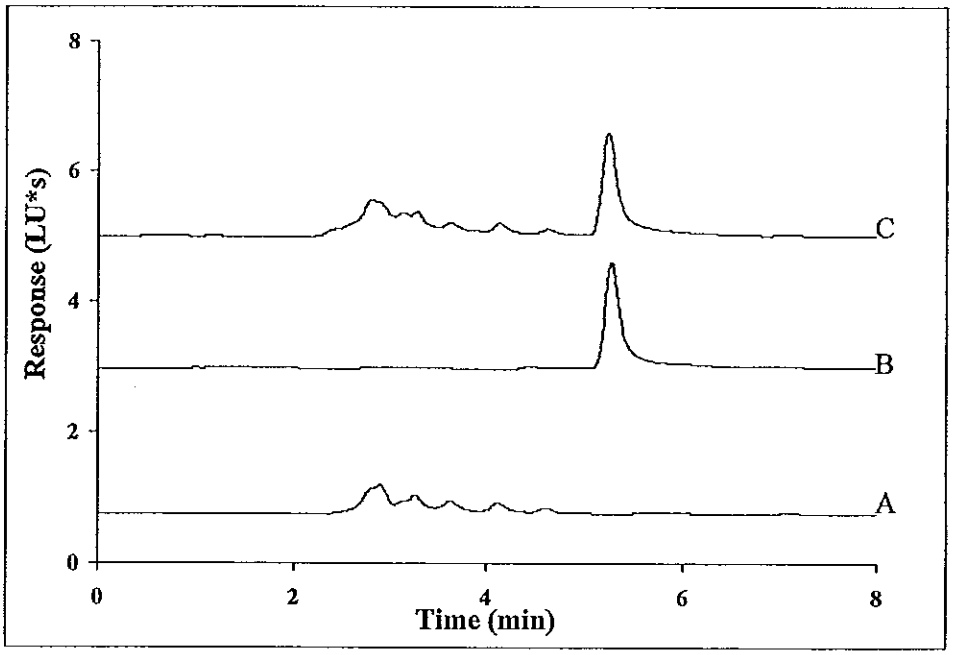


Figure 3.33 HPLC chromatogram (A) blank chicken sample (B) standard oxolinic acid (C) spiked chicken sample with oxolinic acid 5 ng g^{-1}

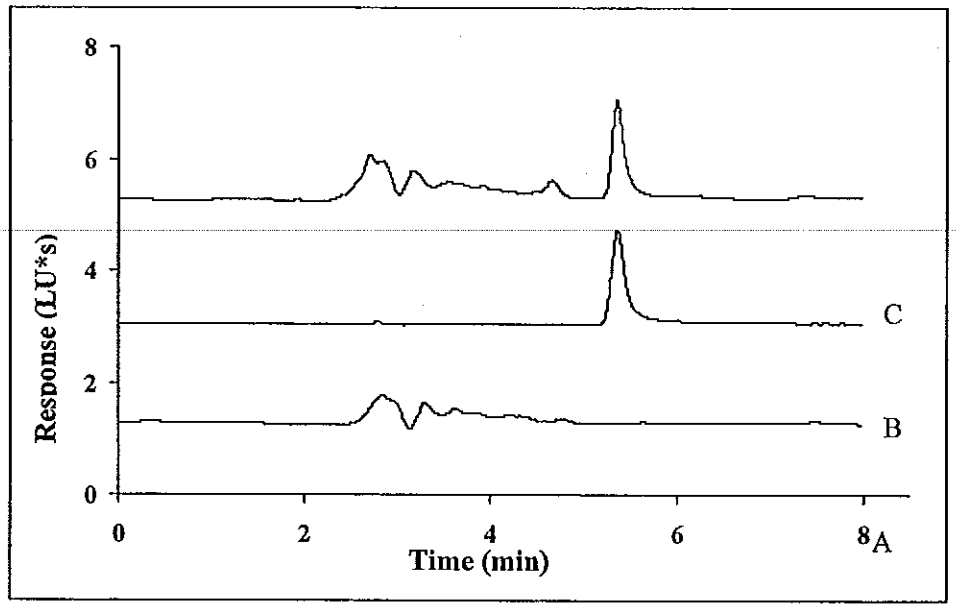


Figure 3.34 HPLC chromatogram (A) blank chicken liver sample (B) standard oxolinic acid (C) spiked chicken liver sample with oxolinic acid 5 ng g^{-1}

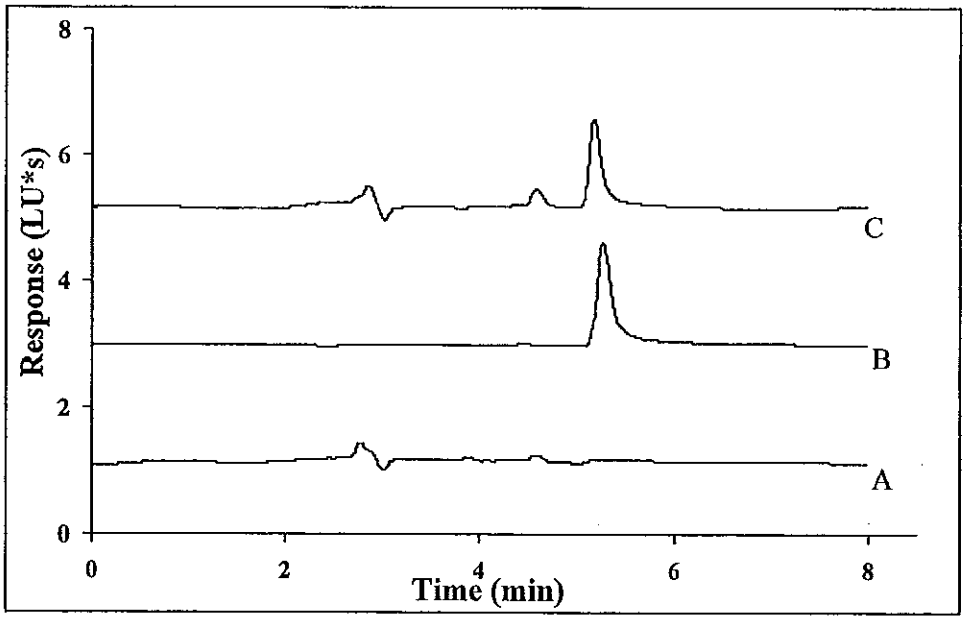


Figure 3.35 HPLC chromatogram (A) blank pork sample (B) standard oxolinic acid (C) spiked pork sample with oxolinic acid 5 ng g^{-1}

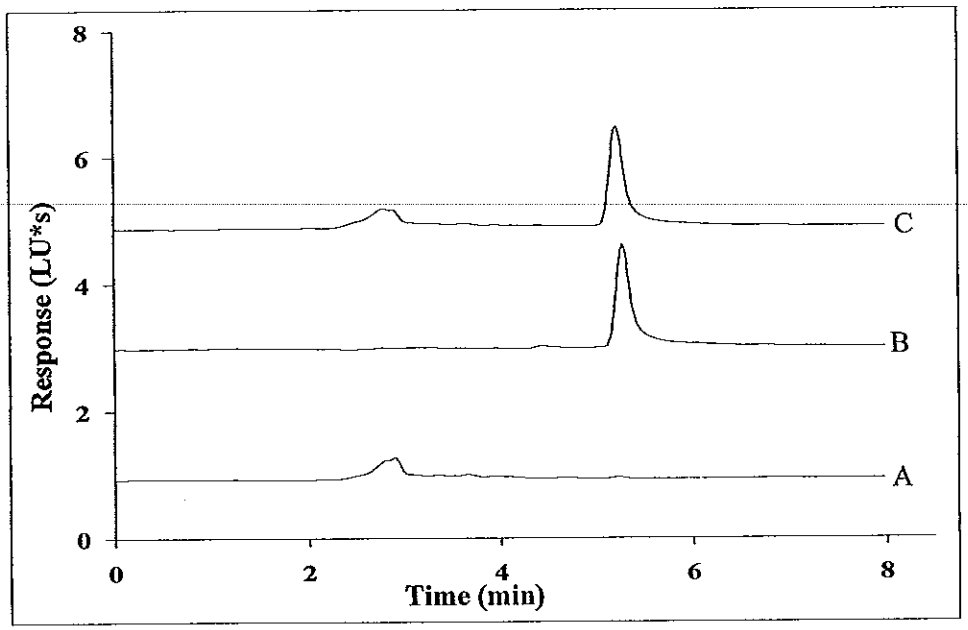


Figure 3.36 HPLC chromatogram (A) blank beef sample (B) standard oxolinic acid (C) spiked beef sample with oxolinic acid 5 ng g^{-1}

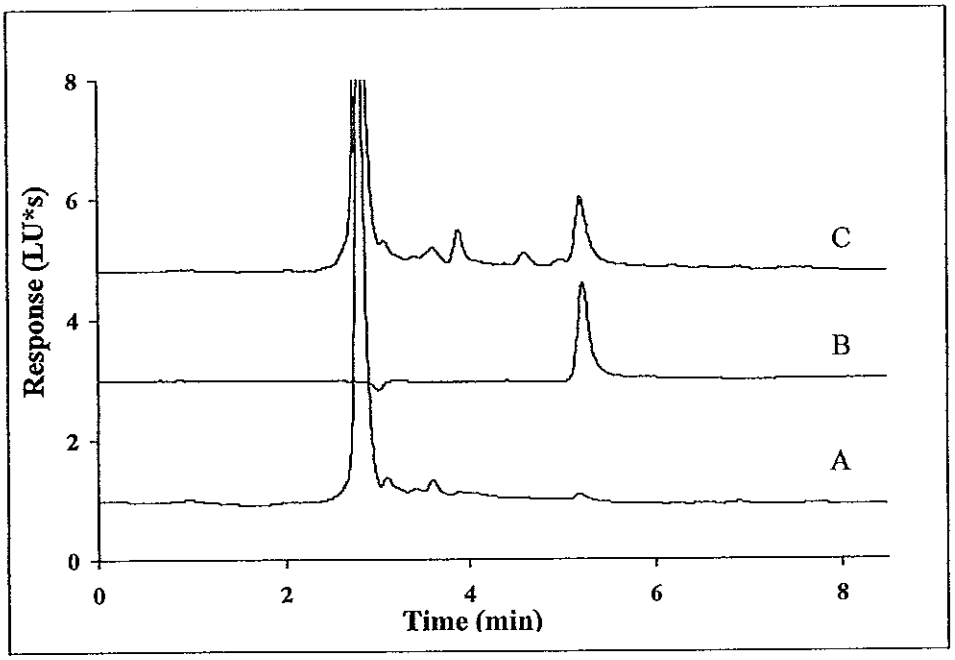


Figure3.37 HPLC chromatogram (A) blank pig liver sample (B) standard oxolinic acid (C) spiked pig liver sample with oxolinic acid 5 ng g^{-1}

3.4.2 Range and linearity

In this proposed analytical chromatographic technique a linear relationship is observed between the detector response (y) and concentration (x) of the analyte in the samples and a linear calibration curve was obtained by least-squares linear regression procedure, $y = ax+b$ where b is the intercept of the calibration curve and a is the slope. The linearity of responses (peak area) versus concentration of oxolinic acid was studied in the range of 5-500 ng g^{-1} to cover the MRL for various sample. The correlation coefficient, intercept and slope values of oxolinic acid are indicated in Table 3.29. The high values of the coefficients of determination (R^2>0.99) indicated good correlation between peak areas and oxolinic acid concentrations from 5 to 500 ng g^{-1}.

Table 3.29 Parameters corresponding to linear regressions obtained from the calibration curves

Sample	Equation	Intercept (LU*s)	Slope (LU*s/ng g^{-1})	R^2
Shrimp	y = 0.2796x-1.0546	-1.0546	0.2796	0.9968
Fish	y = 0.3260x-1.6468	-1.6468	0.3260	0.9963
Chicken (meat)	y = 0.3367x-0.4606	-0.4606	0.3367	0.9968
Chicken (Liver)	y= 0.2870x +0.6918	+0.6918	0.2870	0.9914
Pork	y = 0.2879x+0.5313	+0.5313	0.2879	0.9926
Beef	y= 0.2312x +0.5592	+0.5592	0.2312	0.9969

y = analyte signal (LU*s), x = analyte concentration (ng g^{-1})

3.4.3 Accuracy

Accuracy is the closeness of the test results obtained by the analytical method to the true value (Shabir *et al.*, 2003). An indication of accuracy was based on the calculation of the relative error of the mean observed concentration as compared to the nominal concentration. Relative errors less than 15 % are acceptable according to FAD for the analysis of biological sample. The inter- and intra-day relative errors at three concentrations studied are less than 10 % (Table 3.30) and it is obvious that the method is remarkably accurate which ensures reliable results.

3.4.4 Precision

The precision of the method were studied by measuring the repeatability (intra-day) and intermediate precision (inter-day). The intra-day precision of the HPLC–FLD method was evaluated by the analysis of five shrimp samples fortified at three concentration levels each: 50, 100 and 200 μg kg^{-1}. Inter-day precision was based on the same analysis of shrimp samples on 3 days. The results are shown in Table 3.30. The intra-day relative standard deviations (RSD) were lower than 7.9% and lower than 8.6% for inter-day assays. Repeatability and intermediate precision are lower than 15% in accordance with US-FDA guidance for bioanalytical validation (US-FDA, 2001). These results indicated that the method developed has acceptable precision.

Table 3.30 Precision and accuracy of the analysis of oxolinic acid

	Norminal concentration (ng g^{-1})		
	50.0	**100.0**	**200.0**
Intra-day (n=5)			
Mean (ng g^{-1})	47.1 ± 2.5	99.2 ± 3.5	183.3 ± 6.6
Precision (RSD, %)	5.3	3.5	3.6
Accuracy (error, %)	-5.9	-0.9	-8.6
Inter-day (n=15)			
Mean(ng g^{-1})	46.5 ± 3.8	99.0 ± 5.6	190.0 ± 6.6
Precision (RSD, %)	8.2	5.7	3.5
Accuracy (error, %)	-7.0	-1.0	-5.0

3.4.5 Recovery

The recovery was quantified by calculated the ratio of the slopes of the calibration curves for extracted samples to the corresponding standard calibration curve. The results are shown in Table 3.31. Recoveries of oxolinic acid at the studied concentrations are higher than 85 % for shrimp, fish, chicken meat, chicken liver, pork sample which meet US-FDA regulatory method requirement of 80-110% and higher than 74 % for beef sample. These values are well within the criteria of the Codex for residue analysis (average recovery 70-110%)(Codex Alimentarius Commission, 1993). The recovery of beef sample was lower than other sample due to different characteristic of the meat *e.g.* size of particle, toughness of meat. And the optimum condition of ultrasonic extraction were studied in shrimp sample, when applied to beef sample were not the optimum value. Better recovery might be obtained when optimization of ultrasonic extraction conditions was performed in beef sample.

Table 3.31 Recovery of oxolinic acid from various samples

Sample	Mean recovery (%)
Shrimp	89 ± 4
Fish	87 ± 8
Chicken (meat)	95 ± 5
Chicken (Liver)	86 ± 10
Pork	95 ± 5
Beef	74 ± 3

3.4.6 Limit of detection (LOD) and quantification (LOQ)

The limit of detection (LOD) is the lowest concentration of analyte in a sample that can be detected but not necessarily quantitated under the experimental condition of the method. The limit of quantitation (LOQ) is defined as the lowest concentration of analyte in a sample that can be quantitatively determined with suitable precision and accuracy (Shabir *et al.*, 2003; Xiao *et al.*, 2007). Official guide present different approaches to estimating these limits but leave the analyst completely free to choose (Ribani *et al.*, 2007). The principal approaches for determination of the detection limit and the quantification limit, described in literature and recommended by the international accreditation systems are signal-to-noise ratio and standard deviation of the response (ICH, 1996; Grushka and Grinberg, 2006).

Signal-to-noise ratio: The most commonly used is the signal-to-noise ratio criterion but this method only can be applied in analytical systems that present noise for the baseline. In analytical separation techniques, such as the chromatography, the measurement of noise is not trivial and often subjective. To determine the signal-to-noise ratio, a comparison is made between the signals from sample components of low known concentrations, prepared in the matrix of interest and a component-free

sample in the same matrix. The signal-to-noise ratio can be 3:1 for the detection limit (Touraki *et al.*, 2001) and 10:1 for the quantification limit (Kakumanu *et al.*, 2006; Shao *et al.*, 2007).

Standard deviation (SD) of the response: this approach is based on the standard deviation of a response (S) and the slope of the analytical curve (b).

The standard deviation of the response can be determined based on the standard deviation of a blank (S_B), on the residual standard deviation of the regression line ($S_{y/x}$) or the standard deviation of the y intercept of the regression line (S_a). To get these data, an analytical curve is constructed from pure analyte in the matrix of interest, including concentrations close to the expected quantification limit.

It is possible to determine S_B by performing the blank experiment several times and obtaining an independent value for S_B. However, multiple determinations of the blank are time-consuming and the use of $S_{y/x}$ and S_a are quite suitable in practice (Ribani *et al.*, 2007).

In this work there was no noise in the baseline, also after sample clean up there was no peak on the chromatogram of blank samples. Therefore, detection and quantification limits were evaluated using the standard deviation of the linear plot and the standard deviation of the y intercept which were calculated as described in 2.11.6. The results in Table 3.32 indicated that LOD and LOQ obtained from the standard deviation of the curve ($s_{y/x}$) were higher than obtained from the standard deviation of the y intercept (s_a) in all case. The limits of detection and quantification obtained from both method are below the maximum residue level (MRL) established by the European Union for all sample tissues.

Table 3.32 Limit of detection and quantification of oxolinic acid in various samples

Sample	S_a		$S_{y/x}$	
	LOD	**LOQ**	**LOD**	**LOQ**
Shrimp	5.9 ± 0.2	17.8 ± 0.6	9.0 ± 0.3	27.3 ± 1.0
Fish	8.7 ± 0.4	26 ± 1	13.4 ± 1	41 ± 2
Chicken (meat)	7.2 ± 0.3	22 ± 1	11.0 ± 0.5	33 ± 1
Chicken (Liver)	17 ± 2	56 ± 6	28 ± 3	86 ± 9
Pork	7.3 ± 0.3	22 ± 1	11.0 ± 0.5	37 ± 2
Beef	2.8 ± 0.04	8.4 ± 0.1	4.3 ± 0.1	12.9 ± 0.2

S_a= standard deviation of the *y* intercept of the regression line

$S_{y/x}$= the residual standard deviation of the regression line

3.4.7 Stability

The stability of oxolinic acid in extracted sample was studied at two different concentration levels (10 and 100 ng mL^{-1}) and different experimental conditions which included short-term, freeze-and-thaw, long-term and standard solution were investigated. The accuracy and standard deviations of the concentration found and initial concentration were used for the stability evaluation. The results of the stability studies are shown in Table 3.33.

Short-term stability; five samples were processed and the extracts dissolved in mobile phase were stored at room temperature. They were analysed at the beginning and after 2, 4, 8, 12, 24, 36 and 48 h to ensure that samples are stable at room temperature for at least 2 days. (based on the expected duration that samples will be maintained at room temperature in the intended study in the autosampler). The accuracy from samples ranged from 96 to 109% after short term stability testing.

Freeze-and-thaw stability; five samples were processed, and the evaporated extracts were dissolved in mobile phase were subjected to three freeze-and-thaw cycles. The samples were stored at -20 °C for 24 h and were then thawed at room temperature. Afterwards, the samples were refrozen for 24 h under the same conditions. This cycle was repeated three times. An aliquot of these samples was analysed at the beginning of the process and three aliquots more after each freeze/thaw cycle. The concentrations of oxolinic acid found in each determination were compared to ensure that there is no variation during these processes. Oxolinic acid were stable in extracted sample for three freeze-thaw cycles with <15% variation.

Long-term stability; five samples were stored at -20 °C for 2 months. Afterwards, these samples were analysed and compared with five fresh samples at both concentration level. The accuracy following its storage period was 93-106% of the nominal values of 10 and 100 ng mL^{-1} of oxolinic acid.

Standard aqueous solutions; the standard solutions of oxolinic acid in mobile phase were found to be stable for three months when refrigerated at 4 °C. The samples were analysed every two weeks and were compared with freshly prepared standards. The concentration on comparison with freshly prepared standard after the storage was 96-104 %. The above results indicated that the oxolinic acid was stable in the studied conditions.

Table 3.33 Stability study test

Quality control sample	**Stability (% accuracy) ± SD**	
	10 ng mL^{-1} (Low)	**100 ng mL^{-1} (High)**
Short-term		
2 h	105 ± 4	102 ± 4
4 h	99 ± 4	102 ± 3
8 h	101 ± 4	97 ± 4
12 h	98 ± 2	94 ± 5
24 h	99 ± 2	98 ± 2
36 h	99 ± 1	96 ± 2
48 h	100 ± 2	93 ± 5
Freeze and thaw cycle		
Cycle 1	99 ± 4	95 ± 7
Cycle 2	98 ± 2	97 ± 2
Cycle 3	100 ± 2	93 ± 3
Long-term		
After 2 months at -20 °C	95 ± 2	100 ± 6
Standard aqueous solutions	100 ± 4	100 ± 2

3.4.8 Robustness

As defined by the ICH, the robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. In order to evaluate the robustness of the propose method. The following parameters were altered deliberately: the mobile phase flow rate in the variants: 0.8 and 1.0 mL min^{-1}, the pH of mobile phase in the variants: 3.2 and 3.8, the column temperature in the variants: 20 and 30 °C and percentage of acetonitrile in the variants 42 and 48 %. Other parameters were kept constant at the optimum values during variation in one parameter. The deviations of capacity factor (*k*), plate (*N*), tailing factor (*T*) and peak area were calculated with parameters 0.9 mL min^{-1}, 3.5, 25 °C and 45% as reference values, which is the optimum condition of chromatographic system. The response (peak area) had no significant changes when the parameters were modified (deviations <10%). Method robustness (Table 3.34) shows that the minor changes of the operational parameters have little effect or do not lead to essential changes of the chromatographic separation (capacity factor). But tailing factor of analytes will increase or decrease when the composition of the mobile phase and pH has been changed. However, the values are acceptable, lower than 2 in both cases and these should be careful chosen and set in order to the control chromatographic behavior of oxolinic acid.

Table3.34 Result for robustness test study

Parameter	Variation	*k*	*N* ($\times 10^3$)*	*T*
Mobile phase flow rate (mL min^{-1})	0.8	0.966	30.7	1.45
	0.9	0.912	30.1	1.40
	1.0	0.908	27.2	1.39
Column temperature (°C)	20	0.958	31.1	1.40
	25	0.912	30.1	1.40
	30	0.884	29.5	1.37
Mobile phase pH	3.2	0.914	29.1	1.34
	3.5	0.912	30.1	1.40
	3.8	0.902	27.3	1.50
Acetonitrile percentage (%)	42	1.072	29.0	1.70
	45	0.912	30.1	1.40
	48	0.824	28.3	1.34

*Theoretical plates per meter

Tailing (symmetry) factor (*T*) and number of theoretical plates (*N*) was calculated by formulas: $T = W/(2W\text{a})$ and $N = 5.545(t_R/W_{1/2})^2$, where *W* is the peak width at 5% height from baseline *W*a the peak front edge width at the same height and $W_{1/2}$ is the peak width at half height.

3.5 Qualitative and quantitative analysis of oxolinic acid in edible animal tissues

3.5.1 Qualitative analysis

The optimum conditions of HPLC-FLD were used to analyse oxolinic acid in edible animal tissues. For qualitative analysis, retention time of the spiked sample chromatogram was compared with retention time of the standard chromatogram to identify the oxolinic acid peak (Figure 3.38). The average retention time of oxolinic acid was 5.11 ± 0.01 minutes.

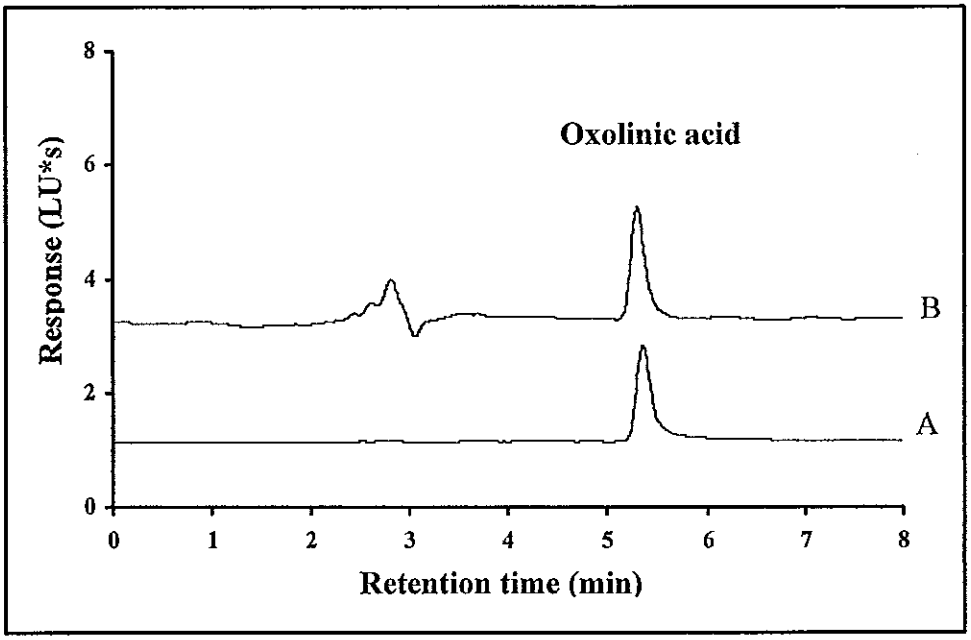


Figure 3.38 Chromatogram of oxolinic acid standard (A) and spiked shrimp sample (B) at the optimum condition

3.5.2 Quantitative analysis

Quantitative analysis of oxolinic acid was done by comparing the response (peak area) of oxolinic acid in the sample to the matrix matched calibration curve (shrimp, pork) and standard calibration curve (fish, chicken, chicken liver and beef). Three samples of shrimp, fish, chicken meat, chicken liver, pork and beef meat were analysed but no response of oxolinic acid was obtained. Therefore, one from each

sample was used to perform the standard addition method. The response and standard addition curve of each sample are shown in Tables 3.35-3.40 and Figures 3.39-3.44. The results are shown in Table 3.42 confirmed that no oxolinic acid presented in shrimp, fish, chicken meat, chicken liver, pork and beef samples. Although oxolinic acid was not found it has been proven that this method can be used to analyte trace residue. It would be useful for testing oxolinic acid residue when there is an epidemic in animals that will require oxolinic acid treatment.

Table 3.35 The results of standard addition calibration curve of oxolinic acid in shrimp sample

Concentration (ng g^{-1})	**Response (LU*s) ± SD**
5	1.46 ± 0.02
10	2.6 ± 0.1
20	4.2 ± 0.2
50	11.5 ± 0.7
100	25.5 ± 0.9

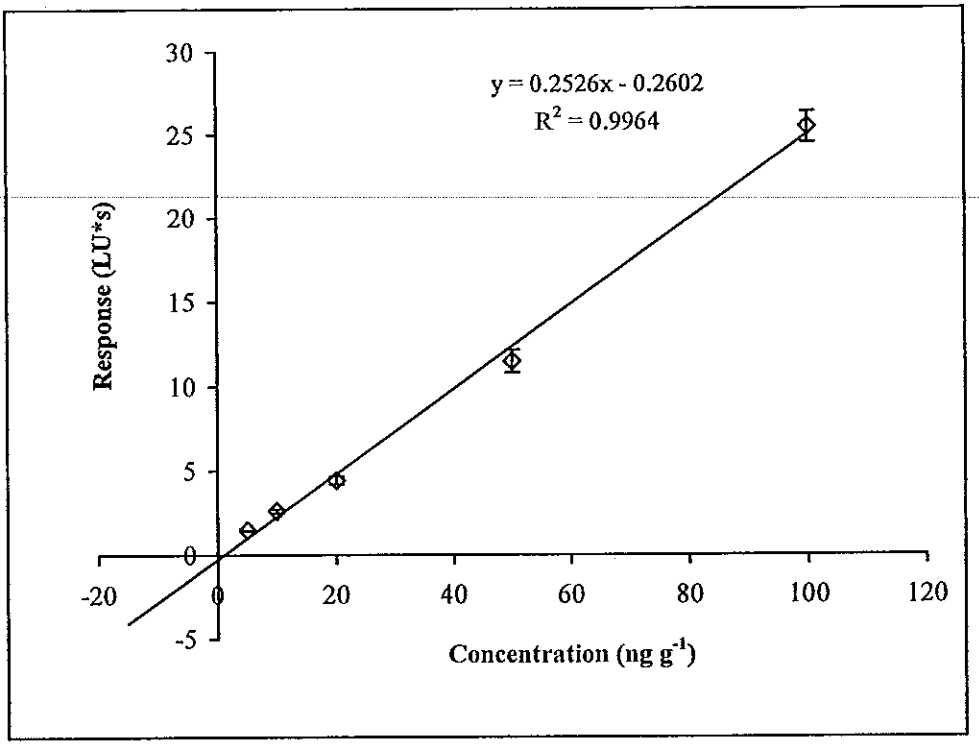


Figure 3.39 The standard addition calibration curve of oxolinic acid in shrimp sample

Table 3.36 The results of standard addition calibration curve of oxolinic acid in fish sample

Concentration (ng g^{-1})	Response (LU*s) ± SD
5	1.52 ± 0.06
10	2.63 ± 0.09
20	4.7 ± 0.1
50	12.4 ± 0.4
100	30 ± 2

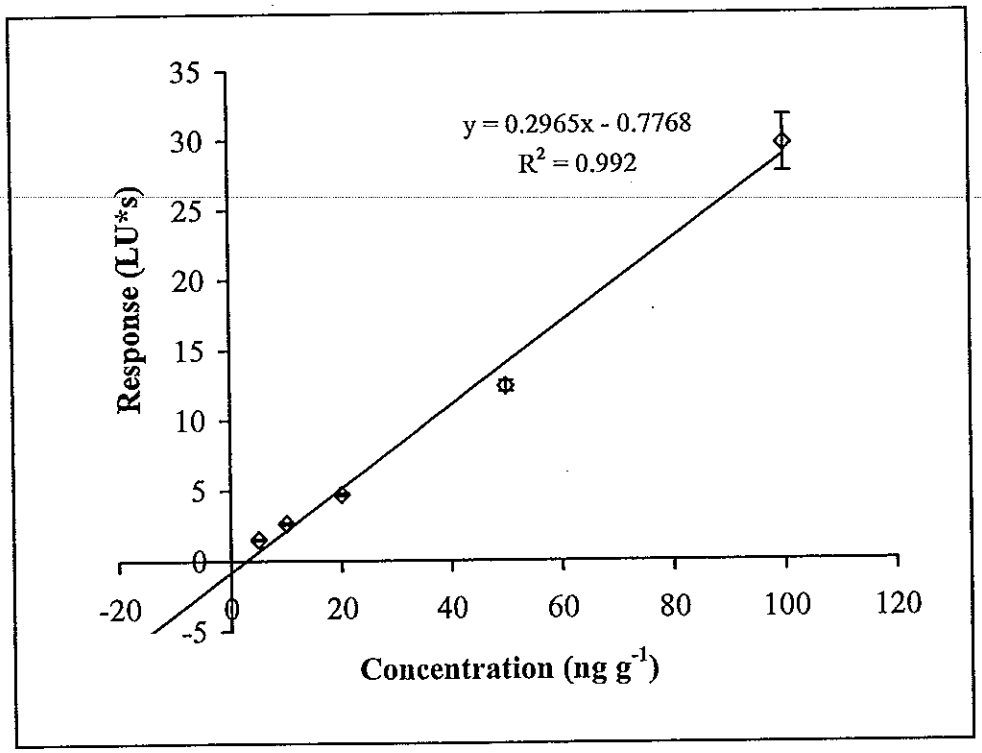


Figure 3.40 The standard addition calibration curve of oxolinic acid in fish sample

Table 3.37 The results of standard addition calibration curve of oxolinic acid in chicken meat sample

Concentration (ng g^{-1})	Response (LU*s) ± SD
5	1.29 ± 0.07
10	2.76 ± 0.09
20	6.1 ± 0.2
50	14.0 ± 0.6
100	32.7 ± 1.1

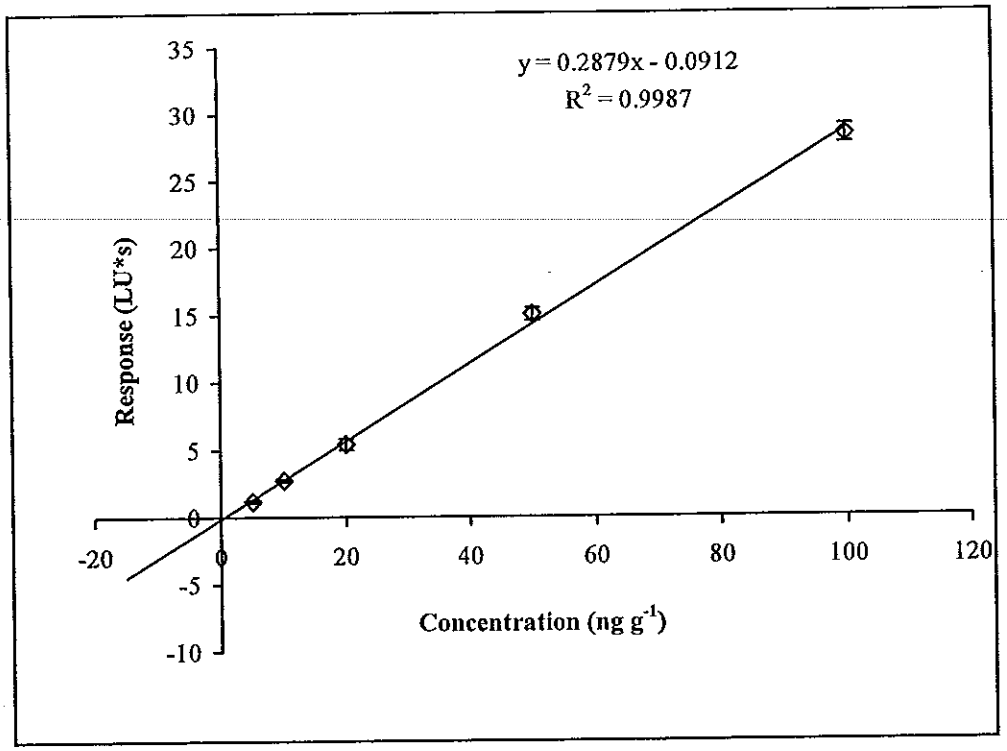


Figure 3.41 The standard addition calibration curve of oxolinic acid in chicken meat sample

Table 3.38 The results of standard addition calibration curve of oxolinic acid in chicken liver sample

Concentration (ng g^{-1})	Response (LU*s) ± SD
5	1.2 ± 0.1
10	2.8 ± 0.1
20	5.4 ± 0.4
50	15.0 ± 0.5
100	28.4 ± 0.7

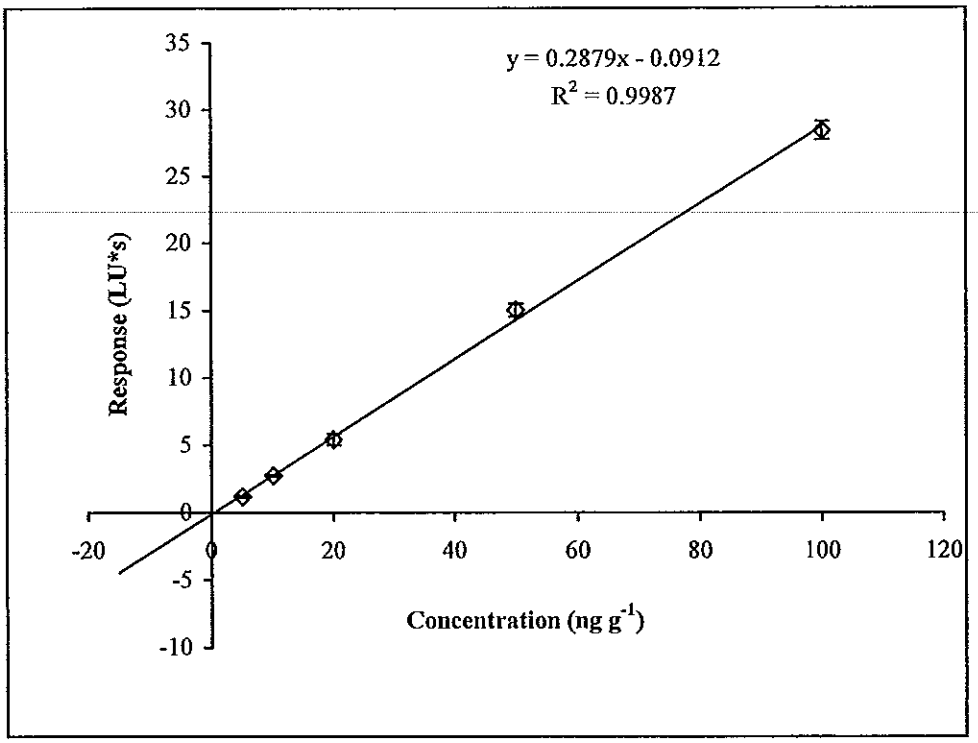


Figure 3.42 The standard addition calibration curve of oxolinic acid in chicken liver sample

Table 3.39 The results of standard addition calibration curve of oxolinic acid in beef sample

Concentration (ng g^{-1})	Response (LU*s) ± SD
5	1.3 ± 0.1
10	2.4 ± 0.1
20	5.1 ± 0.5
50	12.1 ± 0.5
100	25.5 ± 2.1

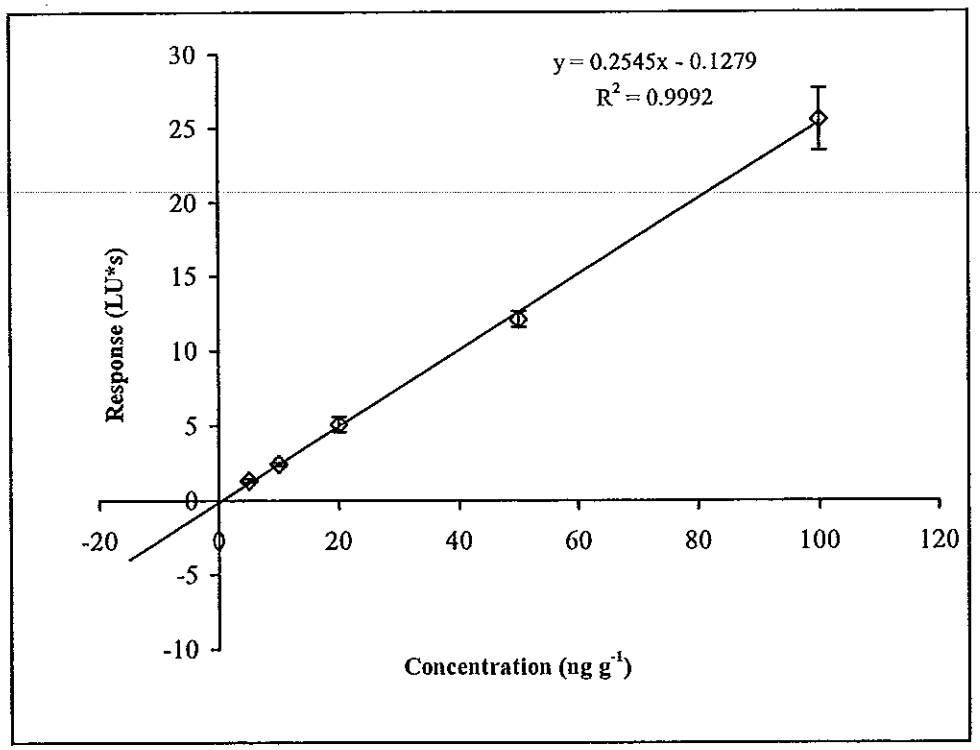


Figure 3.43 The standard addition calibration curve of oxolinic acid in beef sample

Table 3.40 The results of standard addition calibration curve of oxolinic acid in pork sample

Concentration (ng g^{-1})	Response (LU*s) ± SD
5	1.35 ± 0.06
10	2.89 ± 0.08
20	6.2 ± 0.1
50	14.1 ± 0.4
100	32.2 ± 0.9

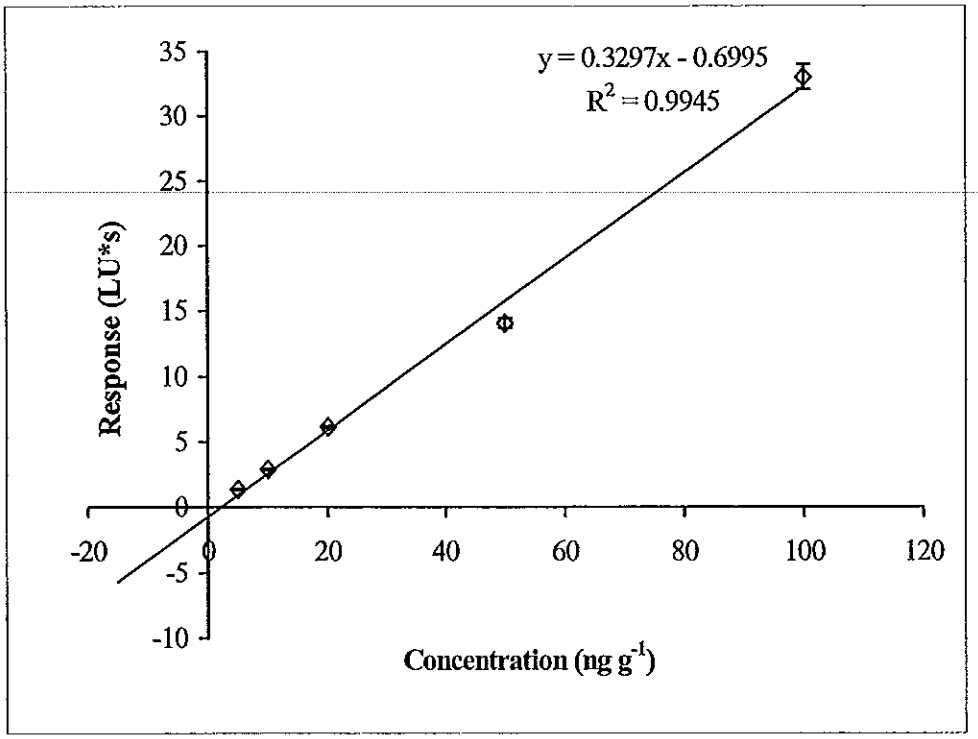


Figure 3.44 The standard addition calibration curve of oxolinic acid in pork sample

Table 3.41 Oxolinic acid concentrations in edible animal tissue by standard addition method

Sample	Oxolinic acid concentration (ng g^{-1})
Shrimp	ND
Fish	ND
Chicken (meat)	ND
Chicken (Liver)	ND
Pork	ND
Beef	ND

ND= Not Detectable

CHAPTER 4

Conclusions

The use of oxolinic acid as veterinary drug in food producing animals to prevent or treat infections and growth promoter can leave residues at trace levels in animal product which has led to a significant increase in antimicrobial resistance and has important effect on public health. Therefore, monitoring of oxolinic acid in edible animal tissues is necessary to ensure that human food is entirely free from potentially harmful substance.

The use of high sensitive high performance liquid chromatography with fluorescence detection was investigated using a Pinnacle II C18, 5 μm, 250×4.6 mm column. System optimization was carried out and the optimum conditions were excitation wavelength 265 nm, emission wavelength 375 nm, the mobile phase was a mixture of acetonitrile and 10 mM oxalic acid buffer (45:55, v/v, pH 3.5), flow rate 0.9 mL min^{-1} and column temperature 25 °C. Under the optimum conditions oxolinic acid was detected in less than 6 minutes.

System suitability parameters were investigated for trace oxolinic acid analysis and the values are acceptable when compared with the recommendation (Table 4.1). This system provided linear dynamic range of 1 ng mL^{-1} to 8 μg mL^{-1} with a coefficient of determination higher than 0.999 and detection limit of 1.03 ng mL^{-1}.

Table 4.1 System suitability test values

Parameter	Recommendation	This work
Capacity factor (k')	$0.5 < k' < 20$	0.912 ± 0.004
Plate (N)	> 2000	7522 ± 113
Tailing factor (T)	≤ 2	1.39 ± 0.03
Peak area (%RSD)	<4	1.1
Retention time (%RSD)	<1	0.39

Oxolinic acid in edible animal tissues was extracted by ultrasonic extraction and clean-up with solid-phase extraction (SPE). The influence of several parameters on the efficiency of the proposed method were investigated. The optimum condition of ultrasonic extraction and solid phase extraction are shown in Table 4.2. The obtained results indicated that the combination of ultrasonic solvent extraction and solid phase extraction can effectively be applied to extract oxolinic acid from edible tissue. Advantages of this method are reduced extraction time, simple, and inexpensive sample preparation.

Table 4.2 Optimum conditions of ultrasonic and solid phase extraction

Parameters	Optimum value
Ultrasonic extraction	
Extraction solvent	Ethyl acetate
Extraction volume (mL)*	10
Extraction time (min)*	15
Stability of oxolinic acid (min)	60
Solid phase extraction	
Type of sorbent	Supelclean LC-18
Type of eluting solvent	3%Trifluoroacetic acid: Acetonitrile (25:75, v/v)
Sample flow rate (mL min^{-1})	4
Volume of eluting solvent (mL)	3
Flow rate of eluting solvent (mL min^{-1})	2
Defatting effect	SPE procedure

*Repeated twice

Validation parameters, such as selectivity, linearity, accuracy, precision, recovery, LOD, LOQ, stability and robustness were investigated. The selectivity of the method was checked by analyzing different types of blank and spiked samples. The results showed no interference substance at the same retention time as oxolinic acid for shrimp, fish, chicken meat, chicken liver, pork and beef. The proposed method was found to be linear in the range 5 to 500 ng g^{-1}. The inter- and intra-day relative errors at three concentrations studied are less than 10% and it is obvious that the method is remarkably accurate which ensures reliable results. The intra-day relative standard deviations (RSD) were lower than 7.9% and lower than 8.6% for inter-day assays. Repeatability and intermediate precision are lower than 15% in accordance with US-FDA guidance for bioanalytical validation. Recoveries of oxolinic acid were higher than 85% for shrimp, fish, chicken meat, chicken liver, pork sample which meet US-FDA regulatory method requirement of 80-110% and

beef sample, these values is well within the criteria of Codex for residue analysis (average recovery 70-110%)(Codex Alimentarius Commission, 1993). The limits of detection and quantification obtained are below the maximum residue level (MRL) established by the European Union. Oxolinic acid was stable in extracted sample for short term, three freeze-thaw cycles and long term with <15% variation. Standard solution was also stable at 4 °C for three months. Method robustness indicated that minor changes of the operational parameters have little effect or do not lead to essential changes of the chromatographic separation (capacity factor). Tailing factor will increase or decrease when the composition of the mobile phase and pH has been changed. However, the values are acceptable, lower than 2 in all cases.

Table 4.3 illustrated the comparison of the proposed method with some other work. When comparing with capillary electrophoresis the proposed technique provided lower limit of detection. For those using HPLC, HPLC-MS-MS provides the lowest limit of detection but the mass detector is susceptible to matrix effect that can suppress ionization of the analyte (King *et al.*, 2000; Mei *et al.*, 2002; Dams *et al.*, 2003). Therefore, determination using HPLC-MS-MS requires a more complex clean-up procedure (two-stage SPE) than other detectors. HPLC-UV and HPLC-FLD (this work) methods offer lower cost than HPLC-MS-MS and oxolinic acid could be detected at concentration levels below the MRL. When comparing with HPLC-UV, HPLC-FLD (this work) gave the same limit of detection. However, the calculations are difference, HPLC-UV used the signal-to-noise method while the LOD of this work was based on the standard deviation of response which is statistically reliable and is suitable in practice since signal-to-noise ratio measurement does not consideration the uncertainty of the baseline variation (Ribani *et al.*, 2007). The LOD calculated from the signal to noise ratio can also be affected by the chromatographic conditions variations *e.g.* lifetime of the column, purity of mobile phase because the presence of noise or baseline unstable. For sample preparation technique, the one used in this work has several advantages, *i.e.*, several extraction can be done simultaneously, is simpler than other works, gives high recovery and no specialized laboratory equipment is required (microwave-assisted extraction)(Ahmed, 2003). The method was also validated by more parameters making this method reliable.

Qualitative and quantitative analysis of oxolinic acid were done in real samples that were purchased from fresh markets. The samples were extracted and analyzed. Oxolinic acid was not found from the normal analysis using the calibration curve. Therefore, standard addition method was carried out to confirm the finding and no oxolinic acid was found. Although oxolinic acid was not detected it has been proven that this method can be used to analyse trace residue. It would be useful for testing oxolinic acid residue when there is an epidemic in animals that will require oxolinic acid treatment.

In conclusion, the proposed method can be used for determination of oxolinic acid in edible animal tissues with selective, reliable, precise and high recovery. This method is simple (not required complex extraction), rapid (several extractions can be done simultaneously), cost effective (the sample preparation not required expensive instrument; <15 Bahts/sample). So, the proposed method is suitable for routine analysis for residue control in different matrix.

Table 4.3 Comparison of the proposed method with other method

Method	Sample	Sample preparation	LOD and LOQ ($\mu g\ kg^{-1}$)		Recovery (%)		Validation	Reference
Proposed method HPLC-FLD	Shrimp, fish, chicken meat, chicken liver, beef and pork	(1) Ex. Ultrasonic (2) SPE (the cartridge were prepared in laboratory)	Shrimp Fish Chicken meat Chicken liver Pork Beef	6;17 9; 26 8; 22 18; 56 7; 22 3; 8	Shrimp Fish Chicken meat Chicken liver Pork Beef	89 87 95 86 96 74	Selectivity, Linearity, Accuracy, Precision, Recovery, LOD, LOQ, Stability Robustness	This work
CE	Fish meat and feeds	(1) Ex. Phosphate buffer (30+30 mL) (2) Centrifuge (15 min) (3) SPE (Commercial cartridge)	80; -		84.8		LOD, linearity, Precision, Recovery	Saad *et al.*, 2002
CE	Chicken meat	(1) Ex. Dichloromethane (20 mL) (2) Centrifuge (5 min) (3) Ex. NaOH (4) SPE (Commercial cartridge)	15; 48		94		LOD, LOQ, Recovery, Accuracy, Precision	Barrón *et al.*, 2002
HPLC-UV	Pork	(1) Microwave-assisted extraction (2) Centrifuge (10 min) (3) SPE (Commercial cartridge)	6; 20		85		LOD, LOQ, Recovery, Accuracy, Precision	Hermo *et al.*, 2005
HPLC-FLD	Chicken meat	(1)Ex: Acetonitrile basic solution (2) Centrifuge (3 min) (3) LLE (hexane) (4) Centrifuge (3 min)	12; -		73 ± 6.5		LOD, Recovery, Accuracy, Precision	Yorke and Forc, 2000
HPLC-MS-MS	Fish meat	(1) Ex. Acetonitrile (2) Two-stage SPE process (Commercial cartridge)	1-3; -		60-80		LOD, LOQ, Recovery, Accuracy, Precision	Johnston *et al.*, 2002

Ex= extraction; SPE= solid phase extraction; LLE= liquid-liquid extraction; CE= capillary electrophoresis; HPLC= high performance liquid chromatography

References

Agilent Technologies. 2002. Agilent 1100 Series Fluorescence Detector. Waldbronn, Germany, Agilent Technologies, Inc.

Ahmed, F. E. 2001. Analyses of pesticides and their metabolites in foods and drinks. *TrAC Trends in Analytical Chemistry* **20** (11): 649-661.

Ahmed, F. E. 2003. Analysis of polychlorinated biphenyls in food products. *TrAC Trends in Analytical Chemistry* **22** (3): 170-185.

Albero, B., Sanchez-Brunete, C. and Tadeo, J. L. 2003. Determination of endosulfan isomers and endosulfan sulfate in tomato juice by matrix solid-phase dispersion and gas chromatography. *Journal of Chromatography A* **1007** (1-2): 137-143.

Altria, K. D. and Elder, D. 2004. Overview of the status and applications of capillary electrophoresis to the analysis of small molecules. *Journal of Chromatography A* **1023** (1): 1-14.

Anastos, N., Barnett, N. W. and Lewis, S. W. 2005. Capillary electrophoresis for forensic drug analysis: A review. *Talanta* **67** (2): 269-279.

Andreu, V., Blasco, C. and Pico, Y. 2007. Analytical strategies to determine quinolone residues in food and the environment. *TrAC Trends in Analytical Chemistry* **26** (6): 534-556.

Arribas, A. S., Bermejo, E., Chicharro, M. and Zapardiel, A. 2007. Application of matrix solid-phase dispersion to the propham and maleic hydrazide determination in potatoes by differential pulse voltammetry and HPLC. *Talanta* **71** (1): 430-436.

Bailac, S., Ballesteros, O., Jimenez-Lozano, E., Barron, D., Sanz-Nebot, V., Navalon, A., Vilchez, J. L. and Barbosa, J. 2004a. Determination of quinolones in chicken tissues by liquid chromatography with ultraviolet absorbance detection. *Journal of Chromatography A* **1029** (1-2): 145-151.

Bailac, S., Ballesteros, O., Jiménez-Lozano, E., Barrón, D., Sanz-Nebot, V., Navalón, A., Vílchez, J. L. and Barbosa, J. 2004b. Determination of quinolones in chicken tissues by liquid chromatography with ultraviolet absorbance detection. *Journal of Chromatography A* **1029** (1-2): 145-151.

Bailac, S., Barrón, D. and Barbosa, J. 2006. New extraction procedure to improve the determination of quinolones in poultry muscle by liquid chromatography with ultraviolet and mass spectrometric detection. *Analytica Chimica Acta* **580** (2): 163-169.

Barker, S. A. 2000. Matrix solid-phase dispersion. *Journal of Chromatography A* **885** (1-2): 115-127.

Barker, S. A. 2007. Matrix solid phase dispersion (MSPD). *Journal of Biochemical and Biophysical Methods* **70** (2): 151-162.

Barriada-Pereira, M., González-Castro, M. J., Muniategui-Lorenzo, S., López-Mahía, P., Prada-Rodríguez , D. and Fernández-Fernández, E. 2007. Comparison of pressurized liquid extraction and microwave assisted extraction for the determination of organochlorine pesticides in vegetables. *Talanta* **71** (3): 1345-1351.

Barrón, D., Jiménez-Lozano, E., Bailac, S. and Barbosa, J. 2003. Simultaneous determination of flumequine and oxolinic acid in chicken tissues by solid phase extraction and capillary electrophoresis. *Analytica Chimica Acta* **477** (1): 21-27.

Belal, F., Al-Majed, A. A. and Al-Obaid, A. M. 1999. Methods of analysis of 4-quinolone antibacterials. *Talanta* **50** (4): 765-786.

Beltrán, J. L., Jiménez-Lozano, E., Barrón , D. and Barbosa, J. 2004. Determination of quinolone antimicrobial agents in strongly overlapped peaks from capillary electrophoresis using multivariate calibration methods. *Analytica Chimica Acta* **501** (2): 137-141.

Benito-Pena, E., Partal-Rodera, A. I., Leon-Gonzalez, M. E. and Moreno-Bondi, M. C. 2006. Evaluation of mixed mode solid phase extraction cartridges for the preconcentration of beta-lactam antibiotics in wastewater using liquid chromatography with UV-DAD detection. *Analytica Chimica Acta* **556** (2): 415-422.

Berge, A. C. B., Atwill, E. R. and Sischo, W. M. 2005. Animal and farm influences on the dynamics of antibiotic resistance in faecal Escherichia coli in young dairy calves. *Preventive Veterinary Medicine* **69** (1-2): 25-38.

Berrueta, L. A., Fernandez-Armentia, M., Bakkali, A., Gonzalo, A., Lucero, M. L. and Orjales, A. 2001. Matrix solid-phase dispersion technique for the determination of a new antiallergic drug, bilastine, in rat faeces. *Journal of Chromatography B: Biomedical Sciences and Applications* **760** (1): 185-190.

Björklund, E., Nilsson, T. and Bowadt, S. 2000. Pressurised liquid extraction of persistent organic pollutants in environmental analysis. *TrAC Trends in Analytical Chemistry* **19** (7): 434-445.

Blackwell, P. A., Holten Lutzhoft, H.-C., Ma, H.-P., Halling-Sorensen, B., Boxall, A. B. A. and Kay, P. 2004. Ultrasonic extraction of veterinary antibiotics from soils and pig slurry with SPE clean-up and LC-UV and fluorescence detection. *Talanta* **64** (4): 1058-1064.

Blasco, C., Pico, Y., Manes, J. and Font, G. 2002. Determination of fungicide residues in fruits and vegetables by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. *Journal of Chromatography A* **947** (2): 227-235.

Blasco, C., Van Poucke, C. and Van Peteghem, C. 2007. Analysis of meat samples for anabolic steroids residues by liquid chromatography/tandem mass spectrometry. *Journal of Chromatography A* **1154** (1-2): 230-239.

Blesa, J., Soriano, J. M., Molto, J. C., Marin, R. and Manes, J. 2003. Determination of aflatoxins in peanuts by matrix solid-phase dispersion and liquid chromatography. *Journal of Chromatography A* **1011** (1-2): 49-54.

Bogialli, S. and Di Corcia, A. 2007. Matrix solid-phase dispersion as a valuable tool for extracting contaminants from foodstuffs. *Journal of Biochemical and Biophysical Methods* **70** (2): 163-179.

Bogusz, M. J., El Hajj, S. A., Ehaideb, Z., Hassan, H. and Al-Tufail, M. 2004. Rapid determination of benzo(a)pyrene in olive oil samples with solid-phase extraction and low-pressure, wide-bore gas chromatography-mass spectrometry and fast liquid chromatography with fluorescence detection. *Journal of Chromatography A* **1026** (1-2): 1-7.

Boone, C. M., Douma, J. W., Franke, J. P., de Zeeuw, R. A. and Ensing, K. 2001. Screening for the presence of drugs in serum and urine using different separation modes of capillary electrophoresis. *Forensic Science International* **121** (1-2): 89-96.

Boyce, P. 2006. Sample preparation. *Filtration & Separation* **43** (7): 36.

Brighty, K. E., Gootz, T. D. and Vincent, T. A. (2000). Chemistry and Mechanism of Action of the Quinolone Antibacterials. The Quinolones (Third Edition). San Diego, Academic Press: 33-97.

Buhrman, D. L., Price, P. I. and Rudewicz, P. J. 1996. Quantitation of SR 27417 in Human Plasma Using Electrospray Liquid Chromatography-Tandem Mass Spectrometry: A Study of Ion Suppression. *Journal of the American Society for Mass Spectrometry* **7** (11): 1099-1105.

Buldini, P. L., Ricci, L. and Sharma, J. L. 2002. Recent applications of sample preparation techniques in food analysis. *Journal of Chromatography A* **975** (1): 47-70.

Camel, V. 2000. Microwave-assisted solvent extraction of environmental samples. *TrAC Trends in Analytical Chemistry* **19** (4): 229-248.

Camel, V. 2003. Solid phase extraction of trace elements. *Spectrochimica Acta Part B: Atomic Spectroscopy* **58** (7): 1177-1233.

Capitán-Vallvey, L. F., Al-Barbarawi, O. M. A., Fernández-Ramos , M. D. and Avidad, R. 2003. Determination of oxolinic acid in cow's milk and human urine by means of a single-use phosphorimetric sensor. *Talanta* **60** (2-3): 247-255.

Careri, M., Corradini, C., Elviri, L. and Mangia, A. 2007. Optimization of a rapid microwave assisted extraction method for the liquid chromatography-electrospray-tandem mass spectrometry determination of isoflavonoid aglycones in soybeans. *Journal of Chromatography A* **1152** (1-2): 274-279.

Castells, C. B., Gagliardi, L. G., Ràfols, C., Rosés , M. and Bosch, E. 2004. Effect of temperature on the chromatographic retention of ionizable compounds: I.

Methanol-water mobile phases. *Journal of Chromatography A* **1042** (1-2): 23-36.

Castro, R., Moyano, E. and Galceran, M. T. 2000. On-line ion-pair solid-phase extraction-liquid chromatography-mass spectrometry for the analysis of quaternary ammonium herbicides. *Journal of Chromatography A* **869** (1-2): 441-449.

Chambers, E., Wagrowski-Diehl, D. M., Lu, Z. and Mazzeo, J. R. 2007. Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. *Journal of Chromatography B* **852** (1-2): 22-34.

Cheng, J., Liu, M., Zhang, X., Ding, L., Yu, Y., Wang, X., Jin, H. and Zhang, H. 2007. Determination of triazine herbicides in sheep liver by microwave-assisted extraction and high performance liquid chromatography. *Analytica Chimica Acta* **590** (1): 34-39.

Chicharro, M., Moreno, M., Bermejo, E., Ongay, S. and Zapardiel, A. 2005. Determination of amitrole and urazole in water samples by capillary zone electrophoresis using simultaneous UV and amperometrical detection. *Journal of Chromatography A* **1099** (1-2): 191-197.

Chu, X.-G., Hu, X.-Z. and Yao, H.-Y. 2005. Determination of 266 pesticide residues in apple juice by matrix solid-phase dispersion and gas chromatography-mass selective detection. *Journal of Chromatography A* **1063** (1-2): 201-210.

Cinquina, A. L., Roberti, P., Giannetti, L., Longo, F., Draisci, R., Fagiolo, A. and Brizioli, N. R. 2003. Determination of enrofloxacin and its metabolite ciprofloxacin in goat milk by high-performance liquid chromatography with diode-array detection: Optimization and validation. *Journal of Chromatography A* **987** (1-2): 221-226.

Claessens, H. A. and van Straten, M. A. 2004. Review on the chemical and thermal stability of stationary phases for reversed-phase liquid chromatography. *Journal of Chromatography A* **1060** (1-2): 23-41.

Clemente, M., Hermo, M. P., Barrón , D. and Barbosa, J. 2006. Confirmatory and quantitative analysis using experimental design for the extraction and liquid chromatography-UV, liquid chromatography-mass spectrometry and liquid chromatography-mass spectrometry/mass spectrometry determination of quinolones in turkey muscle. *Journal of Chromatography A* **1135** (2): 170-178.

Cruces-Blanco, C., Gamiz-Gracia, L. and Garcia-Campana, A. M. 2007. Applications of capillary electrophoresis in forensic analytical chemistry. *TrAC Trends in Analytical Chemistry* **26** (3): 215-226.

Cuadros-Rodríguez, L., García-Campaña, A. M., Almansa-López, E., Egea-González, F. J., Lourdes Castro Cano, M., Garrido Frenich, A. and Martínez-Vidal, J. L. 2003. Correction function on biased results due to matrix effects: Application to the routine analysis of pesticide residues. *Analytica Chimica Acta* **478** (2): 281-301.

Curiel, H., Vanderaerden, W., Velez, H., Hoogmartens, J. and Van Schepdael, A. 2007. Analysis of underivatized gentamicin by capillary electrophoresis with UV detection. *Journal of Pharmaceutical and Biomedical Analysis* **44** (1): 49-56.

Dams, R., Huestis, M. A., Lambert, W. E. and Murphy, C. M. 2003. Matrix effect in bio-analysis of illicit drugs with LC-MS/MS: influence of ionization type, sample preparation, and biofluid. *Journal of the American Society for Mass Spectrometry* **14** (11): 1290-1294.

de Fatima Alpendurada, M. 2000. Solid-phase microextraction: a promising technique for sample preparation in environmental analysis. *Journal of Chromatography A* **889** (1-2): 3-14.

De Liguoro, M., Cibin, V., Capolongo, F., Halling-Sorensen, B. and Montesissa, C. 2003. Use of oxytetracycline and tylosin in intensive calf farming: evaluation of transfer to manure and soil. *Chemosphere* **52** (1): 203-212.

Delépée, R. and Pouliquen, H. 2002. Determination of oxolinic acid in the bryophyte Fontinalis antipyretica by liquid chromatography with fluorescence detection. *Journal of Chromatography B* **775** (1): 89-95.

Delépée, R., Pouliquen, H. and Le Bris, H. 2004. The bryophyte Fontinalis antipyretica Hedw. bioaccumulates oxytetracycline, flumequine and oxolinic acid in the freshwater environment. *Science of The Total Environment* **322** (1-3): 243-253.

Demeestere, K., Dewulf, J., De Witte, B. and Van Langenhove, H. 2007. Sample preparation for the analysis of volatile organic compounds in air and water matrices. *Journal of Chromatography A* **1153** (1-2): 130-144.

Deng, C., Liu, N., Gao, M. and Zhang, X. 2007. Recent developments in sample preparation techniques for chromatography analysis of traditional Chinese medicines. *Journal of Chromatography A* **1153** (1-2): 90-96.

Di Corcia, A. and Nazzari, M. 2002. Liquid chromatographic-mass spectrometric methods for analyzing antibiotic and antibacterial agents in animal food products. *Journal of Chromatography A* **974** (1-2): 53-89.

Diaz-Cruz, M. S. and Barcelo, D. 2007. Recent advances in LC-MS residue analysis of veterinary medicines in the terrestrial environment. *TrAC Trends in*

Analytical Chemistry **26** (6): 637-646.

Dopico-García, M. S., Valentao, P., Jagodzinska, A., Klepczynska, J., Guerra, L., Andrade, P. B. and Seabra, R. M. 2007. Solid-phase extraction versus matrix solid-phase dispersion: Application to white grapes. *Talanta*.

Ebrahimzadeh, H., Yamini, Y., Kamarei, F. and Shariati, S. 2007. Homogeneous liquid-liquid extraction of trace amounts of mononitrotoluenes from waste water samples. *Analytica Chimica Acta* **594** (1): 93-100.

Elizabeth Horne, M. O., Claire Desbrow and Anne Howells 1998. A novel sorbent for the determination of clenbuterol in bovine liver *Analyst* **123**: 2517 - 2520.

EMEA (1998). Committee for vertinary Medicinal Products: Oxolinic Acid. London, UK.

Ermer, J. and Ploss, H.-J. 2005. Validation in pharmaceutical analysis: Part II: central importance of precision to establish acceptance criteria and for verifying and improving the quality of analytical data. *Journal of Pharmaceutical and Biomedical Analysis* **37** (5): 859-870.

Espinosa-Mansilla, A., Pena, A. M. d. l., Gomez, D. G. and Salinas, F. 2005. HPLC determination of enoxacin, ciprofloxacin, norfloxacin and ofloxacin with photoinduced fluorimetric (PIF) detection and multiemission scanning: Application to urine and serum. *Journal of Chromatography B* **822** (1-2): 185-193.

Faria, A. F., de Souza, M. V. N., de Almeida, M. V. and de Oliveira, M. A. L. 2006. Simultaneous separation of five fluoroquinolone antibiotics by capillary zone electrophoresis. *Analytica Chimica Acta* **579** (2): 185-192.

Fattorini, P., Tomasella, F., Albertini, E., Grignani, P. and Previdere, C. 2004. Capillary electrophoresis reveals DNA damage in aged forensic samples. *International Congress Series* **1261**: 559-561.

FDA (2004). Seafood Chemistry. ORA Laboratory Manual. **IV:** 1-16

Feinberg, M. 2007. Validation of analytical methods based on accuracy profiles. *Journal of Chromatography A* **1158** (1-2): 174-183.

Fernández-Fígares, I., Rodríguez , L. C. and González-Casado, A. 2004. Effect of different matrices on physiological amino acids analysis by liquid chromatography: evaluation and correction of the matrix effect. *Journal of Chromatography B* **799** (1): 73-79.

Fernandez, M., Pico, Y. and Manes, J. 2000. Determination of carbamate residues in fruits and vegetables by matrix solid-phase dispersion and liquid chromatography-mass spectrometry. *Journal of Chromatography A* **871** (1-2): 43-56.

Ferrer, C., Gomez, M. J., Garcia-Reyes, J. F., Ferrer, I., Thurman, E. M. and Fernandez-Alba, A. R. 2005. Determination of pesticide residues in olives and olive oil by matrix solid-phase dispersion followed by gas chromatography/mass spectrometry and liquid chromatography/tandem mass spectrometry. *Journal of Chromatography A* **1069** (2): 183-194.

Fierens, C., Hillaert, S. and Van den Bossche, W. 2000. The qualitative and quantitative determination of quinolones of first and second generation by capillary electrophoresis. *Journal of Pharmaceutical and Biomedical Analysis* **22** (5): 763-772.

Flammer, K. 2006. Antibiotic Drug Selection in Companion Birds. *Journal of Exotic Pet Medicine* **15** (3): 166-176.

Fritz, J. S. and Macka, M. 2000. Solid-phase trapping of solutes for further chromatographic or electrophoretic analysis. *Journal of Chromatography A* **902** (1): 137-166.

Fu, I., Woolf, E. J. and Matuszewski, B. K. 1998. Effect of the sample matrix on the determination of indinavir in human urine by HPLC with turbo ion spray tandem mass spectrometric detection. *Journal of Pharmaceutical and Biomedical Analysis* **18** (3): 347-357.

Garcés, A., Zerzaňová, A., Kucera, R., Barrón, D. and Barbosa, J. 2006. Determination of a series of quinolones in pig plasma using solid-phase extraction and liquid chromatography coupled with mass spectrometric detection: Application to pharmacokinetic studies. *Journal of Chromatography A* **1137** (1): 22-29.

Garcinuno, R. M., Ramos, L., Fernandez-Hernando, P. and Camara, C. 2004. Optimization of a matrix solid-phase dispersion method with subsequent clean-up for the determination of ethylene bisdithiocarbamate residues in almond samples. *Journal of Chromatography A* **1041** (1-2): 35-41.

Gilar, M., Bouvier, E. S. P. and Compton, B. J. 2001. Advances in sample preparation in electromigration, chromatographic and mass spectrometric separation methods. *Journal of Chromatography A* **909** (2): 111-135.

Goncalves, C. and Alpendurada, M. F. 2005. Assessment of pesticide contamination in soil samples from an intensive horticulture area, using ultrasonic extraction and gas chromatography-mass spectrometry. *Talanta* **65** (5): 1179-1189.

Granelli, K. and Branzell, C. 2007. Rapid multi-residue screening of antibiotics in muscle and kidney by liquid chromatography-electrospray ionization-tandem mass spectrometry. *Analytica Chimica Acta* **586** (1-2): 289-295.

Graslund, S., Holmstrom, K. and Wahlstrom, A. 2003. A field survey of chemicals and biological products used in shrimp farming. *Marine Pollution Bulletin* **46** (1): 81-90.

Greibrokk, T. and Andersen, T. 2003. High-temperature liquid chromatography. *Journal of Chromatography A* **1000** (1-2): 743-755.

Grobbel, M., Lubke-Becker, A., Wieler, L. H., Froyman, R., Friederichs, S. and Filios, S. 2007. Comparative quantification of the in vitro activity of veterinary fluoroquinolones. *Veterinary Microbiology* **In Press**.

Grushka, E. and Grinberg, N. 2006. Advances in Chromatography, Tay & Francis.

Guerin-Faublee, V., Tardy, F., Bouveron, C. and Carret, G. 2002. Antimicrobial susceptibility of Streptococcus species isolated from clinical mastitis in dairy cows. *International Journal of Antimicrobial Agents* **19** (3): 219-226.

Ha, P. T. T., Hoogmartens, J. and Van Schepdael, A. 2006. Recent advances in pharmaceutical applications of chiral capillary electrophoresis. *Journal of Pharmaceutical and Biomedical Analysis* **41** (1): 1-11.

Hartmann, C., Smeyers-Verbeke, J., Massart, D. L. and McDowall, R. D. 1998. Validation of bioanalytical chromatographic methods. *Journal of Pharmaceutical and Biomedical Analysis* **17** (2): 193-218.

Hassouan, M. K., Ballesteros, O., Taoufiki, J., Vilchez, J. L., Cabrera-Aguilera, M. and Navalon, A. 2007. Multiresidue determination of quinolone antibacterials

in eggs of laying hens by liquid chromatography with fluorescence detection. *Journal of Chromatography B* **852** (1-2): 625-630.

Hendriks, G., Uges, D. R. A. and Franke, J. P. 2007. Reconsideration of sample pH adjustment in bioanalytical liquid-liquid extraction of ionisable compounds. *Journal of Chromatography B* **853** (1-2): 234-241.

Hennion, M.-C. 1999. Solid-phase extraction: method development, sorbents, and coupling with liquid chromatography. *Journal of Chromatography A* **856** (1-2): 3-54.

Hennion, M.-C. 2000. Graphitized carbons for solid-phase extraction. *Journal of Chromatography A* **885** (1-2): 73-95.

Hermo, M. P., Barron, D. and Barbosa, J. 2005. Determination of residues of quinolones in pig muscle: Comparative study of classical and microwave extraction techniques. *Analytica Chimica Acta* **539** (1-2): 77-82.

Hermo, M. P., Barrón, D. and Barbosa, J. 2006. Development of analytical methods for multiresidue determination of quinolones in pig muscle samples by liquid chromatography with ultraviolet detection, liquid chromatography-mass spectrometry and liquid chromagraphy-tandem mass spectrometry. *Journal of Chromatography A* **1104** (1-2): 132-139.

Hernandez-Arteseros, J. A., Barbosa, J., Compano, R. and Prat, M. D. 2002. Analysis of quinolone residues in edible animal products. *Journal of Chromatography A* **945** (1-2): 1-24.

Hernandez-Borges, J., Borges-Miquel, T. M., Rodriguez-Delgado, M. A. and Cifuentes, A. 2007. Sample treatments prior to capillary electrophoresis-mass spectrometry. *Journal of Chromatography A* **1153** (1-2): 214-226.

Hernández-Borges, J., Borges-Miquel, T. M., Rodríguez-Delgado, M. Á. and Cifuentes, A. 2007. Sample treatments prior to capillary electrophoresis-mass spectrometry. *Journal of Chromatography A* **1153** (1-2): 214-226.

Hernandez, F., Sancho, J. V., Ibanez, M. and Guerrero, C. 2007. Antibiotic residue determination in environmental waters by LC-MS. *TrAC Trends in Analytical Chemistry* **26** (6): 466-485.

Hernandez, M., Borrull, F. and Calull, M. 2000. Determination of quinolones in plasma samples by capillary electrophoresis using solid-phase extraction. *Journal of Chromatography B: Biomedical Sciences and Applications* **742** (2): 255-265.

Hirsch, R., Ternes, T., Haberer, K. and Kratz, K.-L. 1999. Occurrence of antibiotics in the aquatic environment. *The Science of The Total Environment* **225** (1-2): 109-118.

Ho, C., Sin, D. W. M., Tang, H. P. O., Chung, L. P. K. and Siu, S. M. P. 2004. Determination and on-line clean-up of (fluoro)quinolones in bovine milk using column-switching liquid chromatography fluorescence detection. *Journal of Chromatography A* **1061** (2): 123-131.

Hoof, N. V., Wasch, K. D., Okerman, L., Reybroeck, W., Poelmans, S., Noppe, H. and Brabander, H. D. 2005. Validation of a liquid chromatography-tandem mass spectrometric method for the quantification of eight quinolones in bovine muscle, milk and aquacultured products. *Analytica Chimica Acta* **529** (1-2): 265-272.

Hooper, D. C. 1999. Mechanisms of fluoroquinolone resistance. *Drug Resistance Updates* **2** (1): 38-55.

Horie, M., Ishikawa, F., Oishi, M., Shindo, T., Yasui, A. and Ito, K. 2007. Rapid determination of cyclamate in foods by solid-phase extraction and capillary electrophoresis. *Journal of Chromatography A* **1154** (1-2): 423-428.

Hu, Q., Zhang, L., Zhou, T. and Fang, Y. 2000. Determination of daunorubicin in human urine by capillary zone electrophoresis with amperometric detection. *Analytica Chimica Acta* **416** (1): 15-19.

Hu, Y.-Y., Zheng, P., He, Y.-Z. and Sheng, G.-P. 2005. Response surface optimization for determination of pesticide multiresidues by matrix solid-phase dispersion and gas chromatography. *Journal of Chromatography A* **1098** (1-2): 188-193.

Hubert, A., Wenzel, K.-D., Manz, M., Weissflog, L., Engewald, W. and Schuurmann, G. 2000. High Extraction Efficiency for POPs in Real Contaminated Soil Samples Using Accelerated Solvent Extraction. *Analytical Chemistry.* **72**: 1294-1300.

Huebra, M. J. G. d. l., Vincent, U. and von Holst, C. 2007. Sample preparation strategy for the simultaneous determination of macrolide antibiotics in animal feedingstuffs by liquid chromatography with electrochemical detection (HPLC-ECD). *Journal of Pharmaceutical and Biomedical Analysis* **43** (5): 1628-1637.

ICH (1996). Validation of Analytical Procedures: Methodology Q2B, International Conference on Harmonasation of Technical Requirements for Registration of pharmaceuticals for Human Use, London.

Injac, R., Kocevar, N. and Kreft, S. 2007. Precision of micellar electrokinetic capillary chromatography in the determination of seven antibiotics in pharmaceuticals and feedstuffs. *Analytica Chimica Acta* **594** (1): 119-127.

Ito, Y., Ikai, Y., Oka, H., Kagami, T. and Takeba, K. 1999. Application of ion-exchange cartridge clean-up in food analysis: II. Determination of benzylpenicillin, phenoxymethylpenicillin, oxacillin, cloxacillin, nafcillin and dicloxacillin in meat using liquid chromatography with ultraviolet detection. *Journal of Chromatography A* **855** (1): 247-253.

IUPAC 1997. IUPAC Compendium of Chemical Terminology. 2nd Edition.

Jafari, M. T., Khayamian, T., Shaer, V. and Zarei, N. 2007. Determination of veterinary drug residues in chicken meat using corona discharge ion mobility spectrometry. *Analytica Chimica Acta* **581** (1): 147-153.

Jarboe, H. H. and Kleinow, K. M. 1992. Matrix solid-phase dispersion isolation and liquid chromatographic determination of oxolinic acid in channel catfish (Ictalurus punctatus) muscle tissue. *Journal of AOAC International* **75**: 428-432.

Johnston, L., Mackay, L. and Croft, M. 2002. Determination of quinolones and fluoroquinolones in fish tissue and seafood by high-performance liquid chromatography with electrospray ionisation tandem mass spectrometric detection. *Journal of Chromatography A* **982** (1): 97-109.

Joshi, S. 2002. HPLC separation of antibiotics present in formulated and unformulated samples. *Journal of Pharmaceutical and Biomedical Analysis* **28** (5): 795-809.

Karbiwnyk, C. M., Carr, L. E., Turnipseed, S. B., Andersen, W. C. and Miller, K. E. 2007. Determination of quinolone residues in shrimp using liquid chromatography with fluorescence detection and residue confirmation by mass spectrometry. *Analytica Chimica Acta* **596** (2): 257-263.

Kataoka, H. 2003. New trends in sample preparation for clinical and pharmaceutical analysis. *TrAC Trends in Analytical Chemistry* **22** (4): 232-244.

Kemper, N. 2007. Veterinary antibiotics in the aquatic and terrestrial environment. *Ecological Indicators* **In Press**.

Kennedy, D. G., McCracken, R. J., Cannavan, A. and Hewitt, S. A. 1998. Use of liquid chromatography-mass spectrometry in the analysis of residues of antibiotics in meat and milk. *Journal of Chromatography A* **812** (1-2): 77-98.

King, R., Bonfiglio, R., Fernandez-Metzler, C., Miller-Stein, C. and Olah, T. 2000. Mechanistic investigation of ionization suppression in electrospray ionization. *Journal of the American Society for Mass Spectrometry* **11** (11): 942-950.

Kiridena, W., Poole, C. F. and Koziol, W. W. 2004. Effect of solvent strength and temperature on retention for a polar-endcapped, octadecylsiloxane-bonded silica stationary phase with methanol-water mobile phases. *Journal of Chromatography A* **1060** (1-2): 177-185.

Kishida, K. and Furusawa, N. 2001. Matrix solid-phase dispersion extraction and high-performance liquid chromatographic determination of residual sulfonamides in chicken. *Journal of Chromatography A* **937** (1-2): 49-55.

Koesukwiwat, U., Jayanta, S. and Leepipatpiboon, N. 2007. Solid-phase extraction for multiresidue determination of sulfonamides, tetracyclines, and pyrimethamine in Bovine's milk. *Journal of Chromatography A* **1149** (1): 102-111.

Kools, S. A. E., Moltmann, J. F. and Knacke, T. 2007. Estimating the use of veterinary medicines in the European Union. *Regulatory Toxicology and Pharmacology* **In Press**.

Kowalski, P., Oledzka, I. and Lamparczyk, H. 2003. Capillary electrophoresis in analysis of veterinary drugs. *Journal of Pharmaceutical and Biomedical Analysis* **32** (4-5): 937-947.

Kristenson, E. M., Brinkman, U. A. T. and Ramos, L. 2006. Recent advances in matrix solid-phase dispersion. *TrAC Trends in Analytical Chemistry* **25** (2): 96-111.

Kristenson, E. M., Haverkate, E. G. J., Slooten, C. J., Ramos, L., Vreuls, R. J. J. and Brinkman, U. A. T. 2001. Miniaturized automated matrix solid-phase dispersion extraction of pesticides in fruit followed by gas chromatographic-mass spectrometric analysis. *Journal of Chromatography A* **917** (1-2): 277-286.

Kulikov, A. U. and Zinchenko, A. A. 2007. Development and validation of reversed phase high performance liquid chromatography method for determination of dexpanthenol in pharmaceutical formulations. *Journal of Pharmaceutical and Biomedical Analysis* **43** (3): 983-988.

Labbozzetta, S., Valvo, L., Bertocchi, P. and Manna, L. 2005. Focused microwave-assisted extraction and LC determination of the active ingredient in naproxen-based suppositories. *Journal of Pharmaceutical and Biomedical Analysis* **39** (3-4): 463-468.

Lambropoulou, D. A. and Albanis, T. A. 2003. Headspace solid-phase microextraction in combination with gas chromatography-mass spectrometry for the rapid screening of organophosphorus insecticide residues in strawberries and cherries. *Journal of Chromatography A* **993** (1-2): 197-203.

Lara, F. J., García-Campaña, A. M., Alés-Barrer, Bosque-Sendra, J. M. and García-Ayuso, L. E. 2006. Multiresidue Method for the Determination of Quinolone

Antibiotics in Bovine Raw Milk by Capillary Electrophoresis-Tandem Mass Spectrometry. *Analytical Chemistry.* **78**: 7665-7673.

LeBlanc, G. 2001. A Review of EPA Sample Preparation Techniques for Organic Compound Analysis of Liquid and Solid Samples. *Sample Prep Perspectives.LCGC.* **19**: 1120-1130.

Lee, J.-B., Chung, H.-H., Chung, Y.-H. and Lee, K.-G. 2007. Development of an analytical protocol for detecting antibiotic residues in various foods. *Food Chemistry* **105** (4): 1726-1731.

Lehotay, G. K. J. 2004. MSPD Extraction of Phenolic Compounds from Fruit-Green Tea Using Various Non-Polar Sorbents *Journal of Liquid Chromatography & Related Technologies* **27** (18): 2837 - 2845.

Leitner, A., Zöllner, P. and Lindner, W. 2001. Determination of the metabolites of nitrofuran antibiotics in animal tissue by high-performance liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A* **939** (1-2): 49-58.

León-González and Pérez-Arribas , L. V. 2000. Chemically modified polymeric sorbents for sample preconcentration. *Journal of Chromatography A* **902** (1): 3-16.

Li, H., Kijak, P. J., Turnipseed, S. B. and Cui, W. 2006. Analysis of veterinary drug residues in shrimp: A multi-class method by liquid chromatography-quadrupole ion trap mass spectrometry. *Journal of Chromatography B* **836** (1-2): 22-38.

Li, J.-k., Wu, R.-n., Hu, Q.-h. and Wang, J.-h. 2007. Solid-phase extraction and HPLC determination of patulin in apple juice concentrate. *Food Control* **18** (5): 530-534.

Li, K., Landriault, M., Fingas, M. and Llompart, M. 2003. Accelerated solvent extraction (ASE) of environmental organic compounds in soils using a modified supercritical fluid extractor. *Journal of Hazardous Materials* **102** (1): 93-104.

Li, S. F. Y. 1993. Capillary electrophoresis; principles, practice and applications.

Li, X.-Z. 2005. Quinolone resistance in bacteria: emphasis on plasmid-mediated mechanisms. *International Journal of Antimicrobial Agents* **25** (6): 453-463.

Li, Z.-Y., Zhang, Z.-C., Zhou, Q.-L., Gao, R.-Y. and Wang, Q.-S. 2002. Fast and precise determination of phenthoate and its enantiomeric ratio in soil by the matrix solid-phase dispersion method and liquid chromatography. *Journal of Chromatography A* **977** (1): 17-25.

Lin, C.-E., Deng, Y., Jr., Liao, W.-S., Sun, S.-W., Lin, W.-Y. and Chen, C.-C. 2004. Electrophoretic behavior and pKa determination of quinolones with a piperazinyl substituent by capillary zone electrophoresis. *Journal of Chromatography A* **1051** (1-2): 283-290.

Liska, I. 2000. Fifty years of solid-phase extraction in water analysis - historical development and overview. *Journal of Chromatography A* **885** (1-2): 3-16.

Lõhmus, M. and Kender, T. 2007. Determination of gestagens in kidney fat by liquid chromatography tandem mass spectrometry. *Analytica Chimica Acta* **586** (1-2): 233-238.

Loos, R. and Niessner, R. 1999. Analysis of atrazine, terbutylazine and their N-dealkylated chloro and hydroxy metabolites by solid-phase extraction and gas chromatography-mass spectrometry and capillary electrophoresis-ultraviolet detection. *Journal of Chromatography A* **835** (1-2): 217-229.

Loveland, P. M., Reddy, A. P., Pereira, C. B., Field, J. A. and Bailey, G. S. 2001. Application of matrix solid-phase dispersion in the determination of dibenzo[a,l]pyrene content of experimental animal diets used in a large-scale tumor study. *Journal of Chromatography A* **932** (1-2): 33-41.

Male, K. B. and Luong, J. H. T. 2001. Derivatization, stabilization and detection of biogenic amines by cyclodextrin-modified capillary electrophoresis-laser-induced fluorescence detection. *Journal of Chromatography A* **926** (2): 309-317.

Maraschiello, C., Cusidó, E., Abellán, M. and Vilageliu, J. 2001. Validation of an analytical procedure for the determination of the fluoroquinolone ofloxacin in chicken tissues. *Journal of Chromatography B: Biomedical Sciences and Applications* **754** (2): 311-318.

Marchesini, G. R., Haasnoot, W., Delahaut, P., Gercek, H. and Nielen, M. W. F. 2007. Dual biosensor immunoassay-directed identification of fluoroquinolones in chicken muscle by liquid chromatography electrospray time-of-flight mass spectrometry. *Analytica Chimica Acta* **586** (1-2): 259-268.

Martínez-Carballo, E., González-Barreiro, C., Scharf, S. and Gans, O. 2007. Environmental monitoring study of selected veterinary antibiotics in animal manure and soils in Austria. *Environmental Pollution* **148** (2): 570-579.

Masqué, N., Marcé , R. M. and Borrull, F. 1998. New polymeric and other types of sorbents for solid-phase extraction of polar organic micropollutants from

environmental water. *TrAC Trends in Analytical Chemistry* **17** (6): 384-394.

McCourt, J., Bordin, G. and Rosa Rodriguez, A. 2003. Development of a capillary zone electrophoresis-electrospray ionisation tandem mass spectrometry method for the analysis of fluoroquinolone antibiotics. *Journal of Chromatography A* **990** (1-2): 259-269.

Mei, H., Hsieh, Y., Nardo, C., Xu, X., Wang, S., Ng, K. and Korfmacher, W. A. 2002. Investigation of matrix effects in bioanalytical high-performance liquid chromatography/tandem mass spectrometric assays: application to drug discovery. *Rapid Communications in Mass Spectrometry* **17** (1): 97-103.

Mitra, S. 2003. Sample Preparation Techniques in Analytical Chemistry. New Jersey, USA, John Wiley & Sons Inc.

Moller, J. G., Sta, H., Heinig, R. and Blaschke, G. 1998. Capillary electrophoresis with laser-induced fluorescence: a routine method to determine moxifloxacin in human body fluids in very small sample volumes. *Journal of Chromatography B: Biomedical Sciences and Applications* **716** (1-2): 325-334.

Monton, M. R. N. and Terabe, S. 2006. Sample enrichment techniques in capillary electrophoresis: Focus on peptides and proteins. *Journal of Chromatography B* **841** (1-2): 88-95.

Nadarassan, D. K., Chrystyn, H., Clark, B. J. and Assi, K. H. 2007. Validation of high-performance liquid chromatography assay for quantification of formoterol in urine samples after inhalation using UV detection technique. *Journal of Chromatography B* **850** (1-2): 31-37.

Nagaraju, D. and Huang, S.-D. 2007. Determination of triazine herbicides in aqueous

samples by dispersive liquid-liquid microextraction with gas chromatography-ion trap mass spectrometry. *Journal of Chromatography A* **1161** (1-2): 89-97.

Nakata, H., Kannan, K., Jones, P. D. and Giesy, J. P. 2005. Determination of fluoroquinolone antibiotics in wastewater effluents by liquid chromatography-mass spectrometry and fluorescence detection. *Chemosphere* **58** (6): 759-766.

Naviner, M., Giraud, E., Thorin, C., Le Bris, H., Pouliquen, H. and Ganiere, J.-P. 2007. Effects of three dosages of oral oxolinic acid treatment on the selection of antibiotic-resistant Aeromonas: Experimental approach in farmed trout. *Aquaculture* **269** (1-4): 31-40.

Neue, U. D. 1997. HPLC Column. USA, Wiley-VCH, Inc.

Núñez, O., Moyano, E. and Galceran, M. T. 2005. LC-MS/MS analysis of organic toxics in food. *TrAC Trends in Analytical Chemistry* **24** (7): 683-703.

O'Connor, S. and Aga, D. S. 2007. Analysis of tetracycline antibiotics in soil: Advances in extraction, clean-up, and quantification. *TrAC Trends in Analytical Chemistry* **26** (6): 456-465.

Pacáková, V. and Stulík, K. 1997. Capillary electrophoresis of inorganic anions and its comparison with ion chromatography. *Journal of Chromatography A* **789** (1-2): 169-180.

Palma, M. S. A., Paiva, J. L., Zilli, M. and Converti, A. 2007. Batch phenol removal from methyl isobutyl ketone by liquid-liquid extraction with chemical reaction. *Chemical Engineering and Processing* **46** (8): 764-768.

Park, H.-R., Kim, T. H. and Bark, K.-M. 2002. Physicochemical properties of quinolone antibiotics in various environments. *European Journal of Medicinal Chemistry* **37** (6): 443-460.

Pecorelli, I., Galarini, R., Bibi, R., Floridi, A., Casciarri, E. and Floridi, A. 2003. Simultaneous determination of 13 quinolones from feeds using accelerated solvent extraction and liquid chromatography. *Analytica Chimica Acta* **483** (1-2): 81-89.

Pena, T., Pensado, L., Casais, C., Mejuto, C., Phan-Tan-Luu, R. and Cela, R. 2006. Optimization of a microwave-assisted extraction method for the analysis of polycyclic aromatic hydrocarbons from fish samples. *Journal of Chromatography A* **1121** (2): 163-169.

Pensado, L., Casais, M. C., Mejuto, M. C. and Cela, R. 2005. Application of matrix solid-phase dispersion in the analysis of priority polycyclic aromatic hydrocarbons in fish samples. *Journal of Chromatography A* **1077** (2): 103-109.

Perez, M. I. B., Rodriguez, L. C. and Cruces-Blanco, C. 2007. Analysis of different [beta]-lactams antibiotics in pharmaceutical preparations using micellar electrokinetic capillary chromatography. *Journal of Pharmaceutical and Biomedical Analysis* **43** (2): 746-752.

Peters, F. T., Drummer, O. H. and Musshoff, F. 2007. Validation of new methods. *Forensic Science International* **165** (2-3): 216-224.

Petersen, J. R., Okorodudu, A. O., Mohammad, A. and Payne, D. A. 2003. Capillary electrophoresis and its application in the clinical laboratory. *Clinica Chimica Acta* **330** (1-2): 1-30.

Petrovic, M., Gros, M. and Barcelo, D. 2006. Multi-residue analysis of pharmaceuticals in wastewater by ultra-performance liquid chromatography-quadrupole-time-of-flight mass spectrometry. *Journal of Chromatography A* **1124** (1-2): 68-81.

Pfenning, A. P., , M., R.K. Turnipseed, S.B., Roybal, J. E., Holland, D. C., A.R., L. and Plakas, S. M. 1996. Determination and Confirmation of Identities of Flumequine and Nalidixic, Oxolinic, and Piromidic Acids in Salmon and Shrimp. *Journal of AOAC International* **79** (5): 1227-12235.

Pichon, V. 2000. Solid-phase extraction for multiresidue analysis of organic contaminants in water. *Journal of Chromatography A* **885** (1-2): 195-215.

Pico, Y., Font, G., Molto, J. C. and Manes, J. 2000. Solid-phase extraction of quaternary ammonium herbicides. *Journal of Chromatography A* **885** (1-2): 251-271.

Picó, Y., Rodríguez, R. and Mañes , J. 2003. Capillary electrophoresis for the determination of pesticide residues. *TrAC Trends in Analytical Chemistry* **22** (3): 133-151.

Poole, C. F. 2003. New trends in solid-phase extraction. *TrAC Trends in Analytical Chemistry* **22** (6): 362-373.

Poole, C. F., Schuette, S.A. 1984. Contemporary practice of chromatography. 1 st ed U.S.A.

Pouliquen, H. and Armand, F. 2000. Determination of oxolinic acid in faeces and urine of turbot (Scophthalmus maximus) by high-performance liquid chromatography using fluorescence detection. *Journal of Chromatography B: Biomedical Sciences and Applications* **749** (1): 127-133.

Pouliquen, H., Gouelo, D., Larhantec, M., Pilet, N. and Pinault, L. 1997. Rapid and simple determination of oxolinic acid and oxytetracycline in the shell of the blue mussel (Mytilus edulis) by high-performance liquid chromatography. *Journal of Chromatography B: Biomedical Sciences and Applications* **702** (1-2): 157-162.

Pouliquen, H., Pinault, L. and Le Bris, H. 1994. Determination of oxolinic acid in seawater, marine sediment and japanese oyster by high performance liquid chromatography. *Journal of Liquid Chromatography* **7**: 929–945.

Prat, M. D., Benito, J., Compañó, R., Hernández-Arteseros , J. A. and Granados, M. 2004. Determination of quinolones in water samples by solid-phase extraction and liquid chromatography with fluorimetric detection. *Journal of Chromatography A* **1041** (1-2): 27-33.

Prat, M. D., Ramil, D., Compano, R., Hernandez-Arteseros, J. A. and Granados, M. 2006. Determination of flumequine and oxolinic acid in sediments and soils by microwave-assisted extraction and liquid chromatography-fluorescence. *Analytica Chimica Acta* **567** (2): 229-235.

Puig, P., Borrull, F., Calull, M. and Aguilar, C. 2007. Recent advances in coupling solid-phase extraction and capillary electrophoresis (SPE-CE). *TrAC Trends in Analytical Chemistry* **In Press**.

Pyrzynska, K. 2007. Determination of molybdenum in environmental samples. *Analytica Chimica Acta* **590** (1): 40-48.

Ramil Criado, M., Hernanz Fernandez, D., Rodriguez Pereiro, I. and Cela Torrijos, R. 2004. Application of matrix solid-phase dispersion to the determination of polychlorinated biphenyls in fat by gas chromatography with electron-capture and mass spectrometric detection. *Journal of Chromatography A* **1056** (1-2):

187-194.

Ramirez, A., Gutierrez, R., Diaz, G., Gonzalez, C., Perez, N., Vega, S. and Noa, M. 2003. High-performance thin-layer chromatography-bioautography for multiple antibiotic residues in cow's milk. *Journal of Chromatography B* **784** (2): 315-322.

Ramos, L., Eljarrat, E., Hernandez, L. M., Rivera, J. and Gonzalez, M. J. 1999. Levels of PCBs, PCDDs and PCDFs in commercial butter samples in Spain. *Chemosphere* **38** (13): 3141-3153.

Ramos, M., Aranda, A., Garcia, E., Reuvers, T. and Hooghuis, H. 2003. Simple and sensitive determination of five quinolones in food by liquid chromatography with fluorescence detection. *Journal of Chromatography B* **789** (2): 373-381.

Reed, L. A., Siewicki, T. C. and Shah, J. C. 2004. Pharmacokinetics of oxytetracycline in the white shrimp, Litopenaeus setiferus. *Aquaculture* **232** (1-4): 11-28.

Rezić, I., Horvat, A. J. M., Babic, S. and Kastelan-Macan, M. 2005. Determination of pesticides in honey by ultrasonic solvent extraction and thin-layer chromatography. *Ultrasonics Sonochemistry* **12** (6): 477-481.

Rezić, I., Krstić, D. and Bokić, L. 2007. Ultrasonic extraction of resins from an historic textile. *Ultrasonics Sonochemistry* **In Press**.

Ribani, M., Collins, C. H. and Bottoli, C. B. G. 2007. Validation of chromatographic methods: Evaluation of detection and quantification limits in the determination of impurities in omeprazole. *Journal of Chromatography A* **1156** (1-2): 201-205.

Ridgway, K., Lalljie, S. P. D. and Smith, R. M. 2007. Sample preparation techniques for the determination of trace residues and contaminants in foods. *Journal of Chromatography A* **1153** (1-2): 36-53.

Rigos, G., Nengas, I., Alexis, M. and Troisi, G. M. 2004. Potential drug (oxytetracycline and oxolinic acid) pollution from Mediterranean sparid fish farms. *Aquatic Toxicology* **69** (3): 281-288.

Rodriguez, I., Llompart, M. P. and Cela, R. 2000. Solid-phase extraction of phenols. *Journal of Chromatography A* **885** (1-2): 291-304.

Romero-González, R., López-Martínez, J. C., Gomez-Milan, E., Garrido-Frenich, A. and Martínez-Vidal, J. L. 2007. Simultaneous determination of selected veterinary antibiotics in gilthead seabream (Sparus Aurata) by liquid chromatography-mass spectrometry. *Journal of Chromatography B* **In Press**.

Rostagno, M. A., Palma, M. and Barroso, C. G. 2007. Microwave assisted extraction of soy isoflavones. *Analytica Chimica Acta* **588** (2): 274-282.

Roudaut, B. and Yorke, J. C. 2002. High-performance liquid chromatographic method with fluorescence detection for the screening and quantification of oxolinic acid, flumequine and sarafloxacin in fish. *Journal of Chromatography B* **780** (2): 481-485.

Rubies, A., Vaquerizo, R., Centrich, F., Compañó, R., Granados, M. and Prat, M. D. 2007. Validation of a method for the analysis of quinolones residues in bovine muscle by liquid chromatography with electrospray ionisation tandem mass spectrometry detection. *Talanta* **72** (1): 269-276.

Saad, B., Mohamad, R., Mohamed, N., Lawrence, G. D., Jab, M. S. and Idiris Saleh, M. 2002. Determination of oxolinic acid in feeds and cultured fish using

capillary electrophoresis. *Food Chemistry* **78** (3): 383-388.

Saavedra, L. and Barbas, C. 2007. Chromatography-based on- and in-line pre-concentration methods in capillary electrophoresis. *Journal of Biochemical and Biophysical Methods* **70** (2): 289-297.

Santalad, A., Teerapornchaisit, P., Burakham, R. and Srijaranai, S. 2007. Capillary zone electrophoresis of organic acids in beverages. *LWT - Food Science and Technology* **40** (10): 1741-1746.

Santos, S. M., Henriques, M., Duarte, A. C. and Esteves, V. I. 2007. Development and application of a capillary electrophoresis based method for the simultaneous screening of six antibiotics in spiked milk samples. *Talanta* **71** (2): 731-737.

Sanz, M. L. and Martinez-Castro, I. 2007. Recent developments in sample preparation for chromatographic analysis of carbohydrates. *Journal of Chromatography A* **1153** (1-2): 74-89.

Schneider, M. J., Braden, S. E., Reyes-Herrera, I. and Donoghue, D. J. 2007. Simultaneous determination of fluoroquinolones and tetracyclines in chicken muscle using HPLC with fluorescence detection. *Journal of Chromatography B* **846** (1-2): 8-13.

Shabir, G. A. 2003. Validation of high-performance liquid chromatography methods for pharmaceutical analysis: Understanding the differences and similarities between validation requirements of the US Food and Drug Administration, the US Pharmacopeia and the International Conference on Harmonization. *Journal of Chromatography A* **987** (1-2): 57-66.

Shao, B., Han, H., Tu, X. and Huang, L. 2007. Analysis of alkylphenol and bisphenol A in eggs and milk by matrix solid phase dispersion extraction and liquid

chromatography with tandem mass spectrometry. *Journal of Chromatography B* **850** (1-2): 412-416.

Silvia Díaz-Cruz, M. S. and Barceló, D. 2007. Recent advances in LC-MS residue analysis of veterinary medicines in the terrestrial environment. *TrAC Trends in Analytical Chemistry* **26** (6): 637-646.

Simonet, B. M., Ríos, A. and Valcárcel, M. 2003. Enhancing sensitivity in capillary electrophoresis. *TrAC Trends in Analytical Chemistry* **22** (9): 605-614.

Singh, S. B., Foster, G. D. and Khan, S. U. 2007. Determination of thiophanate methyl and carbendazim residues in vegetable samples using microwave-assisted extraction. *Journal of Chromatography A* **1148** (2): 152-157.

Smith, R. M. 2003. Before the injection--modern methods of sample preparation for separation techniques. *Journal of Chromatography A* **1000** (1-2): 3-27.

Snyder, L. R. and Kirkland, J. J. 1979. Introduction to Modern Liquid Chromatography. USA, Jhon Wiley & Sons. Inc.

Snyder, L. R., Kirkland, J. J. and Glajch, J. L. 1997. Practical HPLC method development (2nd ed.). USA, Jhon Wiley & Sons, Inc.

Soler, C., Manes, J. and Pico, Y. 2005. Routine application using single quadrupole liquid chromatography-mass spectrometry to pesticides analysis in citrus fruits. *Journal of Chromatography A* **1088** (1-2): 224-233.

Sparr Eskilsson, C. and Bjorklund, E. 2000. Analytical-scale microwave-assisted extraction. *Journal of Chromatography A* **902** (1): 227-250.

Srinubabu, G., Raju, C. A. I., Sarath, N., Kumar, P. K. and Rao, J. V. L. N. S. 2007.

Development and validation of a HPLC method for the determination of voriconazole in pharmaceutical formulation using an experimental design. *Talanta* **71** (3): 1424-1429.

Stolker, A. A. M. and Brinkman, U. A. T. 2005. Analytical strategies for residue analysis of veterinary drugs and growth-promoting agents in food-producing animals--a review. *Journal of Chromatography A* **1067** (1-2): 15-53.

Sun, J., Deguchi, Y., Tauchi, Y., He, Z., Cheng, G. and Morimoto, K. 2006. Distribution characteristics of orally administered olamufloxacin, a newly synthesized fluoroquinolone antibacterial, in lung epithelial lining fluid and alveolar macrophage in rats. *European Journal of Pharmaceutics and Biopharmaceutics* **64** (2): 238-245.

Svec, F. 2006. Less common applications of monoliths: Preconcentration and solid-phase extraction. *Journal of Chromatography B* **841** (1-2): 52-64.

Tagliaro, F., Manetto, G., Crivellente, F. and Smith, F. P. 1998. A brief introduction to capillary electrophoresis. *Forensic Science International* **92** (2-3): 75-88.

Tagliaro, F., Turrina, S. and Smith, F. P. 1996. Capillary electrophoresis: principles and applications in illicit drug analysis. *Forensic Science International* **77** (3): 211-229.

Takatsuki, K. 1992. Gas chromatographic/mass spectrometric determination of oxolinic, nalidixic, and promidic acid in fish. *Journal of the Association of Official Analytical Chemists International* **75**: 982–987.

Teixeira, D. M. and Costa, C. T. d. 2005. Novel methods to extract flavanones and xanthones from the root bark of Maclura pomifera. *Journal of Chromatography A* **1062** (2): 175-181.

Tendencia, E. A. and de la Peña, L. D. 2001. Antibiotic resistance of bacteria from shrimp ponds. *Aquaculture* **195** (3-4): 193-204.

Teuber, M. 2001. Veterinary use and antibiotic resistance. *Current Opinion in Microbiology* **4** (5): 493-499.

Thurman, E. M. and Snavely, K. 2000. Advances in solid-phase extraction disks for environmental chemistry. *TrAC Trends in Analytical Chemistry* **19** (1): 18-26.

Timerbaev, A. R., Hartinger, C. G. and Keppler, B. K. 2006. Metallodrug research and analysis using capillary electrophoresis. *TrAC Trends in Analytical Chemistry* **25** (9): 868-875.

Toldrá, F. and Reig, M. 2006. Methods for rapid detection of chemical and veterinary drug residues in animal foods. *Trends in Food Science & Technology* **17** (9): 482-489.

Tor, A., Aydin, M. E. and Özcan, S. 2006. Ultrasonic solvent extraction of organochlorine pesticides from soil. *Analytica Chimica Acta* **559** (2): 173-180.

Toussaint, B., Bordin, G., Janosi, A. and Rodriguez, A. R. 2002. Validation of a liquid chromatography-tandem mass spectrometry method for the simultaneous quantification of 11 (fluoro)quinolone antibiotics in swine kidney. *Journal of Chromatography A* **976** (1-2): 195-206.

Toussaint, B., Chedin, M., Bordin, G. and Rodriguez, A. R. 2005. Determination of (fluoro)quinolone antibiotic residues in pig kidney using liquid chromatography-tandem mass spectrometry: I. Laboratory-validated method. *Journal of Chromatography A* **1088** (1-2): 32-39.

Turiel, E., Bordin, G. and Rodríguez, A. R. 2003. Trace enrichment of (fluoro)quinolone antibiotics in surface waters by solid-phase extraction and their determination by liquid chromatography-ultraviolet detection. *Journal of Chromatography A* **1008** (2): 145-155.

Turiel, E., Martín-Esteban, A. and Tadeo, J. L. 2006. Multiresidue analysis of quinolones and fluoroquinolones in soil by ultrasonic-assisted extraction in small columns and HPLC-UV. *Analytica Chimica Acta* **562** (1): 30-35.

Ueno, R., Sangrungruang, K. and Miyakawa, M. 1999. A simplified method for the determination of several fish drugs in edible fish and shrimp by high-performance liquid chromatography. *Food Research International* **32** (9): 629-633.

Urraca, J. L., Marazuela, M. D. and Moreno-Bondi, M. C. 2004. Analysis for zearalenone and [alpha]-zearalenol in cereals and swine feed using accelerated solvent extraction and liquid chromatography with fluorescence detection. *Analytica Chimica Acta* **524** (1-2): 175-183.

US-FDA (1994). Reviewer Guidance, Validation of Chromatographic Methods.

US-FDA (2000). Guidance for Industry, Analytical Procedures and Methods Validation.

US-FDA (2001). Guidance for Industry: Bioanalytical Method Validation.

Valenzuela, A. I., Lorenzini, R., Redondo, M. J. and Font, G. 1999. Matrix solid-phase dispersion microextraction and determination by high-performance liquid chromatography with UV detection of pesticide residues in citrus fruit. *Journal of Chromatography A* **839** (1-2): 101-107.

van der Hoff, G. R. and van Zoonen, P. 1999. Trace analysis of pesticides by gas chromatography. *Journal of Chromatography A* **843** (1-2): 301-322.

van Vyncht, G., Jànosi, A., Bordin, G., Toussaint, B., Maghuin-Rogister, G., De Pauw, E. and Rodriguez, A. R. 2002. Multiresidue determination of (fluoro) quinolone antibiotics in swine kidney using liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A* **952** (1-2): 121-129.

Vanhoenacker, G. and Sandra, P. 2005. High temperature liquid chromatography and liquid chromatography-mass spectroscopy analysis of octylphenol ethoxylates on different stationary phases. *Journal of Chromatography A* **1082** (2): 193-202.

Vila, J. 2007. S469 Quinolone resistance in the food chain. *International Journal of Antimicrobial Agents* **29** (Supplement 2): S99.

Wang, S.-P. and Chang, C.-L. 1998. Determination of parabens in cosmetic products by supercritical fluid extraction and capillary zone electrophoresis. *Analytica Chimica Acta* **377** (1): 85-93.

Wells, M. J. M. and Yu, L. Z. 2000. Solid-phase extraction of acidic herbicides. *Journal of Chromatography A* **885** (1-2): 237-250.

WHO (1998). Use of Quinolones in Food Animals and Potential Impact on Human Health. World Health Organization Emerging and other Communicable Diseases, Surveillance and Control, Geneva, Switzerland.

Wu, Y., Wang, Y., Huang, L., Tao, Y., Yuan, Z. and Chen, D. 2006. Simultaneous determination of five quinoxaline-1,4-dioxides in animal feeds using ultrasonic solvent extraction and high-performance liquid chromatography. *Analytica Chimica Acta* **569** (1-2): 97-102.

Xia, X.-R., Monteiro-Riviere, N. A. and Riviere, J. E. 2006. Trace analysis of fullerenes in biological samples by simplified liquid-liquid extraction and high-performance liquid chromatography. *Journal of Chromatography A* **1129** (2): 216-222.

Xiao, H. B., Krucker, M., Albert, K. and Liang, X. M. 2004. Determination and identification of isoflavonoids in Radix astragali by matrix solid-phase dispersion extraction and high-performance liquid chromatography with photodiode array and mass spectrometric detection. *Journal of Chromatography* **1032** (1-2): 117-124.

Yorke, J. C. and Froc, P. 2000. Quantitation of nine quinolones in chicken tissues by high-performance liquid chromatography with fluorescence detection. *Journal of Chromatography A* **882** (1-2): 63-77.

Zhang, L., Liu, Y., Xie, M.-X. and Qiu, Y.-M. 2005. Simultaneous determination of thyreostatic residues in animal tissues by matrix solid-phase dispersion and gas chromatography-mass spectrometry. *Journal of Chromatography A* **1074** (1-2): 1-7.

Zhou, T., Hu, Q., Yu, H. and Fang, Y. 2001. Separation and determination of [beta]-agonists in serum by capillary zone electrophoresis with amperometric detection. *Analytica Chimica Acta* **441** (1): 23-28.

Ziakova, A., Brandsteterova, E. and Blahova, E. 2003. Matrix solid-phase dispersion for the liquid chromatographic determination of phenolic acids in Melissa officinalis. *Journal of Chromatography A* **983** (1-2): 271-275.

Zou, Q.-H., Liu, Y., Xie, M.-X., Han, J. and Zhang, L. 2005. A rapid method for determination and confirmation of the thyreostats in milk and urine by matrix solid-phase dispersion and gas chromatography-mass spectrometry. *Analytica Chimica Acta* **551** (1-2): 184-191.

Vitae

Name Mr. Opas Bunkoed

Student ID 4822124

Education Attainment

Degree	Name of Institute	Year of Graduation
Bachelor of Science (Chemistry)	Thaksin University	2004

Scholarship Awards during Enrolment

The Center for Innovation in Chemistry: Post Graduate Education and Research Program in Chemistry (PERCH-CIC), Commission on Higher Education, Ministry of Education and Graduate School

List of Publication and Proceeding

Oral presentation

Opas Bunkoed, Panote Thavarungkul and Proespichaya Kanatharana. "Development of Sample Preparation Technique for Oxolinic acid residue in Shrimp" The 32nd Congress on Science and Technology of Thailand (STT 32). Queen Sirikit National Convention Center, Bangkok, Thailand, October 10-12, 2006.

Poster presentation

Opas Bunkoed, Panote Thavarungkul and Proespichaya Kanatharana. "Sample Preparation Technique for Trace Analysis of Oxolinic acid in Shrimp" The 5th PERCH-CIC Annual Scientific Congress (PERCH-CIC Congress V), Pattaya, Thailand, May 6-9, 2007.