METHOD DEVELOPMENT FOR THE DETERMINATION OF SULPHONAMIDE RESIDUES IN CHICKEN BY LIQUID CHROMATOGRAPHY ION TRAP TANDEM MASS SPECTROMETRY

by

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DECLARATION

I declare that the work presented in this thesis is an original work except for every part and portion that I had quoted.

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Å = Angstrom

cm = centimeter

g = gram

kg = kilogram

kV = kilovolt

M = molar

mm = milliliter

m/z = mass per charge ratio

pKa = acid dissociation constant

ppb = parts per billion

ppm = parts per million

rpm = ressolutions per minute

µg = microgram

µl = microliter

V = volt

PEMBANGUNAN KAEDAH BAGI PENENTUAN BAKI SULFONAMIDA PADA AYAM MENGGUNAKAN KROMATOGRAFI CECAIR SPEKTROMETER JISIM TANDEM PERANGKAP ION

ABSTRAK

Satu kaedah yang mudah, sensitif dan dipercayai untuk penentuan sisa lima sulfonamida (sulfadiazina, sulfametazina, selfakuinozalina dan sulfadimetoksina) di dalam ayam telah dibangunkan menggunakan gabungan Kromatografi Cecair Berprestasi Tinggi (HPLC) dan Spektrometri Jisim Tandem Perangkap Ion. Pengekstrakan sampel melibatkan pengekstrakan menggunakan asetonitril, proses nyah lemak menggunakan heksana dan diikuti penulinan ekstrak menggunakan penjerap polimer 'Strata X Solid Phase Extraction cartridge' selepas mencairkan semula menggunakan 0.2 M asid fosforik. Ekstrak dialirkan daripada penjerap polimer menggunakan metanol dan dikeringkan di dalam rendaman air yang dialirkan gas nitrogen berterusan. Baki dicairkan semula menggunakan campuran larutan 0.1 % asid asetik dan asetonitril (1:1). Kromatografi Cecair Pengionan Penyemburanelektro Perangkap Ion Spektrometer Jisim Tandem digunakan untuk pengesahan dan pengiraan baki sulfonamida. Suatu turus HPLC yang berdimeter sempit, Genesis C18 (120 Å, 3 µm, 5 sm x 2.1 mm) dan campuran larutan 0.1 % asetik asid di dalam air ultratulin dan asetonitril (65:35) pada kadar aliran 60 µl/min telah digunakan untuk memisahkan sulfonamida tersebut. Validasi kaedah analisis untuk mengesan baki sulfonamida telah dibuat dan pengiraan nilai ketidakpastian pengukuran telah dilakukan untuk memenuhi keperluan sistem kualiti ISO/IEC 17025. Semasa proses validasi spesifisiti, kelinearan, had pengesanan (LOD), had kuantitatif (LOQ), ketepatan dan kecekapan kaedah analisa ditentukan. Dari spektrum jisim, beberapa

ion baru yang boleh digunakan untuk pengesahan dan kuantitasi iaitu pada m/z 174 untuk sulfadiazina, sulfametazina dan sulfakuinozalina, pada m/z 204 untuk sulfametazina dan m/z 226 untuk sulfakuinozalina telah terhasil. Plot graf penentukuran yang dihasilkan adalah lurus pada kepekatan di antara 20 hingga 40 ppb (ng/g) bagi sulfadiazina, sulfakuinozalina dan sulfadimetoksina manakala 10 hingga 40 ppb (ng/g) untuk sulfametazina dengan pekali regrasi untuk setiap julat lengkuk penentukuran adalah 0.999. Kadar had pengesanan (LOD) untuk sulfametazina adalah 2 ppb (ng/g) sementara 5 ppb (ng/g) untuk sulfadiazina, sulfakuinozalina dan sulfadimetoksina. Had pengiraan kuantatif (LOQ) pula adalah 10 ppb (ng/g) untuk sulfametazina dan 20 ppb (ng/g) untuk sulfadiazina, sulfakuinozalina dan sulfadimetoksina. Peratusan ekstrak yang diperolehi semula ke atas sampel yang diperkaya dengan piawai pada paras LOQ adalah 51, 54, 68 dan 83 % sementara pekali variasinya adalah 5, 13, 9 dan 7 % masing-masing bagi sulfadiazina, sulfametazina, sulfakuinozalina dan sulfadimetoksina. Manakala nilai ketidakpastian masing-masing pada kepekatan 100 ppb bagi sulfadiazine, sulfametazina, sulfakuinozalina dan selfadimetoksina ialah 6, 9, 10 dan 4 ppb. Oleh itu daripada nilai ciri-ciri keupayaan yang diperolehi menunjukkan kaedah yang dibangunkan adalah boleh dipercayai untuk digunakan di dalam analisa rutin.

METHOD DEVELOPMENT FOR THE DETERMINATION OF SULPHONAMIDE RESIDUES IN CHICKEN BY LIQUID CHROMATOGRAPHY ION TRAP TANDEM MASS SPECTROMETRY

ABSTRACT

A simple, sensitive and reliable method for the determination of five sulphonamide residues (sulphadiazine, sulphamethazine, sulphaquinoxaline and sulphadimethoxine) in chicken was developed using a combination of high performance liquid chromatography (HPLC) with ion trap tandem mass spectrometry. Sample extraction involvd extraction with acetonitrile, removal of fat with n-hexane followed by purification of the extract with Strata X polymeric sorbent Solid Phase Eextraction cartridge after reconstitution with 0.2 M phosphoric acid. The extract was eluted with methanol and evaporated to dryness in a water bath under constant flow of nitrogen gas. The residue was again reconstituted with a solution mixture of 0.1 % acetic acid in ultra pure water and acetonitrile (1:1). A liquid chromatograph with an electrospray ionization interface to the ion trap tandem mass spectrometrer (LC-MS-MS) was used for simultaneous confirmation and quantitation of the sulphonamide residues. A narrow bore HPLC column, Genesis C18 120 (Å, 3 μm, 5 cm x 2.1 mm) and a solution of 0.1 % acetic acid in ultra pure water and acetonitrile (65:35) with a flow rate 60 μl/min was used to separate the sulphonamides. The analytical procedure for the detection of sulphonamide residues was validated and the measurement of uncertainty was determined for the compliance of the ISO/IEC 17025 quality system requirement. During validation, specificity, linearity, limit of detection (LOD), limit of quantitation (LOQ), precision and accuracy of the method was determined. New product ions that could be used for confirmation and quantitation at m/z 174 for sulphadiazine, sulphamethazine and sulphaquinoxaline, at m/z 204 for

sulphamethazine and m/z 226 for sulphaquinoxaline were observed. A linear plot was obtained for a concentration range between 20 ppb and 400 ppb for sulphadiazine, sulphaquinoxaline and sulphadimethoxine and 10 ppb to 400 ppb for sulphamethazin, respectively, where the regression coefficient for each calibration range obtained was 0.999. The limit of detection (LOD) was 2 ppb for sulphamethazine and 5 ppb for sulphadiazine, sulphaquinoxaline and sulphadimethoxine, respectively. The limit of quantification (LOQ) was 10ppb for sulphamethazine and 20 ppb for sulphadiazine, sulphaquinoxaline and sulphadimethoxine, respectively. The extraction recovery for spiked samples at the LOQ level was 51, 54, 68 and 83 % with coefficient of variation of 5, 13, 9, and 7 % for sulphadiazine, sulphamethazine, sulphaquinoxaline and sulphadimethoxine, respectively and the expanded uncertainty values at concentration of 100 ppb for sulphadiazine, sulphamethazine, sulphaquinoxaline and sulphadimethoxine were 6, 9, 10 and 4 ppb, respectively. Therefore from the performance characteristic obtained the developed method could be reliably used for routine analytical work.

CHAPTER 1

INTRODUCTION

The issue of drug residues in food-producing animals is a common global problem faced by the local health authority. It was reported that shrimp, chicken and chicken egg that were exported to Europe contained chloramphenicol (The New Straits Time, 2002), where this antibiotic was banned to be used in food producing animals.

Drugs especially antibacterials are frequently being used in agricultural practice at subtherapeutically level to maintain health and to promote weight gain, to decrease the amount of feed needed and to prevent disease and in higher dosage forms, for treatment of individual animals for specific disease conditions (Borner, 1997). Such usage may lead to problem of residues in foods which could threaten human health and cause allergic and toxic reactions. Furthermore, antibiotics used as growth promoters may encourage the development of antibiotic-resistance bacteria (Borner, 1997).

Realizing the potential hazard of the antibiotics used in animal production, public health officials and scientists need to examine and make appropriate responses on the usage of antibiotics. In England, due to the major *salmonella* epidemic in calves a committee known as Swann Committee was formed. Following the report by this committee antimicrobials used for animal production in England was regulated differently according to their category of use (Gustafson, 1991). Antimicrobials that were used for the promotion of growth continued to be used under the discretion of

the meat producer but for the treatment of diseases it could only be used under the supervision of the veterinarian. Both types of the antimicrobials should be licensed by The Ministry of Agriculture, Food and Fisheries. In the United States, also following the report by the Swann Committee, the United States Food and Drugs Administration and other agencies as well as interested group appointed a series of committees and task force to study the implication of antibiotic usage in animal feed (Gustafson, 1991).

Since the usage of antibiotics in poultry and livestock industries are unavoidable, it has become the responsibility of regulatory authorities to set maximum residue limits to ensure drug residues in food producing animals are safe to human. In the United States, the approval of the veterinary drug products used in food-producing animals is delegated to the United States Food and Drug Administration (USFDA), Center for Veterinary Medicine, Department of Health and Human Services. The regulatory authority responsible for determining compliance of Maximum Residues Limit (MRLs) is U. S. Department of Agriculture, Food Safety and Inspection Service (Oka *et. al*., 1995).

In Malaysia the regulatory authority responsible for determining compliance of maximum drug residues in food producing animals is the Division of Food Quality Control, Department of Public Health, Ministry of Health (Food Act 1983 & Regulations, 2000). The amount of drug residues in foods had been regulated by Regulation 40 of The Food Regulations (Food Act 1983 & Regulations, 2000). The Maximum Residue Limit (MRLs) of the veterinary drugs including sulphonamides had been set in Table 1 of Schedule 15A of the same regulation.

Sulphonamides are first major class of antibacterial compounds to be discovered and used extensively in food producing animals (Oka *et al*., 1995). It had been widely used for the treatment of diseased animals and the promotion of growth (Corcica, 2002). As a result of the continuous and high dose usage, the possibility of these residues remaining in food producing animals will increase. Due to the weak acid nature of sulfonamides they tend to bind to the basic side of the amino acid, as a result these drugs may remain in the host system for longer period than expected. In Malaysia the maximum permitted sulphonamide residues level was first gazetted in 1998 (Food Regulation 1985 (Amendment), 1998) and the maximum amount residue level was set in the same schedule of the Food Regulation as above. The maximum permitted amount is summarized in Table 1.1. Based on this table, the maximum permitted level for suphadiazine, sulphamethazine, sulphaquinoxaline and sulphadimethoxine was 100µg/kg in edible offal, tissue and muscle of poultry and livestock and 25µg/kg in milk. The residue was defined as its parent compound found in the above matrices.

Determination of sulphonamide residues in food for the enforcement of the Food Regulation is still new. The Ministry of Health has appointed a number of laboratories such as Department of Chemistry, Doping Control Centre, Public Health Laboratory and Veterinary Public Health Laboratory (His Majesty's Government Gazette, 21$^{st.}$ November 2002) as authorized laboratories for the determination of drug residues in foods. Before any analytical method can be used in routine analysis it has to be validated. Method validation is a process of establishing the performance characteristics and limitation of the analytical test method. There are two levels of analytical method validation, first is 'full method validation' where the performance

characteristics are determined by inter-laboratory performance study also known as collaborative study. The second level is called 'single laboratory method validation' where full method validation is not practical or necessary (Thompson *et al*., 2002).

There are several guidelines that can be adopted for the establishment of performance characteristics of analytical test method such as the guidelines by URACHEM Guide – The Fitness for Purpose of Analytical Method : A Laboratory Guide To Method Validation and Related Topics, Thompson and coworkers (2002) and others. The general requirements for the individual performance characteristics for a method validation are discussed below in section 2.4.

Therefore due to the fact that sulphonamides was widely used in food producing animals (Oka *et. al*., 1995) and their potential carcinogenic character (Niessen *et. al*. 1998), it is necessary to ensure that all foods sold in the market contain a safe level of sulphonamides. In addition, to fullfill the demand of law enforcement, the need to provide high sample throughput, relaible, robust and affordable analytical methodology, compared to previously developed method is very important. These requirements can only be met after the methodology has been properly investigated.

Table 1.1 The Maximum Residues Limit (MRLs) of sulphadiazine, sulphamethazine, sulphaquinoxaline and sulphadimethoxine permitted by Food Regulation 1985.

Substance	Drug Definition of residues in which MRL was set	Food	Maximum Residue Limits (MRLs) in food (µg/kg)
Sulphadiazine	Sulphadiazine	Edible offal (mammalian), muscle (mammalian), milk (cattle)	100
Sulphamethazine (sulphadimidine)	Sulphamethazine (sulphadimidine)	Milk (cattle)	25
		Edible offal (chicken and mammalian), muscle (chicken and mammalian), liver, kidney, fat (cattle)	100
		Edible tissue (cattle, turkey, chicken and pig)	100
Sulphaquinoxaline	Sulphaquinoxaline	Edible offal, muscle (poultry)	100
Sulphadimethoxine	Sulphadimethoxine	Milk (cattle)	25
		Edible offal, muscle (cattle and chicken)	100

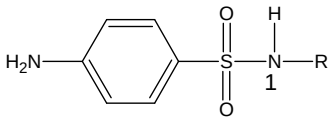
CHAPTER 2

LITERATURE REVIEW

2.1 The chemistry of sulphonamides investigated in this study.

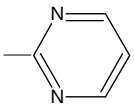
Sulphadiazine, sulphamethazine, sulphaquinoxaline and sulphadimethoxine belong to the class of sulphonamides that have amphotheric behavior because of the inductive properties of SO_2 group and poorly soluble in water, diethyl ether and chloroform but readily soluble in polar organic solvents such as acetone (Guggisberg *et al.*, 1992). It is not regarded as true antibiotics but instead as a synthetic chemical originally derived from the dyestuff industry. The term antibiotic is for agents derived from living organisms, or synthetic or semi-synthetic analogues of such compounds.

Sulphonamides interfere with bacteria growth by affecting the production of dihydrofolic acid, which is essential for the growth of bacteria. The pKa values of sulphadiazine, sulphamethazine, sulphaquinoxaline and sulphadimethoxine are 6.4, 7.4, 5.5 and 6.2, respectively (Agrawal, 1992). Sulphonamides are aromatic amines substituted at the N-1 position. The structure of R for sulphadiazine, sulphamethazine, sulphachloropyridazine, sulphaquinoxaline and sulphadimethoxine are illustrated as in Figure 2.1 and their molecular weights are 250, 278, 284, 300 and 310, respectively.

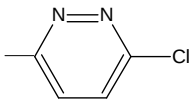


Common sulphonamide structure

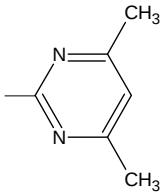
where R represents :



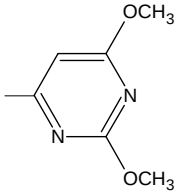
sulphadiazine (mol. wt. = 250)



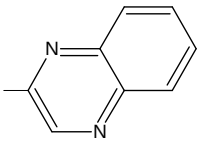
sulphachloropyridazine (internal standard)
(mol. wt. = 284)



sulphamethazine (mol. wt. = 278)



sulphadimethoxine (mol. wt = 310)



sulphaquinoxaline (mol. wt. 300)

Figure 2.1 Structure of sulphonamides investigated in this study.

2.2 Residue analysis of sulphonamides

A general approach for the detection of sulphonamide residues in the foods of animal origin such as meat, milk and eggs involves extraction, purification of sample extract and detection steps. Initially sulphonamides will be extracted with organic solvents such as acetonitrile, chloroform, methylene chloride, acetone, or ethyl acetate and following which the biological extract needs to be further purified; solid phase extraction cartridge (SPE) was widely used for this process. Automated extraction such as by pressurized liquid extraction was also used (Jacobsen *et. al*., 2004). Various SPE cartridges are used for cleaning-up such as normal phase, reverse phase and ion exchange cartridges. Besides the use of prepacked cartridge, self packed cartridge was also used (Hirsch *et. al*., 1998). Some author also used two catrridges for the clean-up (strong anion exchanger and polymeric hydrophilic-lipophilic cartridges) of the extract (Jacobsen *et. al*., 2004). Other than the application of SPE cartridge, liquid-liquid extraction, Matrix Solid Phase Dispersion (MSDP) (Long *et al*., 1990) and lyophilization (Hirsch *et. al*., 1998) was also used to concentrate the extract. Due to the excess usage of organic solvent where the storage of waste solvent will become problematic as well as higher productivity with SPE application, liquid-liquid extraction has become the least preferred technique.

After the cleaning-up, various chromatographic detection techniques were applied such as thin layer chromatography, gas chromatography, liquid chromatography and coupled technique such as liquid chromatography-mass spectrometry. Beside this, non-chromatographic detection technique such as enzyme

immunoassay was also used. The above diversification in the detection of sulphonamides will be presented below.

Horii and coworkers (1990) developed a method for the determination of three suphonamides in animal tissue and egg by liquid chromatography. Ten grams of sample was extracted with acetonitrile. The pH of the concentrated extract was changed to 1-2 with 1% trichloroacetic acid before loading into Bond-Elute C18, a reversed phase SPE cartridge. The sulphonamides were eluted from the SPE cartridge with 0.1 % triethylamine in acetonitrile. After evaporation of the elute, the residue was redissolved with 10 mM potassium dihydrogenphosphate solution. The analyte was analyzed by HPLC using Nucleosil 100 C18 column (5 µm, 250 x 4. 6mm) and 10 mM potassium dihydrogen phosphate-acetonitrile (78:22) as mobile phase and was detected by UV detector at 268 nm. The limit of detection was 0.01 ppm for sulphamethazine (SMZ) and sulphamonomethoxine (SMX) and 0.02 ppm for sulphadimethoxine (SDX). The limit of quantification was 0.02 ppm for SMZ and SMX and 0.04 ppm for SDX.

Furasawa and Mukai (1994) developed a method for the determination of sulphamonomethoxine, sulphadimethoxine and their N^4 – acetyl metabolite in beef, pork, chicken and eggs. Ten grams of sample was homogenized with 90 % acetonitrile and hexane. The acetonitrile layer was applied to an alumina column. Sulphonamides and their N^4 - acetyl metabolite were eluted with 90 % acetonitrile solution. The elute was evaporated to dryness and the residue was dissolved in acetonitrile in 0.05 M phosphate buffer (pH 5.0). The analyte was analyzed by HPLC using LiChrosorb RP-18 column (7 µm, 250 x 4 mm I.D.) and acetonitrile-0.05 M

phosphate buffer (pH 5.0) (25:75) as mobile phase and was detected with UV detector at 270 nm. The detection limit for all compounds by this method was 0.01 ppm.

Roybal and coworkers (2003) developed a method for the determination of six sulphonamides in shrimp. Two gram of sample was extracted with ethyl acetate and the clean-up of sample was done using size-exclusion chromatography column, Sephadex LH-20. Liquid chromatography with UV detector was used for detection of sulphonamides. Phenyl column (5 µm, 150 mm x 4.6 mm) and gradient elution of mobile phase containing methanol, acetic acid and 5 mM sodium hexanesulfonic acid was used for separation of sulphonamides. Recovery of sulphonamides for spiked samples at concentrations of 100 ppb, 50 ppb and 25 ppb was between 70 to 100 %.

Long and coworkers (1990) developed a method for extraction of sulphadimethoxine in catfish muscle tissue by matrix solid phase dispersion technique. Sulphamethoxazole was used as the internal standard. A sample was blended with octadecylsily derivatized silica packing material. A column made from the C18/sample was first washed with hexane and the analyte was eluted with dichloromethane and was evaporated to dryness. The residue was dissolved with the mobile phase and then centrifuged. The clear solution was filtered through 0.45 µm filter and was injected into the HPLC. A 10 µm, 30 cm x 4 mm reversed phase HPLC column was used with 0.017 M aqueous H_3PO_4-acetonitrile (65 + 35, v/v) as mobile phase. The sulphonamides were detected at 270 nm by PDA detector. The recovery of spiked samples obtained was 101 ± 4.2 % and inter assay and intra assay variability was 10.7 ± 8.2 % and 2.2 %, respectively

In the application of liquid chromatography-mass spectrometry, various ionization techniques and types of mass spectrometer were used. Kristiansen and coworkers (1994) made a comparison between flow injection thermospray tandem mass spectrometry (FI/TSI/MS/MS) while liquid chromatography thermospray tandem mass spectrometry (LC/TSI/MS/MS) for the determination of sulphonamide residues in meat. Five sulfonamides were analyzed and sulfapyridine was used as the internal standard. Ten grams of sample was extracted with ethyl acetate after adjusting the sample pH to 5.5 – 6 with 0.1 M HCl. After evaporation of the extract, the residue was dissolved with a solvent mixture of 0.05 M ammonium acetate/methanol (80:20), with no additional clean-up procedure. For these studies, a Finnigan TSQ 700 triple stage quadrupole instrument equipped with thermospray ionization was used for quantitation and confirmation of sulphonamides in the sample. For the LC/TSI-MS/MS analysis the sulphonamides were separated on a Chrompack Microsphere C18 column (3 µm, 100 x 4.6 mm) by using solvent mixture of 0.05 M ammonium acetate-methanol (77:23). The detection limit (LOD) for LC/TSI-MS/MS in meat was 2 ppb for sulphadiazine, sulphamethazine and sulfanilamide and 10 ppb for sulphathiazole and sulphadimethoxine. The LOD for FI/TSI-MS/MS was 2 ppb for sulphamethazine and sulphadimethoxine and 10 and 40 ppb for sulphathiazole and sulphanilamide, respectively.

The method for the determination of sulphadiazine residues in salmon muscle by HPLC and confirmation with atmospheric pressure chemical ionization mass spectrometer (LC-APCI/MS) was developed by Gehring and coworkers (1996). Two different SPE cartridges were used, first with strong cation cartridge and second with reversed phase cartridge. Ten grams of sample was extracted with acetonitrile after

homogenization of the sample with a solution mixture of acetonitrile and 2 % acetic acid (10:90). The extract was then partitioned with methylene chloride and the concentrated extract was loaded to Bond Elute propylsulfonic acid. For HPLC determination, sulphadiazine was eluted with a solution of 10 % acetonitrile in 0.2 M H_3PO_4. For the confirmation, sulphadiazine was first eluted with 0.2 M H_3PO_4 from the Bond Elute propylsulfonic acid SPE cartridge. The eluted solution was loaded to Waters Sep-Pak Vac 6 cc, 1.0 g, trifunctional C18 SPE cartridge and the sulphadiazine was eluted with methanol. For HPLC determination, the Inertsil ODS-2 (5 µm, 150 x 4.6 mm) column was used with acetonitrile-2 % acetic acid (10:90) as the mobile phase. Fluorescence detector with excitation and emission wavelength at 400 and 495 nm, respectively was used. Sulphadiazine was derivatised with fluorescamine solution using post column reaction system before being detected by the fluorescence detector. The limit of detection for this method was 0.2 ppb and limit of quantification was 1.0 ppb. For confirmation, a single quadrupole mass spectrometer equipped with atmospheric pressure chemical ionization interface was used. Positive ions were acquired in full scan or selected ion monitoring modes. The presence of 10 ng sulphadiazine per gram of sample was confirmed by LC/APCI/MS with the presence of sulphadiazine specific ions (m/z 252,158 and 96) and sulphonamide class specific ions (m/z 156, 108 and 92).

Ito and coworkers (2000) developed a simple, rapid and reliable method for the determination of ten sulphonamides in animal liver and kidney. Five grams of sample was extracted with ethyl acetate and was evaporated to dryness. The residue was then dissolved with 50 % ethyl acetate-hexane and was then applied to the Bond Elute PSA cartridge. In order to get optimum recovery the ten sulphonamides were

eluted with a solution mixture of 20 % acetonitrile-0.05 M ammonium formate. The sulphonamides were analyzed by HPLC using L-column ODS column (5 μm, 250 x 4.6 mm) and methanol-acetonitrile-0.05 M formic acid (10:15:75) as mobile phase and detected using UV detector at 277 nm. The detection limit for ten sulphonamides was 0.03 μg/g. For confirmation, the mass spectrometer used was Quatro 11 (Micromass, Altrincham, UK) equipped with electrospray ion source and the instrument was operated in the positive mode with a daughter ion scan. The presence of sulphadimidine (SDD) in the swine kidney and sulphamonomethoxine (SMX) in the bovine kidney was confirmed with the present of m/z 279, 186, 156 and 92 ions for SDD and m/z 281, 188, 156 and 92 ions for SMX, respectively.

Heller and coworkers (2002) developed a method for the determination of 16 sulphonamides in eggs. Ion Trap LC-MS-MS was used for confirmation and quantitation was done with liquid chromatography and UV detector. Five gram sample was extracted with acetonitrile and 3 ml water was added. After evaporation of acetonitrile, the solution was loaded into C18 cartridge. The analyte was eluted with acetonitrile and 1 ml water was added. The solution was concentrated to about 0.5 ml and was made to a final volume of 1 ml with water. Gradient elution was used with a combination of (A) 0.1 % formic acid-methanol (90:10); (B) methanol and (C) acetonitrile. The column used was Symmetry C8 (25 x 4.6 cm) and the UV detector was set at 287 nm. The recovery of 50 ppb, 100 ppb and 200 ppb of fortified sample was between 50 to 100 %. The author reported that the quantitation results with the LC-MS-MS were not satisfactory in terms of linearity, recovery and standard deviation.

The use of electrospray ionization LC-MS-MS for the confirmation and quantitation of 10 sulphonamides in honey was developed by Verzegnassi and coworkers (2002). Sulphonamides in honey were hydrolyzed to liberate sugar-bound sulphonamides followed by liquid-liquid extraction. Analysis was carried out with an 'Alliance' 2690 HPLC system coupled to the Quattro LC-MS-MS. Gradient elution was used with combination of solvent (A) 0.3 % formic acid and 5 % acetonitrile in water and (B) 0.3 % formic acid in acetonitrile at the flow rate 0.2 ml/min. The column used for separation was Nucleosil C18 HD (50 x 2 mm). The recovery of spiked sample at 50 ppb is between 44 to 73 %.

Renew and coworker (2004) developed a method for the detection of sulphonamides, fluoroquinolone and trimethoprim in waste water using tandem SPE cartridges and electrospray LC-MS. In this tandem SPE cartridge, anion exchange cartridge was stacked on the top of a hydrophilic-lipophilic balance cartridge. Sulphamerazine was used as an internal standard for the quantitation of sulphamethazine and sulphamethoxazole. A gradient mobile phase was used and a combination of solvent A contained 1 mM ammonium acetate, 0.007 % (v/v) acetic acid and 10 % acetonitrile and mobile phase B was 100 % acetonitrile. The flow rate was 0.25 ml/min and the column used was 2.1 x 150 mm Zorbax SB-C18. The detection limit for deionized water, final and secondary effluent ranged from 2 to 7 ng/L, 20 to 50 ng/L and 30 to 90 ng/L, respectively. The recovery for 1 ppb spiked sample was between 37 to 129 %.

Beside the purification of the extract with SPE cartridge and detection by HPLC and mass spectrometry as described above, a different method for the detection

of sulphonamides was done. Neidert and coworkers (1986) developed a rapid quantitative determination of sulphathiazole by thin layer chromatography (TLC) and densitometer in honey. Five gram of honey was extracted with dichloromethane and later was evaporated to dryness. The residue obtained was dissolved with acetonitrile and this solution was applied to the TLC plate. The TLC plate was then sprayed with fluorescamine solution. The plate was read by densitometer at excitation and emission wavelength 400 and 510 nm, respectively. In this quantitation method, sulphaquinoxaline was used as the internal standard. The recovery of this method was more than 98 % and the detection limit was 0.02 mg/kg.

Besides the above chromatographic methods for determination of sulphonamides, Sheth and coworker (1990) developed enzyme immunoassay method for the screening of sulphathiazole in honey. The detection limit for this method was 0.3 ppm and an estimated quantitation of sulphathiazole was also done. Capillary zone electrophoresis was also used for the determination of sulphonamides (Ackermans *et. al*., 1992). Sixteen sulphonamides were determined by the authors. The detection limit by this method was between 2 to 9 ppm.

From the above discussion only one method was reported by Ion Trap MS-MS technique for the determination of sulphonamide residues by liquid chromatography mass spectrometer, but the author claimed that the quantitation results obtained was unsatisfactory (Heller *et. al*.,2002). The other authors as mentioned above used single quadrupole or triple stage quadrupole MS-MS. Therefore it is the objective of this study to improve the quantitation results by Ion Trap MS-MS since this technique can offer cheaper alternative for confirmatory analysis. To do this, the method needs to be

evaluated through validation process. From the validation study results, the reliability of the method can be determined.

2.3 Electrospray Ionization Ion Trap Tandem Mass Spectrometer

Thermospray, Fast-Atom Bombardment, Atmospheric Pressure Chemical Ionization, Electospray Ionization and Matrix-Assisted Laser Desorption Ionization are ionization techniques for coupling of liquid chromatography with mass spectrometer (Watson, 1985). Electrospray ionization is one of the most important ionization techniques. Electrospray ionization can ionized small and big molecules at atmospheric pressure and probably one of the most gentle ionization techniques for the mass spectrometers (Bruins, 1998).

The nebulization of the effluent from the liquid chromatography in the electrospray ionization was achieved with the disruption of liquid stream by the high electric field at the spray needle into the small droplet. A potential between 3-5 kV was applied to the spray needle. With this potential and a high velocity of hot nitrogen gas flow, there will be a formation of a fine spray of highly charged aerosol of sample ions at the tip of the capillary (Niessen, 1998). The ions will be transmitted from the atmospheric pressure region to the high vacuum region of the mass analyzer via a low pressure transport region which consists of two or more successive pumps, i.e. rough pump and high vacuum pump (Watson, 1985). Schematic diagram of nebulizer probe for electrospry ionization is given in Figure 2.2. The sensitivity of the electrospray ionization depends on the transmission efficiency of the ions to the mass analyzer. As to improve transmission efficiency, earlier designs used ion lenses, followed by

multipoles (quadrupoles, hexapoles or octapoles) and the latest design used a stack of ring electrodes (Watson, 1985). A typical schematic diagram of electrospray ionization source and interface is shown in Figure 2.3.

The structure and theory behind the ion trap mass analyzer was elobrated in detail by March (1997) and was quoted as follow. Ion trap mass analyzer consist of four electrodes, two end-cap electrodes and another two are ring electrodes. These four electrodes having hyperboloidal geometry shape. The ring electrode is positioned symmetrically between two end-cap electrodes. The two end-cap electrodes can be distinguished by the number of the hole at the center of each electrode. Electrons and/or ions that were transported to the mass analyzer will be gated by the end-cap electrode that has one hole and will be ejected out from the end-cap electrode that has several holes, into electron multiplier. The quadrupole ion trap is a device which functions both as an ion storage in which gaseous ions can be confined for a period of time and as a mass spectrometer. The confinement of gaseous ions permits the study of gas phase ion chemistry and the elucidation of ion structures by the use of repeated stages of mass selection also known as tandem mass spectrometry (MS-MS). Tandem mass spectrometry is a process of carrying out one mass-selective operation after another. The objective of this operation is to isolate an ion species known as the parent ion and the second operation is to determine the mass to charge ratio of fragment ions due to the collision induced dissociation (CID).

The unique feature of ion trap mass analyzer was discussed by Karen and John (1997). The quadrupole ion trap is a mass analyzer with a size of a tennis ball. It was first invented by Wolfgang Paul in 1953 and the quadrupole ion trap mass

spectrometer was first commercialized in 1985. It has the capability for high mass resolution, mass range, and sensitivity and capable to perform MS^n. The main strength of this instrument when compare to the triple stages quadrupole and time-off-flight mass spectrometer is its ability to perform up to twelve stages of tandem mass spectrometry.

The quadrupole ion trap mass spectrometer is also known as tandem-in-time mass spectrometer. Another example on how tandem mass spectrometry experiments can be accomplished is through tandem-in-space instruments. An example of tandem-in-space instrument is the triple stage quadrupole mass spectrometer. Triple stage quadrupole as it name suggest, consists of two quadrupole mass analyzer (Q1 and Q3) and there are linked in between with a collision cell (Q2). The first quadrupole also known as Q1 acts as the mass filter, the second quadrupole (Q2) as collision cell with target gas (argon) admitted to the cell and third quadrupole (Q3) acts as a mass analyzer (Kienhuis, 1993).

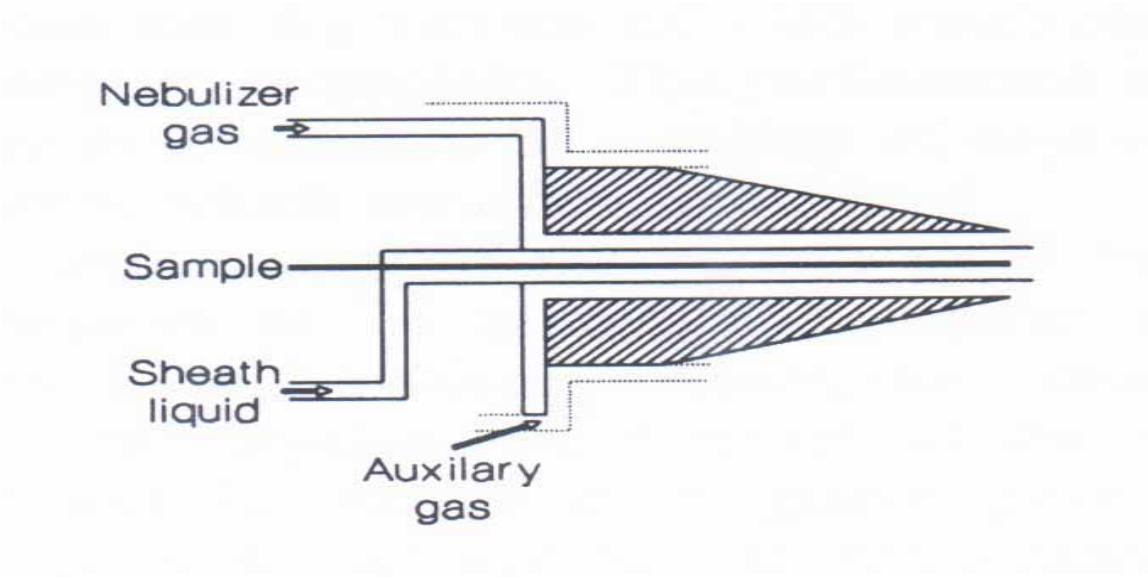


Figure 2.2 – Electrospray Ionization Nebulizer Probe (Niessen, 1998)

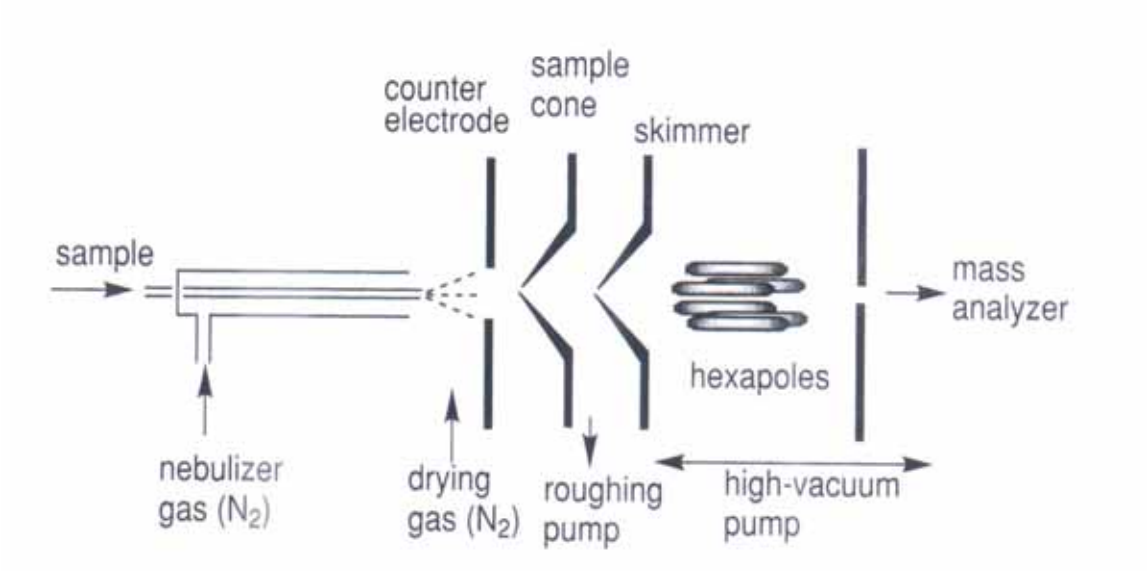


Figure 2.3 – Electrospray Ionization Ion Source and Interface (Watson, 1985)

2.4 Performance characteristics of test method validation

2.4.1 Specificity

Specificity was defined EURACHEM Guide (1998) as 'The ability of the method to determine accurately and specifically the analyte of interest in the presence of other components in a sample matrix under the stated condition of test'. The specificity of the method can be achieved in two ways; first through suitable extraction methods and second through suitable detection techniques.

Microbial growth inhibition assay was the first method for the detection of antimicrobial residue in foods, but this method have major disadvantages such as not specific, limited detection level, only for qualitative assay and may cause false positive results (Mitchell *et al*., 1998) and this reflects the lack of specificity by this method. Gas chromatography and liquid chromatography are the chosen techniques in term of specificity. Even though gas chromatography can provide better sensitivity, this technique requires the sulphonamides to be derivatized before it can be injected into the gas chromatograph (Guggisberg *et. al*., 1992). Nevertheless, the high performance liquid chromatography (HPLC) was normally preferable technique to avoide problem related to the derivatization with gas chromatography. Detection of sulphonamides in food by HPLC was reviewed by Agrawal (1992). The specificity by this technique was obtained through the used of column and mobile phase for the separation and detection at specific wavelength by UV detector. However, HPLC is not regarded as being sufficiently specific for use as a confirmatory technique in the European Union (Kennedy, 1998). In a more recent study, detection of sulphonamides

by HPLC coupled with mass spectrometry has became more popular. This detector is much more specific and provide unambiguous confirmation of the residues by providing the 'finger print' of the investigated compound (Kennedy *et. al*., 1998). The same reason was used for the selection of this technique in the research study.

2.4.2 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD of a method of analysis is the lowest concentration of analyte in the sample that can be detected and confirmed, but not necessarily quantified and the LOQ of a method of analysis is the lowest concentration of the analyte that can be quantified in a sample with an acceptable degree of certainty (EURACHEM Guide, 1998). For the instrumental method a signal to noise ratio of 3:1 is generally acceptable to establish the LOD and 10:1 for the determination of LOQ (ICH Guideline, 1996).

The values of LOD and LOQ are among one of the more important performance characteristics to be determined in method validation as discussed in section 2.2. Thus, logically the sensitivity of the method analysis can be observed from the value of limit of detection (LOD) and limit of quantitation (LOQ). A method with better sensitivity will have lower value of LOD and LOQ. For the determination of drug residues in food, the developed method must have the capability to detect residue below the maximum tolerance limit. For drugs with zero tolerance limits, the most sensitive method for detection of the residue is needed.

For the analysis of drug residue such as chloramphenicol where the tolerance limit was set at zero by the Food Regulation 1985, the detection method with highest sensitivity is needed. For example the LOD for analysis of chloramphenicol in various matrices by gas chromatography and liquid chromatography with UV detector was between 0.1-50 ppb and 0.1-500 ppb, respectively (Oka *et al*., 1995), but in 2002 the United States Food And Drug Administration developed a method for the detection of chloramphenicol in shrimp where the value of LOD and LOQ was 0.08 ppb and 0.3 ppb, respectively by using tandem mass spectrometer (US FDA Laboratory Information Bulletin). Therefore it is necessary to have a method with suitable sensitivity to detect the drug residues to suit with the regulatory requirements.

2.4.3 Linearity study

The linearity of analytical procedure is its ability to obtain test results that are directly, or by means of well-defined mathematical transformation, proportional to the concentration of the analyte in the sample within the given range. Data from calibration line will provide estimation of the degree of linearity. The slope of the regression line and its variance provide mathematical measure of linearity and the intercept is a measure of the potential method bias (Nata Technical Note No. 17, 1998).

2.4.4 Range

The range of analytical method is the interval between the upper and the lower levels (including this level) that have been demonstrated to be determined with precision, accuracy and linearity (Nata Technical Note No. 17, 1998).

2.4.5 Accuracy and Precision

The accuracy of analytical method is the closeness of agreement between the test result and reference value. Accuracy is often normally studied as two component: ‘trueness’ and ‘precision’. The trueness of the method is an expression of how close the mean of a set of results produced by the method, to the true value (EURACHEM Guide, 1998).

Precision refers to the variability between repeated tests and can be measured by the coefficient of variation of the recoveries. Precision normally refers to the three conditions (EURACHEM Guide, 1998);

2.4.5.1 Repeatability

Repeatability refers to close agreement between the results of successive measurement of the same measurand carried out in the same condition of measurement. Repeatability is to assess the variability of test results following execution of the method by one person in one laboratory.

2.4.5.2 Intermediate Precision

Intermediate precision expresses within laboratory variation. The extents to which intermediate precision should be established, depends on the circumstances under which the procedure are intended to be used. The effect of the random events on the precision of the analytical procedure should be established. Typical variation to be studied includes days, analysts, equipment, etc. It is not considered necessary to study these effects individually. This process is to verify the capability the laboratory to produce the same results once the method development is over.

2.4.5.3 Reproducibility

Reproducibility is assessed by means of an inter-laboratory trial. Reproducibility should be considered in case of standardization of an analytical procedure.

As a guideline for the acceptance criteria of the validation of analytical method, a guideline by the Australian Pesticides and Veterinary Medicine Authority (Residue Guideline No. 26, 2003) was followed. The value of coefficient of variation (CV) was accepted if the value has not exceeded the value set in Table 2.1.