



A MULTICLASS METHOD FOR THE ANALYSIS OF ANTIBIOTIC RESIDUES IN MILK POWDER AND WHEY POWDER BY UPLC-MS/MS

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ABSTRACT

An ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) multi-class method was developed and validated for the determination of 27 antibiotic residues from five classes (macrolides, sulfonamids, quinolones, lincosamids, diaminopyrimidines) in milk powder and whey powder which are commonly used as raw material of many products in the food industry. A simple, inexpensive and fast sample preparation based on the extraction of samples with methanol is the main advantage of the validated method. The validation of the method was performed following the requirements outlined in the European Commission Decision 2002/657/EC, which is required procedure for the methods used in analysis of official audits samples in Europe and Turkey. Matrix effect and uncertainties related with sample preparation and instrumental analysis were minimised by using matrix-matched calibration. The method is capable of detecting residues at very low concentrations.

Keywords: antibiotics, residues, UPLC-MS/MS, validation, milk powder, whey powder, 2002/657/EC

SÜT TOZU VE PEYNİRALTI SUYU TOZUNDA ANTİBİYOTİK KALINTILARININ UPLC-MS/MS İLE ANALİZİNDE ÇOKLU GRUP YÖNTEMİ

ÖZ

Gıda endüstrisinde birçok ürünün hammaddesi olarak yaygın bir şekilde kullanılan süt tozu ve peyniraltı suyu tozunda beş gruptan (makrolidler, sülfanamidler, kinolonlar, linkozamidler, diaminopirimidinler) 27 antibiyotik kalıntısının belirlenmesi için bir ultra-performans sıvı kromatografisi-tandem kütle spektrometrisi (UPLC-MS/MS) çok sınıflı bir yöntem geliştirilmiş ve valide edilmiştir. Valide edilmiş bu yöntemin ana avantajı, metanol ile numunelerin ekstraksiyonuna dayanan basit, ucuz ve hızlı numune hazırlamadır. Yöntemin validasyonu, Avrupa ve Türkiye'deki resmi denetim numunelerinin analizinde kullanılan yöntemler için gerekli prosedür olan 2002/657/EC sayılı Avrupa Komisyonu Kararında belirtilen gereklilikler izlenerek gerçekleştirilmiştir. Matriks uyumlu kalibrasyon kullanılarak, numune hazırlama ve enstrümantal

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analiz ile ilgili matris etkisi ve belirsizlikler minimize edilmiştir. Yöntem, kalıntıları çok düşük konsantrasyonlarda tespit etme yeteneğine sahiptir.

Anahtar kelimeler: antibiyotikler, kalıntılar, UPLC-MS/MS, validasyon, süt tozu, peyniraltı suyu tozu, 2002/657/EC

INTRODUCTION

In animal husbandry, the use of antibiotics as to enhance efficiency and to promote animal growth was banned in EU countries and in Turkey since 2006, because of a risk to human health (European Commission, 2005; Ministry of Agriculture and Rural Affairs, 2006). This situation has created undesirable effects such as increased animal infections and decreased animal production, and the total amount of antibiotic use in animals increased due to the significant increase in therapeutic antibiotic and disinfectant use (Cheng et al., 2014). These antibiotics can cause residues in animal origin foods consumed by humans. Consumption of foods containing these residues poses many risks to public health, particularly development of antibiotic resistance, hypersensitivity reaction, carcinogenicity, mutagenicity, teratogenicity, and disruption of intestinal normal flora (Beyene, 2016). These risks emerge the importance of monitoring animal origin foods in terms of antibiotic residues. Although sensitive and reliable analytical methods for the determination of veterinary drugs in food of animal origin are strongly required to ensure food safety, multiclass methods for veterinary drugs are still limited (Dasenaki and Thomaidis, 2015).

In the European Union, the Maximum residue limits (MRL) of antibiotics in foods are determined by the Commission Regulation 37/2010 / EC (European Commission, 2010). The requirements for performance and validation of analytical methods employed in the official residues control are determined by European Decision 2002/657/EC (European Commission, 2002). Turkey legislation is also compatible with these regulations of the European Union.

In the national residue control plans, mostly the primary products are monitored in Europe and in Turkey. However, processed foods derived from products containing antibiotic residues are outside the control plans, thus posing a risk to

food safety. Methods that enable the monitoring of antibiotic residues in processed foods are important tools to ensure food safety by allowing these foods to be included in the National Control Plans.

Whey and milk powder, which have high nutritional value, are used in the production of many foods from floury foods to meat products, from dairy foods to chocolate and confectionery in order to improve the textural properties of foods (Coronado et al., 2002; Secchi et al., 2011; Liang and Hartel, 2004; Barbut, 2006; Serdaroğlu, 2006; González-Martinez et al., 2002). In addition, in recent years, the determination of their positive effects on health has led to the widespread use of whey powder and milk powder in the field of food supplements and functional food production (Hoppe et al., 2008; Ramos et al., 2015; Helkar et al., 2016). So, in order to protect public health, it is necessary that monitoring antibiotic residue in milk powder and whey powder.

Today LC-MS/MS, which has high selectivity and sensitivity, is the most common technique used in the detection and quantification of veterinary drug residues (Blasco et al., 2007; Moretti et al., 2017; Dasenaki and Thomaidis, 2015). The current trend in drug residue analysis is the development of multiple methods that can monitor a wide variety of compounds belonging to different classes. Although published methods for detecting antibiotic residue in milk are relatively abundant, there are a limited number of published studies to detect antibiotic residues in milk powder by LC-MS/MS. Yu and Hu (2012) developed a method of C18-stir bar sorptive extraction (SBSE)-high performance liquid chromatography (HPLC)-tandem mass spectrometry (MS/MS) for the determination of six sulfonamids in milk and milk powder samples. Rezende et al. (2012) developed a method involving ethyl acetate extraction and C-18 dispersive solid phase clean up for the determination of chloramphenicol and florfenicol

in liquid milk, milk powder and bovine muscle by LC–MS/MS and validated it according to the 2002/657/EC decision in liquid milk matrix. A comprehensive study was performed by Dasenaki and Thomaidis (2015) and a method was developed which can determine a wide range of veterinary drugs and pharmaceutical residues in milk powder, butter, fish tissue and eggs using LC–MS/MS with a quite long extraction procedure that includes steps such as ultrasonic bath, defatting with hexane, placing for 12 hours at -23 °C. A method involving acetonitrile extraction and lipid-removing SPE column Oasis PRiME HLB for the determination of 61 veterinary drugs in milk and milk powder by UPLC–MS/MS was developed by Tian et al. (2016) and in validation, parameters such as linearity and precision were evaluated according to 2002/657/EC criteria.

In this study, a simple, fast and inexpensive multiresidue method was validated for the detection of antibiotic residues in whey powder and milk powder which are used as raw material in a wide range of food products. Within the scope of the validated method, 6 parameters from macrolide group, 11 parameters from sulfonamid group, 9 parameters from quinolone group, 1 parameter from lincosamid group, 1 parameter from diaminopyrimidine, totally 27 veterinary drugs were analyzed in whey powder and milk powder. The method is based on the extraction of samples with methanol and the determination and calculation of the residues in the extract by LC–MS/MS. Two product ions obtained from the fragmentation of the molecular ion determined for each analyte were used at the identification of analytes by LC–MS/MS. The highest intensities ions were used for quantification, and the other ions were used for confirmation. The ratios and retention times of the selected ions were also taken into account in the identification of the analytes. Matrix-matched calibration standards were used by the analysis of spiked blank matrix samples to compensate matrix effect. This is the most widely used approach to compensate for signal suppression experienced during MS/MS analysis (Shendy et al., 2016). Since milk powder and whey powder do not have the maximum

residue limits defined in the regulations, the focus was on determining the lowest levels of each analyte that could be detected. And within the scope of the method, the ability to detect analytes at very low values has been obtained. A validation limit (VL) has been defined considering the lowest detectable concentration value for the analytes. And recovery, repeatability and reproducibility studies were performed at three levels, 1, 1.5 and 2 times the VL. Statistical evaluation of all parameters studied was carried out in accordance with the requirements of the Commission Decision 2002/657/EC of the European Union and the results were found within the appropriate value ranges.

MATERIALS AND METHODS

Chemicals and reagents

Erythromycin, Sarafloxacin Hydrochloride, Spiramycin, Tilmicosin, Tylosin, Sulfachloropyridazine, Sulfadiazine, Sulfadimethoxine, Sulfadoxine, Sulfamerazine, Sulfamethazine, Sulfamethoxazole, Sulfapyridine, Sulfathiazole, Sulfaquinoxaline, Trimethoprim, Ciprofloxacin Hydrochloride, Danofloxacin Mesylate, Difloxacin Hydrochloride, Enrofloxacin, Erythromycin, Flumequine, Marbofloxacin, Nalidixic Acid, Oxolinic Acid were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Neospiramycin, Tylvalosin were obtained from TRC (Toronto, Canada). LC–MS grade methanol, formic acid supplied by Merck (Darmstadt, Germany). Ammonium formate (AF) (≥99%) was from Fluka (Buchs, Switzerland, Ultrapure water was produced by using Water Purification System; Elga, Purelab Option Q7.

Equipment

An UPLC–ESI–MS/MS instrument, consisted of a Waters Acquity Ultra-Performance LC with a Waters column manager and heater/cooler, binary system manager, sample manager coupled to a Waters XEVO TQD triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (Waters Corp., Milford, MA, USA) was used for mass spectrometric detection. Chromatographic

separation was effected with BEH C18 1.7 μ m 2.1x50 mm Column.

Sample preparation

5 \pm 0.1 g of sample was weighed in a 50 mL centrifuge tube. 15mL methanol was added and vortexed for 15 min. Then the tube centrifuged for 5 min at 10000 rpm. 7 mL of the supernatant was transferred to a clean glass centrifuge tube and dried at 50 °C in Nitrogen Turbo Evaporator (Biotage, TurboVap®LV). The residue was reconstituted with 1 mL of 50:50 Mobile phase A: Mobile phase B followed by vortex-mixing for 2 min and centrifuged for 5 min at 10000 rpm. The supernatant was filtered through a 0.22 μ m syringe filter into an HPLC vial. Five microliters of the sample was injected into the LC-MS/MS.

Instrument conditions

Water containing 0.1% formic acid and 0.1 mM ammonium formate (A) and methanol containing

0.1% formic acid and 0.1 mM Ammonium formate (B) was utilised as the mobile phase. The gradient conditions were employed as follows: 0–2 min, 1–1% B; 2.0–8.0 min, 1–95% B; 8.0–9.0 min, 95–95% B; 9.01–12.0 min, 1–1% B. The flow rate was set at 0.2 mL/min and column oven temperature was 60°C.

The analyses were performed in electrospray ionization (ESI) positive ion mode using a multiple reaction monitoring (MRM) for quantitative for data acquisition. The mass parameters were as follows: desolvation gas flow, 1000 L h⁻¹; desolvation temperature, 500°C; cone gas flow; cone voltage, 50 V; capillary voltage, 3.5 kV; source temperature, 150°C; extractor voltage, 3 V.

The optimized MRM parameters can be found in Table 1.

Table 1. Optimized parameters of the LC-MSMS system for the 27 selected antibiotics

Group	Name	Dwell time(s)	Molecular Ion	Cone (V)	Product ion 1	Collision (V)	Product ion 2	Collision (V)	Retention time (min)
Quinolones	Ciprofloxacin	0.005	332.1	42	288.1	18	314.1	22	4.55
	Danofloxacin	0.005	358.2	38	96.0	25	314.1	20	4.57
	Difloxacin	0.005	400.3	30	356.2	20	382.2	20	4.76
	Enrofloxacin	0.005	360.3	25	316.3	20	342.3	20	4.60
	Flumequine	0.005	262.1	35	202.0	15	244.0	25	6.24
	Marbofloxacin	0.005	363.1	35	72.0	20	320.0	15	4.30
	Nalidixic Acid	0.005	233.1	30	187.0	25	215.0	15	6.11
	Oxolinic Acid	0.005	262.0	32	216.0	30	244.0	19	5.52
Sulfonamides	Sarafloxacin	0.005	386.2	45	299.1	27	342.1	18	4.81
	Sulfachloropyridazine	0.005	285.1	32	92.0	28	156.0	15	4.75
	Sulfadiazine	0.005	251.0	30	92.0	27	156.0	15	3.78
	Sulfadimethoxine	0.005	311.1	36	92.0	32	156.0	20	5.47
	Sulfadoxine	0.005	311.0	35	92.0	32	156.0	15	5.04
	Sulfamerazine	0.005	265.1	35	92.0	25	156.0	15	4.29
	Sulfamethazine	0.005	279.1	35	124.1	25	186.0	15	4.67
	Sulfamethoxazole	0.005	254.1	30	92.0	25	156.0	15	4.77
	Sulfapyridine	0.005	250.0	33	108.0	25	156.0	16	4.05
	Sulfaquinoxaline	0.005	301.1	32	92.2	30	156.1	16	5.58
Diaminopyrimidines	Sulfathiazole	0.005	256.0	31	92.0	25	156.0	15	3.85
	Trimethoprim	0.005	291.3	40	123.0	30	230.2	30	4.41
Macrolides	Spiramycin	0.005	422.3	30	100.9	22	174.0	22	5.37
	Tilmicosin	0.005	869.6	94	132.0	52	174.0	50	5.86
	Tylosin	0.005	916.5	74	100.9	50	174.1	42	6.45
	Tylvalosin	0.005	1042.6	80	108.9	80	174.0	80	7.11
	Erytromycin	0.005	734.5	30	158.1	30	576.5	20	6.46
	Neospiramycin	0.005	699.5	58	142.1	26	174.1	28	5.18
Lincosamides	Lincomycin	0.005	407.2	40	126.1	25	359.3	20	4.45

Validation

Validation of the method for detection and quantitative analysis of analytes was carried out according to the requirements outlined in Commission Decision 2002/657/EC applicable to all analytes (veterinary medicines, pesticides, minerals, etc.) of animal products (EC 2002). In this validation study, limit of detection (LOD) and limit of quantification (LOQ), Linearity, Trueness/Recovery, Precision (Repeatability, Reproducibility), specificity/selectivity and ruggedness parameters were performed. The matrix-matched extraction procedure was used.

Linearity

The linearity of calibration curves was evaluated by using a five-level matrix matched calibration curve for all analytes. Blank samples were spiked with working standards to obtain the five concentration levels listed in Table 2 for each analyte. Peak areas were used as the analytical response versus concentrations for all target compounds. As the results given in Table 3, acceptable linear regression values ($r^2 > 0.99$) were obtained for all target analytes over the concentration range for the matrix matched calibration curves of milk powder and whey powder.

Table 2. Linear range and concentration levels of each analyte

Analyte	Linear range µg/kg	Level 1 µg/kg	Level 2 µg/kg	Level 3 µg/kg	Level 4 µg/kg	Level 5 µg/kg
Ciprofloxacin	5-30	5	8	12	16	30
Danofloxacin	5-30	5	8	12	16	30
Difloxacin	5-30	5	8	12	16	30
Enrofloxacin	5-30	5	8	12	16	30
Erythromycin	0.5-3	0.5	0.8	1.2	1.6	3
Flumequine	5-30	5	8	12	16	30
Lincomycin	2.5-15	2.5	4	6	8	15
Marbofloxacin	5-30	5	8	12	16	30
Nalidixic Acid	5-30	5	8	12	16	30
Neospiramycin	1.5-9	1.5	2.4	3.6	4.8	9
Oxolinic Acid	5-30	5	8	12	16	30
Sarafloxacin	5-30	5	8	12	16	30
Sulfachloropyridazine	2.5-15	2.5	4	6	8	15
Sulfadiazine	2.5-15	2.5	4	6	8	15
Sulfadimethoxine	2.5-15	2.5	4	6	8	15
Sulfadoxine	2.5-15	2.5	4	6	8	15
Sulfamerazine	2.5-15	2.5	4	6	8	15
Sulfamethazine	2.5-15	2.5	4	6	8	15
Sulfamethoxazole	2.5-15	2.5	4	6	8	15
Sulfapyridine	2.5-15	2.5	4	6	8	15
Sulfaquinoxaline	2.5-15	2.5	4	6	8	15
Sulfathiazole	2.5-15	2.5	4	6	8	15
Spiramycin	0.5-3	0.5	0.8	1.2	1.6	3
Tilmicosin	0.5-3	0.5	0.8	1.2	1.6	3
Trimethoprim	2.5-15	2.5	4	6	8	15
Tylosin	0.5-3	0.5	0.8	1.2	1.6	3
Tylvalosin	0.5-3	0.5	0.8	1.2	1.6	3

Table 3. Linearity and recovery results of analytes for milk powder and whey powder

Analyte	Milk powder				Whey powder			
	Linearity	Recovery(%)			Linearity	Recovery(%)		
		Level 2	Level 3	Level 4		Level 2	Level 3	Level 4
Ciprofloxacin	0.995	92.4	99.9	91.9	0.998	98.1	102.9	100.9
Danofloxacin	0.994	95.9	94.0	98.3	0.991	100.0	101.0	97.8
Difloxacin	0.995	97.5	102.3	98.9	0.993	98.9	105.3	102.2
Enrofloxacin	0.999	98.1	101.9	101.6	0.998	97.1	104.3	99.5
Erytromycin	0.992	98.0	100.9	103.7	0.992	101.7	97.8	95.5
Flumequine	0.996	99.7	108.9	102.5	0.997	99.7	108.9	102.5
Lincomycin	0.995	93.7	99.5	98.0	0.997	93.4	104.7	100.4
Marbofloxacin	0.997	96.5	98.3	100.0	0.995	98.0	99.8	102.3
Nalidixic Acid	0.993	97.9	105.1	102.3	0.993	98.7	106.3	102.2
Neospiramycin	0.991	93.0	99.5	100.1	0.990	97.4	100.8	97.7
Oxolinic Acid	0.997	96.0	103.8	102.1	0.995	101.2	106.4	102.7
Sarafloxacin	0.993	98.0	101.7	99.4	0.990	98.3	104.0	101.7
Sulfachloropyridazine	0.992	99.8	103.6	98.5	0.991	100.2	102.6	99.0
Sulfadiazine	0.992	102.4	101.6	101.4	0.990	95.5	100.9	99.5
Sulfadimethoxine	0.993	102.8	100.8	99.6	0.992	100.6	100.7	101.8
Sulfadoxine	0.995	100.4	98.9	93.5	0.997	100.5	102.3	101.0
Sulfamerazine	0.995	103.8	98.3	104.5	0.992	100.3	100.7	100.8
Sulfamethazine	0.996	106.4	97.4	103.9	0.997	100.9	99.4	99.7
Sulfamethoxazole	0.999	103.0	102.0	102.8	0.995	98.0	101.3	100.9
Sulfapyridine	0.993	103.5	106.1	98.9	0.997	102.0	106.3	101.4
Sulfaquinoxaline	0.996	100.4	96.9	91.5	0.997	100.7	101.8	100.7
Sulfathiazole	0.997	102.0	101.0	97.8	0.997	90.9	103.1	98.5
Spiramycin	0.991	85.4	99.6	101.6	0.990	97.1	96.0	99.2
Tilmicosin	0.990	95.6	105.0	89.4	0.991	99.0	100.8	91.6
Trimethoprim	0.993	100.0	100.7	96.0	0.992	96.0	100.9	103.2
Tylosin	0.990	93.9	99.2	99.3	0.991	100.8	106.9	99.6
Tylvalosin	0.994	101.5	104.0	100.1	0.994	96.2	102.4	103.0

Trueness

The trueness of the method was evaluated using recovery analyses of each analyte in milk powder and whey powder at 1, 1.5, 2 times the VL, (the second-third and fourth concentration levels in Table 2) 6 replicates. Matrix matched calibration curves were used for the evaluation of the recoveries obtained from analyses. Recoveries were calculated as a ratio between the determined concentration and the real concentration. Appropriate recoveries were obtained with the range of 85.4-108.9 % for milk powder and 90.9-108.9 % for whey powder as it seen in Table 3.

Precision (repeatability and within-laboratory reproducibility)

The precision of this method was showed in term of repeatability (intra-day precision) and within-laboratory reproducibility (inter-day precision), which were expressed as the relative standard deviation (RSD) values of set of 6 replicate spiked samples analysis at three different levels in 3 days. Six blank milk powders were spiked with working standard mix for each level, at 1, 1.5, 2 times the VL and analysed. This process repeated three days. In addition, as suggested by Kaufmann, (2009) one set of data was analysed for the other

matrix (whey powder) covered by the analytical method. A sample set was prepared in 6 parallel with blank whey powders, spiked with analyte mix at the same three levels (1, 1.5, 2 times the VL) and analysed. The obtained RSD values of the within-laboratory reproducibility were found below 10 % for the three fortified levels and all measurements did not exceed the acceptable values calculated from the Horwitz equation. These results indicate the sufficient precision of the developed method.

LOD, LOQ

For calculation of LOD and LOQ, the standard deviation (SD) of 20 independent measurements of blank samples fortified at the lowest level of calibration were used. Three times of obtained SD was calculated as LOD and ten times of obtained SD was calculated as LOQ. Very low levels of LOD and LOQ values were calculated especially for analytes in the macrolide group. The obtained values can be found in Table 4.

Table 4. LOD, LOQ, $CC\alpha$ and $CC\beta$ values

Analyte	LOD $\mu\text{g}/\text{kg}$	LOQ $\mu\text{g}/\text{kg}$
Ciprofloxacin	1.24	4.13
Danofloxacin	1.91	6.37
Difloxacin	1.02	3.40
Enrofloxacin	0.76	2.54
Erytromycin	0.22	0.73
Flumequine	1.02	3.40
Lincomycin	0.53	1.75
Marbofloxacin	1.94	6.46
Nalidixic Acid	1.68	5.59
Neospiramycin	0.62	2.08
Oxolinic Acid	0.80	2.67
Sarafloxacin	1.75	5.84
Sulfachloropyridazine	0.48	1.60
Sulfadiazine	0.53	1.78
Sulfadimethoxine	0.49	1.63
Sulfadoxine	0.38	1.26
Sulfamerazine	0.63	2.10
Sulfamethazine	0.46	1.52
Sulfamethoxazole	0.52	1.74
Sulfapyridine	0.88	2.92
Sulfaquinoxaline	0.42	1.40
Sulfathiazole	0.84	2.8
Spiramycin	0.19	0.63
Tilmicosin	0.23	0.78
Trimethoprim	0.52	1.72
Tylosin	0.23	0.78
Tylvalosin	0.20	0.67

Specificity / selectivity

The selectivity of the method was evaluated by the analysis of 20 blank milk powder samples. The absence of any peaks at the retention times of the target analytes indicated that there was no matrix interference that could lead to false positive signals.

Ruggedness

The method was evaluated in terms of ruggedness against changes in Centrifuge Speed in Centrifuge Time, Solvent Brand, Evaporation Temperature, Extraction Vortex Time, Vortex Time After Evaporation, Filter Brand parameters. Approach of Youden was used in the evaluation as indicated in 2002/657/EC (EC 2002). It was determined that there was no significant change at the results.

CONCLUSION

Low-cost, low-solvent consumption and speed are prioritised factors in the multiresidue methods development and the developed method has met all of these priorities. The validation results are in accordance with the European Commission Decision 2002/657/EC. Acceptable validation parameters such as linearity, recovery, precision and LOQs were obtained. It is clear that the present method is capable of detecting low amounts of residues when considering calculated LODs in the range of 0.19 $\mu\text{g}/\text{kg}$ -6.37 $\mu\text{g}/\text{kg}$. The validation results showed that this method is suitable for the determination of antibiotic residues in milk powder and whey powder.

The present method is accredited under ISO 17025 by the Turkish Accreditation Agency (TURKAK) and will allow the control of antibiotic residues in milk powder and whey powder to be included in National Residue Control Plans.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

İ.A. A.Ö and A.Y. designed the research. N.Ü, A.Ö. and İ.A. performed the validation. A.Y., İ.A and N.Ü. analyzed the data. A.Y. prepared the

manuscript. İ.A. edited the manuscript. All authors approved the submitted version.

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