1) **TITLE: QUANTITATIVE DETECTION OF ANTIBIOTICS OF THE TETRACYCLINE GROUP (Oxytetracycline, Tetracycline, Chlortetracycline, Doxycycline) IN MUSCLE (RED MEAT, POULTRY AND FISH) BY LC-MS/ MS**

2) **AUTHOR** : Yasemin KOÇYİĞİT, Dr. Yasemin ÇOŞKUN, A. Turan ERDOĞDU (Toksikoloji Bölümü)

AUTHORIZATION : Dr. Seval ÇETİN (Kalite Yönetim Birimi Şefi)

3) **VERSION : 1**

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1) **Principle**

This method consists of three (3) steps

- Homogenisation of the sample
- Extraction with 70% MeOH
- Detection in LC-MS/MS system with C18 reverse phase colomn and sequential mass spectrometry

2) **SCOPE AND AIMS**

a. Test Matrices : red meat (bovine, sheep) poultry (chicken, turkey) and Muscle and skin in natural proportions (fish)

b. Analytes : This method is applied for detection of residues of tetracycline group (oxytetracycline, tetracycline, chlortetracycline and doxycycline)drugs from animal foodstuffs which are red meat, poultry and fish.

c. Concentration of Interest : 50 - 400 µg/kg

d. MRL, zero tolerance, limit of interest : MRL : oxytetracycline : 100 µg/kg , Tetrasiklin : 100 µg/kg , Klortetrasiklin : 100 µg/kg , doxycycline : 100 µg/kg

e. Screening or confirmatory: Confirmatory

3) **Safety**

i. **Precautions** : Laboratory Safety Procedure (LAS.P.28) should be implemented. Chemicals should not be pipetted orally. Gloves and goggles should be used. Chemical waste bottles should be used. All work must be carried out under a hood.

ii. **Chemical Information** :

Methanol : Highly Flammable, Toxic

Acetonitrile : Highly Flammable, Harmful

Formic acide : Toxic, corrosive

Definitions :

LC : Liquid Chromatography

MS : Mass Spectrometry

MS-MS : Triple Quadrapole Mass Spectrometry

PPM : Parts per milion.

NM : Nanometre

PPB : Parts per billion

MW : Molecular Weight

MRL : Maximum residue limit

MRM : Multiple Reaction Mode,

MeOH: Methanol

Tissue: red meat (bovine, sheep) poultry (chicken, turkey) and Muscle and skin in natural proportions (fish)

Control Sample: Fish sample known to be negative

Spiked sample: Sample obtained by spiking the negative control with analytical standards of test compounds.

Reactive Blank: Reaktive Blank is a sample which is without test sample or is used a suitable solvent (such as water) instead of test sample and is exposed to all analytical procedure.

4) Materials

Chemicals/Reagents/Supplies :

- Acetonitrile, analytical grade
- Formic acid, analytical grade

- Oxalic acid dihydrate, analytical grade
- Methanol, analytical grade
- Titriplex, Na_2EDTA analytical grade

Preparation of The Mobile Phase A

- 0,126 gr oxalic acid is dissolved in 200ml water and solution is transfered volumetric flask which has 1 L volume and then 2 ml formic acid is added. The solution is completed to 1 liter with pure water. It can be stored for 7 days at room temperature.

Preparation of The Mobile Phase B

- 1 ml formic asid is added into the 1000 ml Acetonitrile. It can be stored for 30 days at room temperature.

70% MeOH Solution

- 700 ml methanol is poured in to the volumetric flask which has 1 L volume and then is completed to 1 liter with pure water.

MNa_2EDTA

- 3.72 g Na_2EDTA is dissolved in amount of pure water, and transfered in volumetric flask which has 100ml volume and then is completed to 100ml with pure water.

b. Standard/Standard Solutions :

- Oxytetracycline – hydrochloride , (Riedel De Haen , 46598) , analytical grade
- Tetracycline – hydrochloride (Fluka, 31741) , analytical grade
- Chlorotetracycline hydrochloride (Riedel De Haen, 46133) , analytical grade
- Doxycycline hyclrate (Fluka, 33429) , analytical grade
- Democlocycline Hydrochloride (Sigma Aldrich, D6140) , analytical grade

Main stock standard of oxytetracycline (1 mg/ml – 1000 ppm)

- 10 ± 0,01 mg pure oxytetracycline correspondingly standard of oxytetracycline is weighed into the volumetric flask which has 10 ml volume and then is completed to volume with methanol. It can be stored for 6 months at -20 ºC in dark.

Main stock standard of Tetracycline(1 mg/ml – 1000 ppm)

- 10 ± 0,01 mg pure Tetracycline correspondingly standard of Tetracycline is weighed into the volumetric flask which has 10 ml volume and then is completed to volume with methanol. It can be stored for 6 months at -20 ºC in dark.

Main stock standard of Chlorotetracycline (1 mg/ml – 1000 ppm)

- 10 ± 0,01 mg pure Chlorotetracycline correspondingly standard of Chlorotetracycline is weighed into the volumetric flask which has 10 ml volume and then is completed to volume with methanol. It can be stored for 6 months at -20 ºC in dark.

Main stock standard of Doxycycline (1 mg/ml – 1000 ppm

- 10 ± 0,01 mg pure Doxycycline correspondingly standard of Doxycycline is weighed into the volumetric flask which has 10 ml volume and then is completed to volume with methanol. It can be stored for 6 months at -20 ºC in dark.

Main stock standard of Democlocycline (1 mg/ml – 1000 ppm)

- 10 ± 0,01 mg pure Democlocycline correspondingly standard of Democlocycline is weighed into the volumetric flask which has 10 ml volume and then is completed to volume with methanol. It can be stored for 6 months at -20 ºC in dark.

İntermediate stock standard of Tetracycline Mix (100 µg /ml - 100 ppm)

- 600 µl methanol is completed to 1ml by the addition of 100 µl from main stock standard of tetracycline. It can be stored for 6 months at -20 ºC in dark.

İntermediate stock standard of Tetracycline Mix (10 µg /ml - 10 ppm)

- 900 µl methanol is completed to 1ml by the addition of 100 µl from intermediate stock standard of tetracycline which is 100ppm.

İntermediate stock standard of Tetracycline Mix (2 µg /ml - 2 ppm)

- 800 µl methanol is completed to 1ml by the addition of 200 µl from intermediate stock standard of tetracycline which is 10 ppm.

İntermediate stock standard of Democlocycline (100 µg /ml - 100 ppm)

- 900 µl methanol is completed to 1ml by the addition of 100 µl from main stock standard of democlocycline. It can be stored for 3 months at -20 ºC

İntermediate stock standard of Democlocycline (10 µg /ml - 10 ppm)

- 900 µl methanol is completed to 1ml by the addition of 100 µl from intermediate stock standard of democlocycline which is 100 ppm.

İntermediate stock standard of Democlocycline (2 µg /ml - 2 ppm)

- 800 µl methanol is completed to 1 ml by the addition of 200 µl from intermediate stock standard of democlocycline which is 10 ppm. Amount can be increased.

c. Equipments :

Materials

- Centrifuge Tubes: 50 ml, 15 ml
- Volumetric flasks: 10 ml, 25 ml, 50 ml, 100 ml, 250 ml, 500 ml ,and 1000 ml

Laboratory instruments

- Precision scales, CP 423 S (Sartorius, 1 mg hassasiyette)
- Micropipette, 10–100 µl, 20–200 µl and 100–1000 µl (Eppendorf)
- Dispenser, 50 ml (Optifix Basic)
- Multiple vortex (Heidolph)
- Refrigerated Centrifuge, Rotina 35 R (Hettich)
- Vortex, Velp 2 x^3 (Scientifica)
- Precision scales, SBP31 (Scaltec 0.1 mg sensitive)
- RC Filter, 0.45 µm

- Stainless Steel Knife
- Blender, (Waring)

LC-MS / MS Instruments & Accessories

- Column, ZORBAX SB-C 18, 4,6x 100 mm, 3,5 µm
- Liquid Chromatography, (Agilent 1200)
- Degasser, (Agilent 1200)
- LC Pump, (Agilent 1200)
- Autosampler, (Agilent 1100)
- Mass Dedector, (Agilent 6460)

5) <u>Procedure/Sample Preparation and Extraction</u>

a) **General Remarks :** Extraction of tetracycline antibiotics group from tissue and then defined as qualitative and quantitative by MS detector of LS MS/MS instrument.

i. **Number of Samples:** A reagent blank, a blank four spike and test samples have to be in each working set.

b) Sample Pre-treatment / homogenization :

Adipose and nerve tissue is cutted out from red meat, adipose and nerve tissue and skin is cutted out from poultry and fish samples is washed by water. Samples are chopped. At least 50 grams of tissue samples is used for homogenization

c) Taking an aliquot of the sample addition of internal standards (and addition of normal standard in control samples) :

5 blank samples which are known as negative and 2 ± 0.02 g fish samples which will be analyzed are insert into the <u>polypropylene</u> <u>centrifuge tube</u>s with 50ml volume. Negative samples are named as R (reaktive blank) , B (blank), S1 (0,5 MRL spike) ,S2 (1 MRL spike) , S3 (2 MRL spike), S4 (4 MRL spike). Sample Accept Number is used for test samples.

2 ml pure water is added into the R tube. 50 µL, 100 µL 200 µL, 400 µL from Antibiotic standard working solution are added into the tubes respectively S1, S2, S3 and S4. Then 100 µL from internal standard working solution is added the all tubes.

d) Extraction :

1 - 2 ± 0.02 g from test tissue is weighed into the polypropylene centrifuge tubes with 50ml volume.

2 – 100 µl internal Standard (2ppm) is added and mixed a few seconds by vortex.

3 - 200 µl Na_2EDTA (0.1 M) and 10 ml metanol (% 70) are added on the mix.

4 - mixed fifteen minutes by vortex.

5 – mixture is centrifuged at 4000 rpm for 118 minutes at 3 ° C.

6 - 200 µl supernatant is added into the 1800 µl pure water and mixed a few seonds.

7 - 2 ml extract is filtered into the vial.

8 – 20 µl of sample is injected to LC-MS/MS system.

6) Procedure/Measurement

Conditions of LC,GC,MS etc…

Conditions of Pump: T-flow : 0.8 mL/min, B Con % 10 Pressure limit : P.max : 300 bar

Conditions of Column Oven: Oven Temp : 35°C

Conditions of Auto sampler: Sampler Temperature : 10 ºC, Injection Volume: 20 µl

Pump Rationing Program: (Beginning Mobile Phase A : %90; Mobile Phase B :%10)

Steps	Time (Minute)	Mobile Phase A	Mobile Phase B
1	00.00	90	10
2	01.00	90	10
3	07.50	49	51
4	08.00	90	10

MSQ SOURCE

Gas Temp. : 350 ^{0}C Sheath Gas Temp. : 400 ^{0}C

Gas Flow : 9 L/min. Sheath Gas Flow : 10 L/min.

Nebuliser : 40 psi Capillary : (Positive) 4000 V

MSQ ACQUİSİTİON

Sıra	Start Time	Source Type	DİV Valve	Delta EM V+	Delta EM V-	Stored
1	0,0	MRM	To Waste	0	0	-
2	4,5	MRM	To MS	100	0	+
3	8,0	MRM	To Waste	0	0	-

MSQ ACQUİSİTİON 2

Compound Name	Precursor İon	MS Res	Product ion	MS Res	Dwell	Fragment (V)	Collision energy	Polarity
Chlortetracycline	479	Wide	444	Wide	50	70	20	Positive
Chlortetracycline	479	Wide	154	Wide	50	70	25	Positive
*Demeclocycline	465	Wide	448	Wide	50	70	10	Positive
Oxytetracycline	461	Wide	444	Wide	50	70	10	Positive
Oxytetracycline	461	Wide	426	Wide	50	70	15	Positive
Doxycyline	445	Wide	428	Wide	50	70	15	Positive
Doxycyline	445	Wide	410	Wide	50	70	25	Positive
Tetracycline	445	Wide	410	Wide	50	80	15	Positive
Tetracycline	445	Wide	154	Wide	50	80	25	Positive

* İnternal standard

1) **<u>Evaluation of Results</u>**

   a. **Raw Data – Final Results :**

The work is saved to a folder that contains the date of the same day. Datas and assessment tables have to be in each folder. Samples are charged in the specified order on device. First reactive blank, negative control sample, calibration samples, test samles, negative control sample and charged control sample (Calibration Sample at

the MLR Level). This ordering shouldn't be any changes. If this ordering is changed, reasons to be explained.

Retention times of the compounds should be compatible with the standard. Maximum allowable shift time is 5%.

A single MRM detection is not enough. For each compounds, peak has to be obtained from the two MRM pair. Transition which is named main ion in table is transition which is given result of the quantitative analysis. Ion which is named Verification ion is used for define of compound. Percent rate of between smaller and greater MRM peaks which are obtained have to be compatible with standard.

Relative intensity (% of base peak)	LC-MS, LC-MSn (relative)
> 50 %	± 20 %
> 20 % to 50 %	± 25 %
> 10 % to 20 %	± 30 %
≤ 10 %	± 50 %

b. Calculations

Determination is according to internal standard method. A series standard is prepared that its internal standard amount is fixed while analyte amount is variable (In different mx/ms value) and each chromatogram are identified. Drawn a graph according to volue of Ax/As against volue of Mx/Ms, Curve is a line. slope of curve is respons factor . To the detection of the amount of analyte in the sample, known amount internal standard is added to sample. Volue of Ax/Ais is calculated from chromatogram of the sample positive internal standard mixture, concentration of analyte is calculated from calibration curve.

For examle,

for drawing the calibration curve as shown in the following table it was prepared four standard;

- Each standard has 5.00 mg/ml internal standard.
- Values of Ax/Ais are taken the graph against to volue of Cx/Cis.
- Calibration curve is line whose slope is respond factor.

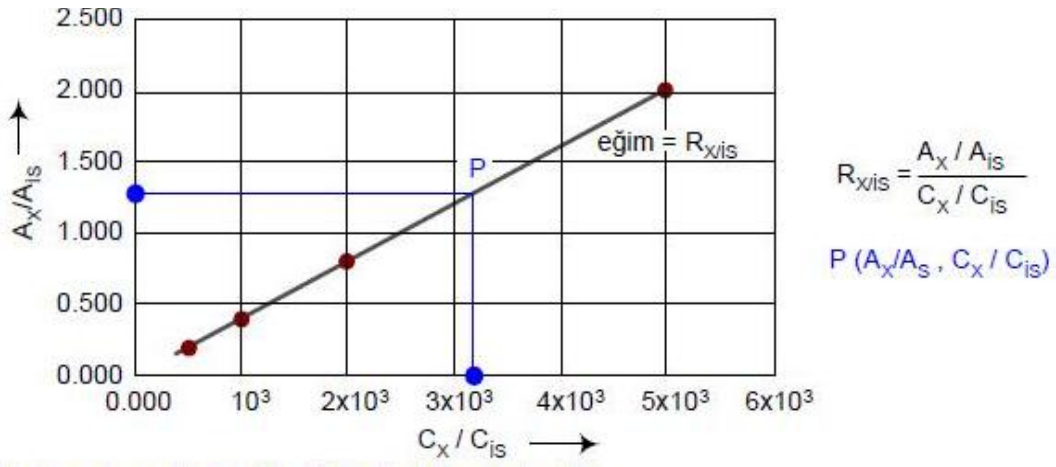
To analyze the sample, known amount internal standard is added to sample and mixture injected to device.

- Value of Ax/Ais is measured.
- Cx/Cis value is determined from calibration data and Cx is calculated.

$$Cx=Cis\ (Cx/Cis)$$

Ax is respons for Ais analyte and internal Standard, As, the field of standards
Mx, mass of internal Standard and Mis injected analyte; Ms mass of Standard
Cx, concentration of Cis injected analyte and internal standard

Standard	X matter, mg /ml	İnternal standard , mg/ml	Cx/Cis	Ax	Aix	Ax/Ais
1	2.50	5.00	0.500	120	600	0.200
2	5.00	5.00	1.000	241	601	0.401
3	10.00	5.00	2.000	480	600	0.800
4	25.00	5.00	5.000	1198	600	1.997



Creation of the Calibration Curve

To calculate the amount of tetracycline group antibiotics in the samples, a calibration curve with five point is created. To create the stated concentrations, Charged samples are prepared from the old negative test samples. And a calibration curve is plotted with the resultant data. The calculated value of charged samples is recorded as coordinated manner µg / kg by system. The calibration curve is plotted using the results obtained from the charged sample. The calibration curve must be linear ($r2 \geq 0{,}98$).

c) Evaluation of Control Samples :

For performance control of device and analysis, negative control and chaced sample are acceped as base. Any data related to analyze compounds should to be obtained from negative control sample. Charged control sample is used as quality control sample. quantitative data of all compounds have to be obtained from loaded control sample and these data should be checked to be compatible with the data on the control board.

d) Final Results/conclusions

Residues of antibiotics of tatracycline groups belong to each sapmle is calculated as µg/kg from calibration curve. system is give results automatically mg/kg level by comparing the peak area of sample values with peak area of samples which are used to creating calibration curve.

e) Reporting the results :

The obtained result is written on analyze result form and is sent to unit of reporting.

2) <u>Acceptability Criteria :</u>

Reaktive blank injection musn't has any positive value. Any compound mustn't be determined at the blank test. MRM pair mustn't be detected from the S1, S2, S3, S4 injection for each compound. The allowed shift amount for retention time is maximum 5%. for the calibration curve should be $r2 \geq 0.98$. Values obtained from S2 should be compatible with the control card for each compound. The validity of the test method is supported by regular participation in proficiency testing.

For validity in this test method, the EU Directive 2002/657 and Test Methods and Validation of Test Methods Procedure (LAS P.17) are used as a guide and determined to criteria of performance. The sustainability of validity of method is provided by quality cart application.

1. **Associated Documents/References**

Internal Source Document List

- Laboratuvar Güvenliği Prosedürü (LAS. P.28)
- Deney Metotları ve Deney Metotlarının Geçerli Kılınması Prosedürü (LAS. P.17)

External Document List

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