APPENDICES

Appendix 1

QUESTIONNAIRE

(for butchers/meat shops)

NOTE:

1. *This questionnaire is designed for a survey on butchers (meat shops) in Hanoi at which meat (muscle) samples are collected for* ***study purpose only****.*
2. *Data and information gathered via this survey are maintained* ***confidential****.*
3. *There is* ***only one appropriate answer*** *to each question unless otherwise specified.*

1- Date of sampling: ……

2- Location (district): ……

3- Wrapping of products:

….. wrapped

…… non-wrapped

4- Type(s) of meat offered at the same shop (more than one type may be selected)

……pork

……beef

……chicken

……processed meat product(s)

5- Origin of meat:

…….slaughterhouse of the city (specify)……

…….private small abattoir(s) in Hanoi

…….province (specify)……..

6- Estimated amount of meat (pork) sold a day……kg

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7- Owner's name: ……

8- Residence:

…..inner-city

…..suburb area

…..other province (specify)……

9- Sex:

……..Male

……..Female

10- Age ……..

11- Educational/professional attainment:

…… primary school

…….junior secondary school

…….senior secondary school

…….high school/professional training course

12- Number of experienced years in business: ……years

……..

Thank you!

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Appendix 2

METHOD FOR DETERMINATION OF TETRACYCLINE RESIDUES IN MEAT

(According to the standard of Agence Française de Sécurité Sanitaire des Aliments (AFSSA) for "Determination of Tetracycline residues in kidney and muscle by high performance liquid chromatography")

1- SCOPE AND FIELD OF APPLICATION

The present method allows the determination of the residues of four compounds and their epimers, oxytetracycline (OTC) and epi-oxytetracycline (epi-OTC), tetracycline (TC) and epi-tetracycline (epi-TC), chlortetracycline (CTC) and epi-chlortetracycline (epi-CTC) and doxycycline (DC). It is suitable for pork and bovine kidney and muscle. The detection limits in kidney are 80 µg/kg, 60 µg/kg, 170 µg/kg and 160 µg/kg for OTC, TC, CTC and DC respectively and 8 µg/kg, 9 µg/kg, 15 µg/kg and 12 µg/kg for OTC, TC, CTC and DC in muscle according to the criteria of the decision 93/256/EEC. Fortified samples from 300 µg/kg to 2400 µg/kg in kidney and from 50 µg/kg to 400 µg/kg in muscle have been analyzed in accordance with the criteria of this decision. Maximum residue limits for the sum of each tetracycline and its epimer (excluding epi-doxycycline) have been set up at 600 µg/kg in kidney and 100 µg/kg in muscle.

2- PRINCIPLE

There are three principal stages in the samples preparation:

- Homogenization and extraction of the sample residues by EDTA/Mc Ilvain buffer,
- Precipitation of proteins using trichloroacetic acid and filtration,
- Cleanup on solid-phase extraction cartridges C_{18} and injection.

Tetracyclines are separated on a C_{18} stationary phase and detected by UV absorption at 355 nm. The amount of tetracycline is calculated by interpolation from a calibration curve determined for each of the 4 compounds, taking into account the calculated recovery.

3- CHEMICALS AND REAGENTS

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Unless otherwise specified, all reagents are of analytical grade. Demineralized water is obtained from an ultra pure water system (Millipore).

3.1- Acetonitrile (Merck, Art. 1.14291).

3.2- Methanol (Merck, Art. 1.06009).

3.3- Trichloroacetic acid (Prolabo, Art. 20742-293): dissolve 50 g in 50 ml ultra pure water to obtain a 1g/ml solution.

3.4- Oxalic acid dehydrate (Prolabo, Art. 28582.291): dissolve 1.20 g in 1 l ultra pure water to obtain a 0.01 M solution. Filter through a 0.45 µm unit under vacuum (4.1.20).

3.5- 0.01 M oxalic acid solution in methanol: dissolve 1.26 g in 1 l methanol.

3.6- Citric acid monohydrate (Merck, Art. 244): dissolve 21 g in 1 l ultra pure water.

3.7- Disodium phosphate anhydrous (Merck, Art. 1.06586): dissolve 28.4 g in 1 l ultra pure water.

3.8- Disodium ethylenediaminetetraacetate dehydrate (EDTA), (Prolabo 20 302 293).

3.9- Mc Ilvain buffer: mix 1 l citric acid solution (3.6) with 625 ml disodium phosphate solution (3.7) and adjust pH to 4.0 ± 0.05 if necessary.

3.10- Mc Ilvain buffer/ETDA solution: prepare a 0.1 M ETDA solution in Mc Ilvain buffer (60.5 g ETDA in 1.625 l).

3.11- Mobile phase: acetonitrile (3.1) and 0.01 M oxalic acid (3.4) in a gradient mode.

3.12- Standards:

3.12.1- Oxytetracycline hydrochloride, potency (Pfizer).

3.12.2- Tetracycline hydrochloride (Virbac).

3.12.3- Chlortetracycline hydrochloride (Vetoquinol).

3.12.4- Doxycycline hyclate (Veprol).

3.12.5- Epi-oxytetracycline (Acros).

3.12.6- Epi-tetracycline (Acros).

3.12.7- Epi-chlortetracycline (Acros).

3.13- Stock solutions:

N.B.: because interferences may occur between some of the standards, they will not be injected simultaneously for the quantification. For example, chlortetracycline standard contains tetracycline. Each stock solution will be prepared with two tetracyclines as here under.

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3.13.1- OTC and CTC stock standard solution: prepare a methanolic solution containing 1 mg/ml of OTC and CTC (+ their respective epimer if these epimers are contained in the standard).

3.13.2- TC and DC stock standard solution: prepare a methanolic solution containing 1 mg/ml of TC and DC (+ their respective epimer if these epimers are contained in the standard).

3.13.3- Epi-OTC, epi-TC and epi-CTC stock standard solution: prepare 3 methanolic solutions containing each 1 mg/ml of epimer.

3.14- Working solutions for analysis of kidney samples:

3.14.1- Two 100 µg/ml intermediate solutions are obtained by diluting the two stock solutions (3.13.1- and 3.13.2-) with methanol. These solutions can be stored two weeks at $+4^0$C

3.14.2- Working solutions are obtained by diluting each of the two intermediate solutions (3.14.1) with 0.01 M oxalic acid in methanol/water solution (30/70) to obtain concentrations of 0.75; 1.5; 3 and 6 µg/ml. These solutions are prepared freshly every day in amber flasks.

3.14.3- Working solutions containing 1 µg/ml of each epimer are obtained by diluting each of the stock standard solutions (3.13.3) with 0.01 M oxalic acid in methanol/water solution (30/70). These solutions will be used only for epimers identification and not for quantification. The amount of tetracycline + the corresponding epimer contained in a kidney sample is calculated by comparison with the standard of tetracycline only.

3.15- Working solutions for the analysis of muscle samples

3.15.1- Two 50 µg/ml intermediate solutions are obtained by diluting the two stock solutions 3.13.1- and 3.13.2- with methanol. These solutions can be stored two weeks at $+4^0$C

3.15.2- Working solutions are obtained by diluting each of the two intermediate solutions (3.15.1) with 0.01 M oxalic acid in methanol/water solution (30/70) to obtain concentrations of 0.125; 0.25; 0.5 and 1 µg/ml. These solutions are prepared freshly every day in amber flasks.

3.15.3- Working solutions containing 1 µg/ml of each epimer are obtained by diluting each of the stock standard solutions (3.13.3) with 0.01 M oxalic acid in

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methanol/water solution (30/70). These solutions will be used only for epimers identification and not for quantification. The amount of tetracycline + the corresponding epimer contained in a kidney sample is calculated by comparison with the standard of tetracycline only.

3.16- Control kidney

3.17- Control muscle

3.18- Spiking solutions

3.18.1- Spiking solutions for kidney samples

Spiking solutions of 6 µg/ml are obtained by diluting the two stock solutions with ultra pure water. These solutions can be stored at $+4^0$C for 24 hours.

3.18.2- Spiking solutions for muscle samples

Two 100 µg/ml intermediate solutions are obtained by diluting the two stock solutions (3.13.1 and 3.13.2) with methanol. Spiking solutions of 1 µg/ml are obtained by diluting with the hundredth these two intermediate solutions. These solutions can be stored at $+4^0$C for 24 hours.

3.19- Spiked control samples

The control samples allow to calculate the recovery and ensure the quality of the analysis.

3.19.1- Kidney spiked samples

Prepared fortified kidney samples by adding 500 µl of spiking solutions (3.18.1) to 5g of control kidney (3.16) to obtain a spiking level of 600 µg/kg. Stir 30 seconds. The kidney sample is frozen until analysis.

3.19.2- Muscle spiked samples:

Prepare fortified muscle samples by adding 500 µl of spiking solutions (3.18.2) to 5g of control muscle (3.17) to obtain a spiking level of 100 µg/kg. Stir 30 seconds. The muscle sample is frozen until analysis.

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4. APPARATUS

4.1- Laboratory equipment

4.1.1- Polypropylene centrifuge tubes, 50 ml capacity, with caps.

4.1.2- Glass tubes, 30 ml capacity.

4.1.3- Polypropylene tubes, 5 ml capacity.

4.1.4- Amber volumetric flasks, 25 ml, 50 ml, 100 ml, 200 ml and 1000 ml.

4.1.5- Graduated glass pipettes, 2 ml, 5 ml, 20 ml and 25 ml.

4.1.6- Automatic pipettes type Gilson P1000.

4.1.7- Blender type moulinette (Moulinex).

4.1.8- Analytic and precision balance model PB302 (Mettler – Toledo).

4.1.9- High precision analytic balance type A120S (Sartorius).

4.1.10- Solvent dispensers (Brandt).

4.1.11- pH-meter (Tacussel).

4.1.12- Electric stirrer type vortex (Bioblock).

4.1.13- Rotary stirrer type Rheax 2 (Heidolph).

4.1.14- Magnetic stirrer type Nuova II (Bioblock).

4.1.15- Cooled centrifuge model GR 4.22 (Jouan).

4.1.16- Solid phase extraction cartridges Bond-Elut C18, 3 cc, 200 mg (Varian).

4.1.17- Solid phase extraction manifold (Supelco), adaptors, needles (Analytichem).

4.1.18- Vacuum pump, 0.4 bar, 12 w (Bioblock).

4.1.19- Whatman disposable filter funnels, 25 mm diameter (Whatman, Art. 1922-1800) or 50 ml reservoirs containing these same filters.

4.1.20- Membrance filter holder with filter paper model HVLP 0.45 μm (Millipore).

4.1.21- Refrigerated ultra-speed centrifuge model MR 1822 (Jouan)

4.2- High Performance Liquid Chromatography equipment

4.2.1- Series 1050 quaternary gradient pump (Hewlett Packard).

4.2.2- Series 1050 UV-VIS detector (Hewlett Packard).

4.2.3- Vectra 486/66VL computer (Hewlett Packard) and HPLC 2D Chemstation software.

4.2.4- Series 1100 autosampler (Hewlett Packard).

4.2.5- Analytical column: Purospher RP 18-e, 5 μm, 4 x 4 mm I.D. guard column (Merck).

5. STORAGE OF SAMLES AND SAMPLING

Sample must be stored at about -20^{0}C. They must be thawed just before the analysis and then ground (4.1.7).

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6. PROCEDURE

NB: Tetracyclines are sensitive to light. Care must be taken to protect solutions from light during the manipulations.

6.1- Extraction

6.1.1- Weigh out 5 ± 0.1 g of ground kidney or muscle into a centrifuge tube (4.1.1).

6.1.2- Add 25 ml Mc Ilvain buffer/ETDA solution (3.10) and stir for about 30 s (4.1.12).

6.1.3- Stir for 15 min at 100 rpm with the rotary stirrer (4.1.13).

6.1.4- Centrifuge 10 min at 4000 g about 4^0C. Do not leave the samples for a long time in this state because of problems of stability.

6.2- Proteins precipitation

6.2.1- Transfer the supernatant in a glass tube (4.1.2), place this tube in a beaker on the magnetic stirrer (4.1.14).

6.2.2- Add slowly 2.5 ml of 1 g/ml trichloroacetic acid solution (3.3) with constant stirring. Then stir more rapidly for a further 1 min. Remove the magnetic stirrer.

6.2.3- Centrifuge 5 min at about 3000 g.

6.3- Cleanup

6.3.1- Activate the cartridge Bond Elut with 1 ml methanol, 1 ml ultra pure water and 1 ml Mc Ilvain buffer. (3.9).

6.3.2- Connect a filter funnel or a reservoir containing a filter (4.1.19) to the cartridge.

6.3.3- Transfer the sample solution into the funnel and pull it through the filter with the vacuum pump (4.1.18) at a flow rate of no more than 2 drops/s. Do not allow the cartridge to dry at this step.

6.3.4- Flush the cartridge with 1 ml ultra pure water.

6.3.5- Dry the cartridge for 5 min using the vacuum pump.

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6.3.6- Remove the filter and elute slowly with 1 ml 0.01 M oxalic acid in methanol (3.5) and next with 1 ml ultra pure water into a polypropylene tube (4.1.3).

6.3.7- The samples are centrifuged 3 min at 20,000 g at about 4^0C before injection of a 100 µl volume into the chromatographic system.

6.4- Chromatographic conditions

6.4.1- Gradient mobile phase

Time	Acetonitrile, %	0.01 M oxalic acid, %
0 min	13	87
15 min	36	64
Post-time: 5 min.		

6.4.2- Flow rate: 0.8 ml/min.

6.4.3- UV detector wavelength: 355 nm.

N.B.: in case of chlortetracycline analysis, the wavelength can be set at 375 nm, which is the more adjusted wavelength for chlortetracycline detection.

6.4.4- Retention times:

Epi-oxytetracycline: 5.9 min
Oxytetracycline: 6.0 min
Epi-tetracycline: 6.1 min
Tetracycline: 7.1 min
Epi-chlotetracycline: 9.3 min
Chlortetracycline: 10.8 min
Doxycycline: 12.1 min

7- CALCULATION OF RESULTS

The following calculations can be executed directly by the HPLC 2D Chemstation software.

7.1- Derive the calibration curve from the results obtained with the working standards solutions. Peaks corresponding to the tetracyclines and to their respective epimer have to be taken into account if possible. Then, determine the curve equation:

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$y = ax + b$

y = peak area (TC + epimer)

x= concentration (ng/ml)

a = slope

b = intercept

7.2- Calculation of the recovery:

This result is obtained from the spiked control sample.

Determine the control sample final concentration (Cf) using the curve equation (7.1) as:

$$Cf = \frac{Yf - b}{a}$$

Cf = final concentration of the injected extract

Yf = tetracycline peak area + epi-tetracycline peak area

a = slope

b = intercept

Calculate the recovery as:

$$R = \frac{Cf}{F.Ct}$$

R = recovery

Cf = final concentration of the injected extract determined above

Ct = true concentration or spiking concentration (600 µg/kg = MRL)

F = concentration factor (2.5 in this case).

Check the quality of the analysis: this last is validated if the calculated recovery is in accordance with the limits establishing during the method validation:

$Rm - 3.SD \leq R \leq Rm + 3.SD$

R = recovery

Rm = mean recovery determined during validation

SD = standards deviation of the mean recovery

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Calculate the concentration of tetracycline + epi-tetracycline present in the sample to be analyzed (Ca) using the calibration curve and taking account the calculated recovery:

$$Ca = \frac{Cf}{F} \times \frac{1}{R}$$

Ca = concentration of tetracycline + epi-tetracycline present in the sample to be analyzed.

Cf = final concentration of the injected extract.

R = recovery

F = concentration factor (2.5 in this case).

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1989 – 1994	Bachelor of science in Veterinary Medicine, Faculty of Animal Science and Veterinary Medicine, Hanoi Agricultural University, VIETNAM
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DECLARATION

I, the undersigned, declare that the thesis is my original work and has not been presented for a degree in any university.

Name: **Duong Van Nhiem**

Signature.....

Date of submission.....

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