

**FLUOROQUINOLONES ELISA  
(Generic test)**

**A competitive enzyme immunoassay for  
screening and quantitative analysis of  
a broad range of fluoroquinolones  
in various matrices**

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8.6 Water samples

The norfloxacin-equivalents read from the calibration curve have to be multiplied by a factor 10 to obtain the fluoroquinolones concentration in ng/g or ng/ml (ppb).

8.7 Honey samples

The norfloxacin-equivalents read from the calibration curve have to be multiplied by a factor 10 to obtain the fluoroquinolones concentration in ng/g or ng/ml (ppb).

8.8 Serum samples

The norfloxacin-equivalents read from the calibration curve have to be multiplied by a factor 10 to obtain the fluoroquinolones concentration in ng/g or ng/ml (ppb).

8.9 Urine samples

The norfloxacin-equivalents read from the calibration curve have to be multiplied by a factor 10 to obtain the fluoroquinolones concentration in ng/g or ng/ml (ppb).

8.10 Feed samples

The norfloxacin-equivalents read from the calibration curve have to be multiplied by a factor 40 to obtain the fluoroquinolones concentration in ng/g (ppb).

**12. LITERATURE**

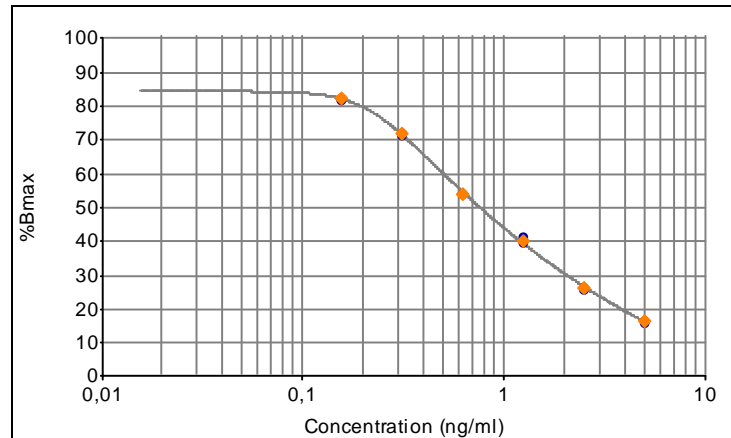
1. R. Verheijen, N. Sajic, I. Hopman and C.J.M. Arts. Detection of fluoroquinolones by Enzyme Immunoassays in biological matrices. VIth International Conference on Agri-Food Antibodies, Uppsala, Sweden, 11-13 September 2003.
2. Giampiero Scortichini, Loredana Annunziata, Valeria Di Girolamo, Roberta Buratti, Roberta Galarini. Validation of an enzyme-linked immunosorbent assay screening for quinolones in egg, poultry muscle and feed samples. Analytica Chimica Acta 637 (2009) 273-278.

**13. ORDERING INFORMATION**

For ordering the fluoroquinolones ELISA kit, please use cat. code 5101FLUQG.

**14. LAST MUTATIONS**

Soft and hard cheese samples and water samples are added.  
Pipette schedule of the standard curve is adapted.



**Figure 1 : Example of a calibration curve**

The Fluoroquinolones concentration in the extracts (ng/ml) corresponding to the % maximal absorbance of each extract can be read from the calibration curve.

#### 8.1.1 Method I: Tissue samples (shrimps, fish and chicken)

The norfloxacin-equivalents read from the calibration curve have to be multiplied by a factor 40 to obtain the fluoroquinolones concentration in ng/g (ppb).

#### 8.1.2 Method II: Tissue samples (shrimps, fish, chicken)

The norfloxacin-equivalents read from the calibration curve have to be multiplied by a factor 2 to obtain the fluoroquinolones concentration in ng/g (ppb).

#### 8.2 Egg samples, raw and pasteurised

The norfloxacin-equivalents read from the calibration curve have to be multiplied by a factor 20 to obtain the fluoroquinolones concentration in ng/g (ppb).

#### 8.3 SPE method tissue and whole egg

The norfloxacin-equivalents read from the calibration curve have to be multiplied by a factor 4.5 for muscle and 2.25 for egg.

#### 8.4 Milk samples

The norfloxacin-equivalents read from the calibration curve have to be multiplied by a factor 10 to obtain the fluoroquinolones concentration in ng/g or ng/ml (ppb).

#### 8.5 Soft and hard cheese samples

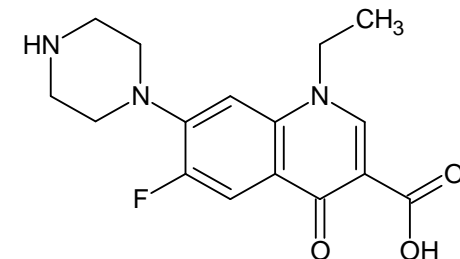
The norfloxacin-equivalents read from the calibration curve have to be multiplied by a factor 40 to obtain the fluoroquinolones concentration in ng/g or ng/ml (ppb).

## BRIEF INFORMATION

The fluoroquinolones ELISA is a competitive enzyme immunoassay for measurement of the concentration of a broad group of fluoroquinolone antibiotics. With this ELISA-kit 96 analyses can be performed. Samples and standards are measured in duplicate which means that in total 40 samples can be analyzed.

The ELISA kit contains all reagents to perform the assay. Reagents for sample preparation are not included in the kit.

## 1. INTRODUCTION



Chemical structure of norfloxacin

Fluoroquinolones are a synthetic class of antibiotics, which all act by inhibition of DNA-gyrase abolishing its activity by interfering with the DNA rejoining reaction. Since gyrase is an essential enzyme in prokaryotes, but is not found in eukaryotes, bacteria are an ideal target for these antibiotics. Fluoroquinolones are mainly active against Gram negative bacteria and have found wide application in both human and veterinary clinical practice. However, the use of fluoroquinolones in animals used for meat production and its use in aquaculture has also generated concern, as fluoroquinolones have contributed to an increasing bacterial resistance for these antibiotics in man, e.g. *Staphylococcus aureus* (MRSA, MRSE), *Campylobacter jejuni* and others. For this reason, effective screening methods for the presence of fluoroquinolones in animal products as well as food products are required [1, 2]. EuroProxima has developed a generic ELISA kit for screening for a broad range of fluoroquinolones. In this ELISA kit an antiserum is used that was raised against a protein bound norfloxacin, the conjugate is a horseradish peroxidase labelled norfloxacin. In the ELISA kit a norfloxacin standard is used, so the fluoroquinolones are expressed as norfloxacin-equivalents.

## 2. PRINCIPLE OF THE FLUOROQUINOLONES ELISA

The microtiter plate based fluoroquinolones ELISA kit consists of one precoated plate (12 strips, 8 wells each). Antibody, horseradish peroxidase (-HRP) labeled norfloxacin and norfloxacin standard solution or sample are added to the wells. Free fluoroquinolones from the samples or standards norfloxacin-HRP conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay).

After incubation step of one hour, non-bound reagents are removed in a washing step. The amount of bound norfloxacin –HRP conjugate is visualized by the addition of a substrate/chromogen solution (peroxide/tetramethylbenzidine, TMB). Bound norfloxacin-HRP conjugate transforms the colourless chromogen into a coloured product.

The substrate reaction is stopped by the addition of sulfuric acid. The colour intensity is measured photometrically at 450 nm. The optical density is inversely proportional to the fluoroquinolones concentration in the sample.

## 3. SPECIFICITY AND SENSITIVITY

The generic fluoroquinolones ELISA utilizes polyclonal antibodies raised in rabbit to protein conjugated norfloxacin. The reactivity pattern of the antibody as tested in buffer is:

Cross-reactivity:	Enrofloxacin	92%
	Ciprofloxacin	124%
	Norfloxacin	100%
	Nadifloxacin	85%
	Pefloxacin	70%
	Enoxacin	57%
	Piromidic acid	62%
	Lomefloxacin	40%
	Ofloxacin	18%
	Danofloxacin	89%
	Fleroxacin	40%
	Oxolinic acid	57%
	Marbofloxacin	16%
	Pipemidic acid	5%
	Sarafloxacin	4%
	Difloxacin	1%
	Levofloxacin	3%
	Gatifloxacin	5%
	Flumequine	2%
	Pazufloxacin	1%
	Cinoxacin	< 0.1%
	Tosufloxacin	< 0.1%
	Nalidixic acid	< 0.1%

- Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate (40 samples; 80 wells).
- Pipette 25 µl conjugate (Norfloxacin-HRP) to all wells, except wells H1 and H2.
- Pipette 25 µl antibody solution to all wells, except wells H1 and H2.
- Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.
- Incubate the plate for 1 hour in the dark at 4°C (2°C – 8°C).
- Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.
- Pipette 100 µl substrate/chromogen solution into each well.
- Incubate 30 minutes at room temperature (approximately 20°C - 25°C).
- Pipette 100 µl of stop solution to each well.
- Read the absorbance values immediately at 450 nm.

## 11. INTERPRETATION OF RESULTS

Subtract the mean optical density (O.D.) of the wells H1 and H2 (Blank) from the individual O.D. of the wells containing the standards and the samples.

The O.D. values of the six standards and the samples (mean values of the duplicates) are divided by the mean O.D. value of the zero standard/ Bmax (wells A1 and A2) and multiplied by 100. The zero standard/ Bmax is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

O.D. standard (or sample)

-----x 100% = % maximal absorbance

O.D. zero standard/ Bmax

### Calibration curve:

The values (% maximal absorbance) calculated for the standards are plotted on the Y-axis versus the analyte equivalent concentration (ng/ml) on a logarithmic X-axis.

### Alternative for calibration curve:

The value of absorption (logit) calculation of the standards are plotted on Y-axis versus the analyte equivalent concentration on a logarithmic X-axis.

Sample dilution buffer\*

Sample dilution buffer is not provided in the kit. Prepare this buffer as follows:

Quantities indicated are for 1 litre of buffer:

Na <sub>2</sub> HPO <sub>4</sub>	0.77 g
KH <sub>2</sub> PO <sub>4</sub>	0.18 g
NaCl	8.94 g
pH	7.4 (7.3-7.5)

**10. ASSAY PROCEDURE**Rinsing protocol

In ELISAs, between each immunological incubation step, unbound components have to be removed efficiently. This is reached by appropriate rinsing. It should be clear that each rinsing procedure must be carried out with care to guarantee good inter- and intra-assay results.

Basically, manual rinsing or rinsing with automatic plate wash equipment can be performed as follows:

Manual rinsing

1. Empty the contents of each well by turning the microtiter plate upside down and remove residual liquid by striking the plate against a paper towel.
2. Fill all the wells to the rims (300 µl) with rinsing solution.
3. This rinsing cycle (1 and 2) should be carried out 3 times.
4. Turn the plate upside down and empty the wells by a firm short vertical movement.
5. Place the inverted plate on absorbent paper towels and tap the plate firmly to remove residual rinsing solution from the wells.
6. Take care that none of the wells dry out before the next reagent is dispensed.

Rinsing with automatic microtiter plate wash equipment

When using automatic plate wash equipment, check that all wells can be aspirated completely, that the rinsing solution is nicely dispensed reaching the rim of each well during each rinsing cycle. The washer should be programmed to execute three rinsing cycles.

Assay Protocol

1. Prepare samples according to chapter 8 (Sample preparation) and prepare reagents according to chapter 9 (Preparations of reagents).
2. Pipette 100 µl of the zero standard in duplicate (wells H1, H2, blank).  
Pipette 50 µl of the zero standard in duplicate (wells A1, A2, maximal signal).  
Pipette 50 µl of each of the norfloxacin standard solutions in duplicate (wells B1,2 to G1,2 i.e. 0.157, 0.313, 0.625, 1.25, 2.5 and 5 ng/ml).

The Limit of detection (LOD) is calculated as:  $X_n + 3SD$  and is determined under optimal conditions.

Matrix	Procedure	LOD
Tissue	8.1.1	12 ng/g
Tissue	8.1.2	0.3 ng/g
Egg	8.2	6 ng/g
Tissue and whole egg	8.3	0.5 ng/g
Milk	8.4	3 ng/ml
Soft and hard cheese	8.5	6 ng/g
Water	8.6	2 ng/ml
Honey	8.7	2 ng/g
Serum	8.8	2.5 ng/ml
Urine	8.9	7 ng/ml
Feed	8.10	5 ng/g

**4. HANDLING AND STORAGE**

- Store the kit at +2°C to +8°C in a dark place. For repeated use store kit components as specified under chapter 9.
- After the expiry date (see kit label) has passed, quality claims are not accepted.
- Before opening the sealed plate, the plate should be at ambient temperature in order to avoid condensation in the ELISA.
- Dilute the kit components immediately before use, but after the components are at ambient temperature.
- After the lyophilised enzyme conjugate (Norfloxacin-HRP) and the lyophilised antibodies have been reconstituted, these reconstituted compounds can be stored in a refrigerator (+2°C to +8°C) for at least half a year. Alternatively, after reconstitution of the conjugate and antibody components, aliquots of these solutions can be prepared. The aliquots can be stored in a freezer (-20°C) until the expiration date has passed (see kit label).
- The substrate chromogen solution can be stored in a refrigerator (+2°C to +8°C) until the expiry date stated on the label.
- Any direct action of light on the substrate chromogen solution should be avoided.

Degeneration of the reagents may have occurred when the following phenomena are observed:

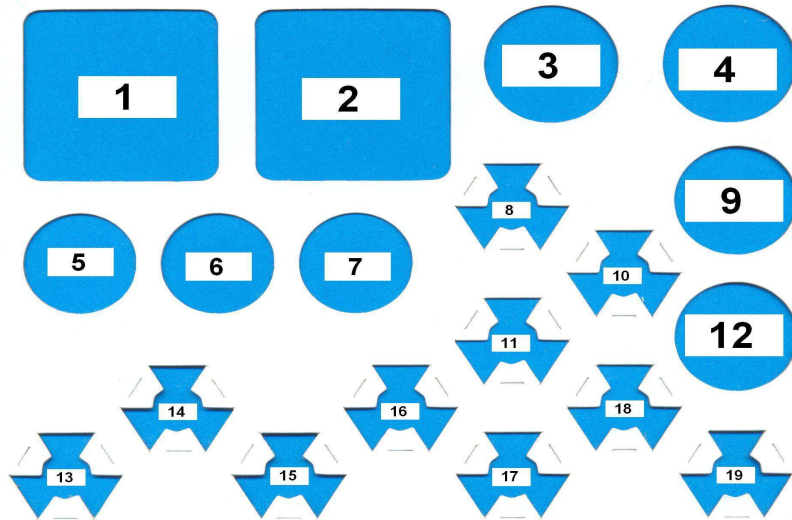
- A blue colouring of the substrate solution before transferring it into the wells.
- A weak or absent colour reaction of the zero standard ( $B_{max}$ ,  $E_{450nm} < 0.8$ ).

## 5. KIT CONTENTS

Manual

One sealed (96 wells) microtiter plate (12 strips, 8 wells each), coated with antibody. Plate is ready-to-use.

Position of the reagents in the kit. For preparation of the reagents see Chapter 9.



1. **Dilution buffer** (20 ml, Ready-to-use)
2. **Rinsing buffer** (30 ml, 20x concentrated)
3. **Substrate solution** (12 ml, Ready-to-use)
4. **Stop solution** (15 ml, Ready-to-use)
5. **Conjugate** (lyophilised, blue cap)
6. **Antibody** (lyophilised, yellow cap)
7. not in use
8. **Standard solution 100 ng/ml** (1ml, Ready-to-use)
9. not in use
10. not in use
11. not in use
12. not in use
13. **Zero standard** (2ml, Ready-to-use)
14. **Standard solution 1** (1ml, Ready-to-use) **0.157 ng/ml**
15. **Standard solution 2** (1ml, Ready-to-use) **0.313 ng/ml**
16. **Standard solution 3** (1ml, Ready-to-use) **0.625 ng/ml**
17. **Standard solution 4** (1ml, Ready-to-use) **1.25 ng/ml**
18. **Standard solution 5** (1ml, Ready-to-use) **2.5 ng/ml**
19. **Standard solution 6** (1ml, Ready-to-use) **5.0 ng/ml**

## 9. PREPARATION OF REAGENTS

Before beginning the test, the reagents should be brought up to ambient temperature. Any reagents not used should be put back into storage immediately at +2°C to +8°C. Prepare reagents fresh before use.

### Microtiter plate

Return unused strips into the resealable bag with desiccant and store at +2°C to +8°C for use in subsequent assays. Retain also the strip holder.

### Rinsing buffer

The rinsing buffer is delivered 20x concentrated. Prepare dilutions freshly before use. For each strip 40 ml of diluted rinsing buffer is required (2 ml concentrated rinsing buffer + 38 ml distilled water).

### Standard solution (100 ng/ml)

To prepare standards in the appropriate matrix or to prepare spikes use the standard solution containing 100 ng FLUOQ per ml. Dilute the standard solution in the appropriate matrix to make a dilution range of 5, 2.5, 1.25, 0.625, 0.313 and 0.157 ng/ml. Also the zero standard should be of the same matrix.

### Dilution buffer for conjugate and antibody

For reconstitution of the enzyme conjugate and antibody, ready-to-use dilution buffer is delivered with the kit.

The buffer may be stored in a refrigerator (+2°C to +8°C) until the expiration date stated on the kit label.

### Conjugate solution

Reconstitute the vial of lyophilised conjugate (norfloxacin-HRP) with 4 ml dilution buffer, mix thoroughly and keep in the dark until use. This reconstituted compound can be stored in a refrigerator (+2°C to +8°C) for at least half a year.

### Antibody solution

Reconstitute the vial of lyophilised antibodies with 4 ml dilution buffer, mix thoroughly and keep in the dark until use. This reconstituted compound can be stored in a refrigerator (+2°C to +8°C) for at least half a year.

### Standard solution

The norfloxacin standard solutions are ready-to-use. The standard solutions contain 5, 2.5, 1.25, 0.625, 0.313 and 0.157 ng/ml norfloxacin in 8% methanol solution. A ready-to-use zero standard is enclosed. Keep these standard solutions in the dark and store at +2°C to +8°C.

### Substrate/chromogen solution

The substrate/chromogen solution (ready-to-use) tends to precipitate at +4°C. Take care that this vial is at room temperature and mix the content well before use. Avoid direct (sun) light.

### 8.5 Soft and hard cheese samples

- Homogenise approximately 10 g of sample.
- Weigh 0,5 g of the homogenised sample and transfer into a test tube.
- Add 1.5 ml of 80% methanol in sample dilution buffer\* (see Chapter 9) and mix (head over head) for 30 minutes.
- Centrifuge (10 minutes, 2000 x g).
- Pipette 100 µl of the supernatant into a clean tube and add 900 µl sample dilution buffer\* (see Chapter 9).
- Add 1 ml hexane, vortex.
- Centrifuge (10 minutes, 2000 x g).
- Remove the upper (hexane) layer with a pipette
- An aliquot of 50 µl layer underneath is used in the ELISA test.

### 8.6 Water samples

- Transfer 0.5 ml of the water sample in a test tube.
- Add 1.5 ml of 40% methanol in sample dilution buffer\* (see Chapter 9) and mix (head over head) for 5 minutes.
- After centrifugation (10 minutes, 2000 x g), 100 µl of supernatant is pipetted into a clean tube and 150 µl of sample dilution buffer\* (see Chapter 9) is added.
- An aliquot of 50 µl is used in the ELISA test.

### 8.7 Honey samples

- Transfer 0.5 ml of the homogenised honey in a test tube.
- Add 4.5 ml of 8% methanol in sample dilution buffer\* (see Chapter 9) and mix (head over head) for 30 minutes.
- After centrifugation (10 minutes, 2000 x g), an aliquot of 50 µl from the liquid part below the fat layer is used in the ELISA test.

### 8.8 Serum samples

- Transfer 0.5 ml of the homogenised serum in a test tube and add 4.5 ml of 8% methanol in sample dilution buffer\* (see Chapter 9).
- Vortex the sample for 2 minutes.
- An aliquot of 50 µl is used in the ELISA test

### 8.9 Urine samples

- Transfer 0.5 ml of the homogenised urine in a test tube and add 4.5 ml of 8% methanol in sample dilution buffer\* (see Chapter 9).
- Vortex the sample for 2 minutes.
- An aliquot of 50 µl is used in the ELISA test.

### 8.10 Feed samples

- Homogenise approximately 10 g of sample.
- Weigh 0,5 g of the homogenised sample and transfer into a test tube.
- Add 1.5 ml of 80% methanol in sample dilution buffer\* (see Chapter 9) and mix (head over head) for 30 minutes.
- Centrifuge (10 minutes, 2000 x g).
- Pipette 100 µl of the supernatant into a clean tube and add 900 µl dilution buffer. Chapter 5 no. 1.
- An aliquot of 50 µl is used in the ELISA test.

## 6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

### General equipment

- Scales and weighing vessels
- Gloves
- Fume hood
- Homogeniser (blender, Ultra Turrax, mixer)
- Centrifuge (for 10 - 15 ml test tubes, 2000 x g)
- Vortex
- Automated microplate washer or 8-channel micropipette 100 – 300 µl
- Magnetic stirrer
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Glass test tubes (10 – 15 ml)
- Micropipettes 20 – 200 µl, 100 – 1000 µl
- Multipipette with 2.5 ml combitips

## 7. PRECAUTIONS

- Fluoroquinolones are toxic compounds. Avoid contact with mouth and skin. Be aware that Fluoroquinolones is not inhaled.
- The stop reagent contains 0.5 M sulfuric acid. Do not allow the reagent to get into contact with the skin.
- Avoid contact of all biological materials with skin and mucous membranes.
- Do not pipette by mouth.
- Do not eat, drink, smoke, store or prepare foods, or apply cosmetics within the designated work area.
- Use the derivative reagent in a fume hood to avoid breathing vapor.
- TMB is toxic by inhalation, in contact with skin and if swallowed; take care when handling the substrate.
- Do not use components past expiration date and do not use components from different lots.
- Each well is ultimately used as an optical cuvette. Therefore, do not touch the under surface of the wells, prevent damage and dirt.
- All components should be completely dissolved before use. Take special attention to the substrate, which crystallises at +4°C.
- Optimal results will be obtained by strict adherence to this protocol. Careful pipetting and washing throughout this procedure are necessary to maintain good precision and accuracy.

## 8. SAMPLE PREPARATIONS

Two methods are specified: Method I is fast and simple and results in an LOD of 12 ppb. Method II has an evaporation step and results in an LOD of 0.3 ppb.

### 8.1 Methods for shrimps, fish and tissue (chicken) samples

#### 8.1.1 Method I

- Homogenise approximately 10 g of sample.
- Weigh 0,5 g of the homogenised sample and transfer into a test tube.
- Add 1.5 ml of 80% methanol in sample dilution buffer\* (see Chapter 9) and mix (head over head) for 30 minutes.
- Centrifuge (10 minutes, 2000 x g).
- Pipette 100 µl of the supernatant into a clean tube and add 900 µl sample dilution buffer\* (see Chapter 9).
- An aliquot of 50 µl is used in the ELISA test.

#### 8.1.2 Method II

- Homogenise approximately 10 g of sample
- Weigh 1 g of the homogenised sample and transfer into a test tube
- Add 3 ml of 80% methanol in sample dilution buffer\* (see Chapter 9)
- Mix for 15 minutes head over head
- Centrifuge (10 minutes, 2000 x g)
- Transfer 2 ml of the supernatant to a glass tube (volume tube 4 ml)
- Evaporate under a mild stream of nitrogen at 50°C
- Reconstitute the residue with 1 ml of 8% methanol in sample dilution buffer\* (see Chapter 9)
- Defat by addition of 1.0 ml hexane
- Vortex for 1 minute and centrifuge (15 minutes, 2000 x g)
- Use 50 µl of the layer underneath in the ELISA test

### 8.2 Egg samples, raw and pasteurised

- Transfer 0.5 g of the homogenised whole egg, the white or yolk, in a test tube.
- Add 1.5 ml of 40% methanol in sample dilution buffer\* (see Chapter 9) and mix (head over head) for 30 minutes.
- After centrifugation (10 minutes, 2000 x g), 100 µl of supernatant is pipetted into a clean tube and 400 µl of sample dilution buffer is added.
- An aliquot of 50 µl is used in the ELISA.

### 8.3 SPE procedure tissue and whole egg

Materials and chemicals required:

- Phosphoric acid
- Oasis HLB Spec disk columns (60 mg/3 ml)
- Methanol 100%
- Ammonia
- Add 8 ml of 100% methanol to 92 ml of sample dilution buffer to obtain an 8% methanol solution (for Sample dilution buffer see Chapter 9)

- Weight 1 g homogenized sample into a 50 ml tube
- Add 4 ml of phosphoric acid 0.6% in water/methanol 60/40 (v/v)
- Vortex the muscle sample 30 seconds and then vigorously shaken for 10 minutes, head over head. The egg sample gently swirling otherwise it becomes jelly.
- Centrifuge 10 minutes 2000 x g
- filter the supernatant into a 15 ml tube
- Repeat the extraction step by adding 4 ml of extraction solution, onto the pellet
- The extracts (8 ml) were combined and mixed
- Pipette 4 ml into a glass tube and evaporated the methanol under a mild stream of nitrogen at 50°C. About 1.6 ml of aqueous phase will remain.
- Dilute the aqueous extract further with 4 ml of phosphoric acid 1%.

Solid phase extraction

- Use Oasis HLB Spec disk columns
- Activate the column:
- Add 1 ml of 100% methanol (flow 1 ml/minute)
- Add 1 ml distilled water

Note: It is important that the disk is not allowed to dry completely during activation and prior to sample addition! If the disk has become dry, repeat the conditioning procedure.

- Load the diluted aqueous extract onto the activated column

Pass the aqueous residual through the column using vacuum; flow 1 ml/minute.

- Washing procedure:
- Add 2 ml of phosphoric acid solution (0.025M, pH 3/methanol 95:5 (v/v))
- Add 2 ml of distilled water allow column to become dry.
- Elution of FLUOQ:
- Add 2 ml of methanol/ammonia 95:5 (v/v) onto the cartridge
- Collect the eluent in a clean glass tube.
- Let the cartridges to be dry for two minutes.
- Evaporate the eluent under a mild stream of nitrogen at 50°C
- Reconstitute the residue for muscle with 2 ml and for egg with 1 ml of 8% methanol in sample dilution buffer\* (see Chapter 9)
- Use 50 µl of this solution in the ELISA

### 8.4 Milk samples

- Transfer 0.5 ml of the homogenised milk in a test tube.
- Add 4.5 ml of 8% methanol in sample dilution buffer\* (see Chapter 9) and mix (head over head) for 30 minutes.
- After centrifugation (10 minutes, 2000 x g), an aliquot of 50 µl from the liquid part below the fat layer is used in the ELISA test.