MULTI-SULFONAMIDE II ELISA

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

EUROPROXIMA MULTI-SULFONAMIDE II ELISA

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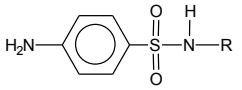
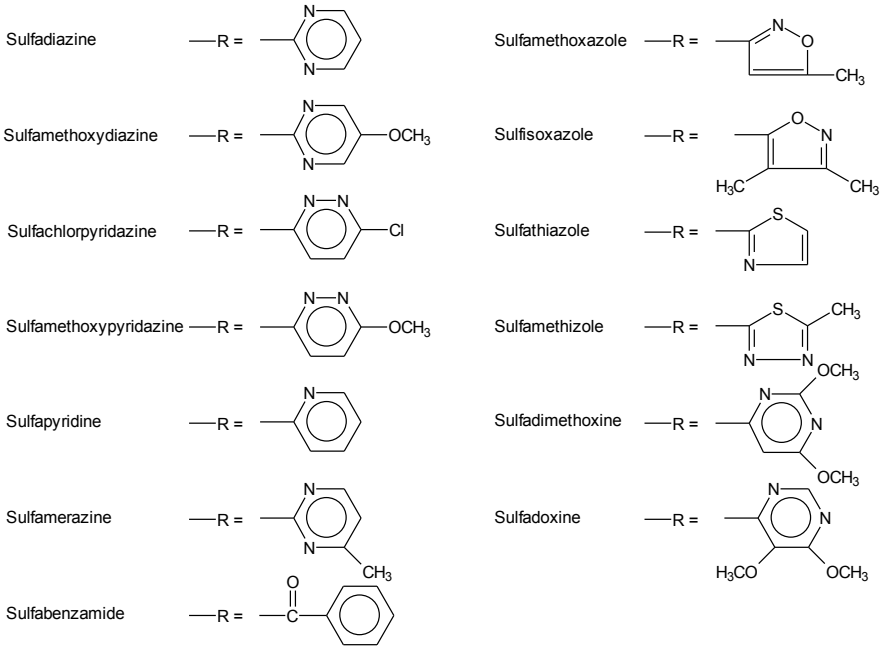
BRIEF INFORMATION

The multi-sulfonamide II ELISA is a competitive enzyme immunoassay for screening and quantitative analysis of sulphonamides in different commodities. With this ELISA kit 96 analyses can be performed. Samples and standards are measured in duplicate which means that a total of 40 samples can be analysed.
The ELISA kit contains all reagents to perform the test. Reagents for sample preparation are not included in the kit.

1. INTRODUCTION



General structure of sulfonamides



Sulfonamides are a group of synthetic drugs of which the molecules share the p-aminobenzenesulfonamide moiety. They act as competitive antagonists of p-aminobenzoic acid, an essential precursor for a lot of bacteria and protozoon of the vitamin folic acid. Sulfonamides are antibiotics that are widely used in veterinary and

human medicine against bacteria and coccidian protozoa. Sulfonamides which are retained in food can result in allergic or toxic reactions in consumers sensitive for these compounds. In addition, there are general concerns that the widespread use of antibiotics may contribute to antibiotic resistance in pathogenic organisms. Normally, tissue residues in animals are controlled by withdrawing the drug from feed 'some time' before slaughter. The concentrations of the sulfonamides are then presumed to deplete to less than the maximum residue level (MRL). However, due to contaminated food or failure to monitor the withdrawal period, a number of animals are reaching slaughterhouses with a substantially excessive amount of drugs still present in their tissues.
The multi-sulfonamide II ELISA can be used to detect residues in various matrices. The following sulfonamides can be detected far below the MRL of 100 µg/kg as stated by the European Union: sulfadiazine, sulfamethoxazole, sulfachlorpyridazine, sulfisoxazole, sulfathiazole, sulfametizole, sulfamethoxypyridazine, sulfamethoxydiazine, sulfapyridine, sulfamerazine, sulfabenzamide, sulfadimethoxine and sulfadoxine.

2. PRINCIPLE OF THE MULTI-SULFONAMIDE II ELISA

The microtiter plate based MULTI-SULFONAMIDE ELISA consists of on precoated plate with antibody against sulfonamides (12 strips, 8 wells each). Horseradish peroxidase (HRP) labeled sulfadiazine and standard solution or sample are added to the wells. Free sulfonamides from the samples or standard and sulfonamide-HRP conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay).
After an incubation step of 30 minutes at room temperature, the non-bound reagents are removed in a washing step. The amount of bound sulfonamide-HRP conjugate is visualized by the addition of a substrate/chromogen solution (tetramethylbenzidine, TMB). Bound sulfonamide-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 sulfonamide concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The multi-sulfonamide II ELISA utilizes antibodies raised in mouse against protein conjugated sulfonamide. The cross-reactivity pattern of the antibody (as tested in buffer) is:

Sulfadiazine	100%	Sulfadoxine	116%
Sulfamethoxazole	62%	Sulfisoxazole	92%
Sulfapyridine	73%	Sulfachlorpyridazine	176%
Sulfathiazole	155%	Sulfamethizole	141%
Sulfamethoxypyridazine	99%	Sulfadimethoxine	70%
Sulfabenzamide	86%	Sulfamethoxydiazine	64%
Sulfamerazine	88%	Sulfamonomethoxine	99%

The cross-reactivities are determined in a buffer system. The reported values may be different in samples due to matrix effects.
The test cannot discriminate between analytes and cross-reactive substances.

The Limit of detection (LOD) and the detection capability (CCβ) are determined under optimal conditions. Cut-off criteria need critical consideration.

Matrix	**Procedure**	**LOD (ppb)**	**CCβ (ppb)**
Tissue	8.1	4.5	6.1
Shrimps	8.2	2.1	n.a.
Honey	8.3	4.5	5.9
Milk	8.4	6.1	7.9
Egg	8.5	6.3	n.a.
Urine	8.6	13.4	18.9

If the sample is found to be non-compliant, the results shall be verified by re-analysis of the sample using a confirmatory method.

4. HANDLING AND STORAGE

- Kit and kit components should be stored 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 of the kit and/or components has passed, no further quality guarantee is valid.
- Bring all kit components including the microtiter plate to ambient (room) temperature before use.
- Dilute the kit components immediately before use, but after the components are brought to ambient temperature.
- Avoid condensation in the wells of the plate. Bring the sealed plate to ambient temperature before opening the plate sealing.
- The substrate chromogen solution can be stored in a refrigerator (2ºC to 8ºC) until the expiry date stated on the label.
- Exposure of the chromogen solution to light should be avoided.

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

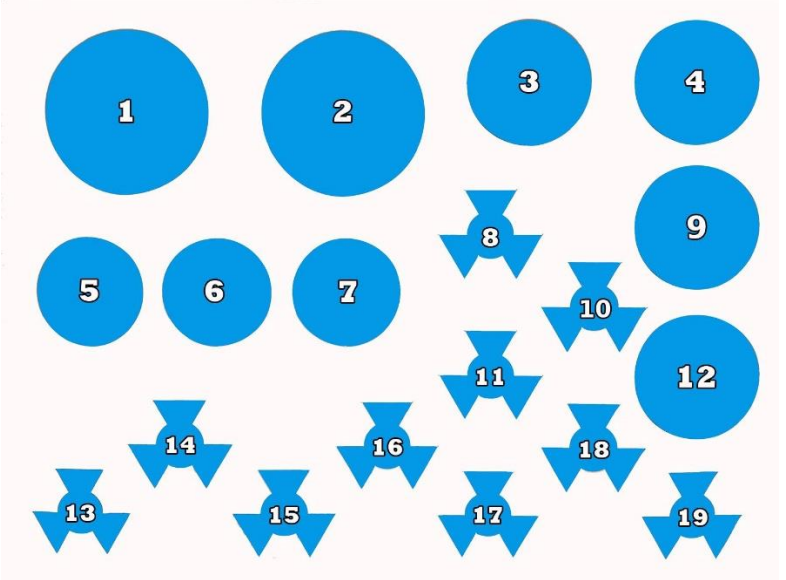
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

Manual

One sealed (96 wells) microtiter plate (12 strips, 8 wells each), coated with specific antibody. 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** (lyophilized)
6. not in use
7. not in use
8. **Standard solution** 100 ng/ml (1ml)
9. not in use
10. not in use
11. not in use
12. not in use
13. **Zero standard solution 1** (2ml, Ready-to-use)
14. **Standard solution 2** (1ml, Ready-to-use) **0.313 ng/ml**
15. **Standard solution 3** (1ml, Ready-to-use) **0.625 ng/ml**
16. **Standard solution 4** (1ml, Ready-to-use) **1.25 ng/ml**
17. **Standard solution 5** (1ml, Ready-to-use) **2.5 ng/ml**
18. **Standard solution 6** (1ml, Ready-to-use) **5.0 ng/ml**
19. **Standard solution 7** (1ml, Ready-to-use) **10.0 ng/ml**

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- 15 ml tubes with screw caps
- Scales and weighing vessels
- Gloves
- Fume hood
- Homogenizer (vortex, mixer)
- Centrifuge
- Automated microtiter plate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Glass test tubes (10 - 15 ml)
- Micropipettes 100 – 1000 µl
- Multipipette with 2.5 ml combi tips
- Aluminum foil or parafilm
- Polypropylene tubes
- Distilled water (bidest)
- Methanol
- Ethylacetate
- N-hexane
- Sodium chloride (NaCl)
- Disodium hydrogenphosphate (Na_2HPO_4)
- Potassium dihydrogenphosphate (KH_2PO_4)
- Milk powder

7. PRECAUTIONS

- This kit may contain hazardous substances. For hazard notes please refer to the appropriate safety data sheets (SDS).
- 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.
- 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 and rinsing buffer, which crystallize at +4ºC.
- 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

8.1 Tissue samples
- Weigh 1 g finely cut and subsequently homogenised sample into a polypropylene tube
- Add 1 ml 100% methanol
- Vortex
- Add 4 ml of ethyl acetate
- Mix well on a vortex followed by mixing head over head for at least 15 minutes
- Centrifuge (5 minutes, 2000 x g)
- Pipette an amount of 1 ml of supernatant (ethyl acetate) into a glass tube
- Evaporate to dryness under a mild stream of nitrogen at 50ºC
- Dissolve the residue in 1 ml of PBS, see chapter 9
- Defat by adding 1 ml of n-hexane
- Mix using a vortex and centrifuge (5 minutes, 2000 x g)
- Remove the upper layer (n-hexane)
- Pipette 100 µl into a tube and add 150 µl of PBS.
- Use 50 µl portions in the ELISA.

8.2 Shrimp samples
- Weigh 1 g finely cut and subsequently homogenised sample into a polypropylene tube
- Add 1 ml 100% methanol
- Vortex
- Add 4 ml of ethyl acetate
- Mix well on a vortex followed by mixing head over head for at least 15 minutes
- Centrifuge (5 minutes, 2000 x g)
- Pipette an amount of 1 ml of supernatant (ethyl acetate) into a glass tube
- Evaporate to dryness under a mild stream of nitrogen at 50ºC
- Dissolve the residue in 1 ml of PBS, see chapter 9
- Defat by adding 1 ml of n-hexane
- Mix using a vortex and centrifuge (5 minutes, 2000 x g)
- Remove the upper layer (n-hexane)
- Use 50 µl portions in the ELISA.

8.3 Honey samples
- Homogenize the sample
- Weigh 1 g homogenised sample into a tube
- Add 2 ml 15% methanol, see chapter 9
- Mix well on a vortex. Take care that the residue is homogenous dispersed before mixing head over head for 15 minutes
- Centrifuge 10 minutes at 2000 x g
- Dilute 45 µl of the upper layer with 255 µl PBS, see chapter 9
- Use 50 µl portions in the ELISA.

8.4 Milk samples

For undiluted defatted milk samples the matrix effect is a critical factor. To avoid fat residues in the sample, the manual should be strictly followed. The pH of the sample is another potential pitfall. Sour milk disturbs the ELISA, neutralization of the pH is essential.

- Centrifuge cold milk samples for 15 minutes at 2000 x g and at 4°C. Remove the upper fat layer using a spatula.
- Pipette 1 ml defatted sample into a tube
- Add 1 ml 30% methanol, see chapter 9
- Mix well on a vortex, followed by mixing head over head for 15 minutes
- Centrifuge 10 minutes at 2000 x g
- Dilute 50 µl of the supernatant with 450 µl PBS, see chapter 9
- Use 50 µl portions in the ELISA.

8.5 Egg samples

- Homogenise the whole egg sample
- Weigh 1 g homogenized sample into a tube
- Add 1 ml 30% methanol, see chapter 9
- Mix well on a vortex. Take care that the residue is homogenous dispersed before mixing head over head for 15 minutes
- Centrifuge 10 minutes at 2000 x g
- Dilute 50 µl of the upper layer with 450 µl PBS, see chapter 9
- Use 50 µl portions in the ELISA.

8.6 Urine samples

- Pipette 0.5 ml of the homogenised sample into a tube
- Add 2 ml milk solution, see chapter 9
- Add 1 ml 100% methanol
- Add 5 ml of ethyl acetate
- Mix well on a vortex, followed by mixing head over head for at least 15 minutes
- Centrifuge (5 minutes, 2000 x g)
- Pipette an amount of 3 ml of the upper layer (ethyl acetate) into a glass tube
- Evaporate to dryness under a mild stream of nitrogen at 50ºC
- Dissolve the residue in 1 ml of PBS, see chapter 9
- Defat by adding 1 ml of n-hexane
- Mix using a vortex and centrifuge (5 minutes, 2000 x g)
- Remove the upper layer (n-hexane)
- Pipette 100 µl of the dissolved residue into a tube and add 300 µl of PBS
- Use 50 µl portions in the ELISA.

9. PREPARATION OF REAGENTS

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

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

Rinsing buffer
The rinsing buffer is delivered 20 times concentrated. Prepare dilutions freshly before use. For each strip 20 ml of diluted rinsing buffer is required (1 ml concentrated rinsing buffer + 19 ml distilled water).

Dilution buffer
The dilution buffer (ready to use) is used to dilute the samples and the conjugate.

Conjugate
Reconstitute the vial of lyophilized conjugate with 6 ml of dilution buffer, mix thoroughly and keep in the dark until use. For prolonged storage freeze aliquots at -20°C.

Substrate/chromogen solution
The substrate/chromogen solution (ready-to-use) tends to precipitate at +4ºC.
Take care that this vial is at room temperature when used (keep in the dark) and mix the content before pipetting into the wells.

Phosphate Buffered Saline (PBS)
Prepare this buffer as follows:
[Quantities indicated are for 1 liter buffer.]

Na_2HPO_4	0.77 g
KH_2PO_4	0.18 g
NaCl	8.94 g

pH 7.4 ± 0.2

Milk solution 10 ml
Prepare freshly for use
Add 1 gram milk powder to 9 ml distilled water
Homogenize the solution.

30% methanol
3 ml methanol to 7 ml distilled water

15% methanol
1.5 ml methanol to 8.5 ml distilled water

10. ASSAY PROCEDURE

Rinsing protocol

Unbound components have to be removed efficiently between incubation steps in ELISA tests. 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.
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 washing solution in 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 and prepare reagents according to Chapter 9.

2. Pipette 100 µl of the zero standard in duplicate (wells H1, H2, blank).
   Pipette 50 µl of the zero standard (Bmax) in duplicate (wells A1, A2, maximal signal).
   Pipette 50 µl of each of the standard solutions in duplicate (wells B1,2 to G1,2 I.e 0.313, 0.625, 1.25, 2.5, 5.0 and 10.0 ng/ml).

3. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate.

4. Pipette 50 µl conjugate (HRP) into all wells, except H1 and H2.

5. Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.

6. Incubate for 30 minutes in the dark at room temperature (20ºC - 25ºC).

7. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.

8. Pipette 100 µl substrate solution into each well.

9. Incubate 15 minutes at 20ºC - 25ºC in the dark.

11. Add 100 µl of stop solution to each well.

12. 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.

$$\frac{\text{O.D. standard (or sample)}}{\text{O.D. zero standard/Bmax}} \times 100 = \text{percentage maximal absorbance}$$

Calibration curve:
The values (percentage 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 the Y-axis versus the analyte equivalent concentration on a logarithmic X-axis.

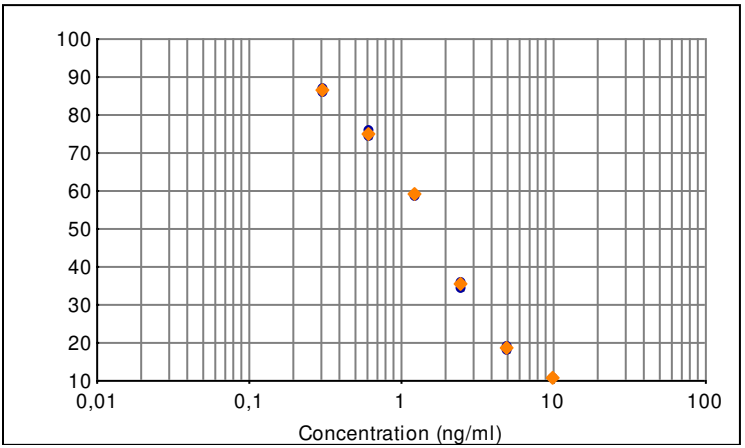


Figure 1: Example of a calibration curve

The amount of sulfonamide in the samples is expressed as sulfonamide equivalents. The sulfonamide equivalents in the extracts (ng/ml) corresponding to the percentage maximal absorbance of each extract can be read from the calibration curve.

8.1 Tissue samples
The sulfonamide equivalents, as read from the standard curve, have to be multiplied by a factor 12.5 to obtain the sulfonamides content in tissue samples.

8.2 Shrimp samples
The sulfonamide equivalents, as read from the standard curve, have to be multiplied by a factor 5 to obtain the sulfonamides content in shrimp samples.

8.3 Honey samples
The sulfonamide equivalents, as read from the standard curve, have to be multiplied by a factor 20 to obtain the sulfonamides content in honey samples.

8.4 Milk samples
The sulfonamide equivalents, as read from the standard curve, have to be multiplied by a factor 20 to obtain the sulfonamides content in milk samples.

8.5 Egg samples
The sulfonamide equivalents as read from the standard curve, have to be multiplied by a factor 20 to obtain the sulfonamides content in egg samples.

8.6 Urine samples
The sulfonamide equivalents as read from the standard curve, have to be multiplied by a factor 10 to obtain the sulfonamides content in urine samples.

12. LITERATURE

W. Haasnoot, F. Cohen, J. du Pré, G. Cazemier, A. Kemmers-Voncken, M. Bienenmann-Ploum and R. Verheijen. Application of generic monoclonal antibodies against sulfonamides in optical biosensors. In: Proceedings of the Euroresidue IV Conference, 8-10 May 2000, Veldhoven, the Netherlands, pp 501-505.

EMEA; The European Agency for the Evaluation of Medicinal Products. EMEA/MRL/026/95. Summary report sulfonamides (2).

M. Bienenmann-Ploum, T. Korpimäki, W. Haasnoot and F. Cohen. Comparison of multi-sulfonamide biosensor immunoassays.
Analytica Chimica Acta, 529, 115-122, 2005.

13. ORDERING INFORMATION

For ordering the multi-sulfonamide II ELISA kit, please use cat. code 5101SULMII.

14. REVISION HISTORY

The manual is adapted to a new layout of the test kit. Several textual changes are added.

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