B ZERO SULFA

Enzyme immunoassay for the detection of sulphonamides (code HU0050006)

***B ZERO* SULFA** is a enzyme immunoassay which allows the quantitative determination of sulphonamides.
The kit contains the procedure and the materials sufficient for 48 determinations, zero standard included. A microtiter plate or strip photometer (manual or automatic ELISA reader) is required for result evaluation.

Samples
Muscle, egg, milk, honey. feed.

Sample preparation
- **Muscle, egg, milk, honey (METHOD I):** homogenization, (skimming for milk), solvent extraction, centrifugation, evaporation, reconstitution.
- **Muscle, egg, honey (METHOD II)** homogenization, (acid hydrolysis for honey) buffer extraction, centrifugation and filtration.
- **Milk(METHOD II)**: centrifugation and dilution.
- **Honey (METHOD III):** acidic hydrolysis, purification on SPE column, elution, evaporation, resuspension.
- **Feed:** extraction with solvent, dilution.

Assay time: 75 minutes (sample preparation not included).
Detection limit
Milk (method I) : 0,25 ppb
Muscle, egg, honey (method I) and honey (method III): 0.5 ppb.
Muscle, egg, honey, milk (method II): 5 ppb.
Feed: 66.6 ppb.

Specificity	
Compound	**Cross-reactivity (%)**
Sulfamerazine	>100
Sulfamonomethoxine	>100
Sulfadiazine	>100
Sulfachloropyridazine	>100
Sulfaclozine (sulfachloropyrazine)	>100
Sulfamethoxydiazine (sulfamethoxine, sulfameter)	>100
Sulfamethoxypyridazine (sulfapyridazine)	>100
Sulfadimethoxine	>100
Sulfaquinoxaline	>100
Sulfathiazole	>100
Sulfamethizole	>100
Sulfamethazine	100
Sulfamethoxazole	90
Sulfisoxazole	50
Sulfapyridine	36
Sulfadoxine	24
Sulfacetamide	2,5
Sulfaphenazole	<2
Sulfabenzamide	<2
Sulfaguanidine	<0,1
Sulfanilamide	<0,1

1. TEST PRINCIPLE
The assay is performed in a polystirene microtiter plate coated with the antigen, which constitutes the solid phase. The zero standard solution, samples and antibody are added in sequence in the microtiter plate.
During the first incubation, free sulphonamides molecules in the sample and the antigen coated on the solid phase compete for the anti-sulphonamide antibody binding sites. After the first incubation, any unbound molecule is removed in a washing step.
The enzyme conjugate is then added; during the second incubation it will bind to the anti-sulphonamides antibodies bound on the solid phase. After a washing step, the enzyme conjugate-antibody bound on the solid phase is detected by the addition of a colourless substrate/chromogen solution, which during the third incubation is converted by the enzyme into a blue coloured reaction product.
After addition of the stop solution, the absorbance is measured at 450 nm with a microtiter plate photometer. The absorbance is inversely proportional to the sulphonamides concentration in the sample.

2. PROVIDED REAGENTS
Microtiter plate: 48 wells (6 strips x 8 wells), coated with the antigen.
As the strips are breakable, the wells can be used individually. For this purpose, it is sufficient to get out the wells from the frame and to break the joint.
Std 0: 1 plastic vial containing 1,5 ml of 0 ng/ml of sulfamethazine standard solution.
ATTENTION: only the standard zero is provided. B/B0 values of calibration curve (0.5-250 ng/ml) are reported in the kit certificate of conformity.
Anti-sulphonamides Antibody: 1 glass vial containing 5 ml of anti-sulphonamides antibody. Blue solution.
Enzyme conjugate: 1 amber plastic vial containing 0,35 ml of enzyme conjugate.
Enzyme conjugate diluent: 2 amber plastic vials containing 17 ml of the enzyme conjugate diluent. Red solution.
Washing-buffer 20X: 1 plastic bottle containing 50 ml of washing buffer.
Developing solution: 1 amber plastic bottle containing 8 ml of developing solution.
Dilution buffer 20X: 1 plastic bottle containing 25 ml of dilution buffer.
Stop solution: 1 glass bottle containing 6 ml of stop solution. White cap.

3. MATERIALS REQUIRED BUT NOT PROVIDED
For sample preparation:
- Balance (muscle, egg, honey)
- Homogenizer (muscle)
- Grinder (feed)
- NaOH, HCl, pH indicator strips (honey)
- Distilled water

METHOD I
- Shaker
- Vortex
- Refrigerated centrifuge
- System to evaporate solvent
- Ethylacetate
- n-hexane (muscle,egg)

METHOD II
- Shaker (muscle,egg)

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- Vortex (honey)
- 20°C Freezer (muscle,egg)
- Refrigerated centrifuge (muscle, egg, honey)
- Incubator or water bath incubator (muscle, egg, honey)
- Filter paper (Whatman 1) (muscle, egg, honey)

METHOD III

- Vortex
- Shaker
- Centrifuge
- System to evaporate solvent
- NH_3
- Methanol HPLC grade
- SPE Column "Strata X-C" 200 mg/6ml (cat.nr.: 8B-S029-FCH) or 100 mg/6 ml (cat.nr. 8B-S029-ECH), Phenomenex.

FEED

- Shaker
- Vortex
- Centrifuge
- Acetonitrile

For assay implementation:

- Micropipette 20-200 µl and 200-1000 µl with suitable tips.
- Multichannel variable pipette (20-200 µl) with suitable tips.
- ELISA plate reader or strip photometer equipped with a 450 nm filter.

4. WARNING AND PRECAUTIONS FOR THE USERS

- For *in vitro* diagnostic use only.
- Some reagents contain solutions that may be identified as dangerous substance by the Regulation (EC) No 1272/2008. Please refer to the Material Safety Data Sheet available on the Eurofins Technologies and Eurofins Tecna web site.
- Handle the reagents with caution, avoiding contact with skin, eyes and mucous membranes.

5. HANDLING AND STORAGE INSTRUCTIONS

- Store the kit at +2/+8 °C and never freeze any component.
- Reseal the unused strips of the microtiter plate in the bag together with the desiccant bag provided.
- Do not use components after the expiration date.
- Do not intermix components between different kit lots.
- Do not use photocopies of the instruction booklet. Keep always to the instruction booklet that is included inside the kit.

6. SAMPLE PREPARATION

6.1 Muscle

METHOD I

- Homogenize about 100 g of defatted sample without fibrous parts.
- Weight 1 g of homogenized sample.
- Add 5 ml of ethylacetate and vortex for 10 seconds.
- Shake for 15 minutes (400 rpm).
- Centrifuge at 3000g for 10 minutes at 15°C.
- Collect 3 ml of supernatant and evaporate to dryness at +50°C under a stream of nitrogen or air.
- Dissolve the residue with 0.6 ml of dilution buffer 1x.
- Add 1 ml of n-hexane, vortex for 30 seconds.
- Centrifuge at 3000g for 5 minutes.
- Collect the lower aqueous phase. The extract is ready for the assay. The dilution factor is 1.

It is suggested to test the samples immediately after preparation.

METHOD II (alternative)

- Homogenize about 100 g of defatted sample without fibrous parts.
- Weight 1 g of homogenized sample.
- Add 10 ml of dilution buffer 1x.
- Shake for 10 minutes (400 rpm).
- Incubate at 80°C for 45 minutes.
- Refrigerate at -20°C for 20 minutes.
- Centrifuge at 4500g for 5 minutes at +2/+8°C
- Collect the supernatant and filtrate with Whatman 1 filter paper. The extract is ready for the assay. The dilution factor is 10.

It is suggested to test the samples immediately after preparation.

6.2 Egg

METHOD I

- Mix well the yolk and the egg white to obtain an homogeneous sample.
- Weight 1 g of sample.
- Add 5 ml of ethylacetate and vortex for 10 seconds.
- Shake for 15 minutes (400 rpm).
- Centrifuge at 3000g for 10 minutes at 15°C.
- Collect 3 ml of supernatant and evaporate to dryness at +50°C under a stream of nitrogen or air.
- Dissolve the residue with 0.6 ml of dilution buffer 1x.
- Add 1 ml of n-hexane, vortex for 30 seconds.
- Centrifuge at 3000g for 5 minutes.
- Collect the lower aqueous phase. The extract is ready for the assay. The dilution factor is 1.

It is suggested to test the samples immediately after preparation.

METHOD II

- Mix well the yolk and the egg white to obtain an homogeneous sample.
- Weight 1 g of sample.
- Add 10 ml of Dilution buffer 1x.
- Shake for 10 minutes (400 rpm).
- Incubate at 80°C for 45 minutes.
- Refrigerate at -20°C for 20 minutes.
- Centrifuge at 4500g for 10 minutes at +2/+8°C.
- Collect the supernatant and filtrate with Whatman 1 filter paper. The extract is ready for the assay. The dilution factor is 10.

It is suggested to test the samples immediately after preparation.

6.3 Milk

METHOD I

- Cool the sample at 4°C.
- Centrifuge a 5 ml sample in a 50 ml Falcon tube for 15 minutes, 4000 rpm at 4°C.
- Eliminate the top fat.
- Transfer 2.5 ml of skimmed milk in a 50 ml Falcon tube.
- Add 5 ml of ethylacetate and mix upside down for 1 minute.
- Let separate the two phases for 5-10 minutes at room temperature.
- Take 4 ml of supernatants and evaporate it under air or N2 stream at 50°C.
- Reconstitute with 1 ml of dilution buffer 1x.
- The dilution factor is 0.5.

METHOD II

- Refrigerate the sample at +2/+8°C and centrifuge at +2/+8°C for 10 minutes at 3000g.
- Separate the fat from the skimmed milk.
- Add to 0.1 ml of skimmed milk 0.9 ml of washing buffer 0.5x (see chapter 7). The sample is ready for the assay. The dilution factor is 10.
- It is suggested to test the samples immediately after preparation.

6.4 Honey

METHOD I

- Weigh 1 g of honey and add 1 ml of 0.5 M HCl.

- Homogenize with wortex.
- Incubate at 37°C for 30 minutes.
- Add 0.5 ml of 1M NaOH and 2.5 ml of dilution buffer 1x.
- Verify that the pH is 7.5. If necessary, adjust with NaOH 1M.
- Add 5 ml of ethylacetate and vortex for 10 seconds.
- Shake for 15 minutes (400 rpm).
- Centrifuge at 3000g for 10 minutes at 15°C.
- Collect 3 ml of supernatant and evaporate to dryness at +50°C under a stream of nitrogen or air.
- Dissolve the residue with 0.6 ml of dilution buffer 1x. The extract is ready for the assay.
- The dilution factor is 1.
- It is suggested to test the samples immediately after preparation.

METHOD II

- Weigh 1 gr of honey and add 1 ml of 0.5 M HCl.
- Homogenize with vortex.
- Incubate at 37°C for 30 minutes.
- Add 0.5 ml of 1M NaOH and 7.5 ml of dilution buffer 1x.
- Adjust the pH at 7.5 with NaOH 0,5M/HCl 0.5M, if necessary, using a pH indicator.
- Filter with Whatman 1. The extract is ready for the assay. The dilution factor is 10.
- It is suggested to test the samples immediately after preparation.

METHOD III

- Weigh 3.0 g of honey.
- Add 3 ml of 2M HCl.
- Shake with vortex.
- Shake on shaker (400 rpm) for 30 minutes.
- Dilute with 17 ml of distilled water.
- Centrifuge at 4500 rpm for 10 minutes
- Purification with SPE 200 mg (100 mg) columns
- Prime the column with 5 ml of methanol and with 5 ml of 0.5 M HCl.
- Apply the diluted sample in the column.
- Wash the column sequentially with: 5 ml (3 ml) of 0,5 M HCl, 5 ml (3 ml) of n-hexane and 5 ml (3 ml) of methanol.
- Elute with 6 ml (4 ml) of methanol/30%NH3 solution in water (90/10, v/v).
- Evaporate under N2 or air stream at temperature of 50°C
- Resuspend with 1 ml of dilution buffer.
- Dilute 3 more times (for example 100 µl + 200 µl of dilution buffer 1x.
- The dilution factor is 1.

6.5 Feed

- Weigh 3.0 g of grinded feed.
- Add 20 ml of a 95/5 acetonitrile/water mixture.
- Vortex for approximately 30 seconds.
- Shake on shaker for 10 minutes (400 rpm)
- Centrifuge at 4000 rpm for 10 minutes
- Dilute 20x with dilution buffer 1x(for example: 100 µl of surnatants + 1900 µl of dilution buffer 1x)
- If necessary, filter with a 0.45 µm filter. The dilution factor is 133.

7. WORKING SOLUTIONS PREPARATION

Std 0: ready to use.
Anti-sulphonamides Antibody: ready to use.
Enzyme conjugate: **PAY ATTENTION**, in order to recover the total conjugate amount, centrifuge the vial briefly at low speed (spin-down) before use. Calculate and prepare the quantity necessary for the experiment. Dilute the conjugate **1/100** with the enzyme diluent (for example, 20 µl of enzyme conjugate concentrated + 1980 µl of enzyme conjugate diluent).
To draw a minimal volume of 20µl of the enzyme conjugate concentrate is recommended.
Gently mix head over head. DO NOT VORTEX.
Enzyme conjugate diluent: ready to use.
Washing buffer: **for microtiter plate washing (buffer 1x):** dilute the concentrated washing buffer 1:20 (1+19) with distilled water; **for milk sample dilution (buffer 0.5x):** dilute the concentrated washing buffer 1:40 (1+39) with distilled water.
ATTENTION: in presence of crystals, bring the solution at room temperature and stir in order to solve them completely.
The diluted washing buffer is stable at room temperature for 24 hours and at +2/+8°C for two weeks.
Developing solution: ready to use; the developing solution is light sensitive: keep away from direct light.
Stop solution: ready to use.
Dilution buffer: dilute the concentrated dilution buffer 1:20 (1+19) with distilled water. **ATTENTION**: in presence of crystals, bring the solution at room temperature and stir in order to solve them completely.

8. ASSAY PROCEDURE

8.1 Preliminary comments

- Before use, bring all reagents at room temperature.
- Return all reagents at +2/+8 °C immediately after use.
- Do not change the assay procedure, particularly:
- do not prolong or shorten the first and incubation time;
- do not incubate the plate at a temperature > 25°C or < 18°C;
- do not shake the plate during the incubations.
- Use always accurate and precise micropipettes with suitable tips;
- Once started, complete all the steps without interruption.
- The reproducibility of ELISA results largely depends upon the efficiency and uniformity of microwells washing; always keep to the described procedure.
- Use a **single disposable tip for each sample** to avoid cross-contamination.
- Do not allow tips to contact the liquid already present in the microwells or the internal microwells surface.
- Avoid direct sunlight during all incubations. Do not use sealing tapes to cover the microtiter plate.
- **ATTENTION**: **we warn to handle the reagents and the sample extracts with care in order to avoid cross-contaminations, particularly in case of spiking experiments.**

8.2 Assay procedure

1. Predispose an assay layout, taking into account that one well is required for zero standard and each sample.
2. Add 50 µl of zero standard /sample into the wells.
3. Using a multichannel pipet, add 50 µl of anti-sulphonamide antibody to each well and shake the plate gently with rotatory motion for few seconds.
4. Incubate 30 minutes at room temperature.

Do not prolong the first incubation time and do not use shaker.

5. Washing sequence:
   - Pour the liquid out from the wells.
   - Fill completely all the wells with working wash solution using a squeeze bottle. Pour the liquid out from the wells.
   - Repeat the washing sequence four (4) times. Remove the remaining droplets by tapping the microplate upside down vigorously against absorbent paper.

   Do not allow the wells to dry out.

6. Using a multichannel pipet, add 100 µl of enzyme conjugate to each well; shake the plate gently with rotatory motion for few seconds.
7. Incubate for 30 minutes at room temperature. *Do not prolong the incubation time and do not use shaker.*
8. Repeat the washing sequence as above.
9. Developing:
   - using the multichannel micropipette, add 100 µl of developing solution to each well;
   - shake the plate gently with rotatory motion for few seconds.
10. Incubate for 15 minutes at room temperature. Protect from light.
11. Using a multichannel pipet, add 50 µl of stop solution to each well and shake the plate gently.
12. Measure the absorbance at 450 nm. Read within 60 minutes.

9. RESULTS INTERPRETATION

- Divide the absorbance value of each sample by the absorbance of the Standard 0 (B_0) and multiply by 100; the Maximum Binding (B_0) is thus made equal to 100% and the absorbance values are quoted as percentage:

$$\frac{\text{Sample absorbance}}{\text{Standard 0 } (B_0) \text{ absorbance}} \times 100 = \frac{B}{B_0}$$

- Enter the B/B_0 provided for each standard (0.5; 1; 10; 20; 50; 250 ng/ml) in the kit lot conformity certificate in a semi-logarithmic system of coordinates against the sulfamethazine standard concentration and draw the standard curve.
- Take the B/B_0 (%) value for each sample and interpolate the corresponding concentration from the calibration curve. The concentration read on the calibration curve must be further multiplied by the corresponding dilution factor, as reported in chapter 6 for each kind of matrix.
  Please note: For the calibration, use the "point to point" curve; Excel spreadsheets are available on website and can be downloaded directly from the bottom of the product page.

WARNING: sample preparation according to method II and milk preparation method can cause high background levels. In this case it is necessary to establish an appropriate decision limit (CCα) for each matrix.

WARNING: By use of preparative methods I and III, the kit is more sensible than the confirmation method HPLC-DAD. Therefore supposed false positives could actually be real positives with concentrations that just LCMS/MS method is able to confirm.

10. EVALUATION OF RESULTS

After processing the results, it is necessary to verify the assay performance. The verification is performed by comparison of obtained data with those given in the kit specifications (chapter 11).
If the values are out of specifications, then the results of the test are not assured, therefore the sulphonamides concentration levels in the samples may not be valid.
In these cases it is advised to check the expiry date of the kit, the wavelength at which the reading was performed, as well as the procedure followed. If operation errors do not emerge, contact our technical assistance.

WARNING: If any kind of substitution is requested, please STORE THE KIT at the temperature defined by this instruction. Replacements are only possible if the original component is returned.

11. KIT SPECIFICATIONS

11.1 Assay specification

Mean Bo absorbance	$\geq$ 0.7 OD_{450nm}

11.2 Assay performance

Detection Capability (CCβ) was calculated as requested by of EU Decision 657/2002.

Detection capability	
Sample	**CCβ**
Muscle (METHOD I)	10 ppb of sulfamethazine 10 ppb of sulfaquinoxaline
Muscle (METHOD II)	50 ppb of sulfamethazine
Egg, milk (METHOD I)	10 ppb of sulfamethazine
Milk (METHOD II)	25 ppb of sulfamethazine
Feed	1 ppm of sulfamethazine
	25 ppb of sulfamethazine
Honey (METHOD II)	8 ppb of sulfathiazole
	2 ppb of sulfamerazine
Honey (METHOD III)	5 ppb of sulfamethazine

12. LIABILITY

Samples evaluated as positive using the kit have to be re-tested with a confirmation method.
Eurofins Technologies Hungary shall not be liable for any damages to the customer caused by the improper use of the kit and for any action undertaken as a consequence of results.
Eurofins Technologies Hungary shall not be liable for the unsafe use of the kit out of the current European safety regulations.

13. LITERATURE

V. Bassani, G. Rosar, F. Diana, R. Galarini, L. Persic Exploiting antibody and receptor cross-reactivities to develop broad range master-curve calibrated assays for tetracyclines and sulfonamides. Poster presentation at 7th International Symposium on Recent Advances in Food Analysis, 2015, November 3-6, Prague, Czech Republic.