BISPHENOL A ELISA
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A competitive enzyme immunoassay for screening and quantitative analysis of bisphenol A (BPA) in various matrices
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5221BPA[3]09.15
12. LITERATURE


13. ORDERING INFORMATION

For ordering the BPA ELISA kit, please use cat. code 5221BPA.

14. LAST MUTATIONS

SPE method also for surface water samples.

BRIEF INFORMATION

The BPA ELISA is a competitive enzyme immunoassay for the screening of water and milk samples. The test is based on antibodies directed against bisphenol A. The ELISA kit contains a 96 well microtiter plate as well as all essential reagents including ready-to-use standards to perform the test.

Methods for a fast and efficient extraction of bisphenol A from different matrices are included in the kit manual.

1. INTRODUCTION

Bisphenol A (BPA) is one of the most produced chemicals worldwide on the order of 6 billion pounds per year. It is mainly used in the production of polycarbonate plastics, a transparent plastic used for among others the production of unbreakable bottles for baby food and drinking water. It is also used in epoxy resins in the form of bisphenol A diglycidyl ether (BADGE), as a protective coating applied to the inside of food packaging, beverage cans, drinking water pipelines.

BPA is a known endocrine disruptor, mimicking estrogens and thyroid hormones. Many studies have found that laboratory animals exposed to low levels of BPA show elevated rates of diabetes, mammary and prostate cancers, decreased sperm count, reproductive problems, early puberty, obesity, and neurological problems. Some scientists believe that humans, especially infants, are currently exposed to levels that are known to cause harm in laboratory animals.

In both the EU and USA, the Total Daily Intake (TDI) of BPA is set at 0.05 mg/kg body weight/day. At present there are no restrictions on the amount of BPA that can be present in a final plastic product, but the tendency of BPA to migrate from food contact materials has been acknowledged in the EU food law. EU legislation [1] sets a Specific Migration Limit (SML) of 0.6 mg BPA per kg food (ppm). The SML in Japan is 2.5 ppm. The manufacture of BPA-containing baby bottles is prohibited since 2011 [2]. The French National Assembly and Senate voted in the end of 2012 to ban BPA from all food contact products by January 2015 (Proposition N°49) and the European Food Safety Authority (EFSA) will soon come with a new opinion [3].
2. PRINCIPLE OF THE BISPHENOL A ELISA

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

After an incubation time of one hour, the non-bound reagents are removed in a washing step. The amount of bound BPA-HRP conjugate is visualized by the addition of a substrate/chromogen solution ($H_2O_2$, TMB). Bound BPA-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 BPA concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The BPA ELISA utilizes antibodies raised in rabbits against protein conjugated bisphenol A. The reactivity pattern of the antibody is:

Cross-reactivity: 2.2-Bis-(4-hydroxyphenyl)-propane (BPA) 100%
Bis-(4-hydroxyphenyl)-methane (BPF) 0.1%
Bis-(4-hydroxyphenyl)-sulfone (BPS) 6%
4.4-Bis-(4-hydroxyphenyl)valeric acid (BVA) 84.9%
Bisphenol A diglycidyl ether (BADGE) < 0.1%
Coumestrol 1.5%
4.4’-Cyclohexylidenebisphenol (BPZ) 3.8%
β-Estradiol 3-benzoate < 0.1%
β-Estradiol 17-acetate < 0.1%
17-α-Ethynylestradiol <0.1%
Dienestrol <0.1%
Diethylstilbestrol (DES) <0.1%
Estriol <0.1%
Estrone <0.1%
Hexestrol <0.1%
Nonylphenol <0.1%
Zearalenone <0.1%

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

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Procedure</th>
<th>LOD ng/ml</th>
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<tbody>
<tr>
<td>Milk</td>
<td>8.1.1</td>
<td>120*</td>
</tr>
<tr>
<td>Milk</td>
<td>8.1.2</td>
<td>0.42</td>
</tr>
<tr>
<td>Water</td>
<td>8.2.1</td>
<td>0.4*</td>
</tr>
<tr>
<td>Water</td>
<td>8.2.2</td>
<td>0.009</td>
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</table>

* lowest standard multiplied by dilution factor

---

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

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

8.1.1 Milk samples: Direct method
The bisphenol A equivalents, as read from the standard curve, have to be multiplied by a factor 600 to obtain the bisphenol A content in milk samples.

The multiplication factor is adapted according to the applied dilution of samples.

8.1.2 Milk samples: Extraction method
The bisphenol A equivalents, as read from the standard curve, have to be multiplied by a factor 1.25 to obtain the bisphenol A content in milk samples.

8.2.1 Water samples: Direct method
The bisphenol A equivalents, as read from the standard curve, have to be multiplied by a factor 2 to obtain the bisphenol A content in water samples.

The multiplication factor is adapted according to the applied dilution of samples.

8.2.2 Water samples: SPE extraction
The bisphenol A equivalents, as read from the standard curve, have to be multiplied by a factor 50 to obtain the bisphenol A content in water samples.
7. Seal the microtiter plate and shake the plate a few seconds on a microtiter plate shaker.

8. Incubate for 1 hour in the dark at 4°C.

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

10. Pipette 100 µl substrate solution into each well.

11. Incubate 30 minutes at 20°C - 25°C.

12. Add 100 µl stop solution to each well.

13. Read the optical density (OD) 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.

\[
\text{OD standard (or sample)} \times 100\% = \% \text{ maximal absorbance}
\]

\[
\text{OD zero standard (Bmax)}
\]

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 absorption value of the standards is plotted on the Y-axis versus the concentration on the X-axis. The Y-axis is in logit, the Y-axis is logarithmic.

4. HANDLING AND STORAGE

- Kit and kit components are stored in a refrigerator (2°C to 8°C) before and immediately after use.
- 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 at ambient (room) temperature before use.
- Avoid condensation in the wells of the plate. Bring the sealed plate at ambient temperature before opening the plate sealing.
- Any direct action of light on the chromogen solution 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 absent colour reaction of the maximum binding (zero standard) (E450nm < 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 solution** (75 µl; 100 times concentrated)
6. **Antibody solution** (75 µl; 100 times concentrated)
7. **Zero standard solution** (2ml, Ready-to-use)
8. **Standard solution 1** (1ml, Ready-to-use) 0.2 ng/ml
9. **Standard solution 2** (1ml, Ready-to-use) 0.5 ng/ml
10. **Standard solution 3** (1ml, Ready-to-use) 1.0 ng/ml
11. **Standard solution 4** (1ml, Ready-to-use) 2.0 ng/ml
12. **Standard solution 5** (1ml, Ready-to-use) 5.0 ng/ml
13. **Standard solution 6** (1ml, Ready-to-use) 10.0 ng/ml

10. **ASSAY PROCEDURE**

**Rinsing protocol**

In ELISA's, 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 washing 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 rim (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 washing 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 (Preparation of reagents).
2. Pipette 100 µl of zero standard in duplicate (blank; well H1, H2).
3. Pipette 50 µl of zero standard in duplicate (zero standard; well A1, A2).
4. Pipette 50 µl of each standard dilution in duplicate (B1,2 to G1,2)
5. Add 25 µl diluted conjugate (Bisphenol A HRP) to all wells, except wells H1 and H2.
6. Add 25 µl diluted antibody solution to all wells, except wells H1 and H2.
9. PREPARATION OF REAGENTS

Before beginning the test, the reagents should be brought up to ambient temperature. Any reagent 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.

Sample dilution buffer**
10% methanol in dilution buffer (necessary for dilution of milk and water). To 1 ml of 100% methanol 9 ml of dilution buffer is added.

Conjugate solution (75 µl)
The conjugate is delivered 100x concentrated. Spin down the conjugate in the vial by a short centrifugation step (1 minute, 1000 x g). Add 5 µl of the concentrated conjugate solution to 495 µl dilution buffer. Per 2 x 8 wells 400 µl is required. Store unused concentrated conjugate at 2°C to 8°C.

Antibody (75 µl)
The antibody is 100x concentrated. Spin down the antibody in the vial by a short centrifugation (1 minute, 1000 x g). Add 5 µl of the concentrated antibody to 495 µl of dilution buffer. Per 2 x 8 wells 400 µl is required. Store concentrated antibody immediately upon use at 2°C to 8°C.

Rinsing buffer (30 ml)
The rinsing buffer is 20x concentrated. Prepare dilutions freshly before use. Per strip 40 ml of diluted rinsing buffer is used (2 ml concentrated rinsing buffer + 38 ml distilled water).

Substrate solution (12 ml)
The substrate solution (ready to use) precipitates at 4°C. Take care that this vial is at 20°C - 25°C (keep in the dark) and mix the content before pipetting into the wells.

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Fume hood
- Homogeniser (vortex, mixer)
- Centrifuge (2000 x g)
- Automated microtiter plate washer or 8-channel micropipette 50 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Micropipettes, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- Methanol 100% (MeOH)
- n-Hexane
- 5 M HCl
- Acetonitril
- Waters OASIS® HLB 5cc 200 mg LP Glass cartridge (186000683)
- Glass tubes

7. PRECAUTIONS

- Bisphenol A is a toxic compound. Avoid contact with mouth and skin. Be aware that bisphenol A 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.
- TMB is toxic by inhalation, in contact with skin and if swallowed; observe care when handling the substrate.
- Do not use components past expiration date and do not intermix components from different serial lots.
- Each well is ultimately used as an optical cuvette. Therefore, do not touch the underside 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

8.1 Milk samples
Milk samples can be analyzed after a 600 times dilution step. Using the direct method the EU Specific Migration Limit (SML) of 0.6 mg BPA can be measured. The measuring range is 6 – 0.12 µg/ml of bisphenol A. A lower detection limit can be obtained using the extraction method.

8.1.1 Milk samples: Direct method
Dilute the milk sample with sample dilution buffer** (chapter 9) in two steps as follows:
- Vortex the sample
- Pipette 100 µl of the milk sample into a glass tube
- Add 900 µl sample dilution buffer** (chapter 9)
- Vortex
- Pipette 15 µl of the 10 times diluted sample to 885 µl of sample dilution buffer** (chapter 9) and mix
- Use an aliquot of 50 µl in the ELISA test.

8.1.2 Milk samples: Extraction method
- Pipette 1 ml milk into a clean glass tube
- Add 4 ml of acetonitrile
- Mix 10 minutes head over head
- Centrifuge 10 minutes 2000 x g at 20°C - 25°C
- Pipette 2 ml of the upper layer into a clean glass tube
- Evaporate to dryness under a mild stream of nitrogen at 50°C
- Dissolve the residue in 0.5 ml sample dilution buffer** (chapter 9) and mix
- Add 0.5 ml n-hexane and vortex for 1 minute
- Centrifuge 2 minutes 2000 x g at 20°C - 25°C
- Remove the upper n-hexane layer
- Use an aliquot of 50 µl of the layer underneath in the ELISA test.

8.2 Water samples
Water samples can be analyzed after a 2 times dilution step. Using the direct method the measuring range is 20 – 0.5 ng/ml of bisphenol A. A lower detection limit can be obtained using the solid phase extraction (SPE) method. With this SPE method also surface water samples can be analyzed.

8.2.1 Water samples: Direct method
- Pipette 250 µl of the water sample into a glass tube
- Add 50 µl of 100% MeOH
- Add 200 µl of dilution buffer and mix
- Use an aliquot of 50 µl in the ELISA test.
To extend the measuring range, samples can be diluted up to 1:80 in sample dilution buffer** (chapter 9).

8.2.2 Water samples: SPE extraction
- Transfer 50 ml water into a clean glass Erlenmeyer flask
- Acidify the water to pH 3 using 5 M HCl
- Enter the SPE procedure

Activate the cartridge*:
- Add 3 ml of acetonitrile
- Add 3 ml of 100% MeOH
- Add 3 ml of demiwater

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

- Bring 50 ml water sample onto the activated cartridge (flow 1 drop/second)
- Add 3 ml of 5% MeOH in water
- Dry the cartridge for 2-5 minutes under vacuum.

Elution of Bisphenol A
N.B. From this step on, all eluents should be collected in glass tubes.
- Add 6 ml of 10% MeOH in acetonitrile (flow 1 drop/second)
- Evaporate the eluent under a mild stream of nitrogen at 50°C
- Dissolve the residue in 1 ml sample dilution buffer** (Chapter 9) and mix.
- Use an aliquot of 50 µl in the ELISA test.

* Water Oasis® HLB 5cc 200 mg LP Glass cartridge (186000683)