13. ORDERING INFORMATION

For ordering the Medroxyprogesterone acetate ELISA kit, please use cat. code 5131MPA.

14. LAST MUTATIONS

Updated “lay out”
Pipette schedule of the standard curve is adapted.

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MEDROXYPROGESTERONE ACETATE ELISA

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A competitive enzyme immunoassay for screening and quantitative analysis of Medroxyprogesterone acetate (MPA) in kidney fat, food and feed samples

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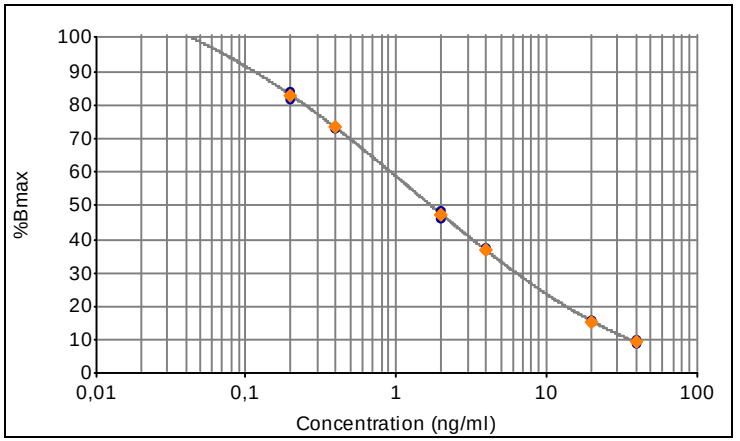


Figure 1: Example of a calibration curve

The amount of Medroxyprogesterone acetate in the samples is expressed as MPA equivalents. The MPA equivalents in the extracts (ng/g) corresponding to the % maximal absorbance of each extract can be read from the calibration curve.

Calculation

The results obtained of the different samples can directly be expressed as ng MPA-equivalents per g or per ml of sample.

12. LITERATURE

1. Lindfors E., Bäckman C. Determination of acetyl gestagens in fat using automated solid phase extraction and normal phase HPLC. Proceedings of the EuroResidue III conference. Edited by N. Haagsma and A. Ruiter. Velhoven, the Netherlands. (1996), 646-649.

2. Hädrich J., Jarvers J., and Podestàt U. Immunoenzymatischer Nachweis von Acetylgestagenen in Fettgewebe. Validierung des Verfahrens gemaβ DIN EN ISO/IEC 17025 und nach den Vorgaben der Entscheidung (EWG) Nr. 93/256 am Beispiel von 6α-Methyl-17α-hydroxyprogesteronacetat. Deutsche Lebensmittel-Rundschau: 97, 11, 409-414, 2001.

7. Incubate for 1 hour in the dark at 37°C.

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

9. Pipette 100 µl substrate solution into each well.

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

11. Pipette 100 µl 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.

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

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.

BRIEF INFORMATION

The Medroxyprogesterone (MPA) ELISA is a competitive enzyme immunoassay for the screening of kidney fat, food and feed samples for the presence of this synthetic progestagen. The test is based on antibodies against MPA. 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 MPA from different matrices are included in the kit manual.

1. INTRODUCTION

Medroxyprogesterone acetate (MPA), megestrol acetate, melengestrol acetate and chlormadinon acetate are synthetic derivatives of progesterone and are also called acetylgestagens. The acetylgestagens can be used as growth promoters in meat production, both in cattle and in pigs. The use of acetylgestagens as growth promoters leads to a faster growth of the animals and to an increase of feed conversion efficiency. Acetylgestagens are permitted as growth promoters in certain countries, e.g. the USA. However, in the EC the use of growth promoters, inclusive acetylgestagens, is prohibited. Furthermore, the use of acetylgestagens as feed additives can affect fertility and development in particular animals.

Acetylgestagens are apolar steroids. These steroids are particularly concentrated in fat tissue within the body of the animals. Therefore, the fat and especially kidney fat is used as matrix for screening for the presence of acetylgestagens. Alternatively, the MPA ELISA is also suitable to screen food and feed samples or feed additives for the presence of acetylgestagens.
The antiserum used in this ELISA is directed against MPA, however the antiserum also shows cross-reactivity against Megestrol acetate (50 %), Melengestrol acetate (25 %) and Chlormadinone acetate (50 %).

2. PRINCIPLE OF THE MEDROXYPROGESTERONE ACETATE (MPA) ELISA

The kit is based on a microtiter plate (12 strips, each 8 wells), precoated with sheep antibodies to rabbit IgG. In one incubation step, specific antibodies (Rabbit anti-MPA), enzyme labelled MPA (enzyme conjugate) and MPA standards or sample are added to the precoated wells. The specific antibodies are bound by the immobilised sheep anti-rabbit antibodies and at the same time free MPA (in the standard solution or in the sample) and enzyme labelled MPA compete for the specific antibody binding sites (competitive enzyme immunoassay).
After an incubation time of one hour, the non-bound (enzyme labelled) reagents are removed in a washing step. The amount of bound enzyme conjugate is visualized by the addition of chromogen substrate (tetramethylbenzidine, TMB). Bound enzyme transforms the 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 and is inversely proportional to the MPA concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The MPA-ELISA utilizes antibodies raised in rabbits against protein conjugated MPA.
The reactivity pattern of the antibody is:

Cross- reactivity:	Medroxyprogesterone acetate	100%
	Megestrol acetate	50%
	Melengestrol acetate	25%
	Chlormadinone acetate	50%

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

Matrix	Procedure	LOD ppb
Kidney fat	8.1	0.5

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).

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 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 treatment) and prepare reagents according to chapter 9.
   Microtiter plate is ready-to-use, do not wash.

2. Pipette 100 µl dilution buffer in duplicate (well H1, H2).
   Pipette 50 µl dilution buffer in duplicate (well A1, A2).
   Pipette 50 µl of each standard dilution in duplicate (well B1,2 to G1,2).

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

4. Pipette 25 µl conjugate (MPA-HRP) to all wells, except wells H1 and H2.

5. Pipette 25 µl antibody solution to all wells, except wells H1 and H2.

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

8.6 Solid phase clean-up

Activate solid phase cartridges (Bond-Elut C18, LRC 10 ml, 500 mg; Varian, art. Nr.: 1211-3027) by washing the cartridges subsequently with 5 ml methanol and with 5 ml 50% methanol in distilled water. Transfer 5.5 ml of the elute obtained after the primary solid phase extraction (paragraph 8.5), or 5.5 ml of the extract from the feed samples (paragraph 8.3), or 5 ml of the extract from the beverage samples (paragraph 8.4) onto an activated solid phase cartridge. Wash the cartridges using 7.0 ml 50% methanol in distilled water. Dry the cartridges under vacuum. Elute the acetylgestagens using 5.5 ml 80% methanol in distilled water with a speed of 1 ml per minute. Evaporate the elute to dryness at a temperature of 50°C under a mild stream of nitrogen. Dissolve the residue in 500 µl dilution buffer at a temperature of 37°C during regular mixing using a vortex or using an ultrasonic bath. An aliquot of 2 times 50 µl is used in the ELISA test.

9. PREPARATION OF REAGENTS

Before starting the test, allow the reagents to come to ambient temperature. Any reagents not used should be put back into storage immediately at 2°C to 8°C.

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 20 times 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

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

Standard

Prepare a dilution range of the Medroxyprogesterone acetate standard.
Reconstitute the lyophilised standard with 2 ml of dilution buffer. The concentration of the reconstituted standard is 40 ng MPA per ml.
Prepare a dilution range of the MPA standard containing 40, 20, 4, 2, 0.4 and 0.2 ng/ml.

Conjugate solution

Reconstitute the vial of lyophilised conjugate (MPA-HRP) with 4 ml dilution buffer, mix thoroughly and keep in the dark until use.
Store the vial immediately after use in the dark at 2°C to 8°C.

Antibody solution

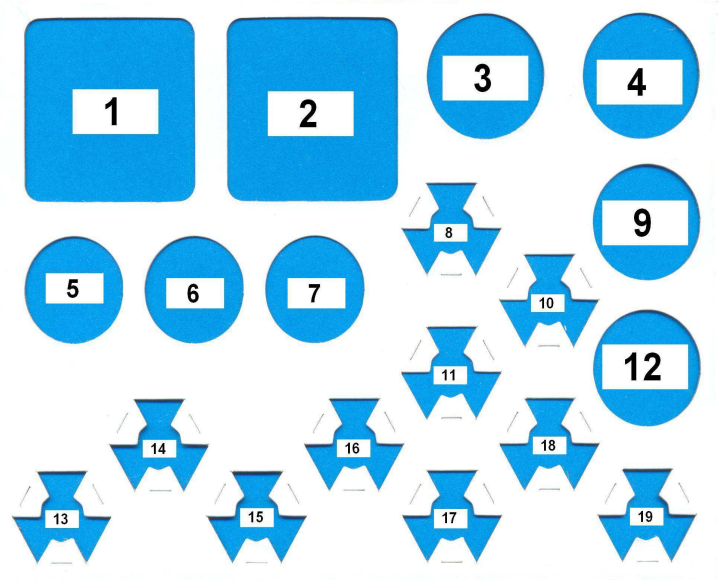
Reconstitute the vial of lyophilised antibodies with 4 ml dilution buffer, mix thoroughly and keep in the dark until use.
Store the vial immediately after use in the dark at 2°C to 8°C.

5. KIT CONTENTS

Manual

One sealed microtiter plate (12 strips, 8 wells each), coated with antibodies to rabbit IgG. Ready-to-use.

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



1. **Dilution buffer** (40 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** (lyophilised, blue cap)
6. **Antibody solution** (lyophilised, yellow cap)
7. **Standard solution** (lyophilised, black cap)
8. not in use
9. not in use
10. not in use
11. not in use
12. not in use
13. not in use
14. not in use
15. not in use
16. not in use
17. not in use
18. not in use
19. not in use

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 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Microwave
- Micropipettes, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- Methanol 100%
- 4 ml glass tubes
- Cyclo-hexane
- 15 ml tubes with screw cap (Greiner, polypropylene)
- Solid phase cartridge (C18)

7. PRECAUTIONS

- MPA is a toxic compound. Avoid contact with mouth and skin. Be aware that MPA 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 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 PREPARATION

Sample preparation will be conducted according to J. Hädrich et al [2]. In this manuscript an ISO validated method is described for screening for acetylgestagens in kidney fat using the EuroProxima MPA ELISA test. A similar method is given for screening of food (e.g. beverages) and feed samples for acetylgestagens.

8.1 Kidney fat samples

Transfer approximately 25 g of kidney fat into a vial and heat at 50°C for approximately 1 hour until the fat is melted. Alternatively, the fat can be melted in a microwave for 3 minutes at 360 Watt. Transfer 0.5 g of the melted fat into a glass tube. Add 5.0 ml of cyclo-hexane. Mix for 30 seconds using a vortex. Conduct the solid phase extraction procedure as described in paragraph 8.5.

8.2 Muscle, meat and sausages

Homogenise approximately 25 g of muscle, meat or sausage sample. Transfer 1.0 g of the homogenised sample into a glass tube. Add 10.0 ml of cyclo-hexane. Mix for 30 seconds using a vortex. Use 5 ml of the clean cyclo-hexane extract for the solid phase extraction procedure as described in paragraph 8.5.

8.3 Feed samples

Homogenise approximately 25 g of feed sample. Transfer 1.0 g of the homogenised sample into a glass tube. Add 7.0 ml of 80% methanol in distilled water. Mix for 30 seconds using a vortex. Mix for another 10 minutes using a shaker. Add 4.0 ml of distilled water and mix again for 30 seconds using a vortex. Transfer 5.5 ml of the clean extract onto an activated C18 cartridge. Conduct the solid phase clean-up procedure as described in paragraph 8.6.

8.4 Beverages

Transfer 0.5 ml of a beverage sample into a glass tube. Extract the acetylgestagens from the sample with 2 times 2.5 ml of cyclo-hexane. Collect the cyclo-hexane phases into a new glass tube, evaporate to dryness at a temperature of approximately 40°C under a mild stream of nitrogen. Dissolve the dry residue in 5 ml 50% methanol in distilled water. Mix for 30 seconds using a vortex. Mix for another 3 minutes using an ultrasonic bath. Conduct the solid phase clean-up procedure as described in paragraph 8.6.

8.5 Primary solid phase extraction

Transfer the cyclo-hexane extracts from the kidney fat samples (paragraph 8.1) or from the muscle, meat or sausage samples (paragraph 8.2) onto a non-activated C18 cartridge (E.g. Bond-Elut C18, LRC 10 ml, 500 mg; Varian, art. Nr.: 1211-3027). Be aware the extracts flow through the cartridges with a speed of 0.5 ml per minute. Wash the cartridges using 3 ml cyclo-hexane. Dry the cartridges for 10 minutes under vacuum. Elute the acetylgestagens using 3.5 ml 80% methanol in distilled water. Add 2 ml of distilled water to the eluate and continue with the second solid phase clean-up procedure as described in paragraph 8.6.