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Immunochemical Method for Ochratoxin A

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1. Introduction

Ochratoxin A (7-L-β-phenylalanylcarbonyl-5-chloro-8-hydroxy-3,4-dihydro-3-R-methylisocoumarin, **Fig. 1**) was first isolated in 1965 from *A. ochraceus* ***(1)***. Meanwhile several *Aspergillus* and *Penicillium* species are known to produce ochratoxin A. The toxin frequently occurs as a contaminant in plant products worldwide, predominantly during storage. Numerous papers have described its occurrence in, for example, cereals, beans, coffee, grapes, beer, and red wine ***(2,3)***. In particular for *Penicillium* spp., cocontamination with ochratoxin A and other *Penicillium* toxins, such as citrinin, is quite common ***(4)***.

Ochratoxin A is a nephrotoxin and a potent renal carcinogen in rodents ***(2)***. It is known to cause mycotoxic porcine nephropathy (MPN) in swine ***(5)***, and is suspected to cause a human disease called "Balkan endemic nephropathy" (BEN) ***(6–10)***. However, no clear proof for the latter hypothesis has been presented so far. The oral acute toxicity (LD_{50}) of ochratoxin ranges from <6 mg/kg bw in female pigs to about 30 mg/kg bw in male rats, with female animals being slightly more sensitive than males ***(2,3)***.

Ochratoxin A binds to blood serum proteins and has a long elimination half-life time in most species ***(11)***. Therefore a carry-over of ochratoxin A via the food chain is possible, the most important source being porcine tissues (kidneys) and products containing porcine blood or serum.

Measurement of ochratoxin A serum levels in humans can be used to estimate the continuous daily intake of ochratoxin A via foods. Breitholtz et al. ***(12)*** made an estimate based on animal data ***(11)***, which gave the following relationship: Ochratoxin A intake (ng/kg bw/day) = 1.34 × ochratoxin A plasma

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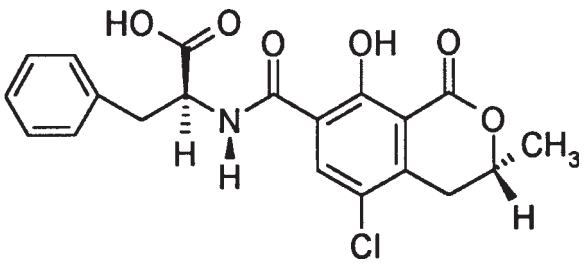

Fig. 1. Structural formula for ochratoxin A.

concentration (ng/mL). However, there are several uncertainties in this estimate, since elimination kinetics and bioavailability of ochratoxin A are not exactly known in humans ***(12)***. Nevertheless, since published data on ochratoxin A in human blood serum are fairly abundant ***(13–16)***, this can be used to compare the overall ochratoxin A intake between countries. As a matter of fact most studies found that 80–100% of all blood samples were ochratoxin positive, with marked regional differences in the mean toxin levels (from <0.5 ng/mL up to 10 ng/mL).

An estimate of the (provisional) tolerable daily intake (TDI) for ochratoxin A made in 1990 by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) resulted in a figure of 112 ng/kg bw per week, corresponding to 16 ng/kg bw per day ***(17)***. This value was based on nephrotoxicity and did not address carcinogenicity. Other calculations of the TDI for ochratoxin A came to figures ranging from 0.2 to 5.0 ng/kg bw per day ***(18)***. According to Scott et al. ***(15)***, the provisional TDI of ochratoxin A in Canada has been set at 3.7 ng/kg bw per day.

Only a few countries have set regulations for ochratoxin A in foods so far ***(19)***. Ochratoxin A in cereals is regulated in Austria, France (5 µg/kg), and in Switzerland (2 µg/kg). Denmark uses the ochratoxin A content in visibly damaged pig kidneys to decide over condemnation of the carcass (10–25 µg/kg). Within the European Union, tolerances for ochratoxin A in the range of 3–5 µg/kg have been under discussion for several years now.

Analytical methods for ochratoxin A are predominantly high-performance liquid chromatography (HPLC) techniques with fluorescence detection ***(20)***, in recent years increasingly employing immunoaffinity chromatography techniques as the extract cleanup method ***(21)***. Several enzyme immunoassay techniques for ochratoxin A, mostly designed as microtiter plate assays, have also been described ***(22,23)***. This chapter gives details about the production of polyclonal rabbit antibodies against ochratoxin A and the development of a

competitive direct enzyme immunoassay for ochratoxin A. This approach uses a mixed anhydride reaction ***(24)*** for immunogen synthesis, and an activated ester method ***(25)*** for production of the labeled antigen. The application of this assay for the determination of ochratoxin A in cereals and in porcine blood serum is described.

2. Materials

2.1. Antigens and Labeled Antigens

1. Ochratoxin A (Sigma-Aldrich Vertriebs GmbH, Deisenhofen, Germany).
2. Human serum albumin (HSA), molecular mass 6.8×10^4 (Sigma-Aldrich) (*see* **Note 1**).
3. Horseradish peroxidase (HRP), enzyme immunoassay grade, molecular mass 4×10^4, (Boehringer Mannheim, Mannheim, Germany).
4. Dimethylformamide (DMF).
5. Isobutyl chloroformate.
6. Tri-n-butylamine.
7. N-hydroxysuccinimide.
8. N,N'-dicyclohexylcarbodiimide.
9. 10 m*M* potassium phosphate, 100 m*M* sodium chloride, pH 7.2–7.3 ("phosphate-buffered saline," PBS).

2.2. Immunizations

1. Complete Freund's adjuvant (Sigma-Aldrich).
2. Sterile pyrogen-free water.

2.3. Assays

2.3.1. Antibody Titer Determination

1. 50 m*M* sodium carbonate buffer, pH 9.6
2. Antirabbit IgG from sheep, affinity-purified (Sigma-Aldrich).
3. Wash solution (8.5 g/L sodium chloride, Tween-20 250 µL/L).
4. Ochratoxin A stock solution in methanol, approx 10 µg/mL (*see* **Note 2**). Stored at –18°C. This stock solution is stable for 2–3 mo. From this stock solution, a dilution with a concentration of 1 ng/mL is prepared with 0.13 *M* $NaHCO_3$ for specific titer determination. Store at 4–8°C for 1–2 wk.
5. Enzyme substrate/chromogen solution ***(26)***:
   a. 65 µL hydrogen peroxide (30% solution) in 200 mL 200 m*M* potassium citrate buffer, pH 3.9. Store at room temperature, protected from light.
   b. 50.4 mg 3,3',5,5'-tetramethylbenzidine in 1 mL acetone plus 9 mL methanol. Store at room temperature, protected from light. Mix 0.5 mL of b) with 10 mL of a) shortly before use.
6. 1 *M* sulfuric acid.

2.3.2. Competitive Direct Enzyme Immunoassay

1. 50 m*M* sodium carbonate, pH 9.6.
2. 0.13 *M* sodium hydrogencarbonate, pH 8.3–8.5.
3. Wash solution (8.5 g/L sodium chloride, Tween-20 250 µL/L).
4. Ochratoxin A stock solution in methanol, approximately 10 µg/mL (*see* **Note 2**). Stored at –18°C, this stock solution is stable for 2–3 mo. This stock solution is diluted with 0.13 *M* $NaHCO_3$ for assay to give final standard concentrations of 16.5 pg/mL to 5000 pg/mL. Store at 4–8°C for 1–2 wk.
5. Enzyme substrate/chromogen solution ***(26)***:
   a. 65 µL hydrogen peroxide (30% solution) in 200 mL 200 m*M* potassium citrate, pH 3.9. Store at room temperature, protected from light.
   b. 50.4 mg 3,3',5,5'-tetramethylbenzidine in 1 mL acetone plus 9 mL methanol. Store at room temperature, protected from light. Mix 0.5 mL of b) with 10 mL of a) shortly before use.
6. 1 *M* sulfuric acid.

3. Methods

3.1. Preparation of Antigens

3.1.1. Preparation of HSA-Ochratoxin A Conjugate

1. Dissolve 5.0 mg ochratoxin A in 2.0 mL DMF in a 10 mL glass vial, add a clean stirring magnet, close vial and cool to 0°C in an ice-water bath.
2. Add 3 µL tri-n-butylamine, and cool down to –10 to –15°C by adding ethanol at a temperature of –18°C to the water bath (*see* **Note 3**).
3. Meanwhile, dissolve 7.5 mg HSA with 2 mL distilled water in a 10 mL glass vial, add 1.5 mL DMF, add a clean stirring magnet, close vial, place in the ethanol-water bath, and let cool down to –10 to –15°C.
4. Add 2 µL isobutyl chloroformate to ochratoxin A solution and stir at –10 to –15°C for 5 min.
5. Using a Pasteur pipet, transfer the ochratoxin A solution into the HSA solution.
6. Incubate conjugation mixture for 1 h at –10 to –15°C under constant stirring. Control pH of the mixture and adjust to approx pH 9 by adding 10–20 µL portions of 0.1 *M* NaOH.
7. Incubate mixture for another 3 h, allowing the temperature of the ethanol-water bath to increase to approx 0°C within the first hour, and maintain at 0 ± 2°C for the next 2 h. Control and adjust pH as above.
8. Add 10 mg $NaHCO_3$ to conjugation mixture.
9. Dialyze the mixture at 4–8°C for 3 d against 5 L of PBS, changing the PBS daily.
10. Determine the protein content of the conjugate by the method of Lowry et al. ***(27)***.
11. Check spectrum of conjugate photometrically for the presence of conjugated ochratoxin A at a wavelength range from 200 nm to 500 nm, using spectra of nonconjugated HSA and ochratoxin A, respectively, for comparison. Conjugation ratio (molecules of ochratoxin A per molecule HSA) may be determined

by using the absorbance at >340 nm of the conjugate solution and calculation from ochratoxin A standard curve in PBS recorded at the same wavelength (*see* **Note 4**).

3.1.2. Preparation of HRP-Ochratoxin A Conjugate

1. Dissolve 5 mg ochratoxin A in 0.5 mL DMF.
2. Dissolve 7.1 mg N-hydroxysuccinimide in 0.25 mL DMF and add to ochratoxin A solution.
3. Dissolve 25.5 mg dicyclohexylcarbodiimide in 0.25 mL DMF and add to ochratoxin A solution.
4. Incubate mixture under slow magnetic stirring at ambient temperature protected from light for 16–20 h (*see* **Note 5**).
5. Dissolve 50 mg HRP in 9 mL 0.13 *M* $NaHCO_3$ solution.
6. Add ochratoxin A reaction mixture dropwise to HRP solution under constant stirring.
7. Incubate at room temperature for 2 h (magnetic stirring at slow speed).
8. Dialyze the mixture at 4°C for 3 d against 5 L of PBS, changing the PBS daily.
9. Dilute an aliquot of the conjugate 1:10 with PBS and record UV spectrum of the conjugate from 200 nm to 500 nm (*see* **Note 6**).
10. Store conjugate in small portions of 0.2 to 0.5 mL at –18°C or lyophilize and store at –18°C (*see* **Note 7**).

3.2. Antibody Production

3.2.1. Immunization of Rabbits

1. Adjust the ochratoxin A-HSA conjugate to a protein concentration of 0.6 mg/mL with sterile distilled water, mix for 30 s on a wrist-action shaker and emulsify per animal 0.5 mL of the conjugate with 1.5 mL of Freund's complete adjuvant (*see* **Note 8**).
2. Inject three or four rabbits (female Chinchilla bastard, 16–20-wk-old) each with 2.0 mL of the mixture intradermally at 20 to 30 sites on shaved backs.
3. Collect blood from the *Arteria auricularis magna,* starting 4 wk after primary injection, and continue to collect blood (20–40 mL per collection) every two weeks over a period of 6–10 mo, depending on the total amount of antiserum required.
4. Depending on the results of the antibody titer determinations (*see* **Subheading 3.2.2.**), perform one or two booster injections using the same amount and composition of immunogen. Either subcutaneous or intramuscular injections (2–4 injection sites) may be given (*see* **Note 9**).

3.2.2. Antibody Titer Determination and Sensitivity Check

1. Dissolve antirabbit IgG in 50 m*M* sodium carbonate, pH 9.6, to give a 10 µg/mL solution. Coat microtiter plate using this solution, 100 µL per well, and incubate overnight in a chamber with >90% relative humidity.
2. Remove the antirabbit IgG solution.

3. Wash each plate with 8.5 g/L sodium chloride containing Tween-20 250 µL/L.
4. Individually prepare serial dilutions (2^n–3^n) in 0.13 *M* $NaHCO_3$ solution of antisera of all rabbits immunized, starting at a 1:100 dilution. Prepare at least 8 dilutions. Always compare antisera with those obtained from the collection before to check whether the titer increases or not.
5. Prepare ochratoxin A standard solution in containing 0.13 *M* $NaHCO_3$ solution at a concentration of 1 ng/mL.
6. Prepare HRP-ochratoxin A solution, diluted 1:10,000 with 0.13 *M* $NaHCO_3$ containing 1% Tween-20.
7. To one half of the microtiter plate, add 35 µL per well ochratoxin A standard solution (positive control); to the other half of the plate, add 35 µL per well 0.13 *M* $NaHCO_3$ solution (negative control).
8. Add 35 µL per well of diluted antiochratoxin A antisera to both the toxin-positive and the toxin-negative half of the microtiter plate, resulting in a positive and a negative control well for each individual antiserum dilution.
9. Add 35 µL per well HRP-ochratoxin A conjugate solution to all wells.
10. Incubate for 2 h at room temperature (*see* **Note 10**).
11. Wash each plate with 8.5 g/L sodium chloride containing Tween-20 250 µL/L.
12. Add 100 µL of enzyme substrate/chromogen solution per well, and incubate for 15 min at room temperature.
13. Add 1 *M* sulfuric acid (100 µL per well) and measure absorbance. On the enzyme immunoassay (EIA) microtiter plate reader (AT400, SLT Labinstruments, Austria, or equivalent) set sample wavelength to 450 nm and reference wavelength to 620 nm.
14. Select antisera which give absorbance values for toxin-negative wells of >1.0 units at high dilutions (usually >1:10,000) and a maximum absorbance for the respective toxin-positive well of <50% of the corresponding toxin-negative well (maximum sensitivity).
15. Individually make checkerboard tritations of the selected antisera versus the HRP-ochratoxin A conjugate, with and without addition of ochratoxin A standard solution, to estimate optimum antiserum dilution and conjugate dilution (*see* **Note 11**).
16. Pool antisera of similar quality to provide a large antiserum stock.
17. Mix an aliquot of antiserum pool with the same volume of 70% saturated ammonium sulfate solution and let stand at room temperature for 4 h.
18. Centrifuge mixture (1500*g*, 15 min, 4°C) and remove supernatant.
19. Reconstitute residue (the immunoglobulin fraction) with PBS to the original antiserum volume, add equal volume of 70% saturated ammonium sulfate solution and incubate at room temperature for 5 min.
20. Repeat **steps 18**, **19**, and **18**.
21. Reconstitute residue with PBS to original antiserum volume and dialyze for three days against 5 L PBS, changing the PBS daily.
22. Store the dialyzed antiserum at –18°C (*see* **Note 12**).

23. Perform checkerboard titrations of antiserum coating versus HRP-ochratoxin A conjugate dilution (with and without standard addition) under the conditions of the competitive direct enzyme immunoassay (*see* **Subheading 3.3.2.**) to determine optimum antiserum dilution for microtiter plate coating and for conjugate dilution.

3.3. Assays

3.3.1. Sample Preparation

3.3.1.1. Cereal Samples

1. Grind grain to pass through 1-mm apertures.
2. Add test sample (2 g) and 5 mL 1 *M* HCl in a 40 mL test tube and mix for 5 min on a magnetic stirrer at full speed.
3. Add 10 mL of dichloromethane and mix at full speed for another 15 min.
4. Centrifuge the mixture (1500*g*, 15 min, 4°C).
5. Remove the upper aqueous layer with a Pasteur pipet and transfer the dichloromethane phase into another 40 mL test tube.
6. Add 10 mL 0.13 *M* $NaHCO_3$ solution to the dichloromethane phase and mix at full speed for 15 min.
7. Centrifuge the mixture again.
8. Remove upper aqueous layer for EIA analysis. If necessary (for high ochratoxin A concentrations) make further dilutions in 0.13 *M* $NaHCO_3$ solution.
9. Recovery checks of the sample extraction procedure in a concentration range from 2.5–10 ng/g should be performed on each day of analysis by adding 25–100 µL methanolic ochratoxin A standard solution to 2-gram portions of toxin-negative sample matrix under analysis and allowing the solvent to evaporate for at least 30 min (*see* **Note 13**). If available, an in-house check-sample, naturally contaminated with ochratoxin A, should also be extracted and analyzed on each day of analysis.

3.3.1.2. Porcine Blood Serum Samples

1. Centrifuge 4–5 mL blood sample at 1200–1500*g* for 10–15 min to separate serum.
2. Pipet 2 mL blood serum in a 20 mL test tube.
3. Add 2.5 mL 1 *M* HCl.
4. Add 4 mL dichloromethane.
5. Mix by magnetic stirring at high speed for 5 min.
6. Centrifuge at 1200–1500*g* for 15 min.
7. Remove upper aqueous phase.
8. Add 3 mL 0.13 *M* $NaHCO_3$.
9. Mix for 30 s (wrist-action shaker).
10. Centrifuge at 1200–1500*g* for 15 min.
11. Collect upper aqueous phase and repeat **steps 8–10**.
12. Mix both aqueous phases in a test tube.

13. Add 1 mL 1 *M* HCl.
14. Add 3 mL dichloromethane.
15. Mix for 30 s (wrist-action shaker).
16. Collect lower dichloromethane phase.
17. Evaporate dichloromethane in a rotary evaporator.
18. Redissolve residue with 2 mL 0.13 *M* $NaHCO_3$ for assay.
19. If necessary, dilute further with 0.13 *M* $NaHCO_3$.
20. Routinely check recovery of this procedure in a concentration range of 0.5–5.0 ng/mL by adding 10–20 µL ochratoxin A standard solution to 5 mL blood serum before extraction (*see* **Note 14**).

3.3.2. Competitive Direct Enzyme Immunoassay

1. Dilute ammonium sulfate-purified antiserum against ochratoxin in 50 m*M* sodium carbonate, pH 9.6 (usually a 1:2000 dilution will work). Coat microtiter plates using this solution, 100 µL per well, and incubate overnight in a chamber with >90% relative humidity.
2. Remove the antiserum solution.
3. Wash each plate with 8.5 g/L sodium chloride containing Tween-20 250 µL/L.
4. By dilution of the ochratoxin A stock solution, prepare seven ochratoxin A standard concentrations in 0.13 *M* $NaHCO_3$ solution, covering a concentration range from 5000 pg/mL to 16.5 pg/mL, including a zero standard (blank).
5. Prepare HRP-ochratoxin A solution (usually a 1:100,000 dilution will work) in 0.13 *M* $NaHCO_3$ solution containing 1% Tween-20.
6. Add 50 µL per well of standard or sample extract solution. Perform at least duplicate analyses of all standards and extracts, fourfold determination should be used if possible.
7. Add 50 µL per well HRP-ochratoxin A conjugate solution to all wells.
8. Incubate for 2 h at room temperature (*see* **Note 10**).
9. Wash each plate with 8.5 g/L sodium chloride containing Tween-20 250 µL/L.
10. Add 100 µL of enzyme substrate/chromogen solution per well, and incubate for 15 min at room temperature.
11. Add 1 *M* sulfuric acid (100 µL per well) and measure absorbance. On the EIA microtiter plate reader (AT400, SLT Labinstruments, or equivalent), set sample wavelength to 450 nm and reference wavelength to 620 nm.
12. If available, evaluate results with immunoassay software capable to calculate competitive immunoassays. Otherwise, calculate mean absorbance for each standard concentration and for all sample extracts. Set absorbance of zero standard (B_0) as 100% and transform absolute absorbance of ochratoxin A standards (B) by using the formula $B/B_0 \times 100$ (relative absorbance). On a semilogarithmic paper, plot concentration versus relative absorbance values. Construct calibration curve by point-to-point interpolation of standards (**Fig. 2**) to determine ochratoxin A concentration of sample extracts. Multiply results with the respective sample extract dilution factors to calculate the ochratoxin A concentrations of sample materials. Do not use the high concentration range corresponding to <20%

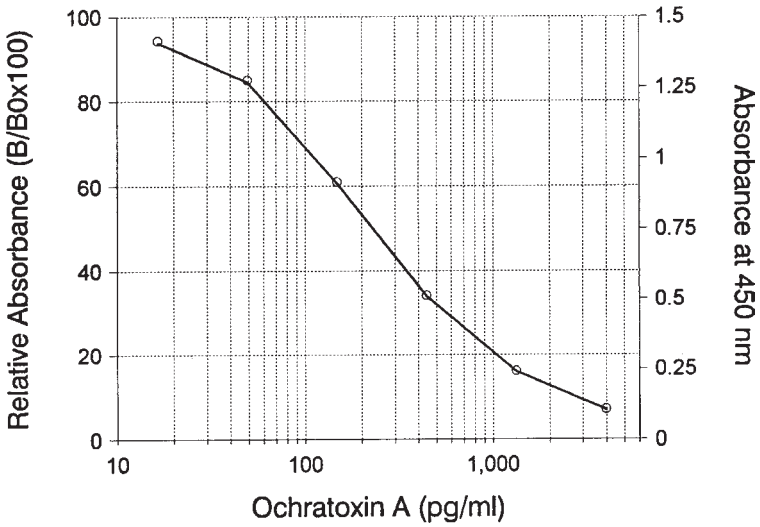

Fig. 2. Standard curve for competitive direct EIA of ochratoxin A with rabbit antiserum and HRP-ochratoxin A conjugate. The left *y*-axis indicates the relative absorbance values ($B/B_0 \times 100$), the right *y*-axis gives the absolute absorbance values (B) measured at 450 nm. Absorbance of the negative control (B_0) was 1.49 units. All standards were performed in quadruplicate. Intraassay coefficients of variation were between 1.5% and 6%. After evaluation of 40 standard curves performed over a period of 2 mo, the 50% inhibition concentration typically was at 250 ± 50 pg/mL, with a standard curve detection limit of 80 ± 30 pg/mL (determined by students T test, one-sided, $n = 4$).

B/B_0 for quantification, but reanalyze using a higher dilution of the extract. Set detection limit of the standard curve to a concentration corresponding to 80% B/B_0; absorbance measurements higher than that should be reported as "not detectable."

13. To check antibody specificity, perform for all available ochratoxin A analogs (for example, ochratoxin B) standard curves under the conditions of the competitive direct enzyme immunoassay, using maximum toxin analogue concentration of at least ten times higher than that routinely used for ochratoxin A. Determine 50% B/B_0 concentrations of the standard curves established for cross-reacting compound and calculate relative cross- reactivities using the formula: "Relative cross-reactivity (%) = 50% B/B_0 concentration of ochratoxin A/50% B/B_0 concentration of test compound × 100". For example, relative cross-reactivity of ochratoxin B was found to be 2%.

4. Notes

1. Instead of HSA, bovine serum albumin (BSA) may also be used with the same reaction protocol.

2. A full UV spectrum should be recorded from 200 to 500 nm to check for impurities. Ochratoxin A in methanol has maximum absorbance at 215 nm and 333 nm, respectively. Concentration should be determined at 333 nm using a molar extinction coefficient of ε =6400 and a molecular mass for ochratoxin A of 403 ***(28)***.
3. This may be a little bit difficult to perform in some labs. It is recommended to put a small (30 × 20 × 5 cm) watertight plastic or metal box on a magnetic stirrer, add ice and a small volume of water, and then add ethanol stored in a –18°C freezer until the required temperature is reached. As the liquid becomes warmer, replace an aliquot with –18°C ethanol from the freezer. In this makeshift waterbath all reaction vials can be placed conveniently.
4. At >340 nm there is no interfering UV absorbance by HSA, i.e., the absorbance measured at this wavelength is only due to ochratoxin A. At the maximum absorbance of ochratoxin A (333 nm) there would be some interference by the 280 nm absorbance maximum of HSA.
5. Precipitates may be visible in the reaction vial, indicating formation of the activated ester intermediate. No attempt to remove these precipitates is required for successful conjugation.
6. Since the absorbance of ochratoxin A interferes with the absorbance maximum of HRP at 403 nm, determination of the conjugation ratio or of the HRP concentration is not possible. However, comparing the spectrum with that of unconjugated HRP and of ochratoxin A solution can be used to qualitatively confirm the success of the conjugation.
7. The HRP-ochratoxin A conjugate is stable, without addition of any additives, at least 5 years when stored at –18°C. Lyophilization may increase shelf-life but also decreases specific activity and is only recommended for longer transport purposes. Repeated (>10 times) thawing and freezing should be avoided. A conjugate predilution of 1:100 in PBS can be stored at 4–8°C for at least 2 wk without loss of specific activity. A typical final working dilution of the conjugate (in 0.13 *M* $NaHCO_3$ solution containing 1% Tween-20) ready for enzyme immunoassay is in the range of 1:100,000 but has to be determined for each conjugate batch prepared.
8. It is essential to prepare a stable emulsion to ensure slow release of the immunogen. It is recommended to mix aqueous phase and oil phase in two syringes of suitable size (5–10 mL) connected by a 40 mm × 0.5 mm id connector with luer fittings on both ends. Mix at least 25 times, starting by as vigorously as possible pressing the aqueous phase into the oil phase. Read also an excellent description of immunogen preparation given by Hurn and Chantler ***(29)***.
9. It is strongly recommended not to follow a predesigned immunization schedule but make booster injections in dependence of the individual immune response. As long as the antibody titer increases, no booster should be given. The first booster may be required any time between weeks 8 and 16 after the primary immunization. Although a terminal bleeding may be taken by cardiac puncture as soon as the antibody titer and the sensitivity are both sufficiently high, it is rec-

Table 1
Recovery of Ochratoxin from Various Cereals at Spiking Levels of 2.5–10 μg/kg

	Ochratoxin A recovery		
Sample type	Mean, %	RSD, %	*n*
Wheat	60	6.3	9
Corn	75	28	14
Barley	69	32	9
Oats	28	6.2	6
Wheat bran	51	18	6

ommended to collect blood samples of 20–40 mL every two weeks. Within 6–8 mo 150–200 mL serum can be harvested, an amount which should be sufficient for most purposes. Stored at –18°C, the antiserum is stable at least 15 yr (probably much longer than that) without any preservatives added.

10. If desired, the incubation time can be reduced as much as a few minutes; however, the concentrations of the antiserum and the HRP-ochratoxin A conjugate have to be increased which results in a lower test sensitivity.
11. If only a small quantity of serum (<10 mL) is needed, the antiserum comparison checks may be not necessary. If there is continuous need for serum however, it is preferable to have a larger stock of the same quality. But never pool any antisera unless you are quite sure that you are doing the right thing! Better to repeat some titrations than to inadvertently reduce the quality of the final product. It may be wise to prepare a small test pool of each 100–200 μL of the sera which are supposed to be pooled and check this test pool under the conditions of the enzyme immunoassay.
12. The purified antiserum is stable for at least 10 yr without any preservatives added. However, thawing and freezing the same vial more than a hundred times may have a deleterious effect. Antiserum should be stored in portions of about 0.5 mL.
13. Recovery may vary with the type of cereal analyzed, and therefore should be checked carefully. Sample matrix may affect extraction of ochratoxin A, further losses could occur during the liquid-liquid partitioning steps (HCl:dichloromethane:$NaHCO_3$). The data presented in **Table 1** were obtained by one technician within a period of six weeks. It should be acknowledged that another factor determining the repeatability of the method is the skillfulness of the person doing these extractions. For example, in a different series of analyses, another technician obtained recoveries for ochratoxin A from spiked wheat and barley of 67–78% and 84–97%, respectively, with relative standard deviations of 7.7% to 18%. In the same analytical series, relative standard deviations obtained after repeated analysis of naturally contaminated barley (ochratoxin A content: 2–57 ng/g) were 11–22%.

Table 2
Recovery of Ochratoxin from Spiked Porcine Blood Serum

	Ochratoxin A recovery		
Ochratoxin added (ng/mL)	Mean, %	RSD, %	*n*
0.5	71.6	19.8	6
1	67.5	12.1	6
2.5	82.5	14.4	6
5	68.7	10.9	6

14. Recovery data for ochratoxin A from porcine blood serum are listed in **Table 2**. The detection limit for ochratoxin A in porcine blood serum was at 0.05–0.1 ng/mL. It may be difficult to find control sera containing ochratoxin A at less than 0.1 ng/mL.

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