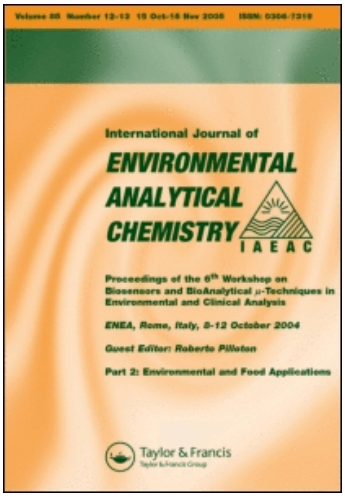

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Development of an Enzyme Immunoassay for the Determination of the Herbicide Metsulfuron-Methyl Based on Chicken Egg Yolk Antibodies

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DEVELOPMENT OF AN ENZYME IMMUNOASSAY FOR THE DETERMINATION OF THE HERBICIDE METSULFURON-METHYL BASED ON CHICKEN EGG YOLK ANTIBODIES

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The development of an indirect competitive enzyme immunoassay for the sulfonylurea herbicide metsulfuron-methyl (MSM) is described. In contrast to traditional antibody generation in mammals, this extremely sensitive method is based on chicken egg yolk antibodies (IgY). They were raised in laying hens using an MSM-derivative-BSA hapten as immunogen. With a 1:10000 dilution of the antibody solution and a coating antigen (MSM-derivative-KLH) concentration of 10 $\mu g\ L^{-1}$ the IC_{50} value achieved for the target analyte was 0.4 $\mu g\ L^{-1}$. The least detectable dose was established at 13 $ng\ L^{-1}$. Cross-reactivity was tested with 5 structurally related compounds, where only sulfometuron showed a significant binding. The ELISA was tested with spiked tap and surface water samples. This paper, for the first time, demonstrates the production of high-affinity IgY antibodies for a herbicide compound.

Keywords: ELISA; egg yolk antibodies; herbicide; metsulfuron-methyl; IgY

INTRODUCTION

Metsulfuron-methyl (2-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]benzoic acid methyl ester) belongs to the group of sulfonylurea herbicides and is used to control broad-leafed weeds and other grasses in cereals at an application rate of less than 0.1 $kg\ ha^{-1}$. This low level requires sen-

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sitive and selective analysis methods. This is usually performed by traditional analytical methods such as gas chromatography (GC), reversed-phase high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) or bioassays.

The great advantage of immunological techniques is their sensitivity, thus not requiring cumbersome preconcentration steps unlike the classical techniques. They represent an inexpensive analytical method for the rapid monitoring of a high number of samples. Many enzyme-linked immunosorbent assays (ELISA) have been developed for pesticides, some of them also for sulfonylurea herbicides. All were based on polyclonal mammalian antibodies from rabbits [1,2] or sheep [3,4] or on monoclonal antibodies from mice [5]. It has been known for more than 100 years, that chickens accumulate large amounts of antibodies in their egg yolks to ensure a primary immunity of their hatch. But only in the early 80ies after Polson's development of a simple and efficient antibody isolation [6], scientists started to use this approach for specific antibody generation. This alternative method is especially used in medical research, as mammals, due to the low phylogenetic distance are normally not very well suited to raise antibodies against human proteins whereas chickens have the ability to recognize the proteins as foreign. In most cases, chickens respond to the immunization with the production of a vast amount of antibodies. Additionally, chickens do not show any adverse effects to the adjuvant, that is essential for obtaining a good immune response. Rabbits are known to develop painful inflammations and rashes at the immunization site, particularly if Freund's complete adjuvant is repeatedly applied [7]. Furthermore, chickens do not have to be bled frequently to test the titer and for the final antibody isolation. These considerate and animal-friendly characteristics of the technique represent an alternative method for antibody production. The importance of the IgY (immunoglobulin of yolk) technique for ethical and responsible use of animal experiments was emphasized at a special conference organized by the European Center for Validation of Alternative Methods (ECVAM) in 1996 [8]. But, until now the use of egg yolk antibodies is only widely spread in medical research [9]. Apart from ELISAs for the mycotoxins ochratoxin A [10], aflatoxin [11], T2-toxin [12], zearalenone [13] and deoxynivalenol [14] the application in environmental trace analysis is limited. So far, only a single herbicide ELISA has been developed on the basis of egg yolk antibodies [15]. In that paper, the authors compared mecoprop-antibodies which were raised either in rabbits or chickens in regard to specificity and affinity. They found that the IgY were less specific and about one order of magnitude less sensitive than the mammalian antibodies. Unfortunately, no details on the immunization protocol were given in that study, as our experience showed, that great care has to be taken in the optimization of the immunization strategy. In the present paper, the

development of an indirect competitive ELISA for metsulfuron-methyl (MSM) based on chicken antibodies is described.

EXPERIMENTAL

Materials

Both the ELISA washer (Columbus) and the reader (SLT Spectra Reader) were purchased from Tecan SLT Laboratory Instruments (Grödig, Austria). The latter was controlled by the Biolise 2.0 for Windows software (SLT). Parafilm "M" American National Can Brand (Mueller-Scherr, Vienna, Austria) was used to cover 96 well flat bottom high binding certified microtiter plates (MTP) from Costar (Szabo Scandic, Vienna).

All sulfonylurea compounds (metsulfuron-methyl 97.6%, amidosulfuron 99.1%, chlorsulfuron 99.5%, cinosulfuron 98.0%, sulfometuron-methyl 99.5% and triasulfuron 99.5%) were obtained from Dr. Ehrenstorfer (Augsburg, Germany).

Perhydrol, skim milk powder and all salts were supplied by Merck (Vienna). Sodium hydroxide 50% solution and organic solvents (HPLC Gradient Grade) were purchased from Mallinckrodt-Baker (Vienna). All water used was of Milli-Q Plus quality (Millipore, Vienna). Tetramethylbenzidine was obtained from Roche Diagnostics (Vienna). Tween 20 and both Freund's complete and incomplete adjuvant as well as poly(ethylene glycol) Av. Mol. Wt. 8000 and Trinitrobenzensulfonic acid (TNBS) were obtained from Sigma (Vienna). Peroxidase-conjugated AffiniPure Rabbit Anti-Chicken IgY* (IgG)(H+L) was from Margaritella (Vienna).

Standards and buffers

Standards of MSM were prepared both in ultrapure water, 0.01 M PBS and pure methanol, respectively. Stock solutions were prepared by dissolving 1 mg of the herbicide in 1 L of water and buffer each, or 10 mg MSM in 100 mL of methanol. Standards of amidosulfuron, chlorsulfuron, cinosulfuron, sulfometuron-methyl and triasulfuron were equally prepared and diluted with water to yield concentrations between 1000 and 10^{-4} $\mu g\ L^{-1}$.

The following buffers were used:

1. Coating buffer, pH 9.6, prepared by dissolving 1.22 g Na_2CO_3, 3.25 g $NaHCO_3$, 0.10 g NaN_3 in 1 L of water.

2. Phosphate-buffered saline (PBS), 0.2 M stock solution, pH 7.5, 32.22 g Na_2HPO_4, 2.62 g NaH_2PO_4 and 21.18 g NaCl dissolved in 1 L of water.
3. Blocking buffer, pH 7.5, 0.05 M PBS, 1% (w/v) milk powder.
4. Washing buffer conc., pH 7.5, 32.22 g Na_2HPO_4, 2.62 g NaH_2PO_4, 21.18 g NaCl and 800 µL Tween 20, dissolved in 1 L of water; for use it was further diluted 1:4 with water.
5. Substrate buffer, pH 4.0, prepared by dissolving 42 g citric acid and 100 mg sorbic acid potassium salt in 1 L of water. The pH was adjusted with 50% sodium hydroxide solution.
6. Tetramethylbenzidine (TMB) stock solution, 375 mg TMB, 5 mL dimethylsulfoxide, 25 mL methanol.
7. Substrate solution for one MTP: 25 mL substrate buffer, 5 µL 30% H_2O_2 and 200 µL TMB stock solution.

Immunization and antibody isolation

For each immunization one Derco brown laying hen was used. The preparation of the hapten-protein conjugates is described elsewhere [1]. The MSM molecule and the hapten structure are shown in Figure 1. About 18% of the available amino groups of the protein were conjugated in the hapten-KLH conjugate and 56% in the hapten-BSA conjugate, as was determined by the TNBS method. Both hapten-protein conjugates were used for immunization and coating of MTPs as well. First, chickens were immunized with the hapten-KLH conjugate. Applied concentrations of the immunogen in PBS were 1 mg mL^{-1} and 0.1 mg mL^{-1}, respectively. 250 µL of the immunogen solution were emulsified with 250 µL Freund's complete adjuvant and injected into the musculus pectoralis of the chicken. At intervals of 6 weeks the hens were boosted with an emulsion of 250 µL immunogen solution and an equal volume of Freund's incomplete adjuvant. Subsequently, immunization of other chickens with MSM-BSA was performed at concentrations of 0.5 mg mL^{-1}, 0.125 mg mL^{-1} and 0.05 mg mL^{-1}, respectively. The immunogen solutions were emulsified with Freund's complete adjuvant or incomplete adjuvant, as described above. Additionally to the change of immunogen, immunization intervals were increased. Chickens were immunized 10, 16 and 31 weeks after priming. Antibodies were isolated after the 2^{nd} booster injection. The laying hens did not show any adverse effects such as the formation of granuloma at the inoculation sites.

The antibody fraction was isolated from egg yolk in sufficient purity by poly(ethylene glycol) (PEG) precipitation based on a modification of Polson's method [6]: The eggs were usually used within two to three weeks after their collection, but they sustain longer storage periods at 4 °C. The egg yolks were sepa-

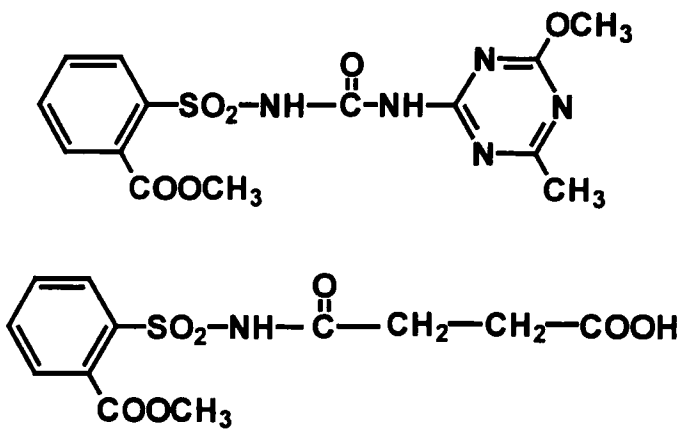

FIGURE 1 Metsulfuron-methyl (above) and the hapten used for immunization (below)

rated from the whites and washed with distilled water to remove any residues. The perivitellin membrane was removed and the yolk was mixed with an equivalent volume of buffer. After stirring for 30 min, 3.5% (w/v) PEG were added. The mixture was stirred and shaken for 15 min and then centrifugated at 15300 × g at 10 °C for 15 min. The supernatant was filtered over several layers of gauze tissue and stored at −70 °C, whereas the precipitate was discarded. Aliquots of 500 µL were mixed with an equal volume of glycerol and stored at − 20°C. Stored under these conditions, they easily sustain frequent thawing and freezing.

The method originally introduced by Polson includes two further precipitation steps with the addition of 12.5% (w/v) PEG to precipitate the proteins including the antibodies. A selected antibody batch was cleaned up according to Polson's instructions.

The antibodies were screened for their sensitivity by checkerboard titration, optimizing the coating antigen concentration using both MSM-BSA as well as MSM-KLH.

Subsequently, concentrations of IgY and secondary antibody (rabbit anti-IgY IgG conjugated to horseradish peroxidase) as well as used buffers were optimized.

ELISA procedure

The assay using IgY that was raised against MSM-BSA was performed as described below. The MTPs were washed with Milli-Q water with an automatic

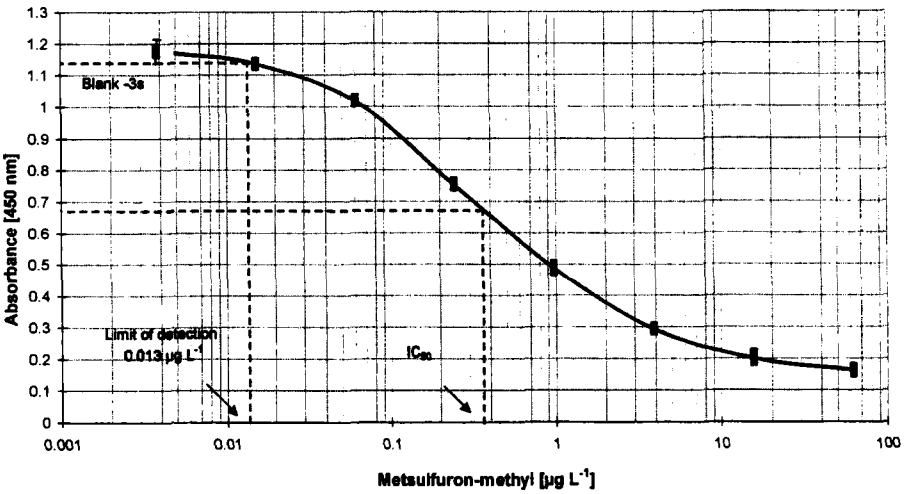

FIGURE 2 Standard curve for MSM

plate washer. Then the plates were coated with 200 µL/well of coating antigen (MSM-KLH) in coating buffer (10 µg L^{-1}) and left to incubate overnight at 4 °C. After washing with washing buffer all sites in the wells not occupied by the coating antigen were blocked for one hour at 37 °C with 300 µL blocking buffer. After another washing step the plates were stored at – 20°C until further use.

150 µL sample or standard and 50 µL of IgY solution (1:10000 in 0.05 M PBS, 0.1% (v/v) Tween) were added to each well and incubated for one hour at room temperature with agitation. This results in a dilution of the IgY of 1:80000 in the wells in respect to the supernatant gained from the antibody isolation. The plates were washed and 200 µL of rabbit anti-IgY IgG conjugated to horseradish peroxidase (1:2500 in 0.05 M PBS, 0.1% (v/v) Tween, 0.1% (w/v) BSA) was added to the wells. After incubating for one hour the plates were washed again. 200 µL of the substrate solution was added to each well and incubated for 15 min at ambient temperature with agitation. Finally, the enzyme reaction was stopped by adding 50 µL of 1 M sulfuric acid to each well. The absorbance was measured at 450 nm with an ELISA reader. All measurements were performed in triplicate.

The assay using IgY that was raised against MSM-KLH employed the following parameters:

Coating: 10 ng mL^{-1} MSM-BSA; IgY dilution: 1:100000; rabbit anti-IgY IgG conjugated to horseradish peroxidase concentration: 1:5000; substrate incubation time: 15 min. Apart from these alterations the ELISA was performed equally as described above.

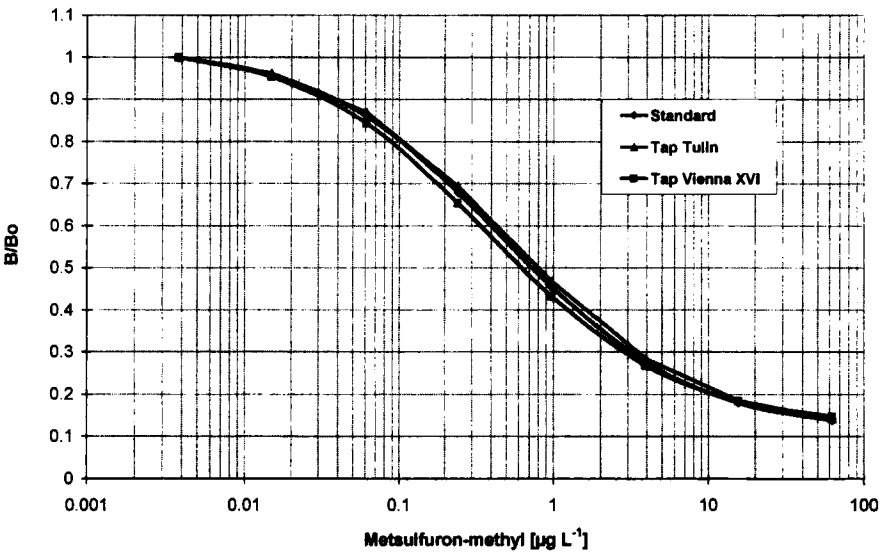

FIGURE 3 Comparison of dose-response curves for MSM calibrators obtained with spiked tap water samples

Cross- reactivity

The specificity of the MSM-IgY was tested with 5 structure-related sulfonylurea compounds. The standard solutions of amidosulfuron, chlorsulfuron, cinosulfuron, sulfometuron-methyl and triasulfuron were prepared in pure methanol and then diluted with ultrapure water to concentrations between 1000 and 10^{-3} µg L^{-1}. The ELISA was performed as described above.

Spiking experiments

Two different tap waters from Vienna (pH 7.95; conductivity 233 µS cm^{-1}) and Tulln (pH 7.15; conductivity 1329 µS cm^{-1}), and two different surface waters from the Kleine Tulln (pH 7.90; conductivity 750 µS cm^{-1}) and the Fischabach (pH 7.97; conductivity 703 µS cm^{-1}), two rural brooks in Lower Austria, were subjected to analysis. Sampling was performed with brown glass containers after rinsing the flasks for several times. The samples were stored at 4 °C in the dark. They were fortified at concentrations between 1000 and 10^{-4} µg L^{-1} and analyzed following the ELISA procedure described above.

RESULTS AND DISCUSSION

Chickens immunized with the MSM-derivative-KLH conjugate yielded a huge amount of antibodies, requiring dilutions of 1:200000, but they were of little value for the ELISA because they had a very poor affinity (IC_{50} ~ 200 μg L^{-1}) for both immunogen concentrations (1 mg mL^{-1} and 0.1 mg mL^{-1}). Subsequently, other chickens were immunized with the MSM-derivative-BSA conjugate. Screening of the antibodies showed, that their quality improved with decreasing immunogen concentration. The IC_{50} of antibodies gained with the highest immunogen concentration of 0.5 mg mL^{-1} was in the range of 500 μg L^{-1} and therefore, not usable for the development of a sensitive ELISA. A reduction of the immunogen concentration to 0.125 mg mL^{-1} lead to a decrease of the IC_{50} down to the range of 20 μg L^{-1}. This showed, that quartering the immunogen concentration improved the sensitivity about 25-fold. The third immunization, as described in the experimental part, was performed using an immunogen concentration that was as low as 0.05 mg mL^{-1}, corresponding to a net amount of 12.5 μg immunogen. Antibodies isolated after the third booster injection were used in the ELISA and showed an even better IC_{50}in the range of 0.4 μg L^{-1}. The standard inhibition curve is shown in Figure 2. Antibodies isolated from egg yolks usually undergo some kind of maturing process with the time after the immunization. This can also be observed in this case, but the improvements are not very pronounced. The IC_{50} for the first 5 egg batches that were collected 5, 15, 25, 35 and 45 days after the 3rd immunization decreased only from 1.0 to 0.3 μg L^{-1}. The modification of Polson's method of antibody clean-up has been justified in an experiment that compared antibodies that were isolated either according to Polson's method or by the shortened procedure. The improvements that were observed with an additional clean-up step were neglectable: IC_{50} of 0.38 μg L^{-1} for the antibodies obtained with the three-fold precipitation procedure as suggested by Polson vs. IC_{50} of 0.50 μg L^{-1} for the antibodies obtained with only a single precipitation step.

TABLE I Cross-reactivity for five structure-related sulfonylurea compounds

Compound	*IC_{50} [μg L^{-1}]*	*Cross-reactivity (%)*
MSM	1	100
Sulfometuron-methyl	1.6	64
Cinosulfuron	188	<1
Triasulfuron	643	<1
Chlorsulfuron	346	<1
Amidosulfuron	Not reactive	Not reactive

The achieved IC_{50} was as low as 0.4 μg L^{-1} and the limit of detection (LOD) 13 ng L^{-1}, which was calculated as the concentration corresponding to the absorbance of a blank sample minus three standard deviations.

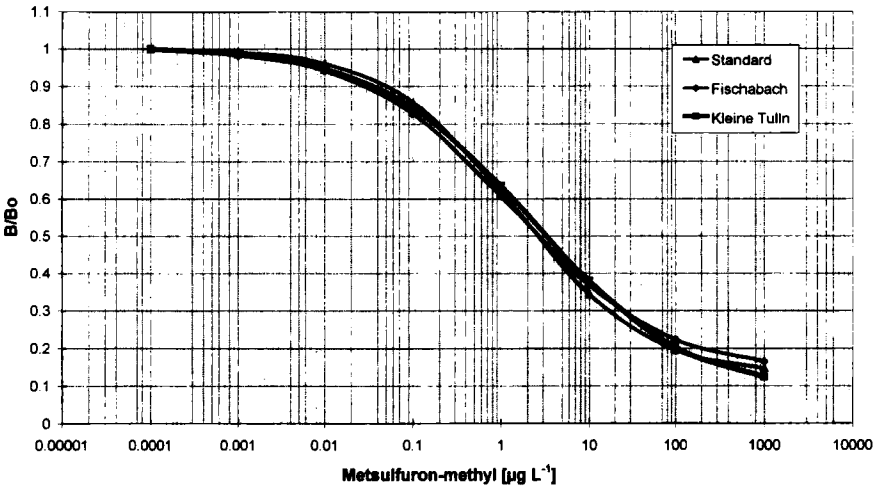

FIGURE 4 Comparison of dose-response curves for MSM calibrators obtained with spiked surface water samples

The figures of merit for the cross-reactivities are quite comparable to those achieved with the rabbit antibodies from earlier experiments [1]. As expected, only the most structure-related compound (sulfometuron-methyl) showed a significant cross-reactivity. All the other sulfonylurea compounds exhibited only a very small (< 1%) cross reactivity or none at all (amidosulfuron). Details of these experiments are given in Table I. The percentage of cross-reactivity was defined as (IC_{50} of MSM/IC_{50} of cross reactant) × 100. Two different tap waters were tested with the ELISA developed in this study, one having its source in the mountains, the other was from a well. For comparison, a calibration curve was set up using standards that were prepared in 0.01 M PBS. The standard curves prepared in the different tap waters showed especially good correspondence at high and low concentration levels. Within the linear range of the curves deviations were higher but, there is no significant difference between standards prepared in PBS and tap water samples (Figure 3). This also applies more or less to the two surface waters that were tested (Figure 4). Intraassay and interassay variation were calculated according to Märtlbauer [16]. For this purpose three-fold determinations of fortified tap water samples (1 μg L^{-1}) were performed on three different plates each. Corresponding variation was 18% for the intraassay and

20% for the interassay. This ELISA clearly meets EC Directives related to water intended for human consumption, which specifies a limit value of 0.1 $\mu g\ L^{-1}$ for any individual pesticide in drinking water as the achieved IC_{50} is close to this value. Besides, the results are well competitive with those achieved in ELISAs based on mammalian antibodies.

Acknowledgements

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