Budapest Technical and Economical University
Faculty of Chemical Engineering

DEVELOPMENT AND APPLICATION OF ENZIME-LINKED IMMUNOSORBENT ASSAYS FOR PESTICIDE ACTIVE INGREDIENTS FENOXYCARB AND ATRAZINE

PhD thesis

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INTRODUCTION

Research activities during my PhD work include two directions: development and characterization of *de novo* enzyme-linked immunosorbent assay (ELISA) systems, and application of ELISA systems, available from commercial or other resources, in various surveys. Although traditionally only the first direction is considered method development, application of commercial ELISA systems also included, although to a lesser extent, certain analytical develpoment tasks.

In the first direction, studies within my doctoral work included the dvelopment of an ELISA system for the detection of the insect growth regulator (insecticide) fenoxycarb and similar work (not discussed in my PhD dissertation) for the detection of the polyaromatic hydrocarbon (PAH), pyerene. Both assay development projects involved international collaboration. This the ELISA system for fenoxycarb was developed together with the Department of Entomology at the University of California, while the ELISA for pyrene was done in cooperation with the Water Chemistry and Chemical Balneology Institut and Munnich (*Institut für Wasserchemie und Chemische Balneologie, Technische Universität München*).

The second part of my work included the use of ELISA systems developed by others. The most important project within this was the application a commercial ELISA kit (ImmunoSystems – Millipore) for the detection of triazine herbicides, mainly atrazine, on soil and surface water samples collected in Hungary. In addition, I applied a magnetic particle based ELISA for the detection of the fungicide captan (not discussed in my PhD dissertation).

In my PhD research I studied other, previously developed ELISA systems as well: I carried out comparative studies with ELISA systems for triazole fungicides, e.g. for an ELISA for myclobutanil developed at the Plant Protection Institute of the Hungarian Academy of Sciences, and for and ELISA for other triazole fungicides (tetrakonazol, penkonazol) developed at the Milano University (*Universitá degli Studi di Milano*). Due to the allowed length of the PhD dissertations, I discuss only the above two directions, and only the work related to the development nad applications of the ELISA systems for the pesticides fenoxycarb and atrazine.

1. Development of an ELISA system for the detection of the insect growth regulator fenoxycarb

The target compound: Insect growth regulators are modern insecticides that disturb the normal development ("growth") of insects, mainly by interfering with the hormones regulating insect metamorphosis. One of these selective active ingredients is fenoxycarb, similar in structure to insect juvenile hormones, disturbing specific metamorphotic processes in insects, and effective at 5-10 g active ingredient/ha dose against several Lepidoptera and scaling insects. The compound blocks transformation of the insect to adult (imago) stage, inhibits molt of the cuticule at early larval stages, and acts against hatching of the insects from eggs. Partly due to these hormonal effects and its physico-chemical properties, the role of fenoxycarb in ecosystems and its effects on non-target organisms has been intensively studied. Although fenoxycarb is much more selective than traditional pesticides, and causes no harmful effects at registered agricultural doses e.g. on ruminants, it is toxic to certain benefitial and non-target insects. An example is the silkworm (*Bombyx mori*), where fenoxycarb causes nonspinning syndrome and economic losses. In addition, fenoxycarb is toxic to aqueous ecosystems, their invertebrates and fishes. Because of this and its slow decomposition (and therefore potential environmental persistence), ecotoxicological concerns apply to certain applications, therefore, we need effective analytical tools (e.g. immunoanalytical methods) for monitoring this active ingredient.

Hapten synthesis and conjugation: The structure of fenoxycarb allowed four modification strategies to prepare hapten derivatives (*Figure 1*). Haptens were prepared at the Plant Protection Institute of the Hungarian Academy of Sciences and at the Department of Entomology at the University of California. Using these haptens, we prepared protein conjugates, and I used these conjugates as immunogens to raise antibodies in animals and as coating antigens for ELISA systems.

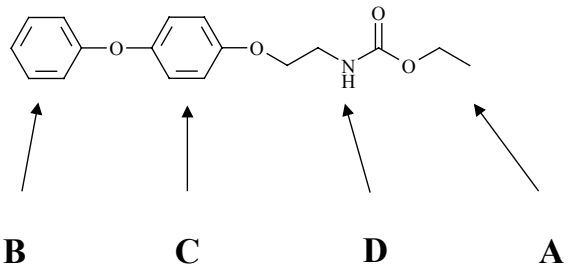

Figure 1 Hapten synthesis strategies for the insect growth regulator fenoxycarb: **A**: replacement of the *O*-etil moiety of the carbamate group with carboxyalkyl group, **B**, **C**: aniline group created at the terminal and the medial benzene by nitration and reduction, **D**: substitution of the *N* atom of the carbamate group by carboxyalkyl group.

Aniline type haptens are bound to protein conjugates by azo-coupling, and this bonding can be verified through specific UV-spectral properties of these azo-compounds. To determine how much aniline hapten has been coupled to protein conjugates, I carried out the conjugation process between three chromophore anilines (similar in structure to the haptens) and 4-cresol to model azo-coupling between diazonium salts from the aniline haptens and the thyrosine groups of the proteins. I measured maximal absorbance wavelengths of the azo-compounds and their molar extinction coefficients (ε), and used the strong UV-VIS absorbances of the azo-part in the hapten–protein conjugates of fenoxycarb to calculate hapten/protein ratios in these conjugates. Using the absorbance intensities of the hapten conjugates at their maximal absorbance wavelengths and the ε values detected for the model compounds, I calculated the molar concentrations of the haptens in the conjugates, and compared these data with the concentration of the carrier protein.

Immunization, ELISA optimization: Using antisera against the hapten conjugates, I prepared immobilized antigen-based, competitive inhibition ELISA systems, and optimized these szstems for their main analytical parameters (the inhibition mid-point (IC_{50}) and the limit of detection (LOD)). I titrated the antisera, determined inhibition mid-points at various method parametes, studied the effect of the pH, organic solvents and various bclocking agents on immunoassay performance. Within the optimzed ELISA szstem, I studied cross-reactivities by 42 compounds, similar in structure to or appearing together with fenoxycarb, and I found the ELISA method specific for fenoxycarb: only its close structural analogs showed significant cross-reactivity.

Practical applications: I applied the developed ELISA method on numerous environmental and biological samples to detect possible matrix effects. Environmental samples included surface and drinking water, and soil, biological samples included fruit homogenates and drinks (apple, pear and grape), insect tissues, fish liver tissue, and bovine urine and blood.

New scientific results:

(A) I developed a *de novo* ELISA system for immunoanalytical detection of the insect growth regulator fenoxycarb. Thus:

1.1. I coupled haptenic derivative of fenoxycarb to various carrier proteins. To verify coupling efficiency in conjugation of the aniline type haptens, I prepared model compounds in the reaction of phenoxyanilines and cresol, and with these compounds verified that hapten coupling strongly depends on the type of the carrier protein. I calculated the hapten /10 kDa protein ratios (HD 10kDa) for various carrier proteins (BSA, CONA, KLH and OVA), and its value was between 0.8 and 4.2, with the highest levels in the BSA- and CONA-conjugates.

1.2. I characterized the antisera raised against hapten conjugates of fenoxycarb, used as immunogens, in immobilized antigen-based ELISA systems. Serum titers in the developed ELISA systems were between 200 and 8000, and the inhibition mid-point (IC_{50}) with fenoxycarb in the optimized ELISA system was 2.7 ± 1.6 ng/ml and 1.1 ± 0.6 ng/ml with various conjugates. The limit of detection (LOD) was 0.2 – 4.6 ng/ml in hapten homolog systems and 0.11 – 0.2 ng/ml in hapten heterolog systems.

1.3. I studied the cross-reactivity of 42 possible co-contaminants in the fenoxycarb ELISA system, and found the ELISA system specific for fenoxycarb. I optimized the ELISA system for its main assay parameters. The assay works the best at pH = 7.4, in a buffer containing 0,5% (v/v) methanol, using gelatin from bovine skin as a blocking reagent.

1.4. I evaluated the applicability of the ELISA system optimized for the detection of fenoxycarb in various environmental and biological samples. Thus, the system is applicable in various water samples (tap water, water from the river Danube), while it can be used, at its present parameters, for the analysis of soil samples only after sample clean-up. I successfully used the optimized ELISA system in insect hemolymph (while strong matrix effects appeared in fat body tissue and in whole body homogenate), animal urine, and in fish liver samples, where I detected increasing fenoxycarb content in the liver of treated animals dependent of the application dose. Using water and fruit drink samples, I compared the optimized ELISA system with an instrumental analytical (GC-MS) method.

2. Application of a commercial ELISA system for the detection of the hericide atrazine

The target compound: The two main calsses of herbicides, ureas and symmetrical triazines, act by inhibition the II. light period of the photosythsesis. A common property of the compound classes is that they block photosynthetic electron transport in the chloroplasts. Symmetrical triazine herbicides are derivatives of 2,4-diamino-1,3,5-triazine, including a large number of compounds with various substituents on the realtively rigid molecule skeleton (the 1,3,5-triazine ring). The herbicide with the highest agricultural use among triazines containing a chlorine atom in the 2 position of the s-triazine ring is atrazine (*Figure 2*), a herbicide selective in corn and applicable to other deep-rooted commodities. (In Hungary it is marketed under the trade name of Aktinit PK.) Atrazine decomposes in soil only very slowly (mainly if conditions are anaerobic), and its slow leaching from the soil makes it a persistent water pollutant. As a result is has been banned in Germany. In additional, due to its endocrine (hormonal) disrupting effects, we decided to minitor the herbicide active ingredient using a commercial ELISA kit (the EnviroGuard® ELISA plate kit by ImmunoSystems–Millipore).

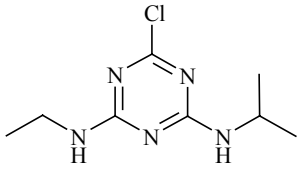

Figure 2 The chemical structure of the *s*-triazine derivative herbicide, atrazine. The compound family containing various substituents on the rigid 1,3,5-triazine skeleton include numerous related compounds.

Cross-reactivity, applicability of the method: I tested the commercial ELISA kit for detection of the herbicide active ingreduients atrazine, cyanazine and prometrine, and I found the method significantly specific for atrazine: the system detected atrazine at 0,2 – 5 ng/ml concentration, while prometrine and cyanazin was detected only at higher (2 – 20 ng/ml) concentrations. I also studied, using the ELISA kit, the decomposition kinetics of atrazine in water and soil. For soil samples, I optimized a solvent extraction process for sample preparation, and I found extraction with methanol as the optimal protocol (by method and economy).

Practical applications: I applied the ELISA kit for the determination of atrazine content in surface water and field soil samples, and the experiments in both cases indicated that the target compound, atrazine is a persistent contaminant in the environment.

New scientific results:

(B) I applied a commercial ELISA kit for the immunoanalytical detection of the herbicide atrazine. Thus:

2.1. I verified the applicability of the ELISA system, marketed for monitoring triazine herbicides, in environmental samples. I optimized the sample preparation process of soil samples for ELISA tests, and found methanol as an optimal solvent. Using the ELISA kit, I studied the decomposition of atrazine in water and soil, and found that in water at room temperature a rapid initial decomposition rate (the active ingredient level of 1 μg/ml decreased to öne-fifth within 12 days) does not decrease further resulting in a persistent residue level, and in soil the 1 μg/g initial dose also remains persistent, both in sand and black (chernoziom) soils, at a level of 0.35 μg/g. The study has verified that atrazine is a persistent contaminant both in water and soil.

2.2. I studied detectable atrazine levels in surface water and field soil samples collected from areas of intensive agricultural use (Békés county) and natural recreation areas (Fejér county). I found that the atrazine level is 0,31-0,40 ng/ml in water samples from agricultural areas, and 0,08-0,30 ng/ml from natural recreation areas. I demonstrated that alarmingly high atrazine contents remain in experimentally treated field soil samples one year after application (31-64% depending on application doses), and residues of atrazine application from previous years was detectable in all agricultural soil samples.

ABBREVIATIONS

ELISA – enzyme-linked immunosorbent assay
IC_{50} – inhibition mid-point (inhibitor concentration causing a 50% decrease of maximal signal)
LOD – limit of detection
HD – hapten density
BSA – bovine serum albumin
CONA – conalbumin
KLH – hemocyanin (from keyhole limpet)
OVA – ovalbumin
GC-MS – gas chromatography – mass spectrometry

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PUBLICATIONS, AS THE BASIS OF THE DOCTORAL THESIS

1. **H.M. Le**; A. Székács; G. Tőkés and B.S. Ferguson (1995) Detection of atrazine in Hungary by immunoanalytical (ELISA) method. *J. Environ. Sci. Health, part B*, **30**, 459-464.
2. F. Szurdoki, A. Székács, **H.M. Le** and B.D. Hammock (2002) Synthesis of haptens and protein conjugates and development of immunoassays for the insect growth regulator fenoxycarb. *J. Agric. Food Chem.*, **50**, 29-40.
3. **H.T.M. Le**; F. Szurdoki and A. Székács (2003) Evaluation of an enzyme immunoassay for the detection of the insect growth regulator fenoxycarb in environmental and biological samples. *Pest Manag. Sci.*, **59**, 410-416.
4. A. Székács; **H.T.M. Le**; F. Szurdoki and B.D. Hammock (2003) Optimization and validation of an enzyme immunoassay for the insect growth regulator fenoxycarb. *Anal. Chim. Acta*, accepted for publication.

COMPLETE LIST OF PUBLICATIONS

Papers in scientific periodicals:

1. **H.M. Le**; A. Székács; G. Tőkés and B.S. Ferguson (1995) Detection of atrazine in Hungary by immunoanalytical (ELISA) method. *J. Environ. Sci. Health, part B*, **30**, 459-464. (I.F. 1,128)
2. A. Székács; S. Cairoli; **H.M. Le** and S. Pagani (1996) Comparative studies on enzyme-immunoassays (ELISA) for the triazole fungicides tetraconazole and myclobutanil. *Acta Phytopathologica et Entomologica Hungarica*, **31**, 293-301. (I.F. 0,050)
3. **H.M. Le**; Gy. Hegedűs and A. Székács (1998) Differential detection of *N*-heterocyclic compounds and their *N*-methylated derivatives by immunoanalysis. *Acta Biologica Hungarica*, **49**, 455-462. (I.F. 0,136)
4. A. Székács; **H.M. Le**; D. Knopp and R. Niessner (1999) A modified enzyme-linked immunosorbent assay (ELISA) for polyaromatic hydrocarbons. *Anal. Chim. Acta*, **399**, 127-134. (I.F. 1,894)
5. F. Szurdoki, A. Székács, **H.M. Le** and B.D. Hammock (2002) Synthesis of haptens and protein conjugates and development of immunoassays for the insect growth regulator fenoxycarb. *J. Agric. Food Chem.*, **50**, 29-40. (I.F. 1,576)
6. V. Krikunova, Gy. Hegedűs, **H.M. Le**, L. Jouravleva, S. Eremin, M. Natangelo, E. Benfenati and A. Székács (2002) Development of an enzyme-linked immunosorbent assay (ELISA) for the herbicide propanil. *Int. J. Envir. Anal. Chem.*, **82**, in press. (I.F. 0,797)
7. **H.T.M. Le**; F. Szurdoki and A. Székács (2003) Evaluation of an enzyme immunoassay for the detection of the insect growth regulator fenoxycarb in environmental and biological samples. *Pest Manag. Sci.*, **59**, 410-416. (I.F. 0,642)
8. A. Székács; **H.T.M. Le**; F. Szurdoki and B.D. Hammock (2003) Optimization and validation of an enzyme immunoassay for the insect growth regulator fenoxycarb. *Anal. Chim. Acta*, közlésre elfogadva (I.F. 2,073)

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Conference presentations and posters:

1. **H.M. Le**, A. Székács, G. Tőkés and B.S. Ferguson (1995) Detection of atrazine in Hungary by immunoanalytical method. Poster presented at the *5th European Conference on Chemistry and the Environment - "Pesticide Chemistry for Sustainable Agriculture" Outlook fore the 21st Century* (Budapest, Hungary, May 15-18, 1995)
2. **H.M. Le**, G. Tőkés, B.S. Ferguson and A. Székács (1995) Application of an enzyme-immunoassay for environmental monitoring of triazine herbicide residues. Poster presented at the *2nd International Conference of the Hungarian Biochemical Society* (Szeged, Hungary, Aug 21-23, 1995)
3. A. Székács, S. Cairoli, **H.M. Le** and S. Pagani (1995) Comparative studies on enzyme-immunoassays for triazole fungicides, tetraconazole and myclobutanil. Poster presented at the *5th Symposium on Chemistry and Fate of Modern Pesticides* (Paris, France, Sep 6-8, 1995)
4. **H.M. Le**, Gy. Hegedűs and A. Székács (1998) Immunodetection of *N*-heterocyclic compounds. Poster presented at the *4th International Conference on the Role of Formaldehyde in Biological Systems - Methylation and Demethylation Processes* (Budapest, Hungary, Jul 1-4, 1998)
5. A. Székács, **H.M. Le**, D. Knopp and R. Niessner (1998) A modified enzyme-linked immunosorbent assay (ELISA) for polyaromatic hydrocarbons. Poster presented at *The Immunochemistry Summit VII and the 3rd Workshop on Biosensors and Biological Techniques in Environmental Analysis* (Las Vegas, Dec 1-3, 1998)
6. Gy. Hegedűs, **H.M. Le**, A. Székács, A. Krasnova, S. Eremin, M.-C. Hennion, M. Natangelo and E. Benfenati (1999) Inter-laboratory trials of GC-MS versus immunoanalytical detection of environmental pollutants. Poster presented at *The 9th Symposium on Handling of Environmental and Biological Samples in Chromatography* (Porto, Portugal, Oct 10-13, 1999)
7. Gy. Hegedűs, A. Székács, **H.M. Le**, V. Krikunova, S. Eremin, M. Natangelo and E. Benfenati (2000) Development of an enzyme-linked immunosorbent assay (ELISA) for the herbicide propanil. Poster presented at *The 4th Euroconference on Environmental Analytical Chemistry* (Visegrád, Hungary, Sep 14-19, 2000)
8. **H.M. Le**, A. Székács, F. Szurdoki, B.D. Hammock (2002) Optimization and validation of an enzyme immunoassay for the insect growth regulator fenoxycarb. Poster presented at *5th Workshop on Biosensors and Bioanalytical Techniques in Environmental Analysis*, IAEAC (Ithaca, USA, May 30 - June 4, 2002)