# APPLICATION OF IMMUNOCHEMICAL TECHNIQUES TO PESTICIDE ANALYSIS IN FOOD AND THE ENVIRONMENT: SEARCHNG FOR DDT RESIDUES IN MEDITERRANEAN HONEY

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**"Pesticides" joint Meetings**
**CONCERNS, CHALLENGES & POSSIBLE SOLUTIONS**

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# Presentation outline

- Introduction: A brief overview on the development and analytical potential of monoclonal antibody-based immunochemical methods for pesticide analysis
- Development of an Enzyme-Linked Immunosorbent Assay (ELISA) for a specially concerning POP group: **DDT** and its derivatives
  - Analytical performance
  - Former applications
- Adaptation of the generic DDT ELISA to the analysis of DDT residues in honey
  - Matrix effects
  - Application to Mediterranean honey samples

# Ci²B:

## Center for Research and Innovation in Bioengineering

Academic Institute of UPV dedicated to scientific and technical research in several areas such as: Bioengineering, Biolectronics and Immunoanalytical Chemistry for Biomedical, Environmental and Food applications

**The "Immunotechnology Group" most classical research line:**

Inmuno Tecnología

Development of **immunochemical methods** (based on the antigen-antibody interaction) **for pesticide analysis**

# Development of immunochemical methods for pesticide analysis

- Synthesis of specific haptens, immunogens (protein-hapten conjugates) and assay conjugates
- Production of specific monoclonal antibodies (homogeneous, standardizable and endless reagents)

**ELISA** (plate Enzyme-Linked ImmunoSorbent Assays)

- High sensitivity (LOD: 0,01 - 0,1 ppb)
- Desired selectivity provided by the antibodies

# Monoclonal antibodies and ELISAs to pesticides and other contaminants developed at Ci²B (UPV)

- **N-Methylcarbamates** (Carbaryl, Carbofuran, Methiocarb, …)
- **Organophosphorus** (Chlorpyrifos, TCP, Azinphos, …)
- **Chlorinated** (DDT and Cyclodiene (Endosulfan) families)
- **Post-harvest fungicides** (Thiabendazole, Conazoles, Dithio-carbamates, …)
- **Plastic components** of food packages (Bisphenol A, 4-nonyl--phenol, phthalate esters)

→ **ELISA Kits**

→ **Bio(Immuno)sensors**

# Application of pesticide Immunoassays and Immunosensors

- **N-Methylcarbamates:** in water, fruit and vegetables, baby food, …
- **Organophosphorus:** in water, soil, fruit and vegetables, human urine (TCP metabolite)
- **Chlorinated cyclodienes:** Endosulfan in water, vegetables, fish fat …
- **Fungicides:** Thiabendazole and Tetraconazoles in fruit (citrus, banana), wheat, …

# A specially concerning POP group: the DDT family

- The insecticidal properties of DDT were discovered in the 1940s. Since then, more than 1 million tons of this product have been used worldwide to control insect pests in agriculture, forestry, and human health.

- DDT has a great stability to physical, chemical, and biological degradation. For this reason, highly persistent DDT residues have accumulated and are frequently found in the adipose tissues of animals and man, as well as in the environment.

# A specially concerning POP group: the DDT family

- DDT and its residues are well know as endocrine disruptors.
- The widespread occurrence of long-term DDT residues, together with their potential toxicological effects, provoked the total ban of the use of DDT in developed countries in the 1970s.
- Nevertheless, DDT is still extensively used in developing countries, where it is very valuable tool in malaria control programs.

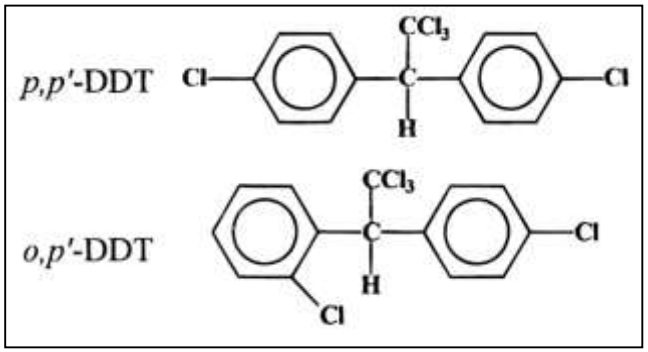
# A specially concerning POP group: the DDT family

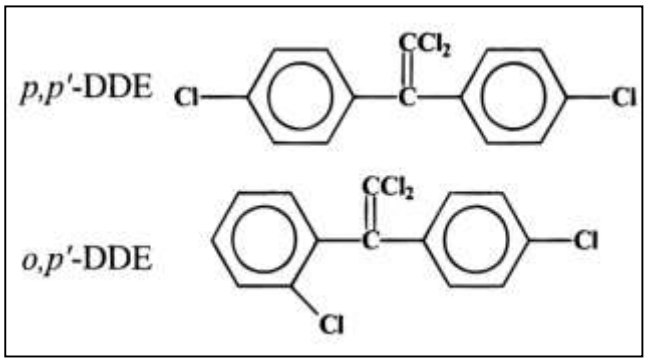
- Monitoring **human and animal exposure** to DDT residues is of great toxicological significance, particularly in geographical areas where DDT is still in use. Monitoring is usually performed through the analysis of biological tissues and fluids (milk, urine and blood)

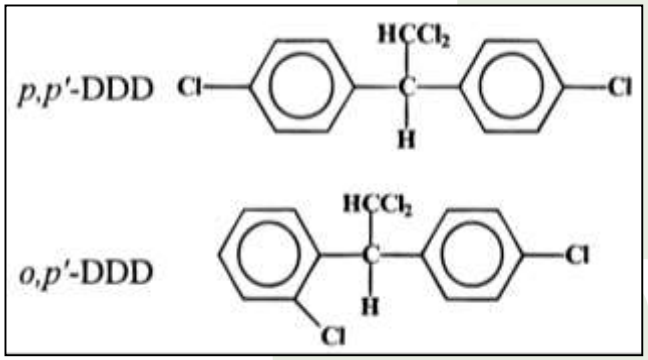
- **Contamination of environment and food** by these so persistent residues is also a serious concern worldwide.

**Developed countries have established very strict regulations on the presence of DDT residues in humans, animals, food and the environment**

# DDT isomers and metabolites

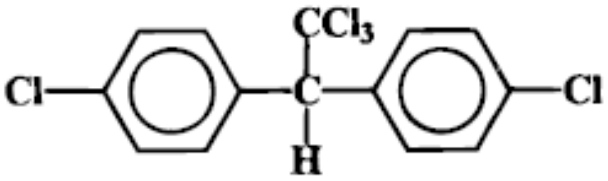


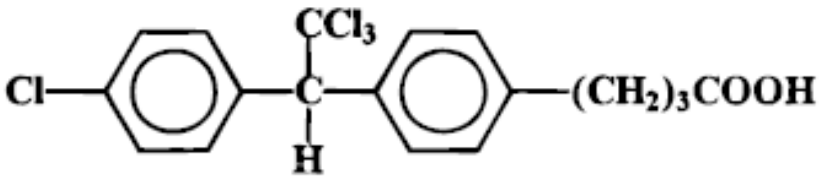




Analytical methods for DDT should be able to determine the six isomers, although method efficiency is normally assessed by the recovery of the most abundant and frequently encountered *p,p'*-isomers

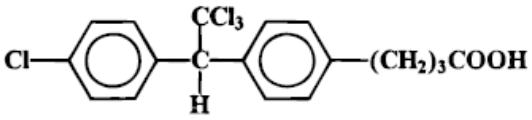
# Development of an ELISA for DDT

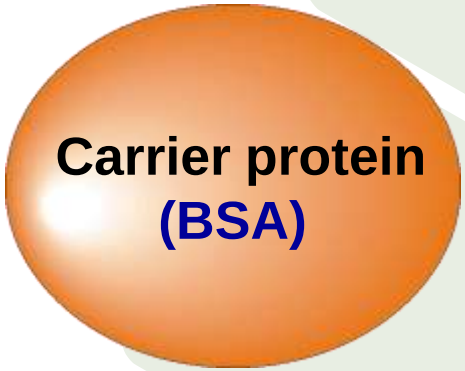



*p,p'*-DDT



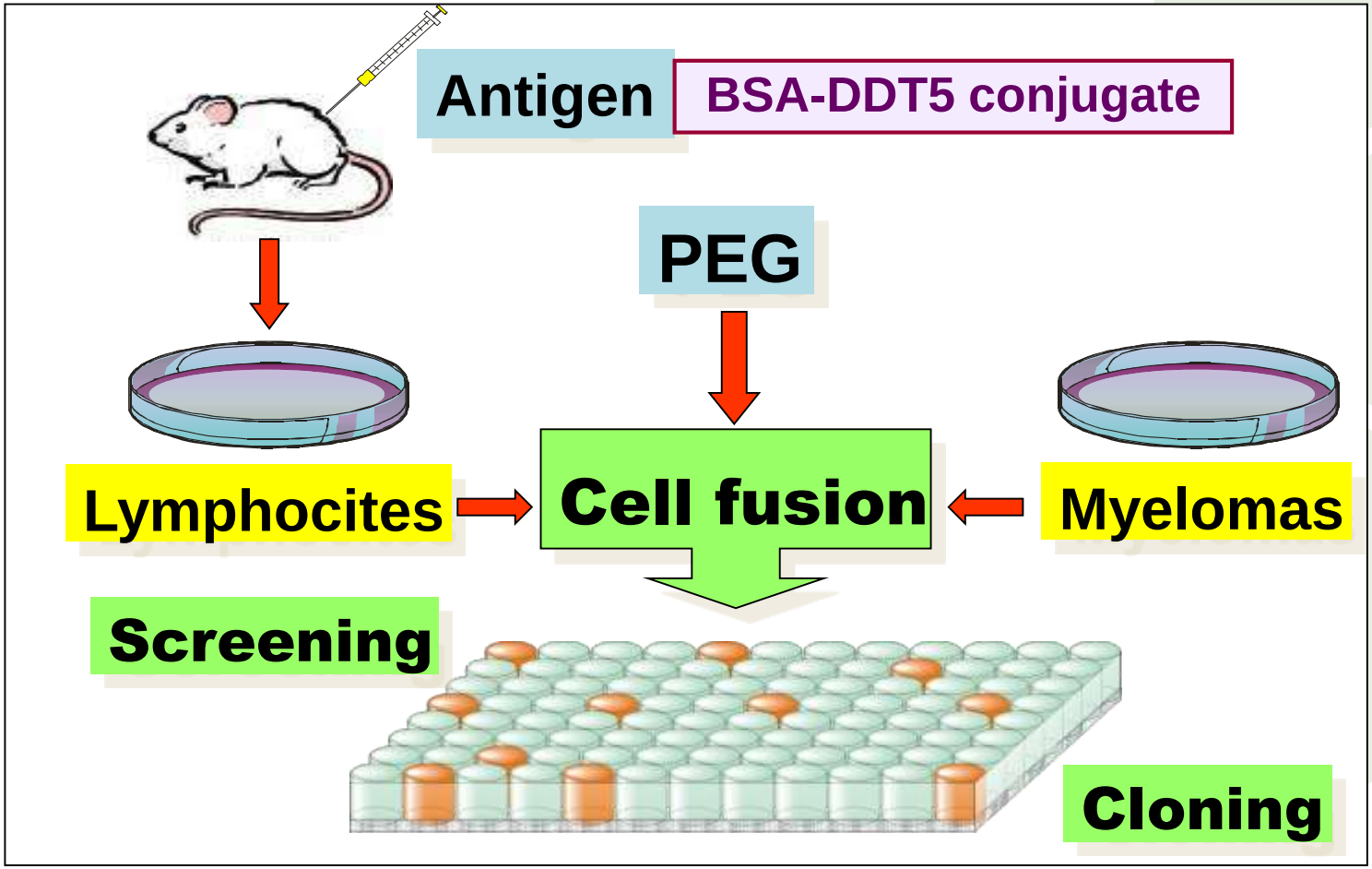

DDT5 hapten

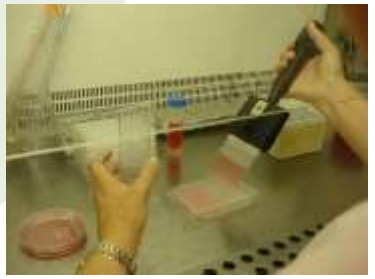


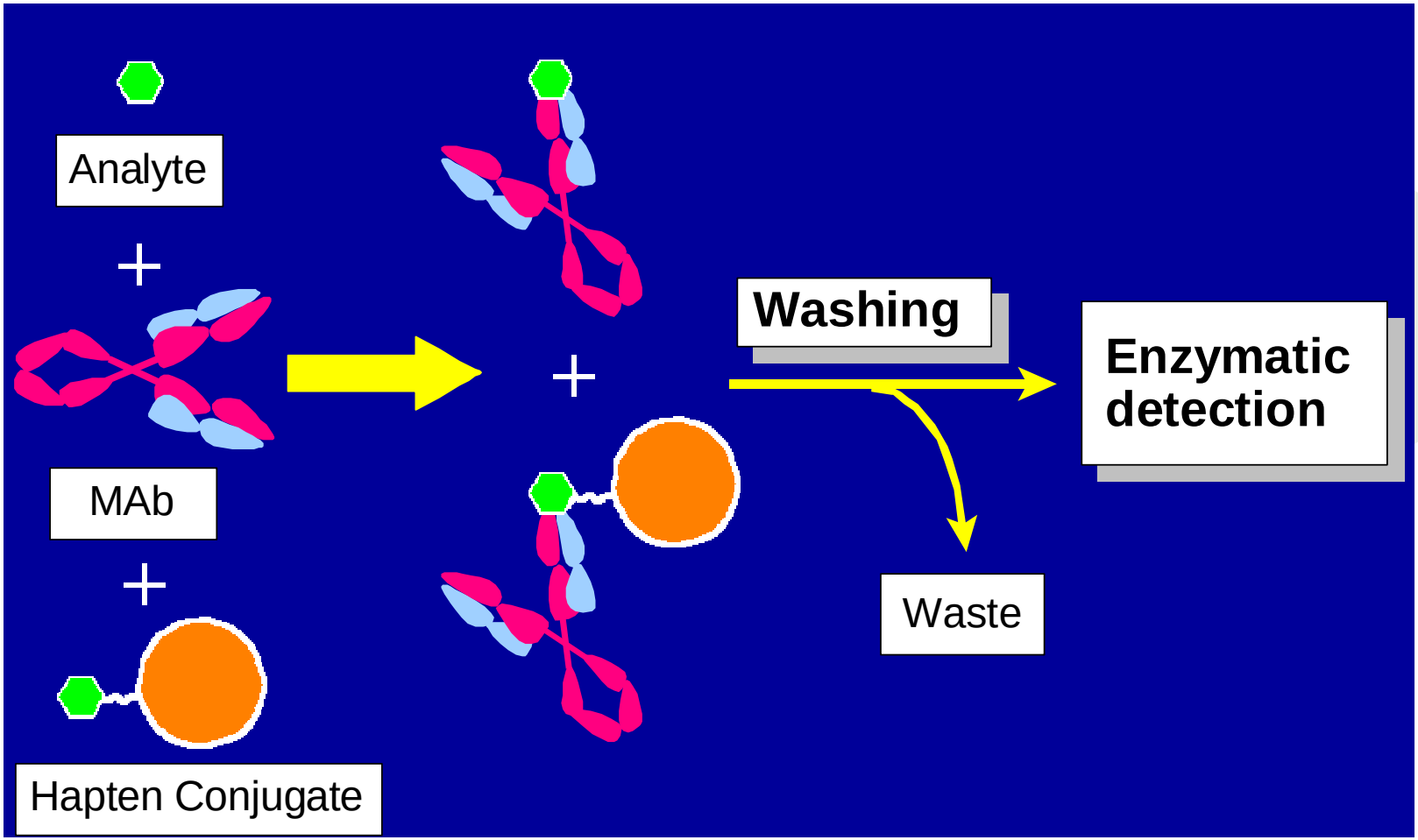

**DDT5-BSA conjugate**

# Monoclonal Antibody production: Hybridoma Technology



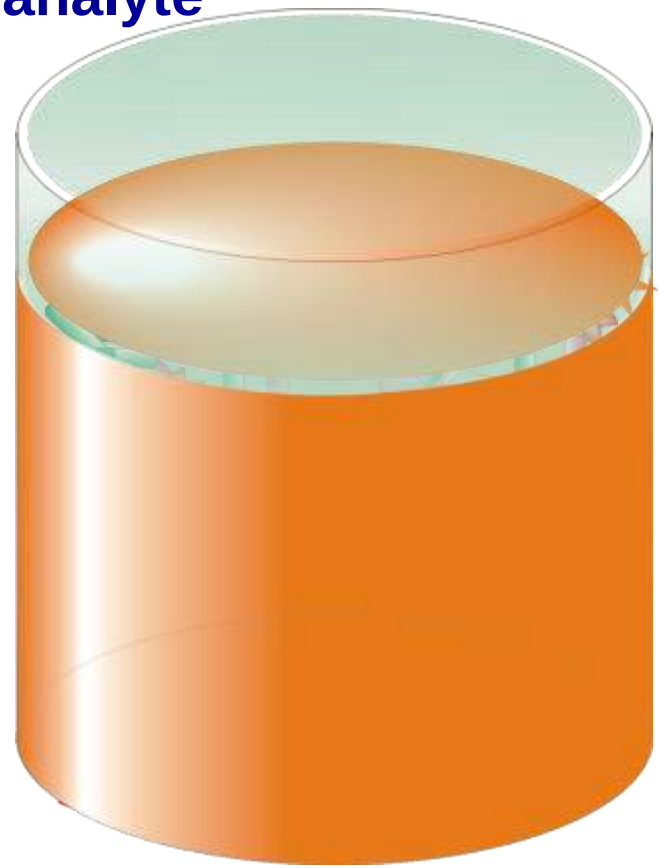



# Principles of the competitive ELISA



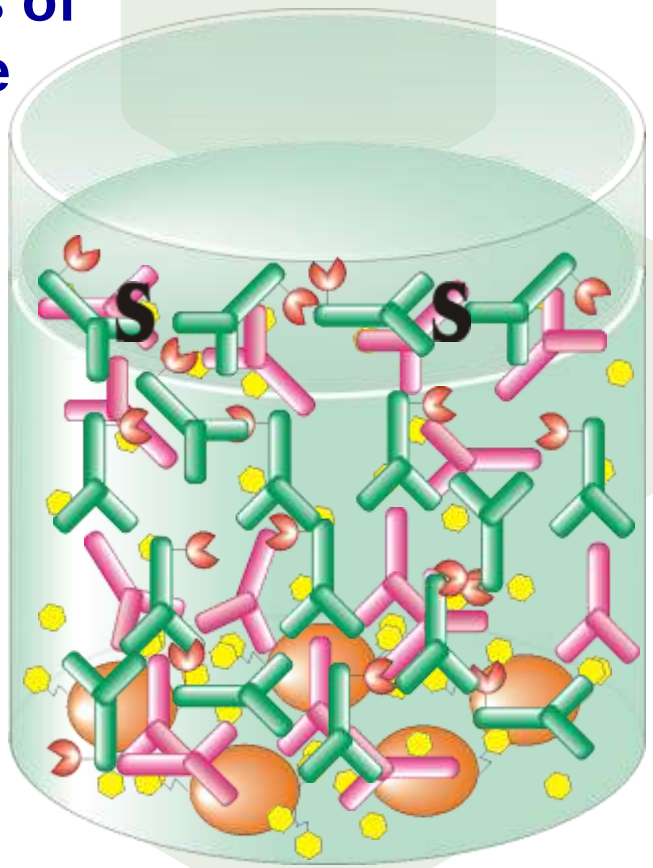
# ELISA in the conjugate-coated format

**no analyte**



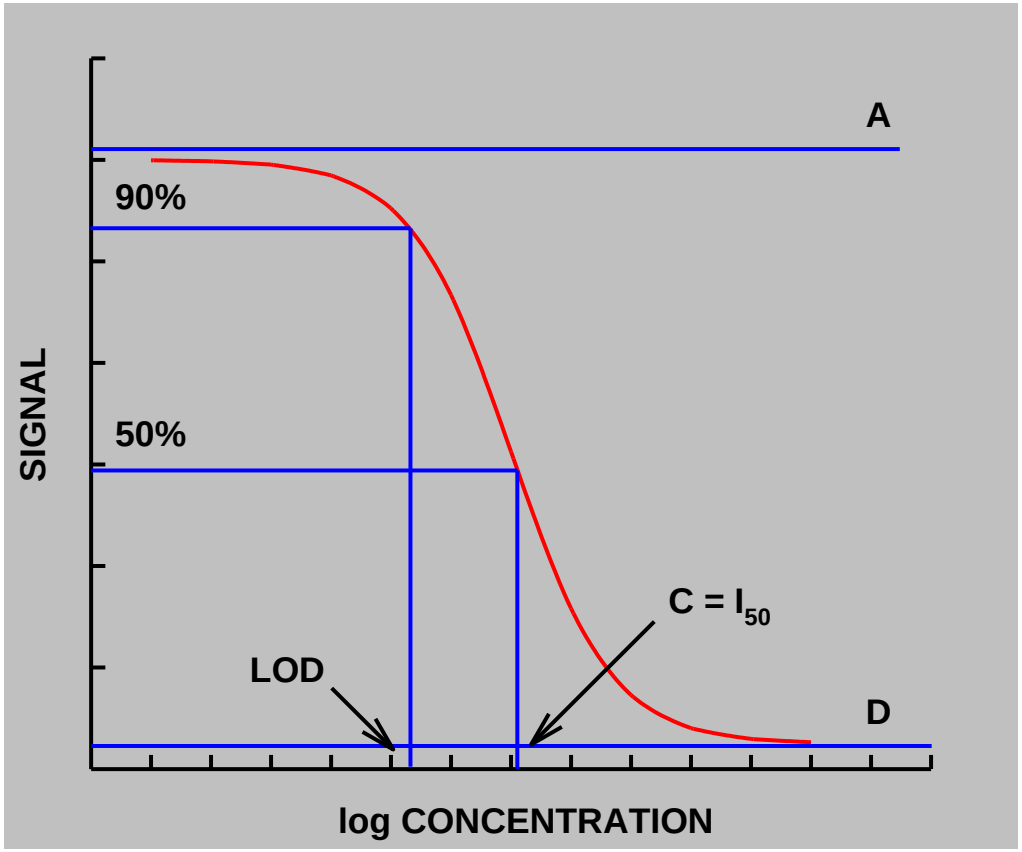
Amax

**excess of analyte**




Amin

# Standard curve for competitive Immunoassays



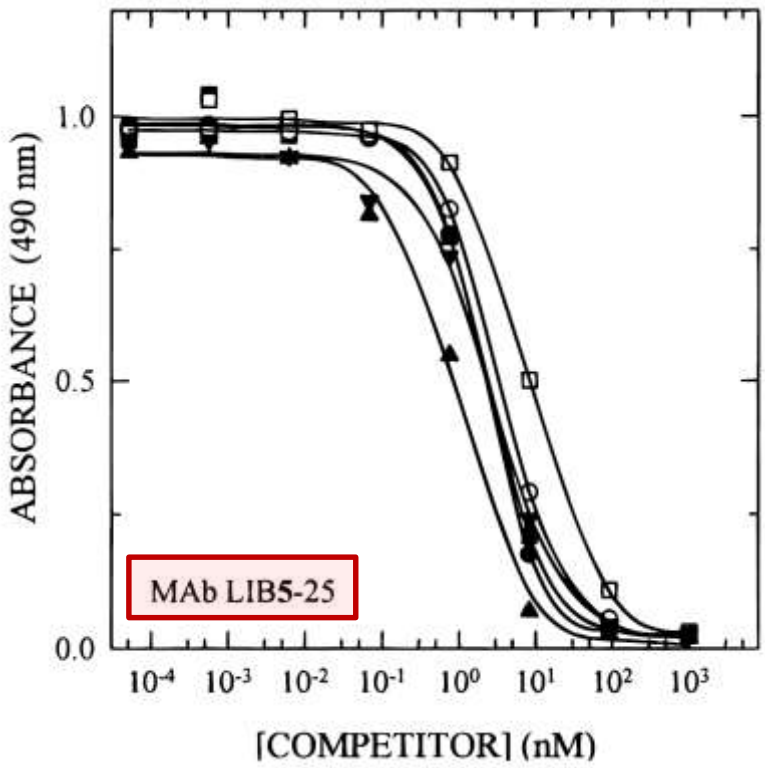

$$y = \frac{A - D}{1 + (x / C)^{B}} + D$$

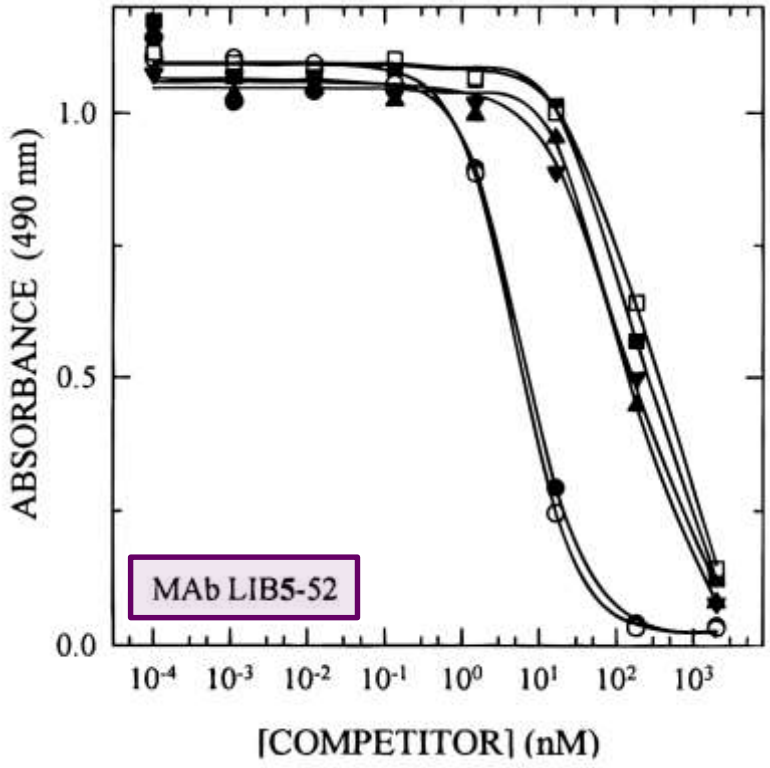
$I_{50}$

**Sensitivity**

Limit of Detection (LOD): $I_{90}$
Working range: $I_{80}$ - $I_{20}$

# Monoclonal ELISA curves for DDT-related compounds




Competitive ELISA standard curves obtained in the conjugate-coated format with the MAbs LIB5-25 (class-selective, left) and LIB5-52 (compound-specific, right).
Analytes: *p,p′*-DDT (●); *o,p′*-DDT (○); *p,p′*-DDE (■); *o,p′*-DDE (□); *p,p′*-DDD (▲); *o,p′*-DDD (▼).

[From: Abad, A. *et al.* (1997) ***J. Agric. Food Chem.*** **45**, 3694−3702]

# Analytical performance of the class-selective ELISA to DDT

## Analytical parameters for *p,p'*-DDT

**$I_{50}$ value:** 2.21 nM = 0.74 µg $L^{-1}$ (ppb)

**LOD:** 0.44 nM = 0.15 µg $L^{-1}$ (ppb)

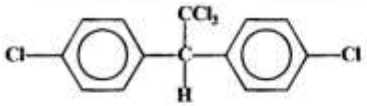
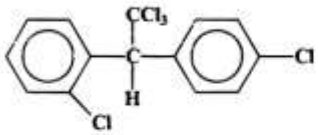
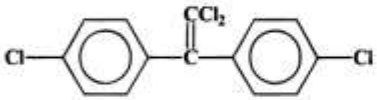
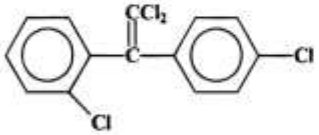
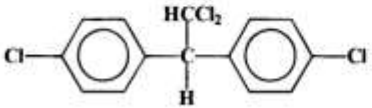
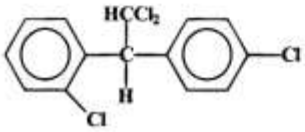
**Working Range:** (0.66 – 7.37) nM
(0.22 – 2.47) µg $L^{-1}$ (ppb)

**High Sensitivity**

# Cross-Reactivity assessment by ELISA

$$\text{Cross-reactivity} = \frac{I_{50}\ (\text{ref. compound})}{I_{50}\ (\text{cross-reactant})} \times 100$$

# Cross-Reactivity of monoclonal antibodies to DDT

| compound | structure | monoclonal antibody cross-reactivity[b] (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | LIB1-11 | LIB5-21 | LIB5-25 | LIB5-28 | LIB5-212 | LIB5-51 | LIB5-52 | LIB5-53 |
| *p,p'*-DDT |  | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |
| *o,p'*-DDT |  | 8.5 | 46.0 | 61.3 | 51.2 | 45.1 | 127.4 | 124.3 | 109.7 |
| *p,p'*-DDE |  | 13.9 | 83.8 | 92.4 | 72.8 | 90.0 | 5.2 | 3.4 | 3.8 |
| *o,p'*-DDE |  | 11.3 | 22.5 | 26.8 | 23.7 | 20.5 | 4.4 | 2.4 | 3.2 |
| *p,p'*-DDD |  | 56.2 | 305.6 | 223.9 | 202.1 | 475.1 | 10.2 | 4.9 | 3.8 |
| *o,p'*-DDD |  | 5.6 | 66.4 | 81.2 | 73.1 | 73.7 | 11.4 | 4.4 | 3.5 |

Class-selective

Compound-specific

[From: Abad, A. *et al. (1997)* ***J. Agric. Food Chem.*** **45**, 3694−3702]

# Application of immunochemical methods for DDT analysis: Immunoassays

- Fluorescence Polarization Immunoassay (FPIA) for the determination of DDT and related compounds in **water** samples
  [Eremin, S.A. *et al. (2002)* ***Anal. Letters*** **35**, 1835-1850]
- Chemiluminescent ELISAs for the analysis of DDT and its metabolites in environmental (**water, soil**) and food (**strawberry, salad, beet root, fish fat**) samples
  [Botchkareva, A.E. *et al*. (2003) ***J. Immunol. Methods*** **283,** 45-57]
- ELISA standardization for DDT detection in **mosquitoes**
  [Penilla, P. et al. In: ***Entomología Mexicana*****, Vol. 7**. Ed. E.G. Estrada, A. Equihua, J.R. Padilla, A. Mendoza. Sociedad mexicana de Entomología A.C., Texcoco, **México 2008**. pp 784-789]

# Application of immunochemical methods for DDT analysis: Immunosensors

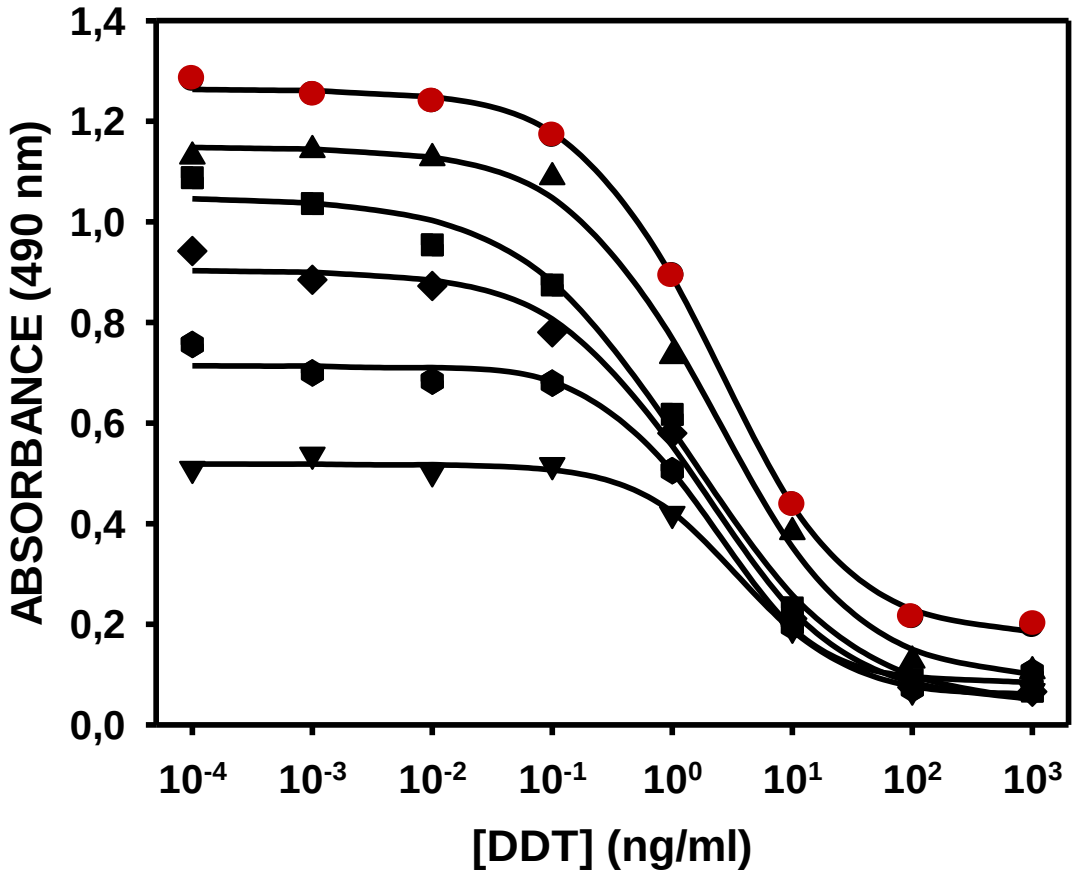
- Development of **nanomechanical biosensors** for the detection of the Pesticide DDT
  [Álvarez, M. *et al.* (2003) **Biosensors & Bioelectronics 18,** 649-653]
- Determination of DDT in **natural water** samples from different origins with a portable **optical immunosensor**
  [Mauriz, E. *et al. (2006)* **Talanta 69,** 359-364]
- **Optical Immunosensor** for fast and sensitive detection of DDT and related compounds in **river water** samples
  [Mauriz, E. *et al.* (2007) **Biosensors & Bioelectronics 22**, 1410-1418].
- Multi-analyte **SPR** immunoassays for environmental biosensing of pesticides, including DDT
  [Mauriz, E. *et al.* (2007) **Anal. Bioanal. Chem. 387,** 1449-1458]

# Our last approach: Adaptation of the generic DDT ELISA to the analysis of DDT residues in honey

- Honey is a natural food with many nutritional and health benefits, very appreciated in Mediterranean countries.
- It can be contaminated by the ubiquitous dissemination of DDT residues.

- DDT is among the POPs for which the EU has established strict regulations in food.
- EU **MRL** for DDT in honey is **0.05 mg $Kg^{-1}$ (50 ppb)**

# Honey matrix effect on the DDT ELISA

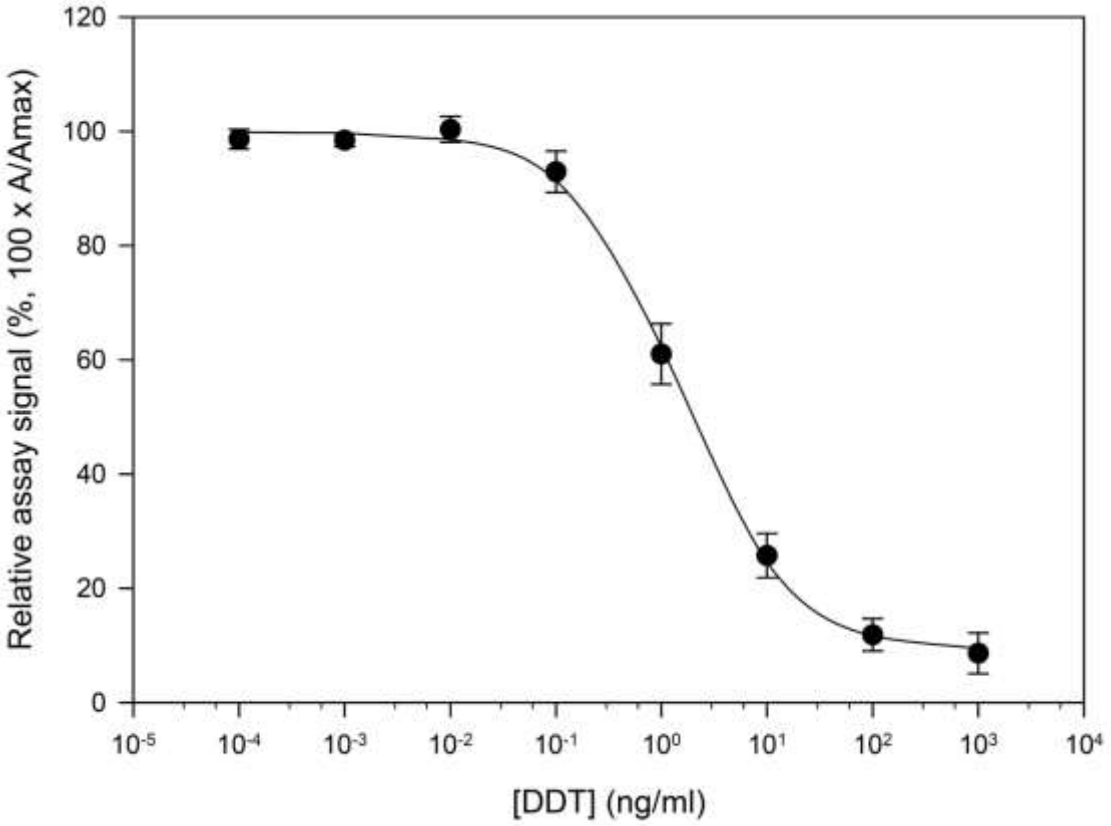



- Standard curve in PBS (without honey)

Honey dilution

- ▲ : 1/500
- ■ : 1/200
- ◆ : 1/100
- ✹ : 1/50
- ▼ : 1/25

# ELISA standard curve for DDT in 1/50 diluted honey




Normalized average of 8 individual standard DDT curves

## Analytical parameters (*p,p'*-DDT)

$I_{50}$ value: 1.52 ng $mL^{-1}$

LOD: 0.11 ng $mL^{-1}$

Working range: (0.29 – 7.95) ng $mL^{-1}$

Precision: CV < 10%

# Analytical performance of the ELISA for DDT in 1/50 diluted honey

Taking into account

- The sample dilution applied to minimize matrix effects (1/50)
- The average honey density (~ 1.4 g $mL^{-1}$)

## Practical analytical parameters of the DDT ELISA in honey

**LOD:** 0.008 mg $Kg^{-1}$ (8 ppb)

**LOQ:** 0.020 mg $Kg^{-1}$ (20 ppb)

Clearly below the MRL

**Working Range:** (0.020 – 0.557) mg $Kg^{-1}$ (20 – 558 ppb)

# ELISA application to DDT analysis in honey

## ➢ Spiked samples

- Commercial mixed-flower honey from a Spanish supermarket (Valencia)
- DDT added at several concentrations (20 - 500 ppb)
- Spiked honey samples diluted 1/50 in PBS for ELISA
- At least 3 replicates assayed per sample
- RESULTS: Good recovery (**85 – 120 %**)
  Good precision (**CV < 12 %**)

# ELISA application to DDT analysis in honey

## ➢ Real samples

- 5 samples from Italy (1 acacia, 1 eucalyptus, 1 orange tree and 2 mixed-flower honey samples)
- 3 samples from Spain (1 rosemary, 1 lavender and 1 mixed-flower honey sample)
- Samples diluted 1/50 in PBS for ELISA
- 9 replicates per sample (3 ELISA plates, 3 replicates per plate)
- RESULTS: Good precision (**CV ~ 13 %**)

**7 samples** were **DDT-free** ([DDT] < LOD)
**1 sample** showed a quantificable concentration of **DDT residues** (**39.6 ppb**,< MRL)

# Conlusions

- We succeeded  in our attempt to adapt the generic ELISA to DDT for its application to the analysis of DDT residues in honey
- As far as its DDT residues content, Mediterranean honey is a safe product that we can continue consuming without major concern

# Acknowledgements


- To all my students at Ci²B (UPV) and my colleagues in Spanish and European Institutions, who made this work possible


- To all of you for your attention