# IN-HOUSE DEVELOPMENT AND CROSS-REACTIVITY EVALUATION OF A POLYCLONAL ANTIBODY FOR THE IMMUNOANALYSIS OF THE POLLUTANT 2,4,6-TRICHLOROPHENOL

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## ABSTRACT

2,4,6-Trichlorophenol (2,4,6-TCP) and other chlorinated phenol derivatives are widely spread in the environment and considered as severe pollutants, predominantly of natural aqueous sources. Exposure to 2,4,6-TCP is considered a carcinogenic risk and determination of 2,4,6-TCP levels in environmental samples is, therefore, of great importance. Immunoanalysis of 2,4,6-TCP is an interesting alternative to the well-established and widely used instrumental analysis of this pollutant; however, development of specific antibodies is a challenging and critical step, since 2,4,6-TCP is a small organic molecule (hapten) that should be suitably conjugated to a carrier protein, in order to elicit an immune response. To our knowledge anti-[2,4,6-TCP] antibodies are not easily available and most immunoassays for 2,4,6-TCP are based on in-house developed immunoanalytical reagents.

In this work we present the in-house development of a polyclonal antibody for 2,4,6-TCP. Commercially available 2,4,6-trichlorophenoxyacetic acid (2,4,6-TCPA) was used as starting material for preparing the immunizing hapten, which was conjugated to the carrier protein keyhole limpet hemocyanin and subsequently administered to New Zealand white rabbits following a well-established immunizing protocol. Immunochemical functionality of the antisera collected (five consecutive bleedings) was evaluated with titer- and displacement experiments in a (biotin/streptavidin)-ELISA system, in which an in-house prepared biotinylated derivative of 2,4,6-TCPA was employed as the immobilized hapten. The ELISA-titer value of the anti-[2,4,6-TCP] antibody was very high (~1:100,000). Cross-reactivity studies with various chlorophenols (2,4,5-trichlorophenol, 2,3,6-trichlorophenol, 3,4,5-trichlorophenol, 2,4-dichlorophenol, 2,6-dichlorophenol, 3,5-dichlorophenol) revealed that the antibody cross-reacted with 3,5-dichlorophenol. The anti-[2,4,6-TCP] antibody developed can be used as a research tool for detecting 2,4,6-TCP in environmental and other samples *via* immunoanalytical systems, including highly sensitive immunosensors, while it might be also suitable for the immunoanalysis of 3,5-dichlorophenol.

**Keywords**: chlorophenols, 2,4,6-trichlorophenol (2,4,6-TCP), polyclonal anti-[2,4,6-TCP] antibody, (biotin-streptavidin) ELISA, cross-reactivity studies.

## 1. Introduction

Chlorophenols are widely spread in the environment and considered as severe pollutants predominantly of natural aqueous sources (Czaplicka, 2004; Olaniran and Igbinosa, 2011). Exposure to 2,4,6-trichlorophenol (2,4,6-TCP) as well as to other chlorophenols is considered a carcinogenic risk (Huff, 2012; Igbinosa *et al.*, 2013) and determination of chlorophenols in environmental samples is, therefore, of great importance. Immunoassays for 2,4,6-TCP and other chlorophenols (Noguera *et al*., 2002; Galve *et al.*, 2002; Nichkova *et al.*, 2003; Nistor and Emnéus, 2003; Abuknesha and Griffith, 2004; Beloglazova *et al.*, 2010); comprise an interesting alternative to the well-established and widely used instrumental analysis of these pollutants. However, development of specific antibodies is a challenging step, since chlorophenols are small organic

molecule (haptens) that should be suitably conjugated to a carrier protein in order to elicit an immune response (Shreder, 2000). To our knowledge anti-[2,4,6-TCP] antibodies are not easily available and most immunoassays for 2,4,6-TCP are based on in-house developed immunoanalytical reagents.

Aim of the present study was to develop a polyclonal antibody for 2,4,6-TCP which could be eventually exploited as a component of various immunoanalytical systems, including immunosensor devices (Jiang *et al.*, 2008; Holford *et al.*, 2012), for detecting 2,4,6-TCA in environmental and other samples of special interest. Depending on its cross-reactivity characteristics, this antibody might be also employed in the immunoanalysis of other chlorophenols.

## 2. Materials and methods

### 2.1. Immunogen and immunizations

2,4,6-trichlorophenoxyacetic acid (2,4,6-TCPA, Sigma-Aldrich) was used as starting material for preparing the immunizing hapten. Briefly, TCPA was conjugated to the carrier protein keyhole limpet hemocyanin (KLH, Thermo Scientific) through a spacer consisting of a suitable combination of amino acids, following a solid-phase chemistry approach previously reported by our team (Papasarantos *et al.*, 2010). Conjugation was performed according to the well-known glutaraldehyde method (Avrameas, 1969), slightly modified.

New Zealand white rabbits were immunized with the aforementioned KLH-conjugate following a well-established procedure (Vaitukaitis, 1981); care of animals was in accordance to the corresponding European legislation.

### 2.2. (Biotin/streptavidin) ELISA

Biotinylated Derivative for ELISA Coating**:** A biotinylated derivative of 2,4,6-TCPA was prepared on a solid support (Rink amide resin, Novabiochem/Merck) and purified with semi-preparative reversed-phase high performance liquid chromatography, following an approach previously reported by our team (Papasarantos *et al.*, 2010). The biotinylated probe was consequently used as the immobilized hapten in the (biotin/streptavidin) ELISA system.

ELISA Buffers: *Coating buffer*: 0.01 M phosphate buffer pH 7.4 (PB); *Washing buffer*: 0.01 M phosphate buffered saline pH 7.4, containing 0.05 % (v/v) Tween-20 (PBS-T); *Diluting Buffer A*: PBS-T containing 0.2 % (w/v) BSA; *Diluting Buffer B*: Diluting Buffer A containing 10 % (v/v) ethanol; *Diluting Buffer C*: Diluting Buffer A containing 5 % (v/v) ethanol.

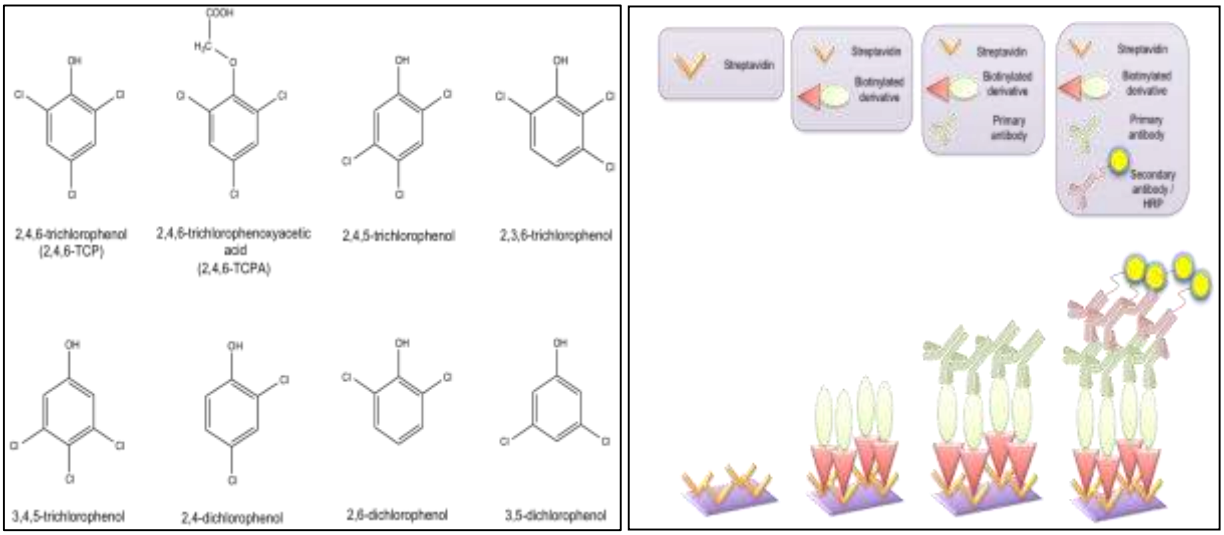
ELISA titration experiments: ELISA microwells were coated with streptavidin (Sigma, 10 μg/mL in coating buffer, overnight, 37°C). The following day, the wells were washed with 0.01 M PB, pH 7.4, blocked with a 2% BSA solution in PBS-T (1 h, room temperature), washed with PBS-T and incubated with the biotinylated derivative of 2,4,6-TCPA (100 ng/mL in diluting buffer A, 2 h, 37°C); then, the wells were washed as above described, incubated with serial dilutions of the anti-[2,4,6-TCP]-antisera (five consecutive bleedings) in diluting buffer C (2 h, 37°C), washed, incubated with anti-rabbit IgG/HRP (Sigma-Aldrich), diluted 1:1,000 in diluting buffer A (2 h, 37°C), washed, and finally incubated with ABTS (30 min, 37°C). The OD was measured (405 nm) in a microtiter plate reader (Sirio S, SEAK) and the titer curves were plotted.

ELISA displacement experiments: ELISA microwells were coated, blocked and incubated with the biotinylated derivative of 2,4,6-TCPA as described above. Then, the wells were washed and incubated (2 h, 37°C) with a 1:1, v/v, mixture of the anti-[2,4,6-TCP]-antiserum, suitably diluted in diluting buffer A, and a solution of either 2,4,6-TCP or of putative cross-reacting chlorophenols in diluting buffer B, at increasing concentrations; all chlorophenol-solutions were prepared by properly diluting a 10 mg/mL stock solution in ethanol. Afterward, the procedure described in the ELISA-titration experiments was followed and finally the displacement curves were plotted.

## 3. Results and discussion

Commercially available 2,4,6-trichlorophenoxyacetic acid (2,4,6-TCPA, Figure 1A) was used as starting material for preparing the immunizing hapten, following an approach previously reported by our team (Papasarantos *et al.*, 2010).

An in-house prepared biotinylated derivative of 2,4,6-TCPA was used for the development of a (biotin/streptavidin) ELISA system (Bayer and Wilchek, 1996; Neokosmidi *et al.*, 2004), the format of which is schematically described in Figure 1B; this assay was consequently employed for evaluating the immunochemical functionality of the anti-[2,4,6-TCP] antibody through titration and displacement experiments.




**(A)** **(B)**

**Figure 1: A.** Chemical structures of 2,4,6-TCPA, 2,4,6-TCP and various chlorophenols tested for cross-reactivity. **B.** Schematic representation of the main reagents and steps comprising the (biotin/streptavidin) ELISA system that was employed for evaluating the titer of the anti-[2,4,6-TCP] antibody.

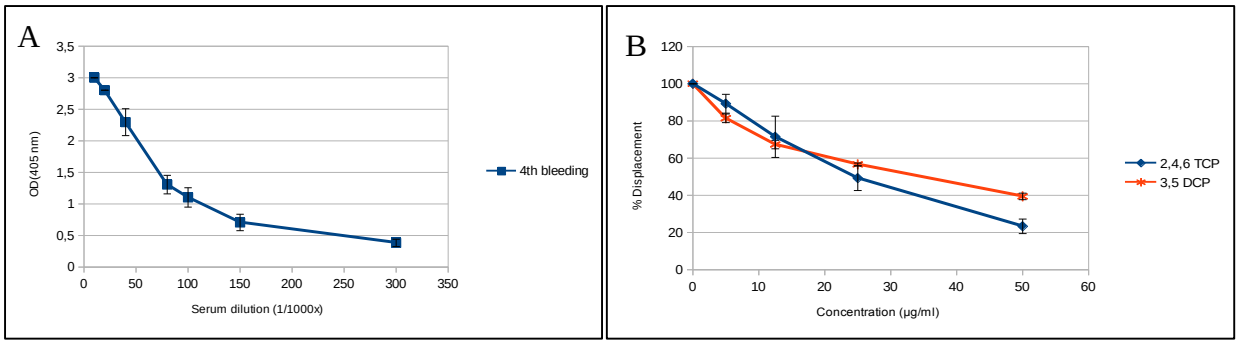



**Figure 2: A.** A typical ELISA-titer curve obtained with the anti-[2,4,6-TCP] antiserum (fourth bleeding). **B.** ELISA-displacement curves obtained with the anti-[2,4,6-TCP] antiserum in the presence of 2,4,6-TCP and 3,5-dichlorophenol at increasing concentrations.

As shown with the ELISA-titration experiments, the anti-[2,4,6-TCP] antibody developed has shown very high titer values; the antiserum corresponding to the fourth bleeding, which led to the highest titer value (~1:100,000, Figure 2A), was further used in the ELISA-displacement experiments. Cross-reactivity studies with various chlorophenols (2,4,5-trichlorophenol, 2,3,6-trichlorophenol, 3,4,5-trichlorophenol, 2,4-dichlorophenol, 2,6-dichlorophenol, 3,5-dichlorophenol, presented in Figure 1A) revealed that the antibody cross-reacted with 3,5-dichlorophenol (Figure 2B), while, practically, it did not cross-react with any of the trichlorophenols

tested; a tendency for cross-reaction with 2,4-dichlorophenol and 2,6-dichlorophenol was also observed, but only at the highest concentrations used. Cross-reactivity studies with few more chlorophenols as well as other pollutants bearing structural similarity with 2,4,6-TCP are currently underway.

## 4. Conclusions

In this work we present the in-house development and cross-reactivity evaluation of a polyclonal antibody for 2,4,6-TCP, a pollutant possibly carcinogenic to human and widely spread in the environment for which there are no easily available antibodies. The antibody developed may be used as a research tool for detecting 2,4,6-TCP in environmental and other samples *via* immunoanalytical systems, including highly sensitive immunosensors. Moreover, this antibody might be also suitable for the immunoanalysis of other chlorophenols, such as 3,5-dichlorophenol.

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## REFERENCES

1. Abuknesha R.A. and Griffith H.M.T. (2004) Evaluation of a polyclonal antiserum to pentachlorothiophenol-acetic acid-KLH immunogen: binding properties and use with heterologous PCP derivatives in ELISA for pentachlorophenol, Anal. Bioanal. Chem., **379**, 411-418.
2. Avrameas S. (1969) Coupling of enzymes to proteins with glutaraldehyde: use of the conjugates for the detection of antigens and antibodies, Immunochemistry, **6**, 43-52.
3. Bayer E.A. and Wilchek M. (1996) The Avidin-Biotin System. In: Immunoassay (E.P. Diamandis and T.K. Christopoulos, Eds), Academic Press, London, pp. 237-267.
4. Beloglazova N.V., Goryacheva I.Yu., Rusanova T.Yu, Yurasov N.A., Galve R., Marco M.-P. and De Saeger S. (2010) Gel-based immunotest for simultaneous detection of 2,4,6-trichlorophenol and ochratoxin A in red wine, Anal. Chim. Acta, **672**, 3-8.
5. Czaplicka M. (2004) Sources and transformations of chlorophenols in the natural environment, Sci. Total Environ., **322**, 21-39.
6. Galve R., Sanchez-Baeza F., Camps F. and Marco M.-P. (2002) Indirect competitive immunoassay for trichlorophenol determination – rational evaluation of the competitor heterology effect, Anal. Chim. Acta, **452**, 191-206.
7. Holford T.R.J., Davis F. and Higson S.P.J. (2012) Recent trends in antibody based sensors, Biosensors and Bioelectronics, **34**, 12-24.
8. Huff J. (2012) Long-term toxicology and carcinogenicity of 2,4,6-trichlorophenol, Chemosphere, **89**, 521-525.
9. Igbinosa E.O., Odjadjare E.E., Chigor V.N., Igbinosa I.H., Emoghene A.O., Ekhaise F.O., Igiehon, N.O. and Idemudia O.G. (2013) Toxicological profile of chlorophenols and their derivatives in the environment: the public health perspective, Sci. World J., 1-11.
10. Jiang X., Li D., Xu X., Ying Y., Li Y., Ye Z. and Wang J. (2008) Immunosensors for detection of pesticide residues, Biosensors and Bioelectronics, **23**, 1577-1587.
11. Neokosmidi A., Ragoussis V., Zikos C., Paravatou-Petsotas M., Livaniou E., Ragoussis N. and Evangelatos G. (2004) Synthesis of haptens and development of an immunoassay for the olive fruit fly pheromone, J. Agric. Food Chem., **52**, 4368-4374.
12. Nichkova M., Feng J., Sanchez-Baeza F., Marco M.-P., Hammock B.D. and Kennedy I.M. (2003) Competitive quenching fluorescence immunoassay for chlorophenols based on laser-induced fluorescence detection in microdroplets, Anal. Chem., **75**, 83-90.
13. Nistor C. and Emnéus J. (2003) A capillary-based amperometric flow immunoassay for 2,4,6-trichlorophenol, Anal. Bioanal. Chem., **375**, 125-132.
14. Noguera P., Maquieira A., Puchades R., Brunet E., Carramolino M.M. and Rodríguez-Ubis J.C. (2002) Development of an enzyme-linked immunosorbent assay for screening contamination by chlorophenols in environmental waters, J. Environ. Monitor., **4**, 442-448.

15. Olaniran A.O. and Igbinosa E.O. (2011) Chlorophenols and other related derivatives of environmental concern: properties, distribution and microbial degradation processes, Chemosphere, **83**, 1297-1306.
16. Papasarantos I., Klimentzou P., Koutrafouri V., Anagnostouli M., Zikos C., Paravatou-Petsotas M. and Livaniou E. (2010) Solid-phase synthesis of a biotin-derivative and its application to the development of anti-biotin antibodies, Appl. Biochem. Biotechnol., **162**, 221-232.
17. Shreder K. (2000) Synthetic haptens as probes of antibody response and immunorecognition, Methods, **20**, 372-379.
18. Vaitukaitis J.L. (1981) Production of antisera with small doses of immunogen: multiple intradermal injections, Methods Enzymol., **73**, 46-52.