Articles

Biological Monitoring of 2,4,5-Trichlorophenol (I): Preparation of Antibodies and Development of an Immunoassay Using Theoretical Models

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Antibodies against 2,4,5-trichlorophenol have been prepared after theoretical and molecular modeling chemical studies of three potential immunizing haptens with the aim to find out the one mimicking best the target analyte. Competitive direct and indirect ELISAs have been developed after screening a battery of haptenized enzyme tracers and coating antigens, respectively. The relation between the degree of heterology of the competitor and the resulting immunoassay detectability has been investigated according to the electronic similarities of the competitor haptens with the target analyte taking in consideration their pK_a values. These studies have been performed using theoretical and molecular modeling tools to find out their electronic distribution at their minimum energetic levels. The results suggest that the competitors should have a high homology to produced assays with good detectability values. On the other hand detectability improves when lowering the hapten density of the competitors. An indirect competitive ELISA has been finally selected for further investigation. The immunoassay has an IC_{50} value of 0.6 μg L^{-1} and a limit of detection of 0.084 μg L^{-1}. The selectivity of the assay is high in relation to other chlorophenols frequently present in real samples. In contrast, the brominated analogues may also be recognized with this assay.

Introduction

Chlorophenol-related compounds have been used for over 35 years as fungicides, pesticides, and for many other agricultural, industrial, and commercial uses. Particularly, 2,4,5-trichlorophenol (2,4,5-TCP) has been an intermediate in the manufacture of industrial and agricultural chemicals, such as the phenoxy acid herbicides 2,4,5-T (2,4,5-trichlorophenoxyacetic acid), Silvex [2-(2,4,5-trichlophenoxy)propionic acid, 2,4,5-TP], Ronnel (*o,o*-dimethyl-*o*-2,4,5-trichlorophenyl phosphorothionate), and sodium 2,4,5-trichlorophenate (*1*). Additionally, 2,4,5-TCP and its salts have been used as antifungic and antimicrobial preservatives for adhesive products, synthetic textiles, rubber, wood, paints, in the leather industry and in pulp and paper mills. All technical and formulated products are contaminated in varying degrees by the most toxic dioxin isomer, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), at levels sometimes exceeding 1 ppm in chlorophenoxyacid herbicides (*2*) and 2,4,5-TCP products (*3*). Chlorophenols are also formed during combustion of organic material, in pulp and paper industries using chlorine-bleaching processes, and as byproducts during water chlorinating procedures.

Even though the discharge of chlorophenols to the environment has decreased in the past decade for the developed countries, their persistence has determined its widespread distribution in the environment still today and the repeated exposure of the general population to low concentrations of these chemicals. Thus, chlorophenols have been identified as usual contaminants of surface (*4*−*7*) and groundwater (*8*, *9*) samples. Similarly, trichlorophenols have been found to contaminate soils and sediments (*7*, *10*−*12*). Consequently, drinking water may also contain 2,4,5-TCP between other chlorinated phenolic compounds at low levels (*7*, *9*, *13*−*15*) and significant levels accumulate in fish and seafood (*5*, *16*). Thus, a bioconcentration factor of 250−310 has been estimated in fish and concentrations around 35−59 μg L^{-1} have been detected in the tap water of certain locations in Finland (*15*).

The general population is exposed through contaminated environment, textiles, leather goods, domestic preservatives, and edible products (*17*, *18*). Intake may occur dermally, orally or via respiratory tract. Several studies performed in Germany and in the U.S. show that 2,4,5-TCP can be found the in the urine of a high percentage of the population (*18*−*20*). Thus, in 1994 the frequency of detection of 2,4,5-TCP in the urine of adults living in the U.S. was 20% with a 95th percentile and maximum urinary concentrations of 3 and 25 μg L^{-1} (*21*). Occupational exposure occurs through inhalation and dermal contact with this compound at workplaces where 2,4,5-TCP is used or produced. The NIOSH (National Institute of Occupational Safety and Health) statistically

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estimated that 758 workers were potentially exposed to 2,4,5-TCP during a 3 years period NOES survey 1981−1983 (*22*).

2,4,5-TCP is within the five chlorophenols out from 19 considered to have significant toxicological effects and potential carcinogenicity (*23*). An oral Minimal Risk Level (MRL) of 0.003 mg/kg/day would be applicable for an intermediate exposure duration according to the updated toxicological profile for chlorophenols of the Agency for Toxic Substances and Disease Registry (ATSDR) of the U.S. Department of Health and Human Services (*24*). A recent study among U.S. men aged 30−60 demonstrated that the occupational exposure to chlorophenol is a risk factor for nasal and nasopharyngeal cancers closely associated with the duration of exposure (*25*). On the other hand, long-term use of chlorophenol polluted household drinking water and consumption of contaminated fish have been correlated with certain symptoms, related to those occurred after chlorophenol occupational exposure, such as gastrointestinal and skin symptoms (*15*).

2,4,5-TCP may also appear in urine of people exposed to hexachlorobenzene (HCB) (*26*), hexachlorocyclohexane (HCH, lindane) (*27*), certain organophosphorus insecticides (*21*, *28*), and chlorophenoxyacid herbicides (*29*). Thus, detection of 2,4,5-TCP in urine may be an indicator of exposure to chlorophenols and to the above-mentioned substances frequently used in industry, commerce, agriculture and in private households for a variety of reasons (*30*). On the other hand, a continuous excretion of 2,4,5-TCP may also indicate a risk for dioxin exposure (*3*, *31*, *32*).

Determination of the actual chlorophenol intake by the population would provide a reliable estimation of the individual health risk due to environmental and occupational exposure. Laboratory methodsfor 2,4,5-TCP determination are based on chromatographic techniques, usually gas chromatography coupled to electron capture (ECD) or mass spectrometry (MS). Thus, these methodologies have allowed detecting 2,4,5-TCP in human urine and blood serum (*33*−*35*) at low ppb level, but lack of the necessary speed for rapid screening the large number of samples involved on continuous biomonitoring programs. Rapid, inexpensive, and reliable techniques are essential for the routine risk assessment of human and environmental exposure to hazardous chemicals. Immunochemical methods may fulfill the efficiency requirements due to their simplicity, specificity, high detectability, and high throughput sample processing capabilities.

The key step of the development of an immunoassay for small molecules is the hapten design (*36*−*39*). Both chemical structures, of the immunizing hapten and of the competitor, may play an important role on the immunoassay features. The introduction of the spacer arm in the molecule may cause deformations of the molecular geometry as well as changes in its electronic distribution. On the other hand, often the chemical synthesis of the hapten is one of the most time-consuming steps of the antibody production process. Therefore, the synthetic effort necessary to produce antibodies against an organic molecule should be carefully evaluated in relation to the chances to obtain a good antibody. Nowadays, it is possible to make computer-assisted theoretical and molecular modeling studies in order to predict the suitability of a particular chemical structure as hapten to raise antibodies against the target analyte (*39*−*43*). In a previous paper, we demonstrated the convenience of using theoretical studies to choose the most appropriate immunizing hapten to raise antibodies against 2,4,6-trichlorophenol (*44*). Similarly, we have demonstrated that the immunoassay specificity and detectability can also be modulated by the chemical structure of the competitor hapten (*45*, *46*). With these precedents, we report here the preparation of antibodies for 2,4,5-TCP and the development of an immunoassay. Theoretical models have been used with the aim to achieve sufficient specificity and detectability to assess human exposure in environmental and biological monitoring programs.

Experimental Section

Molecular Modeling and Theoretical Calculations. Molecular modeling was performed using the Hyperchem 4.0 software package (Hyperube Inc, Gainesville, FL). Theoretical geometries and electronic distributions were evaluated for 2,4,5-trichlorophenol and the haptens using semiempirical quantum mechanics MNDO (*47*) and PM3 (*48*) models. All the calculations were performed using standard computational chemistry criteria. Theoretical calculations regarding pK_a values were carried out using the ACD/pK_a 1.2 software package (Advanced Chemistry Development Inc., Toronto, ON, Canada) at the Department of Analytical Chemistry (University of Lund, Sweden).

Synthesis of the Haptens. The preparation of hapten **A** [*3-(2-hydroxy-3,5,6-trichlorophenyl)propanoic acid*] and haptens **1** (*2,4,6-trichlorophenoxyacetic acid*), **2** (*2,4,5-trichlorophenoxyacetic acid*), **3** [*3-(2-hydroxy-3,5,6-trichlorophenyl)-2-propenoic acid*], **5** [*3-(3-hydroxy-2,4,6-trichlorophenyl)propanoic acid*], **7** [*3-(4-hydroxy-3,5-dichlorophenyl)propanoic acid*], **8** [*3-(2-hydroxy-3,6-dichloropheny)propanoic acid*], **9** (*3-hydroxy-2,6-dichlorophenylacetic acid*), and **10** (*3-hydroxy-4,6-dichlorophenylacetic acid*) has already been reported by our group (*36*, *45*, *49*). Hapten **B** (*2,4,5-trichlorophenoxypentanoic acid*) was synthesized following the procedure described by Kramer et al. (*50*). Hapten **4** (*2-hydroxy- 3,5,6-trichlorobenzoic acid*), as well as other chemical reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI). Finally haptens **11** and **12** were prepared as follows.

(1) General Methods and Instruments. Thin layer chromatography (TLC) was performed on 0.25 mm, precoated silica gel 60 F254 aluminum sheets (Merck, Gibbstown, NJ). Unless otherwise indicated purification of the reaction mixtures was accomplished by "flash" chromatography using silicagel as stationary phase. ^{1}H and ^{13}C NMR spectra were obtained with a Varian Unity-300 (Varian Inc., Palo Alto, CA) spectrometer (300 MHz for ^{1}H and 75 MHz for ^{13}C). Infrared spectra were measured on a Bomen MB120 FTIR spectrophotometer (Hartmann & Braun, Québec, Canada).

(2) 3-Chloro-2-methylphenoxyacetic Acid 11. A solution of methyl bromoacetate (0.35 mL, 3.7 mmol) was added dropwise to a mixture of 3-chloro-2-methylphenol (500 mg, 3.51 mmol) and anhydrous K_2CO_3 (600 mg, 4.35 mmol) in dry acetone (15 mL). The mixture was refluxed for 5 h until the initial product was no more detected by TLC. The solution was acidified with 1 N HCl and extracted with Et_2O. The organic phase was washed with saturated NaCl, dried with $MgSO_4$, filtered and distilled under vacuum to obtain 680 mg of *methyl 3-chloro-2-methylphenoxyacetate* (90% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.33 (s, 3H, -CH_3), 3.79 (s, 3H, -OCH_3), 4.59 (s, 2H, -CH_2-), 6.66 (dd, J = 8.8 Hz, J = 3 Hz, 1H$_{Ar}$ *ortho*), 6.79 (d, J = 3 Hz, 1H$_{Ar}$ *ortho*), 7.22 (d, J = 8.7 Hz, 1H$_{Ar}$ *meta*). ^{13}C NMR (75 MHz, $CDCl_3$) δ 20.2 (-CH_3), 52.1 (-OCH_3), 65.4 (C-2), 113.1 (C-2′), 117.3 (C-6′), 126.9 (C-4′), 129.6 (C-3′), 137.2 (C-5′), 156.3 (C-1′), 169.1 (C-1). A solution of 0.5 N NaOH (17 mL, 8.5 mmol) was added to a solution of the ester (300 mg, 1.39 mmol) in THF (20 mL). The reaction mixture was stirred for 5 h at room temperature until the complete disappearance of the starting material by TLC. The solvent was evaporated and the residue dissolved in

0.5 N NaOH (aq), washed with Et_2O and acidified with 1 N HCl. The aqueous layer was extracted with ethyl acetate, dried with $MgSO_4$, filtered, and evaporated to dryness under reduced pressure to obtain 270 mg of the desired acid **11** (96% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.28 (s, 3H, -CH_3), 4.64 (s, 2H, -CH_2-), 6.76 (dd, J = 9 Hz, J = 3 Hz, 1H_{Ar} *ortho*), 6.92 (d, J = 3 Hz, 1H_{Ar} *ortho*), 7.27 (d, J = 9 Hz, 1H_{Ar} *meta*). ^{13}C NMR (75 MHz, DMSO-d_6) δ 19.5 (-CH_3), 64.6 (C-2), 113.5 (C-2′), 117.2 (C-6′), 126.9 (C-4′), 129.3 (C-3′), 136.3 (C-5′), 156.4 (C-1′), 169.7 (C-1).

(3) 2-Chlorophenoxyacetic Acid 12. As described for the compound **11**, a solution of methyl bromoacetate (0.38 mL, 3.9 mmol) was added to a mixture of 2-chlorophenol (500 mg, 3.89 mmol) and anhydrous K_2CO_3 (650 mg, 4.71 mmol) in dry acetone (16 mL) to obtain 740 mg of *methyl 2-chlorophenoxyacetate* (95% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.80 (s, 3H, -OCH_3), 4.61 (s, 2H, -CH_2-), 6.79 (d, J = 8.2 Hz, 1H_{Ar} *ortho*), 6.90 (s, 1H_{Ar} *ortho*), 6.97 (d, J = 8 Hz, 1H_{Ar} *para*), 7.20 (dd, J = 8 Hz, J = 8 Hz, 1H_{Ar} *meta*). ^{13}C NMR (75 MHz, $CDCl_3$) δ 52.2 (-OCH_3), 65.3 (C-2), 113.0 (C-2′), 115.2 (C-6′), 122.0 (C-4′), 130.3 (C-3′), 135.0 (C-5′), 158.4 (C-1′), 168.9 (C-1). As described before, a solution of 0.5 N NaOH (18 mL, 9 mmol) was added to a solution of the ester (300 mg, 1.5 mmol) in THF (22 mL), and 250 mg of the desired acid **12** were obtained (90% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.70 (s, 2H, -CH_2-), 6.89 (d, J = 8.5 Hz, 1H_{Ar} *ortho*), 6.98 (s, 1H_{Ar} *ortho*), 6.99 (d, J = 8.5 Hz, 1H_{Ar} *para*), 7.29 (dd, J = 8.5 Hz, J = 8.5 Hz, 1H_{Ar} *meta*). ^{13}C NMR (75 MHz, DMSO-d_6) δ 64.7 (C-2), 113.5 (C-2′), 114.6 (C-6′), 120.9 (C-4′), 130.6 (C-3′), 133.6 (C-5′),, 158.6 (C-1′), 169.6 (C-1).

Preparation of Protein Conjugates. *Immunogens*: Haptens **A** and **B** were covalently attached through their carboxylic group to the lysine residues of KLH (keyhole limpet hemocyanin) by the mixed anhydride (MA) method as described by Ballesteros et al. (*43*). Briefly, each hapten (hapten **A**, 15.5 mg, 0.04 mmol and hapten **B** 15.5 mg, 0.04 mmol) was reacted with tributylamine (10.5 μL, 0.04 mmol) and isobutylchloroformate (6.4 μL, 0.048 mmol) in anhydrous DMF (dimethylformamide, 200 μL) and added to KLH (10 mg). Simultaneously, BSA (bovine serum albumin) conjugates were prepared by the same procedure reacting hapten **A** (20 μmol) and hapten **B** (20 μmol) with 10 mg of the protein. *Enzyme tracers (ET)*: Haptens **1**−**5** and **7**−**12** (10 μmol) were coupled to HRP (2 mg) using the active ester (AE) method as reported (*43*, *44*). *Coating antigens (CA): Active Ester (AE) Method*. Following described procedures (*45*, *49*, *51*) the haptens **1**−**3**, **5**, and **7**−**12** (10 μmol) were covalently attached to BSA, OVA (chicken egg ovalbumin) and CONA (chicken egg conalbumin) (10 mg/each) in a hapten:protein (lys) molar ratio 2:1. Haptens **5** and **7** were conjugated to BSA and OVA using different hapten:protein (lys) ratios (1:1, 1:2.5, 1:5, and 1:10). *Mixed Anhydride (MA) Method*. Hapten **5** was conjugated to the same proteins at different hapten:protein (lys) molar ratios (2:1, 1:1, 1:2.5, 1:5). In both methods, the protein conjugates were purified by dialysis against 0.5 mM PBS (4 × 5 L) and milliQ water (1 × 5 L) and stored freeze-dried at −40 °C. Unless otherwise indicated working aliquots were stored at 4 °C in 10 mM PBS at 1 mg mL^{-1}.

Hapten Density Analysis. Hapten densities of the protein conjugates were determined by matrix-assisted laser-desorption ionization time-of-fly mass spectrometry (MALDI-TOF-MS) by comparing the molecular weights of the unreacted proteins with those of the conjugates. The MALDI-MS (matrix-assisted laser desorption ionization mass spectrometer) used for analyzing the protein conjugates was a Perspective Biosystems Time-of-Flight (TOF) Mass Spectrometer Voyager-DE RP equipped with a laser unit which operates with an intensity of 2800. The instrument is controlled by a BioSpectrometry Workstation provided with the software Voyager-DE-RP (version 4.03) developed by Perspective Biosystems Inc. (Framingham, MA) and GRAMS/386 (for Microsoft Windows, version 3.04, level III) developed by Galactic Industries Corporation (Salem, NH). MALDI spectra were obtained by mixing 2 μL of the matrix (*trans*-3,5-dimethoxy-4-hydroxycinnamic acid, 10 mg mL^{-1} in CH_3CN/H_2O 70:30, 0.1% TFA) with 2 μL of a solution of the conjugates or proteins (10 mg mL^{-1} in CH_3CN/H_2O 70:30, 0.1% TFA).

Polyclonal Antisera. Three female New Zealand white rabbits (As53−As55) were immunized with A-KLH and other three rabbits (As56−As58) were immunized with B-KLH as reported (*43*). Evolution of the antibody titer was assessed by measuring the binding of serial dilutions of the antisera to microtiter plates coated with A-BSA and B-BSA. After an acceptable antibody titer was observed, the animals were exsanguinated and the blood collected on vacutainer tubes with a serum separation gel. Antiserum was obtained by centrifugation and stored at −80 °C in the presence of 0.02% NaN_3. The antisera (As) were used without further purification. Working aliquots were stored at 4 °C.

Immunochemistry. (1) General Methods and Instruments. The pH and the conductivity of all buffers and solutions were measured with a pH-meter pH 540 GLP and a conductimeter LF 340, respectively (WTW, Weilheim, Germany). The centrifuge, model 5415D was from Eppendorf (Germany). Polystyrene microtiter plates were purchased from Nunc (Maxisorp, Roskilde, D.K.). Washing steps were carried out using a SLY96 PW microplate washer (SLT Labinstruments GmbH, Salzburg, Austria). Absorbances were read on a SpectamaxPlus microplate reader (Molecular Devices, Sunnyvale, CA). The competitive curves were analyzed with a four-parameter logistic equation using the software SoftmaxPro v2.6 (Molecular Devices) and GraphPad Prism (GraphPad Software Inc., San Diego, CA). Unless otherwise indicated, data presented correspond to the average of at least two well replicates.

(2) Chemicals and Immunochemicals. Immunochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Standards for cross-reactivity studies were purchased from Aldrich Chemical Co. (Milwaukee, WI).

(3) Buffers. PBS is 10 mM phosphate buffer, 0.8% saline solution and unless otherwise indicated the pH is 7.5. Borate buffer is 0.2 M boric acid−sodium borate, pH 8.7. Coating buffer is 50 mM carbonate-bicarbonate buffer pH 9.6. PBST is PBS with 0.05% Tween 20. Citrate buffer is a 40 mM solution of sodium citrate pH 5.5. The substrate solution contains 0.01% TMB (tetramethylbenzidine) and 0.004% H_2O_2 in citrate buffer.

(4) Noncompetitive Direct ELISA. The avidity of the As53−As58 versus the enzyme tracers was determined by measuring the binding of serial concentrations (1 to 0.015 μg mL^{-1} in PBST, 100 μL/well) of each enzyme tracer (ET) to microtiter plates coated overnight at 4 °C with 12 different dilutions (1/1000 to 1/1024000 in coating buffer, 100 μL/well) of each of As. The ET solutions were incubated in the coated plates for 30 min at RT. The plates were then washed four times with PBST, and the substrate solution (100 μL/well) was added and incubated 30 min at room temperature before the enzymatic reaction was stopped by adding 4 N H_2SO_4 (50 μL/well).

(5) Noncompetitive Indirect ELISA. The avidity of the different As53−As58 for the coating antigens was determined by measuring the binding of serial dilutions (from 1/1000 to 1/64000 in PBST; 100 μL/well) of each As to microtiter plates coated overnight at 4°C with 12 different concentrations (from 10 to 0.005 μg mL^{-1} in coating buffer; 100 μL/well) of the **1**−**5** and **7**−**12** −BSA, −CONA, and −OVA conjugates. The As solutions were incubated in the coated plates for 30 min at room temperature. After that, the plates were washed four times with PBST, and a solution of goat anti-rabbit IgG coupled to horseradish peroxidase (antiIgG-HRP, 1/6000 in PBST) was added to the wells (100 μL/well) and incubated for 30 min at room temperature. The plates were washed again and the substrate solution (100 μL/well) was added. Color development was stopped after 30 min at RT with 4 N H_2SO_4 (50 μL/well) and the absorbances were read at 450 nm. Optimal concentrations for coating antigens and As dilution were chosen to produce around 0.7 to 1 units of absorbance in 30 min.

(6) Competitive Indirect ELISA. Microtiter plates were coated with **7**-OVA (AE) (1:2.5) (0.6 μg mL^{-1} in coating buffer,

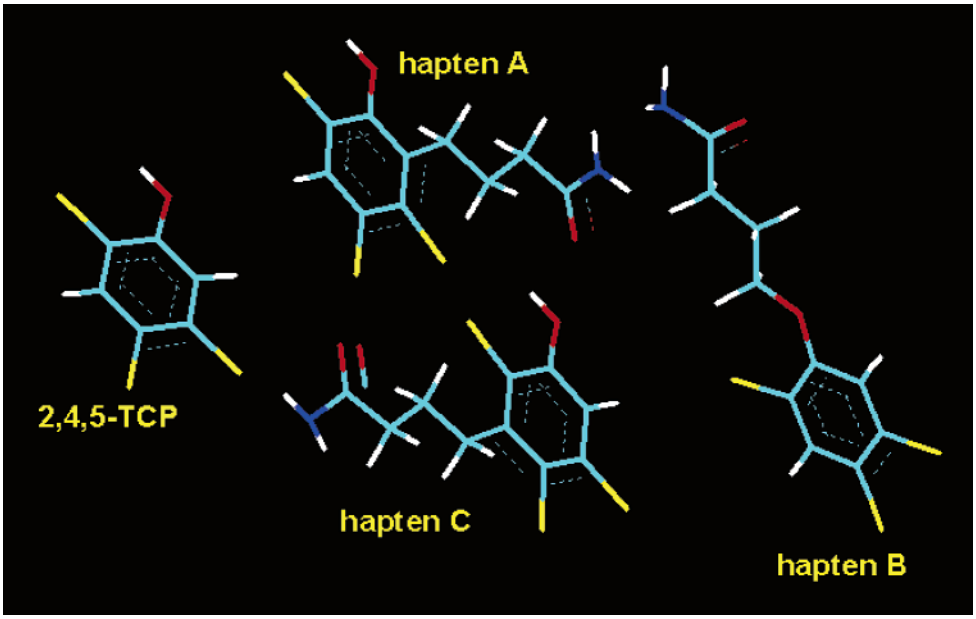


Figure 1. Stick display of the optimized geometries of 2,4,5-TCP and haptens A, B, and C according to MNDO models. Calculations have been made using the corresponding amide derivatives to mimic the conjugated haptens. The elements are presented in the following manner: light blue, carbon; dark blue, nitrogen; white, hydrogen; red, oxygen; yellow, chlorine.

100 μl/well) overnight at 4 °C covered with adhesive plate sealers. The following day the plates were washed with PBST (five times, 300 μL/well). 2,4,5-TCP standards (400 to 0.04 nM, prepared in PBS) or samples were added to the coated plates (50 μL/well) followed by the sera As53 (1/1000 in PBST, 50 μL/well). After 30 min of incubation at RT, a solution of anti IgG-HRP (1/6000 in PBST) was added (100 μL/well) and the mixture incubated for 30 min more at RT. The plates were processed as described above.

(7) Specificity Studies. Stock solutions of different phenolic compounds were prepared (1 mM in DMSO) and stored at 4 °C. Standard curves were prepared in PBS (10 000 nM to 0.04 nM) and each IC_{50} determined in the competitive experiment described above. The cross-reactivity values were calculated according to the following equation: (IC_{50} trichlorophenol/IC_{50} phenolic derivative) × 100.

Results and Discussion

Hapten Design. The spacer arm necessary to link the molecule to the protein could be introduced in three different positions of the chemical structure of 2,4,5-TCP. Figure 1 shows these chemical structures at their minimum energetic levels after using MNDO models. The introduction of the spacer arm does not produce significant conformational changes since the three chemicals conserve their planar geometries. Thus, in hapten **A**, the spacer arm substitutes the hydrogen atom of the *ortho* position. Although the linker is apparently too close to the phenolic group, this hapten keeps the three chlorine atoms at the same positions as the target analyte and the phenolic group free. In hapten **B**, the aromatic ring does not suffer any apparent geometric modification, and the spacer arm is placed through the oxygen atom thus maximizing the recognition of the aromatic group and the particular distribution of the chlorine atoms. Finally, hapten **C** respects the phenolic group by introducing the spacer arm at the *meta* position of the aromatic ring.

As demonstrated with theoretical studies in our previous paper (*44*), the real differences between these haptens and the target analyte are observed on the electronic distribution of these molecules, especially considering the acid−base equilibrium of the phenolic compounds in aqueous media ($ArOH \rightarrow ArO^- + H^+$). Thus, punctual charges in the aromatic ring for both species (acid and conjugated base) were calculated at their minimum energetic conformations. Figure 2 shows the results obtained for each atom of the ring and the sum of those charges. It can be observed that there are almost no differences in the punctual charges between the analyte and haptens **A**, **B**, and **C** when we do consider them as organic acids. Only the balance of the total charge is slightly more positive for hapten B (see Figure 2, left). Quite the opposite, the punctual charges vary significantly considering the haptens as conjugated bases. As phenoxides, the charges of the target analyte and haptens **A** and **C** follow the same pattern and the total charge is more negative. In contrast, hapten B having the hydroxyl group blocked cannot participate in this equilibrium, keeping the same punctual distribution independently from the pH of the media and the total charge remains positive (see Figure 2, right).

Haptens **A** and **C**, thus, seem to better mimic the analyte regarding both geometry and punctual charges independently from a media with a pH higher or lower than the respective pK_a. Nevertheless, to apply these considerations the tendency of these organic acids to give protons to the media should be the same, that means all three compounds must have similar pK_a values. According to the Gibbs equation, on proton transfer reactions, the deprotonation enthalpy (DPE) is directly related to the pK_a. Dewar et al. proved that semiempirical theoretical models provide reliable data regarding PA (proton affinities) and DPE values if compared with experimental data (*52*). Using MNDO semiempirical models, we have been able to determine the formation enthalpies (ΔH_0) of 2,4,5-TCP, hapten **A** and hapten **C** as organic acids [$Hf_p(ArO^-)$] and as conjugated bases [$Hf_r(ArOH)$]. Since,

$$\Delta H_0 = \Delta Hf_p - \Delta Hf_r$$

where ΔHf_r is the formation enthalpy of the reagents and ΔHf_p is the formation enthalpy of the products, the DPE values of the different proton-transfer equilibria can be

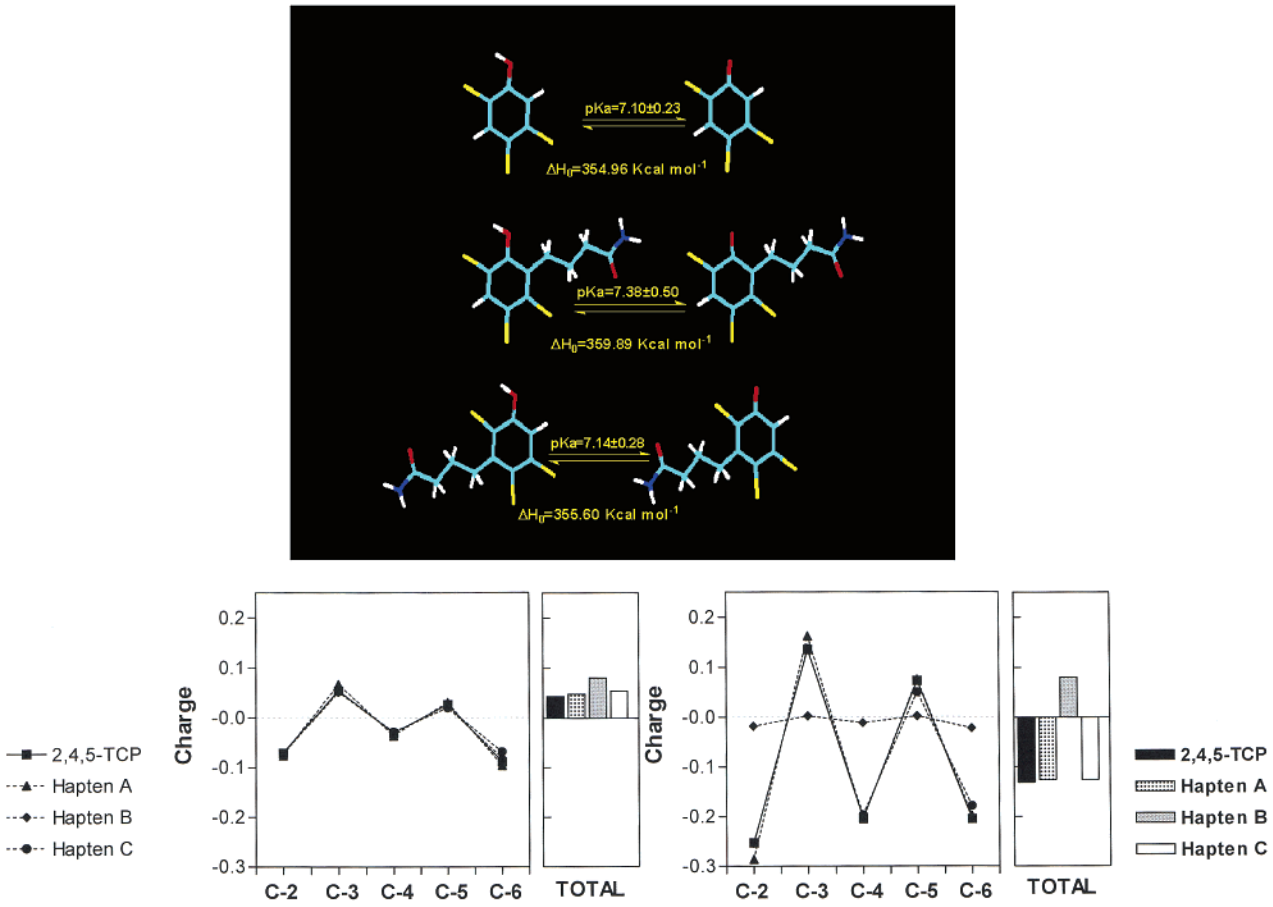


Figure 2. Total charge and punctual charge of 2,4,5-TCP and haptens A, B, and C as phenols (left) and phenoxides (right). The relative amounts of these two forms depend on the corresponding pK_a values (see Table 3). The bars show the total charge. Haptens A and C mimic better the behavior of the target analyte in both situations. On top there are shown the acid−base equilibria and the calculated deprotonation enthalpies (DPE) using MNDO semiempirical models and pK_a values. The elements are presented in the following manner: light blue, carbon; dark blue, nitrogen; white, hydrogen; red, oxygen; yellow, chlorine. For the graphs, the carbon atoms have been numbered clockwise starting with the atom that supports the oxygen.

calculated according to the following equation:

$$\Delta H_0 = [\Delta Hf_p(H^+) + \Delta Hf_p(ArO^-)] - \Delta Hf_r(ArOH) = DPE$$

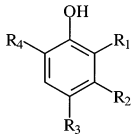
According to the literature the formation enthalpy of the H^+ is 367.2 kcal/mol (*52*, *53*). Figure 2 (top) shows the DPE calculated for 2,4,5-TCP and haptens **A** and **C** deprotonation equilibrium reactions using a MNDO semiempirical models. The DPE values clearly indicate the acidic character of these compounds, but the differences encountered between the calculated values did not show large variations. This fact was additionally supported by theoretical calculations made for the pK_a values using a suitable software (see Figure 2, top). Although hapten **C** and 2,4,5-TCP had a slightly closer pK_a and DPE values, overall we can conclude that both haptens **A** and **C** mimic reasonably well the properties of the target analyte.

From the synthetic point of view, the preparation of hapten **C** introducing the spacer arm at the *meta* position of the target analyte is more difficult. Regioselective nucleophilic substitution at *meta* positions of phenolic substances has been reported by preparing first arene-tricarbonyl-chromium (0) complexes (*54*) of the corresponding aromatic compound. Another strategy could go beyond using 3(3-hydroxyphenyl)-propenoic acid as starting material (possessing already the spacer arm in *meta*) and introducing the chlorine atoms in the last step (*44*). However, the idea of introducing a chlorine atom at the less active *meta* position of the aromatic ring seemed also tricky. In contrast, the introduction of an alkylamino group in *ortho* of 2,4,5-trichlorophenol, as it appears in hapten **A**, had already been reported by Stokker et al. for the preparation of a new class of saluretic agents (*55*). Thus, following a similar procedure, we were able to accomplish the preparation of 3-(2-hydroxy-3,5,6-trichlorophenyl)propanoic acid (hapten **A**) with a moderate yield (*45*, *49*)

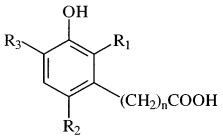
Polyclonal Antisera. Hapten **A** was coupled to KLH and BSA following the mixed anhydride method (MA) and the KLH conjugate was used to raise antibodies in three white New Zealand rabbits. The obtained antisera were named As53, As54, and As55. Antibodies against hapten B (As56, As57, and As58) were already available in our group. Thus, with the aim to prove the results of the theoretical studies we decided to evaluate both groups of antisera for their ability to bind 2,4,5-TCP on a competitive ELISA.

Direct ELISA. Haptens **A**, **1**−**5**, and **7**−**12** (see Table 1 for chemical structures) were coupled to HRP and tested in a noncompetitive direct ELISA format. Recognition was observed only for the homologous or quasi-homologous enzyme tracers. Thus, As53−As55 (**A**-KLH) did only recognize **A**-HRP with an acceptable titer while 5-HRP was only recognized by As55. As56−As58 (**B**-

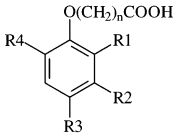
Table 1. Chemical Structures of 2,4,5-TCP and Phenolic Haptens



Hapten	R_1	R_2	R_3	R_4
2,4,5-TCP	H	Cl	Cl	Cl
A	$(CH_2)_2COOH$	Cl	Cl	Cl
3	CH=CHCOOH	Cl	Cl	Cl
4	COOH	Cl	Cl	Cl
7	Cl	H	$(CH_2)_2COOH$	Cl
8	$(CH_2)_2COOH$	Cl	H	Cl



Hapten	n	R_1	R_2	R_3
5	2	Cl	Cl	Cl
9	1	Cl	Cl	H
10	1	H	Cl	Cl



Hapten	n	R_1	R_2	R_3	R_4
B	2	H	Cl	Cl	Cl
1	1	Cl	H	Cl	Cl
2	1	H	Cl	Cl	Cl
11	1	H	CH_3	Cl	H
12	1	H	Cl	H	H

Table 2. Features of the Competitive Direct ELISAs

As[a]	ET[b]	A_{max}	A_{min}	slope	IC_{50}[c]	r^2
53	**A**-HRP	0.81	0	−0.43	18.87	0.989
54	**A**-HRP	0.63	0.06	−0.89	165.48	0.985
55	**A**-HRP	1.27	0.21	−1.03	21.62	0.981
	5−HRP	0.94	0.14	−0.97	15.84	0.971

[a] As, antisera. [b] ET, enzyme tracer. [c] IC_{50} values are expressed in μg L^{-1}. Antisera 56−58 raised against hapten B-KLH did not afford any competitive immunoassay.

KLH) did only recognize **2**-HRP that only differs from hapten B in the length of the spacer arm. However these last As/enzyme tracer combinations produced only a maximum absorbance of 0.3 units when the As was diluted 1/500 times and the enzyme tracer was used at a concentration of 2 μg mL^{-1}. Thus, we focused on the antisera raised against hapten **A** to test the ability of this antiserum to bind 2,4,5-TCP on a competitive assay. All the combinations gave competitive assays although the detectability was too poor for our purposes. Table 2 shows the features of these immunoassays. The best assay gave a detectability of 16 μg L^{-1} in the middle point of the assay. Therefore, we decided to explore the indirect ELISA format with the aim to find a coating antigen/antiserum combination with a better detectability. Although the indirect format introduces an additional step in the procedure, the absence of the enzyme during the competition made it more promising regarding possible matrix effects from the real biological samples (*36*).

Indirect ELISA. (1) Screening of the Antiserum Avidity for the Immunoreagents. Haptens **1**−**4** and **7**−**12** were initially conjugated to BSA, OVA, and CONA by the active ester (AE) method, and hapten **5** was conjugated to the three proteins by the mixed anhydride (MA) method. The hapten densities of the BSA conjugates determined by MALDI-TOF-MS were quite high (see Table 3).

Table 3. Relative Similarities between the Target Analyte and the Haptens Regarding Punctual Charge Distribution

hapten	pK_a[a]	RO^- (%)[b]	RMSE,[c] pH 7.5	hapten density[d]
1	-	-	0.436	30
2	-	-	0.425	25
3	6.59	89.05	0.081	14
4	5.51	98.99	0.113	16
5	6.63	88.11	0.120	7
7	7.37	57.43	0.157	25
8	7.82	32.37	0.224	17
9	7.99	24.45	0.320	21
10	7.99	24.45	0.249	26
11	-	-	0.411	27
12	-	-	0.421	28
A	7.38	56.86	0.093	10
2,4,5-TCP	7.1	71.5		-

[a] pK_a values of the haptens are calculated in their amide form. [b] Percentage of the phenoxide species when the pH is 7.5. [c] The similarity between the haptens and the analyte has been expressed as the root-mean-square error (RMSE) of the differences between the punctual charges of the corresponding atoms of both molecules (hapten vs analyte) at pH 7.5. The punctual charge on each atom was calculated considering the percentage of the neutral molecule and their anion form at pH 7.5 according to their pK_a values. [d] Hapten densities of the BSA conjugates measured by MALDI-TOF-MS. The data corresponds to conjugates synthesized by the active ester method, except for hapten 5 that corresponds to the conjugate prepared by the mixed anhydride method. In both methods the hapten:protein molar ratio employed during the conjugation reaction 2:1 in terms of lysine residues of the protein. These data has only been included as reference.

The avidity of As53−As58 for the protein conjugates was evaluated by two-dimensional checkerboard titration experiments (data non shown). Similarly to the direct ELISA, it was observed that As56−As58 (immunizing hapten **B**) had lower avidity for the protein conjugates than As53−As55 (immunizing hapten **A**). Taking into account that the pH value of the assay buffer in these experiments was 7.5, we can attribute this divergence to the marked differences in the punctual charges of the aromatic ring when the hydroxyl group is blocked (see above, Figure 2). Thus, the competitors of phenyl-akyl ether type, such as haptens **1**, **2**, **11**, and **12,** were the best recognized by As56−As58. Quite the opposite and as expected, As53−As55 showed higher avidity for the haptens with the free phenolic group, especially if the chlorine atoms were at the same positions as in the target analyte. According to the pK_a values of most of the competitor haptens (see Table 3) an important fraction of molecules are ionized at the assay pH. Only haptens **8**, **9**, and **10** had a pK_a sufficiently high to remain mainly in their protonated forms.

(2) Competitive ELISA. Those As/coating antigen combinations showing reasonable titers were used for screening the ability of 2,4,5-TCP to inhibit binding of the antibodies to the coated plates. In agreement with the results from the molecular modeling studies, only As53−55, raised against hapten **A**, were able to provide competitive assays within the analyte concentration interval investigated. Table 4 shows the features of the immunoassays with IC_{50} values lower than 10 μg L^{-1}. Almost all the assays have slope values close to 1 that proves the high avidity of the antibodies for the target analyte.

Using theoretical models, on a previous paper we reported that the best immunoassay detectability was accomplished using a competitor hapten with the highest

Table 4. Immunoassay Features of the Competitive Indirect ELISA[a]

As[b]	CA[c]	method[d]	A_{max}	A_{min}	slope	IC_{50}[e]	r^2
53	1-BSA	AE	0.61	0.03	−1.2	9.28	0.985
	5-BSA	MA	1.02	0.08	−0.63	6.94	0.980
	5-OVA	MA	0.68	0.03	−0.64	2.42	0.989
	7-OVA	AE	0.94	0.06	−1.2	0.83	0.990
	8-OVA	AE	0.75	0.01	−0.6	6.47	0.992
	9-CONA	AE	0.70	0.02	−0.96	5.35	0.994
	10-CONA	AE	0.86	0.01	−0.71	7.93	0.989
	12-OVA	AE	0.57	0.13	−0.86	4.38	0.995
54	5-BSA	MA	1.3	0.21	−0.68	8.49	0.995
	5-CONA	MA	1.33	0.07	−0.84	10.04	0.990
	5-OVA	MA	0.56	0.03	−0.90	4.56	0.998
	7-BSA	AE	1.05	0.01	−0.78	3.36	0.996
	7-OVA	AE	0.69	0.01	−1.0	3.05	0.990
	8-BSA	AE	1.07	0.001	−0.5	6.71	0.959
	10-OVA	AE	0.63	0.01	−1.0	4.87	0.990
55	1-BSA	AE	0.98	0.2	−0.96	4.05	0.910
	2-BSA	AE	0.82	0.02	−1.2	8.66	0.990
	2-OVA	AE	1.01	0.04	−1.3	9.64	0.940
	8-OVA	AE	0.73	0.04	−1.2	8.53	0.970

[a] Only those assays reaching IC_{50} values below 10 μg L^{-1} are shown in the table. [b] As, antisera. [c] CA, coating antigen. [d] Conjugation method used to prepare the coating antigens: AE is active ester; MA is mixed anhydride. [e] IC_{50} values are expressed in μg L^{-1}. In bold are the best As/coating antigen combinations.

degree of heterology regarding punctual charge distribution and structural geometry (*45*). Persisting on the aim to rationalize immunoassay development, we tried also to provide objective data about the hapten heterologies and their correlation with the IC_{50} values of the immunoassays obtained. Thus, all haptens **1**−**12** and the target analyte were optimized to find their minimum potential energies using a semi-empiric PM3 model. The ratio phenol:phenoxide was calculated for each hapten according to their pK_a values and used to determine the ultimate punctual charges of the molecule. The heterology, as the difference between the charge distribution of each hapten and the analyte, has been expressed as the root-mean-square error (RMSE) of the addition of the errors for each equivalent position of the aromatic rings (competitor vs target analyte, see Table 3). With these considerations the following heterology order according to the electronic distribution on these molecules can be defined: hapten **1** > hapten **2** ≥ hapten **12** > hapten **11** ≫ hapten **9** > hapten **10** > hapten **8** > hapten **7** > hapten **5** > hapten **4** > hapten **A** > hapten **3**. The greatest homology of hapten **3** in relation to hapten A and 2,4,5-TCP can be explained considering the pK_a values of these substances. While hapten **A** has approximately only half of the molecules ionized at pH 7.5, hapten **3** and 2,4,5-TCP have 89 and 71%, respectively, in the ionized form. However, it must be noticed that using hapten **3** as immunizing hapten would had the risk of introducing additional undesirable epitopes for antibody recognition.

According to these results, we can observe that the best competitive assays were afforded by the competitors with a high homology with the target analyte (see Table 4). Except for the As55 in all other cases, haptens **5** and **7** afforded the most sensitive assays. Unfortunately, we did not have sufficient hapten **A** to prepare coating antigens by the AE method to prove if this was also true for this competitor. However, in addition to the hapten homologies, other factors are also influencing the availability of the competitor for the antibody interaction, when covalently forming part of the protein conjugate. Thus, the hapten density or the protein nature, are also other aspects to consider. Similarly the hydrophobic or hydrophilic nature of the compound may also determine its tendency to be hidden or not inside the tertiary structure of the protein.

Table 5. Effect of the Hapten Density of the Competitor on the Immunoassay Features[a]

CA	hapten:protein[b]	δ[c]	A_{max}	A_{min}	slope	IC_{50}	r^2
5-BSA (MA)	2:1	7.5	1.02	0.08	−0.63	6.94	0.980
	1:1	9	1.04	0.01	−0.46	1.30	0.980
	1:2.5	5	0.92	0.01	−0.52	1.94	0.987
	1:5	3	0.92	0.02	−0.45	1.56	0.989
5-OVA (MA)	2:1	n.d.[e]	0.68	0.03	−0.64	2.42	0.989
	1:1	6	0.82	0.01	−0.56	1.76	0.990
	1:2.5	3	0.87	0.02	−0.54	1.92	0.990
	1:5	3	0.61	0.01	−0.5	0.45	0.990
5-BSA (AE)	1:1[d]	n.d.					
	1:2.5	13	1.13	0.01	−0.48	15.52	0.981
	1:5	8	0.8	0.01	−0.64	11.1	0.988
	1:10	5	1.1	0.01	−0.56	9.13	0.989
5-OVA (AE)	1:1	20	1.19	0.01	−0.65	16.81	0.997
	1:2.5	12	1.28	0.02	−0.72	9.70	0.980
	1:5	6	1.24	0.01	−0.60	10.30	0.985
	1:10	3	0.79	0.01	−0.68	4.80	0.988
7-BSA (AE)[f]	2:1	25					
	1:1	22	0.37	0.03	−1.69	0.24	0.964
	1:2.5	13	0.59	0.01	−0.98	0.28	0.985
	1:5	7	0.3	0.01	−0.71	0.1	0.986
	1:10	4	0.1	0.01	−1.19	0.06	0.919
7-OVA (AE)	2:1	n.d.	0.94	0.06	−1.20	0.9	0.990
	1:1	12	0.77	0.03	−1.24	0.57	0.990
	1:2.5	10	0.72	0.01	−1.13	0.29	0.990
	1:5	3	0.65	0.02	−1.00	0.21	0.990
	1:10	3	0.74	0.02	−1.20	0.23	0.990

[a] In all cases the antiserum used was As 53. [b] Hapten:protein molar ratio used for the conjugation reaction. The molar ratio of the protein has been calculated in terms of the lysine residues available. [c] Hapten densities (δ) are expressed as mol of hapten per mol of protein, and the data reported has been measured by MALDI-TOF-MS. [d] 5-BSA (AE) (1:1) was not soluble. [e] nd, not determined. [f] The conjugate with the highest density was not recognized by the antibody.

ELISA Optimization and Characterization. Immunoassays **5**-OVA/As53 and **7**-OVA/As53 were selected for further optimization and evaluation because of their detectability, good signal-to-noise ratio, acceptable slope, and reproducibility (see Table 4). The initial studies were aimed to improve immunoassay detectability. It has been reported that the degree of *hapten density of the competitors* can affect the immunoassay sensitivity (*44*, *56*, *57*). The carrier protein OVA has 20 lysine residues, most of which are available for hapten coupling (*58*). In this case, the hapten densities of the corresponding 5-BSA and 7-BSA conjugates measured by MALDI-TOF-MS were 7 and 25 mol/mol of protein (see Table 3). The difference encountered between those two conjugates can be explained by the fact that hapten **5** had been conjugated by the MA method, which usually gives lower yields. To study the effect of the hapten density, hapten **5** was conjugated again to BSA and OVA by both methods (MA and AE) and hapten 7 by the AE method at different hapten:lys residues molar ratios. The resulting degree of conjugation was determined by MALDI-TOF-MS (see Table 5). For the case of hapten **5**, the assays reached lower IC_{50} values (higher detectability) when the MA was the conjugation method used. In general for both haptens, it was observed that, the lower the hapten density, the higher the immunoassay detectability (lower IC_{50} values). The same effect has been observed on other immunoassays such as sulfamerazine (*56*), chlorpyrifos (*57*), or

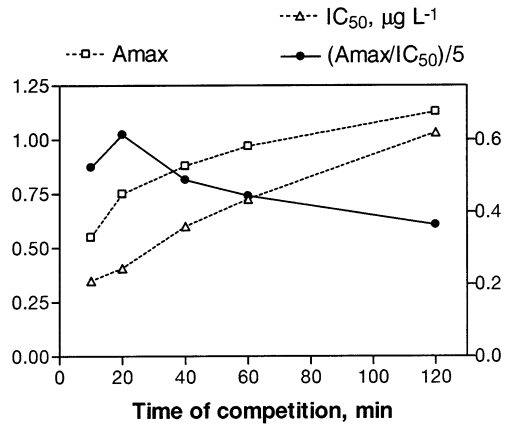


Figure 3. Variation of the immunoassay parameters (IC_{50} and A_{max}) as function of the length of the competitive step. The analyte, 2,4,5-TCP and the antibody were incubated at different times (between 10 and 120 min) in the antigen-coated plates. The results reported are extracted from the four-parameter equation used to fit the standard curves. Each standard curve was run in duplicate.

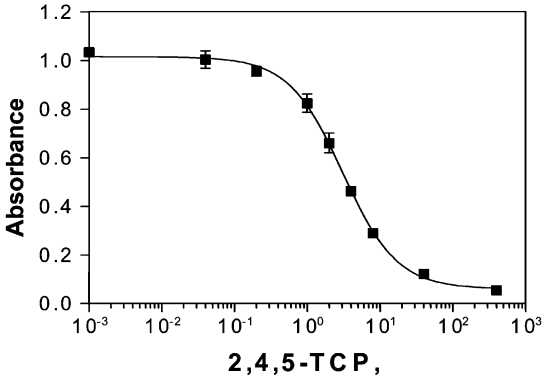
2,4,6-TCP (*44*). This is consistent with the statement that the least hapten centers are available in the coating antigen, the least amount of analyte in solution is needed to compete for the limited number of antibody binding sites. The detectability improved significantly by diminishing the hapten density for the As53/**7**-OVA assays. Finally, the coating antigen **7**-OVA (AE 1:2.5 molar ratio) was selected for further studies, since lower molar ratios did not increase significantly the immunoassay detectability, and it was necessary to employ higher concentrations of immunoreagents to obtain the same maximum absorbance in the assay (see Table 5).

Although it has been reported that the sensitivity of an immunoassay can be improved by *preincubating* of the analyte with the As prior to the competition (*43*, *59*), in this case no significant effect on the detectability was observed after incubating the As with the analyte overnight at 4 °C (IC_{50} 0.29 μg L^{-1} versus 0.31 μg L^{-1}). The same effect was observed for other ELISAs developed in our group for phenolic compounds (*44*−*46*). On the other hand, shortening the *length of the competitive step* improved the immunoassay detectability (the lowest IC_{50}). Thus, an increase of the IC_{50} value of three times was observed when varying the incubation length from 10 min to 2 h. However, a concomitant increase of the maximum assay signal (A_{max}) was also produced. Therefore, a competitive incubation time of 20 min was chosen as compromise because of the greater A_{max}/IC_{50} ratio encountered at this time (see Figure 3).

Finally, we must mention that the *concentration of Tween 20* did not significantly influence the features of the 2,4,5-TCP immunoassay. Only when the concentration reached values of 0.5 and 2.5% a significant increase of the IC_{50} was observed, achieving values close to 6 μg L^{-1} without affecting A_{max}. This result is in agreement with the idea suggested by some authors that polar analytes are less influenced by the presence of detergent because of their inability to establish nonhydrophobic interactions with it (*45*, *46*, *49*, *60*). Next experiments were performed at 0.025% Tween 20 final concentration by preparing the standards in PBS and the As in PBST (0.05% Tween 20).

As result of this evaluation we obtained an assay using As53 and 7-OVA (molar ratio 1:2.5, hapten density 10 mol of hapten/mol of protein) as coating antigen. The assay did not introduce any preincubation of the As with the analyte and had a competitive step of 20 min. The assay buffer was 10 mM PBS, and the As was diluted in the same buffer containing 0.05% Tween 20 (final concentration in the assay 0.025%). Using these conditions, the assay showed an IC_{50} of 0.61 μg L^{-1} and a LOD of 0.084 μg L^{-1} (see Figure 4).

Immunoassay *specificity* was evaluated by testing 22 structurally related compounds as competitors. The results of the crossreactivity studies shown in Table 6 demonstrate that the assay is quite specific. It can be observed that the degree of recognition was directly related to the presence of two chlorine atoms (one at *ortho* and another at *para* position) and the hydrogen in *meta* as in the target analyte. For example, 2,3,4,6-tetrachlorophenol (TtCP) possessing these three atoms on one of its moieties was the most recognized compound with 19% crossreactivity value, while 2,3,4,5-TtCP lacking the hydrogen atom in *meta* was less recognized (4.6%) and 2,3,5,6-TtCP lacking the chlorine atom at *para* position and possessing two chlorine atoms at *meta* positions cross-reacted only 0.5%. Similarly, among the trichlorophenols 2,4,6-TCP is the most recognized (17%) possessing both *ortho* and *para* chlorine atoms and a *meta* hydrogen, in the same range as 2,3,4,6-TtCP. The lack of the hydrogen in *meta* (2,3,4-TCP) or of the chlorine atom in *para* (2,3,5-TCP and 2,3,6-TCP) decreases significantly the recognition. Analytes with only two, one or none chlorine atoms had a negligible (≤3.5%) or void interference in the assay. As reported (*44*, *45*, *49*),



A*max*	1.02
A*min*	0.06
Slope	1.16
IC_{50}, µg L^{-1}	0.61
LOD, µg L^{-1}	0.084
Dynamic Range, µg L^{-1}	0.18-2.07
r^2	*0.990*

Figure 4. Calibration curve of the 2,4,5-TCP immunoassay using As53 and **7**-OVA (hapten density, 10). The assay is performed in 10 mM PBST (0.025% Tween 20). The curves were run in duplicate. On the right side of the graph are shown the features of this immunoassay.

Table 6. Interference Caused by Structurally Related Chemicals, Expressed by Their IC_{50} and the Percentage of Crossreactivity[a]

no.[b]	phenolic compds	IC_{50} (nM)	% CR	brominated analogues	IC_{50} (nM)	% CR
5	PCP	101.3	1.5	PBP	361.9	0.42
4	2,3,4,6-TtCP	8	18.9			
	2,3,4,5-TtCP	33	4.6			
	2,3,5,6-TtCP	304	0.5			
3	2,4,5-TCP	1.52	100	2,4,6-TBP	5.5	27.7
	2,4,6-TCP	8.9	17			
	2,3,4-TCP	168.9	0.9			
	2,3,5-TCP	1520	0.1			
	2,3,6-TCP	380	0.4			
2	2,4-DCP	43.4	3.5	2-B-4-CP	14.9	10.2
	2,5-DCP	168.9	0.9	2,4-DBP	14.5	10.5
	2,6-DCP	337.8	0.45	2,6-DBP	108.6	1.4
	3,4-DCP	>10000	<0.01			
1	2-CP	2533.3	0.06			
	3-CP	>10000	<0.01			
	4-CP	>10000	<0.01	4-BP	475	0.32

[a] Crossreactivity is expressed as % of the IC_{50} of 2,4,5-TCP/IC_{50} phenolic compound. [b] Number of halogens. B, bromo; C, is chloro; DCP, dichlorophenol; TCP, trichlorophenol; TtCP, tetrachlorophenol; PCP, pentachlorophenol; BP, bromophenol; DBP, dibromophenol; TBP, tribromophenol; PBP, pentabromophenol.

brominated phenols were also highly recognized in this assay. Thus, 2,4-dibromophenol (DBP) cross-reacted 10.5% while its homologous chlorinated analyte 2,4-DCP was only recognized 3.5%. Similarly, 2,4,6-tribromophenol (TBP) was recognized 27.7% while 2,4,6-TCP interfered only 17%. Unfortunately, at the time of writing this paper, we have not been able to test 2,4,5-TBP, but we would expect recognition greater than 100%.

Conclusions

An immunoassay has been developed for the detection of 2,4,5-TCP making use of molecular modeling tools to assist on the hapten design of the chemical structures of the immunogen and the competitors. The theoretical studies have been useful to rationalize certain aspects related to the avidity of the different antisera obtained. Regarding the chemical structure of the immunizing hapten, the antibodies raised against hapten **B** did not afford any usable assay, while several immunoassays were obtained with the antisera raised against hapten **A**. From the synthetic point of view hapten **B** was easy to prepare however the theoretical studies provide rational criteria to understand the lack of avidity of As56−As58 for the target analyte. Thus, 2,4,5-TCP, hapten **A** and hapten **C** are organic acids that in the aqueous media of the assay buffer are in equilibrium with their corresponding phenoxide forms. These two forms differ considerably on their punctual charge distribution. It is therefore very important to know their actual ratio in the assay conditions (see Figure 2). However, the absence of a free hydroxyl group in hapten B prevents it from behaving as an organic acid, and its punctual charge distribution is more close to the protonated form of the analyte than to the corresponding conjugated base. Thus, at pH 7.5, 71% of the molecules of 2,4,5-TCP are ionized and similarly occurs with haptens **A** and **C**. Therefore, the higher frequency of the anionic forms and the strong differences encountered in the punctual charges with the neutral form of hapten B explains the lack of recognition showed by these antibodies. In general, the results obtained are in agreement with previous studies (*44*) and seem to indicate that for this type of analyte the punctual charge distribution of the molecule is one of the most important parameters.

On the other hand a degree of heterology has been established for a battery of 13 different haptens (including the immunizing hapten) used as competitors according to their punctual charge distribution calculated at their minimum energetic level and considering the pK_a value of these substances (see Table 3). By correlating this parameter with the detectability afforded by the different assays obtained, we have arrived to the conclusion that the best performance occurs when the competitor has a moderate-to-high degree of homology. However, this result should not be taken as a general rule since other factors determining the way the competitor haptens are available for antibody recognition also play an important role. Thus, in this aspect we must notice that the work presented here proves once more that detectability improves with competitors showing a moderate hapten density.

The indirect ELISA reported here shows a limit of detection of 0.086 $\mu g\ L^{-1}$ and it is quite specific for 2,4,5-TCP. As occurred with other reported assays (*44*, *45*, *49*) the brominated analogues may also be recognized. The detectability accomplished by this immunoassay in the assay buffer is sufficient for environmental and biological monitoring studies. As mentioned in the Introduction, the concentrations found in water and urine samples are often above the limit of detection (0.086 $\mu g\ L^{-1}$). Further work has been focused on the evaluation of this assay to analyze chlorophenols in body fluids (*61*).

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