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Development of an Immunochemical Technique for the Analysis of Trichlorophenols Using Theoretical Models

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An immunoassay has been developed for trichlorophenol analysis on the basis of theoretical chemistry modeling studies. These data have allowed us to choose the optimum chemical structure of the immunizing hapten according to realistic similarities with the target analyte. The synthesis of this hapten and the subsequent application of an appropriate immunization protocol have lead to the production of polyclonal antibodies against the target analyte. A homologous direct competitive ELISA has been developed that can be carried out in about 1 h. It has a limit of detection of 0.2 ± 0.06 μg/L (1.01 ± 0.3 nM) and it has been proven to tolerate a wide range of ionic strengths and pH values. Thus, the assay has acceptable features in samples with ionic strength between 4 and 56 mS/cm and pH values between 5.5 and 9.5. Studies on the selectivity of this immunoassay have demonstrated a high recognition of the corresponding brominated analogues. Other phenolic compounds do not interfere significantly in the analysis of 2,4,6-trichorophenol using this immunochemical technique. The accuracy of the assay has been evaluated using certified and spiked samples.

Chlorophenols are widely distributed in many compartments of the ecosystem such as soil,[1−4] water,[2,5,6] tissues,[7] and body fluids.[7−14] Their presence is especially due to their use as antistain agents for wood products since the early 1930s and also as intermediates in the production of phenoxy herbicides and other important chemicals. The generalized use of bleaching processes in the past decades has also contributed to their presence. Pulp and paper factories have been important sources of the occurrence of trichlorophenols in the environment. For example, between 0.6 and 1.6 mg/L of AOX (adsorptive organic halogen) were estimated to be emitted daily by the Finish paper industries in the late 1980s.[6] Chlorophenols are also formed in incinerators during the combustion of organic matter in the presence of chlorine or chlorine-containing compounds.[15,16]

Although the use of chlorophenol derivatives as preservatives has recently dropped down in many developed countries and whitening and disinfection procedures use alternatives to hypochlorite processes, exposure of the population still occurs via a contaminated environment,[6,17] domestic preservatives, edible products,[11,18] etc. Thus, chlorophenols were found in Swedish riverine sediments at low microgram per gram levels, 40−50 km downstream from cellulose-plant discharges.[19] In fact, it is worth noting that the amount of AOX found in several American and European pulp and paper samples was significantly high (6 −1265 mg/L), even if in some occasions these were marketed as TCF (total chlorine free) or ECF (elemental chlorine free) products.[20,21] Another important fact relative to the widespread exposure to chlorophenols is that polychlorinated dibenzodioxins (PCDD), including the most toxic dioxin congener 2,4,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and polychlorinated dibenzofurans

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(PCDFs) are common impurities. The presence of chlorophenols has been considered as a prerequisite for the formation of dioxins. Thus, on a German plant the estimated median concentration of TCDD in 1955−1970 was 5 ppm on 2,4,5-T products, but could reach values between 10 and 50 ppm in some processes. Measurements made in the 1970s revealed that the median concentration of TCDD was about 1 ppm and lower than 0.1 ppm after the introduction of a new dioxin extraction procedure in 1981.[8] As a consequence, TCDD has been detected in the lipid fraction of the serum of the production workers. In an Austrian plant, in 1990 an average level of 340 pg/g of blood lipid was determined and all of workers showed symptoms of chloracne and neurological diseases.[22−24] Chlorophenols have thus been named as *predioxins*[25] and can be contemplated as indicators of the formation of PCDDs. Therefore, routine monitoring of chlorophenols in urine or other environmental and food matrixes may be important not only because of their own risk effects but also owing to their association with dioxins.

Because of the toxicity and persistence of these compounds in the environment, chlorophenols are included in the priority list of the EU and US environmental protection agencies. Determination of phenolic compounds in water is especially difficult as reported in a few interlaboratory studies[26] due to their wide range of polarities and high vapor pressure that enhances volatilization during the extraction and cleanup procedures. There is a need for developing more efficient methods for extraction from environmental matrixes. Usual methods of analysis require acidification of the sample, liquid−liquid or solid-phase extraction, derivatization, and cleaning up the sample, and the analysis finally takes place by GC/ECD or GC/MS, reaching limits of detection between 0.2 and 1 μg/L (1.01 and 5.06 nM, respectively), starting from significant amounts of sample.[1−5] In this context, the advantages of immunochemical techniques are well-known because of their reliability, low cost, speed of analysis, easy of use, portability, selectivity, and detectability.[27] Additionally, immunochemical methods can detect extremely small quantities of the target analyte with little sample preparation.

The key step in developing an immunoassay for small molecules is the design and preparation of optimum haptens as immunogens and competitors. Recently it has been demonstrated that theoretical molecular models and calculations can be extremely useful tools for the immunochemists to develop assays under rational bases and to adequately direct the immunoassay features.[28−31] In this paper we present the way in which the application of some of these theoretical criteria to the development of an immunoassay for determination of trichlorophenol has led to a highly sensitive and reliable analytical immunochemical technique.

EXPERIMENTAL SECTION

Thin Layer Chromatography (TLC) was performed on 0.25-mm, precoated silica gel 60 F254 aluminum sheets (Merck, Gibbstown, NJ) and unless otherwise indicated the mobile phase employed was hexane/Et_2O, 1:1.^{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 FT−IR spectrophotometer (Hartmann & Braun, Québec, Canada). Gas chromatography−mass spectrometry was performed on a MD-800 capillary gas chromatograph with MS quadrupole detector (Fisons Instruments, VG, Manchester, UK) and data are reported as m/z (relative intensity). The ion-source temperature was set at 200 °C, a 15 m × 0.25 mm i.d. × 0.15 μm (film thickness) DB-225 fused capillary column (J&W, Folsom, CA) was used; He was the carrier gas employed at 1 mL/min. GC conditions were as follows: temperature program, 80−220 °C (10 °C/min), 220 °C (10 min); injector temperature, 250 °C. The MALDI-MS (matrix-assisted laser desorption ionization mass spectrometer) used for analyzing the protein conjugates was a time-of-flight (TOF) mass spectrometer Bruker Biflex III (Bruker, Kalsruhe, Germany) equipped with a laser unit that operates at a wavelength of 337 nm and a maximum output of 6 mW. 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). Polystyrene microtiter plates were purchased from Nunc (Maxisorp, Roskilde, Denmark).Washing steps were carried out using a SLY96 PW microplate washer (SLT Labinstruments GmbH, Salzburg, Austria). Absorbances were read using a Multikskan Plus MK II microplate reader (Labsystems, Helsinki, Findland) at a single wavelength mode of 450 nm or on a SpectamaxPlus (Molecular Devices, Sunnyvale, CA). The competitive curves were analyzed with a four-parameter logistic equation using the software Genesys (Labsystems), SoftmaxPro v2.6 (Molecular Devices), and GraphPad Prism (GraphPad Sofware Inc., San Diego, CA). Unless otherwise indicated, data presented correspond to the average of at least two well replicates. Immunochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Chemical reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI).

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. 2X PBST is PBST double-concentrated. 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.

Molecular Modeling and Theoretical Calculations. Molecular modeling was performed using the Hyperchem 4.0 software package (Hyperube Inc, Gainesville, FL). Theoretical geometries

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and electronic distributions were evaluated for 2,4,6-trichlorophenol and potential haptens (all of them as amide derivatives) using semiempirical quantum mechanics MNDO[32] and PM3[33] models. All the calculations were performed using standard computational chemistry criteria. Theoretical calculations regarding pK_a values were carried out using the ACL/P*K*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 Hapten. *3-(3-Hydroxyphenyl)-propanoic acid* ***2***. An alloy of Na−Pb (5g, 10% Na, 21.75 mmol, 3.6 equiv in terms of Na) was added slowly to a stirred solution of (3-3-hydroxyphenyl) propenoic acid **1** (5 g, 3.05 mmol) in 5% aqueous NaOH (4 mL) at RT. A slight N_2 stream was used to remove the hydrogen formed during the reaction. After 4 h at RT the color of the reaction mixture had changed from an intense to a pale yellow. The mixture was acidified to pH 1 with 0.1 N HCl and extracted with ethyl acetate. The organic layer was washed with NaCl sat solution, dried with $MgSO_4$ anhyd, filtered, and evaporated to dryness under reduced pressure to obtain the acid **2** (0.45 g) with an 89% yield. ^{1}H NMR (200 MHz, DMSO-d_6);δ 2.49 (t, J = 7.5 Hz, 2H, $-CH_2COO-$), 2.73 (t, J = 7.5 Hz, 2H, $PhCH_2-$), 3.37 (bs, 1H, −OH), 6.56−6.65 (ca, $2H_{Ar}$ ortho, $1H_{Ar}$ para), 7.06 (dd, J = 7.9 Hz, J = 8 Hz, $1H_{Ar}$ meta), 9.25 (bs, 1H, COOH).

Methyl 3-(3-hydroxyphenyl)-propanoate **3**. Three drops of H_2SO_4 concentrated were added to a solution of the acid **2** (400 mg, 2.41 mmol) in MeOH (5 mL), placed on a round-bottom flask provided with a $CaCl_2$ tube. After 6 h at RT the reaction was completed according to the TLC analysis. The solvent was evaporated under reduced pressure and the oily residue suspended with 5% aqueous $NaHCO_3$ and extracted with ethyl ether. The organic layer was dried over $MgSO_4$ anhyd, filtered, and evaporated to dryness to afford the desired ester **3** (430 mg) with an 88% yield. ^{1}H NMR (200 MHz, $CDCl_3$); δ 2.63 (t, J = 7.6 Hz, 2H, $-CH_2COO-$), 2.90 (t, J = 7.6 Hz, 2H, $PhCH_2-$), 3.68 (s, 3H, $-OCH_3$), 6.03 (s, 1H, −OH), 6.68−6.77 (ca, $2H_{Ar}$ ortho, $1H_{Ar}$ para), 7.15 (dd, J = 7.7 Hz, J = 7.6 Hz, $1H_{Ar}$ meta).

Methyl 3-(3-hydroxy-2,4,6-trichlorophenyl)-propanoate **4**. A mixture of SO_2Cl_2 (1.5 mL, 18.7 mmol) and Et_2O anhyd (2.5 mL, 23.92 mmol) was added dropwise to a stirred solution of the ester **3** (0.35 g, 1.94 mmol) in CH_2Cl_2 anhyd (2.5 mL) under an Ar atmosphere. The reaction mixture was stirred at RT for 5 h and additional amounts of SO_2Cl_2 (150 μL, 1.86 mmol) and Et_2O anhyd (200 μL, 1.91 mmol) were added. After 1 h more the reaction was completed according to the TLC analysis, affording the ester **4** (0.51 g) with a 93% yield. Melting point: 86 °C. IR, ν (KBr, cm^{-1}): 3250 (−OH st), 1710 (C═O), 1458 (COO^- st), 1166 (C−O st), 869 (ArC−H δ oop). ^{1}H NMR (200 MHz, $CDCl_3$) δ: 2.56 (t, J = 8.4 Hz, 2H, $-CH_2COO-$), 3.23 (t, J = 8.4 Hz, 2H, $PhCH_2-$), 3.72 (s, 3H, $-OCH_3$), 5.90 (s, 1H, −OH), 7.34 (s, $1H_{Ar}$ meta). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 27.1 ($-OCH_3$), 31.9 (C-3), 51.9 (C-2), 119.4 (C-4′), 122.1 (C-2′), 125.7 (C-6′), 128.2 (C-5′), 135.8 (C-1′), 147.1 (C-3′), 172.6 (C-1). EM, m/z (%): 282 (M^+, 13), 247 (100), 223 (17), 222 (10), 209 (40), 205 (47). Anal. Calcd for $C_{10}H_9Cl_3O_3$: C, 42.36; H, 3.20; Cl, 37.51. Found: C, 42.45; H, 3.17; Cl, 37.57.

3-(3-Hydroxy-2,4,6-trichlorophenyl)-propanoic acid ***5***. A solution of 0.5 N NaOH (9 mL, 4.5 mmol, 3 equiv) was added to a solution of the ester **4** (200 mg, 0.7 mmol) in THF (11.3 mL). The reaction mixture was stirred for 12 h at RT until the complete disappearance of the starting material was judged to have occurred by TLC. The solvent was evaporated and the residue was dissolved in 0.5 N aq NaOH, 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 170 mg of the hapten **5** with a 90% yield. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 2.40 (t, J = 8.4 Hz, 2H, $-CH_2COO-$), 3.07 (t, J = 8.4 Hz, 2H, $PhCH_2-$), 7.59 (s, $1H_{Ar}$ meta), 10.43 (bs, 1H, COOH). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 26.8 (C-3), 31.7 (C-2), 120.5 (C-4′), 123.6 (C-2′), 123.7 (C-6′), 127.8 (C-5′), 135.6 (C-1′), 148.5 (C-3′), 172.6 (C-1).

Preparation of the Protein Conjugates. *Immunogen*. Following described procedures,[34] the hapten (21.6 mg, 0.08 mmol) was reacted with tributylamine (21 μL, 0.08 mmol) and isobutylchloroformate (2.4 μL, 0.08 mmol) in DMF (dimethylformamide 200 μL) and added to KLH (keyhole limpet hemocyanin 20 mg).

Antigen. Simultaneously, a BSA (bovine serum albumin) conjugate was prepared by the same procedure but using 10.8 mg of the hapten for 20 mg of the protein. This conjugate was used to assess conjugation by MALDI-TOF-MS.

Enzyme Tracer. According to described procedures,[35] the hapten (2.7 mg, 10 μmol) was reacted with NHS (*N*-hydroxysuccinimide, 5.7 mg, 50 μmol) and DCC (dicyclohexylcarbodiimide, 20.6, 100 μmol) in DMF (200 μL) and added to HRP (horseradish peroxidase, 2 mg).

Polyclonal Antisera. Three female New Zealand white rabbits (rabbits 43, 44, and 45) weighing 1−2 kg were immunized with **5**−KLH according to the immunization protocol previously described.[35] Evolution of the antibody titer was assessed by measuring the binding of serial dilutions of the antisera to microtiter plates coated with **5**−BSA. After an acceptable antibody titer was observed, the animals were exsanguinated and the blood collected on vacutainer tubes provided with a serum separation gel. Antiserum was obtained by centrifugation and stored at −80 °C in the presence of 0.02% NaN_3.

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Immunochemistry. The appropriate dilutions of the antisera and the enzyme tracer were established after a 2D checkerboard titration assay performed as described with the three antisera obtained.[28] Finally antiserum 43 was selected for further optimization and evaluation of the resulting competitive assay.

Optimized ELISA Protocol. Microtiter plates were coated with the antiserum 43 (1/8000 in coating buffer 100 μL/well) overnight at 4 °C. The following day the plates were washed four times with PBST and the different concentrations of the analyte standard, crossreactants, or samples in 5 mM PBS buffer (50 μL/well) were added followed by the solution of the enzyme tracer 5HRP (1/8000 diluted in 5mM PBS, 50 μL/well). After 30 min at room temperature, plates were washed again four times with PBST. The substrate solution was added (100 μL/well) and the enzymatic reaction stopped after 30 min at room temperature with 4N H_2SO_4 (50 μL/well). The absorbances were measured at 450 nm. The standard curve was fitted to a four-parameter logistic equation according to the following formula: $y = (A - B/[1 - (x/C)^D]) + B$, where A is the maximal absorbance, B is the minimum absorbance, C is the concentration producing 50% of the maximal absorbance, and D is the slope at the inflection point of the sigmoid curve.

Cross-Reactivity Determinations. Stock solutions of different phenolic compounds were prepared (1 mM in DMSO) and stored at 4 °C. Standard curves were prepared in PBS (10 000−0.64 nM) and each IC_{50} determined in the competitive experiment described above. The cross-reactivity values were calculated according to the following equation: (I_{50} trichlorophenol/I_{50} phenolic derivative) × 100.

Accuracy. (*a*) *Blind Spiked Samples.* This parameter was assessed by preparing 10 different blind spiked samples in milliQ water and measuring them in the ELISA. Measurements were performed in triplicate and in three separate plates run on different days.

(*b*) *Certified water Samples.* Two certified water samples, Group 18B and A29, provided by Aquacheck WRC (Medmenham, UK) and Yorkshire Environmental (LEAP Scheme, Bradford, UK), respectively, were used to measure 2,4,6-TCP and compare the results with the target value and the average value obtained by several laboratories which analyzed this sample by chromatographic methods. The Aquacheck sample was a mixture of chloroform, bromodichloromethane, dibromochloromethane, bromoform, trichloroethylene, tetrachloroethylene, carbon tetrachloride, pentachlorophenol, trichlorophenol, phenol, 2- and 4-chlorophenol, 2,4-dichlorophenol, 2-bromophenol, benzene, toluene, ethylbenzene, styrene, *o*-xylene, *m*-xylene, and *p*-xylene. The Yorkshire Environmental sample contained 4-chloro-3-methylphenol, 2-chlorophenol, 2,4-dichlorophenol, 2,4-dimethylphenol, 2,4-dinitrophenol, 2-methyl-4,6-dinitrophenol, 2- and 4-nitrophenol, pentachlorophenol, phenol, 2,4,5-trichlorophenol, and 2,4,6-trichlorophenol.

RESULTS AND DISCUSSION

Hapten Design and Synthesis. The production of antibodies against a small molecule such as 2,4,6-TCP requires preparation of a hapten which mimics as much as possible the analyte.[36−38] The introduction of a linker in the molecule may cause deformations of the molecular geometry as well as changes in its electronical distribution. On the other hand, often the chemical synthesis of the hapten is laborious and one of the most time-consuming steps of the antibody-development process. Therefore, one must thoroughly evaluate the synthetic effort before starting to produce antibodies against an organic molecule. Nowadays, it is possible to make computer-modeling studies in order to predict the suitability of a particular chemical structure of a hapten for raising antibodies against the target analyte.[28−31]

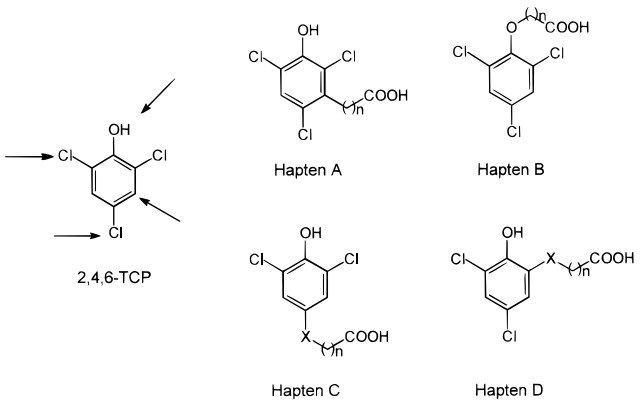

Figure 1. Chemical structures of the target analyte and four potential immunizing haptens used to raise antibodies against 2,4,6-TCP. The arrows indicate possible derivatization positions.

In our search for the best hapten molecule to obtain high-quality antisera against 2,4,6-TCP, we considered four different potential haptens (see Figure 1, A−D). From the synthetic point of view, hapten B can be easily prepared in just one step using the analyte as starting material. However, the hydroxyl group is blocked in this hapten and, additionally, certain chlorophenoxyacid herbicides could be then also recognized by the antibodies raised against that hapten. Observing the planar chemical structures, one can already be aware that hapten A is the only one preserving all the functional groups of the target analyte (chlorine atoms and hydroxyl group), since in haptens C and D one of the chlorine atoms has been replaced by the spacer arm at either the para or the ortho position, respectively. From both of them, hapten C appeared as the most suitable one since the area covered by the phenolic group and the two chlorine atoms in ortho was more exposed to the immune system.

In a retrosynthetic analysis for the preparation of hapten A we considered the possibility of introducing an alkyl chain at the meta position. However, it is well-known in organic chemistry that it is difficult to derivatize this position in phenolic compounds using the traditional Friedel−Crafts alkylation or acylation reactions. The opposite reactivity, which means the nucleophilic substitution in the aromatic ring, has been described if electron-withdrawing groups are present.[39−41] Thus, nucleophilic substitution at the meta position with a high regioselectivity has been reported by preparing first arene-tricarbonyl-chromium (0) complexes[41] of the corresponding aromatic compound.

After these previous considerations and before starting any synthetic effort, we decided to perform theoretical studies on the suitability of haptens A−C for antibody production against 2,4,6-TCP. Using MNDO models we could really see that the geometries of the haptens and the target analyte, at their minimum energetic levels, did not vary significantly (see Figure 2). The charge density showed, in contrast, slight differences as we will discuss later. Working with phenolic compounds both the neutral compound and the corresponding conjugated base coexist to-

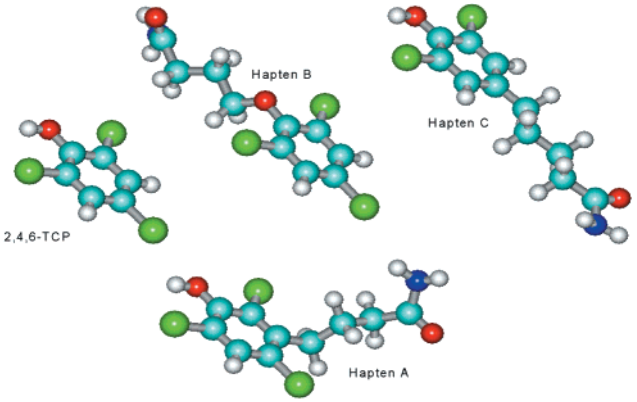

Figure 2. Stick and wedges display of the optimized geometries of 2,4,6-TCP and haptens A−C according to MNDO models. Calculations have been made using the corresponding amide derivatives to mimic the conjugated haptens. The elements are presented in the following manner: Light blue, carbon; dark blue, nitrogen; gray, hydrogen; red, oxygen; green, chlorine.

gether in an aqueous media, their ratio depending on the pH. For example, with a pK_a of 6.23,[42] one can assume that both the acid and conjugated base of 2,4,6-TCP will coexist in a physiological media or in the assay buffer (see Figure 3).

Charge distribution in the aromatic ring for both species was calculated at their minimum energetic conformations for haptens A−C and the target analyte. Figure 3 shows the results obtained on each atom of the ring positions and the sum of those charges. Thus, as organic acids, the differences between the analyte and the haptens were only small and the balance of the total charge was positive for all compounds except for hapten C, due to the lower inductive effect produced by the alkyl chain replacing the chlorine atom. However, the differences were greater when considering the charge distribution in a situation where the compounds would exist as conjugated bases. The balance of charge in the aromatic ring was, in this case, negative for all molecules except for hapten B, which cannot participate in this equilibrium. Looking at the graphed differences between this hapten and the target analyte, it is thus clearly demonstrated that hapten B was not an appropriate hapten to raise antibodies against 2,4,6-TCP, since it cannot mimic the analyte regarding electronic distribution. Hapten C has, again, a more negative total charge and especially at the para position because of the effect of the linker. In contrast, hapten A mimicked almost perfectly the behavior of the 2,4,6-TCP in both situations.

However, all these studies are only valid if the tendency of these organic acids to give protons to the media is the same. This fact is indicated by their pK_a values, but it is not described in the literature for all of them. The pK_a values can be estimated theoretically using the information available in the databases for other similar compounds. However, another strategy that has the same meaning is to determine the deprotonation enthalpies (DPE) of the proton-transfer reactions of these compounds. According to the Gibbs equation

$$\Delta G_0 = \Delta H_0 - T\Delta S_0$$

$$\Delta G_0 = -2.303RT \log K_{eq}$$

$$\Delta H_0 = -2.303RT \log K_{eq} + T\Delta S_0$$

on proton-transfer reactions

$$K_{eq} = K_a \text{ and } -\log K_a = pK_a$$

thus

$$\text{DPE} = +2.303\ RTpK_a + T\Delta S_0$$

Therefore, DPE is directly related to the pK_a value in the gas phase. Dewar et al. proved that semiempirical theoretical models provide reliable data regarding PA (proton affinities) and DPE values if compared with experimental data.[43] Since

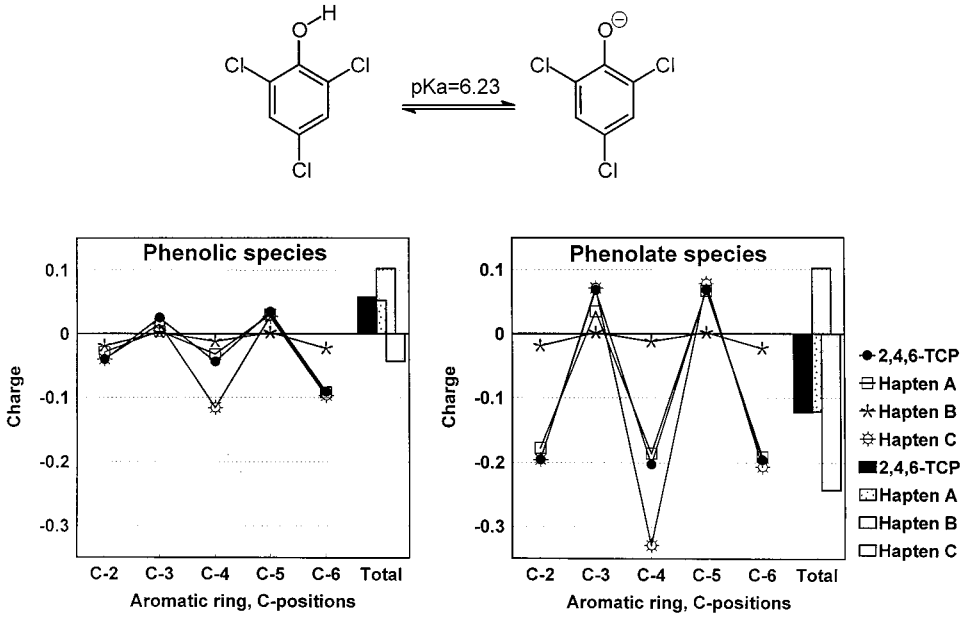

Figure 3. Total charge and punctual charges in the phenolic forms and as the corresponding conjugated bases. The relative amount of these two species depends on their pK_as. Hapten A is the one which mimicks the behavior of the target analyte better in both situations.

Table 1. Calculated DPE and PKa Values[a]

compound	ΔH_f(ArOH)	ΔH_f(ArO^-)	DPE	pK_a
2,4,6-TCP	−1373	−1362	378	6.59 ± 0.23
hapten A	−2638	−2631	374	6.63 ± 0.28
hapten C	−2937	−2922	383	7.37 ± 0.25

[a] The enthalpies and pK_a values were calculated using appropriate software as described in the Experimental Section. Enthalpies are expressed in kcal/mol. The formation enthalpy considered for the proton was 367.2 kcal/mol.

$$\Delta H_0 = \Delta H_{fp} - \Delta H_{fr}$$

by calculating the formation enthalpy of the reagents (organic acids, ΔH_{fr}) and the products (conjugated bases and proton, ΔH_{fp}) for 2,4,6-TCP and haptens A and C, we determined the DPE values of the different equilibria according to the following equation

$$\Delta H_0 = [\Delta H_{fp}(H^+) + \Delta H_{fp}(ArO^-)] - \Delta H_{fr}(ArOH) = DPE$$

The ΔH_{fp} of the H^+ was considered to be 367.2 kcal/mol, according to the literature.[43,44] Table 1 shows the formation enthalpies calculated for the studied phenols and their conjugated bases using a MNDO semiempirical model as well as the DPE values of the corresponding deprotonation equilibrium reactions. The calculated DPE values indicate clearly 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 of the analyte and haptens A and C using suitable software (see Table 1). All these data demonstrate the validity of the theoretical model used to choose the hapten which best mimics our target analyte. In summary, hapten A mimics well the geometry, charge distribution, and acidic character of the target analyte—parameters that can be considered as appropriate descriptors of its physicochemical behavior.

With all these precedents in mind we addressed to the preparation of hapten A. A more accurate retrosynthetic analysis suggested the possibility to start from a commercially available compound lacking the chlorine atoms in the aromatic ring, but possessing the appropriate function at the *meta* position. Thus, 3(3-hydroxyphenyl)-propenoic acid **1** was selected as starting material. Reduction of the double bound was accomplished by treating the acid with Na−Pb alloy (10% Na) in basic aqueous media (5% NaOH).[45] Next step consisted on introducing the chlorine atoms avoiding chlorination of the benzylic position. The direct chlorination of phenols using Cl_2 has been described (g).[46] Similarly, the use of *N*-halosuccinimides (NCS, *N*-chlorosuccinimide) appears in the literature as a source of halonium ions that react as electrophiles in front of the aromatic ring in solvents with a high dielectric constant (i.e., DMF) or in the presence of perchloric acid.[47,48] Masilamani and Rogic[49] reported the controlled monochlorination of phenols using sulfuryl chloride with the advantage that the reaction takes place at room temperature in CH_2Cl_2 using ethyl ether as a base. Additionally, this approach was successfully employed by Corey et al.[50] for the chlorination of 3,5,-dimethylphenol with a high yield and the benzylic positions were not affected. These precedents prompted us to apply this procedure to the chlorination of the methyl ester **3** using three equivalents of SO_2Cl_2. The reaction was completed after 1.30 h with a yield close to 90%. The hydrolysis of the ester gave the desired final product, **5** (hapten A), with a global yield of 70% from the starting acid **1** (see Figure 4).

Table 2. Features of the Competitive Immunoassays Obtained by Immunizing with 5KLH and Using 5HRP as Tracer[a]

antiserum	A_{max}	A_{min}	slope	IC_{50} (μg/L)	r^2
43	0.747	0.062	−0.97	5.5	0.997
44	0.526	0.105	−0.86	29.9	0.991
45	0.830	0.108	−0.82	10.1	0.994

[a] The parameters are extracted from the four-parameter equation used to fit the standard curve. Curves were run in duplicate using twelve different concentrations.

IMMUNOCHEMISTRY

The 3-(3-hydroxy-2,4,6-trichlorophenyl)-propanoic acid **5** was conjugated to KLH and to BSA following the mixed anhydride method. The coupling reaction was verified by analyzing the BSA derivative by MALDI-TOF-MS. By comparing the observed molecular weight with that of the intact protein we could estimate an average of 7.4 haptens covalently attached to each molecule of BSA, which means a yield ranging from 21 to 25% (considering that the amount of accessible lysine residues is between 30 and 35). This yield was considered sufficient to start the immunization protocol. Thus, three rabbits were inoculated with 100 μg of the 5-KLH conjugate and boosted each month for six months until no significant increase in the antibody titer was observed.

The same hapten was conjugated to HRP and used as an enzyme tracer (5-HRP). We found it more convenient to use, in this case, a different coupling procedure to avoid potential interferences due to side reactions.[51] We used the active ester method and verified conjugation by analyzing the recognition of the enzyme tracer by the antisera.

Once the necessary immunoreagents had been prepared, we established the appropriate concentrations by a 2D-checkerboard titration experiment where the avidities of different dilutions of the antiserum immobilized on a microtiter plate versus different concentrations of the enzyme tracer were measured. Using these immunoreagent concentrations we could obtain three different competitive immunoassays for each antiserum. Table 2 shows the features of the direct ELISAs obtained; all of them showed a good signal and an appropriate assay slope. Similarly, the regression coefficient of the four-parameter equation used to fit the standards

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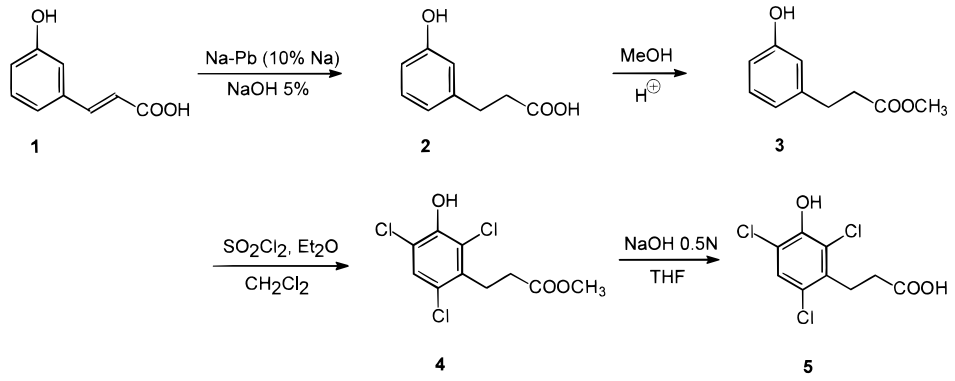

Figure 4. Synthetic pathway leading to hapten A (product 5). The hapten is obtained with a global yield around 70% by using this procedure.

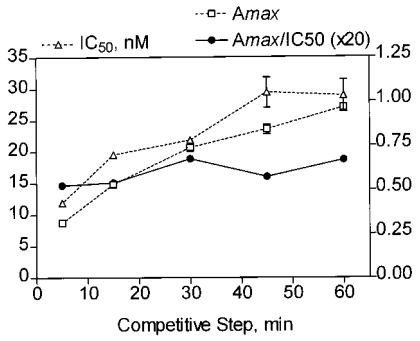

Figure 5. The length of the competitive step did not produce any improvement in the immunoassay performance (A_{max}/IC_{50} ratio). A decrease of the incubation time produced an enhancement of the detectability but it was accompanied by a significant reduction of the immunoassay signal. The data presented correspond to the average of two assays run on different plates. Each standard curve was run in duplicate.

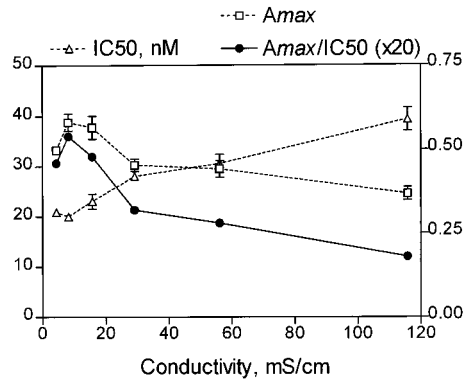

Figure 6. Variation of the immunoassay features with the ionic strength (expressed in mS/cm) of the media. The best immunoassay performance occurs with a conductivity of 8.16 mS/cm. Detectability slowly diminishes when the ionic strength is increased. An inflection point is produced when the media has a conductivity of 29.1 mS/cm. The data presented are extracted from the four-parameter equation used to fit the standard curve. Data correspond to the average effect observed for each pH value, on two assays run in different microtiter plates. Each standard curve was run in duplicate for each assay.

was very good for all assays. From all of them, antiserum 43 showed the best performance regarding detectability (IC_{50} 5.5 μg/L, 27.83 nM) when compared with the antisera 44 and 45 (IC_{50} 29. 9 and 10 μg/L, respectively). Thus we continued investigating the combination *antiserum 43/5-HRP* for further optimization and evaluation.

IMMUNOASSAY OPTIMIZATION AND EVALUATION

Preincubation Time. The analyte was incubated in the antiserum coated plates at different periods of time (0, 10, 20, 40, and 60 min) before adding the enzyme tracer to proceed with the competitive step. This strategy has proven to be useful in this and other laboratories to improve immunoassay detectability;[28,35,52] however, in this case no significant variation was observed on any of the parameters of the assay (i.e., IC50 4.8, 4.2, 4.2, 4.3, 4.4 μg/L at different incubation times from 60 to 0 min).

Length of the Competitive Step. As observed with other assays,[28] a reduction of the duration of the competitive step led to a decrease of the assay signal simultaneous with an increase in the detectability (see Figure 5). Incubation periods around 30 min were chosen as a compromise between detectability and maximal absorbance of the assay, although in further experiments we tried to find out conditions in which the signal could be improved using short incubation periods, without success.

Ionic Strength. As reported for other immunoassays,[28,53,54] an increase in the ionic strength produced a concomitant decrease of the maximal absorbance of the assay. In contrast, an improvement of the assay detectability was observed when diminishing the ionic strength of the media (see Figure 6), indicating perhaps that ionic species may compete in establishing electrostatic interactions with the antibody for the target analyte, due to its polar nature.[55] A similar effect has been described for other polar analytes such as 3,5,6-trichloro-2-pyridinol,[56] while opposite results have been reported for nonpolar analytes, which behavior can be explained by an enhancement of the hydrophobic interactions caused by the high ionic strength of the media.[57,58] Anyway, it is worth noting that the assay performed well under a wide range of ionic-strength values, which is a positive issue regarding the potential application of this assay to the analysis of complex matrixes (wastewater, urine, etc.) with high ionic-strength values. Thus, from 8.16 to 56.1 mS/cm (5−50 mM in terms of PBS), the

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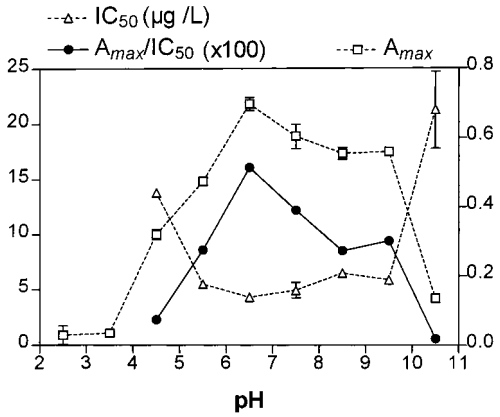

Figure 7. Effect of the pH in the 2,4,6-TCP immunoassay. Several standard curves were prepared using 5 mM PBS at different pH values and added to the Ab coated plates. The results reported are extracted from the four-parameter equation used to fit the standard curve. Data correspond to the average effect observed for each pH value, on two assays run in different microtiter plates.

IC50 varied only from 3.95 ± 0.07 μg/L (n = 2) to 6.03 ± 0.5 μg/L (n = 2) and the A_{max} from 0.582 ± 0.034 (n = 2) to 0.445 ± 0.034 (n = 2) units of absorbance, respectively. With an ionic strength of 116 (100 mM) mS/cm, the immunoassay still worked with acceptable features (A_{max}= 0.368 ± 0.028; slope 0.90 ± 0.07, IC_{50} = 7.76 ± 0.6 μg/L, r^2 = 0.997). Because of the higher detectability encountered and good assay signal, a PBS 5 mM with an ionic strength of 8.16 mS/cm was employed for subsequent experiments.

Concentration of Tween 20. It has been reported that low concentration values or the absence of detergent may enhance the affinity of the antibody for analytes such as chlorpyrifos,[59] permethrin[60] or endosulfan.[30] This is why we decided to examine the influence of Tween 20 in the analytical characteristics of the 2,4,6-TCP immunoassay. An improvement of 38% on the assay detectability was observed in the absence of the detergent without affecting the A_{max}. Since no significant differences were observed in terms of the coefficient, subsequent experiments were thus carried out in the absence of the detergent.

Effect of pH. The assay performed better in neutral or basic media, and it was inhibited below pH 4. The IC_{50} did not significantly vary between pH 5.5 and 9.5, but the maximal absorbance of the assay reached a maximum at pH 6.5 and then slowly decreased until pH 9.5. Higher pHs inhibited, again, the assay signal (see Figure 7). Although optimum conditions regarding detectability and maximal signal are placed around pH 7, the results show that the assay may be useful in a range between 5.5 and 9.5. Similar behavior has been observed with other immunoassays for phenolic compounds.[53,54] Because of their acidic character, pH changes cause a shift of the phenol/phenolate ratio present in the solution and consequently in the antibody−analyte equilibrium reaction. The greater tolerance to the basic conditions (pH > pK_a) indicates that participation of the phenolate in the stabilization of the immunocomplex may be larger than that of the phenol. These results support, again, the idea that electrostatic interactions may play an important role in the recognition of these analytes by the antibody.

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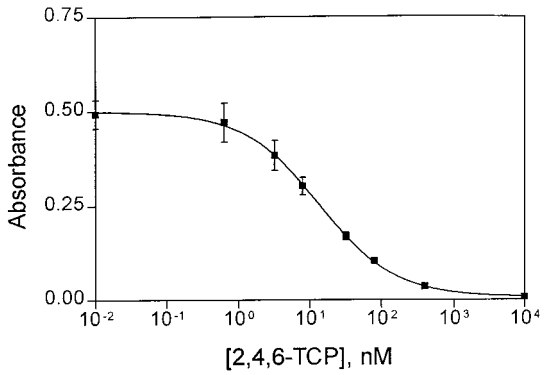

Figure 8. Calibration curve of the optimized 2,4,6-TCP immunoassay. The data presented correspond to the average and the standard deviation of nine assays run on three different plates. The curves were run in triplicate. See Table 3 for the features of the optimized immunoassay.

Table 3. Features of the Optimized Immunoassay *Antiserum 43/5HRP*[a]

A_{max}	0.501 ± 0.042
A_{min}	0.006 ± 0.003
IC_{50}, μg/L	2.76 ± 0.26
slope	−0.82 ± 0.07
dynamic range	0.53 ± 0.11 to 14.88 ± 1.68
LOD, μg/L	0.22 ± 0.07
r^2	0.993 ± 0.003

[a] The parameters are extracted from the four-parameter equation used to fit the standard curve. The data presented correspond to the average of nine calibration curves run in three different plates. Each curve was build using three-well replicates.

Figure 8 shows the standard curve, and Table 3 summarizes the parameters defining the calibration graph of the immunoassay *antiserum 43/5HRP* carried out after the evaluation of the above-mentioned immunoassay characteristics. The assay can be run in about 1 h in 5 mM PBS and in the absence of detergent. The limit of detection (90% of the assay response at zero dose) is 0.21 ± 0.06 μg/L (1.06 ± 0.03 nM), and the working range (20−80% of the assay response at zero dose) is between 14.88 ± 1.68 and 0.53 ± 0.11 μg/L (75.29 ± 8.50 to 2.68 ± 0.55 nM).

Immunoassay Specificity. We evaluated the potential interference of a group of 23 structurally related compounds by preparing calibration curves of all of them and measuring them in the assay. The degree of recognition was directly related to the presence of the three chlorine atoms at the ortho and para positions as in the target analyte, and as expected, any change introduced at the opposite site where the spacer arm was placed affected much more strongly the binding to the antibody (see Table 4). Thus, 2,3,4,6-tetrachlorophenol, possessing the three necessary chlorine atoms, was recognized 86% (considered 100% the recognition of the analyte), while 2,3,5,6-tetrachlorophenol, lacking the chlorine atom at the para position and possessing two chlorine atoms at the meta positions cross-reacted only 3%. Similarly, 2,4,5-trichlorophenol, mimicking half of the chemical structure of the immunizing hapten, was recognized 41%, while 2,3,4-trichlorophenol and 2,3,6-TCP were not recognized at all. Analytes with only two, one, or no chlorine atoms had a negligible (≤3%) or void interference in the assay. It is also worth noting that blocking the hydroxy group as an ether reduced drastically the recognition; thus, 2,4,6-

Table 4. Interference Caused by Structurally Related Chemicals, Expressed by Their IC50 and the Percentage of Cross-Reactivity[a]

no.[b]	phenolic cmpds	IC_{50} (nM)	% CR	brominated analogues	IC_{50} (nM)	%CR
5	PCP	92.3	9.8	PBP	95.6	24
4	2,3,4,6-TtCP	15.2	86			
	2,3,5,6-TtCP	463.1	3			
3	2,4,6-TCP	15.7 ± 2.59	100	2,4,6-TBP	0.8	1550
	2,4,6-TCA	>10 000	<0.001			
	2,3,4-TCP	1730	0.5			
	2,4,5-TCP	39.7	41			
	2,3,5-TCP	130.7	12			
	2,3,6-TCP	>10 000	<0.001			
2	2,4-DCP	259.5	3.5	2-B-4-CP	67.2	33.5
				2,4-DBP	11.1	136
	2,5-DCP	2666.6	0.7			
	2,6-DCP	827.4	2.3	2,6-DBP	291	5.2
	3,4-DCP	>10 000	<0.001			
1	2-CP	>10 000	<0.001			
	4-CP	>10 000	<0.001	4-BP	117.1	14
	4-C-3-MeP	>10 000	<0.001			
0	4-NP	>10 000	<0.001			
	phenol	>10 000	<0.001			

[a] Cross-reactivity is expressed as a percent of the IC50 of the 2,4,6-TCP/IC50 phenolic compound. [b] Number of halogens. B, bromo; C, chloro; Me, methyl; CP, chlorophenol; DCP, dichlorophenol; TCP, trichlorophenol; TtCP, tetrachlorophenol; PCP, pentachlorophenol; BP, bromophenol; DBP, dibromophenol; TBP, tribromophenol; PBP, pentabromophenol; TCA, trichloroanisol.

trichloroanisol was not recognized in the assay at concentrations up to 10 000 nM (2.11 mg/L), even if it had the three chlorine atoms in the right position. The reason for this lack of recognition should be found again in the incapability to produce the phenolate species, which seems to be the one participating the most in the stabilization of the immunocomplex. As it was demonstrated by theoretical models, blocking of the hydroxy group produced great electronic differences with the target analyte. Surprisingly, brominated phenols were highly recognized in this assay, especially those keeping the geometry of the immunizing hapten, and in the same degree as the chlorinated analytes. Hence, 2,4,6-tribromophenol cross-reacted 1550% and 2,4-dibromophenol 136%. When the bromine atom in para was a chlorine, the crossreactivity dropped to 33%. If only the *ortho* positions had bromines the interference was only 5.2%. In every case the recognition of the brominated analytes was always greater than that of the chlorinated analyte analogues (see Table 4). We have not yet found an explanation for this behavior; that will be the object of further studies using computer models and other series of halogenated analytes.

Immunoassay Accuracy. To perform this study, 10 blind samples and two certified samples were analyzed with the optimized ELISA.

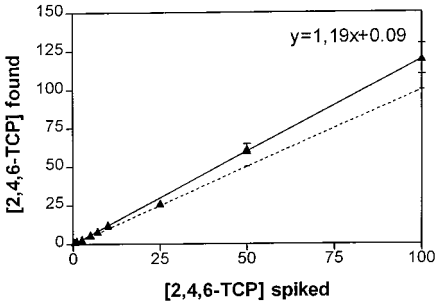

Figure 9. Graph showing the correlation between the spiked and the measured values. Blind samples were prepared by spiking milliQ water with different concentrations of 2,4,6-TCP. The data shown correspond to the average and the standard deviation of the results obtained from the immunochemical analysis of the blind samples run in triplicate during three different days. The dotted line corresponds to a perfect correlation (slope = 1).

For the spiked blind samples, a slight overestimation of the concentration was observed (see Figure 9), as evidenced by the slope of the linear regression equation that is slightly higher than 1 (slope = 1.19). The consequence of this is a small increase in the risk of false positives. As a screening technique it is desirable to avoid false negatives, and this never occurred. The correlation between spiked and measured values had a very good coefficient of correlation ($r^2 = 0.992$). Certified samples were a mixture of different analytes as described in the Experimental Section. Table 5 shows the sample composition and the 2,4,6-TCP concentration value measured by ELISA and by liquid chromatography. The overestimation observed for sample A was attributed to the presence of significant concentrations of other chlorinated phenolic compounds such as 2,4-dichlorophenol, pentachlorophenol, and 2,4,5-trichlorophenol (see above for cross-reactivity). Similarly, sample B contained pentachlorophenol, 2,4-dichlorophenol, and 2-bromophenol that could also contribute to the ELISA measured value. Nevertheless, the chromatographic measured values did not match the target value either, probably because of the difficulties for those highly polar compounds to be adequately extracted and quantitated as mentioned in the introductory section.[1,5,61,62]

CONCLUSIONS

Molecular geometry and charge distributions are important parameters determining the ability of these analytes to establish noncovalent binding forces. For ionized molecules, it is also interesting to consider the changes produced in the above physicochemical parameters as a consequence of this property. An initial knowledge of some of these features helps the immunochemist to rationalize hapten design. Thus, using molecular

Table 5. Results Obtained from the Analysis of Certified Samples

	composition (μg/L)[a]							2,4,6-TCP results	
sample	2-BP	2-CP	2,4-DCP	PCP	2,4,5-TCP	4-CP	2,4,6-TCP (target value)	chromatography	ELISA
A		113	99.1	149	139		123	203.3	244
B	2.59	1890	1.27	2.14		4.2	2.98	2.15	3.88

[a] Only the concentration values of those analytes potentially interfering in the assay are reported in the table. Sample A was provided by Yorkshire Environmental. Sample B is from Aquacheck.

modeling tools we have been able to develop an immunoassay for the detection of 2,4,6-TCP, reaching reasonable detectability limits, and have found explanations for the behavior of the assay. Thus, it has been demonstrated by theoretical calculations that blocking the phenolic group in order to introduce a linker is a bad strategy for raising antibodies against a phenolic compound. Similarly, analytes where this function is protected such as the corresponding anisole, are not recognized by the antibodies raised. The ELISA reported can simultaneously process many samples in about 1 h. The assay is reliable and shows accuracy in the measurement of 2,4,6-trichlorophenol in water that is similar to that of the chromatographic method. Only brominated phenolic compounds have shown a significant interference in the assay, although their presence in environmental water samples has rarely been reported.[63]

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