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Development and Evaluation of an Immunoassay for Biological Monitoring Chlorophenols in Urine as Potential Indicators of Occupational Exposure

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Trichlorophenols (TCP) eliminated by the urine can be considered as potential biomarkers of exposure of many chemicals (chlorophenols, chlorophenoxy acid herbicides, prochloraz, lindane, hexachlorobenzene, etc). High-throughput screening methods are necessary to carry out efficient monitoring programs that may help to prevent certain occupational health diseases. For this purpose, an indirect enzyme-linked immunosorbent assay (ELISA) for 2,4,6-trichlorophenol detection has been developed using polyclonal antisera raised against 3-(3-hydroxy-2,4,6-trichlorophenyl)propanoic acid (hapten 5) covalently coupled by the mixed anhydride (MA) method to keyhole limpet hemocyanin (KLH). The indirect ELISA uses a heterologous coating antigen prepared by conjugation of 3-(2-hydroxy-3,6-dichlorophenyl)propanoic acid (hapten 4) to bovine serum albumin (BSA) using the active ester (AE) method. The optimum hapten density for the coating antigen was found to be 3 mol of hapten/mol of protein. The assay shows a limit of detection of 0.245 ± 0.116 μg L^{-1}, and it is performed on 96-well microtiter plates in about 1.5 h. The ELISA reported recognizes on a much less extent other chlorinated phenols, such as 2,3,4,6-tetrachlorophenol (2,3,4,6-TtCP, 21%), 2,4,5-TCP (12%) and 2,3,5-TCP (15%); however, brominated phenols (BP) are even more recognized than the corresponding chlorinated analogues (ex. 2,4,6-TBP, 710%; 2,4-DBP, 119%). With the aim of finding an explanation for this behavior, theoretical calculations have been performed for those and other halogenated phenols (2,4,6-triiodophenol and 2,4,6-trifluorophenol) to clarify which physicochemical parameter can explain better the recognition pattern observed. Finally, the assay has been adapted to the analysis of urine samples. The studies have shown that a limit of detection of 1 μg L^{-1} can be accomplished on this biological matrix by combining the ELISA procedure with a C18 solid-phase extraction method.

Despite the fact that the use of chlorophenols as preservatives has dropped in many developed countries and bleaching procedures use methods other than chlorination processes, chlorophenols are widespread in many environmental and biological compartments. They have been used as antistain agents for wood products since the early 1930s, and they are also important synthetic intermediates of many chemicals such as chlorophenoxy herbicides (i.e., 2,4,5-T), dyes,[1] fungicides (i.e., prochloraz), or bleaching agents (i.e., chloranil). The importance of this industry is evidenced by the great amount of chlorophenoxy herbicides and chlorophenols produced.[2] Chlorophenols also appear in the environment when water resources containing organic materials, such as phenolic derivatives (i.e., derived from lignine), or certain aromatic acids are disinfected using chlorinating agents. Wastewaters from wood pulp and paper mills contain important levels of chlorinated organic compounds.[3−5] Finally, chlorophenols are also formed during the combustion of organic matter in the presence of chlorine or chlorine-containing compounds.[6,7]

Chlorophenols have, thus, been identified as usual contaminants of surface waters. Particularly, 2,4,6-trichlorophenol (2,4,6-TCP) has been reported to contaminate several rivers and lakes in Europe, Canada, and South Africa.[8,9] Contamination of the groundwater near sawmills or wastesites at levels ranging from 0.03 to 91.3 ppb has also been reported.[10] Similarly, trichlorophenols have been noticed as contaminants in soils and sediments in Sweden,[11] Finland,[12] British Columbia in Canada,[13] and New Zealand.[14] As a consequence, significant levels of 2,4,6-TCP may

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accumulate in fish and seafood.[9,15] Concentrations varying from 0.03 to 0.7 μg L^{-1} have also been detected in the drinking water of Janakka (Finland), Ville Mercier (Quebec, Canada), and in Utah.[16−18]

2,4,6-TCP is one of the 5 chlorophenols out of 19 thought to have significant toxicological effects and potential carcinogenicity. Although epidemiological human studies have led to inconclusive results, those carried out on animals have provided sufficient evidence of the 2,4,6-TCP carcinogenic effects.[19] An oral minimal risk level (MRL) of 0.003 mg/Kg/day would be applicable to 2,4,6-trichlorophenol 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.[20] Similarly, in Finland, an ADI (average daily intake) of 50 μg/day has been established.[21]

Exposure of the general population has been noted via contaminated environment,[3] domestic preservatives, and edible products.[22,23] Chlorophenols may also be detected in the urine as a consequence of the exposure to other chlorinated substances, such as the bleaching agent prochloraz, chlorophenoxy acid herbicides, hexachlorocyclohexanes, and chlorobenzenes.[1,22,24−26] Thus, by analyzing the urine of adults in the U.S. who have no known occupational contact with chlorophenols or related substances, it was found that 2,4,6-TCP was present in a significant number of all of the samples analyzed, with a mean value of 10.1 nM (2 μg L^{-1}).[22,23] Several studies have been performed in Germany that reveal that the 95th percentile concentration of 2,4,6-TCP in the urine of children (10−12 years old) was 1.74 μg L^{-1},[27] but for adults the mean concentration of 2,4,6-TCP in urine could reach values around 4.7 μg L^{-1} [28] in some regions. In a recent study, significant levels have been found in the plasma of German adults, despite the fact that the use of chlorophenols as preservatives has been prohibited by law in that country.[29,30]

Occupational exposure may occur through inhalation and dermal contact with this compound at workplaces where 2,4,6-TCP or the above-mentioned substances are used or produced. The NIOSH (National Institute of Occupational Safety and Health) had statistically estimated that 851 workers were potentially exposed to 2,4,6-TCP during a three-year NOES survey.[31] Worker exposure has been reported in plants producing chlorinated pesticides or fungicides,[2,32,33] in pesticide spray operators,[2] in Finnish saw mills,[34] industrial incinerator waste plants,[7,35] and electrical utility linemen in contact with chlorophenol-treated poles used in electrical line construction.[36] As an example, levels of 2,4,6-TCP ranged between 1 and 12 μM (199−2369 μg L^{-1}) in the urine of workers of a Finnish saw-mill factory where chlorophenols had been used as preservatives.[37] In a German waste incinerator, the levels of chlorophenols were significantly higher in the urine of workers in contact with the incinerator than in the urine of those in the administrative section, ranging from 0.16 to 28.30 μg L^{-1} .[7]

Another important fact of the widespread exposure to chlorophenols is that polychlorinated dibenzodioxins (PCDD), including the most toxic dioxin congener, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and polychlorinated dibenzofurans (PCDFs) are common impurities, because chlorophenols are the precursors of these substances. The median concentration of TCDD in workers in a German production plant of 2,4,5-T products was about 0.1 ppm in 1981.[2] Thus, TCDD has been detected in the lipid fraction of the serum of trichlorophenol plant production workers.[2] Similarly, in an Austrian production plant, an average level of 340 pg/g blood lipid was determined, and all plant production workers showed symptoms of chloracne and neurological diseases.[38−40] Chlorophenols have been named *predioxins*[28] and can be thought of as indicators of the formation of PCDDs.

Therefore, routine monitoring of chlorophenols in urine, environmental, and food matrixes may be important not only because of the risk effects of the parent compounds but also because of its association with dioxins. Establishment of reliable

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biomarkers for human exposure assessment for toxic substances requires specific analytical methods reaching very low limits of detection (LOD)[41,42] in order to carry out appropriate toxicological studies. Additionally, conducting efficient biological monitoring programs for exposure assessment requires rapid, quantitative, and high-throughput screening techniques.[43,44] At present, no efficient screening method exists to analyze clorophenols in urine as indicators of exposure of the general population or occupationally exposed risk groups to the above-mentioned compounds. Immunoassays may fulfill these requirements as a result of their simplicity, specificity, and high detectability.[43,45−47] Thus, the objective of this work has been the development and evaluation of a heterologous indirect immunoassay for the determination of 2,4,6-TCP in urine samples as a potential tool for the assessment of human exposure to polluted environments. Furthermore, an evaluation of the physicochemical parameters governing antibody recognition has been performed using molecular modeling and theoretic studies in order to find an explanation of the observed specificity of the optimized assay.

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 2,4,6-trichlorophenol (2,4,6-TCP), 2,4,6-tribromophenol (2,4,6-TBP), 2,4,6-triiodophenol (2,4,6-TIP), and 2,4,6-trifluorophenol (2,4,6-TFP) using semiempirical quantum mechanics MNDO[48] and PM3[49] models. All of the calculations were performed using standard computational chemistry criteria. Theoretical calculations regarding pK_a and log *P* values were carried out using the ACD/Log *P* and the ACD/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 Haptens. The preparation of hapten **5,** 3-(3-hydroxy-2,4,6-trichlorophenyl)propanoic acid, has already been reported.[50] Hapten **7**, 2-hydroxy-3,5,6-trichlorobenzoic acid, as well as other chemical reagents, were obtained from Aldrich Chemical Co. (Milwaukee, WI). The synthesis of haptens **2**−**4** and **6** is described below.

General Methods and Instruments. Thin layer chromatography (TLC) was performed on 0.25-mm, precoated silica gel 60 F254 aluminum sheets (Merck, Darmstadt, Germany). Unless otherwise indicated, purification of the reaction mixtures was accomplished by "flash" chromatography using silica gel as the stationary phase. ^{1}H and ^{13}C NMR spectra were obtained using a Varian Unity-300 (Varian Inc., Palo Alto, CA) spectrometer (300 MHz for ^{1}H and 75 MHz for ^{13}C) or a Gemini 200 (199.975 MHz for ^{1}H and 50.289 for ^{13}C). Infrared spectra were measured on a Bomen MB120 FTIR spectrophotometer (Hartmann & Braun, Québec, Canada). Gas chromatography/mass spectrometry (GC/MS) was performed on a MD-800 capillary gas chromatograph equipped with a MS quadrupole detector (Fisons Instruments, VG, Manchester, U.K.) with an electronic impact (EI) source of 70 eV provided by a capillary column (HP-5, 25 mm). The positive ions were recorded in SCAN mode. The spectroscopic and spectrometric data are given as Supporting Information.

2-Hydroxy-3,5,6-trichlorobenzaldehyde ***1***. $CHCl_3$ (6.32 mL, 79 mmol, 3.12 equiv) was added dropwise for about 1 h to a mixture of 2,4,5-trichlorophenol (5 g, 25.3 mmol), $Ca(OH)_2$ (8.3 g, 0.112 mol), and Na_2CO_3 (9.1 g, 85.85 mol) in H_2O (63.25 mL, 3.5 mol) previously heated to 65 °C. A white paste was initially formed that later became yellow. The reaction was stirred for 16 h at 65 °C. The mixture was then acidified with 1 N HCl to pH 1 and was distilled under vapor current. The compound obtained was dissolved in CH_2Cl_2, dried with anhydrous $MgSO_4$, filtered, and evaporated to dryness to obtain 3 g of a mixture containing the starting trichlorophenol and the aldehyde **1**. Separation of these two compounds was accomplished by column chromatography using hexane:CH_2Cl_2 9:1 as the mobile phase. Aldehyde **1** was obtained pure as a yellow solid (870 mg, 15% yield) and identified by its spectroscopic and spectrometric data.

3-(2-Hydroxy-3,5,6-trichlorophenyl)-2-propenoic Acid ***2***. A mixture of the aldehyde **1** (0.4 g, 1.77 mmol) and methyl diethylphosphonacetate (0.4 mL, 2.18 mmol, 1.2 equiv) was added to a suspension of KOH powder (0.4 g, 7.13 mmol, 4 equiv) in THF (10.7 mL) under argon atmosphere and magnetic stirring at room temperature (RT). Solubilization of the KOH followed by the appearance of a solid was observed. The reaction was followed by TLC (hexane:CH_2Cl_2, 7:3), until disappearance of the starting aldehyde **1** after 1.5 h. The mixture was acidified with 1 N HCl to obtain a white solid that was separated by filtration. The solid was dissolved in ethyl acetate, dried with anhydrous $MgSO_4$, filtered, and evaporated to dryness under reduced pressure to obtain 0.43 g of a white solid of the acid **2** (90% yield) identified by its spectroscopic and spectrometric data and elemental analysis.

3-(2-Hydroxy-3,5,6-trichlorophenyl)propanoic Acid ***3***. The carboxylic group was first esterified by dissolving the acid **2** (200 mg, 0.75 mmol) in MeOH (2.5 mL, 63.5 mmol) with few drops of concentrated H_2SO_4. The mixture was stirred for 6 h until complete disappearance of the starting material was observed by TLC (hexane:ethyl ether, 1:1). The solvent was evaporated, and the residue was treated with aqueous 5% $NaHCO_3$ and extracted with ethyl acetate. The organic layer was washed with saturated NaCl, dried with $MgSO_4$, filtered, and evaporated to obtain 210 mg of methyl 3-(2−hydroxy-3,5,6−trichlorophenol)-2-propenoate (95% yield). For the reduction of the double bond, a solution of 99.99% CuBr (10.87 mmol, 15.3 equiv) in anhydrous THF (2 mL) was placed in a three-neck flask with a completely inert Ar atmosphere, a magnetic stirrer was added, and the solution was cooled to an inner temperature between −5 and 0 °C. A portion

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of 2.75 M Red-Al (8 mL, 22 mmol, 31 equiv) was added slowly. The suspension, which soon acquired a black color, was stirred for 1 h at 0 °C. The temperature was then decreased to −78 °C, and anhydrous 2-butanol (2.4 mL, 26.16, 36.8 equiv) was added, followed (about 5 min later) by the solution of the methyl ester of the acid **2** (190 mg, 0.67 mmol) in anhydrous THF (5 mL). After 15 min, the temperature was allowed to reach −20 °C, and the reaction mixture was stirred for 4 h, when it was added to a solution of saturated NH_4Cl and stirred for 2 h at RT until the color changed from black to dark blue. The mixture was then extracted with ethyl ether, and the organic layer was washed with saturated NaCl, dried with anhydrous $MgSO_4$, filtered, and evaporated to dryness under reduced pressure to obtain 200 mg of a yellow-brownish oil that was purified by column chromatography using mixtures of hexane:ethyl ether with increased polarity as the mobile phase to obtain 80 mg of the methyl ester of the desired compound methyl 3-(2-hydroxy-3,5,6-trichlorophenol)-propanoate (40% yield) that was identified according to its spectroscopic and spectrometric data. The ester (45 mg, 0.16 mmol) was then dissolved in THF (3 mL), and a solution of 0.5 N NaOH (2 mL, 1 mmol) was added, which turned the reaction mixture yellow. After 4 h at RT, the hydrolysis of the methyl ester was complete, as observed by TLC (hexane:ether, 1:1). The solvent was evaporated, and the residue was treated with 0.5 N NaOH, washed with ethyl acetate, and acidified with 1 N HCl to produce a precipitate. The aqueous layer was then extracted with ethyl acetate, dried with $MgSO_4$,and evaporated to dryness to obtain 35 mg of a pale yellow solid corresponding to the desired acid **3** (82% yield), which was identified according to its spectroscopic and spectrometric data.

3-(2-Hydroxy-3,6-dichlorophenyl)propanoic Acid ***4***. A Na−Pb (10% Na−Pb) alloy (0.6 g, 2.61 mmol, 3.7 equiv) was added to a solution of the acid **2** (95 mg, 0.36 mmol) in 5% NaOH (600 μL). The reaction was stirred for 5 h at RT with a slight N_2 current to eliminate the H_2 that was formed. The mixture was acidified with 0.1 N HCl to pH 1 and extracted with ethyl acetate. The organic layer was washed with a saturated NaCl solution, dried with $MgSO_4$, filtered, and evaporated to dryness to obtain 80 mg of a white solid containing a mixture of compounds. The crude mixture was, thus, esterified as described above by dissolving the solid in MeOH (4 mL), adding a few drops of concentrated H_2SO_4, and stirring the mixture for 12 h at RT. The isolation of the desired compound was accomplished by column chromatography using mixtures of hexane:ethyl ether with increased polarity as mobile phase to obtain 50 mg (57% yield) of methyl 3-(2-hydroxy-3,6-dichlorophenyl)-propanoate, which was identified according to its spectroscopic and spectrometric data. As described above, the ester (50 mg, 0.2 mmol) was hydrolyzed by dissolving the compound in THF (3.25 mL), adding a solution of 0.5 N NaOH (2.5 mL, 1.25 mmol), and stirring the mixture for 12 h until the complete disappearance of the starting material was observed by TLC (hexane:ethyl acetate, 1:1). Treatment of the reaction afforded 40 mg (85% yield) of a white solid of the acid **4**, according to its spectroscopic data.

3-(4-Hydroxy-3,5-dichlorophenyl)propanoic Acid ***6*****.** Following the procedure described for hapten **4,** the reduction of the double bond was carried out by dissolving the commercial acid, 3-(4-hydroxyphenyl)-2-propanoic acid (0.5 g, 3.04 mmol) in 5% NaOH (4 mL) and by slowly adding a Na-Pb alloy (10% Na, 5 g; 21.75 mmol; 3.6 equiv). The mixture was stirred for 3 h at RT to obtain 0.47 g (93% yield) of a white solid corresponding to 3-(4-hydroxyphenyl)-2-propanoic acid, according to its spectroscopic data. The reduced acid (0.4 g, 2.4 mmol) was then esterified as described before in MeOH (4 mL, 0.1 mol) and few drops of concentrated H_2SO_4 for 12 h at RT to obtain 400 mg of methyl 3-(4-hydroxyphenyl)-propanoate (92% yield). The introduction of the chlorine atoms was then accomplished by dissolving the ester (300 mg, 1.67 mmol) in anhydrous CH_2Cl_2 (1 mL). To this solution, SO_2Cl_2 (300 μL, 3.73 mmol) was added, followed by the dropwise addition of anhydrous ethyl ether (525 μL, 5.02 mmol, 3 equiv). The reaction, which acquired an intense yellow color, was kept at RT for 12 h under a nitrogen atmosphere. The mixture was brought to basic pH with 5% $NaHCO_3$ and extracted with ethyl ether. The organic layer was then washed with saturated NaCl, dried with $MgSO_4$, filtered, and evaporated under reduced pressure to obtain 350 mg of a yellow oil that was purified by column chromatography using mixtures of hexane and ethyl acetate with increasing polarity to isolate 170 mg (24% yield) of methyl 3-(4-hydroxy-3,5-dichlorophenyl)-propanoate that was identified according its spectroscopic and spectrometric data. The hydrolysis of the ester was carried out as described above by adding a solution of 0.5 N NaOH (3.5 mL, 1.75 mmol) to the ester (70 mg, 0.28 mmol) dissolved in THF (4.5 mL). The mixture was stirred for 12 h at RT to obtain 50 mg (76% yield) of a yellowish solid corresponding to the desired hapten **6**, identified according to its spectroscopic data.

Preparation of the Protein Conjugates. *Active Ester (AE) Method.* Following described procedures,[51] haptens **2**−**4** and **6** (10 μmol) were reacted with NHS (*N*-hydroxysuccinimide, 5.75 mg, 50 μmol) and DCC (dicyclohexylcarbodiimide, 20.63 mg, 100 μmol) in DMF (dimethylformamide, 200 μL) for about 1 h at RT. The urea that was formed as a secondary product was precipitated and removed by centrifugation, and the supernatant was divided into three equivalent fractions and added to three vials containing bovine serum albumin (BSA), ovalbumin (OVA), and conalbumin (CONA) (10 mg/each) dissolved in 0.2 M borate buffer (1.8 mL). For certain conjugates, the molar ratio hapten:lysine residues of the protein were 2:1, 1:1, 1:2.5, and 1:5.

Mixed Anhydride (MA) Method. According to described procedures,[52] the haptens **2**−**4** and **6** (20 μmol) were reacted with tributylamine (5.3 μL, 22 μmol) and isobutylchloroformate (3.2 μL, 24 μmol) in anhydrous DMF (200 μL). The solution containing the activated hapten was then divided into three equivalent fractions and added to the BSA, OVA and CONA (20 mg/each) dissolved in 0.2 M borate buffer (1.8 mL). In both methods, the protein conjugates were purified by dialysis against 0.5 mM PBS (four times, 5 L each) and Milli-Q water (1 time, 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.

Hapten Density Analysis. Hapten densities of **2**−**4** and **6**−**BSA** conjugates were determined by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-

(51) Gascón, J.; Oubiña, A.; Ballesteros, B.; Barceló, D.; Camps, F.; Marco, M.-P.; González-Martinez, M.-A.; Morais, S.; Puchades, R.; Maquiera, A. *Anal. Chim. Acta* **1997**, *347*, 149−162.

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TOFMS) by comparing the molecular weight of the standard BSA and that 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 that operates with an intensity of 2800. The instrument is controlled by a BioSpectrometry Workstation equipped 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 1 μL of the matrix [(*E*)-3,5-dimethoxy-4-hydroxycinnamic acid; 10 mg/mL in CH_3CN:H_2O 70:30; 0.1% TFA] with 1 μL of a solution of the conjugates or proteins (5 mg/mL in CH_3CN:H_2O, 70:30; 0.1% TFA).

Immunochemistry. *General Methods and Instruments.* The pH and the conductivity of all buffers and solutions were measured using a pH meter 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 Multiskan Plus MK II microplate reader (Labsystems, Helsinki, Findland) or on a SpectramaxPlus (Molecular Devices, Synnyvale, CA) at a single wavelength mode of 450 nm. 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 Software Inc., San Diego, CA). Unless otherwise indicated, data presented correspond to the average of at least two well replicates.

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). The preparation of the antisera (As43, As44, and As45) against TCP by immunizing with hapten 5-KLH conjugate (3(3-hydroxy-2,4,6-trichlorophenyl)propanoic acid coupled to keyhole limpet hemocyanin by the mixed anhydride method) has already been reported.[50] The antisera (As) were used without further purification and were stored frozen in the presence of 0.02% NaN_3. Working aliquots were stored at 4 °C. Protein conjugates were prepared as described below.

Buffers. Unless otherwise indicated, 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. 2× 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.

Non-Competitive Indirect ELISA. Screening of the coating antigens and the antisera was performed by a noncompetitive indirect ELISA protocol as described below. For this purpose, the avidity of the different As (As43, As44 and As45) was determined by measuring the binding of serial dilutions (1/1000 to 1/64 000) of each antisera to microtiter plates coated with twelve different 2-fold dilutions (from 10 μg/mL to 9 ng/mL) of the **2**−**4** and **6**-BSA, -CONA,and -OVA conjugates. Optimal concentrations for coating antigens and As dilution were chosen to produce around 0.7−1 units of absorbance in 30 min. The microplates were coated with the antigens dissolved in coating buffer (100 μL/well) overnight at 4 °C. The next day, the plates were washed four times with PBST, and the antiserum appropriately diluted in PBST was added to the wells (100 μL/well) and incubated for 30 min at RT. The plates were washed as described before, and a solution of goat anti-rabbit IgG coupled to horseradish peroxidase (anti-IgG-HRP) in PBST (1/6000) was added to the wells (100 μL/well) and incubated for 30 min at RT. The plates were washed again, and the substrate solution was added (100 μL/well). 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.

Optimized Competitive Indirect ELISA. Microtiter plates were coated with 4-BSA(1:5) in coating buffer (0.625 μg/mL, 100 μL/well), covered with adhesive plate sealers, and allowed to sit overnight at 4°C. The following day, the plates were washed with PBST (four times, 300 μL/well), and 2,4,6-TCP standards (1000−0.625 nM, prepared in PBS) or samples were added to the coated plates (50 μL/well), followed by the sera As43 (1/2000 in PBST, 50 μL/well). The plates were washed again, and 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 was incubated for 30 min more at RT. The plates were processed as described above.

ELISA Evaluation. The specificity of the immunoassay as well as the effect of physicochemical features of the media, such as ionic strength and pH value, were evaluated as previously reported.[50,53]

Correlation Studies between ELISA and Gas Chromatography with Electron-Capture Detector (GC-ECD). PBS solutions were spiked with 2,4,6-TCP at different concentration levels and split into two parts for ELISA and GC-ECD analysis. Correlation was performed by linear regression analysis of the results obtained by both methods. For ELISA analysis, the PBS solutions were directly added to the microtiter wells and measured using the protocol described above. For GC-ECD analysis, tetrachloronitrobenzene was added to a concentration of 5 μg L^{-1}, and the PBS solutions were extracted with toluene (PBS:toluene, 5:1) on an shaker for 30 min at 300 rpm. The extraction efficiency is 77.78 ± 8.35%. The extracts were derivatized by adding of BSTFA (bis-(trimethylsilyl)trifluoroacetamide, 2 μL) and incubated for 2 h at RT. Injections (1 μL) were made on a HP 5890A gas chromatograph equipped with a HP 7673A autosampler and with an ECD detector ($^{63}_{28}$Ni), and HP 3396 Series II integrator (recorder). A capillary column (25 m × 0.22-mm i.d. × 0.25-μm film thickness) BPX35 (35% Phenyl(equiv)polysilphenylene-siloxane; SGE Europe Ltd, U.K.) was used. GC conditions were set as follows: temperature program, 100 °C (1 min) to 250 °C (at 5 °C/min), 250 °C to 300 °C (at 15 °C/min); injection temperature, 250 °C; detector temperature, 300 °C; He, the carrier gas, employed at 130 kPa (20 cm/sec); make up gas, N_2 5.0.

Matrix Effect Studies.. Urine samples of the same individual were collected over 2 days, stored in a refrigerator, and mixed to have a large and homogeneous urine sample. The urine was alkalized to pH 8 with 5 N NaOH and centrifuged (3000 rpm, 10 min) to eliminate the particulate matter. The supernatant was then

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aliquoted and stored frozen at −40 °C. The samples were thawed and were diluted at least two times with Milli-Q water prior to the ELISA studies.

Sample Cleanup. A C18 cartridge (Sep-Pak Plus C18 cartridge, Waters) was conditioned by passing MeOH (5 mL) followed by 0.1% aqueous acetic acid (5 mL). Urine samples (10 mL) were then loaded into the column. The cartridge was washed with 5 mL of 50:50 MeOH:H_2O (0.1% acetic acid) and 2,4,6-TCP eluted with 5 mL of 85:15 MeOH:H_2O (0.1% acetic acid). About 1-mL fractions were collected and analyzed. All of the steps were performed at a flow rate of 1 mL/min. Under these conditions, 98% of the TCP loaded was eluted in the first fraction. Prior to the ELISA studies, the fractions were diluted 10 times (initial urine volume) with PBS.

Analysis of the Urine by GC/MS. Urine samples (100 mL) were extracted with toluene (1 mL) and derivatized by adding BSTFA as mentioned above. For the GC/MS analysis, injections (1 μL) were splitless (48 s) solvent delay (5 min). A HP-5MS (cross-linked 5% Ph Me siloxane, 30 m × 0.25-mm i.d. × 0.25-μm film thickness) column was used. The ion source temperature was set at 200 °C, and He was the carrier gas employed at 1 mL/min. The ionization energy was 70 eV. GC conditions were as follows: temperature program, 100−300 °C (7 °C/min); injector temperature, 250 °C; and ions 253/268 (2,4,6-TCP and 2,4,5-TCP), 309/324 (2,4-DBP), 387/402 (2,4,6-TBP), 289/304 (2,3,4,6-TtCP) were monitored in the SIM mode. For the SCAN mode, the mass range explored was 45−550. All of the data are reported as m/z (relative intensity).

ELISA Matrix Effect Studies. Standard curves of 2,4,6-TCP were prepared in PBS and in PBS-diluted urine (1/2−1/256) and run in the competitive ELISA using the protocol described above to compare parallelism of the calibration curves.

RESULTS AND DISCUSSION

In a previous article, we reported the merit of theoretical calculations and molecular modeling studies for assessing the selection of hapten **5** as the most appropriate immunizing hapten to raise antibodies against 2,4,6-TCP.[50] A homologous direct ELISA was then developed and validated for the analysis of water samples. Urine samples have often been quantitated by ELISA; however, accurate quantitation of small organic analytes requires the use of cleanup procedures or the application of high dilution factors to overcome the effect of the matrix[45,54,55]. Direct ELISA formats are more likely to suffer nonspecific interferences from the sample matrix as a result of the fact that the enzyme label coexists with the sample, whereas in the indirect ELISA format, the label is added in a second step. On the other hand, although it cannot be considered as a general behavior, an improvement of the immunoassay detectability, by using a competitive hapten showing a certain degree of heterology from the immunizing hapten, has often been observed.[56,57] In light of these considerations, our aim has been the development of a heterologous indirect ELISA format suitable for the analysis of chlorophenols in urine samples.

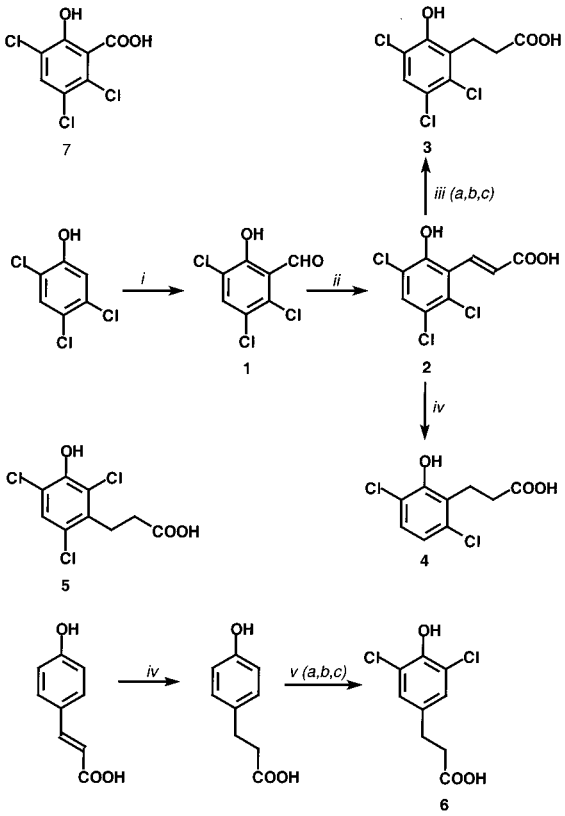

Figure 1. Chemical structures of the haptens **1**−**6** and synthetic pathways used for their preparation. Hapten 7 was commercially available. Preparation of hapten 5 has already been described (50). (i),$CH_3Cl/Ca(OH)_2/Na_2CO_3/H_2O$; (ii) methyl diethylphosphonacetate/KOH in THF; (iii) a, H_2SO_4/MeOH; b, Red-Al/CuBr/2-BuOH; c, aq NaOH/THF; (iv) Na−Pb (10% Na)/aq NaOH; (v) a, H_2SO_4/MeOH; b, SO_2Cl_2/Et_2O in CH_3Cl; c, aq NaOH/THF.

Competitive Hapten Design and Synthesis. Several chemical structures differing in the position of the spacer arm and the number and location of the chlorine atoms were initially proposed (see Figure 1). The haptens possessing the linker at the ortho position were prepared from the aldehyde **1**. Initial attempts to obtain this aldehyde moved toward the reduction of the carboxylic acid of the corresponding commercially available salicylic acid using DIBAH. However, we did not succeed in controlling the reduction only to the aldehyde, but most of the time, the reaction proceeded until the corresponding benzylic alcohol. Reexamining classical organic chemistry procedures draws to our attention the direct introduction of the aldehyde at the ortho position of a phenolic group. Thus, both Duff and Reimer−Tieman reactions had been used to prepare the aldehyde **1**, although with very low yields.[58−61] Following the procedure described by Stokker et al.,[58] we were able to prepare the desired aldehyde at a 15% yield in a pure form. The introduction of the spacer arm was accomplished using the Wittig−Horner reaction following the procedure de-

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scribed by Texier-Bouller and Foucaud,[62] affording hapten **2** at a 95% yield. Preparation of hapten **3** by reduction of the double bond of **2** was initially approached by using a Na−Pb alloy.[63] This method that had afforded excellent results during the preparation of the immunizing hapten **5**[50] produced the hydrogenolysis of the chlorine atom at the para position, leading to the saturated acid **4** (≈60% yield), instead of the expected acid **3**. All attempts using described procedures[50,64,65] to chlorinate the para position of hapten **4** to obtain **3** were unsuccessful, leading to complex reaction mixtures. In light of these results, we decided to explore alternative reduction methods for the double bond present in the spacer arm of the acid **2**. From all methods explored (H_2/Pd, $Na_2S_2O_4$, etc) only Red-Al (sodium bis(2-methoxyethoxy aluminum hydride)[66] in the presence of CuBr and 2-butanol accomplished the reduction of the double bond of the methyl ester of **2** (40% yield). The ester hydrolysis afforded the desired hapten **3**.

Synthesis of hapten **6** used the commercially available 4-hydroxycinnamic acid as the starting material. Reduction of the double bond was accomplished using a Na−Pb alloy in NaOH solution at a very high yield. The introduction of the chlorine atoms was performed in the corresponding methyl ester with SO_2Cl_2 and Et_2O in CH_2Cl_2, as previously reported.[50,65] However, the expected methyl 3-(4-hydroxy-3,5-dichlorophenyl)propanoate was obtained with only a 24% yield isolated from a complex mixture of compounds that could not be identified. We suspect that the reason for this low yield was the chlorination of the activated benzylic position in para versus the hydroxy group. Previously, we had used this method with a very good yield to chlorinate an aromatic ring that also had a benzylic group, but in a less activated meta position.[50] The desired hapten **6** was obtained after the hydrolysis of the ester.

Screening of the Antiserum Avidity for the Immunoreagents and Their Influence on the Immunoassay Detectability. Haptens **2**−**4** and **6**, initially conjugated to BSA, OVA, and CONA using the active ester (AE) method, afforded assays with acceptable detectability but showed low solubility in aqueous buffers, causing stability and reproducibility problems. Analyses made by MALDI-MS revealed a high, but not unusual, degree of conjugation (see Table 1). The lipophilic character conferred by the chlorine atoms of the analytes to the conjugates or to the undesired side conjugation reactions produced by the carbodiimide[67] could be some of the reasons for the lack of stability of the immunoreagents. In contrast, the conjugates prepared by the MA method were water-soluble and had lower conjugation yields (see Table 1); however, only those prepared with hapten **4** and the homologous antigen 5BSA afforded competitive assays, although with a low detectability and background noise (see Table 2), which can be attributed to the use of a homologous coupling method.[68]

Table 1. Hapten Densities Measured for the 1−6BSA Conjugates

conjugate	conjugation method[a]	hapten:protein[b]	hapten density[c]
2BSA	AE	2:1	14
	MA	2:1	8
3BSA	AE	2:1	10
	MA	2:1	10
4BSA	AE	2:1	17
	MA	2:1	18
5BSA	MA	2:1	7
6BSA	AE	2:1	25
	MA	2:1	12

[a] Method of conjugation: AE active ester, MA, mixed anhydride. [b] Hapten:protein molar ratio employed during the conjugation reaction. The ratio is calculated in terms of lysine residues of the protein. [c] Hapten density was measured by MALDI-TOFMS.

Table 2. Immunoassay Features of the Competitive ELISA[a]

As[a]	CA[b]	conj. method	A_{max}	A_{min}	slope	IC50[c]	r^2
43	**4**BSA	AE	1.021	0.001	−0.54	0.96	0.984
		MA	1.292	0.09	−1.08	10.08	0.982
	4CONA	AE	1.509	0.011	−1.13	3.57	0.991
		MA	0.946	0.136	−1.67	21.31	0.988
	4OVA	AE	1.233	0.009	−1.45	1.72	0.979
		MA	0.697	0.035	−1.36	5.29	0.996
	5BSA	MA	0.940	0.335	−1.33	237.7	0.894
	6BSA	AE	0.971	0.047	−1.46	9.47	0.964
	6OVA	AE	1.334	0.068	−1.42	14.58	0.987
44	**4**BSA	AE	1.747	0.001	−0.78	24.21	0.988
	4CONA	AE	1.469	0.001	−0.70	23.31	0.984
	5BSA	MA	1.305	0.01	−0.7	186.6	0.971
	6BSA	AE	0.951	0.051	−0.81	21.94	0.967
45	**4**BSA	AE	1.848	0.017	−1.23	39.37	0.975
	4CONA	AE	1.075	0.001	−0.99	19.19	0.981
	5BSA	MA	1.244	0.244	−1.17	29.48	0.964
	6BSA	AE	1.155	0.213	−1.28	76.43	0.864
	6OVA	AE	0.849	0.001	−0.89	64.46	0.980

[a] As, antisera. [b] CA, coating antigen. [c] IC50 values are expressed in $\mu g\ L^{-1}$.

All of these considerations encouraged us to rationally prepare new coating antigens with hapten 4 that had afforded the best competitive immunoassays, using again the AE method by controlling very well the hapten density on one side to avoid the solubility problems that were observed but also to improve immunoassay detectability. Table 3, shows the features of the competitive assays obtained. A higher detectability can be observed when using a coating antigen with the lower hapten density, which supports the hypothesis of the existence of a certain hapten density value for optimum immunoassay performance.[69,70] Thus, the combination As43/4BSA(1/5) was selected for further evaluation of an immunoassay to analyze chlorophenols in urine samples (see Figure 2 and Table 4 for immunoassay parameters).

Immunoassay Evaluation. *Physicochemical Properties of the Media.* Considering the particular properties of the urine samples (pH ≤ 6 and conductivity between 25 and 30 mS cm^{-1}), it was

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Table 3. Effect of the Hapten Density on the Immunoassay Features

CA	hapten:protein[a]	hapten density[b]	A_{max}	A_{min}	slope	IC50	r^2
4BSA	2:1	13	0.771 ± 0.214[c]	0.075 ± 0.097	−1.16 ± 0.13	2.25 ± 0.04	0.991 ± 0.006
	1:1	10	0.820 ± 0.263	0.066 ± 0.096	−1.07 ± 0.11	3.05 ± 0.13	0.991 ± 0.009
	1:2.5	7	1.209 ± 0.355	0.056 ± 0.106	−1.19 ± 0.07	3.12 ± 0.13	0.939 ± 0.003
	1:5	3	1.018 ± 0.301	0.046 ± 0.092	−1.15 ± 0.06	1.67 ± 0.16	0.977 ± 0.007

[a] 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. [b] Hapten density calculated by MALDI-TOFMS. [c] Average and standard deviation of two experiments performed on different days. The IC50 is expressed in μg L^{-1}.

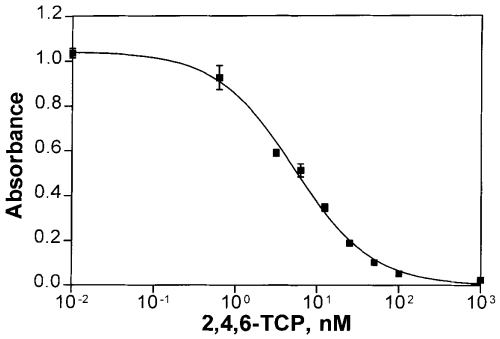

Figure 2. Calibration curve of the 2,4,6-TCP heterologous indirect immunoassay. The data presented correspond to the average and the standard deviation of nine assays run on three different plates. The curves were run in triplicate. See Table 4 for the features of the optimized immunoassay.

Table 4. Features of the Optimized Immunoassay Antiserum 43/4BSA[a]

A_{max}	0.893 ± 0.177
A_{min}	0.041 ± 0.042
IC50, μg L^{-1}	1.53 ± 0.35
slope	−1.24 ± 0.19
dynamic range, μg L^{-1}	4.49 ± 0.79 to 0.45 ± 0.24
LOD, μg L^{-1}	0.24 ± 0.12
r^2	0.991 ± 0.006
N	18

[a] The parameters are extracted from the four-parameter equation used to fit the standard curve. The data presented correspond to the average of 18 calibration curves run in different plates through a 1-month period. Each curve was built using three-well replicates.

necessary to know the behavior of the immunoassay when varying these parameters. As reported in other immunoassays for phenolic compounds,[50,53,71] a greater tolerance to basic conditions was also observed in this case. The assay performs well between pH 7.5 and 9.5, although the detectability slowly decreased from pH 6.5 to pH 10.5. At pH values lower than 6, the assay is inhibited. Regarding the effect of the ionic strength of the media, detectability of the assay did not change significantly from 12 to 25 mS cm^{-1}, although the maximum signal slowly decreased in this range from 1.3 to 0.7 units of absorbance. These experiments demonstrated the necessity of buffering and lowering the ionic strength of the urine sample before its measurement by ELISA.

ELISA Specificity. The degree of recognition was directly related to the presence of the three chlorine atoms at the ortho and para position, as in the target analyte as previously reported,[50] but now the specificity of this assay was even greater (see Table 5). The best-recognized analyte, 2,3,4,6-tetrachlorophenol (TtCP), possessing the three necessary chlorine atoms at the right positions, was only recognized 20% of the time, but in the previously developed assay, it crossreacted 86%[50] of the time, which means that now the fourth chlorine atom at the meta position disfavors competition with hapten **4** for the antibody binding. Other trichlorophenols, such as 2,4,5- and 2,3,5-TCP crossreacted only 12 and 13%, respectively. 2,4-DCP was the only dichlorophenol that was recognized, demonstrating again that the presence of the chlorine atom in the para position was necessary. Thus 2,6-DCP did not crossreact at concentrations higher than 2000 nM. Pentachlorophenol (PCP) and 2,3,5,6-TtCP were not recognized, either. Previously, we had demonstrated by experimental and theoretical studies that a free phenol group was also essential for antibody recognition.[50] As in the homologous direct format, brominated phenols were highly recognized in this assay. Hence, 2,4,6-tribromophenol (TBP) crossreacted 710%, and 2,4-dibromophenol (DBP), 119%. When the bromine atom in the para position was replaced by a chlorine atom, the crossreactivity dropped to 31%. As before, the bromine at position 4 was necessary; if only the ortho positions had bromine atoms, the analyte was not recognized. In every case, the recognition of the brominated analytes was greater than that of the chlorinated analyte analogues.

Influence of the Physicochemical Features of the Analytes in the Antibody Recognition. We have been discussing several hypotheses to explain this high recognition of the brominated analogues. One reason could be the size of the bromine atoms, that being greater than the chlorine atoms (van der Waals radii of 1.95 and 1.77 Å, respectively), they could fit better into the antibody binding cavities. A second explanation could be the differences in the pK_a or the acidity of the phenol group. Lower pK_a values would induce the preferential existence of the deprotonated form of the analyte in assay media, favoring its electrostatic interaction with the antibody. As it has been demonstrated in this and other previously reported immunoassays for phenolic compounds,[50,53,71] these kinds of interactions may have a large participation in the formation of the complex. A third explanation could be found in the differences of the electronic distribution between chlorinated and brominated analogues, which could create differences in dipole−dipole interactions in the antigen−antibody complex. Finally, we have also been considering the hydrophobic interactions that, according to published works, may also play an important role in the stabilization of the immunocomplex.[72,73]

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(72) Van Oss, C. J. *J. Mol. Recognit.* **1997**, *10*, 203−216.

Table 5. Interference Caused by Structurally Related Chemicals, Expressed by Their IC50 and the Percentage of Crossreactivity[a]

no. halogens[b]	phenolic compds	IC50 (nM)	% CR	brominated analogues	IC50 (nM)	% CR
5	PCP	>2000	<0.05	PBP	>2000	<0.05
4	2,3,4,6-TtCP	32.5	21			
	2,3,5,6-TtCP	>2000	<0.05			
3	2,4,6-TCP	8.54 ± 1.44	100	2,4,6-TBP	1.5	710
	2,4,5-TCP	56.5	12			
	2,3,5-TCP	52.1	13			
2	2,4-DCP	410.1	2	2-B-4-CP	25.1	32
				2,4-DBP	6.7	119
	2,6-DCP	>2000	<0.05	2,6-DBP	>2000	<0.05
1				4-BP	232	4

[a] Crossreactivity is expressed as % of the IC50 of 2,4,6-TCP/IC50 phenolic compound. [b] B, bromo; C, chloro; DCP, dichlorophenol; TCP, trichlorophenol; TtCP, tetrachlorophenol; PCP, pentachlorophenol; BP, bromophenol; DBP, dibromophenol; TBP, tribromophenol; PBP, pentabromophenol.

Table 6. Crossreactivity Data of Trihalogenated Phenolic Compounds Compared to Some of Their Physicochemical Features

A

analyte[a]	%CR	pK_a	α (PhO$^-$)[b]	log *P*
2,4,6-TBP	710	6.34 ± 0.23	0.98	4.33 ± 0.49
2,4,6-TIP	205	6.47 ± 0.23	0.97	3.88 ± 0.50
2,4,6-TCP	100	6.59 ± 0.23	0.96	3.58 ± 0.33
2,4,6-TFP	<0.05	7.47 ± 0.23	0.77	2.24 ± 0.50

B

analyte[a]	%CR	size[c]	surface[d]	volume[e]
2,4,6-TBP	710	1.95	342	338
2,4,6-TIP	205	2.10	364	422
2,4,6-TCP	100	1.77	315	280
2,4,6-TFP	<0.05	1.30	265	158

C

analyte[a]	%CR	ACS[f]	HAC[g]	HCD[h]
2,4,6-TBP	710	−0.449	−0.100	−3.21
2,4,6-TIP	205	−0.430	−0.111	−2.86
2,4,6-TCP	100	−0.609	−0.026	−1.12
2,4,6-TFP	<0.05	−0.321	−0.125	−13.5

[a] 2,4,6-TBP, 2,4,6-tribromophenol; 2,4,6-TIP, 2,4,6-triiodophenol; 2,4,6-TCP, 2,4,6-trichlorophenol; 2,4,6-TFP, 2,4,6-trifluorophenol. [b] Relative fraction of the phenoxide at pH = 8.0. [c] van der Waals radii of the halogen atom expressed in angstroms. [d] Accessible molecular surface for phenoxide ion in Å^2. [e] Volume of the minimal box containing the molecule expressed in Å^3. [f] Sum of the aromatic carbons point charges expressed in e$^-$ units. [g] Average of the halogen atoms point charge in e$^-$ units. [h] Halogen atom charge density in (e$^-$/Å^3) × 10^3.

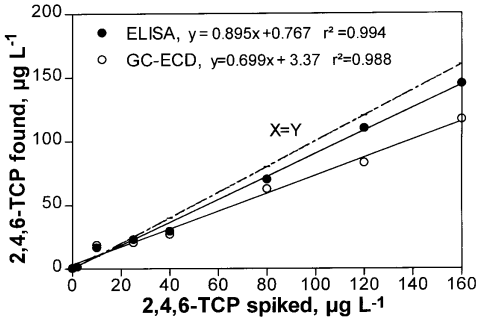

Figure 3. Accuracy of the ELISA method compared to the GC-ECD method. Spiked samples were prepared in PBS and split into two parts to be analyzed by both methods. Samples were directly measured for ELISA, but for GC-ECD, samples were extracted in toluene in the presence of an internal standard, derivatized, and then injected into the chromatograph. ELISA shows better accuracy than the GC-ECD method. Correlation between both techniques agrees to the following equation $y = 1.26x - 4.27$ and has a regression coefficient of $r^2 = 0.993$.

To assess all of these hypotheses, the recognition of several 2,4,6-halogenated phenolics in the assay was investigated. This set of compounds has comparable directional interactions with the receptor pocket in the antibody. Thus, the observed changes in the affinity could be attributed to the variations in the strength of the molecular interactions and the molecular volume and surface. Table 6 shows some of these molecular parameters, macroscopic and microscopic, that could explain the observed relative affinities.

The acidity of these compounds is similar, with the exception of the fluorinated derivative (see Table 6, section A). If it is expressed as the dissociated fraction at the working pH, we could see that almost all of the compounds are in the phenolate form, with the exception of the trifluoroderivative in which only 77% exists as phenolate. This factor could have some influence in the poor recognition of the last compound but could not justify the differences among the other three. On the other hand, it indicates clearly that the recognized compound is actually the phenolate and not the phenol structure. The log *P* (*n*-octanol/water partition coefficient) calculated values for the four studied phenols (see Table 6, section A) show a good correlation with the observed recognition values. This parameter is related, at the microscopic level, with the differential (solute:water, solute:octanol) hydrogen bond interactions, the hydrophobic−hydrophilic interactions with the solvents, and the energy needed to open a cavity by the solute molecule in the solvent. All of these interactions are similar to those involved in the antigen/antibody recognition event[73] except for the fact that in the last case, they are spatially oriented. Thus the *n*-octanol/water partition could be a good model for the antibody recognition.

The size of the halogen atoms, their van der Waals radii, moves in a short range (only a 16% from chlorine to iodide) and could have only a little influence, considering the dimensions (molecular

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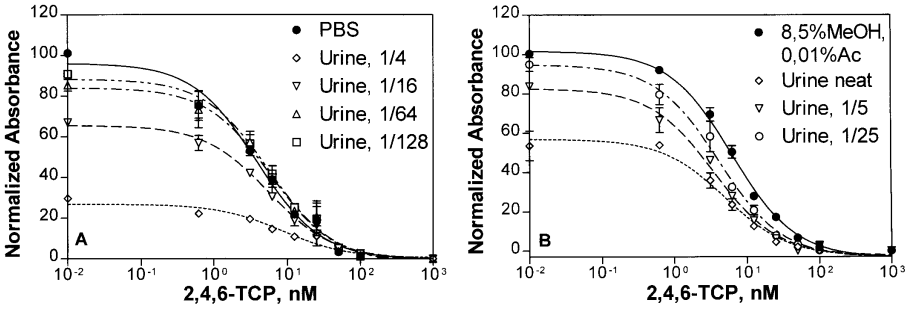

Figure 4. Calibration graphs showing the interference of the urine in the ELISA method that was developed. Under the present immunoassay conditions, the urine has to be diluted at least 50 times to ensure accurate results, which results on a LOD of ~10 μg L^{-1}. A cleanup step consisting of a C18-SPE procedure allows direct measurement of the urine samples by just diluting the sample five times with the assay buffer. A, calibration curves obtained with untreated urine; B, calibration graphs obtained after C18-SPE cleanup step.

Table 7. Concentration Levels of Chlorophenols Found in Urine by GC/MS and Their Equivalents in 2,4,6-TCP Immunoreactivity (IR)[a]

analyte	GC/MS, μg L^{-1}	% CR	2,4,6-TCP IR, μg L^{-1}
2,4,6-TCP	0.06	100	0.06
2,4,5-TCP	nd[b]	12	-
2,3,4,6-TtCP	nd	21	-
2,4,6-TBP	nd	710	-
2,4-DBP	0.1	119	0.119
total IR			0.179

[a] 2,4,6-TCP immunoreactivity equivalents was calculated considering the percentage of cross-reactivity in the assay. [b] nd, not detected.

surface and volume) of the analytes (see Table 6, section B) in respect to those for the binding pocket in the antibody. Furthermore, we take into account that the protein side chains in this domain have some flexibility.[74] Finally, partial atomic charges could be related with dipole−dipole interactions (permanent or induced), which are the main forces under the hydrophobic−hydrophilic interactions. We have considered the sum of the charges over the aromatic carbons and the average of the halogen atoms charge (see Table 6, section C). These two parameters hardly correlate with the recognition observed, but point charges are poor descriptors for dipole interactions, whereas atomic charge densities (expressed as the relation between point charges and van der Waals atomic volume) are more realistic descriptors and could be better related to the observed affinities.

Correlation between GC-ECD and ELISA. Spiked PBS samples ($n = 8$) were analyzed by ELISA and by GC-ECD. The correlation between both techniques is good enough and conforms to the equation $y = 1.26x - 4.27$ with a regression coefficient of $r^2 = 0.993$. With respect to the spiked values, the results are shown in Figure 3. It is possible to observe that the ELISA results (slope = 0.89) are closer to the spiked values than the GC-ECD results (slope = 0.70), which can be explained by the null sample treatment necessary to analyze these aqueous samples by ELISA.

Matrix Effect Studies. A blank urine sample would be desirable to assess matrix effect in the ELISA; however, as was mentioned in the Introduction, the general population is exposed to xenobiotics that are eliminated as halophenols in urine. For this reason, the urine used in this study was first characterized by GC/MS in order to detect the presence of the most important phenolic compounds interfering with the ELISA (2,4,6-TCP, 2,4,5-TCP, 2,3,4,6-TtCP, 2,4,6-TBP, and 2,4-DBP; see above results from the crossreactivity studies). As is shown in Table 7, some of these compounds were detected at the trace level. Considering the crossreactivity values of those compounds in the ELISA, we estimated that the total interference of this "blank urine" could be equivalent to 0.18 μg L^{-1} of TCP immunoreactivity (TCP-IR). Because the LOD of the present ELISA is around 0.24 μg L^{-1}, the urine sample used in this study could be considered to be a real "blank matrix".

To assess the effect of the urine on the immunoassay, the urine was collected and alkalized to enhance the solubility of the chlorophenols, and the pH and conductivity were adjusted to place the sample within the working conditions of the immunoassay. Standard curves were prepared in raw urine and PBS-diluted urine. Figure 4A shows a significant effect of the matrix interfering with the assay. The urine has to be diluted over 50 times to obtain reliable results when measuring urine samples. As was mentioned in the Introduction, levels of chlorophenols of occupational exposed workers can be high enough to directly analyze their urine after diluting the sample with the assay buffer; however, many samples containing significant levels of chlorophenols would be considered as negative samples, because by diluting the sample 50 times, the LOD of the assay would have diminished to 10 μg L^{-1}, instead of the 0.2 μg L^{-1} shown when analyzing buffered samples.

In light of these results, a C18-based solid-phase extraction (SPE) method was applied to extract 2,4,6-TCP from the urine in order to diminish its interference in the ELISA. Figure 4B shows that an important improvement can be achieved by introducing this cleanup method, because urine analysis can be accomplished after just 5-fold dilution with PBS. Thus, by combining C18-SPE and ELISA, urine samples containing TCP-IR levels over 1 μg L^{-1} may now be detected. The LOD that is reached is sufficient for occupational exposure assessment if we consider, as mentioned in the Introduction, that the general population can have background levels of TCP-IR up to this value or even higher.

CONCLUSIONS

Biological monitoring requires rapid and efficient high-throughput screening methods. Analysis of xenobiotics or their metabolites in urine can be used as biomarkers of exposure to

(74) Van Regenmortel, M. H. V. *Biomer. Pept. Prot. Nucleic Acids* **1995**, *1*, 109−116.

certain toxic compounds of industrial origin. Chlorophenols are widespread in the environment as a result of their extensive use as wood and textile preservatives and also because of their industrial use as intermediates for many other chemicals (pesticides, bleaching agents, etc.). Biological monitoring of trichlorophenols as indicators of exposure is important not only because of their own toxicological risk effects as the parent compounds (trichlorophenols, lindane, hexachlorobenzene, prochloraz, etc) but also because of their association with dioxins. Occupationally exposed people constitute important risk groups because of their contact with high concentrations of these compounds. Therefore, it would be desirable to provide occupational practitioners and laboratories with screening tools to assess levels of exposure in order to provide appropriate health surveillance to the exposed population. Because of its simplicity and its capacity to process many samples simultaneously, the immunochemical technique described in this paper could provide a useful tool to carry out biological monitoring studies in target industrial sectors where exposure to the mentioned chlorinated substances may occur. Despite the complexity of the urine samples, the protocol described in this paper allows measurement of chlorophenols in this matrix with a LOD of around 1 μg L^{-1}. To reach this detection level in urine, the immunochemical method reported here needs a cleanup step. A C18-SPE procedure has been shown to eliminate most of the interferences from the matrix. Despite this, the ELISA reported here allows the simultaneous measurement of many samples. Furthermore, many commercial suppliers provide SPE methods on a 96-well configuration to allow high-throughput sample treatment on a format easily adjustable to the microplate-based ELISA methods. We must also consider that chlorophenols may appear in urine not only as free phenols but also as glucuronides and sulfate conjugates; therefore, further studies will be addressed to validate the applicability of the method presented here to the analysis of hydrolyzed urine from individuals.

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SUPPORTING INFORMATION AVAILABLE

Spectroscopic data (^{1}H and ^{13}C NMR, IR), spectrometric data (MS), and elementary analysis of the compounds described in the paper are available as Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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