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Evaluation of the carbonyl metallo immunoassay (CMIA) for the determination of traces of the herbicide atrazine

Nathalie Fischer-Durand, Anne Vessières *, Jan-Martin Heldt, Franck le Bideau, Gérard Jaouen

Ecole Nationale Supérieure de Chimie de Paris, Laboratoire de Chimie organométallique, UMR CNRS 7576, 11 rue P. et M. Curie, 75231 Paris Cedex 05, France

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Abstract

The non-isotopic immunoassay (CMIA) was investigated for the quantification of the herbicide atrazine. This assay combines transition metal carbonyl complex and Fourier transform infrared spectroscopy. We describe the synthesis of three dicobalt hexacarbonyl tracers, derivatives of atrazine. Their relative binding affinity towards two purified IgG fractions of polyclonal rabbit anti-sera (anti-atrazine and -simazine) was evaluated by ELISA and CMIA. The best tracer **9** (bearing the longest alkyl chain between atrazine aromatic ring and metal carbonyl complex) was used for further study by CMIA. Reproducible competitive standard curves were obtained by using anti-simazine antibodies in PBS buffer pH 7.4 with pre-incubation of various amounts of atrazine and antibodies for 1 h at 4 °C before addition of the tracer. This study demonstrates the feasability of using CMIA for pesticide determination, although the sensitivity of current CMIA format does not reach the 0.1 μg l^{-1} level required by E.U.

Keywords: Atrazine; Metal carbonyl complex; Immunoassay; Fourier-transform infrared spectroscopy (FT-IR)

1. Introduction

Environmental protection is a growing concern today, and government policies under public and health organization pressures, are harsher towards residues rates released in water and soil. In Europe, the council directive 80/778/EEC *relating to the quality of water intended for human consumption* fixed maximum allowable levels of pesticides in drinking water at 0.1 μg l^{-1} for an individual pesticide and no more than 0.5 μg l^{-1} for total pesticides in a given sample.

Some sophisticated analytical methods are available for the determination of residues in drinking or surface waters, including gas chromatography coupled with mass spectrometry (GC–MS) [1] and high performance liquid chromatography coupled with MS (LC–MS) [2], but they require cumbersome sample preparation, owing to the complexity of water matrices. With the pioneer development of antibodies production against small molecules in the medical diagnostic domain, immunochemical approach is now widely used in pesticide monitoring: antibodies are designed to selectively trap a given analyte or a group of structurally related compounds in complex matrices. These immunochemical methods are mostly based on immunoaffinity chromatography (IAC) followed by on-line or off-line LC or LC–MS quantification [3–6], and immunoassays on microtitre plates [6–8] with detection by UV–vis (ELISA technique: enzyme linked immunosorbent assay) or fluorescence (FIA: fluorescence immunoassay).

Our previous work in both the clinical diagnostic (carbonyl metallo immunoassay or CMIA) [9–12] and the environmental domain (IAC coupled with LC) [13–16], prompted us to evaluate the determination of pesticides such as chlortoluron [17] and atrazine using CMIA which uses metal-carbonyl complexes as tracers and Fourier-transform infrared spectroscopy (FT-IR) as the detection method. Atrazine, a member of the chlorotriazine family which also includes simazine, was chosen

* Corresponding author. Tel.: +33-144-276729; fax: +33-143-260061
E-mail address: vessiere@ext.jussieu.fr (A. Vessières).

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because of its widespread application as a herbicide in fields of corn and sorghum, due to its low cost and high efficacy. As a consequence atrazine is frequently detected in ground and surface waters at higher than authorized levels.

This paper presents our results for the quantification of atrazine following the CMIA format, exploring the use of two polyclonal antibodies (anti-atrazine and -simazine), three cobalt-carbonyl tracers and two different buffer solutions.

2. Experimental

2.1. General

Analytical grade atrazine (ATZ) and simazine (SIM) were purchased from Riedel-de Haën. Cyanuric chloride, ethylamine, isopropylamine, ε-amino-*n*-caproic acid, glycine, 1-ethyl-3-(3-(dimethylaminopropyl)carbodiimide (EDAC) and BSA (bovine serum albumin) were from Sigma. Hydroxysuccinimide and propargylamine were purchased from Aldrich and dicobalt octacarbonyl $Co_2(CO)_8$ from Strem Chemicals. Solvents were dried and purified using standard procedures. Flash chromatography was performed on silica gel 60 (Merck, 40–63 μm). NMR spectra were recorded on BRUKER AC 200 and BRUKER Avance 400 spectrometers. Standard Greiner flat-bottomed polystyrene microplates were used in ELISA experiments. Microplates were washed with BIO-RAD Model 1575 immunowash, and read with a BIO-RAD Model 550 microplate reader. Melting points were determined on a Kofler hot stage and are uncorrected. Mass spectra were recorded on a NERMAG R10-10C mass analyser. Microanalyses were performed by CNRS (ICSN, Gif sur Yvette, France) or UPMC (SIAR, Paris, France).

2.2. Buffers

CMIA buffer (pH 8.7) contained glycerol (100 ml), $Na_2HPO_4 \cdot 12\ H_2O$ (10.7 g), NaCl (9.5 g) and NaN_3 (2 g) per liter of deionized water. Carbonate–bicarbonate buffer (coating buffer pH 9.6) contained Na_2CO_3 (1.4 g) and $NaHCO_3$ (2.93 g) per liter. Phosphate-buffered saline (PBS pH 7.4) contained KH_2PO_4 (0.2 g), $Na_2HPO_4 \cdot 12\ H_2O$ (1.78 g), NaCl (8 g) and KCl (0.2 g) per liter. Citrate–phosphate buffer (pH 5.0) contained citric acid (5.1 g) and $Na_2HPO_4 \cdot 12\ H_2O$ (7.3 g) for 500 ml solution.

2.3. FT-IR spectroscopy

FT-IR spectra were recorded on a bench-top Bomem Michelson MB 100 FT spectrometer equipped with a liquid nitrogen cooled MCT detector for characterization of compounds, a liquid nitrogen cooled 1.0 mm^2 InSb (indium antimoine) detector and microbeam (1.0 mm-diameter) for quantitative analysis. FT-IR data were manipulated on a Windows-equipped PC using the WinBomemEasy program. Routinely, 44 scans were coadded in about 1 min and resulting interferogram was apodized using a cosine function and then Fourier-transformed to yield a 4 cm^{-1} resolution spectrum. The absorbance value was baseline corrected using the Quant method included in the program. The IR cell used was an ultralow volume, gold light-pipe cell with a fill volume of 30 μL and an optical pathlength of 20 mm [18].

2.4. Production of rabbit antibodies and purification of the IgG fraction

Anti-atrazine and -simazine antibodies were obtained by injecting immunogens BSA–ATZ and BSA–SIM intradermally into New Zealand White rabbits according to a previously described procedure [14]. Crude antisera were stored at −20 °C and were stable for years. Throughout this study we worked with the immunoglobulin G (IgG) fractions of antisera which were obtained by affinity chromatography purification of the crude sera on Avid Chrom gel (Unisyn Technologies Inc, Hopkinton, MA, USA) according to the manufacturer's instructions.

2.5. Synthesis of organometallic tracers of atrazine

2.5.1. 2-Chloro-4-(alkylamino)-6-(carboxymethylamino)-s-triazine (*1*) and (*2*): general procedure

Cyanuric chloride (5 g, 27.1 mmol) was dissolved in $C_6H_5CH_3$ (60 ml) and cooled to 0 °C. NaOH (30%, 5 ml) was added, followed by ethylamine (33% in water, 4.3 g, 1.15 equivalents) for **1** or isopropylamine (2.66 ml, 1.15 equivalents) for **2**. The mixture was stirred for 15 min at 0 °C, then for 3 h at room temperature (r.t.). This was followed by dropwise addition of glycine (2.32 g, 1.14 equivalents) dissolved in 5 M NaOH (7 ml), and the resulting mixture was left to stir overnight at r.t. Diethylether (100 ml) and water (100 ml) were added. The aqueous phase was acidified with concd. HCl until precipitation occurred (pH 3). The precipitate was filtered off, washed with H_2O, and dried. The crude product was dissolved in Me_2SO (75 ml) at 50 °C, then H_2O was added (10 ml) and the solution was allowed to stand at r.t. until crystallization occurred. The compound was filtered off, washed with H_2O and dried to yield a white powder.

2.5.1.1. 2-Chloro-4-(ethylamino)-6-(carboxymethylamino)-s-triazine (1). Yield: 2.8 g, about 44%; m.p.: 215 °C (dec.); ^{1}H-NMR (200 MHz,

Me_2SO): δ 1.05 (3H, t, $J = 6.5$ Hz, CH_3 *Et*); 3.2 (2H, q, $J = 6.4$ Hz, CH_2 *Et*); 3.86 (2H, m, CH_2 *Gly*); 7.9 (2H, m, NH); 12.5 (1H, broad s, CO_2H); ^{13}C-NMR (50 MHz, Me_2SO): δ 14.6; 35.45; 42.6; 135.35; 165.3; 165.8; 168; 171.6. MS (CI+NH_3) *m*/*z*: 232–234 [MH^+].

*2.5.1.2. 2-Chloro-4-(isopropylamino)-6-(carboxymethylamino)-s-triazine (**2**).* Yield: 1.4 g, about 22%; m.p.: 205 °C (dec.); ^{1}H-NMR (200 MHz, Me_2SO): δ 1.08 (6H, d, $J = 6.5$ Hz, CH_3 *iPr*); 3.85 (2H, s large, CH_2 *Gly*); 3.94 (1H, m, **H**C(CH_3)$_2$); 7.7–8.0 (2H, m, NH); 12.5 (1H, broad s, COOH); ^{13}C-NMR (50 MHz, Me_2SO): δ 22.1; 22.5; 42.4; 164.5; 171.5. MS (CI+CH_4) *m*/*z*: 246–248 [MH^+].

*2.5.2. 2-Chloro-4-(isopropylamino)-6-(carboxypenthylamino)-s-triazine (**3**)*

Previously described [7,19].

*2.5.3. 2-Chloro-4-(alkylamino)-6-[N(3-propynamido)alkylamino]-s-triazines (**4**), (**5**), and (**6**): general procedure*

The previously prepared acid **1**, **2** or **3** (0.907 mmol) was dissolved in anhydrous DMF (12 ml) under Ar. The resulting solution was cooled to −15 °C, then hydroxysuccinimide (0.136 g, 1.3 equivalents), EDAC·HCl (0.226 g, 1.3 equivalents), and *i*Pr_2EtN (0.205 ml, 1.3 equivalents) were added. The mixture was stirred for 1 h at −15 °C, then for 2 h at 0 °C, and allowed to stand at r.t. overnight. The colored solution (yellow to orange depending on the starting acid) was cooled to 0 °C, and propargylamine (74.7 μL, 1.2 equivalents) was added under Ar. The solution was stirred at 0 °C for 3 h.

For compound **4**, the solution was poured into a flask cooled on an ice bath, and cold H_2O was added (15 ml). The precipitate was filtered off, washed with cold water and dried to yield an off-white powder (0.65 g, 65%).

For compounds **5** and **6**, the solutions were diluted with EtOAc (20 ml), and H_2O was added (20 ml). The aqueous phases were extracted with EtOAc (3 × 50 ml). The organic phases were dried (Na_2SO_4), and evaporated to dryness under reduced pressure. The crude products were purified on SiO_2 (EtOAc, $R_f = 0.6$ for both compounds).

Compound **4**: m.p.: 264 °C; ^{1}H-NMR (200 MHz, Me_2SO): δ 1.0 (3H, t, $J = 7.1$ Hz, CH_3 *Et*); 3.1 (1H, broad s, C≡CH); 3.2 (2H, q, $J = 6.7$ Hz, CH_2 *Et*); 3.8 (4H, m, CH_2 *Gly*+C**H$_2$**–C≡CH); 7.8–7.9 (2H, broad m, NH amines); 8.32 (1H, broad m, NH amide); ^{13}C-NMR (50 MHz, Me_2SO): δ 14.5; 28.2; 35.3; 43.9; 73.0; 81.4; 165.2; 166.0; 168.1; 168.9. MS (CI+CH_4) *m*/*z*: 269–271 [MH^+]; Anal. Calc. for $C_{10}H_{13}ClN_6O$: C, 44.69; H, 4.87; N, 31.27. Found: C, 45.05; H, 5.22; N: 30.52%.

Compound **5**: Off-white powder (0.1 g, 39%); m.p.: 213 °C; ^{1}H-NMR (200 MHz, Me_2SO): δ 1.07 (6H, m, CH_3 *iPr*); 3.1 (1H, m, C≡CH); 3.8 (4H, m, CH_2 *Gly*+C**H$_2$**–C≡CH); 3.9 (1H, m, **H**C(CH_3)$_2$); 7.7–7.9 (2H, m, NH); 8.3 (1H, t, $J = 5.5$ Hz, NH amide); ^{13}C-NMR (50 MHz, Me_2SO): δ 22.2; 28.2; 40.5; 42.3; 73.0; 81.3; 164.5; 165.9; 168.1; 168.9. MS (CI+CH_4) *m*/*z*: 283–285 [MH^+]; Anal. Calc. for $C_{11}H_{15}ClN_6O$: C, 46.73; H, 5.35; N, 29.72. Found: C, 46.96; H, 5.61; N, 29.17%.

Compound **6**: Colorless oil which crystallized at 4 °C (0.17 g, 50%); m.p.: 104 °C; ^{1}H-NMR (250 MHz, $CDCl_3$): δ 1.14 (6H, m, CH_3 *iPr*); 1.31 (2H, m, CH_2); 1.60 (4H, m, CH_2); 2.13 (3H, m, C**H$_2$**–CONH and C≡CH); 3.31 (2H, m, NH–C**H$_2$**–R); 3.97 (2H, dd, $J = 5.25$ and 2.5 Hz, C**H$_2$**–C≡CH); 4.08 (1H, m, **H**C(CH_3)$_2$); 5.62 (1H, d, $J = 7.75$ Hz, NH amine); 6.13 (1H, m, NH amine); 6.68 (1H, t, $J = 5.87$ Hz, NH amide); ^{13}C-NMR (60 MHz, $CDCl_3$): δ 22.7; 23.2; 25.6; 26.8; 29.5; 36.5; 41.0; 43.3; 71.8; 80.1; 165.1; 166.1; 168.3; 173.0. MS (CI+CH_4) *m*/*z*: 339–341 [MH^+]; Anal. Calc. for $C_{15}H_{23}ClN_6O$: C, 53.14; H, 6.84; N, 24.80. Found C, 53.33; H, 6.88; N, 24.52%.

*2.5.4. Complexation of propargylic compounds **4** and **5** with $Co_2(CO)_8$: general procedure*

In a three-neck round bottomed flask protected from light and under argon, a propargylic derivative (0.409 mmol) was dissolved in anhydrous DMF (9 ml). $Co_2(CO)_8$ (0.15 g, one equivalent) was added three times at 15 min intervals and the reaction was monitored by TLC (EtOAc, $R_f = 0.8$ for **7**, $R_f = 0.9$ for **8**). After 90 min the reaction mixture was directly flash chromatographed under Ar on SiO_2 (15 cm length, EtOAc). The combined fractions containing the expected compound were concentrated and again flash chromatographed to yield an orange powder.

Compound **7**: 50 mg (22%); IR ($CHCl_3$): 2031.6; 2058.6; 2097; ^{1}H-NMR (400 MHz, Me_2SO): δ 1.0 (3H, m, CH_3); 3.2 (2H, m, CH_2 *Et*); 3.8 (2H, broad s, CH_2 *Gly*); 4.5 (2H, broad d, C**H$_2$**–C≡CH); 6.6 (1H, s, C≡CH); 7.8 (2H, m, NH amines); 8.6 (1H, NH amide); ^{13}C-NMR (100 MHz, Me_2SO): δ 14.3; 35.0; 40.9; 43.5; 73.0; 95.2; 165.1; 165.8; 167.7; 168.8; 200.0. MS (FAB^+) *m*/*z*: 555–557 [MH^+].

Compound **8**: 69 mg (30%)+50 mg of starting compound; IR ($CHCl_3$): 2031.9; 2058.7; 2097.2; ^{1}H-NMR (400 MHz, Me_2SO): δ 1.2 (6H, broad s, CH_3 *iPr*); 4.1 (3H, m, CH_2 *Gly*+**H**C(CH_3)$_2$); 4.7 (2H, broad s, C**H$_2$**–C≡CH); 6.4 (1H, broad s, C≡CH); 6.7–6.9 (2H, m, NH amines); 8.1 (1H, broad s, NH amide); ^{13}C-NMR (100 MHz, Me_2SO): δ 22.3; 42.0; 43.1; 44.6; 73.4; 95.5; 165.7; 167.1; 169.2; 169.5; 200.6; MS (CI+NH_3) *m*/*z*: 569–571 [MH^+].

*2.5.5. Complexation of propargylic compound **6** with $Co_2(CO)_8$*

In a three-neck round bottomed flask protected from light and under Ar, propargylic compound **6** (72 mg, 2.13×10^{-4} mol) was dissolved in anhydrous THF (4

ml). Co_2CO_8 (0.12 g, 1.5 equivalents) was added and the reaction was monitored by TLC (EtOAc–C_5H_{12}: 9/1, $R_f = 0.9$ for **9**). After 1 h, THF was evaporated under reduced pressure and the crude product was flashed chromatographed under Ar on SiO_2 (15 cm length, EtOAc–C_5H_{12}: 9/1) to yield compound **9** as a red powder (0.1 g, 80%); IR ($CHCl_3$): 2096; 2058; 2031; ^{1}H-NMR (400 MHz, CD_2Cl_2): δ 1.2 (6H, m, CH_3 *iPr*); 1.4 (2H, m, CH_2); 1.6 (2H, m, CH_2); 1.7 (2H, m, CH_2); 2.2 (2H, t, $J = 7.5$ Hz, C**H$_2$**–CONH); 3.4 (2H, m, C**H$_2$**–NHAr); 4.1 (1H, m, **H**C(CH_3)$_2$); 4.6 (2H, d, $J = 6$ Hz, C**H$_2$**–C≡CH); 5.2 (1H, broad s, NH amine); 5.5 (1H, broad s, NH amine); 5.9 (1H, broad s, NH amide); 6.1 (1H, s, C≡CH); ^{13}C-NMR (100 MHz, CD_2Cl_2): δ 21.8; 24.5; 26.1; 28.6; 35.7; 40.4; 41.2; 42.6; 72.1; 93.3; 164.8; 165.7; 168.0; 171.8; 199.3; MS (CI + NH_3) *m*/*z*: 625–627 [MH^+].

2.6. Immunoassays

2.6.1. Plate coating procedure

An atrazine–β-lactoglobulin (βLG–ATZ) conjugate was prepared using the way previously described [14] to coat ELISA plates. βLG–ATZ (100 μl, 1 μg ml^{-1} solution in freshly prepared $NaHCO_3$–Na_2CO_3 buffer) were added to each well of microtiter plates. The plates were covered with parafilm and stored at 4 °C overnight. Plates were then washed three times with PBS buffer, incubated for 1 h at r.t. with 200 μl of 4% skimmed milk in PBS buffer to block unoccupied sites and avoid further non-specific binding of proteins, and finally washed four times with PBS containing 0.1% Tween 20 (PBS-T). They can be stored prior use for several months at 4 °C covered with parafilm.

2.6.2. Titre determination of anti-ATZ and -SIM antibodies

Serial dilutions of IgG fractions in PBS + 2% skimmed milk (PBS-BR) were prepared at 100 μl/well and allowed to react with βLG–ATZ bound to the wells at r.t. for 2 h. Plates were then washed four times with PBS-T. Peroxidase-goat *anti*-rabbit conjugate at a 1:8000 dilution in PBS-BR was added at 100 μl/well and allowed to stand at r.t. for 2 h. Plates were then washed four times with PBS-T. Substrate consisting of *o*-phenylenediamine dihydrochloride (OPD, 14 mg) in citrate–phosphate buffer pH 5.0 (20 ml) with 8 μl H_2O_2 30%, was added at 100 μl/well and allowed to stand at r.t. in the dark for 10–20 min. The reaction was stopped by addition of 50 μl/well of H_2SO_4 2.5 M. After 10 min in the dark, the plate was read at 492 nm on a microplate reader. 1:5000 dilutions for both anti-ATZ and -SIM IgG fractions, giving optical densities around 1–1.5, were chosen for the competitive inhibition by ELISA.

2.6.3. Competitive immunoassays for the different tracers

IgG fraction (125 μl) diluted 1:2500 in PBS-BR was added to 125 μl of 10 dilutions of ATZ from 0.1 to 1500 ng ml^{-1} in PBS-BR (thus giving a twofold dilution of concentrations of both ATZ and antibodies). After mixing and incubation for 1 h at 4 °C, 100 μl of each solution was added in duplicate to the wells of the coated plate. Incubation at r.t. was continued for 2 h, then the plate was washed four times with PBS-T. Addition of the HRP-conjugated secondary antibody and plate reading were performed as described above (titre determination). Standard curves were obtained by plotting the B/Bo ratios against the concentration of ATZ. B was the O.D. at a given concentration and Bo the O.D. read without ATZ.

2.6.4. Titration curve by CMIA

500 μl fractions consisting of 30 pmol of tracer **7**, **8**, or **9** (30 μl of a 10^{-6} M solution in EtOH) and eight dilutions of antibody (from 1:20 to 1:300) in CMIA buffer were incubated for 2 h at 20 °C. The free fraction of the tracer was then extracted from the aqueous phase by addition to each tube of 1 ml CMIA buffer-saturated isopropylether. The mixtures were vortexed for 30 s, then 750 μl of each organic phase was transferred to 1.5 ml Eppendorf tube and evaporated to dryness on a Speed Vac (Savant concentrator). IR analysis were obtained by dissolving dry residues in 30 μl CCl_4. The FT-IR spectra were immediately recorded on the spectrometer using the gold light-pipe cell previously described [18]. The absorbance value of the 2058 cm^{-1} peak was proportional to the free fraction (F) of the tracer. The absorbance of the total quantity of tracer (T) present in the incubation step was obtained by extracting 500 μl fraction containing 30 pmol of tracer without antibodies. The bound fraction (B) was calculated from the difference T–F. Titration curves were constructed by plotting the B/T ratio versus the inverse of antibody dilutions. Titre value was the antibody dilution that bound 50% of tracer (%B/T = 50).

2.6.5. Standard curve by CMIA (optimized procedure)

500 μl fractions consisting of antibody (IgG fraction) in PBS buffer at the titre value and 10 amounts of ATZ (from 12.5 to 1000 pmol) dissolved in 50 μl EtOH were incubated for 1 h at 4 °C. Then 30 pmol of tracer **9** (30 μl of a 10^{-6} M solution in EtOH) was added and incubation was continued for 2 h at 20 °C. Separation of the free and bound fractions of the tracer and determination of the bound fractions were performed as described for the titration curve. Bo was the value obtained without atrazine in the incubation step. Standard curves were then obtained by plotting the B/Bo ratios versus the amounts of ATZ.

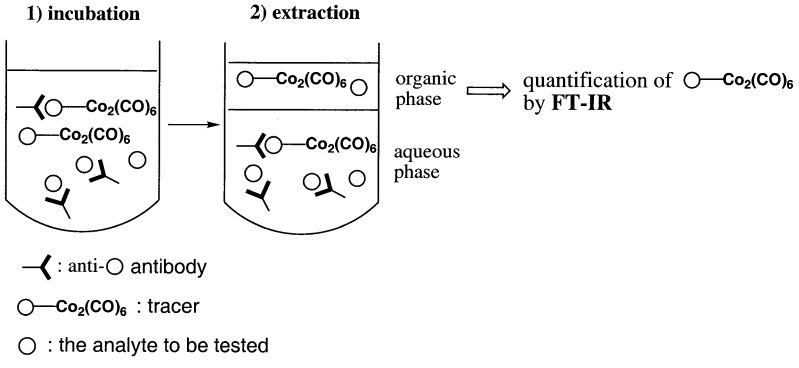
3. Results and discussion

The CMIA method is a homogenous competitive immunoassay between the compound to be quantified, in this case ATZ, and a metal carbonyl tracer, here an alkyne derivative of atrazine bearing a dicobalt hexacarbonyl moiety (Scheme 1). After incubation with specific antibodies, the free fraction of the tracer is extracted by isopropylether. After evaporation of the solvent, the residue is taken in 30 μl CCl_4 and quantified by FT-IR. Quantitative analysis of the tracer is performed by direct measurement of the absorbance of the central peak at 2058 cm^{-1}, one of the three characteristic ν_{CO} vibration bands of the metal-carbonyl unit (Fig. 1) [12,20]: it has been demonstrated that this peak gives the lowest detection limit. Antibody–antigen interactions were performed with polyclonal antibodies raised against atrazine and simazine immunoconjugates (Scheme 2) [14]. The two herbicides are too small to be immunogenic, therefore they were coupled to the lysine residues of protein, here BSA, by means of a C_5-alkyl chain linker. ELISA and CMIA analyses were performed with the IgG fraction of sera obtained by purification on affinity chomatography of crude sera. Our previous results [15] had shown that atrazine was well recognized by both anti-ATZ and -SIM antibodies. In a preliminary study, rabbit antisera collected every 2 weeks were screened by ELISA on plates coated with βLG–ATZ in order to select sera exhibiting the highest concentrations of specific anti-ATZ antibodies.

The three dicobalt hexacarbonyl tracers were prepared following the general strategy depicted in Scheme 3. Cyanuric chloride was first reacted with ethylamine (for the simazine tracer) or isopropylamine (for the atrazine tracer) at 0–20 °C, then either ε-amino-*n*-caproic acid or glycine was added at 20 °C, leading to acid derivatives **1**, **2**, **3**. Activation of acid moieties with hydroxysuccinimide, then nucleophilic addition of propargylamine at 0 °C yielded the three propargylic derivatives **4**, **5**, **6**, which were then complexed with dicobalt octacarbonyl to give the three expected tracers **7**, **8**, **9**. Tracer **9**, ATZ–C_5–$Co_2(CO)_6$, was prepared with the same aminocaproic linker used for the preparation of immunogens injected into rabbits for antibody production, and therefore **9** would be expected to be better recognized by antibodies than the two other tracers which bear a smaller glycine linker. On the other hand, a sensitive CMIA assay requires that the tracer–antibody equilibrium constant not be too high, so as not to overwhelm the atrazine–antibody equilibrium when working with trace quantities of atrazine.

Tracers ATZ–C_5–$Co_2(CO)_6$ and ATZ–C_1–$Co_2(CO)_6$ were first evaluated by ELISA technique on microplates coated with 1 μg ml^{-1} of βLG–ATZ conjugate. The results of competitive inhibition assays are given in Table 1. IC_{50} value is the amount of ATZ or tracer needed to displace 50% of antibodies from binding to ATZ coated on plate. The lower this quantity is, the more specific the antibodies are for the given compound. Anti-simazine IgG exhibited a higher reactivity towards ATZ than anti-atrazine IgG: the concentration of free ATZ required to achieve 50% inhibition was almost three times lower than with anti-atrazine IgG. Cobalt carbonyl complexes were recognized by either anti-atrazine and -simazine IgG. Competitive inhibition assays carried out with anti-ATZ IgG gave 50% inhibition at a concentration of 120 pmol ml^{-1} for tracer **9** which is twofold less reactive than the analyte, but 16.5-fold more reactive than tracer **8** which behaves as a weak inhibitor. Experiments with anti-SIM IgG yielded inhibitory concentrations in the same order of magnitude, but the difference in reactivity between the two tracers was weaker (5.5 rather than 16.5). These results would suggest that the dicobalt hexacarbonyl entity was too close to the chlorotriazine moiety in tracer **8** and therefore weakened the binding affinity of both anti-ATZ and -SIM antibodies.

In another set of experiments, tracers **7**, **8**, and **9** were evaluated in the determination of the titre value of antibodies to be used in the competitive assay by CMIA. A titration curve by CMIA was obtained after incubating 30 pmol of organometallic tracer with decreasing amounts of antibodies in CMIA buffer. The amount of free tracer extracted by isopropylether increased as the



Scheme 1. Principle of the CMIA method.

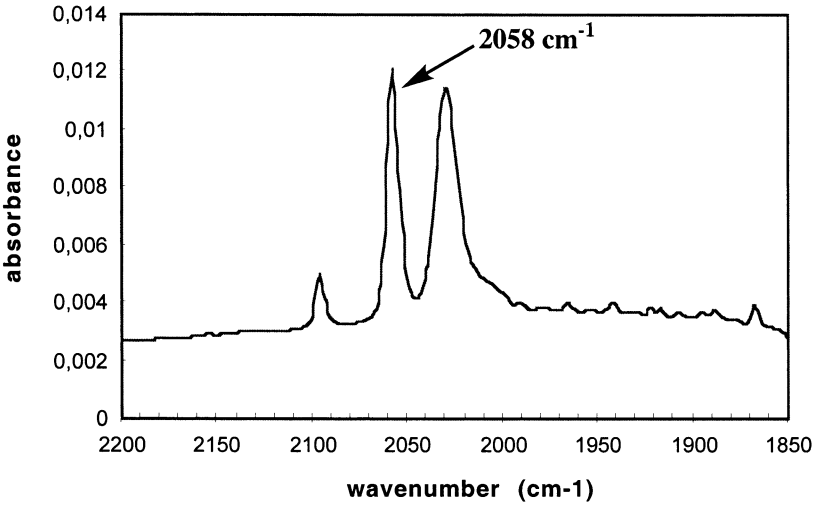
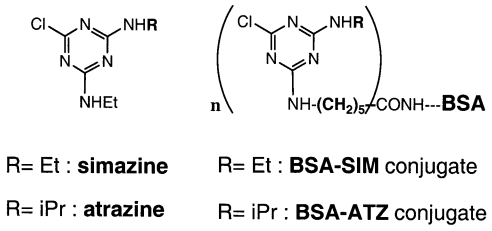


Fig. 1. FT-IR spectrum of the dicobalt hexacarbonyl complex of ATZ (**9**). 30 pmol of tracer+50 μl EtOH+420 μl CMIA buffer. Extraction with 1 ml iPr_2O. 750 μl were transferred to 1.5 ml Eppendorf microtube and evaporated to dryness, then taken in 30 μl CCl_4 for FT-IR quantification.



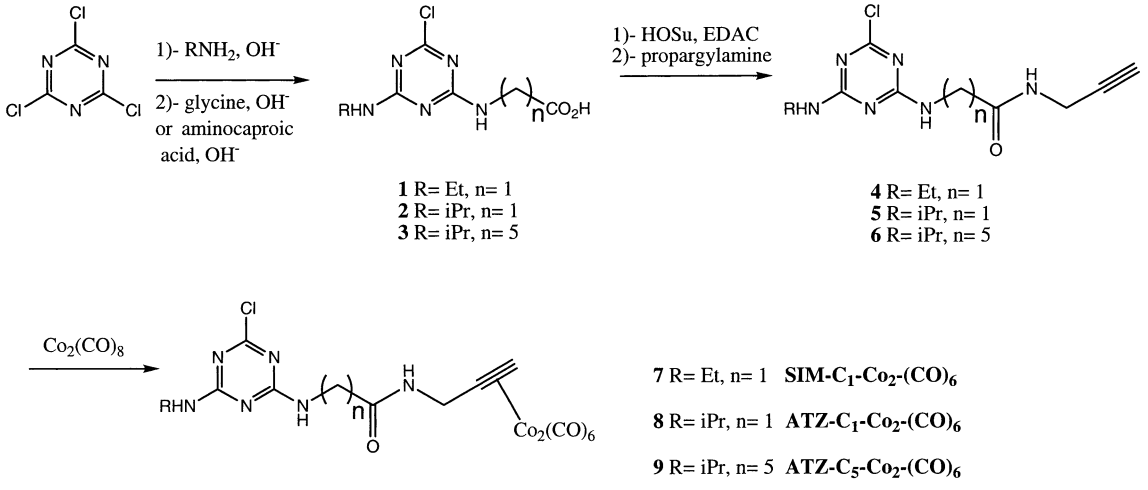
Scheme 2. Chemical structures of atrazine, simazine and immunoconjugates.

Table 1
IC_{50} [a]

	IC_{50} (pmol ml^{-1})	
	Anti-ATZ antibody [b]	Anti-SIM antibody [b]
ATZ	65	25
ATZ–C_5–$(Co)_2(CO)_6$ **9**	120	200
ATZ–C_1–$(Co)_2(CO)_6$ **8**	2000	1100

[a] Concentration of ATZ, tracers **9** and **8** required to displace 50% of antibodies from binding to ATZ coated on plate.
[b] IgG fraction.

quantity of antibodies decreased. As a result, the height of the peak at 2058 cm^{-1} increased proportionally to the free fraction of the tracer. The titre is defined as the antibody dilution that binds 50% of the tracer. The highest the dilution is, the more specific the antibodies are for binding to a given tracer. Only tracer ATZ–C_5–$Co_2(CO)_6$ exhibited sufficient binding affinity towards both anti-ATZ and -SIM antibodies to reach 50% binding, at dilutions 100 for anti-ATZ and 74 for anti-SIM. In the case of tracers with short spacer, even with high concentration of antibodies (i.e. dilution 20), we did not reach 50% binding. Moreover, we could not perform the assay at higher antibody concentration without encountering strong emulsions, preventing



Scheme 3. General synthesis of tracers.

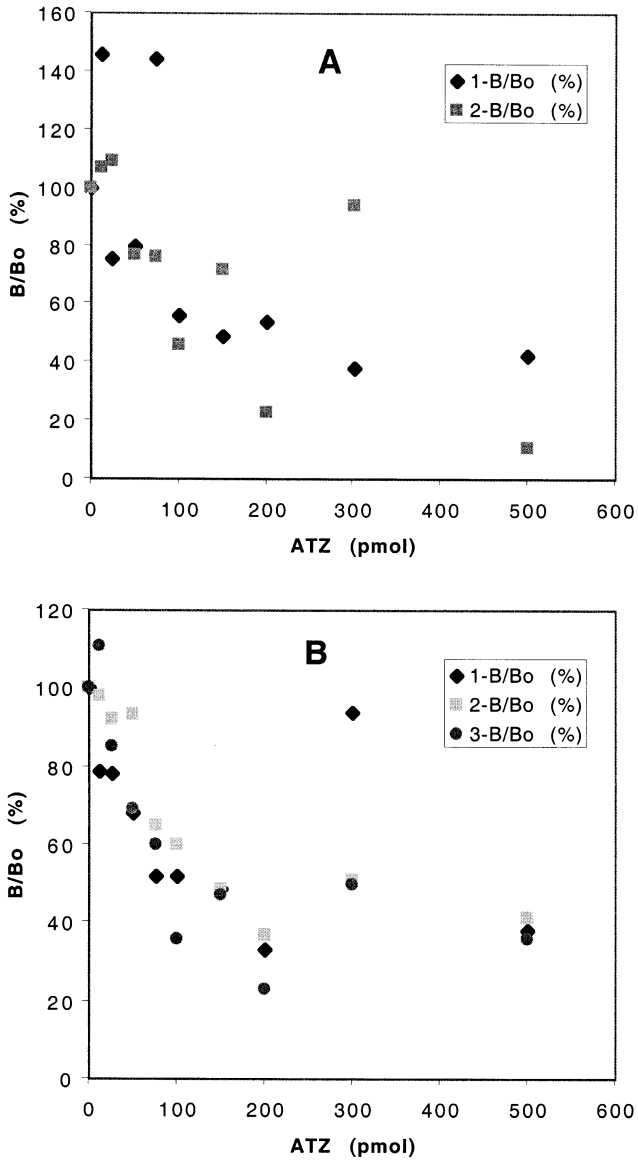


Fig. 2. Competition curves by CMIA, CMIA buffer pH 8.7, without pre-incubation: (A) anti-ATZ IgG fraction, dilution 1:100; (B) anti-SIM IgG fraction, dilution 1:74.

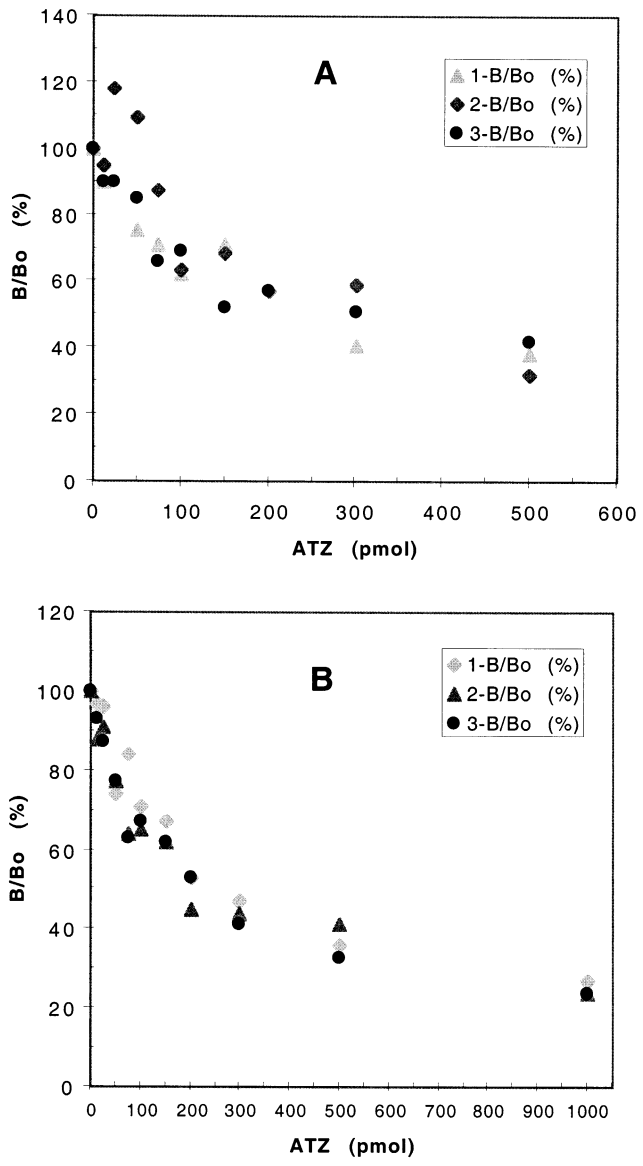


Fig. 3. Competition curves by CMIA: (A) anti-ATZ IgG fraction, pre-incubation for 1 h at 4 °C, CMIA buffer ph 8.7; (B) anti-SIM IgG fraction, pre-incubation for 1 h at 4 °C, PBS buffer pH 7.4.

proper extraction of the tracer. This drop in binding affinity observed with the glycine spacer was consistent with the first results obtained by the ELISA technique (see above), and proved the need for at least a medium-length spacer arm between the triazine ring and the metal carbonyl moiety in order to retain tracer–antibody affinity.

Accordingly, calibration curves for atrazine quantitation were conducted with ATZ–C_5–$Co_2(CO)_6$.

In previous CMIA for antiepileptic drugs [11,12], the incubation step was carried out in CMIA buffer pH 8.7 without pre-incubation of the analyte with antibodies. Our first experiments were performed under these conditions. Tracer (30 pmol), ATZ (12.5–500 pmol) and antibodies at the titre dilution previously determined in a 500 μL solution assay were incubated for 2 h at room temperature. Extraction of the free tracer and quantification by FT-IR gave the standard curves shown in Fig. 2. The competition curves obtained for anti-ATZ antibodies showed that ATZ and tracer **9** did not efficiently compete for binding to the antibodies when added at the same time, while in the case of anti-SIM antibodies, the two compounds competed well in the range 0–200 pmol/tube of ATZ, but at higher concentrations, ATZ did not displace tracer **9**, suggesting that the antibodies recognized the spacer arm common to immunogen BSA–ATZ and tracer **9**.

In light of these results, we decided to perform a pre-incubation step, which is commonly used in immunoassays [21,22]. The assays were run with anti-ATZ

antibodies in CMIA buffer pH 8.7. IgG fraction at 1:100 dilution was incubated for 1 h at 4 °C with 12.5–500 pmol/tube of ATZ. Then 30 pmol of tracer **9** was added, and incubation continued for 2 h at room temperature before extraction of the free tracer. Standard curves plotted in Fig. 3A showed an improvement in the shape of the curve, though high unspecific interactions still existed (about 40%).

Finally, changing from CMIA buffer pH 8.7 to PBS buffer pH 7.4, and using anti-SIM antibodies led to reproducible standard curves (Fig. 3B). IC_{50} was about 200 pmol/tube (86 μg l^{-1}) and the sensitivity (IC_{80}) was about 50 pmol/tube (21 μg l^{-1}). These values are far from the sensitivity required by European regulations to reach 0.1 μg l^{-1} but our results were obtained without the help of any sample pre-concentration which is routinely used with HPLC or GC analyses. On the other hand, an interesting feature of this immunoassay compared to standard ones is its capacity for simultaneous multiple assays as previously described [11], when using tracers with different metal carbonyl moieties.

4. Conclusion

In this study, we have shown the feasibility of using the non-isotopic CMIA method in environmental monitoring, by modification of the procedure already developed for the clinical diagnostics. Pre-incubation in PBS buffer pH 7.4 of anti-SIM antibodies with increasing amounts of atrazine to be quantified for 1 h at 4 °C, followed by addition of 30 pmol of dicobalt hexacarbonyl tracer **9** and incubation for 2 h at room temperature, led to optimized standard curves as shown in Fig. 3B. At present the carbonyl metallo immunoassay is not sensitive enough to satisfy the European Guidelines, but our first results are encouraging, bearing in mind that this assay is carried out without sample pretreatment.

Increasing the sensitivity by amplification of the FT-IR signal is under investigation and will be reported in due course.

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