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First carbonyl metallo immunoassay in the environmental area: application to the herbicide chlortoluron

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We report a new, non-isotopic, immunoassay (CMIA) for chlortoluron, a pesticide of the phenylurea family. The novel feature of this assay is the use of metal carbonyl complexes as tracers and Fourier-transform infrared (FT-IR) spectroscopy as the detection method. We describe the synthesis of three tracers, derivatives of chlortoluron with an attached cyclopentadienyl manganese tricarbonyl moiety. Quantitative analysis of these tracers, by FT-IR spectroscopy was performed by simple measurement of the absorbance peak at 2033 cm^{-1}, characteristic of the ν_{CO} of the metal-carbonyl unit. Immunoassays were carried out using the IgG fraction of rabbit antiserum. Standard curves obtained by CMIA for the three tracers showed the feasibility of this technique for chlortoluron assay. However, the IC_{50} and IC_{90} values obtained with the three tracers varied widely, with the long-chain tracer 12 giving the most sensitive assay for chlortoluron, with an IC_{50} value of 50 pmol (21 $\mu g\ l^{-1}$) and an IC_{90} of 6.5 pmol (2.7 $\mu g\ l^{-1}$).

KEYWORDS: metal carbonyl complex; immunoassay; pesticide; chlortoluron; Fourier-transform infrared spectroscopy

INTRODUCTION

Intensive agricultural methods, with their increasing use of a wide variety of pesticides, have become the subject of public concern focused on their potential environmental impact. This has led to a need for environmental analysis methods that are reliable, rapid and capable of detecting many different possible pollutants in increasingly low concentrations. A European Community directive of 1980 (80/778/EEC), for example, set maximum allowable levels of pesticides in drinking water at 0.1 $\mu g\ l^{-1}$ for any individual pesticide and no more than 0.5 $\mu g\ l^{-1}$ total for all pesticides present in the sample. Until recently, the most commonly used analytical techniques were based on separative methods, such as high-performance liquid chromatography, gas chromatography or capillary electrophoresis. These methods all demand sample preparation, which increases processing time and thus limits the number of samples that can be analysed. Moreover, to reach the very low detection levels required by the legislation, a preliminary preconcentration of the sample may be necessary.[1,2] A parallel development over the last few years has demonstrated the potential of the immunoassay as an alternative method, permitting reliable, rapid, sensitive and cost-effective analysis. A number of tests using the enzyme-linked immunosorbent assay (ELISA) technique have been reported for the detection of pesticides,[2] in particular isoproturon,[3,4] atrazine,[5] simazine,[6] and chlortoluron.[7] Over the last few years in our own laboratory we have been developing a new type of non-isotopic immunoassay termed the carbonyl metallo immunoassay (CMIA), which uses metal carbonyl complexes as tracers and FT-IR spectroscopy as the detection method. This technique has proved successful in the assay of drugs such as the antiepileptics carbamazepine, phenobarbital and DPH[8,9] and of an endogenous hormone, cortisol.[10] We also showed that the real advantage of the method is that it allows the simultaneous assay of several analytes, such as for example the double or triple immunoassay of the antiepileptic drugs mentioned above.[11,12] This simultaneous multi-assay capability of CMIA seems particularly well suited to the requirements of pollution detection, and led us to consider whether it would offer advantages over the existing analytical methods. To examine the potential of the CMIA method in this application, we chose to assay a phenylurea, chlor-

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toluron, a herbicide used extensively in the control of the four types of wild oats and black-grass in winter barley.[7]

EXPERIMENTAL PROCEDURES

Materials and methods

All syntheses were carried out under argon. Solvents were dried and purified using standard procedures. Chlortoluron ([3-(3-chloro-4-methylphenyl)-1,1-dimethylurea], Pestanal grade), was purchased from Fluka, *N,N′*-dicyclohexylcarbodiimide (DCC), *O*-(*N*-succinimidyl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate (TSTU) and diisopropylethylamine (*i*-Pr_2EtN) were purchased from Acros Organics. Flash chromatography was performed on silica gel 60 (Merck, 40–63 mesh). Other compounds were commercially available and were used as received. NMR spectra were run on a Bruker AC 200 spectrometer. Microanalyses were performed by CNRS (ICSN, Gif sur Yvette, France) or UPMC (SIAR, Paris, France). Microanalytical data are quoted as measured. Low carbon values are sometimes found with such systems because of the formation of metal carbides, leading to incomplete combustion. Mass spectra were determined on a Nermag R 10-10C instrument (ENSCP, Paris, France).

FT-IR spectroscopy

FT-IR spectra were recorded on a bench-top Bomem Michelson MB 100 FT spectrometer. For quantitative analysis the spectrometer was equipped with a liquid-nitrogen-cooled 1.0 mm^2 InSb (indium antimonide) detector and the measurements were performed in solution, in an ultralow volume, gold light-pipe cell with a fill volume of 30 µl and an optical pathlength of 20 mm.[13] For characterization of the compounds the spectrometer was equipped with a liquid-nitrogen-cooled MCT detector and the analyses were recorded as KBr pellets. FT-IR data were manipulated on a Windows-equipped PC using the WinBomemEasy program. Routinely, 44 scans were coadded (collection time 1 min) and the 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.

Production of rabbit antibodies and purification of the IgG fraction of the antisera

Anti-chlortoluron polyclonal antibodies were obtained by injection into rabbits of the immunogen (**2**), prepared as described previously by coupling of the corresponding acid onto bovine serum albumin (BSA)[3,14] (Scheme 1). The IgG fraction was obtained by passing the crude sera through an AvidChrom column (Unisyn Technologies, MA, USA). Typically, 2.5 ml of crude serum are diluted with 8 ml of buffer A (10 mM sodium phosphate, 0.1 NaCl M, pH 7.4) and percolated through the column at a rate of 0.5 $mlmin^{-1}$. Proteins strongly absorb at 280 nm, so the column is first washed out with buffer A at a rate of 1 $ml\,min^{-1}$ until $DO_{280} = 0$ is reached, thus indicating that all unbound proteins have been removed. Then, the IgG fraction is eluted with buffer B (0.1 M sodium acetate, pH 3) at a rate of 1 $mlmin^{-1}$. The 3 ml fractions collected are neutralized with 0.3 ml Tris buffer (1 M, pH 9). Fractions with a DO_{280} higher than 0.2 are pooled (typically 35–40 ml) and concentrated by centrifuge (Beckman J2-21, 4500 rpm, 4 °C, 2 h cycles) on Amicon® Centriplus 30 membranes to a volume of 1.5–2.0 ml. The recovered fraction containing 10–15 mg ml^{-1} IgG is then dialysed against NaCl 9/1000, and stored at −20 °C as 100 µl aliquots.

Synthesis of the organometallic tracers of chlortoluron

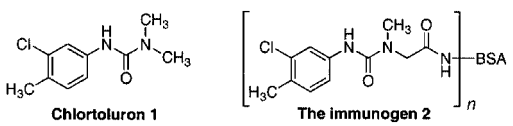
β-Cymantrenoyl-propionic acid (**3**) was prepared according to the published method.[15]

Synthesis of **5** *(short-chain tracer)*

DCC (136 mg, 0.66 mmol) was added to a solution of **3** (200 mg, 0.66 mmol) and 3-chloro-4-methyl-aniline (**4**; 160 µl, 1.32 mmol) in dichloromethane (1 ml). The reaction mixture was stirred for 1 h at room temperature and allowed to stand overnight at −5 °C. The precipitate was eliminated by filtration and the organic layer evaporated. The residue thus obtained was crystallized in hexane–dichloromethane to give **5** as a yellow solid (185 mg, 70%). M.p. = 132 °C. IR (KBr): 2035, 1937, 1664, 1667, 1588 cm^{-1}. ^{1}H NMR (200 MHz, C_6D_6) *δ*: 7.92 (br s, 1H), 7.61 (br s, 1H), 7.3–7.0 (m, 2H), 5.49 (br s, 2H), 4.89 (br s, 2H), 3.2–3.0 (m, 2H), 2.9–2.6 (m, 2H), 2.32 (br s, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) *δ*: 136.7, 134.3, 131.7, 130.9, 120.5, 118.1, 90.8, 86.8, 83.9, 34.2, 30.9, 19.4. MS, *m/z*: 343, 289, 195, 174, 159, 119, 106, 93, 77, 65, 55.

Synthesis of **6**

To a solution of **3** (1.955 g, 6.4 mmol) in DMF (40 ml), TSTU (2.147 g, 7.1 mmol) and *i*-Pr_2EtN (250 µl, 7.1 mmol) were added at room temperature. The mixture was stirred for 2 h at room temperature, diluted with AcOEt (50 ml), and washed with saturated NaCl (3 × 50 ml). The organic layer was dried over anhydrous $MgSO_4$, and evaporated. The crude mixture (2.53 g) was purified by flash chromatography using diethyl ether as eluant to give **6** as a yellow powder. ^{1}H NMR (200 MHz, $CDCl_3$) *δ*: 5.49 (t, *J* = 2.2 Hz, 2H), 4.88 (t, *J* = 2.2 Hz, 2H), 3.04 (br s, 4H), 2.84 (br s, 4H). ^{13}C NMR (50 MHz, $CDCl_3$) *δ*: 193.9, 168.9, 90.5, 86.7, 83.8, 33.3, 25.6,



Scheme 1.

25.0. IR (KBr): 2023, 1937, 1824, 1785 cm^{-1}. MS, *m/z*: 419 (MNH_4^+), 402 (MH^+). Anal. Found: C, 47.93; H, 2.87; N, 3.40. Calc. for $C_{16}H_{12}MnNO_8$ (401.2): C, 47.90; H, 3.01; N, 3.49%.

Synthesis of **9** *and* **10**

Thionyl chloride (0.40 ml, 5.5 mmol) and DMF (0.1 ml) in CH_2Cl_2 (5 ml) were added dropwise to a suspension of 5 mmol of **7** (glycine) or **8** (aminocaproic acid) in 10 ml of CH_2Cl_2. The reaction mixtures were stirred for 1 h at room temperature and 1 h at 40 °C. 3-Chloro-4-methyl-aniline (**4**) in 10 ml CH_2Cl_2 was added and the mixtures stirred at reflux for 3 h. After filtration, the brown solids obtained were washed with acetone (100 ml) to give **9** and **10** as white powders.

10: 905 mg, 62% M.p. = 157 °C. IR (KBr): 3427, 3271, 3018, 2942, 1656, 1599, 1530, 1498 cm^{-1}. ^{1}H NMR (200 MHz, D_2O) δ: 7.23 (s, 1H), 6.95 (m, 3H), 2.80 (t, J = 7.3 Hz, 2H), 2.19 (t, J = 7.3 Hz, 2H), 2.05 (s, 3H), 1.46 (m, 4H), 1.22 (m, 2H). ^{13}C NMR (50 MHz, D_2O) δ: 176.1, 136.7, 134.6, 132.1, 133.8, 122.6, 120.7, 40.3, 37.1, 27.5, 26.2, 25.7, 19.7. Anal. Found: C, 52.5; H, 6.93; N, 9.88. Calc. for $C_{13}H_{20}Cl_2N_2O$ (291.2): C, 53.61; H, 6.92; N, 9.62%.

Synthesis of **11** *and* **12**

The activated ester **6** (400 mg, 1 mmol) was dissolved in DMF (20 ml). The corresponding ammonium salt **9** or **10** (1 mmol) and *i-Pr*EtN (175 µl, 1 mmol) were successively added and the resulting yellow mixtures were stirred for 45 min at room temperature, diluted with AcOEt (50 ml), and washed with saturated NaCl (3 × 50 ml). The organic layer was dried over anhydrous $MgSO_4$, and evaporated. The crude mixtures obtained were purified by flash chromatography (AcOEt–hexane: 80/20) to give **11** as yellow powder and **12** as brown solid.

11: 180 mg, 57%. M.p. = 167 °C. IR (KBr): 2928, 2028, 1962, 1650 cm^{-1}. ^{1}H NMR (200 MHz, C_6D_6) δ: 8.6 (br s, 1H), 7.5–7.7 (m, 2H), 7.0–7.2 (m, 1H), 6.50 (br s, 1H), 5.50 (br s, 2H), 4.91 (br s, 2H), 4.2–4.0 (m, 2H), 3.2–3.0 (m, 2H), 2.7–2.5 (m, 2H), 2.35 (br s, 3H). ^{13}C NMR (50 MHz, acetone-d_6) δ: 198, 138.2, 134.3, 131.8, 131.0, 120.8, 118.9, 92.3, 88.1, 85.4, 44.0, 35.0, 19.3. MS, *m/z*: 400 $[M–(CO)_3]^+$.

12: 498 mg, 92%. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.69 (br s, 1H), 7.62 (br s, 1H), 7.30 (bd, J = 8.3 Hz, 1H), 7.12 (d, J = 8.3 Hz, 1H), 5.90 (br s, 1H), 5.43 (t, J = 1.6 Hz, 2H), 4.84 (t, J = 1.6 Hz, 2H), 3.2–1.2 (m, 14H), 2.32 (s, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 222.9, 166.9, 172.2, 171.3, 137.1, 134.4, 131.6, 131.0, 120.5, 118.2, 91.1, 86.8, 83.8, 39.2, 37.4, 34.4, 29.1, 26.1, 24.8, 22.7, 19.5. MS, *m/z*: 541 $[MH]^+$. Anal. Found: C, 55.51; H, 4.80. Calc. for $C_{25}H_{26}ClMnN_2O_6$ (540.9): C, 55.52; H, 4.84%.

Immunoassays

For CMIA, stock solutions (1×10^{-3} M) of the metal carbonyl tracers were prepared in ethanol. These solutions remained stable for at least 1 month provided they were kept in the dark at −20 °C. Serial dilutions were performed in CMIA buffer (30 mM Na_2HPO_4, 160 mM NaCl, 30 mM sodium azide, glycerol 10% v/v) just prior to use.

Titration curve by CMIA

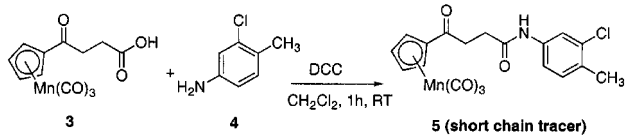
500 µl fractions containing 30 pmol of one of the organometallic tracers of chlortoluron (**5**, **11** or **12**), anti-chlortoluron antibody (1/50 to 1/2500; pure antiserum or purified IgG fraction) in CMIA buffer were incubated for 2 h at 0 °C. At the end of the incubation period the fraction B of the tracer bound to antibodies was separated from the fraction not bound to antibodies (F, the free fraction) by selective solvent extraction of the free fraction with isopropyl ether in the following manner. 1 ml of the organic solvent saturated in buffer was added to each tube; the mixtures were vortexed at the maximum speed for 1 min; then 750 µl of the organic phase containing the free tracer was withdrawn and transferred immediately to 1.5 ml Eppendorf tubes. The solutions were quickly evaporated to dryness on a Speed Vac (Savant concentrator). Samples for IR analysis were obtained by dissolving the dry residues in 30 µl CCl_4. The FT-IR spectra were immediately recorded on the spectrometer using the gold light-pipe cell described previously.[13] For each tracer, the absorbance value of the 2033 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 buffer was obtained following extraction of a 500 µl fraction containing an identical quantity of tracer but no antibodies. The bound fraction B was calculated from the difference $T - F$. Titration curves were then constructed by plotting the B/T ratio for each sample versus the inverse of the dilution of the antibodies. (Note: care should be exercised when CCl_4 is used, as it is a possible carcinogen.)

Standard curve by CMIA

Fractions of 500 µl of CMIA buffer containing a dilution of anti-chlortoluron (purified IgG fraction) at a dilution equal to the titre value, 30 pmol of one of the tracers **5**, **11** or **12** and various quantities of chlortoluron (25–300 pmol) were incubated at 0 °C for 2 h. Separation of the free and bound fractions of the tracer and determination of the bound fractions B was performed as described for the titration curve. B_0 is the B value obtained in the absence of chlortoluron. Standard curves were then obtained by plotting the B/B_0 ratios against the amounts of chlortoluron added. Linearization of the curves was, obtained by plotting ln(amount of chlortoluron in the tube) versus logit Y, with $Y = \ln\{(B/B_0)/[100 - (B/B_0)]\}$.

RESULTS AND DISCUSSION

The first step in the development of a CMIA analysis for chlortoluron is to obtain specific anti-chlortoluron antibodies and metal carbonyl tracers suitable for quantitative analysis by IR spectroscopy of small amounts of tracer.



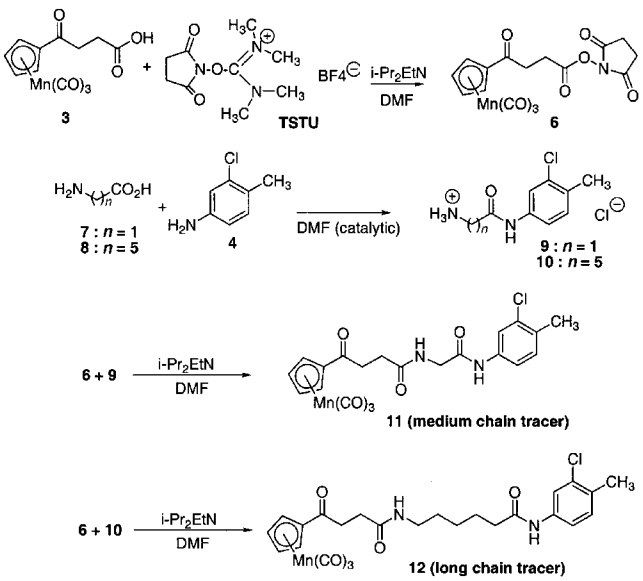
Scheme 2.

Preparation of anti-chlortoluron polyclonal antibodies

For our study we chose to work with rabbit anti-chlortoluron polyclonal antibodies. Since chlortoluron (Scheme 1) is a molecule of low molecular weight without immunogenic properties, the anti-chlortoluron immunogen (**2**) must first be synthesized as described previously by coupling the corresponding acid to BSA lysines.[3] This immunogen is then injected into rabbits to obtain crude antisera. Since the first attempts to obtain standard curves for the CMIA of chlortoluron did not give satisfactory results, we chose to work with a purified fraction of the antibodies, specifically the IgG fraction of the antisera obtained by passing the crude sera through a commercially available AvidChrom column.

Preparation of the organometallic tracers

For our study we selected the organometallic moiety cyclopentadienyl manganese tricarbonyl. We have previously shown that molecules containing this moiety can be quantified by IR spectroscopy in a range suitable for immunological analysis.[16] Three different tracers, **5, 11** and **12**, were synthesized (Schemes 2 and 3) that differ in the length and type of chain linking them to the chlortoluron molecule.



Scheme 3.

Synthesis of 5 (short-chain tracer)

The short chain tracer **5** is prepared as shown in Scheme 2 by a peptide coupling using DCC, in CH_2Cl_2 between β-cymantrenoyl-propionic acid (**3**) obtained via the protocol described in the literature[15] and 3-chloro-4-methyl-aniline (**4**).

Synthesis of 11 (medium-chain tracer) and 12 (long-chain tracer)

Tracers **11** and **12** were prepared via an identical protocol (Scheme 3). During the first step, β-cymantrenoyl-propionic acid (**3**) is activated in the presence of TSTU and *i*-Pr_2EtN in DMF to give the activated ester **6**. The amino acids **7** (glycine) or **8** (6-aminocaproic acid) are transformed *in situ*, using thionyl chloride, into the corresponding acid chloride, to give, in the presence of the substituted aniline **4** and a catalytic amount of DMF in dichloromethane at reflux, the ammonium salts **9** and **10** respectively. Finally, the corresponding amines were generated by addition of *i*-Pr_2EtN, then coupled to the activated ester **6** to give **11** and **12**.

IR study of the organometallic tracers 5, 11 and 12

The CMIA method is based on the IR quantification of the characteristic $\nu_{(CO)}$ vibration bands of the metal carbonyl tracers. These bands, appearing in the 1850–2200 cm^{-1} region, have the double advantage of being seven to ten times more intense than all the other bands of the spectrum, and of occurring in a region where other organic molecules do not absorb. It is important to note that the positions of the bands are a characteristic of the organometallic moiety, in this case the cyclopentadienyl manganese tricarbonyl, and are essentially unaffected by the organic entity to which the organometallic tripop is attached.[16] As an example, Fig. 1

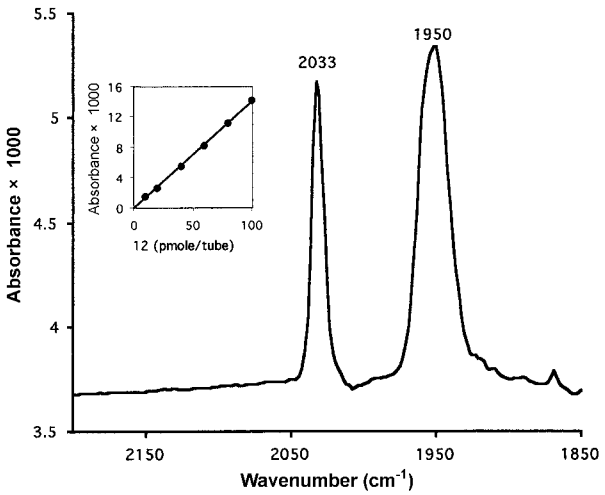


Figure 1. FT-IR spectrum of **12** (100 pmol in CCl_4). Expansion of the ν(CO) region between 2200 and 1850 cm^{-1}. **Inset**: Beer's law plot for **12** ranging from 10 to 100 pmol/tube. Absorbance value at 2033 cm^{-1} (r^2 = 0.999).

shows the IR spectrum of the tracer **12** (long-chain tracer), in the 1850–2200 cm^{-1} region. As expected with this type of tracer, two peaks are seen in this region of the spectrum, one fine and intense (symmetry A_1) at 2033 cm^{-1}, and the other wide and intense at 1951 cm^{-1} (symmetry E). During our development of the quantitative analysis by IR of metal carbonyl tracers, we showed that the height of these peaks is proportional to the quantity of tracer present in the medium, and that the best sensitivity is found with the fine, intense peak at 2033 cm^{-1}. This peak, termed the analytical peak, is the one which will be used in the rest of this study. As can be seen from the inset to Fig. 1, the absorbance value (height of the peak) of **12** at 2033 cm^{-1} is proportional to the quantity of tracer for a range of 10–100 pmol/tube of tracer. For the CMIA study of chlortoluron, we used a 'light-pipe'-type measurement cell. This cell has an optical pathlength of 20 mm for a fill volume of only 30 µl,[13] and we chose to work routinely with 30 pmol tracer per tube. With this cell the absorbance value of the peak at 2033 cm^{-1} is 3.23×10^{-3} (mean of eight experiments) for **5**, 4.46×10^{-3} (mean of ten experiments) for **11**, and 2.82×10^{-3} (mean of six experiments) for **12**. Taking into account the level of noise at this wavelength, this represents a signal/noise ratio of around 100, permitting reliable quantification of the tracer.

Determination of the titre value of the antibodies

The starting point for a titration curve by CMIA is a series of IR spectra of the free fraction of the organometallic tracer obtained after incubation in the presence of decreasing amounts of antibodies. As the quantity of antibodies present in the medium is reduced, the height of the peak at 2033 cm^{-1} increases proportionally to the free fraction of the tracer. The titre value obtained in the presence of 30 pmol tracer for the IgG fraction of the antiserum varies between 25 and 50, according to the tracer used.

Standard curve

The titre value of an antiserum is an indication of the quantity of antibodies present in the fraction, but it gives no indication of the feasibility or otherwise of the assay, particularly as far as the relative affinities of the antibody–tracer and antibody–analyte pairs are concerned. To make this assessment, a standard curve must be acquired to permit quantitative analysis of the tracer. This curve is obtained by incubating a fixed quantity of tracer (30 pmol of one of the three chlortoluron organometallic tracers) in the presence of a fixed quantity of antibodies at a dilution equal to the titre value, and increasing quantities (25–300 pmol) of chlortoluron. After incubation, separation of the free and bound fractions of tracer, and quantification of the tracer by FT-IR, three standard curves are obtained, as shown in Fig. 2. It will immediately be noted that the curves for the three tracers are very different in appearance. With **5**, the short-chain tracer, the non-specific binding rate is too high to permit a sensitive assay for chlortoluron. With **11**, which possesses a chain of medium length, the non-specific binding rate is low but the IC_{50} value is high (140 pmol, 59 µg l^{-1}). It is with **12**, the long-chain tracer, that the most sensitive assay is obtained, with an IC_{50} of 50 pmol (21 µg l^{-1}), an IC_{90} of 6.5 pmol (2.7 µg l^{-1}) and a very low non-specific binding rate. However, these values are insufficient to reach the 0.1 µg l^{-1} maximum authorized concentration (MAC) set by the EU. Preconcentration of the samples will therefore be necessary to reach this threshold, but this is often required in any case, to eliminate interference effects from organic substances present in the water sample that may cause inaccurate quantification of pesticide levels. The available solid-phase extraction or immuno-concentration techniques are well adapted to perform this function of purification and concentration of samples.[1,14,17]

Conclusion and future directions

This study demonstrates for the first time the possibility of using the non-isotopic CMIA method in the area of environmental analysis. The example used was that of chlortoluron, for which a series of three organometallic tracers, **5**, **11** and **12**, were synthesized. The results obtained show that the long-chain tracer, **12**, accompanied by use of the IgG fraction of rabbit antibodies, permits the most sensitive assay by the CMIA method, with an IC_{50} of 50 pmol (21 µg l^{-1}) and an IC_{90} of 6.5 pmol (2.7 µg l^{-1}). Since this level is higher than the MAC set by the EU for drinking water (0.1 µg l^{-1}), a purification/preconcentration step, frequently required in any case for this type of analysis, is necessary.[1] This necessity is hardly surprising, however, since at its current stage of development the CMIA method does not employ any means of signal amplification, an important component of the most sensitive immunoassays, such as ELISA. Our future aim, therefore, is to increase the sensitivity of the CMIA technique by carrying out multi-labelling of antibodies by metal

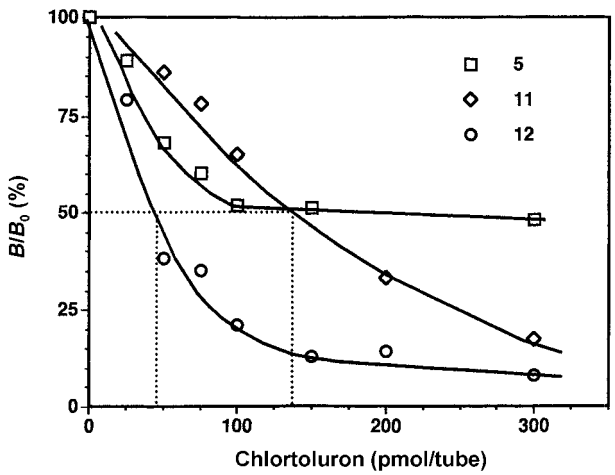


Figure 2. Standard curves of chlortoluron with the organometallic tracers (**5**, **11**, **12**) by CMIA (30 pmol of the tracers, quantification of the free fraction of the tracer by FT-IR).

carbonyl tracers. We have already shown that one of the advantages of the CMIA method is its capacity for double[12] or even triple simultaneous assays.[11] For this reason we have developed, in parallel to the single assay by CMIA of chlortoluron, that of atrazine, with in this case the dicobalt hexacarbonyl entity $Co_2(CO)_6$ whose $\nu_{(CO)}$ peaks in IR spectroscopy lie between 2055 and 2033 cm^{-1}. Since we know that the simultaneous quantitative analysis of these two tracers is within the range of this immunoassay method, we propose in the next stage of our work to study the feasibility of a simultaneous double assay of these two potential pollutants, atrazine and chlortoluron.

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