The Determination of *N*-Methylcarbamate Pesticides Using Enzyme Immunoassays with Chemiluminescent Detection

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Abstract: In the present work, enzyme-linked immunosorbent assays (ELISAs) with chemiluminescent detection for the determination of carbofuran, carbaryl and methiocarb were developed and the analytical parameters of these assays were compared with those of ELISAs with colorimetric detection. The sensitivity of immunochemical methods was expressed as detection limit, linear working range, and I_{50} value. In comparison with colorimetric ELISA, the ability of the chemiluminescent reagents to detect lower concentrations of HRP allowed to decrease the optimal antibody and conjugate concentrations and to reach better analytical parameters. The experimental comparison of the analytical performance of the ELISAs was carried out by analysing simply diluted fruit juices, spiked at different concentration levels with the above mentioned pesticides. Recovery values for both ELISAs were around 100% and no matrix effects were observed when fruit juices were diluted 1:20 or more.

Keywords: *N*-methylcarbamate pesticides; chemiluminescent and colorimetric ELISA; fruit juices

INTRODUCTION

N-methylcarbamates are an important class of pesticides widely used in agriculture instead of organochlorine pesticides as insecticides, acaricides, nematocides and molluscicides for crop protection [1]. Carbaryl, carbofuran and methiocarb are the main compounds belonging to this class of pesticides, which are acetylcholinesterase inhibitors; their residues may occur in fruits and vegetables and, therefore, poses a potential hazard for consumers [2]. As a result, international organisations relatively strict regulate maximum residue limits for pesticides in foods.

Traditional methods of analysis (e.g. HPLC/MS), although sensitive, are expensive and time-consuming and require specialised instrumentation. In this respect, enzyme immunoassays are gaining importance as analytical techniques in the agrochemical field, since they provide the analytical chemists with a rapid, sensitive and cost-effective alternative to chromatographic methods [3, 4].

Recently, we reported the application of ELISA format with colorimetric detection based on monoclonal antibodies for the determination of carbofuran, carbaryl and methiocarb in apple-strawberry baby foods [5]. In the present study, we modified the ELISA format introducing a chemiluminescent detection and we applied the optimised CL assays for the determination of the above mentioned carbamates in diluted, unextracted fruit juices. The introduction of chemiluminescent (CL) reagents to reveal the immunocomplexes formation in ELISA assays already led to an improvement of the sensitivity when compared with the colorimetric end-point detection. The advantages of the chemiluminescent (CL) assay over the colorimetric one are the detection of pesticides in a wider range of concentrations and lower consumption of immunoreagents [6, 7]. The CL reagents, ready to use from commercial sources, are absolutely not toxic and the instrumentation required for luminescent assay is cheap, simple, portable and automatable in comparison with the reference chromatographic techniques.

EXPERIMENTAL

Immunoreagents. Monoclonal antibodies (MAbs) specific for carbaryl, carbofuran and methiocarb, as well as the corresponding OVA-hapten conjugates, were prepared in the Centro de Investigación e Innovación en Bioingenieria (Universidad Politécnica de Valencia, Spain) [8–10].

Fruit samples. *N*-methyl carbamates free tomato and fruit juices, as verified by LC/MS analysis, were used. For matrix effect and recovery studies they were used without any sample treatment. For ELISA determination both were spiked with the mixed standard solution of the three carbamates to obtain concentration levels of 0.5, 1 and 5 ng/ml for methiocarb, carbaryl and carbofuran, respectively. To be analysed the juices were simply diluted at the 1:5, 1:10, 1:20, 1:50, and 1:100 ratios with PBS buffer, pH 7.4.

ELISA determinations. For determinations of carbaryl, carbofuran and methiocarb in samples was chosen an indirect competitive ELISA format that could work with colorimetric end-point detection (absorbance at 492 nm) [6] or with chemiluminescent detection (HRP catalysed luminol emission). Both were conjugate-coated formats based on identical monoclonal antibodies and homologous protein conjugates. Samples were quantitatively analysed for a single pesticide in each plate, irrespective of the presence of the two other analytes.

The colorimetric assay was performed as already described [5]. In the CL one, the peroxidase activity was revealed by adding the CL mixture (1mM luminol, 0.5mM *p*-iodophenol, 1mM H_2O_2 in borate buffer, pH 8.5), 100 µl per well. The chemiluminescence emission was measured, immediately after the addition, for 1 s/well.

Absorbance values or chemiluminescence intensity values from standards were mathematically fitted to a four-parameter logistic equation [7]. The analyte concentration in samples was determined by interpolation of the absorbance values or chemiluminescence intensity values on the appropriate standard curve.

Comparison study. To compare the analytical performance of the ELISA with colorimetric detection and ELISA with chemiluminescent detection, both were used to analyse the fruit juices at all spiked concentrations. The limit of detection (LOD) for ELISAs was calculated as the analyte concentration that reduced signal to 90% of the maximum. The linear working ranges were determined as the concentration causing 20–80% inhibition of the maximal assay signal. The I_{50} values were also calculated.

RESULTS AND DISCUSSION

In our previous paper [5], the application of MAbs-based ELISA formats with colorimetric detection for the determination of carbofuran, carbaryl and methiocarb was described. In order to improve the sensitivity of these immunoassays the luminol enhanced luminescent reaction was adapted as end-point detection system.

Both colorimetric and chemiluminescent (CL) assays were performed by using black microplates with transparent bottom, i.e. the kind required for the chemiluminescent detection. For ELISA I (colorimetric detection) and ELISA II (chemiluminescent detection) the optimum concentrations of the monoclonal antibodies specific for carbaryl, carbofuran and methiocarb and of the corresponding OVA-hapten conjugates were found using a checkerboard titration. The optimum reagent concentrations were defined as those which gave the maximum intensity of assay signal with minimum reagent expense. The obtained results clearly show that the chemiluminescent assay is able to detect lower amount of HRP, requires in quite all cases lower concentrations of both the immunoreagents and that it is able to measure samples diluted from 3 to 10 times more than the colorimetric assay.

During the experiments the detection limits, the linear working ranges and the I_{50} values obtained by ELISAs I or ELISAs II were compared: all analytical parameters were greatly improved by using the CL detection. For example, the detection limits for carbofuran ELISA I and ELISA II were 1.3 ng/ml and 0.03 ng/ml, respectively. For carbaryl ELISA I and ELISA II were obtained the detection limits 0.04 ng/ml and 0.007 ng/ml, respectively. For methiocarb ELISA I and ELISA II were obtained the detection limits 0.016 ng/ml and 0.004 ng/ml, respectively. By the chemiluminescent assay here described is possible to detect the mentioned carbamates even at the low levels established by the European legislation for drinking water, which in case of carbofuran is 0.1 ng/ml.

In order to asses the possibility to analyse non-fatty samples without pre-treatment or extraction procedures we simply diluted by PBS buffer tomato and orange juices as representative examples of

Table 1. Reproducibility and accuracy of the carbamate ELISA with chemiluminescent detection for spiked juice samples

	Mean[a] ± S.D	R.S.D. (%)	Recovery (%)
Carbofuran 5 ng/ml			
Tomato juice	5.1 ± 0.2	4.9	101.1
Orange juice	5.0 ± 0.3	5.2	99.5
Carbaryl 1 ng/ml			
Tomato juice	1.1 ± 0.1	9.7	109.0
Orange juice	1.0 ± 0.1	8.5	98.0
Methiocarb 0.5 ng/ml			
Tomato juice	0.49 ± 0.04	8.1	98.8
Orange juice	0.52 ± 0.03	5.3	104.0

[a] (ng/ml)– data are the average of 6 determinations

possible contaminated products. The concentration ranges of the added pesticides were chosen taking into account the maximum residue limit (MRL) for pesticides in baby foods (10 μg/kg, corresponding to the 10 ng/ml) and the MRLs established for fruit and vegetables (about 3–0.3 mg/kg and 1–0.1 mg/kg, respectively). Table 1 shows the recovery values obtained by chemiluminescent ELISA for fruit juices for the three carbamates. Nearly identical results were obtained by colorimetric ELISA. A slight matrix effect can be observed in tomato juice samples at the lower dilution ratios (1:5 and 1:10). This effect leads to false positive results when non-spiked samples are analysed and resulted in low or too high recovery values in spiked juices. These effects can be ascribed to the darker color and turbidity of the tomato juice in comparison with the orange juice that, in fact, allows to obtain good results at any dilution ratio. At the higher dilution ratios all matrix effects disappear and an accurate determination of the pesticides content is still possible.

The reported results show how the chemiluminescent detection can improve the analytical performances of carbamates ELISAs. Moreover, the employment of assays with lower detection limits, such as the CL ones, can allow to analyse various kinds of non-fatty samples simply diluting them, avoiding the time-consuming pre-treatment or extraction procedures, since the problems connected with possible matrix effects can be easily overcome by using highly diluted samples. The time required to prepare the samples can be greatly reduced and their number increased significatively in each analytical session, as well as the reagents cost per assay is reduced.

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References

[1] Molina M., Wiedmer S.K., Jussila M., Silva M., Riekkola M.-L. (2001): J. Chromatogr. A, **927**: 191.

[2] Yang S.S., Goldsmith A.I., Smetana I. (1996): J. Chromatogr. A, **754**: 3.

[3] Dankwardt A. (2001): In: Meyers R.A. (ed.): Immunochemical Assays in Pesticide Analysis. Encyclopedia of Analytical Chemistry. John Wiley & Sons Ltd., Chichester: 1.

[4] Hennion M-C., Barcelo D. (1998): Anal. Chim. Acta, **362**: 3–34.

[5] Mickova B., Zrostlikova J., Hajslova J., Rauch P., Moreno M.J., Abad A., Montoya A. (2003): Anal. Chim. Acta, **495**: 123.

[6] Dzgoev A.B., Gazaryan I.G., Lagrimini L.M., Ramanathan K., Danielsson B. (1999): Anal. Chem., **71**: 5258.

[7] Botchkareva A.E., Eremin S.A., Montoya A., Manclus J.J., Mickova B., Rauch P., Fini F., Giroti S. (2003): J. Immunol. Methods, **283**: 45.

[8] Abad A., Montoya A. (1997): J. Agric. Food Chem., **45**: 1495.

[9] Abad A., Moreno M.J., Montoya A. (1999): J. Agric. Food Chem., **47**: 2475.

[10] Abad A., Moreno M.J., Montoya A. (1998): J. Agric. Food Chem., **46**: 2417.