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Analytical performances of validated chemiluminescent enzyme immunoassays to detect *N*-methylcarbamate pesticides

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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. Both were conjugate-coated formats based on identical monoclonal antibodies and homologous protein conjugates. In comparison with colorimetric ELISA, the ability of the chemiluminescent reagents to detect lower concentrations of horseradish peroxidase 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 extracts of apple-strawberry baby food and simply diluted fruit juices, both 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. Results obtained by ELISAs correlated well, both in terms of accuracy and precision, with those obtained by a liquid chromatography–electrospray mass spectrometry (LC/ESI/MS/MS) analysis, used as reference method to validate the immunoassays results. The limits of detection reached by using the chemiluminescent assay were 0.03, 0.007 and 0.004 ng ml^{-1} for carbofuran, carbaryl and methiocarb, respectively.

Keywords: *N*-methylcarbamate pesticides; Chemiluminescent and colorimetric enzyme-linked immunosorbent assay; Liquid chromatography–electrospray mass spectrometry; Baby foods; Fruit juices

1. Introduction

N-methylcarbamates are an important class of pesticides now 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 that are acetylcholinesterase inhibitors; their residues may occur in fruits and vegetables and, therefore, pose a potential hazard for consumers [2]. As a result, international organisations relatively strict regulate maximum residue limits for pesticides in foods. Baby foods have a special status in legislative bodies to the issue of pesticides, since children are an extremely sensitive and vulnerable consumers group. In EU countries the total amount of pesticide residues in baby foods must be under the maximum residue limit of 10 μg kg^{-1} [3]. Because of the polarity and thermal instability of most of *N*-methylcarbamates, their determination by traditional gas chromatography (GC) techniques is difficult, then to detect the carbamates contamination the most widely used analytical technique is liquid chromatography (LC), using fluorescence, ultraviolet, diode array, and mass spectrometry detectors [4–7].

Liquid chromatography (LC) is a very sensitive and selective technique, but it involves some important drawbacks,

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such as the employment of complex and expensive instrumentation, highly qualified personnel, and time-consuming procedures for cleaning and concentrating the sample. All of these requirements often hinder the analysis of large number of specimens. The large scale monitoring activities imposed in several countries by environment concerned legislation requires the availability of rapid, sensitive and cost-effective analytical techniques [8]. Among others the immunoenzymatic assays aroused intense interest, complying with the characteristics mentioned above [9,10] and the enzyme-linked immunosorbent assay (ELISA) has been found to be a rapid, sensitive and cost-effective alternative to chromatographic methods [11]. Various polyclonal (PAb) or monoclonal antibody (MAb)-based assays have been developed to determine the carbamates presence in water and soil [12,13].

Colorimetric immunoassays for the determination of methiocarb, carbaryl and carbofuran in vegetables, contaminated water, fruits and fruit juices were previously developed. They demonstrated to be not only rapid and specific but also as suitable as the reference method for the quantitative determination of these compounds [14–17], which represents an analytical performance of remarkable importance.

The introduction of chemiluminescent (CL) reagents to reveal the formation of immunocomplexes in ELISAs already led to an improvement of the sensitivity, to a wider range of detected concentrations and to a lower consumption of immunoreagents when compared with the colorimetric endpoint detection [18,19].

The CL reagents, ready to use from commercial sources, are absolutely not toxic and the advantages related to the specific, sensitive and reliable immunoassays are further enhanced by the cheapness, simplicity, availability of portable and automatable of the instrumentation required for the luminescent detection.

Recently, we reported the application of colorimetric, MAb-based ELISAs for the determination of carbofuran, carbaryl and methiocarb in apple-strawberry baby foods [16]. In the present study, with the aim of demonstrating the possibility to perform very sensitive ELISAs of these *N*-methylcarbamates in non-fatty samples without any sample treatment, we introduced and optimised the chemiluminescent detection of our assays and we applied them to the determination of the above mentioned analytes both in extracts of non-fatty baby foods and in simply diluted, untreated fruit juices. The validation of the data obtained both by colorimetric and luminescent ELISAs on the extracts was done analysing them also by liquid chromatography–electrospray mass spectrometry (LC/ESI/MS/MS).

2. Experimental

2.1. Chemicals and immunoreagents

Certified standards of the three pesticides (purity 95–99%) were purchased from Dr. Ehrenstoffer (Germany). Individual stock standard solutions (concentration range 3.00–3.50 mg ml^{-1}) were prepared by dissolving neat pesticide standard in methanol. A stock standard solution of all analytes was made by mixing these individual pesticide solutions. Mixed working standard solution for ELISA and LC determinations was prepared fresh every day.

Gradient grade methanol was obtained from Merck, Germany. Deionised water for mixing of a mobile phase was produced in Milli-Q apparatus (Millipore, Germany). All other reagents were of analytical grade.

Bovine serum albumin (BSA), Tween 20, *o*-phenylenediamine (OPD), luminol and *p*-iodophenol (PIP) were obtained from Sigma Chemical Company (St. Louis, USA). Horseradish peroxidase (HRP)-labelled swine anti-mouse IgG (SwAM, HRP/SwIgG = 1.81, concentration, 8.9 mg ml^{-1}) was obtained from Seva Pharma, s.r.o. (Czech Republic).

MAbs specific for carbaryl, carbofuran and methiocarb, as well as the corresponding ovoalbumin (OVA)–hapten conjugates, were prepared in the Centro de Investigación e Innovación en Bioingenieria (Universidad Politécnica de Valencia, Spain). LIB-CNH45 MAb and OVA-2NAH conjugate were used for carbaryl analysis [20], LIB-BFNB67 MAb and OVA-BFNH conjugate were used for carbofuran analysis [21], and LIB-MXNB31 MAb and OVA-DPNH conjugate were used for methiocarb analysis [22].

2.2. Materials and instrumentation

Black, high binding, 96-well ELISA microplates with transparent bottom (Costar, cat. no. 3601) for chemiluminescent and colorimetric measurements were from Corning Incorporated (USA). ELISA plates were washed with a Labsystem Multiwash (USA) apparatus. Absorbances were read in a Labsystem Multiscan MCC/340 (Finland) and CL intensity was measured in a microplates reader "Victor" from Wallac (Turku, Finland). Data were processed using Microsoft Excel software (Microsoft Corporation, USA).

Liquid chromatography separation was carried out using a HP1100 liquid chromatograph (Hewlett Packard, USA) as previously described [16]. MS/MS analysis was performed as previously described [16] by LCQ Deca ion trap instrument and acquired data were processed by XCalibur software, both from Finnigan (USA).

2.3. Samples

Apple-strawberry baby foods and fruit juices were from a retail market.

Baby food extracts: The apple-strawberry baby foods, in which the absence of *N*-methylcarbamate residues had been verified by LC/MS analysis, were spiked with the mixed solution of the three carbamates at 1, 10 and 100 ng ml^{-1} and 12.5 g were mixed with 50 ml of acetonitrile and homogenised for 2 min in a Turrax tissumiser. The homogenate was evaporated and the residue was dis-

solved in methanol–water (1:4, (v/v)) and used for the analysis.

Fruit juices: *N*-methylcarbamates-free tomato and fruit juices, as verified by LC/MS analysis, were used without any sample treatment. For matrix effect and recovery studies both were spiked with the mixed standard solution of the three carbamates to obtain concentration levels of 0.5, 1 and 5 ng ml^{-1} for methiocarb, carbaryl and carbofuran, respectively. These concentrations were selected according to the respective LOD previously determined by ELISA on baby foods. For the analysis, the juices were simply diluted 1:5, 1:10, 1:20, 1:50 and 1:100 with phosphate-buffered saline solution (PBS: 0.14 M NaCl, 1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4, 2.7 mM KCl, pH 7.4).

2.4. ELISA determinations

An indirect competitive ELISA format was chosen, that could work with colorimetric end-point detection (absorbance at 492 nm) [18] or with chemiluminescent detection (HRP catalysed luminol light emission). To compare the analytical performances of the colorimetric ELISA with those of the chemiluminescent one, both were used to analyse the crude extracts of baby foods and the fruit juices at all added concentrations. Samples were quantitatively analysed for a single pesticide in each plate, irrespective of the presence of the two other analytes. Working standard solutions for each immunoassay were prepared from the mixed standard stock solution by serial dilution in PBS. Each primary test sample was subjected to three independent dilutions to fit into the linear part of the calibration curve and subsequently analysed by ELISA. The dilution factors applied for the *N*-methylcarbamate analysis are shown in Table 1.

The immunoassays were performed by the following procedure: microplates were coated with the OVA–pesticide conjugate solution in coating buffer (0.05 M carbonate–bicarbonate buffer, pH 9.6; 100 μl/well) by overnight incubation at laboratory temperature, and then washed four times with washing solution (PBS containing 0.05% Tween 20). 50 μl/well of pesticide standards or sample extracts, diluted in PBS, and 50 μl/well of specific MAbs, diluted in PBS containing 0.1% BSA, were added to each well. After 2 h incubation at room temperature the microplates were washed as described above. The second antibody (SwAM) conjugated with peroxidase was added (100 μl/well) and incubated for 1 h at room temperature, then washed again. In the ELISA with photometric detection, 100 μl/well of a OPD solution in reaction buffer (4.6 mM OPD in 0.1 M phosphate–citrate buffer, pH 5.0, containing 0.1% (v/v) of 30% H_2O_2) was added. After 10 min incubation at room temperature, the reaction was stopped by adding 50 μl/well of 2.5 M sulfuric acid, and the absorbance at 492 nm measured. In the chemiluminescent ELISA, the peroxidase activity was revealed by adding 100 μl/well of the CL mixture (1 mM luminol, 0.5 mM *p*-iodophenol, 1 mM H_2O_2 in borate buffer, pH 8.5). Immediately after the addition, the chemiluminescent emission was measured for 1 s per well.

Table 1
Concentrations of immunoreagents and baby food sample dilutions applied in the ELISAs

Detection mode	Carbaryl	Carbofuran	Methiocarb
	Antibody concentration (μg ml^{-1})		
	LIB-CNH45	LIB-BFNB67	LIB-MXNB31
Colorimetric	0.060	0.030	0.060
Chemiluminescent	0.030	0.008	0.125
	Conjugate concentration (μg ml^{-1})		
	OVA-2NAH	OVA-BFNH	OVA-DPNH
Colorimetric	0.130	0.250	1.00
Chemiluminescent	0.130	0.030	0.250
	Pesticide added (ng ml^{-1})	Sample dilution	
Colorimetric			
	1	1/5	1/1	1/5
	10	1/25	1/5	1/50
	100	1/500	1/50	1/500
Chemiluminescent			
	1	1/15	1/5	1/20
	10	1/150	1/50	1/200
	100	1/1500	1/500	1/2000

Absorbance values or chemiluminescence intensity values from standards were mathematically fitted to a four-parameter logistic equation [19]. The analyte concentration in samples was determined by interpolation of the obtained values on the appropriate standard curve.

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 concentrations producing 20–80% inhibition of the maximal assay signal. The I_{50} values were also calculated as the analyte concentrations that reduced the assay signal to 50% of the maximum one.

3. Results and discussion

Both colorimetric and chemiluminescent assays were performed by using black microplates with transparent bottom, i.e. those required for the chemiluminescent detection. Since the adsorption capacities of the various kinds of microplates could be different, the reagent concentrations were again selected for the colorimetric ELISA and optimised first time for the chemiluminescent one, to achieve the best immunoassay performances. The optimum concentrations of the specific monoclonal antibodies and of the corresponding OVA–hapten conjugates were found using a checkerboard titration. The optimum reagent concentrations, listed in Table 1, were defined as those giving the maximum inten-

Table 2
Comparison between chemiluminescent and colorimetric assays

	Linear working range (ng ml^{-1})	Limit of detection (ng ml^{-1}) ± S.D.	I_{50} (ng ml^{-1}) ± S.D.
Carbofuran			
Colorimetric	50 – 2	1.3 ± 0.4	6 ± 1
Chemiluminescent	0.96 – 0.04	0.03 ± 0.02	0.28 ± 0.09
Carbaryl			
Colorimetric	0.83 – 0.06	0.04 ± 0.01	0.15 ± 0.05
Chemiluminescent	0.52 – 0.02	0.007 ± 0.003	0.063 ± 0.004
Methiocarb			
Colorimetric	0.56 – 0.02	0.016 ± 0.004	0.08 ± 0.02
Chemiluminescent	0.18 – 0.01	0.004 ± 0.001	0.038 ± 0.005

sity of assay signal with minimum reagent expense. These concentrations were used to perform all the experiments. It is clear from Table 1 that: (i) the chemiluminescent assay, as it is able to detect lower amount of HRP, requires in quite all cases lower concentrations of both the immunoreagents; and (ii) CL-ELISA is able to measure samples diluted from 3 to 10 times more than those used in the colorimetric immunoassay.

In Table 2 the detection limits, the linear working ranges and the I_{50} values obtained by colorimetric and chemiluminescent ELISAs are compared: all analytical parameters were greatly improved by using the CL detection. In particular, the detection limits were significantly lower in comparison with those previously obtained by colorimetric ELISAs for fruit juices [23,24]. By using the chemiluminescent assay here described it is possible to detect the mentioned carbamates even at the low levels established by the European legislation as maximum residue limits (MRL) for drinking water, that in the case of carbofuran is 0.1 ng ml^{-1}.

The concentration ranges of the added pesticides were chosen taking into account the MRLs for pesticides in baby foods (10 μg kg^{-1}, corresponding to the 10 ng ml^{-1}) and the MRLs established for fruit and vegetables (about 3–0.3 mg kg^{-1} and 1–0.1 mg kg^{-1}, respectively). The influence of extracts purification on the analytical performance of ELISAs was assessed in our previous work on baby food samples [16] and negligible differences were observed in results obtained from purified and not-purified extracts. The latter were also suitable to be analysed by LC/MS/MS. For these reasons crude extracts were directly used in this work.

Table 3 summarises the repeatability and recovery values obtained for baby food extracts. Irrespective of the spiked level, mean recovery values obtained by both ELISAs were in the range 80–120% for all the analytes. The precision of the assay was better for the chemiluminescent ELISA, since coefficients of variation (CV) ranging from 1.2 to 19.2% were obtained. These values were lower than those of the colorimetric assay, and similar to those of LC/ESI/MS/MS determinations (range 1.4–18%).

In order to assess the possibility to analyse non-fatty samples without pre-treatment or extraction procedures, tomato and orange juices, as representative examples of possible contaminated products, were simply diluted with PBS. Table 4 shows the recovery values obtained for both fruit juices at the different dilutions applied: these values were very good also in this case. The samples were analysed only by chemiluminescent ELISA, since the better sensitivity of this detection method was already established during the assays of the baby food extracts. A slight matrix effect can be observed in tomato juice samples at the lower dilution factors (1:5 and 1:10). This effect led to false positive results with non-spiked samples and to low or too high recovery values in spiked juices when analysed at low dilutions. These effects could be ascribed to the turbidity of the tomato juice in comparison with the orange juice that, in fact, allows to obtain good results at any dilution assayed. Anyhow, this is not a problem, since at higher dilutions all matrix effects disappeared and an accurate determination of the pesticides content was still possible. Data showing the reproducibility and

Table 3
Overview of the results obtained by analysis with colorimetric and chemiluminescent ELISAs and LC/ESI/MS/MS of crude strawberry baby food extracts spiked with carbaryl, carbofuran and methiocarb at 1, 10 and 100 ng ml^{-1} (n = 3)

ng ml^{-1}	Colorimetric			Chemiluminescent			LC/ESI/MS/MS		
	Mean	Recovery (%)	CV (%)	Mean	Recovery (%)	CV (%)	Mean	Recovery (%)	CV (%)
Carbaryl									
1	0.8	80.0	24.2	1.0	97.5	10.8	1.2	121.0	5.2
10	9.3	92.5	14.8	10.0	100.0	10.0	8.9	89.3	7.8
100	91.4	91.4	9.4	110.3	110.3	8.7	100.1	100.1	2.5
Carbofuran									
1	1.2	119.6	26.4	1.0	100.0	14.1	1.1	112.4	8.7
10	9.5	95.0	22.3	9.0	90.0	19.2	8.8	88.0	18.0
100	93.0	93.0	3.0	114.0	114.0	1.2	99.7	99.7	1.4
Methiocarb									
1	0.9	85.5	23.2	1.0	96.0	10.9	1.1	105.4	14.2
10	9.2	92.0	23.2	11.0	110.0	18.1	9.3	93.2	8.3
100	100.3	100.3	4.9	100.3	100.3	6.1	98.6	98.6	3.1

Table 4
Influence of the matrix dilution on reliability of the determinations by CL-ELISA

Dilution factor	Tomato juice				Orange juice			
	0 ng ml^{-1}	5 ng ml^{-1}			0 ng ml^{-1}	5 ng ml^{-1}		
	Found[a]	Found[a]	Recovery (%)	CV (%)	Found[a]	Found[a]	Recovery (%)	CV (%)
Carbofuran								
5	3	2.6	51.0	13.9	n.d.	5.1	102.0	4.2
10	2	3.4	68.2	4.1	n.d.	4.7	94.7	3.9
20	n.d[b]	5.0	99.6	3.2	n.d.	5.4	108.2	3.1
50	n.d	5.1	102.2	5.1	n.d.	4.9	97.0	6.2
100	n.d	5.1	101.4	8.4	n.d.	4.9	98.9	9.7
		1 ng ml^{-1}				1 ng ml^{-1}		
		Found[a]	Recovery (%)	CV (%)		Found[a]	Recovery (%)	CV (%)
Carbaryl								
5	0.5	2.5	250	23.0	n.d	0.9	92.1	6.4
10	0.5	1.5	150	9.4	n.d.	1.1	105.6	9.2
20	n.d.	1.1	110.1	6.1	n.d.	1.0	100.9	4.5
50	n.d.	1.0	97.0	5.6	n.d.	0.9	94.3	4.1
100	n.d.	1.1	109.5	5.9	n.d.	1.0	98.1	6.0
		0.5 ng ml^{-1}				0.5 ng ml^{-1}		
		Found[a]	Recovery (%)	CV (%)		Found[a]	Recovery (%)	CV (%)
Methiocarb								
5	n.d.	0.44	87.5	8.3	n.d.	0.52	103.1	9.9
10	n.d.	0.51	101.5	5.4	n.d.	0.56	111.1	5.1
20	n.d.	0.48	95.0	6.3	n.d.	0.50	99.1	5.0
50	n.d.	0.55	109.3	7.4	n.d.	0.53	105.7	8.9
100	n.d.	0.50	99.5	14.1	n.d.	0.49	98.4	10.6

[a] (ng ml^{-1}); data are the average of 4 independent determinations.
[b] not detectable.

accuracy of the CL immunoassay on juices are reported in Table 5.

The correlation between the LC/ESI/MS/MS data, or the added amounts of carbamates, and ELISA results was very good: correlation coefficients and regression slopes were very close to 1. For methiocarb, the linear regression equations for the whole concentration range in crude extracts were: $y = 0.98x + 0.25$ for data obtained by colorimetric ELISA and $y = 0.99x + 0.69$ for the chemiluminescent one. For carbaryl the corresponding equations were $y = 1.10x + 0.46$ and $0.91x + 0.07$, respectively. For carbofuran the equations: $y = 1.08x + 0.80$ and $y = 1.01x + 0.56$ were obtained for the colorimetric and the chemiluminescent ELISA, respectively.

Table 5
Reproducibility and accuracy of the carbamate CL-ELISAs for spiked juice samples

	Mean (ng ml^{-1})	CV (%)	Recovery (%)
Carbofuran (5 ng ml^{-1})			
Tomato juice	5.1	4.9	101.1
Orange juice	5.0	5.2	99.9
Carbaryl (1 ng ml^{-1})			
Tomato juice	1.1	9.7	109.0
Orange juice	1.0	8.5	98.0
Methiocarb (0.5 ng ml^{-1})			
Tomato juice	0.49	8.1	98.8
Orange juice	0.52	5.3	104.0

Data are the average of 6 determinations. Spiked samples were measured at the 1:20 dilution ratio.

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 chemiluminescent ones, can allow to analyse various kinds of non-fatty samples simply by diluting them. This fact could avoid the time-consuming pre-treatment or extraction procedures, since the problems related 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 significantly in each analytical session, as well as the reagents cost per assay is reduced.

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