ELSEVIER

Journal of Chromatography A, 823 (1998) 109–120

JOURNAL OF CHROMATOGRAPHY A

Validation of an immunoassay method for the determination of traces of carbaryl in vegetable and fruit extracts by liquid chromatography with photodiode array and mass spectrometric detection

G.S. Nunes[a], M.P. Marco[b], M.L. Ribeiro[c], D. Barceló[d,*]

[a]Department of Technological Chemistry, Federal University of Maranhão/UFMA, Av. Portugueses, s/n, 65080-040 São Luís (Ma), Brazil

[b]Department of Biological Organic Chemistry, CID/CSIC, Jordi Girona 18–26, 08034 Barcelona, Spain

[c]Institute of Chemistry/UNESP, C.P. 355, 14800-900 Araraquara (SP), Brazil

[d]Department of Environmental Chemistry, CID/CSIC, Jordi Girona 18–26, 08034 Barcelona, Spain

Abstract

A competitive enzyme-linked immunosorbent assay (ELISA) method for carbaryl quantitation in crop extracts was validated by liquid chromatography (LC) with diode array detection (DAD). For this purpose, six crops (banana, carrot, green bean, orange, peach and potato) were chosen for recovery and reproducibility studies. The general sample preparation included extraction with methanol followed by liquid–liquid partitioning and clean-up on Celite–charcoal adsorbent column of the vegetable extracts. ELISA samples consisted of a diluted LC extract in assay phosphate buffer (pH 7.5). The potential effect of methanol in these samples was evaluated. It was observed that a maximum content of 10% methanol present in the assay buffer could be tolerated without expressive losses in the ELISA performance. Under these conditions, a IC_{50}~1.48 μg l^{-1} was obtained. A minimum matrix effect with a 1:50 dilution of the methanolic extracts in assay buffer was noticed, except for green bean samples that inhibited completely the assay. For the vegetable extracts, the ELISA sensitivities varied from 3.9 to 5.7 μg l^{-1}, and good recoveries (82–96%) with R.S.D.s ranging from 5.7 to 12.1% were found. An excellent correlation between the LC–DAD and ELISA techniques was obtained. The confirmation of the carbaryl in less concentrated samples was achieved by LC–mass spectrometry interfaced with atmospheric pressure chemical ionisation. The $[M+H]^+$= 202 and $[M+H-57]^+$=145 ions, equivalent to the protonated molecular and 1-naphthol ions, respectively, were used to carbaryl identification in these samples. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Vegetables; Fruits; Food analysis; Carbaryl; Pesticides

1. Introduction

Carbaryl, trade name Sevin and chemical name 1-naphthol *N*-methylcarbamate, is a wide-spectrum carbamate insecticide which controls over 100 species of insects on citrus, fruit, cotton, forests, lawns, nuts, ornamentals, shade trees and other crops, as well as on poultry, livestock and pets. It is also used as a molluscicide and as an acaricide [1].

Carbaryl is moderately toxic, and it can produce adverse effects in humans by skin contact, inhalation and ingestion [1,2]. It is an inhibitor of the acetyl-

*Corresponding author.

0021-9673/98/$ – see front matter © 1998 Elsevier Science B.V. All rights reserved.
PII: S0021-9673(98)00111-3

cholinesterase, and although several adverse effects have been reported in the literature [2,3], it is considered a safe insecticide because of its low mammalian toxicity. The oral LD_{50} of carbaryl ranges from 250 mg kg^{-1} to 850 mg kg^{-1} for rats, and from 100 mg kg^{-1} to 650 mg kg^{-1} for mice [4,5]. No reproductive or fetal effects were observed during a long-term study in rats which were fed with high doses of carbaryl [4] and the US Environmental Protection Agency (EPA) has concluded that carbaryl does not pose a teratogenic risk to humans if used properly [5]. Carbaryl has a relatively short residual life on treated crops. The insecticide remains at the application site, where it is slowly taken into the plant and metabolised. Despite this, residues of carbaryl in foods must be continuously controlled due its great degradation capacity to 1-naphthol which is several times more toxic than its precursor. Degradation of carbaryl in crops occurs by hydrolysis inside the plants or vegetables. Numerous studies indicate that carbaryl shows only a slight mutagenic risk [4,6]. However, with the continuous exposition and indirect consume of the pesticide through contaminated foods, carbaryl can react with nitrite (such compound can be found in food additives and in human saliva) under certain conditions to give rise to *N*-nitrosocarbaryl. This transformation product has been shown to be highly mutagenic at low levels in laboratory test systems [7]. Moreover, *N*-nitrosocarbaryl has been shown to be carcinogenic in rats at high doses [8]. Despite its lower persistence in the environment, monitoring of carbaryl residues in crops is necessary due to the above mentioned negative health effects.

Traditional analytical methods for determining the carbaryl contamination level in agricultural products involve time-consuming procedures of extraction and cleanup procedures, followed by concentration of the extracts to obtain the sensitivity desired. The final analysis of the pesticide usually occurs by high-performance liquid chromatography (HPLC) with ultraviolet (UV) [9,10], diode array (DAD) [11,12], fluorescence spectrometry [13–16] and mass spectrometry (MS) [17–19] detection techniques. In contrast with other techniques, the HPLC method involving a post-column is sufficient and the sample preparation does not need extensive clean-up procedures. Unfortunately, the required instrumentation is still expensive and most of the laboratories have until now employed more universal UV detection. In general, the main inconvenience of HPLC analysis is the necessary time required for cleaning the extracts prior to the chromatographic analysis which is usually much higher than for any biorecognising technique – which are commonly less subject to matrix interferences exhibiting lower detection limits. In this respect, immunochemical methods are gaining importance as analytical techniques for environmental monitoring. Enzyme-linked immunosorbent assays (ELISAs) have been found to be simple and cost-effective alternatives to instrumental analysis specially when numerous samples are being analysed.

This paper describes the validation of an immunochemical method for the quantification of carbaryl in vegetable and fruit extracts. The method proposed here is based in an indirect competitive ELISA format for carbaryl analysis developed by Marco et al. [20]. They have produced high-affinity rabbit polyclonal antibodies for carbaryl and they have studied the cross-reactivity of the 1-naphthol, the major metabolite of this pesticide, on the format ELISA [21]. Marco et al. [22] applied the immunoassay method to determine the carbaryl in ground water samples. In the present study this same anti-carbaryl antibody was used to analyse the pesticide in crops. The effect of the different vegetable matrices as well as of the organic solvents used for extraction have been evaluated. Validation was achieved analysing the sample extracts by HPLC–DAD and HPLC–MS.

2. Experimental

2.1. Chemicals, immunoreagents, buffers and solutions

(1) A 10 n*M* stock solution of carbaryl for immunoassays was prepared by dissolution of the solid standard pesticide (Dr. Ehrenstorfer, Augsburg, German, 99.4% purity, M_r=201) in dimethylsulfoxide (DMSO, Merck, Augsburg, Germany) and stored at 4°C; working solutions (1.0 n*M*) were prepared by dilution of the stock solution in DMSO before use. For preparation of the standard solutions of the

calibration curve, a sequential 1:5 dilution was performed in order to obtain concentrations varying from 25 000 to 0.013 n*M*. Carbaryl stock solution (200 $\mu g\ ml^{-1}$) used in the chromatographic analyses was prepared by dissolving of the solid standard with methanol; diluted solutions were prepared with acetonitrile.

(2) Anticarbaryl polyclonal antibody and coating antigen immunoreactives were provided by Professor Bruce D. Hammock at the Department of Entomology of the University of California, Davis CA, USA. The polyclonal antibody R2114 had been raised in white New Zealand rabbits immunised with 8-KLH, a conjugate of 1-(5-carboxypentyl)-3-(1-naphthyl)urea and keyhole limpet hemocyanin (KLH). The coating antigen 5-CONA is a conjugate of *N*-(2-naphthol)-6-amino-hexanoic acid covalently coupled to conalbumin (CONA). The synthesis of compounds is described by Marco et al. [20].

(3) Goat anti-rabbit IgG-horseradish peroxidase (AntilgG-HRP) was provided by Sigma (cat. No. R2004, St. Louis, MO, USA). (4) Substrate was consisted in a hydrogen peroxide solution. (5) Chromogen substance TMB (3,3′,5,5′-tetramethylbenzidine) was purchased from Sigma. Working solutions were prepared in DMSO. (6) Coating buffer was consisted in a carbonate buffer solution, pH 9.5. (7) PBS (phosphate-buffered saline, pH 7.5). (8) PBST assay buffer (also used as washing solution) consisted of PBS buffer containing Tween 20 (Sigma). (9) Substrate buffer was a citrate buffer, pH 5.5. (10) Substrate–chromogen mixture for the enzyme peroxidase was prepared immediately before use by mixing TMB, H_2O_2 and citrate–acetate buffer (pH 5.5).

(11) The enzymatic reaction was interrupted with a stopping solution of H_2SO_4 aqueous solution. All buffer solutions were prepared with Milli-Q water. Salts, acids and H_2O_2 were from analytical grade and they were obtained from Merck.

2.2. Apparatus and instrumental

2.2.1. LC–DAD analysis

The chromatographic system was composed of a Waters Model 600-MS quaternary LC pump coupled to a Waters Model 996 photodiode array detector. The injector system was a 20-μl fixed volume Rheodyne type (CA, USA). The chromatographic column was a Zorbax SB-C_{18} (15 cm×4.6 mm I.D.) packed with 5-μm particles supplied by Rockland Technologies, Nuenen, Netherlands. An acetonitrile–water mixture at a flow-rate of 1.0 $ml\ min^{-1}$ was used as mobile phase, and a gradient program consisting of decreasing polarity from 30% to 100% acetonitrile during 30 min was applied.

2.2.2. LC–atmospheric pressure chemical ionization (APCI)-MS analysis

In order to avoid false positives, presence of carbaryl at lower contents was confirmed by a LC–MS system (VG Platform mass spectrometer, Micromass, Manchester, UK) operating at APCI mode. Briefly, this interface consists of a heated nebuliser probe and the standard atmospheric pressure source configured with a corona discharge needle. The LC eluent enters the probe, where it is pneumatically converted into an aerosol and rapidly heated into the vapour–gas phase at the probe tip (at 300°C). The resulting vapour is carried toward the counter electrode by a nitrogen gas flow (~300 $l\ h^{-1}$). After gas expansion, an excess of energy (potential of 20 V) between collisions can be gained by the ions. Mobile phase molecules rapidly react with ions from the corona discharge (3.5 kV) to produce stable reagent ions. Then, analyte molecules introduced into the mobile phase react with the reagent ions at atmospheric pressure and typically become protonated and followed quantitatively countered into the mass spectrometer. The LC–APCI-MS system was controlled by the MassLynx data system and the chromatograms were recorded under time-scheduled selected ion recording (SIR) conditions. For chromatographic separation, the same C_{18} Zorbax column was used, and an acetonitrile–water (both containing 0.1% acetic acid) mixture at 1.0 $ml\ min^{-1}$ was used as mobile phase. The elution process started at 5% acetonitrile, increasing linearly during 30 min until 100% acetonitrile. The eluent was delivered by a gradient system from Waters 616 pumps coupled to a Waters 600S controller (Waters, Milford, MA, USA).

2.2.3. Microtiter-plate ELISA analysis

Analysis was performed on 96-well polystyrene microtiter plates from NUNC (MaxiSorb, Roskilde, Denmark). Ninety-six-well polystyrene plates for

mixing and prepare dilutions and calibration curves were used. The absorbance values were read at 450 nm in a microtiter-plate ELISA Titertek Multiskan PLUS MK 11 (Labsystem, Helsinki, Finland). For data acquisition and processing a commercial software package Genesis (Labsystems) was employed. The data adjusting of the calibration curves was performed through a four-parameter logistic equation. ELISA plates were washed with an automatic washer Model SLT 96PW (Salegurb, Austria).

2.3. Preparation of the crop extracts

Six vegetable products (banana, carrot, green bean, orange, peach and potato) were chosen for recovery and reproducibility studies and stored at −20°C before analysis. Fig. 1 shows the extraction and clean-up steps for the preparation of the extracts destined to chromatographic and ELISA analyses. The general procedure is based in the analytical methodology adopted by the US Food and Drug Administration (FDA) for *N*-methylcarbamate [23] determination, but several modifications were included in order to simplify the general methodology [24]. Extraction and clean-up procedures were substantially simplified. To cleaning the extracts, previously established reversed-phase elution procedure on a silanized Celite–charcoal (4:1) adsorbent column was performed, and a methylene chlorine–methanol (99:1) mixture was employed as eluent system. After the eluate have been collected, it was concentrated until dryness at 35°C and the residue was finally redissolved with 1 ml methanol for LC–DAD and LC–APCI-MS analysis. For the ELISA determinations, the final extract was diluted in PBST assay buffer (the dilution factors were after all studied).

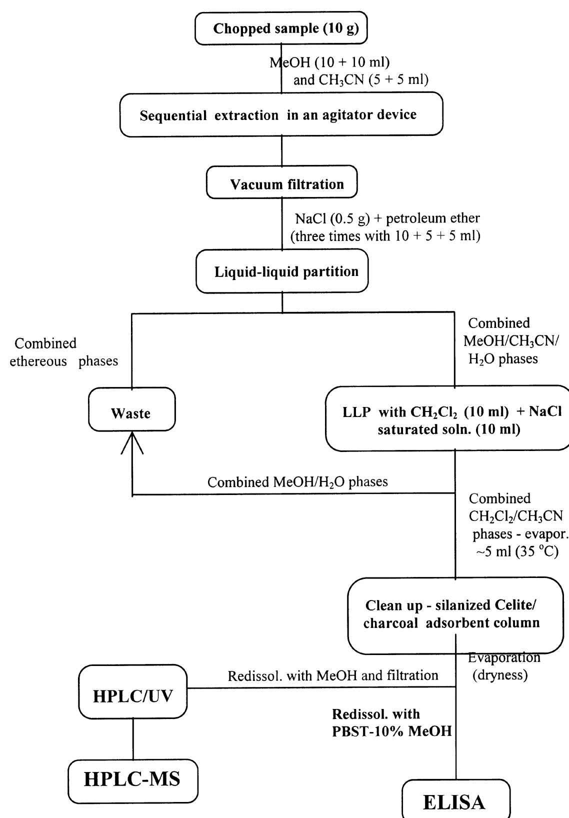

Fig. 1. Preparation of vegetable and fruit extracts for chromatographic and ELISA analyses.

2.4. Immunoassays

After each incubation step, the plates were washed five times with 300 μl PBST/well and blotted dry on a paper towel. Initially, plates were coating with 100 μl/well of antigen solution (0.4 μg 5-CONA per ml coating buffer), followed by overnight incubation at 4°C. Samples and standard solutions (ten concentrations varying from 25 000 n*M* to 0.001 n*M*) and spiked or vegetable samples were pre-incubated overnight at 4°C with the polyclonal antibody R2114 (1/32 000 in PBST with 10% methanol) into polystyrene plates or polypropylene tubes. The day after, a volume of 100 μl/well of these solutions was added to each well of the pre-coated plate and incubated for 30 min at room temperature and followed by 100 μl of a goat antiIgG-HRP (the antibody anti-rabbit IgG, developed in goat and covalently coupled to horseradish peroxidase, 1/6000 diluted with PBST) was added to each well and incubated for 1 h more at room temperature. Finally, 100 μl/well of the substrate-chromogen mixture was added and, after 30 min incubation at room temperature, the enzymatic reaction stopped with 50 μl/well 4 *M* H_2SO_4. Absorbances were read immediately at 450 nm. The concentration of the coating antigen and the dilution of antiserum were previously optimized through two-dimensional experiments.

2.5. Recovery studies

The goal of these experiments was to evaluate the efficiency of the ELISA for the measurement of residues of the carbaryl insecticide in vegetable and fruit samples. Before ELISA analysis, crop extracts were spiked at three concentration levels: 5, 20 and 100 n*M* (1, 4 and 20 ppb, respectively) of carbaryl, and the ELISA was performed according to the method described above.

2.6. Effect of the organic solvent

Standard curves of carbaryl were prepared in PBST containing different concentrations of methanol (1%, 5%, 10%, 20%, 30%, 40% and 50%, v/v). The effect of the organic solvent on the colour developed was investigated using ELISA.

2.7. Matrix effects studies

LC methanolic extracts of banana, carrot, green bean, orange and peach (0.5 ml) were diluted with PBST–10% methanol (0.5 ml) and used to prepare standard curves of carbaryl. Then, standard curves were prepared with diluted extracts in PBST–10% methanol, performing total dilution factors of 1:20 and 1:50. The ELISA was carried out as described above and the curves adjusted to a four-parameter logistic equation to compare them for parallelism with the standard curve prepared in assay buffer containing 10% methanol.

2.8. Correlation studies

In order to compare the ELISA and HPLC methods for carbaryl analysis, extracts of banana obtained according to Fig. 1 were spiked at 2.5, 5,10, 50, 100 and 200 ppb in five replicates. Filtered and diluted extracts (with PBST containing 10% methanol) were analysed by ELISA within 48 h. After storage at −20°C, the extracts were analysed by LC within two weeks, and the quantification was based on triplicate injections.

2.9. Statistical evaluation

Fig. 2 shows a model of a plate containing the calibration curves for two vegetable samples and spiked samples. Two replicates represented for two wells were routinely analysed per calibration curve per plate. For the spiked samples, four replicates were performed per plate in each fortification level. Five extracts were produced and 20 replicates per fortification level was performed for each crop sample. The efficiency of the proposed immunoassay was evaluated according the accuracy and reproducibility parameters, through the recovery results and relative standard deviations (R.S.D.s), respectively. In order to determine the repeatability of the ELISA method, R.S.D.s between assays were calculated. Correlation studies between the immunoassay method and the classical LC technique were performed using linear regression.

3. Results and discussion

In five of the vegetable products (banana, carrot, green bean, orange and peach) carbaryl insecticide is extensively applied in Brazilian agriculture. Although since 1987 Brazilian legislation has not permitted the use of the carbaryl in potato, eventually it can be detected in some samples. Thus, the immunoassay behaviour with this kind sample was also studied.

3.1. Operating conditions for ELISA analysis

The ELISA described here uses a competitive assay format. The coating antigen competes with the unlabeled carbaryl present in the sample for the antibody sites, and a colour is developed. This colour is inversely proportional to the pesticide concentration in the sample. The original format for analysis in water have shown that the antibody does not recognize 1-naphthol, and only a negligible (<5%) cross-recognition is observed to other carbamates [20,21]. Indubitably, the development of a highly sensitive ELISA method for pesticide depends to a great extent on the hapten used that must preserve as far as possible the analyte structure. The immunoassay described here uses an antisera (R2114) developed against a carbaryl mimic coupled to KLH. The carbaryl present in the samples competes with

	1	2	3	4	5	6	7	8	9	10	11	12	
A													
B	B	5000	1000	200	40	8	1.6	0.32	0.064	0.013	0.003	0.00	**V1**
C	"	"	"	"	"	"	"	"	"	"	"	"	(ppb)
D	B	5000	1000	200	40	8	1.6	0.32	0.064	0.013	0.003	0.00	**V2**
E	"	"	"	"	"	"	"	"	"	"	"	"	(ppb)
F	FL_1	FL_1	FL_2	FL_2	FL_3	FL_3	FL_1	FL_1	FL_2	FL_2	FL_3	FL_3	**CS**
G	FL_1	FL_1	FL_2	FL_2	FL_3	FL_3	FL_1	FL_1	FL_2	FL_2	FL_3	FL_3	**CS**
H													

fortification levels (FL) for the vegetable 1 (V1) — fortification levels (FL) for the vegetable 2 (V2)

Fig. 2. ELISA plate layout to the recovery studies for carbaryl. The upper wells were designated to the calibration curves for two vegetable extracts V1 and V2 (two replicates for each point of curve) prepared with the methanolic PBST. Control samples (CSs) are in the inferior wells (four replicates for each fortification level). The border wells were despised.

the immobilized antigen for a fixed amount of antibody.

3.2. Effect of the organic solvent in the ELISA

In order to obtain the vegetable extracts in solution, and to compare the LC and ELISA results, the use of an organic solvent was necessary. Recent studies in the area of immunoassay in non-aqueous or slightly organic media have led to a variety of exciting possibilities for practical applications in pesticide analysis. Therefore, an analytical method initially validated for analysis in aqueous matrixes, e.g., the ELISA assay for carbaryl [18], can be perfectly adapted to analysis on other kinds of samples, since that it is studied the ability of the enzyme in accept different nucleophiles, such as alcohols, amines, etc. Particularly for horseradish peroxidase, this enzyme catalyses a variety of reactions in a number of different organic solvents without considerable losses in its performance [23,24]. In the present work, we evaluated the potential effect of the presence of methanol on the competitive ELISA format. Fig. 3 shows the competitive standards curves obtained in the presence of varying amounts of methanol. It can be observed that an amount equal or superior to 10% methanol in the final extract should not interfere in immunoassay performance. This data agrees with other reported works on immunoassay tolerance to organic solvents [21] and such conditions were used for further investigations. The IC_{50} values (averages of triplicate determination) were as follows: 1.48 $\mu g\ l^{-1}$ for control PBST; 0.97 $\mu g\ l^{-1}$ for PBST–5% methanol; 0.86 $\mu g\ l^{-1}$ for PBST–10% methanol; 0.80 $\mu g\ l^{-1}$ for PBST–20% methanol and 0.98 $\mu g\ l^{-1}$ for PBST–30% methanol. In general, the curve slopes varied from 0.5 to 0.8, but the higher slopes were obtained at lower contents of solvent. To evaluate the accuracy of the carbaryl ELISA under these conditions employing PBST buffer, carbaryl spiked samples at concentrations varying from 0.0 n*M* to

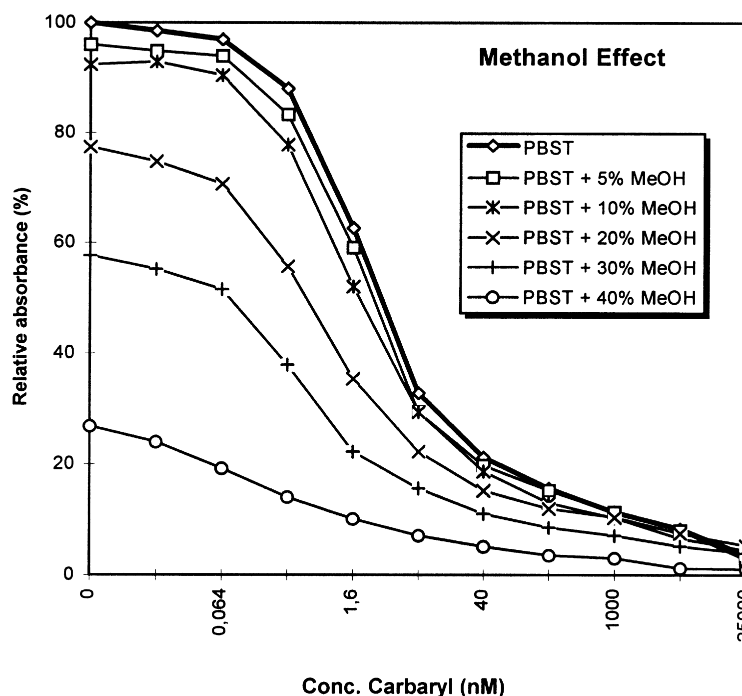

Fig. 3. Effect of methanol content of the vegetable extracts on the ELISA for carbaryl analysis. Assay buffer (PBST, pH 7.5) was compared to six PBST buffer solutions containing methanol in concentrations varying from 10 to 50% (v/v). Absorbance data were normalised to percentage.

40.0 n*M* were measured. A good correlation ($R=0.998$) between the spiked and the measured values was observed, indicating that samples containing about 10% methanol can directly be measured by ELISA. The regression equation was: add. conc. carbaryl (ppb)=0.1744+1.1225×estim. conc. carbaryl (ppb).

3.3. Dilution of the vegetable extracts and matrix effects

To determine the interference of the vegetable matrices used in this study, the final vegetable extracts were 20- and 50-fold diluted in PBST containing 10% methanol. These extracts were used to study the parallelism of the standard curves. The curves shown in Fig. 4 evidence the necessity of diluting the samples with PBST buffer to reduce matrix effects in the competitive ELISA format; 1:20 dilution factor was not sufficient, and effectively changed the immunoassay parameters, mainly for banana, peach and orange extracts in which this effect was more pronounced. A minimum 1:50 dilution appears to be adequate for this purpose, since the standard curve did not differ significantly from the one obtained in buffer, except for green bean extract that inhibited completely the assay. Perhaps there is some component in the composition of this matrix that inhibits the antibody–antigen binding. Due the parallelism of the curves observed by the carrot, banana and potato 1:50 diluted extracts, the immunoassay could be used in future studies as a method of analysis. Table 1 shows the sensibilities for carbaryl ELISA in the vegetable extracts. The IC_{50} values varied from 3.9 $\mu g\ l^{-1}$ to 5.7 $\mu g\ l^{-1}$ and higher R.S.D.s were observed for banana and potato extracts, due possibly to the difficulty of pesticide extraction in such samples.

3.4. ELISA accuracy

Table 2 lists the recovery results for the spiked crop extracts measured by ELISA. In this experiment, only the ELISA efficiency has been evaluated,

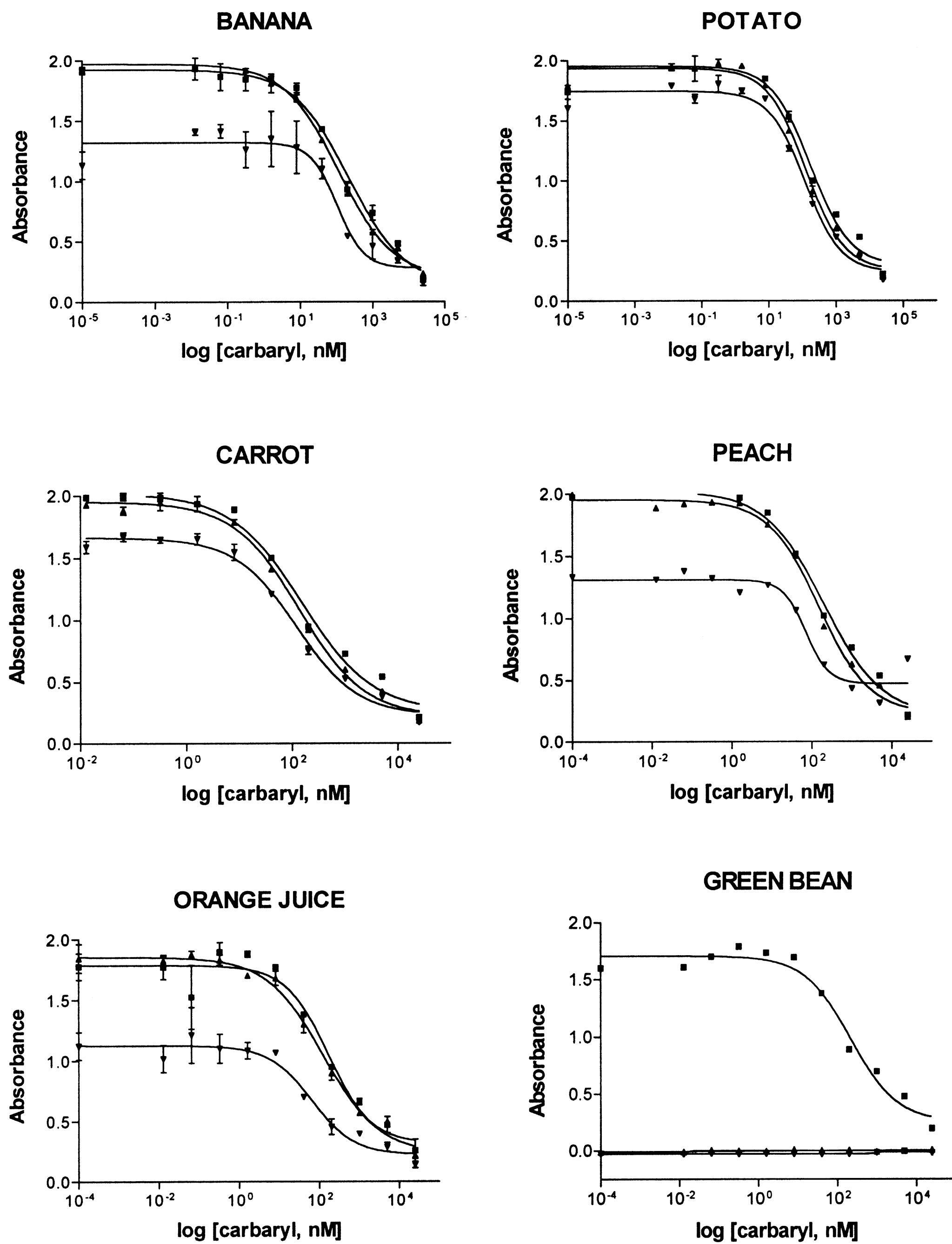

Fig. 4. Matrix effect of the vegetable extracts. ELISA assay performed with PBST buffer containing 10% MeOH (■) and the vegetable extract diluted 50-fold (▲) and 20-fold (▼) with PBST. Dilution of the antibody: 1/32 000; concentration of the coating antigen: 0.4 μg ml^{-1}.

Table 1
Sensitivity of the ELISA method for carbaryl analysis in vegetable extracts ($n=5$)

Vegetable sample[a]	Linear range (μg l^{-1})	IC_{50} (μg l^{-1})	R.S.D. (%)
Banana	1.5-90	5.7	5.6
Carrot	0.2-100	4.2	3.5
Orange juice	0.15-150	3.9	4.0
Peach	0.08-170	2.7	3.0
Potato	0.1-100	4.6	4.6

[a] Fifty-fold diluted (in PBST with 10% methanol) extracts, after preparing as for chromatographic analysis.

since that recoveries of the extraction/clean-up treatments were previously determined by LC, and the results varied from 82 to 96% with R.S.D.s ranging from 5.7 to 12.1%. [24]. Thus, the accuracy of the carbaryl assay to crop analysis was evaluated by adding known amounts of the pesticide to the PBST-diluted extracts and by plotting obtained results in the calibration curve prepared with original extract (see Fig. 2). The spiking levels were into the linear range of the assay. Recoveries were high indicating the accuracy of the carbaryl immunoassay with only a weak matrix effect influencing the final recoveries. The average assay recovery was 95% with R.S.D.s ranging from 5.0 to 11%. It should be mentioned that when the clean-up procedure on adsorbent Celite–charcoal column is not performed, the R.S.D.s inter-assays are considerably increased (in some cases, as for example for potatoes, the average R.S.D. reach to be three-fold higher than the original result). Although charcoal retains efficiently certain co-extractives of plants, as pigments (xantophylls, chlorophylls, etc.) and other substances which eventually could interfere the ELISA analysis, C_{18}- and a CN-bonded solid-phase extraction (SPE) cartridge can improve method performance reducing the time of analysis. Alternatively, for simplification

Table 2
Recovery results of carbaryl ELISA for crop extract[a] analysis ($n=5$)

Vegetable	Carbaryl spike	Recovery	Carbaryl result	
sample	(μg l^{-1})	(%)	Mean (μg l^{-1})	R.S.D. (%)
Banana	1	87.0	0.87	10.3
	4	111.0	4.54	5.4
	20	113.6	22.72	7.3
Carrot	1	96.0	0.96	8.6
	4	93.0	4.69	5.2
	20	105.0	22.00	9.0
Orange juice	1	71.0	0.71	7.3
	4	88.0	3.52	5.5
	20	86.1	17.22	8.2
Peach	1	85.6	0.86	10.2
	4	87.2	3.49	7.9
	20	90.3	18.10	7.8
Potato	1	90.0	0.90	11.1
	4	98.2	3.93	9.3
	20	119.8	23.97	6.9

[a] Fifty-fold diluted (in PBST with 10% methanol) extracts, as shown in Fig. 1.

of the analytical methodology, the elimination of the liquid–liquid partitioning (LLP) and/or clean-up steps could be tested, but this approaches require future work to verify the absence of the matrix effects.

3.5. Correlation studies between ELISA and LC analysis

Fig. 5 shows a typical chromatogram obtained by LC–DAD analysis of an extract of carrot. Experimental comparison between both analytical techniques was performed by analysing spiked extracts of banana at increased carbaryl contents. Although for ELISA higher R.S.D.s and a discreet over-estimation at high spiked concentrations had been observed, an excellent correlation between the real and measured carbaryl contents was obtained. The regression analysis resulted in adequate coefficients (R=0.986 to ELISA and R=0.995 to HPLC–DAD). Both techniques, HPLC and ELISA, showed slope values near to the unity. The regression equations were: estim. conc. carbaryl (ppb)=−0.3655+1.1104×spiked conc. carbaryl (ppb) and estim. conc. carbaryl (ppb)=−0.6011+0.99014×spiked conc. carbaryl (ppb) for ELISA and LC curves, respectively.

3.6. LC–MS confirmation analysis

The confirmation of the carbaryl peaks in samples spiked at lower contents was carried out by LC–APCI-MS. Under the chromatographic conditions described in Section 2.2.2, the pesticide was eluted before than 16 min (t_r~15.6 min). Carbaryl followed the general patterns previously indicated in literature [25], and the ions used for identification in the vegetable extracts were: $[M+H]^+$=202 and $[M+H-57]^+$=145 (57=CH_3NCO), equivalent to the protonated molecular peak and to the acid hydrolysis

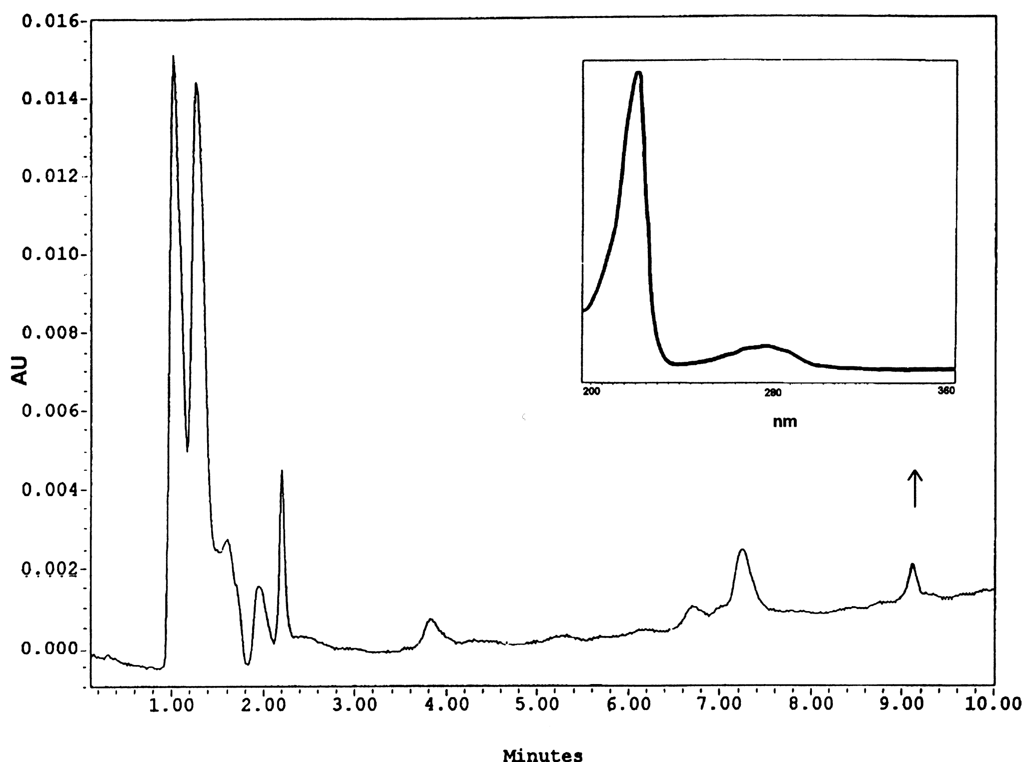

Fig. 5. Typical LC–DAD chromatogram obtained of an extract of carrot spiked at 5 ppb of carbaryl. Identification of the carbaryl peak was confirmed by the UV spectrum shown in the upper detail. Chromatographic conditions as described in Section 2.2.1.

reaction of the carbaryl, with consequent formation of the protonated 1-naphthol $[C_{10}H_8O]^+$, respectively. 145 and 202 were the base and the second most abundant peaks, respectively, at an extraction voltage of 20 V. Fig. 6 presents the total ion chromatogram (TIC) and the SIR to m/z values of 145, 202 and 177. In most samples, 145 and 202 peaks have predominated, but in some of them a 177 peak appeared also. A detail of the SIR of the 177 peak (t_r~18.4 min) is also shown in Fig. 6. This $[145+32]^+$ peak is associated with the protonated 1-naphthol together the intact methanol molecules. The TIC indicates the presence of the 1-naphthol, and the confirmation of the 177 peak was achieved later by performing an LC–MS chromatographic analysis of a 1-naphthol standard. Volmer et al. [26] founded that at lower temperatures of both probe and source ionic adducts of the molecules of the analyte and/or its degradation product with the organic solvent (in case, methanol) could be formed. The specificity and selectivity which were afforded for direct mixture analysis by this technique greatly enhanced the utility of the APCI source for unambiguous compound identification in complex matrices.

4. Conclusions

Within the general objective of validating an ELISA method for carbaryl analysis in crop extracts, a systematic study was undertaken, starting with the influence of the methanol content present in the extracts, and finishing with the correlation study between a conventional LC technique and the immunoassay method. In the present work we confirmed the great sensitivity and selectivity of the

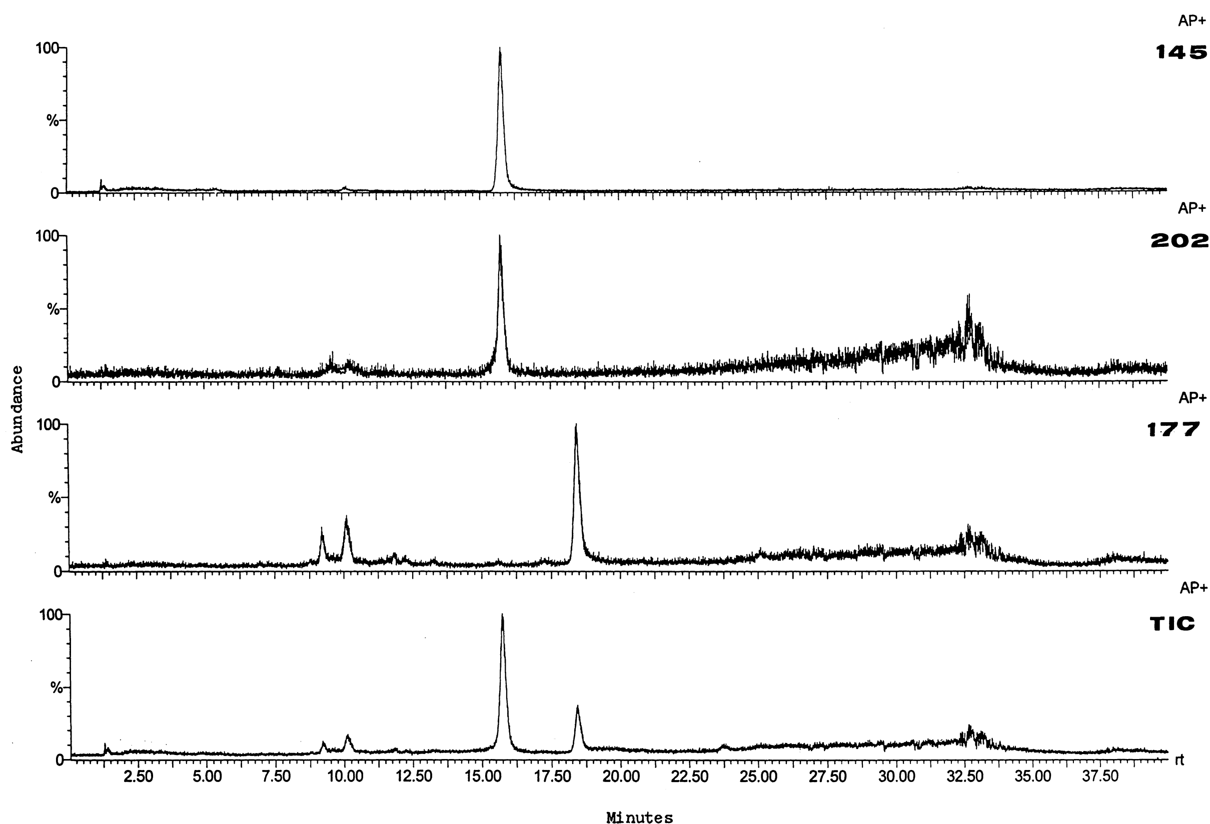

Fig. 6. LC–APCI-MS chromatograms of carbaryl and 1-naphthol present in a peach extract spiked at 10 ppb of carbaryl. Above, the m/z ions at 145 and 202 (to carbaryl identification) and 177 (to 1-naphthol identification, if it is present); below, the TIC with carbaryl (t_r~15.6 min) and 1-naphthol (t_r~18.4 min).

immunoassay techniques, and the possibility of their use in pesticides residue monitoring on food and crop samples. The assay compares favourably to HPLC determinations and exhibits excellent precision and accuracy, assuring consistent monitoring of the carbaryl residue. The ELISA is also free from interferences from commonly found vegetable components, but a rigorous clean-up procedure was carried out for this purpose.

The use of LC–APCI-MS has permitted the detection of the pesticide present in the samples spiked at lower carbaryl contents. This technique showed levels of sensibility and selectivity comparable to the GC–MS, making it possible to analyse the unstable carbaryl and also to verify the degradation of the carbaryl to 1-naphthol in some extracts. Finally, the ELISA method presented here have shown to be adequate to analyze crop samples and the principal advantages are that it is rapid, very sensitive, selective and cost effective for large sample loads. Additionally, the proposed method constitutes an exceptional screening tool.

Acknowledgements

This study has been funded by the Commission of the European Communities Environmental Programs, Contract No. ENV4-CT97-0384 and CICYT (AMB96-0926). G.S.N. would like thank the CNPq and ICI/AECI (Brazilian and Spanish research organizations, respectively) for the scholarship and financial support. Sincere appreciation is expressed to Roser Chaler and Teresa Cristina Santos for help with LC–MS analysis, and Rosa Maria Alonso for helpful discussions. We also thank Professor Bruce D. Hammock for supplying the immunoreactives used in this work.

References

[1] R.L. Baron, in W.H. Hayes, Jr. and E.R. Laws, Jr. (Editors), Handbook of Pesticide Toxicolgy, Vol. 3, Academic Press, San Diego, CA, 1991, pp. 1125–1189.
[2] M. Farage-Elawar, W.D. Blaker, J. Appl. Toxicol. 12 (1992) 421.
[3] G.P. Casale, J.L. Vennerstrom, S. Bavari, T.L. Wang, Immunopharmacol. Immunotoxicol. 15 (1993) 199.
[4] National Library of Medicine, Hazardous Substances Databank, Carbaryl, 4 February 1992.
[5] US Environmental Protection Agency, Office of Drinking Water, Carbaryl Health Advisory, Draft Report, August 1987.
[6] D. Siebert, G. Eisenbrand, Mutat. Res. 22 (1974) 121.
[7] R. Elespuru, W. Lijinsky, J.K. Setlow, Nature 247 (1974) 386.
[8] J.D. Regan, R.B. Setlow, A.A. Francis, W. Lijinsky, Mutat. Res. 38 (1976) 293.
[9] M.C. Pietrogrande, G. Blo, C. Bighi, J. Chromatogr. 349 (1985) 63.
[10] T.D. Spittler, R.A. Marafiot, G.W. Helfman, R.A. Morse, J. Chromatogr. 352 (1986) 439.
[11] R.J. Bushway, J. Chromatogr. 457 (1988) 437.
[12] B.D. McGarvey, J. Chromatogr. 642 (1993) 89.
[13] R.T. Krause, J. Assoc. Off. Anal. Chem. 68 (1985) 726.
[14] A. De Kok, M. Hiemstra, C.P. Vreeker, J. Chromatogr. 507 (1990) 459.
[15] A. De Kok, M. Hiemstra, J. Assoc. Off. Anal. Chem. 75 (1992) 1063.
[16] M.J. Page, M. French, J. Assoc. Off. Anal. Chem. 75 (1992) 1073.
[17] D. Barceló, G. Durand, V. Bouvot, M. Nielen, Environ. Sci. Technol. 27 (1993) 271.
[18] R.M. Marcé, H. Prosen, C. Crespo, M. Calull, F. Borrull, U.A.Th. Brinkman, J. Chromatogr. A 696 (1995) 63.
[19] W.H. Newsome, B.P.Y. Lau, D. Ducharme, D. Lewis, J. Assoc. Off. Anal. Chem. 78 (1995) 1312.
[20] M.P. Marco, S.J. Gree, H.M. Cheng, Z.Y. Liang, B.D. Hammock, J. Agric. Food Chem. 41 (1993) 423.
[21] P.M. Kramer, M.-P. Marco, B.D. Hammock, J. Agric. Food Chem. 42 (1994) 934.
[22] M.-P. Marco, S. Chiron, J. Gáscon, B.D. Hammock, D. Barceló, Anal. Chim. Acta 311 (1995) 319.
[23] US Food and Drug Administration, Pesticide Analytical Manual, 1992, Ch. 4, pp. 400-1/401-b1.
[24] G.S. Nunes, M.L. Ribeiro, L. Polese, D. Barceló, J. Chromatogr. A 795 (1998) 43.
[25] W.M.A. Niessen and J. van der Greef, Liquid Chromatography–Mass Spectrometry – Principles and Applications, Marcel Dekker, New York, 1992, pp. 229–245.
[26] D. Volmer, K. Levsen, M. Honing, D. Barceló, J. Abián, B.L.M. van Baar, E. Gelpí, U.A.Th. Brinkman, J. Am. Soc. Spectrom. 6 (1995) 656.