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# Detection of coumaphos in honey using a screening method based on an electrochemical acetylcholinesterase bioassay

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ABSTRACT

An analytical protocol based on an electrochemical assay for the detection of acetylcholinesterase (AChE) inhibitors has been optimised for the detection of coumaphos in honey. Coumaphos is a phosphotionate insecticide requiring transformation in the corresponding oxo-form to act as an effective AChE inhibitor. The inhibition assay was based on the electrochemical detection of the product of AChE, choline, via a choline oxidase biosensors obtained using prussian-blue modified screen printed electrodes. A simple procedure for the oxidation of coumaphos via N-bromosuccinimide (NBS) and AChE inhibition was optimised. A calibration curve for coumaphos (8–1000 ng/ml) was obtained in buffer; the intra electrode CV ranged between 8 and 12% whereas the inter electrode CV was comprised between 12 and 14%. A detection limit (LOD) of 8 ng/ml was achieved, with an $I_{50\%}$ of 105 ng/ml. The assay was then applied to detect coumaphos in honey samples. Despite the solubility of the samples in buffer, the assay was affected by many electrochemical interferences present in this sample matrix A simple C18 based solid phase extraction procedure has been then optimised and used for the assay. This allowed to eliminate all the electrochemical interferences with a satisfactory coumaphos recovery (around 86%) for a final LOD of 33 ng/g. The developed assay applied to detect coumaphos in different honey samples gave data well correlated with LC–MS detection.



## 1. Introduction

Coumaphos (O-3-chloro-4-methyl-2-oxo-2H-chromen-7-yl O,O diethylphosphorothioate), is an organothiophosphate pesticide which is active by contact, ingestion, and vapour action, and causes phosphorylation of the acetylcholinesterase enzyme of tissues, allowing accumulation of acetylcholine at cholinergic neuro-effector junctions (muscarinic effects), and at skeletal muscle myoneural junctions and autonomic ganglia [1]. As well as other thiophosphate molecules, coumaphos is active in its oxon product which appears to be an important active metabolic intermediary. The use of this chemicals to combat honeybee's diseases or parasite infestations is a common practice among beekeepers that has led to contamination of honey and other hive products, ultimately affecting human health [2–4]. Coumaphos along with amitraz, bromopropylate, cymiazole and fluvalinate is among the most commons acaricides used by the beekeepers to combat the parasitic mites *Varroa jacobsoni* and *Ascophera apis* [3,4]. In addition to the environmental concern, the presence of the aforementioned substances in honey decreases its quality. This situation has forced the European Union (EU), and also other countries to establish different regulations limiting maximum residual levels (MRLs) of acaricides in honey. For instance, the Commission Regulation 508/1999/EC of EU and subsequent modifications has established MRL in honey for coumaphos at 0.1 mg $kg^{-1}$ [5]. The same MRL for coumaphos has been established by the Environmental Protection Agency (USEPA) [6].

Most of the multiresidues analytical procedures consist in an extraction step and clean-up followed by GC-ECD or HPLC-DAD analysis [7,8]. These procedures are expensive, time consuming and require specialised personnel, therefore they are not easily adaptable for the screening of the several lots that are usually brought to industrial producers by small beekeepers. Thus, there is a need for fast and inexpensive testing devices for pesticide detection in honey. Inhibition based biosensors have been widely exploited in this field and a review has been recently published [9]. Nevertheless biosensing of these toxic agents still remain an open issue as witnessed by recent publications [10,11] One of the most used approaches assumes the determination of pesticide inhibition by acetylcholinesterases (AChE) coupled with the electrochemical determination of the enzyme product. Low cost, Prussian Blue based hydrogen peroxide electrodes, as screen printed electrodes or carbon resistors have been succefully used with immobilised

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oxidase enzymes in real food samples [12,13]. They appear very promising because of the robustness of the redox mediator and the favourable potential window used for the detection. This type of sensors have been also recently proposed for the measurement of acetylcholinesterase activity in conjunction either with immobilised [14,15] or free Ache [16–19]. In this paper we describe the use of AChE in solution that has been reported to provide lower detection limits with respect to immobilised enzyme [20] and appears a more useful approach for systematic use in real samples use avoiding cumbersome regeneration steps [21] or expensive disposable use of probes.

Most of the paper related to the application of AChE inhibition assays for pesticide detection reports matrix limitation; thus they require a multi-step preparation of samples or exhibited problems affecting accuracy of results [22,23]. Furthermore literature data are generally limited to carbamate or oxo-forms of organophosphate pesticides, whereas many insecticides are sulfur containing molecules. Coumaphos contains a P=S moiety thus belonging to the family of phosphorothionate. The metabolic pathway of the target organisms oxidises the P=S moiety to the oxo form which shows a dramatically higher inhibiting activity. Bromine and N-bromosuccinimide (NBS) are reported as oxidising agents to be used in in-vitro test with a high yeld [23,24], alternatively direct in situ oxidation has been proposed [25].

In the present paper we report the use of a choline oxidase amperometric biosensor as measuring device for an inhibition assay for coumaphos based on AChE.

The biochemical–electrochemical pathway used to determine the inhibition consisted of two enzymatic reactions (I and II) generating a chemical oxidation (III) determined by cathodic chronoamperometry (IV).

(I) Acetylcholine + $H_2O$

→ Acetic acid + Choline (Enzyme I : AChE)

(II) Choline + $2O_2 + H_2O$

→ $2H_2O_2$ + Betaine (Enzyme II : Choline oxidase)

(III) $PB_{red} + H_2O_2 \rightarrow PB_{ox} + H_2O$ (Chemical oxidation)

(IV) $PB_{ox} + e^- \rightarrow PB_{red}$ (Electrochemical reduction)

Experimental parameters for coumaphos oxidation (e.g. reagents concentration and incubation time) were optimised. We also reported the limitations affecting the use of the bioassay in honey and the optimisation of a simple SPE procedure that allowed to overcome such limitations. Finally the overall procedure, was applied to honey samples obtained by local producers; data were confirmed by LC–MS-MS.

## 2. Materials and methods

Acetylcholinesterase (EC 3.1.1.7) from electric eel (type IV V-S, 970 U/ml), choline oxidase from *Alcaligenes* sp. (EC 1.1.3.17), potassium phosphate, potassium chloride, potassium ferricyanide, ferric chloride, hydrochloric acid, bovine serum albumin (BSA), nafion, glutaraldehyde, n-bromosuccinimide (NBS), ascorbic acid (AA), methanol, acetonitrile, HPLC water, and coumaphos were obtained from Sigma–Aldrich (Milan, Italy). Filter paper was purchased from Whatman International Ltd. (Maidstone, UK). C18-isolute phase was purchased from Stepbio, Bologna, Italy, Screen printed electrodes were obtained from Ecobioservice and Research, Firenze.

### 2.1. Biosensor preparation and testing

The choline oxidase biosensor was constructed using disposable graphite screen printed electrodes made of three printed conducting pads resulting in: a graphite working electrode (3 mm diameter), a pseudo-reference silver/silver chloride electrode and a graphite counter electrode. The working electrode was modified with Prussian Blue catalyst (PB) and with the biorecognition element (choline oxidase). PB modification of SPE was made by addition of a 40 μl mixture (1:1, v/v) of potassium ferricyanide ($K_3Fe(CN)_6$) and ferric chloride ($FeCl_3$) in 10 mM HCl, both 0.1 M. Choline oxidase was immobilised onto the PB-SPE surface using a cross-linking method that required 8 μl mixture of glutaraldehyde, Nafion, and bovine serum albumin (BSA). To prepare 150 μl of the mixture, 20 μl of glutaraldehyde (2.5%, v/v, diluted in water) and 30 μl of Nafion (5%, v/v, in ethanol) were mixed with 100 μl of a solution prepared by dissolving 40 mg of BSA and 10 mg of choline oxidase in 1 ml of 0.05 M phosphate buffer + 0.1 M KCl, pH 7.4 according to the procedure described by Ricci et al. [26] and successfully adopted by our group in a previous works [27]. Measurement was carried out at an applied potential of −50 mV vs pseudo-Ag/AgCl reference electrode using a PalmSens instrument (Palm Instruments BV, Houten, Netherlands). Efficiency of the Choline biosensor was tested checking the steady state signal (2 min) of 200 μmol of choline; sensors giving signals lower than 500 nA were discarded.

### 2.2. AChE inhibition assay

The inhibitory effect of coumaphos on cholinesterase was evaluated by monitoring the decrease in the current produced by the reduction of the electrochemical mediator Prussian Blue.

Both standard and sample solutions were measured according to the following experimental scheme: first a blank sample was measured and the current recorded; then the pesticide – either a standard or sample solution – was measured in duplicate; finally, a second blank measurement completed the protocol. The average current of the blank sample ($I_0$) and the average current of the contaminated sample (II) were used to calculate the inhibition percentage according to Eq. (1):

$$I\% = 100x\left(\frac{I_0 - I_I}{I_0}\right) \quad (1)$$

The assay consisted in the addition of 100 μl of sample solution, either the methanolic standard or extract solution, to an AChE solution (0.125 U/ml), final volume 975 l. The incubation was allowed to proceed for an optimised period of time (30 min) and then 25 μl of acetylcholine was added to the solution, final concentration 0.3 mM. 2 min later, 100 μl of the solution was placed on the choline oxidase biosensor surface and the steady-state current recorded for 2 min.

The biosensor was polarised for 10 min in phosphate buffer solution, pH 7.4, KCl 100 mM, at −50 mV vs Ag/AgCl pseudo-reference electrode. After each measurement, the biosensor surface was rinsed with phosphate buffer. Potential remained applied during the entire experimental procedure. All calibrations, recovery and sample measurements were carried out according to this protocol. Current/time records for this kind of protocol have been already reported in [19].

### 2.3. Chemical activation of coumaphos

The oxidation was carried out modifying the procedure described by Shulze Holger et al. [28] and optimised for the use in food by Del Carlo et al. [27]. The procedure was based on the use of NBS as an oxidising reagent followed by the use of ascorbic

**Table 1**
recoveries of carbaryl, paraoxon and dichlorovos with the proposed SPE procedure.

| Spiking (ng/g) | Calc. conc. (ng/g) | *R*% | SD *n* = 3 | Calc. conc. (ng/g) | *R*% | SD *n* = 3 | Calc. conc. (ng/g) | *R*% | SD *n* = 3 |
|---|---|---|---|---|---|---|---|---|---|
| 50.0 | 44.5 | 88.9 | 7.5 | 43.8 | 87.6 | 6.0 | 44.6 | 89.2 | 8.0 |
| 100.0 | 88.8 | 88.8 | 6.0 | 82.4 | 82.4 | 7.4 | 87.2 | 87.2 | 6.8 |
| 200.0 | 182.2 | 91.1 | 8.3 | 185.2 | 92.6 | 8.0 | 180.4 | 90.2 | 6.0 |

acid as an antioxidant to remove the excess of NBS. The 200 ng/ml coumaphos solution in phosphate buffer (pH 7.4) was used to assess the production of the oxo form. On the basis of our experience a fixed concentration (5 mg/l) of both NBS and AA was used whereas the incubation times were optimised varying the incubation time of the oxidant and antioxidant and AChE inhibition, respectively, by 10, 20, and 30 min. The procedure consisted of two separate steps: first, the coumaphos solution was treated with the NBS for a particular incubation period (10, 20, or 30 min); secondly, the AA was added (10, 20, or 30 min).

Once the optimum conditions were obtained in the buffer solution, they were adapted to cope with the honey matrix. In fact, the optimised oxidation conditions in buffer solutions were not satisfactory once they were applied to the SPE sample extract, so the conditions were re-optimised by increasing the NBS and AA in the 5–100 mg/l concentration range.

### 2.4. Evaluation of matrix effect on the sensor response

In order to evaluate potentially occurring interferences on the electrochemical choline oxidase biosensor honey samples were dissolved in phosphate buffer (pH 7.4) in different w/v ratios: 1:10, 1:5, 1:2. The electrochemical biosensor was polarised at the working potential for 10 min, afterward the freshly prepared honey solution was placed on the electrode surface and the current monitored over a three hours period at 15 min intervals. A similar experiment was also carried out using the prussian blue modified screen printed electrode, in this case just a dissolution ratio 2:10 (w/v) was used, and pH dependence was also investigated (pH 7.4, pH 8.5, pH 9.5).

### 2.5. SPE procedure for the extraction of AChE inhibitors from honey

A C18 SPE purification strategy was evaluated in terms of recovery by using three well known AChE inhibitors as model analytes (carbaryl, paraoxon and dichlorvos). The SPE procedure was as follows; 0.5 g of C18 phase was packed in 5 ml syringe bodies; the solid phase was conditioned with 2 × 2.5 ml of methanol; 3 g of the fortified honey samples were dissolved in 10 ml of 30% methanol solution and loaded onto the column; the polar compounds were removed by 2 × 2.5 ml water and finally the analyte of interest was eluted by 2 × 500 μl of methanol. The extraction efficiencies were calculated at 50 ng/g, 100 ng/g and 200 ng/ml for all the tested pesticides from the AChE inhibition value obtained after SPE extraction vs the AChE inhibition value obtained before the SPE step. In a second step the same SPE procedure was used for the extraction of coumaphos, both from fortified (50, 100, 150 ng/g) and naturally contaminated honey samples.

### 2.6. Liquid chromatography–tandem mass spectrometry

In order to compare the data obtained with the inhibition assay an LC–MS method for coumpahos analysis was used. Liquid chromatography was carried out using a HPLC/Autosampler (equipped with a 100 μl loop)/vacuum degasser system PerkinElmer Series 200 (PerkinElmer, Norwalk, CT, USA). Analytes were separated with a reverse phase C18 column (25 cm × 4.6 mm I.D.) Alltima (Alltech, Deerfield, IL, USA) packed with 5 μm diameter particles equipped with an Alltima guard column. The mobile phase was composed by acetonitrile and water (60:40, v:v) added with formic acid 5 mM at a flow rate of 1 ml $min^{-1}$, but only 150 μl $min^{-1}$ was passed into the mass spectrometer source. A PE Sciex API 2000 tandem triple quadrupole mass spectrometer (PerkinElmer), equipped with a TurboIonSpray (Applied-Biosystems pneumatically assisted Electrospray) source, was used. Nitrogen gas was used as a curtain gas and collision gas with a flow rate of 1.1 l $min^{-1}$, while air was used as a nebulizer and drying gas with a pressure setting of 50 psi. Mass axis calibration of each mass-resolving quadrupole Q1 and Q3 was performed by infusion of a polypropylene glycol solution at 10 μl $min^{-1}$. Unit mass resolution was established and maintained in each mass-resolving quadrupole by keeping a full width at half maximum of approximately 0.7 ± 0.1 Da. For quantitative analysis, two precursor ion/product ion transitions for the Selected Reaction Monitoring (SRM) acquisition mode were chosen after observing collision-induced dissociation (CID) spectra, obtained by full scan product ion experiments: 363 → 227 (*m*/*z*) 363 → 307 (*m*/*z*). All source and instrument parameters for monitored analytes were optimized by standard solutions of 100 pg $ml^{-1}$ (containing 1 mmol $l^{-1}$ of formic acid) infused at 10 μl $min^{-1}$ by a syringe pump.

Coumaphos was detected in the positive ionization (PI) mode using TurboIonSpray source, with a capillary voltage (ISV) of 5000 V, Turbo gas temperature at 300 °C and dwell time 600 ms.

Peak areas for selected ions were determined using PE Sciex package Multiview 1.4. The calibration curve was prepared by fortifying a blank sample extract by adding suitable amounts of working standard solutions using six calibration levels (10, 50, 100, 150, 300, 600, 1000 ng/ml).

### 2.7. Matrix matched calibration of coumaphos via AChE inhibition assay

A 8 points matrix matched calibration of coumaphos in honey was performed in the concentration interval 5–1000 ng/g. An honey blank extract obtained according to the optimised SPE procedure was spiked at different concentration levels and underwent to chemical oxidation via the described NBS-AA oxidation. Then the solution containing the oxo-form of coumaphos were used according to the usual inhibition assay scheme. Three replicates were used for each concentration level.

### 2.8. Honey sample analysis

Thirteen honey samples obtained from local producers were finally analysed using the established SPE-NBS-AA-AChE inhibition bioassay procedure and compared with data obtained via LC–MS.

3 g of each honey samples were dissolved in 5 ml of 30% methanol solution and extracted according to the SPE procedure. The methanolic extract was then split in three aliquots, one was analysed via the AChE inhibition assay to detect the presence of anticholinesterasic activity, the second was analysed via NBS-AA-AChE inhibition bioassay whereas the third was analysed via LC–MS procedure. For coumaphos quantification with the electrochemical bioassay, the matrix matched calibration was used. Moreover two negative samples (S10 and S11) were spiked with coumaphos at 50 ng/g, 100 ng/g and 150 ng/g and analysed as above described.

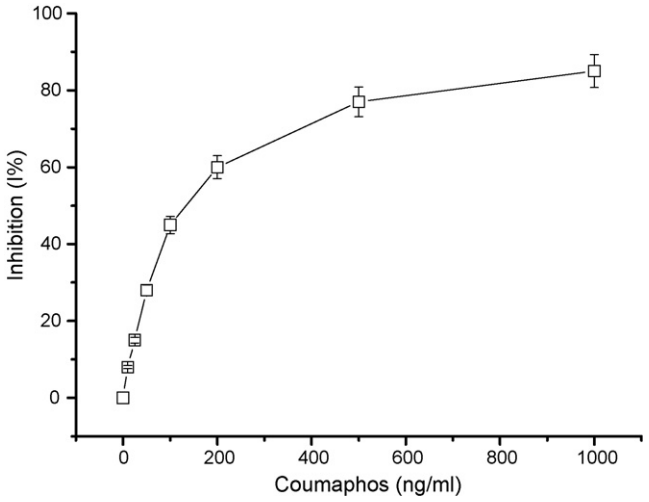


**Fig. 1.** Calibration curve of coumaphos in phosphate buffer pH 7.4 in the 8–1000 ng/ml range.

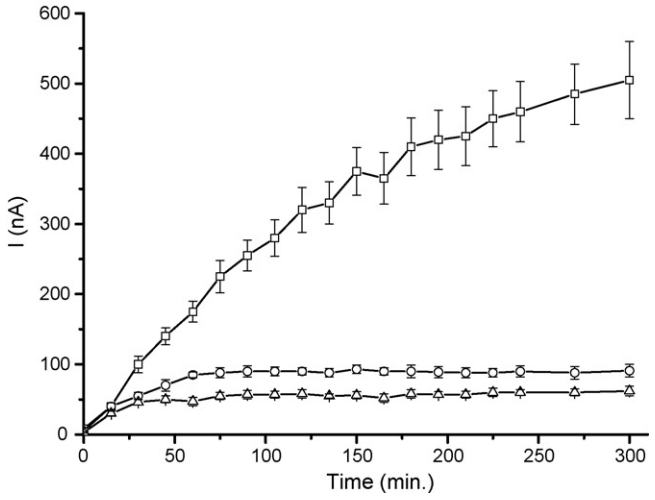


**Fig. 3.** Cathodic current produced at the Prussian blue modified screen printed electrode prepared without the enzymatic (choline oxidase) layer polarised at −50 mV vs Ag/AgCl reference electrode by the honey sample at different pHs; pH 7.4 (□), pH 8.5 (○), pH 9.5 (△).

## 3. Result and discussion

### 3.1. AChE inhibition assay

The choline oxidase biosensor was prepared and used to measure the inhibitory effect of coumaphos in standard solutions. Typical calibration curve for choline were linear in the 1–200 μmol range with a sensitivity of 2.6 nA/μM. Coumaphos is a organothiophosphate pesticide that needs oxidation to be able to inhibit acetylcholinesterase. The conditions for the chemical oxidation of coumaphos were initially optimized in buffer by using a fixed concentration of NBS and AA (5 mg/l each) and varying the incubation times (10, 20 and 30 min). Using a coumaphos solution of 200 ng/ml the best results as a compromise between the highest inhibition ($I\%$ = 60.0) and repeatability (RSD = 8.0%) were obtained using 30′ min of incubation for all the steps. During this period no significant change of AChE activity was observed. In fact enzyme activity was stable during the working day, with no significant time related activity decrease. The coumaphos calibration curve obtained in PBS pH 7.4 is reported in Fig. 1. The dynamic range was 8–1000 ng/ml, the linear range 8–100 ng/ml ($r^2$ = 0.998) and the calculated limit of detection was 8 ng/ml; the sensitivity expressed as $I_{50\%}$ was 105 ng/ml. These analytical figures are slightly better than those reported previously by Ivanov et al. [15] for coumaphos (i.e LODs for different electrodes from 18 to 36 ng/ml). Moreover, considering the oxidation of coumaphos, the milder oxidation conditions used should allow a more robust assay despite the longer incubation time selected. The choline oxidase biosensors resulted stable to the reaction conditions over 60 consecutive measurements, a 20% decrease in current was in fact observed with respect to the first assay; enzyme electrodes with such a sensitivity were anyway discarded.

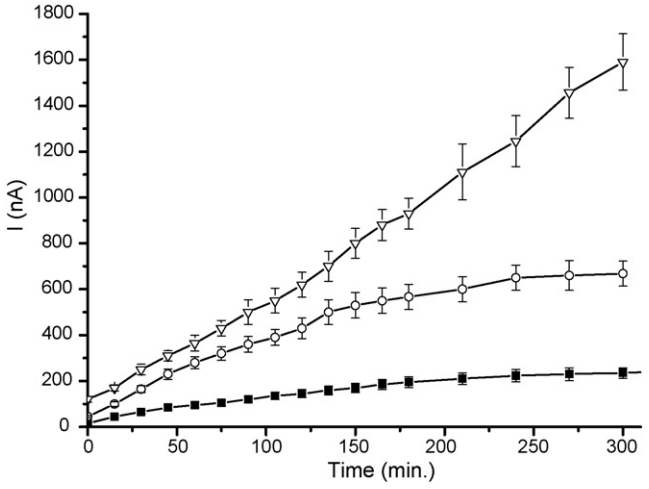


**Fig. 2.** Cathodic current produced at the choline oxidase biosensors polarised at −50 mV vs Ag/AgCl reference electrode by the honey sample at different dilution ratios (1:2 (△), 1:5 (○), 1:10 (□), w/v, respectively).

### 3.2. Evaluation of matrix effect on the sensor response

One of the main features of biosensing based analysis is the possibility to use minimally treated samples. In order to evaluate such opportunity for the detection of coumaphos in honey, the electrochemical response of buffer diluted samples was tested at different dilution ratios (Table 1).

A cathodic current which appeared dependent on the amount of honey was observed over a 5 h experiment (Fig. 2). The higher current was produced when a ratio 1:2 (w/v) was used to dilute the sample, whereas lower current values were recorded when higher dilution ratios were used (1:5, 1:10, w/v, respectively). The occurring electrochemical interferences were most likely due to the glucose/glucose oxidase and to choline naturally present in honey. In fact, choline has been reported to be present in honey at levels of 0.3–25.0 mg/kg [29]. Looking at the continuosly increasing signals the interfering compounds seems continuously produced as in an enzymatic reaction, (no clear steady state is formed). In our opinion, small amounts of glucose oxidase, reported to be present in honey, not active because of the viscosity, high concentrations of substrates and poor oxygen availability, become active vs glucose after the dilution in buffer producing hydrogen peroxide detected by the sensor. The same effect, at different extent, was observed for different samples of honey. We, indirectly confirmed this, investigating the behaviour of diluted honey of at different pH values (pH 7.4, pH 8.5, pH 9.5) using only the Prussian blue hydrogen peroxide sensor (Fig. 3 modified screen printed electrode prepared without the enzymatic (choline oxidase) layer). The continuous increase in current was still evident and strongly dependent on pH. Moreover, comparing the current measured in the same condition (pH 7.4 and 1:5, w/v, honey dilution, Fig. 3) the contribution due the choline

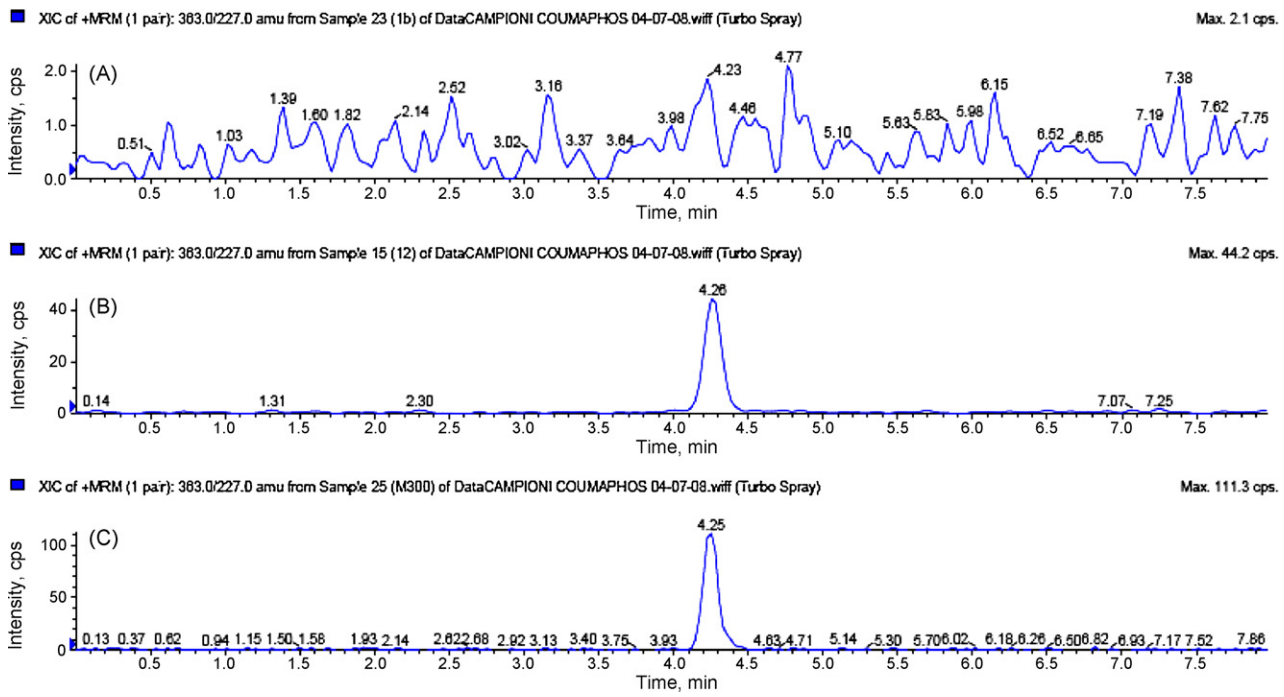


**Fig. 4.** Typical chromatograms obtained for blank, positive (0.040 mg/kg) and fortified (0.100 mg/kg) samples.

appears negligible glucose/glucose oxidase system. Due to the drift of the signal and differences among samples this kind of interfering signal appeared difficult to handle; for this reason an extraction procedure was optimised.

### 3.3. SPE procedure for the extraction of AChE inhibitors from honey

A C18 SPE purification strategy was evaluated in terms of recovery by using three well known AChE inhibitors as model analytes (carbaryl, paraoxon and dichlorvos) which do not need chemical activation. Using the optimised SPE procedure no electrochemical interference was observed, thus demonstrating that the procedure was fitted to the purpose. The recoveries for carbaryl, paraoxon, and dichlorovs from spiked honey, calculated using the calibration curve for each of the analyte are presented in Table 2. They were satisfactory for all substances and concentrations tested with values ≥89% for carbaryl, ≥82% for paraoxon, and ≥87% for dichlorvos. These results demonstrated the suitability of the SPE procedure. The repeatability of each analyte at each tested concentration level was measured using three separate SPE extractions (Table 2), the average standard deviation was 7.1%. The presence of methanol was limited to 10% of the total volume in order to have the best compromise between residual activity of the enzyme (still 85% compared to buffer solution) and an appropriate amount of inhibitor from the SPE procedure.

**Table 2**
S1–S13 honey samples analysed via the SPE-NBS-AA-AChE assay and SPE-LC–MS, two negative samples, S10 and S11, were fortified at 1/2 MRL, MRL and 1.5 MRL.

| Sample | LC–MS (ng/g) | Bioassay (ng/g) | Error (%) |
|---|---|---|---|
| S1 | 56 | 70 | 25 |
| S2 | 84 | 88 | 5 |
| S3 | 69 | 54 | −22 |
| S4 | 12 | <LOD | N.A. |
| S5 | 3 | <LOD | N.A. |
| S6 | 59 | 40 | −32 |
| S7 | 48 | 35 | −33 |
| S8 | 3 | <LOD | N.A. |
| S9 | 5 | <LOD | N.A. |
| S10 | <LOD | <LOD | N.A. |
| S11 | <LOD | <LOD | N.A. |
| S12 | <LOD | <LOD | N.A. |
| S13 | <LOD | <LOD | N.A. |
| S10_A | 49 | 40 | −18 |
| S10_B | 102 | 95 | −7 |
| S10_C | 148 | 140 | −5 |
| S11_A | 51 | 55 | 8 |
| S11_B | 98 | 103 | 5 |
| S11_C | 147 | 145 | −1 |

### 3.4. Cross-validation by HPLC-MS/MS

In order to evaluate the analytical performances of the electrochemical bioassay a selected number of samples were monitored by means of a LC–MS/MS method. First of all the performances of the latter method have been evaluated: limit of detection (LOD)

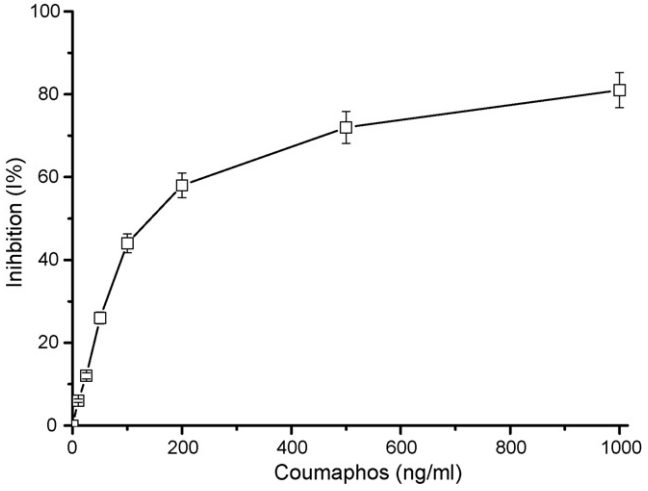


**Fig. 5.** Calibration curve of coumaphos in matrix extract diluted 1:10 in phosphate buffer pH 7.4 over 10–1000 ng/ml range.

and limits of quantitation (LOQ), linearity and carryover, precision, accuracy and matrix effect. The calibration curve, prepared by fortifying a blank sample at six calibration levels (10, 50, 100, 150, 300, 600, 1000 ng/ml) had a correlation coefficient ≥0.99. Typical chromatograms obtained in the conditions described in Section 2 are reported in Fig. 4 for blank, positive (0.040 mg/kg) and fortified (0.100 mg/kg) samples. In order to evaluate the presence of any degree of carryover the calibration points were analyzed both at increasing and decreasing concentration: the results showed a non significant carryover phenomena. LOD and LOQ, calculated on the calibration curve as $\mu + 3\sigma$ and $\mu + 10\sigma$ (where $\mu$ is the average signal value of the noise) were respectively 3 ng/ml and 10 ng/ml. Precision was expressed as RSD (%), where the sample standard deviation ($s$) was calculated on six replicates for each level for the within-day (intraday) precision and over five days for the between day (interday) precision. Instrumental intraday RSD was below 2% and interday was below 5%. Accuracy, evaluated using the percentage of the measured value vs the reference value, was always higher than 95%. In order to evaluate matrix effect, the instrumental calibration curve ("calibration curve in solvent") was compared with calibration curves obtained by blank extracts, spiked with the analytes ("calibration curve in matrix"). No significant effect of the matrix was registered both on retention time and analytical signal.

The recovery rates were calculated at three concentration levels (1/2 MRL, MRL, 1.5 MRL) as required by UE legislation [30] and it has been proved that they are not influenced by concentration (Table 2, S10_A,B,C and S11_A,B,C). Repeatability (intra-day) and reproducibility (inter-day) were calculated at the same concentration levels and the CV has demonstrated to be concentration independent in the selected range.

### 3.5. Matrix matched calibration of coumaphos via AChE inhibition assay

The oxidation procedure was then optimised for honey samples. A comparison among 5 mg/l, 50 mg/l and 75 mg/l NBS and AA using 30′ incubation time for all the steps was performed. 50 mg/l was the optimal amount of reagents considering the rate and repeatability of oxidation (data not shown). Using these parameters a matrix matched calibration of coumaphos was performed and is reported in Fig. 5. The dynamic range was 10–1000 ng/ml, the linear interval was 10–100 ng/ml ($r^2 = 0.996$) and the calculated limit of detection was 10 ng/ml. No evident fouling effect by matrix co-extract compounds was observed for the choline oxidase biosensors. The $I_{50\%}$ was 120 ng/ml showing a slight decrease in the sensitivity of the assay when the honey matrix was analysed. The linear portion of the calibration curve was used for the evaluation of coumaphos concentration in honey samples.

### 3.6. Honey sample analysis

The calculated LOD of the SPE-NBS-AA-AChE inhibition bioassay was 33 ng/g; this value is well below the 100 ng/g MRL of coumaphos in honey and, thus, allows the analysis of honey samples at the MRL level.

The analysis performed using the AChE bioassay showed that no anticholinesterasic activity was found in the methanolic extract prior to chemical oxidation, demonstrating that no AChE inhibiting pesticides were present. In Table 2 the comparison between data obtained by the SPE-NBS-AA-AChE inhibition bioassay and LC–MS procedure are reported. These data were also confirmed by LC–MS analysis. For all samples a good agreement between data was observed with a calculated error for the bioassay of ranging from −33 to +25%. Moreover no false negative, nor false positive samples were detected by the proposed screening method. This study demonstrate that the application of biosensor based technology to real samples analysis needs a dedicated approach regarding sample preparation depending on the investigated matrix. Moreover, we have shown that the developed simple SPE procedure can quantitatively recover coumaphos from honey and that the extract is readily usable with both the electrochemical bioassay and a sophisticated instrumental techniques as LC–MS.

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