Detection of DDT and carbaryl pesticides in honey by means of immunosensors based on High Fundamental Frequency Quartz Crystal Microbalance (HFF-QCM)

Short running title:

Highly sensitive HFF-QCM immunosensors for DDT and carbaryl detection in honey

Lourdes Cervera-Chiner[a], Carmen March[b], Antonio Arnau[b,c], Yolanda Jiménez[b,c] and Ángel Montoya[b*]

[a] Instituto de Ingeniería de Alimentos para el Desarrollo. Universitat Politècnica de València. Camino de Vera s/n, 46022 Valencia, Spain.

[b] Centro de Investigación e Innovación en Bioingeniería. Universitat Politècnica de València. Camino de Vera s/n, 46022 Valencia, Spain.

[c] Advanced Wave Sensors SL. Paterna, Valencia, Spain.

* Corresponding author: amontoya@ci2b.upv.es

Abstract

BACKGROUND: In the last years, there is a concern about the presence of pesticides in honey since residues of DDT and carbaryl were found in honey samples. The traditional techniques, such as chromatography, reach the required limits of detection (LOD) but are not suitable for in-situ implementation in honey packaging industry due to their high cost and the

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jsfa.10267

need of highly qualified staff for routine operation. Biosensors offer simplicity, low cost and easy handling for analytical purposes in food applications.

RESULTS: Piezoelectric immunosensors based on High Fundamental Frequency Quartz Crystal Microbalance (HFF-QCM) have been developed for detection of carbaryl and DDT in honey. The biorecognition was based on competitive immunoassays in the conjugate-coated format, using monoclonal antibodies as specific immunoreagents. The assay LODs attained by the HFF-QCM immunosensors were 0.05 µg L^{-1} for carbaryl and 0.24 µg L^{-1} for DDT, thus reaching a similar detectability to that of the usual reference techniques. The practical LODs in honey samples were 8 µg kg^{-1} for carbaryl and 24 µg kg^{-1} for DDT. The immunosensors analytical performance allow the detection of these pesticides in honey at EU regulatory levels with good accuracy (recovery percentages ranging from 94 to 130% within the working range of each pesticide standard curve) and precision (coefficients of variation in the 9-36% range).

CONCLUSION: The proposed immunosensor is a promising analytical tool that could be implemented for quality control in the honey packaging industry, in order to ease and to cheapen the routine pesticide analysis in this appreciated natural food.

Keywords: Immunosensors; HFF-QCM; pesticides; carbaryl; DDT; honey

INTRODUCTION

The control of chemical residues in honey marketing is an essential requirement to ensure consumer safety. In the last years there is a concern about the presence of pesticides in honey

since residues of DDT and carbaryl were found in honey samples[1–3], with the subsequent potential health risk. The available equipment and techniques, such as high-performance liquid chromatography (HPLC), reach the required limits of detection (LOD)[4] but are not suitable for in-situ implementation in honey packaging industry due to their high cost and the need of highly qualified staff for routine operation. The use of biosensors for analytical purposes in food applications is increasing because of their simplicity, low cost and easy handling. In particular, piezoelectric immunosensors are becoming an interesting alternative to classical immunoassays for pesticide detection since they offer the advantages of real-time output, sensitivity, simplicity, and cost-effectiveness, without the requirement of reagent labeling[5]. We have recently developed piezoelectric immunosensors based on High Fundamental Frequency Quartz Crystal Microbalance (HFF-QCM) for the detection of pesticides[6,7] and disease biomarkers[8]. This powerful technology, which combines the high sensitivity of HFF-QCM transducers with the extreme selectivity provided by monoclonal antibodies involved in antigen-antibody interactions, is consolidating as a real option for the analytical control of chemical residues in food. As compared to traditional methods, HFF-QCM immunosensors offer lower cost, less sample and reagent consumption, label-free detection and direct, real time signal transduction.

The aim of the present work was the application of the HFF-QCM immunosensor technology to pesticide detection in honey. Two insecticides were chosen as model analytes due to the relevance of their presence in honey: the N-methylcarbamate carbaryl and the organochlorine DDT. The analytical performance of these immunosensors to reveal carbaryl and DDT residues in honey was assessed.

MATERIAL AND METHODS

Honey samples, reagents and immunoreagents

Two pesticide-free honey samples, as determined by Gas Chromatography-Mass Spectrometry (GC-MS) for DDT detection[1] and Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS) for carbaryl detection[9], were employed for analysis. A carbaryl-free thyme honey stock from the Spanish cooperative (Valencia, Spain) was used for carbaryl experiments. A DDT-free honey sample (Bee Natura, S.L.) from an ecological lavander grown in Sierra Calderona region (Valencia, Spain) was employed for DDT experiments.

Immunoreagents were high affinity anti-carbaryl and anti-DDT monoclonal antibodies (MAbs LIB-CNH45 and LIB-DDT5.25, respectively) used in combination with specific assay conjugates (BSA-CNH and BSA-DDT5, respectively). All of them had been previously produced as reported[10,11].

HFF-QCM immunosensor set up.

100 MHz AT-cut quartz crystals with a 1.00 mm diameter gold electrode were used as transducer elements for the immunosensor (AWSensors, Valencia, Spain). For the experiments, HFF-QCM sensors were placed into a flow-cell suitable for operation at high frequencies (AWSensors, Valencia, Spain). All assays were performed in the AWS A20 platform combined with the AWS F20 flow module (AWSensors) for real-time recording of the frequency and resistance produced as sensor responses during the assays. A uniform flow

of PBST (PBS: 10 mM phosphate-buffered saline solution, 0.9% NaCl, pH 7.4, with 0.005% Tween 20) was maintained through the sensor cell by an automated flow-through equipment provided with a degasser.

Sensor functionalization

Sensors were functionalized by covalent immobilization of the BSA-CNH or BSA-DDT5 assay conjugates onto the gold electrode surface, using mixed self-assembled monolayers (mSAM) of alkane-thiols and acids as intermediate layers for conjugate attachment, as described by Cervera-Chiner *et al.*[7]. The concentrations of the assay conjugates were previously optimized in order to get maximum assay signals ($S_{max} \geq 1000$ Hz) with minimum reagent consumption. They were 20 µg mL^{-1} of the BSA-CNH conjugate for carbaryl and 10 µg mL^{-1} of the BSA-DDT5 conjugate for DDT.

Immunoassay format and protocol

For each pesticide, a MAb-based competitive immunoassay was integrated as the sensing specific bio-recognition event coupled to the HFF-QCM transducer. To perform the competitive immunoassays, a fixed and limiting concentration of each specific monoclonal antibody was used.: 2.0 µg mL^{-1} of LIB-CNH45 MAb and 1.0 µg mL^{-1} LIB-DDT5.25 MAb for carbaryl and DDT assays, respectively. Monoclonal antibody preparations were mixed (1:1 v/v) with different concentrations of pesticide standard solution or with spiked honey samples. This way, the final MAb concentrations were 1.0 µg mL^{-1} in carbaryl immunoassays and 0.5 µg mL^{-1} in DDT immunoassays. The sample-antibody mixtures were pre-incubated

for 10 minutes at 25ºC, and 250 µL was pumped over the functionalized immunosensor. The running buffer was PBST at a flow rate of 20 µL min^{-1}. A competition was subsequenttly established between the analyte present in the sample and the immobilized conjugated hapten for binding to the limiting recognition sites provided by the antibody. Only the antibody molecules that remained free in the mixture was able to bind to the immobilized conjugate, thus decreasing the resonance frequency of the sensor. Immunosensor regeneration between assays was achieved by flowing 0.1 M HCl for 4 min at 250 µL min^{-1} throught the surface of the sensor, in order to break the antibody-conjugate binding, followed by 5 min with PBST at 250 µL min^{-1} and 5 min with PBST at 20 µL min^{-1}.

Standard calibration curves

Calibration curves for the determination of carbaryl and DDT were performed in honey with the respective HFF-QCM immunosensors by assaying different pesticide concentrations in the 10^{-4} – 10^{3} µg L^{-1} range. Standards were prepared from a 1 mM carbaryl stock solution in N-N´-dimetylformamide and from a 2.85 mM DDT stock solution in 1,4- dioxane. In order to minimize matrix effects, honey was diluted in PBS: 1/200 for carbaryl assays and 1/140 for DDT assays. Each standard concentration was measured in triplicate and calibration curves were subsequently obtained by plotting the normalized assay signal (frequency) *vs* analyte concentration.

Frequency signals were normalized as the percentage of the frequency shift provided by each standard concentration with respect to the maximum response in the absence of analyte

(maximum assay signal, S_{max}=100%). The experimental values were fitted to the usual four-parameter logistic equation as previously described[7].

Analysis of honey samples

Pesticide-free (as previously determined by LC-MS/MS) thyme honey samples were spiked with carbaryl at 0, 20, 50, 100, 200 and 500 µg kg^{-1}. Pesticide-free (as previously determined by GC-MS) lavender honey samples were spiked with DDT at 0, 20, 50, 100, 200, 500 and 1000 µg kg^{-1}. No sample pre-treatment other than PBS dilution (1/200 and 1/140 for carbaryl and DDT determinations, respectively) was required for pesticide analysis in honey samples. Assay signals produced by samples were interpolated in their respective standard curves performed in honey, and recovery and coeficient of variation were subsequently evaluated.

Moreover, six non-spiked honey samples from different origin (lavender, rosemary, eucalyptus, orange tree, oak tree and mixed-flower) and commercial source (supermarkets, honey cooperatives, etc) were analyzed by HFF-QCM in order to detect carbaryl and DDT.

RESULTS

Standard calibration curves in honey

Each complete cycle took around 35 minutes, including regeneration with 0.1 M HCl and washing with PBST. The S_{max} taken as the reference signal (≥ 1000 Hz ≡ 100%) was consistently reliable during 80-100 cycles. After that, a progressive S_{max} decrease was

observed and, although sensors were still usable, their sensitivity was lower probably due to the damage of the surface caused by the regeneration treatment with HCl. This phenomenon has also been reported in previous works using biosensors when many assay cycles were performed[12–14].

The normalized carbaryl and DDT calibration curves obtained in honey with the respective immunosensors are shown in Figure 1. They follow the decreasing sigmoidal shape typically associated to competitive immunoassays (ELISA and immunosensors). DDT assays showed less dispersion (standard deviations (SD) in the 0.3–5.0 % range) than carbaryl assays (SD in the 1–18 % range).

The analytical parameters obtained for the HFF-QCM immunosensors are summarized in Table 1. The I_{50} value is the analyte concentration that produces 50% inhibition of the maximum assay signal. This value is usually accepted as an estimate of the sensitivity in competitive immunoassays. In the present study, I_{50} values were 0.41 µg L^{-1} for carbaryl and 1.94 µg L^{-1} for DDT. In HFF-QCM immunosensors with competitive standard curves, the limits of detection (LOD) and quantification (LOQ) are defined as the pesticide concentrations that produce 10% and 20% inhibition of the maximum signal, respectively. In this case, the immunosensor LODs were 0.05 µg L^{-1} for carbaryl and 0.24 µg L^{-1} for DDT. The working range is defined as the region comprised between the analyte concentrations providing 20% and 80% inhibition of the maximum signal, which in a decreasing sigmoidal model roughly coincides with the linear portion of the calibration curve. For these immunosensors, the LOQs were 0.11 µg L^{-1} and 0.52 µg L^{-1} for carbaryl and DDT, respectively.

The detectability attained by the HFF-QCM pesticide immunosensors in standard assays is similar to that reported for other reference techniques. LODs for DDT obtained by GC-MS/MS were around 0.7 µg L^{-1} [15]. LODs reached by ELISA were 0.11 µg L^{-1} for DDT and 1.2 µg L^{-1} for carbaryl,[16], and SPR immunosensors reached LODs of 1.38 µg L^{-1} for carbaryl and 0.032 µg L^{-1} for DDT[14,17].

Taking into account the dilution factor of honey required to minimize matrix effects in the immunosensors (1/200 for carbaryl, 1/140 for DDT) and the average honey density (1.4 g mL^{-1}), the practical immunosensor LODs in honey were 8 µg kg^{-1} for carbaryl and 24 µg kg^{-1} for DDT. The respective LOQs were 16 and 52 µg kg^{-1}, and the assay working ranges were 16 – 206 µg kg^{-1} for carbaryl and 52 – 726 µg kg^{-1} for DDT. This results show that HFF-QCM immunosensors allow the determination of carbaryl and DDT in honey samples at the levels established by the EU (Maximum Residue Limit: MRL = 50 µg kg^{-1}).

Analysis of spiked and non-spiked honey samples

An example of a real record of the frequency signals obtained in honey analysis with the DDT immunosensor is shown in Figure 2. Arrows indicate the injection of honey samples and brackets indicate the regeneration cycles with 0.1 M HCl. The two first assays producing the reference maximum signal (maximum frequency decrease) correspond to the mixture of non-spiked honey with the anti-DDT MAb (1.0 µg mL^{-1} LIB-DDT5.25). The next three assays correspond to a honey sample spiked with 200 µg kg^{-1} DDT and mixed with 1.0 µg mL^{-1} of anti-DDT MAb. As it can be appreciated, a significant signal inhibition (less frequency decrease) was produced by the presence of the analyte. The last assay is again the reference

one (non-spiked, DDT-free honey sample) to verify the maintenance of the maximal assay signal.

Results obtained when carbaryl- and DDT-spiked honey samples were analyzed with the HFF-QCM immunonsensors are presented in Table 2. In samples spiked with carbaryl at concentrations within the carbaryl immunosensor working range (16 – 206 μg kg^{-1}), recoveries from 94 to 117% were obtained with coefficients of variation ranging from 20 to 33%. For the fortification level out of the working range (500 μg kg^{-1}), the recovered concentration was clearly underestimated (26% recovery with 21% coefficient of variation). This is caused by the extremely low slope of the asymptotic stretch of the calibration curve in this area, which produces a dramatic loss of assay sensitivity. Regarding honey samples fortified with DDT, recoveries from 98 to 130% were obtained for concentrations within the DDT immunosensor working range (52 – 726 μg kg^{-1}), with coefficients of variation below 20% with the exception of the 500 μg kg^{-1} fortification level (36% coeficient of variation). For concentrations out of the working range (1000 μg kg^{-1} DDT), similar behaviour to the carbaryl assay was observed, though in this case the recovered concentration was overestimated (260% recovery). In both immunosensors, analyte concentrations lower than the respective LODs were undetectable. Moreover, no false positives were found in non-spiked, pesticide-free honey samples.

Regarding the analysis of non-spiked real honey by means of the HFF-QCM imunosensor, neither DDT nor carbaryl were detected in any of the six measured samples.

CONCLUSION

Highly sensitive HFF-QCM immunosensors for the determination of carbaryl and DDT insecticides in honey were developed using functionalized 100 MHz quartz crystals as transducers and specific monoclonal antibodies as bio-recognition elements. The limits of detection and quantification and the assay working range of the immunosensors are in the same order of magnitude than those previously reported for reference techniques. When applied to the analysis of the target pesticides in honey samples, HFF-QCM immunosensors showed good accuracy and acceptable precision for immunochemical methods. In consequence, the immunosensor analytical performance allows the detection of the pesticides in honey at EU regulatory levels.

ACKNOWLEDGEMENTS:

This research was funded by the Spanish Ministry of Economy and Competitiveness with FEDER funds (AGL Project 2013-48646-R). L.C.C. was the recipient of a PhD fellowship from the European Social Fund and the Generalitat Valenciana (ACIF / 2016/132).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Blasco C, Fernández M, Pena A, Lino C, Silveira MI, Font G, *et al*., Assessment of Pesticide Residues in Honey Samples from Portugal and Spain. *J Agric Food Chem* **51**:8132-8138 (2003).

2. Rissato SR, Galhiane MS, De Almeida MV, Gerenutti M and Apon BM, Multiresidue determination of pesticides in honey samples by gas chromatography-mass spectrometry and application in environmental contamination. *Food Chem* **101**:1719–1726 (2007).

3. Ruiz-Toledo J, Vandame R, Castro-Chan RA, Penilla-Navarro RP, Gómez J and Sánchez D, Organochlorine pesticides in honey and pollen samples from managed colonies of the honey bee apis mellifera linnaeus and the stingless bee scaptotrigona mexicana guérin from southern, Mexico. *Insects* **9**:54 (2018).

4. Juan-Borrás M, Domenech E and Escriche I, Mixture-risk-assessment of pesticide residues in retail polyfloral honey. *Food Control* **67**:127-134 (2016).

5. Marrazza G, Piezoelectric biosensors for organophosphate and carbamate pesticides: A review. *Biosensors* **4**:301–317 (2014).

6. March C, García JV, Sánchez Á, Arnau A, Jiménez Y, García P, *et al*., High-frequency phase shift measurement greatly enhances the sensitivity of QCM immunosensors. *Biosens Bioelectron* **65**:1–8 (2015).

7. Cervera-Chiner L, Juan-Borrás M, March C, Arnau A, Escriche I, Montoya Á, *et al.*, High Fundamental Frequency Quartz Crystal Microbalance (HFF-QCM) immunosensor for pesticide detection in honey. *Food Control* **92**:1-6 (2018).

8. Montoya A, March C, Montagut YJ, Moreno MJ, Manclús JJ, Arnau A, *et al*., A High Fundamental Frequency (HFF)-based QCM Immunosensor for Tuberculosis Detection. *Curr Top Med Chem* **17**:1623-1630 (2017).

9. Venu S, Santhi K, Rawson A, Paranthaman R and Sureshkumar K, Liquid

Chromatography–Mass Spectrometry/Mass Spectrometry Method Development for the Determination of Carbaryl Residue in Honey. *Pharmacogn Mag* **15**:205-211 (2019).

10. Abad A, Primo J and Montoya A, Development of an Enzyme-Linked Immunosorbent Assay to Carbaryl. 1. Antibody Production from Several Haptens and Characterization in Different Immunoassay Formats. *J Agric Food Chem* **45**(4):1486–1494 (1997).
11. Abad A, Manclús JJ, Mojarrad F, Mercader J V, Miranda MA, Primo J, *et al.*, Hapten Synthesis and Production of Monoclonal Antibodies to DDT and Related Compounds. *J Agric Food Chem* **45**(9):3694–3702 (1997).
12. González-Martínez MA, Morais S, Puchades R, Maquieira A, Abad A and Montoya A, Monoclonal Antibody-Based Flow-Through Immunosensor for Analysis of Carbaryl. *Anal Chem* **69**:2812–2818 (1997).
13. March C, Manclús JJ, Jiménez Y, Arnau A and Montoya A, A piezoelectric immunosensor for the determination of pesticide residues and metabolites in fruit juices. *Talanta* **78**:827–833 (2009).
14. Mauriz E, Calle A, Manclús JJ, Montoya A, Hildebrandt A, Barceló D *et al*., Optical immunosensor for fast and sensitive detection of DDT and related compounds in river water samples. *Biosens Bioelectron* **22**:1410–1418 (2007).
15. Sapozhnikova Y, Simons T and Lehotay SJ, Evaluation of a fast and simple sample preparation method for polybrominated diphenyl ether (PBDE) flame retardants and dichlorodiphenyltrichloroethane (DDT) pesticides in fish for analysis by ELISA compared with GC-MS/MS. *J Agric Food Chem* **63**:4429–4434 (2015).
16. Sun J, Dong T, Zhang Y and Wang S, Development of enzyme linked immunoassay

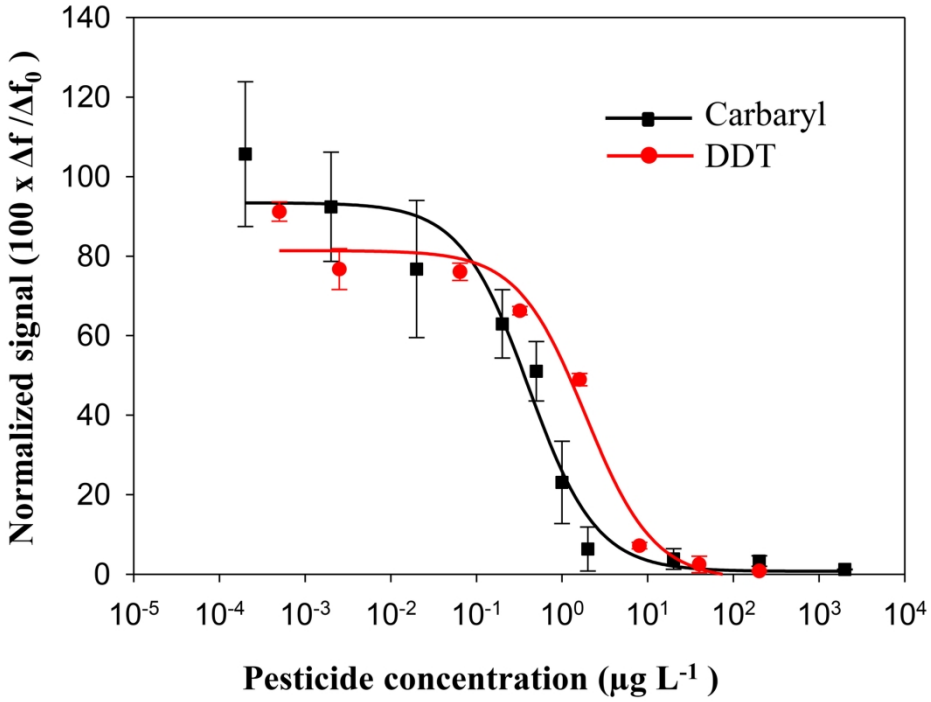
for the simultaneous detection of carbaryl and metolcarb in different agricultural products. *Anal Chim Acta* **666**:76–82 (2010).

17. Mauriz E, Calle A, Abad A, Montoya A, Hildebrandt A, Barceló D *et al*., Determination of carbaryl in natural water samples by a surface plasmon resonance flow-through immunosensor. *Biosens Bioelectron* **21**:2129–2136 (2006).

Figure captions

Figure 1. Calibration standard curves of the HFF-QCM immunosensors for carbaryl and DDT. Curves were performed in PBS-diluted honey: 1/200 for carbaryl and 1/140 for DDT. Each point is the mean ± SD of three replicates.

Figure 2. HFF-QCM immunosensor response: Two replicates of non-spiked honey (reference signal), followed by three replicates of honey spiked with 200 µg kg^{-1} DDT and a new reference, non-spiked honey sample. The blue line shows the frequency variation during the assays. Arrows indicate the injection of honey samples and the horizontal brackets (lower part) indicate the regeneration step.



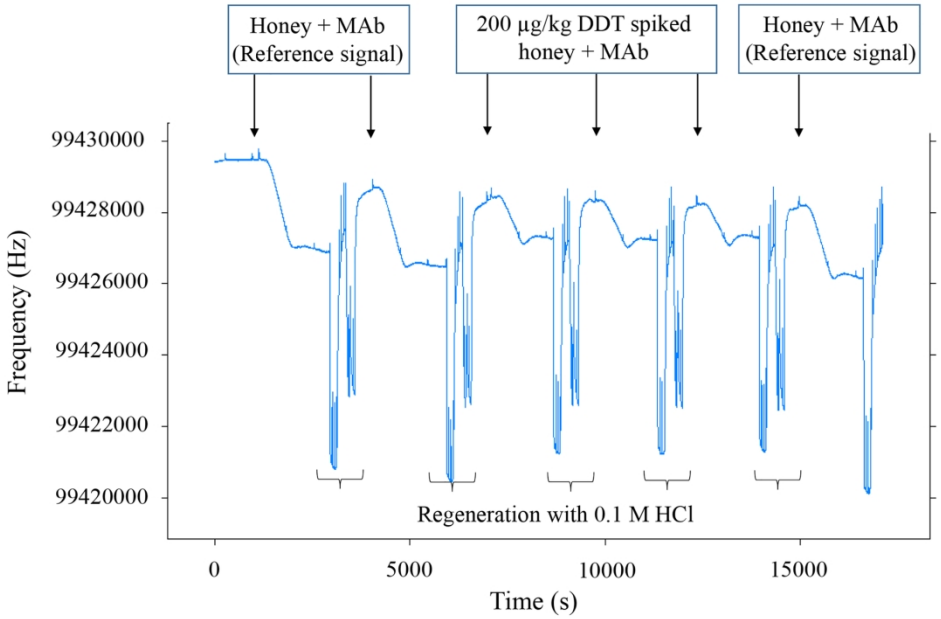


Table 1. Analytical parameters obtained for the HFF-QCM immunosensor of carbaryl and DDT in standard assays and in real honey samples.

Analytical parameters in assay (µg L^{-1})	**Carbaryl**	**DDT**
I_{50}	0.41	1.94
LOD	0.05	0.24
LOQ	0.11	0.52
Working range	0.11 – 1.44	0.52 – 7.26
Analytical parameters in honey (µg kg^{-1})	**Carbaryl**	**DDT**
LOD	8	24
LOQ	16	52
Working range	16 – 206	52 – 726

Table 2. Analysis of spiked honey samples (carbaril and DDT) by means of the HFF-QCM immunosensors.

Pesticide	Fortified level in honey (µg kg^{-1})	Dilution factor	Found* (µg kg^{-1})	CV (%)	Recovery (%)
Carbaryl	0	200	< LOD	-	-
	20	200	22 ± 7	32	110
	50	200	57 ± 19	33	115
	100	200	117 ± 29	25	117
	200	200	188 ± 37	20	94
	500	200	128 ± 27	21	26
DDT	0	140	< LOD	-	-
	20	140	< LOD	-	-
	50	140	50 ± 5	9	100
	100	140	101 ± 10	10	101
	200	140	197 ± 37	19	98
	500	140	648 ± 233	36	130
	1000	140	2603 ± 214	8	260

* Mean ± standard deviation of three replicates.