Cervera-Chiner, L.; Juan Borras, MDS.; March, C.; Arnau Vives, A.; Escriche Roberto, MI.; Montoya, Á.; Jiménez Jiménez, Y. (2018). High Fundamental Frequency Quartz Crystal Microbalance (HFF-QCM) immunosensor for pesticide detection in honey. Food Control. 92:1-6. doi:10.1016/j.foodcont.2018.04.026

Accepted Manuscript

High Fundamental Frequency Quartz Crystal Microbalance (HFF-QCM) Immunosensor for Pesticide Detection in Honey

Lourdes Cervera-Chiner, Marisol Juan-Borrás, Camen March, Antonio Arnau, Isabel Escriche, Ángel Montoya, Yolanda Jiménez

PII:	S0956-7135(18)30189-0
DOI:	10.1016/j.foodcont.2018.04.026
Reference:	JFCO 6088
To appear in:	*Food Control*
Received Date:	05 March 2018
Revised Date:	12 April 2018
Accepted Date:	13 April 2018

Please cite this article as: Lourdes Cervera-Chiner, Marisol Juan-Borrás, Camen March, Antonio Arnau, Isabel Escriche, Ángel Montoya, Yolanda Jiménez, High Fundamental Frequency Quartz Crystal Microbalance (HFF-QCM) Immunosensor for Pesticide Detection in Honey, *Food Control* (2018), doi: 10.1016/j.foodcont.2018.04.026

MANUSCRIPT TITLE:

High Fundamental Frequency Quartz Crystal Microbalance (HFF-QCM) Immunosensor for Pesticide Detection in Honey

AUTHORS and AFFILIATIONS

Lourdes Cervera-Chiner[a], Marisol Juan-Borrás[a], Camen March[b], Antonio Arnau[b], Isabel Escriche[a,c], Ángel Montoya[b*], Yolanda Jiménez[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 (Ci2B). Universitat Politècnica de València. Camino de Vera s/n, 46022, Valencia, Spain

[c] Food Technology Department (DTA), Universitat Politècnica de València, Camino de Vera s/n, 46022, Valencia, Spain

* Correspondence to: Ángel Montoya (amontoya@ci2b.upv.es)

High Fundamental Frequency Quartz Crystal Microbalance (HFF-QCM) Immunosensor for Pesticide Detection in Honey

Abstract

Quantification of chemical residues in honey is a market requirement to ensure consumer safety. The most common method used to analyze these compounds is the LC/MS/MS methodology, which requires highly qualified technicians and a tedious pre-treatment of the sample. The honey-packaging industry needs cheaper and faster alternatives for routine control. HFF-QCM (High Fundamental Frequency Quartz Crystal Microbalance) sensors are becoming a good option due to their high sensitivity, fast detection and low cost, while avoiding complex sample pre-treatment. The HFF-QCM technology is based on piezoelectric sensors with frequencies in the range from several tenths of MHz to hundreds of MHz. In this work a 100 MHz HFF-QCM sensor was used in a monoclonal antibody-based competitive immunoassay for specific bio-recognition of carbaryl pesticide as testing contaminant. The work intends to validate the use of HFF-QCM technology, in comparison with liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique, for the detection of contaminants in honey. For this purpose, the validation criteria required by SANCO 12571/2013 guidance document were considered. The precision and accuracy (recovery) of both methods were determined by comparison of 5 replicates at 4 different concentrations (from 0 to 100 µg/kg) using the same honey matrix. HFF-QCM technology showed good accuracy, with recovery percentages always between 110 and 120%. As regards to precision, HFF-QCM coefficients of variation (CV) were around 10% higher than those recommended by GC SANCO 12571/2013. HFF-QCM limits of detection

(LOD) and quantification (LOQ) were in the same order of magnitude as those for LC-MS/MS, which allows the analysis of carbaryl residues in honey under the established maximum residue limits (MRL), without sample pre-treatment. These results show that biosensors based on HFF-QCM technology has become a serious alternative to the traditional analytical techniques for food quality and safety applications.

Keywords

High-fundamental-frequency QCM; Piezoelectric immunosensors; Carbaryl; Pesticides; Honey

1.-Introduction

Despite honey is a highly appreciated natural food with numerous properties and benefits, lately it has gone through many and frequent food alerts and consequently its health attributes have been devalued. This is due to the extensive use of antibiotics and pesticides in veterinary and agricultural practices (Juan-Borrás, Domenech, & Escriche, 2016). In order to protect human health, these chemical hazards must be controlled to prevent pesticides reaching the food chain (Barganska, Slebioda, & Namiesnik, 2013). Quantification of chemical residues in honey imposed by specific regulation (EC regulation 396/2005) is a market requirement to ensure consumer safety. The most common analytical method used to quantify these residues is the liquid chromatography-tandem mass spectrometry (LC-MS/MS) methodology (Juan-Borrás et al., 2016; Masiá, Suarez-Varela, Llopis-Gonzalez, & Picó, 2016; Souza Tette, Guidi, De Abreu Glória, & Fernandes, 2016). Although chromatographic methodology has key advantages such as sensitivity and

accuracy, its high cost prevents its routine use in local laboratories. LC-MS/MS requires tedious sample pretreatment, which makes it unsuitable for on-line analysis. Moreover, it requires highly qualified technicians and high volumes of toxic solvent (Souza Tette et al., 2016). ELISA (enzyme-linked immunosorbent assay) is another frequently used method. It is based on antibody-antigen recognition, which offers high specificity and sensitivity, as well as cost-effectiveness (Abad & Montoya, 1997; González-Martínez et al., 1997; Marco, Gee, Cheng, Liang, & Hammock, 1993; Nunes, Toscano, & Barceló, 1998; Qian et al., 2009). However, ELISA often requires long incubation periods and repeated washing steps that make difficult their automation for on-line sample analysis (Mauriz, García-Fernández, & Lechuga, 2016). Routine control of pesticides in honey is carried out by the honey-packaging industry, which needs simpler, cheaper and faster screening methods than those currently used, while preserving their high sensitivity.

In this regard, piezoelectric immunosensors based on HFF-QCM (High Fundamental Frequency Quartz Crystal Microbalance) transducers are becoming a good alternative to on-line screening methods in food control due to their high sensitivity and specificity, fast real time detection and low cost, while avoiding complex sample pre-treatment. Disadvantages of the method are its high sensitivity to external disturbances such as pressure or temperature (Gaudin, 2017) and its low throughput. The latter is a key remaining challenge for QCM to be competitive with other immunological methods such as ELISA (Tatsuma, Watanabe, Oyama, Kitakizaki, & Haba, 1999). HFF-QCM immunosensor technology is based on the combination of highly specific antigen-antibody recognition with highly sensitive HFF-QCM transducers

(March et al., 2015; Montoya et al., 2017).The transducer converts the bio-recognition events, which take place near its surface due to the presence of the pesticide in the sample, into a measurable electrical signal (electrical phase). This way, the concentration of the pesticide in the sample can be quantified by monitoring transducer phase shifts, (March et al., 2015; Montagut et al., 2011). Usually, a second electrical parameter related to dissipation is monitored to ensure that other events different from bio-recognition, such as changes in viscosity or elasticity, do not significantly contribute to the sensor response (Jiménez *et al*., 2006). This technology provides highly sensitive devices, able to improve about one order of magnitude the limits of detection (LOD) for pesticides such as carbaryl provided by optical transducers based on Surface Plasmon Resonance (García et al., 2014; March et al., 2015). Moreover, the carbaryl LOD achieved by HFF-QCM was in the same order of magnitude as that for ELISA. Therefore, HFF-QCM could be considered a suitable and reliable technique for the analysis of contaminants in complex matrices such as honey.

The aim of the present work was the application of the HFF-QCM technology, for the first time, to the detection of pesticides in honey. For this purpose, the N-methylcarbamate pesticide carbaryl was used as a model analyte, and the validation criteria required by GC SANCO 12571/2013 (European Commission, 2013) were followed. The analytical performance of the proposed method in terms of LOD, limit of quantification (LOQ), accuracy and precision, was compared to LC-MS/MS as reference technique.

2. Material and methods

99 *2.1 .Honey samples, reagents and immunoreagents*

100 A carbaryl-free honey stock from the Spanish cooperative Melazahar (Valencia,
101 Spain) was used as a “blank honey” and was fortified with the pesticide when
102 required.

103 The reagents used for immobilization were: thiol compounds 11-mercapto-1-
104 undecanol 97% (MUOH) and 16-mercaptohexadecanoic acid 90% (MHDA)
105 from Sigma-Aldrich Chemie (Steinheim, Germany); 1-ethyl-3-(-3-dimethyl-
106 amino-propyl) carbodiimide hydrochloride (EDC) and n-hydroxysuccinimide
107 (NHS) from Pierce (Rockford, IL), and ethanolamine blocking agent from Sigma
108 (St Louis, Mo). Immunoreagents [BSA-CNH protein-hapten conjugate used as
109 assay conjugate and LIB-CNH45 monoclonal antibody (MAb)] were produced
110 as described (Abad, Primo, & Montoya, 1997). Tween 20 surfactant was
111 acquired from Fluka-Aldrich Chemie (St Louis, Mo).

112 Reagents used for LC-MS/MS were: formic acid (99%), acetonitrile and
113 methanol, all of them from Prolabo (Fontenay-sous-Bois, France). The
114 composition of Quechers reagents was; Quechers I: 4 g of anhydrous
115 magnesium sulfate, 1 g of sodium citrate tribasic dihidrate, 0,5 g of sodium
116 citrate dibasic sesquihidrate, the three from Sigma Aldrich (Steinheim,
117 Germany) and 1 g of sodium chloride from Prolabo (Fontenay-sous-Bois,
118 France). Quechers II: 100 mg of bonded silica (PSA) from Supelco (Bellefonte,
119 USA) and 600 mg of anhydrous magnesium sulfate. All reagents were MS,
120 HPLC or analytical grade.

The carbaryl standard, was purchased from Dr. Ehrenstorfer (Augsburg, Germany). Ultrapure water was generated in-house from a Milli-Q 82 system (Millipore Corp., Billerica, MA).

2.2. HFF-QCM methodology

2.2.1. HFF-QCM immunosensor set-up

Piezoelectric sensors were supplied by AWSensors (Valencia, Spain, www.awsensors.com). They were square shaped 100 MHz AT-cut, inverted mesa crystals, with 36 mm^2 of total surface and with an etched area thickness of approximately 17 μm. The gold electrode active surface was 1 mm in diameter. The resonators were permanently fixed to a support of polyether ether ketone (PEEK) with a conical hole to expose the active surface of the gold electrode where interfacial events such as immunoassays will take place.

For the experiments, HFF-QCM sensors were placed into a flow-cell, designed and manufactured by AWSensors, taking into account the mechanical, electrical and chemical application requirements.

AWS A20 platform (AWSensors) was used for real-time characterization of the sensor response through the experiments performed in flow conditions. This platform consists of an electronic characterization system based on the fixed-frequency phase-shift measurement technique previously described (Montagut et al., 2011). The platform provides two electrical voltages directly related with the sensor phase and amplitude (u_Φ and u_A). The AWS F20 platform (AWSensors) was used to generate a uniform flow through the sensor cell. This platform consists of an automated flow-through equipment controlled by syringe pumps (Hamilton, Bonaduz, GR, Switzerland) and thermostatized at 25 °C.

Sample injection was performed by an injection valve and a 250 µl loop. A degasser DEGASi® Compact from Biotech (Onsala, Sweden) was connected to the AWS F20 platform to prevent bubbles generation. The AWSuite software interface (AWSensors) was used to control both platforms and to register and process the acquired data.

2.2.2. Sensor functionalization

Covalent immobilization of HFF-QCM sensors was performed employing mixed self-assembled monolayers (mSAM) of alkane thiols as intermediate layers for surface functionalization. This allowed the covalent attachment of the assay conjugate onto the gold electrode surface in a more orderly and stable way than with simple SAMs. With this aim, freshly cleaned crystals were placed in especially made immobilization cells (AWSensors). These cells were designed to expose only the active area of the sensors to functionalization reagents. The immobilization protocol was based on that previously described by March *et al.* (2015) with minor modifications: a) 250 µL of 0.25 mM solution of thiol compounds MUOH and MHDA in ethanol (50:1 molar ratio) was added to the immobilization cell cavity where the sensor active surface was confined; b) 250 µL ethanolic solution of EDC/NHS was incubated for 3.5 h; and c) 50 µL of BSA-CNH assay conjugate (20 µg/mL) diluted in 0.1 M sodium phosphate buffer, pH 7.5 was placed onto the gold electrode active surface for 2.5 h, instead of 5 h.

2.2.3. Immunoassay format and protocol

The working conditions for carbaryl immunoassays were defined in the previous work by March *et al*. (2015). An indirect competitive immunoassay in the

conjugate-coated format was employed. For the inhibition assays, a fixed concentration of 2 µg/mL of LIB-CNH45 MAb was mixed (1:1 v/v) with the carbaryl standard solution or with the spiked honey samples. The mixture was pre-incubated for 1 h at 25 °C and 250 µL was pumped over the previously functionalized immunosensor surface. As the binding between free antibody and the immobilized conjugate took place, the variations in phase and amplitude were monitored in real time. The regeneration of the reactive surface was carried out with 0.1 M HCl to break the antibody-hapten conjugate binding.

Standard curve. Carbaryl calibration curves were performed in buffer solution (PBS: 10 mM phosphate-buffered saline solution, 0.9% NaCl, pH 7.4.) and in honey diluted with PBS (1:200). From a 1 mM carbaryl stock solution in N-N'-dimetylformamide, carbaryl standards in the $2 \cdot 10^{3}$ to $2 \cdot 10^{-4}$ µg/mL range were prepared by serial dilutions in PBS and in honey diluted with PBS. Standards were run four times, and calibration curves were subsequently obtained by plotting the phase shift vs analyte concentration. The experimental points were fitted to the four-parameters logistic equation:

$$y = D + {(A - D)}/{\left(1 + \left({x}/{C}\right)^{B}\right)} \quad (1)$$

Where: *x* is the analyte concentration; *y* is the HFF-QCM signal (phase variation at the fixed fundamental frequency Δu_{ϕ}). *A* is the asymptotic maximum (maximum signal in the absence of analyte); *B* is the slope of the sigmoidal curve at the inflection point; C is the analyte concentration giving 50% inhibition (I_{50} value) and *D* is the asymptotic minimum (background signal).

Standard curves were normalized by expressing the phase shift provided by each standard concentration as the percentage of the maximum response (maximum signal, S_{max}=100%) in the absence of analyte.

Immunoassay protocol. A 20 µL/min continuous flow rate of working buffer (PBST: PBS containing 0.005% Tween 20) was pumped through the sensor. When a nearly constant baseline was reached (signal variation less than 1 mV/min), the sample (250 µL of the pre-incubated antigen-antibody mixture) was injected. The interaction process was allowed to proceed for 20 min. Sensor regeneration was achieved by flowing 0.1 M HCL (4 min) followed by PBST (5 min), at 250 µL/min in both cases. Finally, the flow was returned to 20 µL/min in order to recover the baseline.

2.2.4. Honey sample preparation

The only requirement to make honey suitable for HFF-QCM immunosensor analysis was a 1/200 dilution in PBS. No other sample pre-treatment was needed.

2.3. Chromatographic methodology

2.3.1. Analytical standards

From a 1000 mg/L stock solution of carbaryl in methanol, stored at -20°C, a 1 mg/L working solution was prepared and stored at 4 °C. The working solution was used to obtain the carbaryl standards (from 0.5 to 100 µg/L in methanol) for the calibration curve, and also to prepare the spiked honey samples at 20, 50 and 100 µg/kg.

2.3.2. *LC-MS/MS procedure*

Analytical determinations by the LC–MS/MS reference method were performed with an Agilent 1200 LC system coupled to a triple quadrupole mass spectrometer (Agilent 6410 triple Quad LC/MS) with electrospray ionization source. The column used was Atlantis T3-C18 (100 mm x 2.1 mm, 3 µm particle size), from Waters (Mildford, Massachusetts), kept at 30°C. Chromatographic separation was carried out with a mobile phase composed by 0.5% formic acid (phase A) and methanol (phase *B*), with a flow rate of 0.3 mL/min. The elution program used was as follows: 5% *B* at 0 min and held for 0.3 min, increased to 20% *B* at 0.5 min, reaching 100% *B* at 6 min, where it was held during 2 min, then the percentage of *B* was decreased to 5% over 8.1 min where it was held for 5 min (13 min total run time). The injection volume was 5 µL. The operating parameters for the mass spectrometer were as follows: capillary voltage 4 kV; source temperature 350°C; nebulization gas (nitrogen) at a flow rate of 12 L/min and collision gas (nitrogen) at a 40 psi.

The monitored transitions (MRM) were 202>145.1 (qualitative information) and 202>117.1 (quantitative) with a collision energy of 5 and 10 respectively, setting the fragmentor to 80. The confirmation of the compounds in the samples was made taking into account: a) the analyte retention time, b) the presence of both transitions, and c) the ratio of both transitions.

2.3.3. Honey sample preparation

To extract the pesticide and to remove impurities from honey samples, a dispersive solid phase extraction technique following the QuEchERS procedure was performed. The protocol applied was: To 5 g of honey placed in a conical centrifuge tube, 10 mL of Milli-Q water was added and it was shaken manually until homogenization. Then, 10 mL of 0.1% formic acid in acetonitrile was added

and shaken for 5 min. Subsequently, Quechers I was added and the mixture was shaken manually for 1 min and in a vortex for a further 1 min. The extract was centrifuged at 5000 rpm for 5 min. The obtained supernatant (4 mL) was transferred to a 15 mL centrifuge tube containing Quechers II. This mixture was vortexed for 30 s and centrifuged again at 5000 rpm for 5 min. An aliquot of the supernatant was collected for subsequent LC-MS/MS analysis.

2.4. Method comparison

The comparison between HFF-QCM and LC-MS/MS methods was performed in accordance with SANCO 12571/2013 guidance document. The analytical performance of the methods was assessed in terms of precision (reproducibility and repeatability), accuracy (% recovery), limit of detection (LOD), limit of quantification (LOQ) and working range. The maximum residue limit (MRL) established by the European Food Safety Authority (EFSA) for carbaryl in honey is 50 µg/kg (Commission Regulation 1096/2014).

In analytical methods for compounds with established LMRs, it is recommended that the LOQ should be as low as (or even lower than) the MRL, i.e., the MRL should be included in the operative working range. For this reason, 20 µg/kg was included as a fortification level.

3. Results and discussion

3.1. Standard calibration curves: Immunoassay sensitivity and matrix effect.

An immunoassay cycle performed in the HFF-QCM immunosensor is shown in Fig. 1. After baseline stabilization sample was injected and changes in both phase (u_Φ) and amplitude (u_A) were produced due to the specific binding of the MAb to the assay conjugate immobilized on the sensor surface. As shown, the

phase voltage shift was evident and significant enough, whereas the amplitude voltage shift was negligible. Therefore, the phase voltage shift (Δu_Φ) was selected as the suitable immunosensor signal to quantify the antibody-antigen interaction. After sensor regeneration, the baseline returned to its initial level.

Determination of pesticide residues in honey is a challenge, due not only to their very low concentrations, but also to the interferences of the complex matrix on the analysis. (Souza Tette, Guidi, De Abreu Glória, & Fernandes, 2016). To assess the possible interference of the honey composition on HFF-QCM measurements and to minimize matrix effects (Caldow et al., 2005), the calibration curve was performed both in PBS and in PBS-diluted honey (1/200 p/V).

Both calibration curves were performed with carbaryl standards ranging between $2 \cdot 10^{-4}$ and $2 \cdot 10^{3}$ µg/L. The results are shown in Fig. 2. Signals (phase voltage shifts) were normalized by expressing them as 100 x $\Delta u_\Phi / \Delta u_{\Phi 0}$, where Δu_Φ is the phase change produced by a given carbaryl concentration and $\Delta u_{\Phi 0}$ is the phase change obtained at zero analyte concentration (maximum signal).

The experimental values were fitted to the mathematical logistic function according to Eq. (1). As shown in Fig. 2, standard curves had a typical decreasing sigmoidal shape, as expected for competitive immunoassays (Osterloh et al., 1989). Since a competition was established between the immobilized assay conjugate and the pesticide in the sample for binding to the limited free MAb, lower analyte concentrations produced the higher assay signals, whereas higher analyte concentrations provided the smaller ones. In both sigmoidal regressions, the *D* parameter (lower asymptote) was nearly zero, thus indicating a total inhibition at high carbaryl concentrations.

Furthermore, no statistical differences were found for parameters *A*, *B* and *C* in both curves according to the t-test ($p<0.05$).

The analytical parameters obtained for the standard curves in PBS and in diluted honey are shown in Table 1. The I_{50} value is the analyte concentration that produces the signal at the midpoint between the two asymptotes, and it is considered as an estimate of assay sensitivity. LOD and LOQ are concepts used to quantify the smallest concentration of the analyte that can be reliably detected and measured, respectively, by an analytical method. In HFF-QCM immunosensors, LOD and LOQ are defined as the pesticide concentrations that produce 10% and 20% of maximum signal inhibition, respectively. Finally, the working range is the linear portion of the calibration curve and is defined by the analyte concentrations providing 20% and 80% inhibition of the maximum signal.

As shown, the I_{50} values were quite similar in PBS and in diluted honey (the calculated I_{50} value in 1/200-diluted honey was in fact lower than that in PBS). Therefore, the dilution process applied to honey seemed to be effective to minimize matrix effects without reducing the assay sensitivity.

As for I_{50} value, LOD and LOQ seemed to improve (lower values) in diluted honey as compared to those obtained in PBS. Nevertheless, the differences observed were not statistically significant, so they were probably due to the assay variability. Likewise, the assay working range was very similar in both conditions, although a tendency to expand was found in diluted honey. This would probably facilitate the analysis of real honey samples.

In summary, the developed HFF-QCM immunosensor for carbaryl in 1/200 diluted honey showed a LOD of 0.035 µg/L and a LOQ of 0.083 µg/L in the

assay, corresponding to 7 and 17 µg/L, respectively, in undiluted honey. The LOD and LOQ of the LC-MS/MS method used as reference, calculated according to reference (Litte, 2015), were in the same order of magnitude (2 and 10 µg/L, respectively). Therefore, both methods were comparable in terms of detectability.

3.2. Analysis of spiked honey samples: comparison with the LC-MS/MS method

A preliminary validation of the HFF-QCM immunosensor method was conducted in accordance with SANCO 12571/2013 guidelines. Five independent replicates of commercial honey were spiked at three different carbaryl concentrations, and the pesticide was subsequently analyzed with the HFF-QCM immunosensor. The honey stock used as blank matrix for fortification had a density of 1.4 g/mL. Therefore, potential honey matrix effects or interferences could be due to the high sample density, together with its high viscosity and sugar content. Accuracy was evaluated as the mean recovery percentage and precision was estimated from the coefficient of variation at each pesticide concentration.

Fig. 3 shows a real screen record of the phase signals obtained for the consecutive injections of five honey samples spiked with 50 µg/kg of carbaryl. As a reference, an initial injection of a non-spiked honey sample was run to show the maximum assay signal. The injection of honey samples and the regeneration reagent are indicated by continuous and dashed arrows, respectively. The measurement repetitiveness and the baseline stability can be observed.

The reference method for carbaryl determination specified by the Food and Agriculture Organization of the United Nations (FAO) and by the World Health Organization (WHO) is reverse phase high performance liquid chromatography

using UV detection and external standardization (FAO specifications and evaluations for carbaryl, 2006). Nevertheless, in this work LC-MS/MS was used for comparison with HFF-QCM because of its higher sensitivity (Debayle, Dessalces, & Grenier-Loustalot, 2008).

In Table 2 the results obtained with HFF-QCM and LC-MS/MS methods for carbaryl-spiked honey samples are compared in terms of accuracy and precision. Honey samples were fortified in the 20-100 µg/kg range and blank honey (zero carbaryl level) was included to account for false positives. As shown, HFF-QCM technology showed good accuracy, with recovery percentages ranging from 110 to 120%. No false positives were detected in non-spiked honey,

To compare the accuracy of the HFF-QCM immunosensor and the LC-MS/MS method, their respective results when applied to honey samples spiked with the mentioned carbaryl concentrations were correlated with the fortification levels (Fig. 4). Both methods provided good linear regressions, with correlation coefficients of 0.999 and 0.992 for HFF-QCM and LC-MS/MS, respectively. *Y* intercept was near zero for both models, which is in agreement with the absence of false positives. The linear regression slopes were 1.14 for HFF-QCM and 1.02 for LC-MS/MS, without any statistically significant difference according to the t-test. Therefore, we could assume that both techniques are statistically equivalent.

As regards to precision, at any of the assayed fortification levels HFF-QCM technology gave coefficients of variation (CV) around 10% higher than those established by SANCO. To this respect, dispersion has been pointed out in the literature (Gaudin, 2017) as a drawback of traditional QCM sensors working at

low frequencies (5-10 MHz). Piezoelectric transducers are very sensitive to external disturbances such as thermal variations or pressure changes (evidenced as bubbles). In our experiments, the temperature was continuously controlled and kept at 25ºC by AWS A20 and F20 platforms. Moreover, an external degasser was incorporated to the set-up to prevent bubbles on the sensor surface. As previously reported (Johannsmann, 2015), measurements made on the fundamental mode of the sensor have greater dispersion than those made at its third overtone, being energy trapping and electric fringe fields putative sources of this behavior. This possibility will be checked in future work by using 50 MHz fundamental frequency sensors working at its third harmonic.

Other possible sources of dispersion could be the variability in the manual process of sensor functionalization when measurements are made with different sensors, or differences in surface regeneration when measurements are performed with the same sensor.

Conclusions

To our knowledge, this is the first report dealing with pesticide detection in honey using the HFF-QCM technology. The developed HFF-QCM immunosensor is able to determine carbaryl in honey with a limit of quantification 17 µg/L without any sample pre-treatment. Only a 1/200 sample dilution is required to minimize matrix effects. Therefore, this method allows the analysis of carbaryl residues in honey down to the levels established by the current European legislation (MRL= 50 µg/kg, (Commission Regulation 1096/2014).

A preliminary validation of the immunosensor method was conducted in accordance with SANCO 12571/2013 guidelines. The HFF-QCM immunosensor

has proved to be accurate enough, with recovery percentages between 110 and 120% and the absence of false positives. As regards to precision, coefficients of variation ranged from 25 to 33%, not reaching the high standards recommended by SANCO 12571/2013 criteria. Further work with the aim of improving the method precision is going on, including the use of 50 MHz fundamental frequency sensors working at its third harmonic.

Immunosensors based on HFF-QCM technology could be a reliable alternative to current techniques for pesticide quantification in honey, since they are able to reach the limits of detection and quantification offered by traditional chromatographic methods such as LC-MS/MS, without the need of sample pre-treatment.

Acknowledgments

The authors are grateful for financial support from the Ministry of Economy and Competitiveness of Spain under the project AGL2013-48646-R. We also want to thank "Generalitat Valenciana" for the financial support to hire research personnel through the grant “Ayudas para la contratación de personal investigador en formación de carácter predoctoral” to carry out this research (DOC Num.7615/15.09.2015).

References

Abad, A., & Montoya, A. (1997). Development of an Enzyme-Linked Immunosorbent Assay to Carbaryl. 2. Assay Optimization and Application to the Analysis of Water Samples. *Journal of Agricultural and Food Chemistry*, *45*(4), 1495–1501. https://doi.org/10.1021/jf950691w

Abad, A., Primo, J., & Montoya, A. (1997). Development of an Enzyme-Linked

412 Immunosorbent Assay to Carbaryl. 1. Antibody Production from Several
413 Haptens and Characterization in Different Immunoassay Formats. *Journal*
414 *of Agricultural and Food Chemistry*, *45*(4), 1486–1494.
415 https://doi.org/10.1021/jf9506904

416 Barganska, Z., Slebioda, M., & Namiesnik, J. (2013). Pesticide residues levels
417 in honey from apiaries located of Northern Poland. *Food Control*, *31*, 196-
418 201.

419 Caldow, M., Stead, S. L., Day, J., Sharman, M., Situ, C., & Elliott, C. (2005).
420 Development and Validation of an Optical SPR Biosensor Assay for Tylosin
421 Residues in Honey Development and Validation of an Optical SPR
422 Biosensor Assay for Tylosin Residues in Honey. *Work*, 7367–7370.
423 https://doi.org/10.1021/jf050725s

424 Commission Regulation (EU) No 1096/2014 of 15 October 2014 amending
425 Annexes II, III and V to Regulation (EC) No 396/2005 of the European
426 Parliament and of the Council as regards maximum residue levels for
427 carbaryl, procymidone and profenofos in or on certain products Text with
428 EEA relevance

429 Debayle, D., Dessalces, G., & Grenier-Loustalot, M. F. (2008). Multi-residue
430 analysis of traces of pesticides and antibiotics in honey by HPLC-MS-MS.
431 *Analytical and Bioanalytical Chemistry*, *391*(3), 1011–1020.
432 https://doi.org/10.1007/s00216-008-2003-2

433 European Commission 396/2005. EU legislation on MRLs.

434 European Commission. (2013). Guidance document on analytical quality control

435 and validation procedures for pesticide residues analysis in food and feed.
436 *Sanco/12571/2013*, 1–46.

437 FAO specifications and evaluation for Carbaril (2006).
438 http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticid
439 es/Specs/Carbaryl07.pdf

440 García, J. V., Rocha, M. I., March, C., García, P., Francis, L. A., Montoya, A., …
441 Jimenez, Y. (2014). Love Mode surface acoustic wave and high
442 fundamental frequency quartz crystal microbalance immunosensors for the
443 detection of carbaryl pesticide. *Procedia Engineering*, *87*, 759–762.
444 https://doi.org/10.1016/j.proeng.2014.11.649

445 Gaudin, V. (2017). Advances in biosensor development for the screening of
446 antibiotic residues in food products of animal origin – A comprehensive
447 review. *Biosensors and Bioelectronics*, *90*(September 2016), 363–377.
448 https://doi.org/10.1016/j.bios.2016.12.005

449 González-Martínez, M. A., Morais, S., Puchades, R., Maquieira, A., Abad, A., &
450 Montoya, A. (1997). Development of an automated controlled-pore glass
451 flow-through immunosensor for carbaryl. *Analytica Chimica Acta*, *347*(1–2),
452 199–205. https://doi.org/10.1016/S0003-2670(97)00003-2

453 Jiménez, Y., Otero,M.&Arnau, A. (2008) Piezoelectric transducers and
454 aplications. Ed. Springer Velag.ISBN:978-3-540-77507-2

455 Johannsmann, D. (2015) The Quartz Crystal Microbalance in Soft Matter
456 Research. Fundamentals and Modeling .Ed:Springer .ISBN: 978-3-319-
457 07836-6

458 Juan-Borrás, M., Domenech, E., & Escriche, I. (2016). Mixture-risk-assessment
459 of pesticide residues in retail polyfloral honey. *Food Control*, *67*, 127–134.
460 https://doi.org/10.1016/j.foodcont.2016.02.051

461 Little, T. (2015). Method Validation Essentials, Limit of Blank, Limit of Detection,
462 and Limit of Quantitation. *BioPharm International*, 28 (4).

463 March, C., García, J. V., Sánchez, Á., Arnau, A., Jiménez, Y., García, P., …
464 Montoya, Á. (2015). High-frequency phase shift measurement greatly
465 enhances the sensitivity of QCM immunosensors. *Biosensors and*
466 *Bioelectronics*, *65*, 1–8. https://doi.org/10.1016/j.bios.2014.10.001

467 Marco, M. P., Gee, S. J., Cheng, H. M., Liang, Z. Y., & Hammock, B. D. (1993).
468 Development of an Enzyme-Linked Immunosorbent Assay for Carbaryl.
469 *Journal of Agricultural and Food Chemistry*, *41*(3), 423–430.
470 https://doi.org/10.1021/jf00027a015

471 Masiá, A., Suarez-Varela, M. M., Llopis-Gonzalez, A., & Picó, Y. (2016).
472 Determination of pesticides and veterinary drug residues in food by liquid
473 chromatography-mass spectrometry: A review. *Analytica Chimica Acta*,
474 *936*, 40–61. https://doi.org/10.1016/j.aca.2016.07.023

475 Mauriz, E., García-Fernández, M. C., & Lechuga, L. M. (2016). Towards the
476 design of universal immunosurfaces for SPR-based assays: A review.
477 *TrAC - Trends in Analytical Chemistry*, *79*, 191–198.
478 https://doi.org/10.1016/j.trac.2016.02.006

479 Montagut, Y., García, J. V., Jiménez, Y., March, C., Montoya, Á., & Arnau, A.
480 (2011). Validation of a phase-mass characterization concept and interface

for acoustic biosensors. *Sensors*, *11*(5), 4702–4720. https://doi.org/10.3390/s110504702

Montoya, A., March, C., Montagut, Y., Moreno, M., Manclus, J., Arnau, A., … Torres, R. (2017). A High Fundamental Frequency (HFF)-based QCM Immunosensor for Tuberculosis Detection. *Current Topics in Medicinal Chemistry*, *17*(14), 1623–1630. https://doi.org/10.2174/1568026617666161104105210

Nunes, G. S., Toscano, I. A., & Barceló, D. (1998). Analysis of pesticides in food and environmental samples by enzyme-linked immunosorbent assays. *TrAC Trends in Analytical Chemistry*, *17*(2), 79–87. https://doi.org/10.1016/S0165-9936(97)00116-7

Osterloh K.R.S., Smith G.D., Peters T.J. (1989) Interpolation in Assay Systems with a Sigmoid Response Curve. In: Pal S.B. (eds) Reviews on Immunoassay Technology. Palgrave Macmillan, London.

Qian, G., Wang, L., Wu, Y., Zhang, Q., Sun, Q., Liu, Y., & Liu, F. (2009). A monoclonal antibody-based sensitive enzyme-linked immunosorbent assay (ELISA) for the analysis of the organophosphorous pesticides chlorpyrifos-methyl in real samples. *Food Chemistry*, *117*(2), 364–370. https://doi.org/10.1016/j.foodchem.2009.03.097

Souza Tette, P. A., Guidi, L. R., De Abreu Glória, M. B., & Fernandes, C. (2016). Pesticides in honey: A review on chromatographic analytical methods. *Talanta*, *149*, 124–141. https://doi.org/10.1016/j.talanta.2015.11.045

504 Tatsuma, T., Watanabe, Y., Oyama, N., Kitakizaki, K., & Haba, M. (1999).
505 Multichannel quartz crystal microbalance. *Analytical Chemistry*, *71*(17),
506 3632–3636. https://doi.org/10.1021/ac9904260

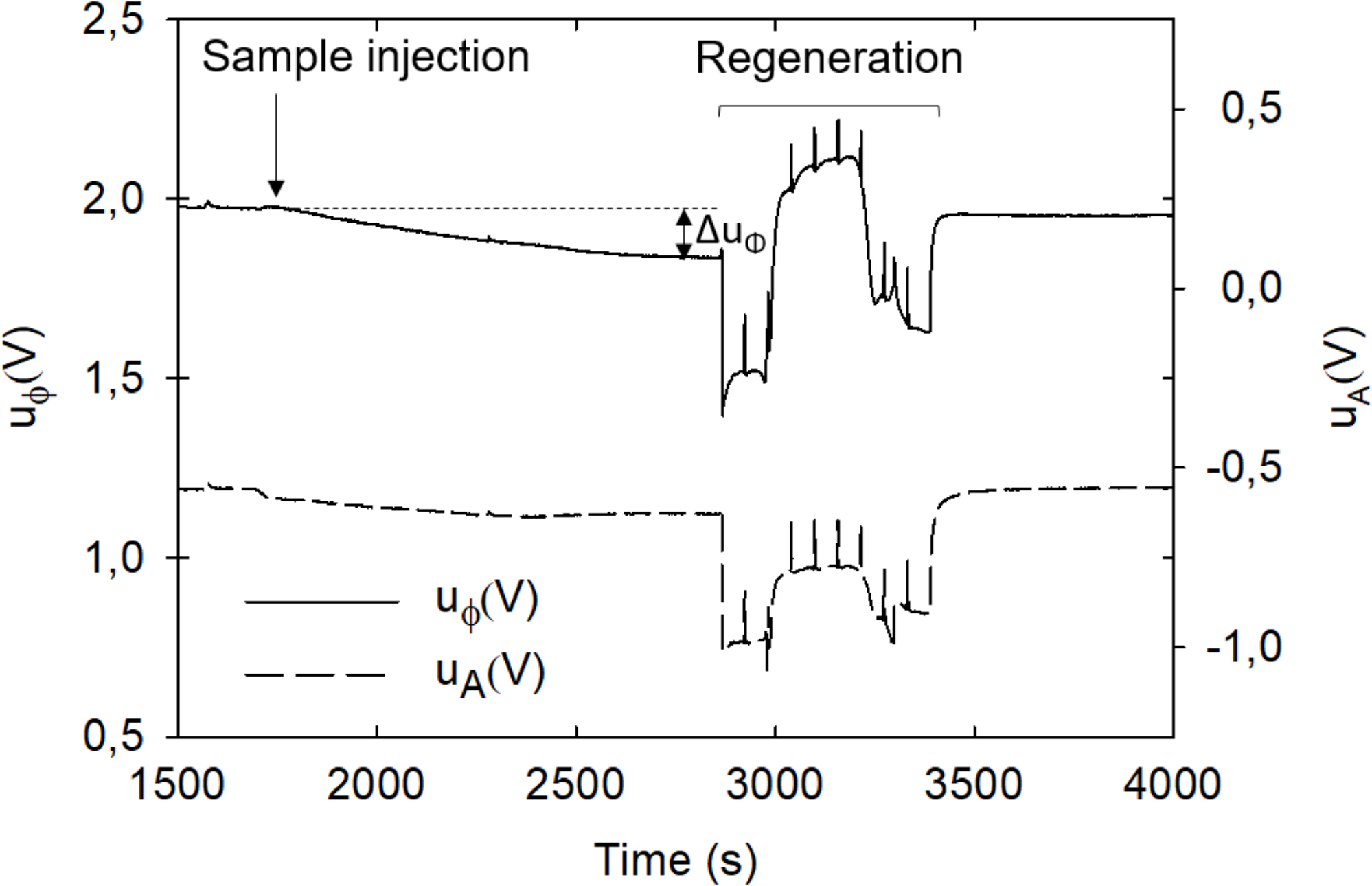
507 **Figure captions**

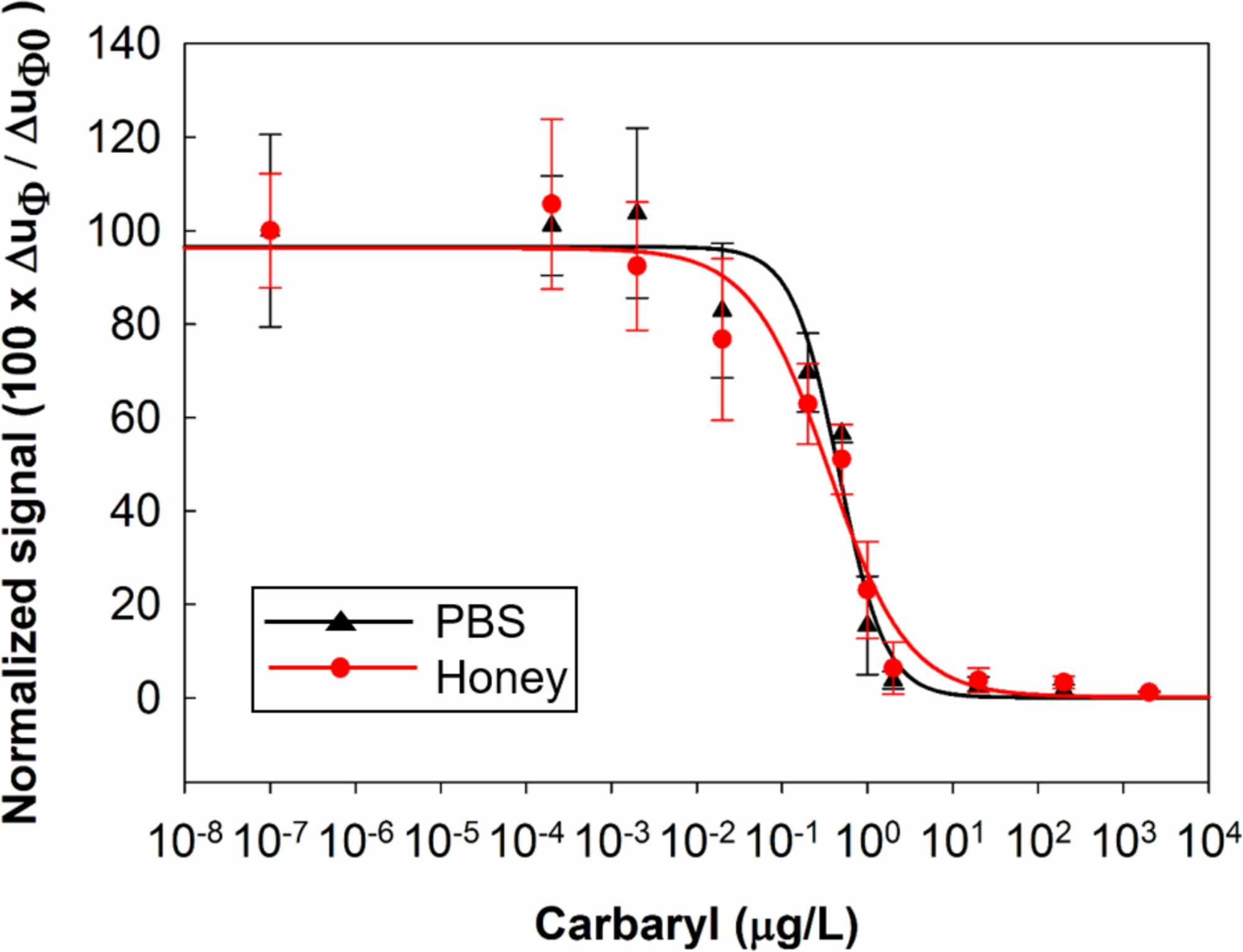
508 **Figure 1**.HFF-QCM Immunoassay cycle.

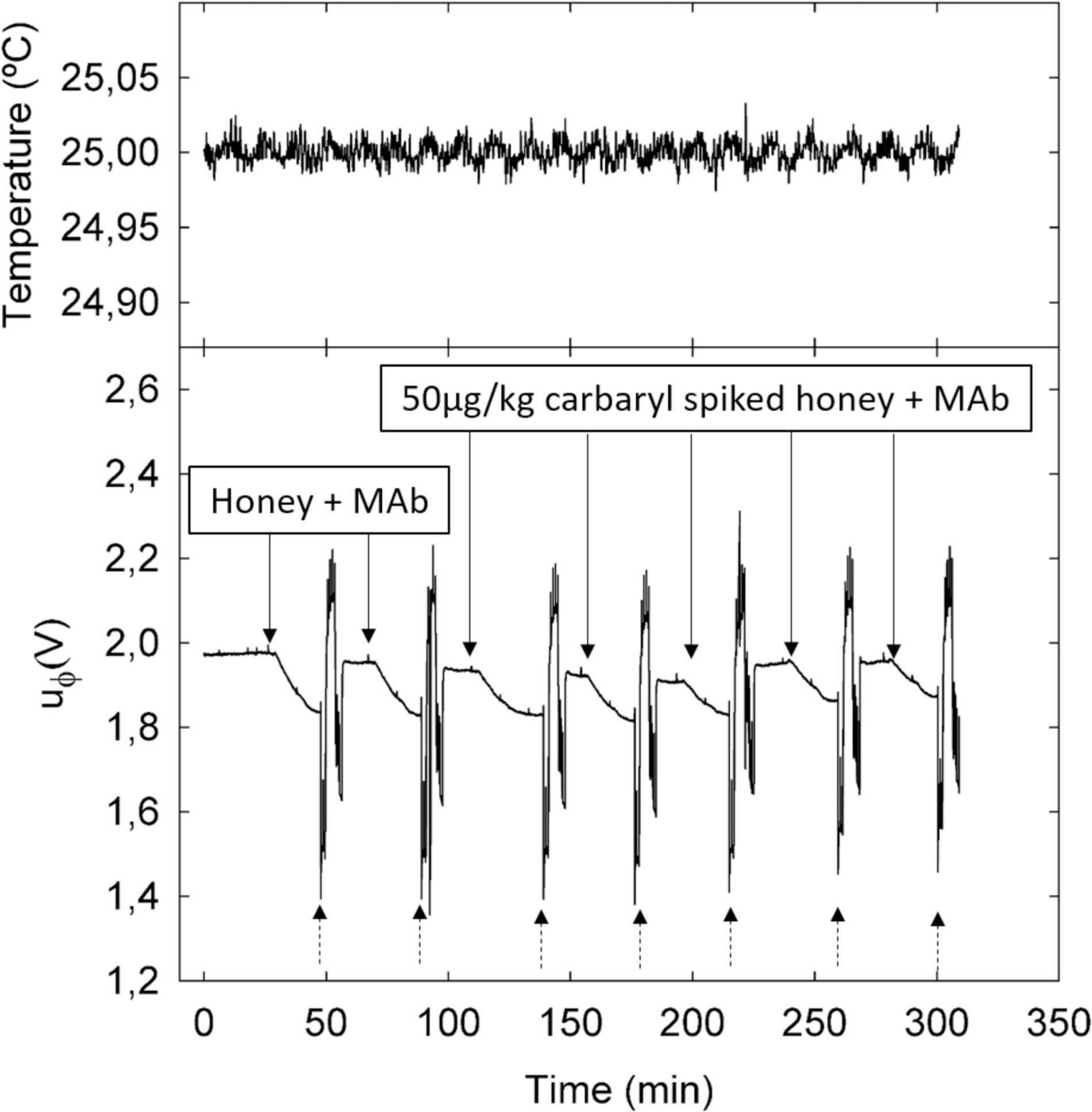
509 **Figure 2**. HFF-QCM carbaryl calibration curves in PBS and in honey diluted
510 1/200 in PBS. Each point is the average of 4 determinations. Vertical bars
511 represent standard deviation.

512 **Figure 3**. HFF-QCM immunosensor response of five independent honey
513 samples spiked with carbaryl at 50 µg/kg. Continuous and dashed arrows mark
514 the injection of honey samples and regeneration steps, respectively. The upper
515 panel shows the temperature during the assays.

516 **Figure 4**. Comparison of HFF-QCM and LC-MS/MS in the analysis of fortified
517 honey samples.


Sample injection
Regeneration
Δu_Φ
u_ϕ(V)
u_A(V)
u_ϕ(V)
u_A(V)
2,5
2,0
1,5
1,0
0,5
0,5
0,0
-0,5
-1,0
1500
2000
2500
3000
3500
4000
Time (s)


Normalized signal (100 x ΔuΦ / ΔuΦ0)
140
120
100
80
60
40
20
0
PBS
Honey
10-8 10-7 10-6 10-5 10-4 10-3 10-2 10-1 100 101 102 103 104
Carbaryl (μg/L)


Temperature (ºC)
25,05
25,00
24,95
24,90
50µg/kg carbaryl spiked honey + MAb
Honey + MAb
u_ϕ(V)
2,6
2,4
2,2
2,0
1,8
1,6
1,4
1,2
0
50
100
150
200
250
300
350
Time (min)

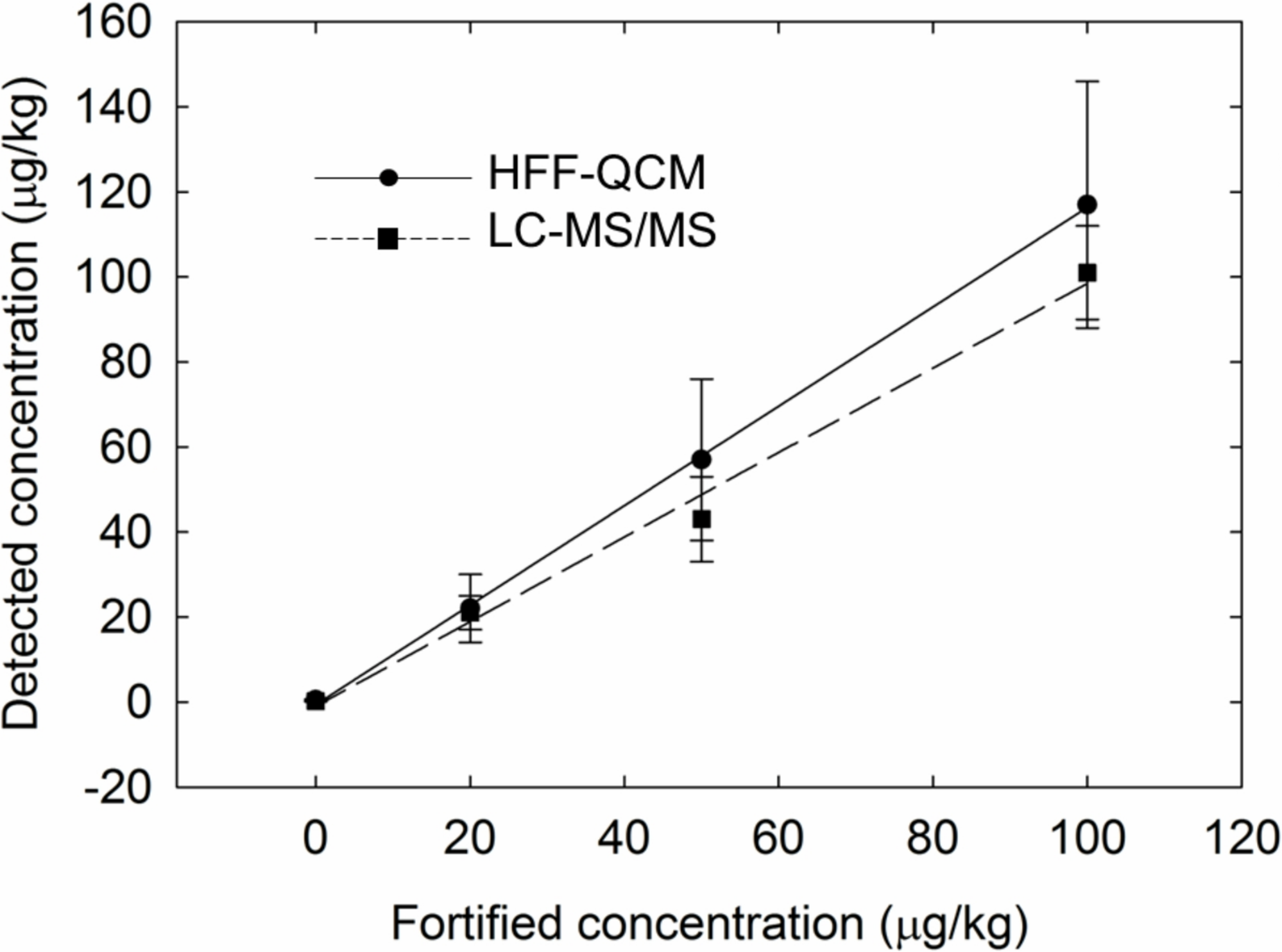

HFF-QCM
LC-MS/MS
Detected concentration (μg/kg)
Fortified concentration (μg/kg)
160
140
120
100
80
60
40
20
0
-20
0
20
40
60
80
100
120

Table 1. Analytical parameters obtained for the HFF-QCM standard curves of carbaryl in PBS and in 1/200 diluted honey.

Analytical parameter (µg/L)	PBS	1/200 diluted honey
I_{50}	0.465	0.360
LOD	0.118	0.035
LOQ	0.195	0.083
Working range	0.195 - 1.108	0.083 - 1.572

Table 2. Analysis of carbaryl spiked honey samples. Comparison of HFF-QCM technology with LC-MS/MS.

Analysis of carbaryl in spiked honey samples*						
Fortified level (µg/kg)	**Detected (µg/kg)**	**Recovery (%)**	**CV (%)**	**Detected (µg/kg)**	**Recovery (%)**	**CV (%)**
	HFF-QCM[1]			**LC-MS/MS[2]**		
0	<LOD	No false positives		<LOD		
20	22 ± 7	110	32	21 ± 4	106	20
50	57 ± 19	115	33	43 ± 10	85	23
100	117 ± 29	117	25	101 ± 11	101	11

*Average of 5 independent replicates. All of the replicates for HFF-QCM and LC-MS/MS analysis came from the same original honey sample.
[1] Sample dilution factor 1/200
[2]Sample dilution factor 1/2