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Immunosensor for detection of sulfathiazole in honey**

	Credit Author Statement
Lourdes Cervera-Chiner	Investigation; Formal analysis; Writing - Original Draft;
Yolanda Jiménez	Funding acquisition; Project administration; Conceptualization, supervision, Writing - Review & Editing
Ángel Montoya	Project administration, supervision, Writing - Review & Editing
Marisol Juan-Borrás	Validation; Formal analysis
Nuria Pascual	Resources
Antonio Arnau	Conceptualization
Isabel Escriche	Project administration; supervision, Writing - Review & Editing

High Fundamental Frequency Quartz Crystal Microbalance (HFF-QCMD) Immunosensor for detection of sulfathiazole in honey

Lourdes Cervera-Chiner ^a, Yolanda Jiménez ^b, Ángel Montoya ^b, Marisol Juan-Borrás ^{a,d}, Nuria Pascual ^c, Antonio Arnau ^b, Isabel Escriche ^{a,d,*}

^a Instituto de Ingeniería de Alimentos para el Desarrollo (IIAD), 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 Nb4D group, Instituto de Química Avanzada de Cataluña (IQAC-CSIC), CIBER-BBN, Barcelona, Spain

^d Food Technology Department (DTA), Universitat Politècnica de València, Camino de Vera s/n, 46022, Valencia, Spain

*Corresponding author: Isabel Escriche (iescrich@tal.upv.es)

Abstract

In this study, a piezoelectric immunosensor based on High Fundamental Frequency Quartz Crystal Microbalance (HFF-QCMD) technology was developed for detection of sulfathiazole in honey. The biorecognition was based on a competitive immunoassay in the conjugate-coated format, using monoclonal antibodies as specific immunoreagents. The quantification of sulfathiazole was performed by building the corresponding calibration standard curve in diluted honey (1/140). Due to the competitive nature of the immunoassay, the standard curve showed a sigmoidal pattern with limits of detection (LOD) and quantification (LOQ) of 0.10 µg/kg and 2 µg/kg honey, respectively. The

LOD reached by this immunosensor is 40-50 times lower than those reported by other techniques for antibiotic detection. Moreover, this method requires minimum honey pre-treatment, making it faster and simpler than other methods. This immunosensor meets the precision and accuracy requirements established by SANCO guidelines, when sulfathiazole concentration in honey is not lower than 10 µg/kg. These findings could be the basis for reaching enough reliability for lower concentrations. Therefore, HFF-QCMD immunosensors can be considered a feasible alternative to current techniques for rapid and highly sensitive determination of sulfathiazole in honey with minimum sample preparation.

Keywords: Immunosensor; HFF-QCMD; LC-MS/MS; antibiotic; sulfathiazole; honey

1. Introduction

The presence of antimicrobial agents in foodstuff is currently considered a serious public health problem since their residues can cause allergies, alterations in the intestinal microbiota and even the development of multibacteria resistance (ECDC/EFSA/EMA 2015; WHO, 2018). However, the use of these chemicals is sometimes a necessary and widespread practice for the treatment of infectious pathologies in different types of livestock such as the honeybees in apiculture production. The European Union, in order to ensure food safety, has established Maximum Residue Limits (MRLs) for different pharmacologically active substances in foods of animal origin (European Commission, 2010). However, referring to honey, limits have been set only for coumaphos (100 mg/kg) and amitraz (200 mg/kg); both are pesticide compounds applied to control the highly dangerous *Varroa destructor* mite, the most damaging enemy of honeybee colonies. This European Commission regulation includes only the list of approved active substances; therefore, no other substance is allowed in beekeeping. However, sometimes beekeepers improperly use prohibited

substances to prevent the death of bees when they are affected by other diseases. Among them, of special significance are the American and European Foulbrood (AFB and EFB) diseases, caused by *Paenibacillus larvae* and *Melissococcus plutonius*. When these bacteria affect colonies beekeepers should mandatorily burn the beehive, but this is not always occurring and by applying bad apicultural practices, they use different antibiotics for this purpose. Hence, the need to control the presence of these compounds, mainly sulfathiazole since this is the most commonly used. For this reason, this sulphonamide is on the list of compounds that are routinely evaluated in the quality control of honey during commercialization and packaging.

Liquid chromatography-Mass spectrometry tandem (LC-MS/MS) is the most applied technique for this purpose due to its high sensitivity and selectivity. However, it is an expensive analytical method, with a long and multistage sample preparation that makes the analysis time-consuming, labor-intensive, and unsuitable for routinely monitoring sulphonamide levels (Guillén, Guardiola, Almela, Núñez-Delicado, & Gabaldón, 2017; Juan-Borrás, Periche, Domenech, & Escriche, 2015; Louppis, Kontominas, & Papastephanou, 2017).

The honey packaging industry needs on-line and low-cost screening methods as an alternative to overcome the drawbacks of traditional chromatographic techniques. One of the first and most extensively used screening method in food control analysis has been ELISA. Its main strengths are its high sensitivity, together with its ability to analyze several food samples simultaneously with a relatively low cost (Ricci, Volpe, Micheli, & Palleschi, 2007). However, its main drawbacks concern the use of molecular labels, which can compromise the bio-chemical activity (Gaudin, 2017; Hawkins, Cooper, & Campbell, 2006), and its difficulty of automation, which prevents its use for on-line analysis (Mauriz et al., 2006).

Immunosensors have emerged as feasible candidates to overcome the identified weaknesses of ELISA. The more extensively used for antibiotic control in food have been those based on Surface Plasmon Resonance (SPR) and Quartz Crystal Microbalance with dissipation (QCMD) technologies (Gaudin, 2017). Both immunosensors are direct label-free and real-time techniques, so they can easily and quickly provide the characteristics of binding reactions involved in the bio-recognition. Low-frequency (5-10 MHz) QCMD immunosensors, have successfully been used for detection in food safety applications, such as pesticides in fruit juices (March, Manclús, Jiménez, Arnau, & Montoya, 2009), toxins in red wine (Karczmarczyk, Haupt, & Feller, 2017), hormones in milk (Ito et al., 2017) and antibiotics, such as chloramphenicol in milk, meat, egg and honey (Karaseva & Ermolaeva, 2012). In all these cases, the sensitivity was around 1-2 orders of magnitude above the MRLs, when established (Regulation (EC) N° 396/2005; Regulation (EC) N° 470/2009; Regulation (EC) N° 37/2010).

The lack of sensitivity of low-frequency QCMD (its main drawback) has been overcome by High-Fundamental-Frequency QCMD technology (HFF-QCMD) (50–150 MHz). The reduced size of HFF-QCMD sensors and the simplicity in the instrumentation needed to characterize them are other important advantages of this technology, in comparison with the previously mentioned techniques such as SPR (Janshoff, Galla, & Steinem, 2000). This provides a cost-effective solution that will enable the simultaneous detection of several samples in a single analysis by integrating tens of these sensors, thus, saving time, and minimizing sample consumption (Deng, Chen, Wang, & Wei, 2018; Tao et al., 2016; Vaughan et al., 2018). The advantages of HFF-QCMD technology such as screening method have been tested in previous studies (March et al., 2015) and specifically in the detection of pesticides in honey (Cervera-

Chiner et al., 2018). Bearing in mind that this technology has not yet been used to identify sulfathiazole in foodstuff, the aim of this work was the development of a suitable HFF-QCMD immunosensor for the detection of this sulfonamide in honey. The analytical performance of this method will be compared with LC-MS/MS as reference technique, in terms of precision, accuracy, limit of detection (LOD), limit of quantification (LOQ) and working range.

2. Material and methods

2.1. Honey samples, reagents and immunoreagents

A mixture of 5 polyfloral honey samples (supplied by “Cooperativa Melazahar”, Valencia, Spain) without sulphonamides (checked by chromatographic analysis, Juan-Borrás et al., 2015) was used as a “blank honey” for spiking with sulfathiazole (Sigma Aldrich, Steinheim, Germany) when required. In addition, 6 more polyfloral honey samples (purchased from different supermarkets and honey cooperatives) were used to verify the reliability of the HFF-QCMD technology for detecting sulfathiazole. These last honey samples were kept frozen until analysis to minimize losses of the target compound.

The reagents used for sensor immobilization were: thiol compounds 11-mercapto-1-undecanol 97% (MUOH) and 16-mercaptohexadecanoic acid 90% (MHDA) (Sigma-Aldrich Chemie, Steinheim, Germany); 1-ethyl-3-(3-dimethyl-amino-propyl) carbodiimide hydrochloride (EDC) and n-hydroxysuccinimide (NHS) (Pierce, Rockford, IL, USA), and ethanolamine blocking agent (Sigma, St Louis, Mo, USA). The immunoreagents (Custom Antibody Service, U2-ICTS-NANBIOSIS; Nb4D group-IQAC-CSIC/CIBER-BBN, Barcelona, Spain) were the following: SA2-BSA AE1 B28 protein-hapten conjugate (used as assay conjugate) and purified monoclonal antibody against sulfonamide 6C11 batch 8678. Tween 20 surfactant was acquired from Fluka-

Aldrich Chemie (St Louis, Mo, USA). The buffer used as mobile phase in HFF-QCMD experiments was PBST (PBS: 10 mM phosphate-buffered saline solution, 0.9% NaCl, pH 7.4, with 0.005% Tween 20). Ultrapure water was produced in-house using a Milli-Q 82 system (Millipore Corp., Billerica, MA, USA).

2.2. HFF-QCM methodology

2.2.1. HFF-QCM immunosensor set-up

100 MHz HFF-QCMD sensors were supplied by AWSensors (AWSensors, Valencia, Spain, www.awsensors.com). The sensors consist of a 66 μm -thick AT-cut quartz 6×6 mm-square wafer with a 17 μm -thick and 2.92 mm-diameter circular double-sided etched region in the center. The etched region is sandwiched between two concentric 1 mm-diameter circular electrodes with a 67 nm-thick gold layer on a 5 nm Cr-adhesion layer. To make the handling of the sensors easier, they are assembled on a Polyether Ether Ketone (PEEK) support. AWS flow-through cell housing was used as crystal holder for in-liquid measurements (AWSensors). The cell creates a chamber of around 2.75 μL over the sensor.

The AWS A20 platform (AWSensors) was used for real-time characterization of the sensor response during the experiments carried out in flow conditions. This platform records variations in the resonance frequency, Δf , and energy dissipation, ΔD . Measurement of Δf provides information about the antibiotic concentration in the sample, while ΔD monitoring serves to ensure that other events different from bio-recognition, such as changes in stiffness or viscosity in the sample, do not significantly contribute to the sensor response (Jiménez, Otero, & Arnau, 2009).

The AWS F20 platform (AWSensors) was used to generate a uniform flow through the sensor cell. Moreover, a degasser DEGASi® Compact from Biotech (Onsala, Sweden) was connected to the AWS F20 platform for preventing bubbles. Sample injection was

carried out by an injection valve and a 250 μ L loop. Both platforms allow carrying out thermostated experiments at 25 °C. They are controlled by means of the software interface AWS Suit 2.5.0 version (AWSensors), which also allows registering and processing the acquired data.

2.2.2. Sensor functionalization and detection format

An indirect competitive immunoassay in the conjugate-coated format was applied. Mixed self-assembled monolayers (mSAM) of alkane thiols were used as intermediate layers for covalent immobilization of the hapten conjugate to the sensor surface. The mSAMs allow more orderly and stable distribution of the hapten conjugate molecules on the sensor surface than simple SAMs. Previously to the mSAM formation, the sensors were rinsed with bidistilled water and ethanol, dried with nitrogen gas, exposed for 15 minutes to UV/Ozone ProCleaner from BioForce Nanosciences (Utah, USA), rinsed again with ethanol and dried with nitrogen gas. The cleaning and immobilization processes were carried out in a cell especially made for immobilization (AWSensors) as described in detail by Cervera-Chiner et al., 2018.

2.2.3. Immunoassay protocol and standard curves

For the determination of the optimal concentrations of immunoreagents to perform the competitive immunoassays of sulfathiazole, several concentrations of SA2-BSA conjugate from 5 to 50 μ g/mL were first immobilized on the HFF-QCM sensor surface and tested in combination with different concentrations (1 and 2 μ g/mL) of monoclonal antibody against sulfonamide (6C11 MAb).

Competitive sulfathiazole immunoassays were carried out by mixing (1:1 v/v) a fixed concentration of 6C11 MAb (2 μ g/mL) with sulfathiazole standard solution or with the spiked honey samples. The mixture was pre-incubated for 10 minutes at 25 °C and, subsequently, 250 μ L were pumped over the previously functionalized sensor surface.

The AWS A20 platform recorded Δf and ΔD in real time as the binding between free antibody and the immobilized conjugate took place. Once the assay reached the equilibrium, the surface sensor was regenerated by pumping 0.1 M HCl on the sensor surface, this breaks the antibody-hapten conjugate binding and makes the sensor ready for the next assay. The assay time, including sensor surface regeneration, was around 30 min. For further details of the immunoassay protocol performed, see the work reported by Cervera-Chiner et al., 2018.

Two standard sulfathiazole calibration curves were performed, one in PBS and the other in honey diluted with PBS (1/140 w/v). A 24 mg/mL stock solution of sulfathiazole was prepared in 0.5 M sodium hydroxide. From this stock, a working solution of 240 mg/L was obtained in bidistilled water. From this solution, sulfathiazole standards in the 2×10^3 to 2×10^{-4} $\mu\text{g/mL}$ range were prepared by serial dilutions in PBS and in honey diluted with PBS (see section 2.2.4 below). The calibration curves were performed running the sulfathiazole standards in quadruplicate. The frequency shifts generated by the binding (Δf) were acquired and, subsequently, processed to be expressed as a percentage of the maximum signal frequency shift ($\Delta f_{\max}$) registered in the absence of the analyte. Finally, these normalized frequency signals were plotted vs each standard concentration and fitted to the four-parameters logistic equation shown below:

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

Where x is the analyte concentration, y is the acquired normalized frequency ($\Delta f \times 100 / \Delta f_{\max}$), A is the asymptotic maximum (maximum signal registered without 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).

2.2.4. Honey sample preparation for HFF-QCMD

To study honey matrix effects, different honey dilutions in PBS: 1/25, 1/50, 1/100, 1/140, 1/150 (w/v) were tested. Each dilution was mixed with 2 $\mu\text{g/mL}$ of MAb and injected on the sensor. The measured values for Δf and ΔD were compared with those provided by the sensor when a mixture of PBS with the same amount of MAb was injected. Dilution factors below 1/140 provided dissipation shifts (ΔD) higher than those obtained with PBS, thus confirming the effect of the viscoelastic properties of honey on the sensor response (matrix effect) for those dilutions. Matrix effect minimization was achieved only for 1/140 and 1/150 dilutions, which provided Δf and ΔD values similar to those obtained with PBS. Between them, 1/140 dilution was selected since higher dilution factors reduce the target concentration, thus impairing its detection.

To evaluate the accuracy and precision of the developed immunosensors, spiked honey samples were prepared by adding the working solution of sulfathiazole (1 mg/L) to the “blank honey” in order to obtain the suitable levels (0.5, 2, 10, 50, 100 and 1000 $\mu\text{g/kg}$).

2.3. LC-MS/MS methodology

A 1000 mg/L stock solution of sulfathiazole was prepared in methanol. Then, a 1 mg/L working solution was carried out in bidistilled water from the stock solution. Both solutions were stored at 4°C. The working solution was used to obtain the sulfathiazole standards (from 0.02 to 2000 $\mu\text{g/L}$ in bidistilled water). The calibration curves were built in solvent and matrix honey at this range of concentrations.

The fortified honey samples were prepared in the same way as for HFF-QCMD. A solid phase extraction (SPE) performed with Strata X-CW cartridges (33 μm polymeric strong cation 100 mg/3mL, Phenomenex, California, USA) was applied to extract the antibiotic and to remove impurities from honey samples. In each case, a 1 g of honey was weighed, spiked at the corresponding level and subjected to acid hydrolysis (2 M HCl, 1 mL) and then left for 30 min at room temperature. Then, 5 mL of 0.3 M citric

acid solution were added and mixed. The cartridges were conditioned with 3 mL of methanol and were rinsed with 3 mL of bidistilled water. Then, the samples were passed through the cartridge. Subsequently, the cartridges were rinsed twice with 3 mL of bidistilled water, followed by two rinses with 3 mL of methanol/acetonitrile solution (50:50, v/v) and were allowed to dry for 2 min. Then, the extract was eluted with 3 mL of 2% ammonium hydroxide/methanol solution. The eluates were evaporated until completely dry under stream of nitrogen while being maintained at 40°C in a thermostatic bath (Grant GR, Cambridge, England). Finally, 100 µL of bidistilled water: acetonitrile (95:5) was added and mixed with a vortex to re-dissolve the extract and transferred into a LC-MS/MS vial for subsequent analysis. The chromatography procedure was carried out following as described by Juan-Borrás et al., 2015.

2.4. Methodology comparison

In order to evaluate the analytical performance of the proposed new methodology (HFF-QCMD), five independent replicates of blank honey were spiked at seven levels: 0, 0.5, 2, 10, 50, 100 and 1000 µg/kg, and analyzed with both methodologies (HFF-QCMD and LC-MS/MS). The measurements performed with HFF-QCMD technology were compared with LC-MS/MS as reference method for sulfathiazole determination. Both methodologies were applied in accordance with SANCO 12571/2013 guidance document in terms of precision (reproducibility and repeatability), accuracy (% recovery), limit of detection (LOD), limit of quantification (LOQ) and working range.

Since there is no maximum residue limit (MRL) established by the European Food Safety Authority (ECDC/EFSA/EMA, 2015) for antibiotics in honey, the current requirement is the absence of antibiotics. Thus, the maximum limit of antibiotic residues in honey should be established based on the detection limit of the technique used (Maudens, Zhang, & Lambert, 2004).

3. Results and discussion

3.1. Immunoassay development: selection of the optimal monoclonal antibody concentration

The sensitivity and the LOD of an immunosensor are strongly dependent on antibody and immobilized conjugate concentrations (Chauhan et al., 2015). The optimal combination of the immobilized conjugate and monoclonal antibody concentrations was selected to obtain a commitment to ensure a good signal-to-noise ratio for the highest analyte concentrations in the competitive assay, with the lowest immunoreagent consumption. Previous works developed by the research group showed that values of $\Delta f_{\max}$ parameter (see section 2.2.3) of at least 1 kHz are enough to achieve the signal to noise criterion (Fernández-Benavides et al., 2019; March et al., 2015). The values of Δf signals provided by several combinations of immunoreagent concentrations are summarized in Table 1. As expected, higher signals were observed as MAb and conjugate concentrations increased. The optimal concentrations selected to accomplish the above described tradeoff were 5 $\mu\text{g/mL}$ of SA2-BSA conjugate with 2 $\mu\text{g/mL}$ of monoclonal anti-sulfonamide antibody 6C11. Subsequent immunoassays were performed using these concentrations.

3.2. HFF-QCM sulfathiazole standard curves: matrix effect

The construction of standard calibration curves was performed in order to quantify the sulfathiazole in samples. With the aim of evaluating the matrix effect, calibration standard curves in PBS (sulfathiazole from 2×10^{-4} to 2×10^3 $\mu\text{g/L}$) and in diluted honey (1/140, w/v) were performed. Figure 1 shows, as an example, a sensorgram of the HFF-QCMD response to different concentrations of sulfathiazole in diluted honey. As the concentration of sulfathiazole increased, a greater signal inhibition was registered, caused by less availability of free antibody.

Figure 2 depicts the sulfathiazole standard curves obtained in PBS and in diluted honey. The competitive nature of the immunoassay is reflected in the sigmoidal behavior of the standard curve (Osterloh, Smith, & Peters, 1989), i.e. the signal decreases as an inverse function of the analyte concentration. The higher the analyte concentration the lower the quantity of free available antibody molecules in the assay, thus leading to a proportional signal inhibition (Fernández-Benavides et al., 2019).

In both standard curves the experimental data showed excellent fitting with the logistic equation ($R^2 = 0.9918$ for PBS and $R^2 = 0.9866$ for honey).

Table 2 shows the mathematical parameters (A, B, C and D) of equation (1) resulting from the fitting of both curves. No significant statistical differences for any parameter were found between both curves (t-test; $p < 0.05$), demonstrating the absence of matrix effect for 1/140 diluted honey. This result is in agreement with the great similarity between the small ΔD values measured for both PBS and diluted honey, thus confirming that viscoelastic properties of diluted honey can be neglected. This simple honey pre-treatment used with HFF-QCMD immunosensors makes this method faster and simpler than LC-MS/MS which, in turns, allows saving time in the analysis procedure.

In addition to the mathematical parameters A, B, C and D, the standard curve has several analytical parameters that allow the comparison among different conditions. The analytical parameters of the calibration curves run in PBS and in diluted honey are summarized in Table 3. The I_{50} parameter provides the analyte concentration corresponding to the inflection point between the two asymptotes, and it is considered as an estimation of the assay sensitivity. The limit of detection (LOD) corresponds to the analyte concentration that produces 10% inhibition of the maximum signal. The limit of quantification (LOQ) is obtained as the analyte concentration that produces

20% inhibition of the maximum signal. Finally, the working range is calculated as the range of concentrations that provide 20 and 80% of signal inhibition.

Despite the absence of statistical differences in the mathematical parameters of the logistic equation (1), the curve in diluted honey was selected for the analyte quantification in fortified samples since the assay LOD and LOQ were lower in this case.

The LOD reached by the developed HFF-QCMD immunosensor was $0.0010\text{ }\mu\text{g/L}$ in diluted honey. Taking into account the dilution factor in the assay (1/140), this value corresponds to a LOD in honey of $0.10\text{ }\mu\text{g/kg}$. The LOQ was $0.02\text{ }\mu\text{g/L}$ in diluted honey ($2\text{ }\mu\text{g/kg}$ in honey sample), and the working range ranged from 0.02 to $1000\text{ }\mu\text{g/L}$ (2 to $100,000\text{ }\mu\text{g/kg}$ honey).

Regarding the sensor surface regeneration, the HFF-QCMD immunosensor developed was able to properly work for around 100 assay cycles without significant decrease in the signal. After 100 cycles the repetitiveness of the measures was highly compromised (the assay signal was around 50% of that obtained in the first assays), and a new immobilization of the assay conjugate was necessary.

The LOD achieved both in diluted honey and in honey samples, by different reported techniques for sulfathiazole detection in honey are summarized in Table 4.

As shown in Table 4, the LOD achieved by the immunosensor developed in the present work ($0.10\text{ }\mu\text{g/kg}$) is up to forty times lower than that reported for ELISA ($4\text{ }\mu\text{g/kg}$) (Pastor-Navarro et al., 2007) and fifty times better than LC-MS/MS ($5\text{ }\mu\text{g/kg}$) (Juan-Borrás et al., 2015). These results indicate that this immunosensor can be more sensitive than the current antibiotic detection techniques. The high sensitivity achieved by the immunosensor is mainly due to the sensitivity enhancement provided by HFF-QCMD technology and to the low noise characterization system used (March et al., 2015).

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

In order to evaluate the analytical performance of the proposed new methodology (HFF-QCMD), in comparison with the most commonly used (LC-MS/MS) for sulfathiazole determination, the accuracy and precision were evaluated for both methods. To this aim, five independent replicates of “blank honey” spiked at seven levels: 0, 0.5, 2, 10, 50, 100 and 1000 $\mu\text{g/kg}$ were measured with both techniques. The comparison of recovery and coefficient of variation (CV) of spiked samples obtained with both techniques are listed in Table 5. No false positives were detected since the blank honey samples were below the LOD in both techniques. In this table, fortified concentrations higher than 2 $\mu\text{g/kg}$, showed good recoveries and CV in all cases: recoveries from 100% to 113% (HFF-QCMD) and from 94% to 103% (LC-MS/MS), whereas CVs ranged from 14% to 17% and from 0.3 to 11%, for HFF-QCMD and LC-MS/MS, respectively. This proves that, only in this range of concentrations (from 10 $\mu\text{g/kg}$ to 1000 $\mu\text{g/kg}$), the new developed HFF-QCMD immunosensor meets the requirements established by SANCO 12571/2013 guidelines (recovery percentages between 80% and 120% as well as CV lower than 20%). Looking more in detail the CV values for concentrations in the range 10-1000 $\mu\text{g/kg}$, it can be observed that these values were always higher in HFF-QCMD than in LC-MS/MS. This shows that the new method is precise enough only in this range. Consequently, to be usable at lower concentrations it will need to be fine-tuned in future developments to try to avoid small uncontrolled or involuntary variations in its operating conditions

These findings could be expected, since the spiked concentrations (from 10 to 1000 $\mu\text{g/kg}$) lay within the working range of HFF-QCMD immunosensor (see Table 3). However, for lower levels (0.5 and 2 $\mu\text{g/kg}$) it showed poor accuracy and precision, because these concentrations were below the immunosensor LOQ.

With the aim of checking the performance of this new technology for detecting sulfathiazole in real (non-fortified) samples, six honey samples from different sources were analyzed by LC-MS/MS and HFF-QCMD, following the same steps and in the same way as detailed before for the spiked samples. Each sample was analyzed in parallel, first by the conventional procedure and after by the new methodology. None of them revealed the presence of sulfathiazole, neither by using HFF-QCMD nor LC-MS/MS, thus confirming the reliability of the immunosensor for sulfathiazole detection. Nevertheless, this is to be considered as a preliminary result and it would be necessary to extent the scope to other types of honey with the aim of taking into account further external factors that might create variations in the results.

4. Conclusions

This work reports the first HFF-QCMD immunosensor for quantification of sulfathiazole in honey by using a functionalized 100 MHz quartz sensor as transducer and specific monoclonal antibodies as bio-recognition elements. This immunosensor based method requires simple honey pre-treatment, making it faster and simpler than other methods. Moreover, it is highly sensitive, achieving LODs 40-50 times lower than other reported techniques for sulfathiazole detection in honey.

The requirements of precision and accuracy set by SANCO 12571/2013 are met by this HFF-QCMD immunosensor for concentration of sulfathiazole in honey equal or higher than 10 µg/kg. These findings are promising and a starting point for future developments in order to achieve a better LOQ and enough reliability for lower concentrations. Therefore, this new technique can be considered a good alternative for faster determination of sulfathiazole in honey with minimum sample preparation.

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Figure Caption

Figure 1. Sensorgram of HFF-QCMD response to the biorecognition events of different sulfathiazole concentrations in diluted honey during the performance of inhibition competitive assays.

Figure 2. HFF-QCMD sulfathiazole calibration curves in PBS and in honey diluted 1/140 in PBS. Each point is the average of four determinations. Vertical bars represent standard deviation.

Table 1. Frequency signal shifts (Δf) obtained with the HFF-QCMD sensor in the checkerboard titration of several concentrations of the immobilized SA2-BSA conjugate and monoclonal anti-sulfonamide antibody 6C11. Each value is expressed as the average and the standard deviation of three measurements.

SA2-BSA conjugate concentration ($\mu\text{g/mL}$)	Frequency signal shift (Δf , Hz)	
	1 $\mu\text{g/mL}$ MAb	2 $\mu\text{g/mL}$ MAb
5	660 $\pm$ 140	1050 $\pm$ 200
10	900 $\pm$ 40	1100 $\pm$ 180
20	855 $\pm$ 160	1560 $\pm$ 30
50	2200 $\pm$ 300	3800 $\pm$ 200

1 **Table 2.** Mathematical parameters of logistic equation.

Parameter	PBS curve		1/140 diluted honey curve	
	Coefficient	Std. Error	Coefficient	Std. Error
A	104.4	16.5	129.0	40.3
B	0.4	0.1	0.3	0.1
C	3.4	3.1	5.4	11.9
D	1.8	13.2	12.3	32.2
R^2	0.9918		0.9866	

2

- 1 **Table 3.** Analytical parameters obtained for the HFF-QCMD standard curves of
 2 sulfathiazole in PBS and in 1/140 diluted honey.

Analytical parameter	PBS ($\mu\text{g/L}$)	1/140 diluted honey ($\mu\text{g/L}$)	Relevant parameters in honey samples ($\mu\text{g/kg}$)
I₅₀	3	5	-
LOD	0.007	0.0010	0.10
LOQ	0.07	0.02	2
Working range	0.07 to 100	0.02 to 1000	2 to 100,000

3

Table 4. Comparison of LODs reported by different techniques for sulfathiazole detection in honey.

Technique	LOD in diluted honey ($\mu\text{g/L}$)	LOD in honey sample ($\mu\text{g/kg}$)	Reference
<ul style="list-style-type: none"> High fundamental quartz crystal microbalance (HFF-QCMD) 100 MHz 	0.0010	0.10	Present work
<ul style="list-style-type: none"> ELISA 	0.25	4	Pastor-Navarro, Gallego-Iglesias, Maquieira, & Puchades, 2007
<ul style="list-style-type: none"> Immunocomplex capture fluorescence-based immunosensor device 	0.11	-	Jornet, González-Martínez, Puchades, & Maquieira, 2010
<ul style="list-style-type: none"> High-pressure liquid chromatography/tándem mass spectrometry 	-	5	Juan-Borrás et al., 2015

- 1 **Table 5.** Comparison of HFF-QCMD immunosensor and LC-MS/MS for the analysis of
 2 sulfathiazole-spiked honey samples (n=5).

HFF-QCMD				LC-MS/MS		
Fortified	Detected	Recovery	CV	Detected	Recovery	CV
($\mu\text{g/kg}$)	($\mu\text{g/kg}$)	(%)	(%)	($\mu\text{g/kg}$)	(%)	(%)
0	<LOD	-	-	<LOD	-	-
0.5	0.6 ± 0.4	120	55	0.5 ± 0.01	100	2.6
2	2.7 ± 0.6	135	23	2.0 ± 0.3	100	15
10	11.3 ± 1.9	113	17	10.0 ± 1.1	100	11
50	52 ± 8	104	17	47.0 ± 0.2	94	0.4
100	100 ± 15	100	14	103.0 ± 0.3	103	0.3
1000	1100 ± 170	110	16	1000 ± 60	100	6

3

Highlights

- A HFF-QCMD immunosensor was developed for sulfathiazole analysis in honey
- This method requires minimum honey pretreatment and no false positives were detected
- The LOD reached is 40-50 times lower than those provided by other techniques
- This immunosensor meets SANCO guidelines for concentrations up to 10 $\mu\text{g/kg}$

Conflicts of interest

The authors declare that they have no conflict of interest

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