



A smartphone aptasensor for fipronil detection in honey samples

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Abstract

In this study, an electrochemical smartphone-based aptasensor for the determination of fipronil was developed by modifying a screen-printed carbon electrode (SPCE). Fipronil is a broad-spectrum insecticide that has been widely used in various applications such as agriculture, veterinary, and household pest control. Recently, its use has raised concerns over the potential impact on the environment and human health. The absence of effective methods for this purpose poses a significant obstacle. To tackle this problem, we have developed a cutting-edge aptamer-based portable sensor capable of rapidly and conveniently detecting fipronil in situ. Considering that the detection of small molecules, such as fipronil, can be a challenging task, a competitive replacement assay was set up based on the aptamer's preference for the free form of fipronil over the immobilized one on the electrode. The analytical performance provided by the sensor on standard solutions of a known fipronil content made it possible to estimate a limit of detection (LOD) equal to $1.07 \mu\text{g kg}^{-1}$ and a limit of quantification (LOQ) of $3.21 \mu\text{g kg}^{-1}$. Selectivity tests were conducted using atrazine as a possible interferent. The use and performance of the developed portable aptasensor was assessed on honey samples, which were simultaneously analyzed using an HPLC–MS method. This aptasensor could be an affordable and effective tool for accurately quantifying fipronil not only in honey samples but also in other food products.

Keywords Biosensors · Aptamer · Smartphone · Honey · Food analysis · Aptasensor

Introduction

The increased food demand, combined with the necessity of controlling insect-borne diseases, has stimulated the development of pesticides [1]. For many decades, the use of pesticides has been steadily increasing on agricultural land, especially in developing countries [2–4]. In Europe, the approval of the active substances is entrusted to the European Commission, which considers the scientific evaluation and peer reviews of the European Food Safety Authority (EFSA) [5]. In this context, legal limits and their control in marketed products are the main tools of risk management [6, 7]. European Commission promotes a road to the sustainable

use of pesticides, an objective that is pursued within the Farm to Fork strategy, one of the central pillars of the Green Deal [8]. The concrete objective, to be achieved through the application of the Common Agricultural Policy, by 2030, consists of a 50% reduction in the use and risk of the most dangerous pesticides in the EU [9].

Hence, pesticides are among the analytes that attract the greatest attention in the field of food control [10, 11]. Among pesticides, fipronil garnered significant attention due to its involvement in the contamination of eggs in 2017 [12, 13]. Fipronil is a broad-spectrum insecticide that has been widely used in various applications such as agriculture, veterinary, and household pest control [14]. Recently, its widespread use has raised concerns over the potential impact on the environment and human health [15–18]. In the European Union, the maximum residue limit (MRL) for fipronil in food products is $5 \mu\text{g kg}^{-1}$ [19]; this limit applies to a wide range of food products, including eggs, meat, dairy, and certain fruits and vegetables [20].

Currently, the main analytical techniques exploited for the control of fipronil in different food matrices are gas chromatography and liquid chromatography, most of the time

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coupled to mass spectrometry [21–23]. However, traditional analytical methods require complex sample preparation and analysis procedures, specialized personnel, long times, and high costs and are not suitable as methods for in situ analysis [24]. Simpler alternative analytical tools could provide better monitoring of fipronil residues. Several techniques have been suggested as potential solutions, with notable examples such as Raman spectroscopy and enzyme-linked immunosorbent assays (ELISA), while these methods offer distinct advantages of analysis; they also exhibit certain limitations, some of which can be likened to those encountered in traditional methodologies [25, 26].

In this context, there is a significant opportunity for the application of biosensors, with aptasensors standing out as a noteworthy choice [27, 28]. Biosensors are incredibly versatile and powerful tools due to their low cost, ease of manufacture, rapid analysis, compact size, and exceptional detection capabilities [29–32]. However, the detection of small molecules, such as fipronil, can be a challenging task due to several factors [33, 34]. In fact, small molecules typically have low molecular weights and simpler structures compared to larger biomolecules such as proteins or nucleic acids. At the same time, when it comes to the most common detection strategies, sandwich assays cannot be employed due to the requirement of the target having at least two epitopes [35], which is an unrealistic condition in the case of small molecules [36]. On the other hand, a label-free assay, involving a signal directly correlated to the selective recognition of the molecule, runs the risk of exhibiting low sensitivity due to the small size of the target [37]. For these reasons, the use of simple and effective approaches, such as competitive assays, is of high interest [38, 39]. Among them, competitive replacement assays stand out as a compelling and effective method for detecting small molecules, as evidenced by their successful application in quantifying small compounds [40, 41].

Motivated by the potential benefits that a portable pesticide sensor could bring, here, we present a smartphone aptasensor that employs a competitive replacement assay approach to quantify fipronil. The primary impact of this research could be reflected in consumer safety, as well as in monitoring the potential environmental repercussions of pesticide residues in the ecosystem. To demonstrate the effectiveness of our work, we chose to test the aptasensor on honey samples.

Materials and methods

Reagents and materials

Two aptamers were used in the present work, one previously described in literature [42], and its corresponding form without primers, both biotinylated at the 5' end and produced by Sigma-Aldrich (Milan, Italy). Table 1 shows the names and their respective sequences.

For the preparation of all the solutions and the cleaning operations, ultrapure water ($R > 18 \text{ M}\Omega \text{ cm}$) was used, obtained with the Elga Purelab flex 4 system, Veolia Water Technologies (Italy). All reagents used were of analytical grade. Salts for buffer solutions, 2-aminobenzoic acid (2-ABA), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), fipronil, bovine serum albumin (BSA), tris(hydroxymethyl)aminomethane hydrochloride (Tris), and 3, 3', 5, 5'-tetramethylbenzidine (TMB) were purchased by Sigma-Aldrich (Italy). Streptavidin conjugated horseradish peroxidase (HRP) was purchased by Merck (Italy). Ethanol was purchased from J.T. Backer (Italy), and methanol for HPLC–MS was manufactured by Honeywell, Riedel-de Haën (France). Atrazine was purchased by LGC Standards (UK). Screen-printed carbon electrodes (SPCEs) were purchased from DropSens (Metrohm, Italy).

Electrochemical measurements were performed by a portable potentiostat SensiSMART (Palmsens, Netherlands) managed via the PStouch app (version 2.8) and an Autolab PGSTAT204 potentiostat (Metrohm, Italy), managed by the Nova software (version 2.1). For the HPLC–MS analysis, it was necessary the purification of the samples through solid-phase extraction (SPE) cartridge C18, Isolute (1 g / 6 mL) of Biotage (Italy). The UHPLC instrument used was a Dionex Ultimate 3000 (Thermo Scientific, San Jose, CA, USA) equipped with a thermostatic autosampler and an AGILENT InfinityLab Poroshell 120 EC-C18 column (4.6 × 150 mm, particle diameter 2.7 μm) with guard column (4.6 × 5 mm, particle diameter 2.7 μm) made up of the same stationary phase and thermostated at 30 °C. The mass spectrometer employed was a Finnigan LXQ linear ion trap (Thermo Scientific, San Jose, CA, USA) equipped with an electrospray ion source (ESI) operating in negative mode.

Table 1 Nucleotide sequence of aptamers

Name	Sequence
FPAP (fipronil aptamer with primers)	5'-[Bt _n]TGTACCGTCTGAGCGATTTCGTACAGTTTCTGGAGGA CTG GGCGGGGTGACGGTTATGAGCCAGTCAGTGTTAAGG AGTGC-3'
FPAS (fipronil aptamer Short)	5'-[Bt _n]AGTTTCTGGAGGACTGGGCGGGGTGACGGTTATG-3'

Binding assay — K_D determination protocol

All SPCEs, before being used, were washed with 1 mL of ethanol and 1 mL of ultrapure water and finally dried with compressed air. Subsequently, 60 μL of the 2-ABA (50 mM) and KCl (0.1 M) solution prepared in H_2SO_4 (1 M) were deposited on the SPCE. The polymerization was carried out by applying 10 cycles of cyclic voltammetry starting from an initial potential of 0 V to a final potential of 1 V, at a scan rate equal to 0.05 V/s, cyclic voltammograms of the electropolymerization are shown in Figure S1. This process led to the formation on the working electrode (WE) of a polymer presenting carboxyl groups, in accordance with the proposed mechanism shown in Figure S2. Subsequently, the carboxyl groups were activated by incubating 10 μL of an EDC–NHS reaction mix, in PBS pH 5.6, for 1 h. Afterwards, the electrode surface was incubated overnight with 10 μL of a fipronil solution 40 μM . At the end of this step, fipronil was covalently linked to the electrode surface thank to the formation of amidic bonds. Next, to minimize non-specific binding, the electrode surface was incubated for 15 min with 10 μL of a 1% bovine serum albumin (BSA) solution, prepared in PBS pH 7.4. In order to control the fabrication of the sensor, we performed EIS measurements after each modification step; see Figure S3. Finally, the electrode was ready to carry out the measurements aimed at establishing the apparent dissociation constant (K_D) of fipronil aptamer. Figure 1 shows a schematic representation of the various steps of SPCE modification.

At this point, 10 μL of the solutions at different concentrations of aptamers FPAP e FPAS prepared in fipronil buffer solution (F-BS: NaCl 100 mM, MgCl_2 2 mM, Tris–HCl 20 mM) were incubated for 30 min on modified SPCEs. Then, the SPCEs were incubated for 10 min with the streptavidin–HRP solution ($0.75 \mu\text{g mL}^{-1}$). The electrochemical transduction was performed by adding on the electrode 60

μL of TMB solution, and after 1 min of enzymatic reaction, a chrono-amperometric measurement at 0 V was performed, sampling the reduction current of the oxidized TMB after 55–60 s.

Electrochemical competitive replacement assay for fipronil detection

The procedure for modifying the SPCE for the fipronil determination assay aligns with the modification process described above for measuring K_D . In this case, to minimize non-specific binding, after optimization, 0.5% BSA solution was used, prepared in PBS at pH 7.4, and incubated on the WE for 5 min. Afterwards, the aptamer was incubated at a concentration of 1 μM . After 30 min of incubation, the electrode surface was rinsed and dried, thus resulting ready for the subsequent assay. The calibration of the sensor response to fipronil was performed by incubating 10 μL of each standard solution at different concentrations for 1 h on the modified SPCEs as previously reported. At the end of this step, the SPCEs were incubated for 10 min with the streptavidin–HRP solution ($0.75 \mu\text{g mL}^{-1}$). The electrochemical transduction was performed by adding on the electrode 60 μL of TMB solution, and after 1 min of enzymatic reaction, a chrono-amperometric measurement at 0 V was performed, sampling the reduction current of the oxidized TMB after 55–60 s. Figure 2 shows a schematic representation of the fipronil assay procedure using the competitive replacement assay, and Figure S4 shows the measurement device including the smartphone and Sensit/SMART potentiostat connected to a SPCE.

Selectivity and stability evaluation

For the evaluation of the selectivity, the procedure adopted for the analysis was followed by replacing the solutions of

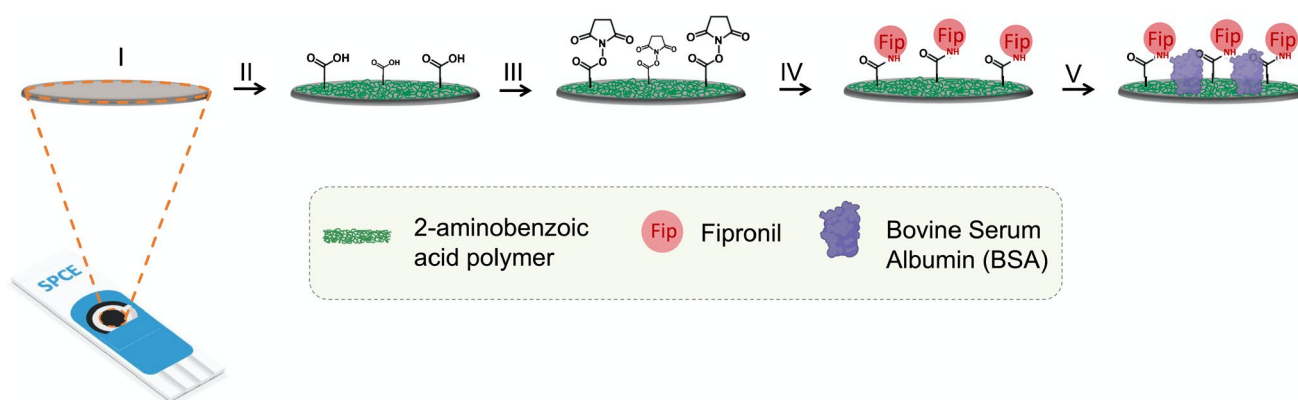


Fig. 1 Schematic representation of the different modification steps of the SPCE surface for the determination of the K_D of the aptamer–fipronil adduct. I, bare electrode; II, 2-aminobenzoic acid electropo-

lymerization; III, incubation with EDC–NHS reagent; IV, incubation with fipronil; V, incubation with BSA

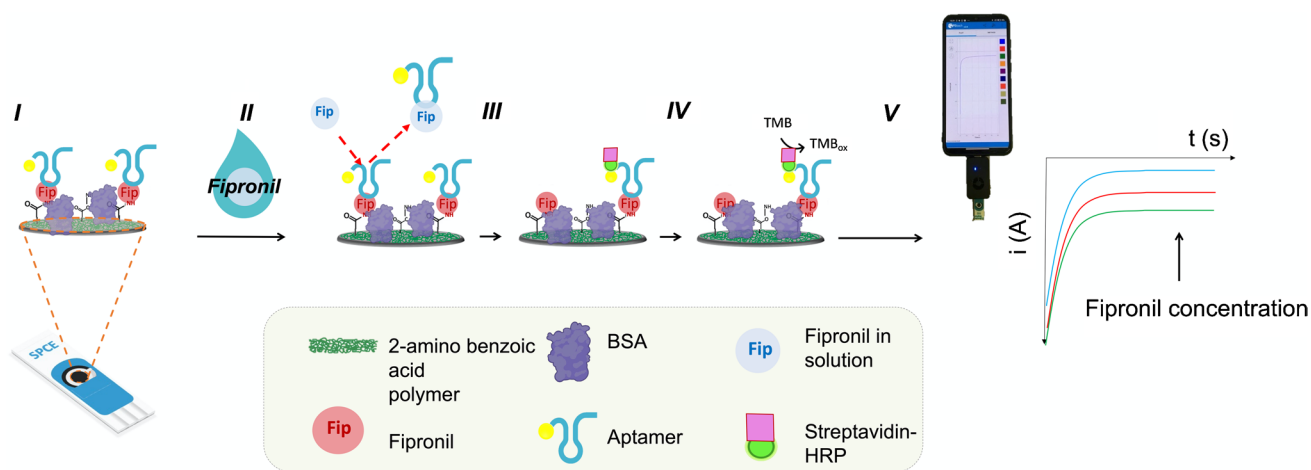


Fig. 2 Schematic representation of the competitive replacement assay. I, SPCE modified and ready for the analysis; II, incubation with solution containing fipronil (when the aptamer comes in contact with a sample containing fipronil, it detaches from the electrode

surface to bind fipronil in solution); III, incubation with streptavidin-HRP; IV, TMB substrate incubation; V, chrono-amperometric measurement performed with a portable potentiostat managed by a smartphone (increasing fipronil concentration from green to blue)

fipronil with solutions of known concentration of atrazine, which is a commonly used herbicide and one of the interferent reported in the literature in the quantification of fipronil [42]. Therefore, to determine the selectivity, the assay was conducted with the same modalities adopted for fipronil. Furthermore, the stability of the sensor stored at 4 °C was evaluated. A batch of sensors was prepared and stored ready for the use; the signal at the fixed concentration of 7 $\mu\text{g kg}^{-1}$ was evaluated for 10 days.

Real samples

In this study, we analyzed two distinct types of honey: one sourced from organic farming and the other one from non-organic farming. The samples were conveniently diluted 1:2 with F-BS prior to analysis and spiked with gradually increasing concentrations of fipronil. To prevent any potential contamination, the sample preparation was conducted in a separate laboratory from the one used for the subsequent analyses. Sample measurements were always accompanied by blank measurements (i.e., matrix without analyte) to evaluate and take into account the matrix effect.

HPLC–MS analysis

Sample preparation procedure

Two-hundred fifty milligrams of each honey sample was purified by solid-phase extraction with an SPE C18 cartridge. For this purpose, the honey samples were diluted in water and loaded onto the SPE cartridge previously conditioned with methanol and water. This was followed by washing with water (10 mL) and elution of fipronil with methanol

(5 mL). Finally, after vacuum distillation of the organic solvent with Rotavapor ($T < 35\text{ °C}$) and solubilization of the residue in 1 mL of methanol/water 80/20%, the sample was then subjected to HPLC–MS analysis.

HPLC–MS² procedure

The chromatographic separation was carried out on a C18 reverse phase column using a mixture of methanol and water as the mobile phase, at a flow rate of 0.3 mL min⁻¹ and with the concentration gradient shown in Table S1. Fipronil was identified in negative mode, isolating the ion having an m/z ratio of 435 and corresponding to $[\text{M}-\text{H}]^-$, whose spectrum is shown in Figure S5. This ion was subsequently fragmented obtaining the fragment ions at m/z 399 $[\text{M}-\text{H}-\text{HCl}]^-$ and at m/z 330 $[\text{M}-\text{H}-\text{HCl}-\text{CF}_3]^-$ as described in literature [43, 44]. The quantification of fipronil was carried out using the standard additions method and selecting in MS² the most intense fragment ion at m/z 399 (Figure S6a). By way of example, Figure S6b shows the chromatograms and the mass spectrum obtained in the HPLC–MS² analysis of a solution at known fipronil content.

Results and discussion

K_D determination

Binding assays were performed to establish the dissociation constants of the fipronil aptamer adducts.

The sequence described in the literature, which comprises the primers used during the selection procedure, has been taken into consideration. Simultaneously, the same sequence

was evaluated without primers, considering that the variable portion, i.e., the portion without primer, is typically involved in binding with the target.

In Table 2 are reported the K_D values, while Fig. 3 and Figure S7 show the related titration curves. The best fittings, characterized by coefficients of determination (R^2) = 0.99, were obtained with the logistic model.

The K_D data show that the affinity of FPAS towards fipronil is higher than that calculated for FPAP. These results confirm the advantage of utilizing the aptamer without the primer regions in terms of affinity. Consequently, based on the K_D values, FPAS was selected for the subsequent experimental activities.

Fipronil quantification by a competitive replacement assay

Encouraged by the results of the binding assay of FPAS, an electrochemical aptasensor for fipronil quantification using a competitive replacement assay was developed. The principle of the assay is illustrated in Fig. 2. This type of approach has already been successfully employed in literature for the quantification of small molecules such as neomycin or aflatoxin B [40, 41]. Here, we exploited the competitive replacement of the aptamer bound to the target immobilized on the surface with the free fipronil molecules in solution. Aptamers exhibit a higher preference for binding targets in solution rather than immobilized on a surface, because of accessibility, conformational flexibility, and more favorable electrostatic interactions [45]. Hence, this preference was exploited in the design of this assay, and when the aptamer comes into contact with a sample containing fipronil, it detaches from the electrode surface to bind fipronil in solution. Subsequently, through a washing step, the aptamer is eliminated from the electrode surface, causing a decrease in the reduction current of the oxidized TMB as the concentration of fipronil in solution increases.

Figure 4 shows the calibration curve obtained with increasing concentration of fipronil; the percentage signal decreases as the fipronil concentration increases. The model that best fits the data obtained is a dose–response model represented, with an R^2 of 0.999, by Eq. 1.

$$y = 52.90 + \frac{100.09 - 52.90}{1 + 10^{(6.41-x)(-0.40)}} \quad (1)$$

The limit of detection (LOD), calculated as three times the standard deviation of the blank signal divided by the slope

Table 2 Dissociation constants (K_D) of the fipronil aptamer adducts

Aptamer	K_D (nM)
FPAP	94.3 ± 0.5
FPAS	24.9 ± 1.7

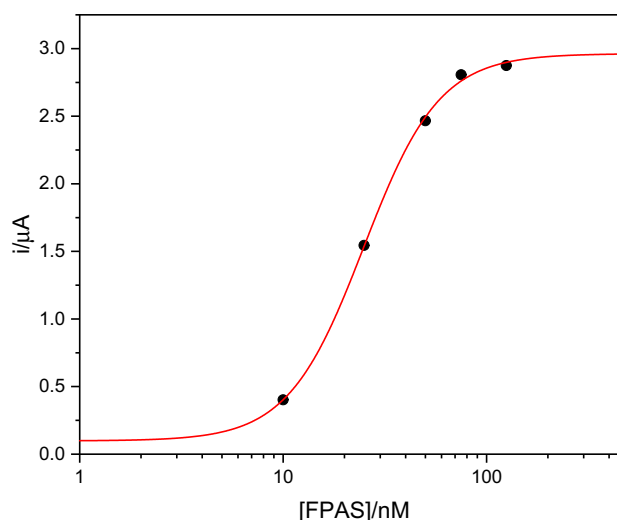


Fig. 3 Binding curve of FPAS vs. fipronil (10, 25, 50, 75, 125 nM)

calculated for the linear range, was $1.07 \mu\text{g kg}^{-1}$, while the limit of quantification (LOQ) was $3.21 \mu\text{g kg}^{-1}$. This confirms the high sensitivity of the response of the developed aptasensor, suitable for detecting quantities of fipronil even below the maximum residual level established by the European Union in the main food products, which is $5 \mu\text{g kg}^{-1}$. The selectivity of the aptasensor with respect to an herbicide product such as atrazine was evaluated. Atrazine was selected due to its resemblance to fipronil, characterized by its small molecular size and stereochemical arrangement, making it one of the most frequently employed compounds to test the selectivity of the developed sensor. The results obtained by comparing the response of the sensor in solutions of known concentration of

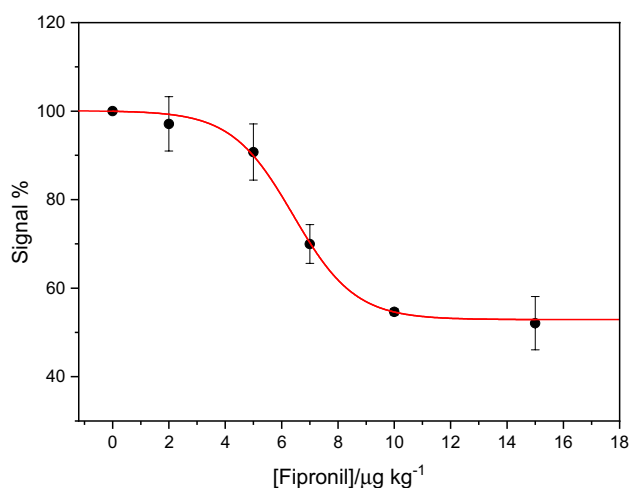


Fig. 4 Calibration curve of the signal obtained from chrono-amperometric measurements with the aptasensor on standard solutions with increasing fipronil concentration. The error bar is the standard deviation from a minimum of three measurements for each concentration

fipronil and atrazine are summarized in Fig. 5. The aptasensor stability was tested measuring its activity during 10 days of refrigerated storage. We recorded a maximum of 10% of sensor response decrease, indicating that the sensor can be used for up to 10 days after fabrication without major loss of efficiency.

Regarding the advances in the field of fipronil analysis, Table 3 compares the aptasensors for the detection of fipronil proposed by the literature in terms of analytical performance. It can be seen that the smartphone aptasensor here reported successfully competes with the other present in literature; moreover, it advances a portable analysis of fipronil, compared to the other approaches which still employ classic instrumentation with the correlated limitations.

Application of the competitive assay to food samples

To verify whether the developed biosensor could be employed in the analysis of real food samples, we proceeded to the analysis of fipronil in samples of honey.

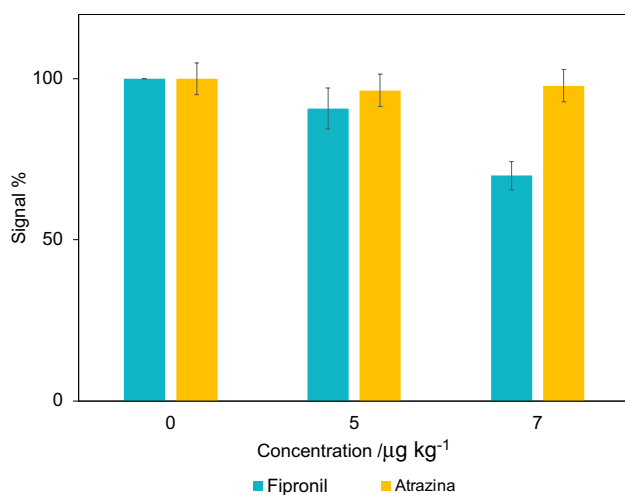


Fig. 5 Decrease of the signal obtained from chrono-amperometric measurements with the aptasensor on standard solutions with increasing concentrations of fipronil and atrazine

First, the two honey samples were subjected to HPLC–MS analysis to determine the initial fipronil content. The honey sample sourced from organic farming did not show any detectable presence of fipronil, or if present, it was below the limit of detection ($0.19 \mu\text{g kg}^{-1}$) of the HPLC–MS method. In contrast, the non-organic farming honey exhibited a fipronil content of $2.35 \pm 0.35 \mu\text{g kg}^{-1}$. These data were used as starting point to evaluate the results provided by the biosensor.

The honey samples underwent parallel analysis using a biosensor, employing the standard addition method. Fipronil was incrementally added to the samples, ensuring concentrations that fell within the dynamic response range of the sensor and were comparable to the maximum residue level (MRL) mandated by European legislation for honey.

The summarized results for the organic farming honey and non-organic farming honey are presented in Table 4.

These results confirm the reliability of the biosensor as it consistently produces results that align with those obtained through the HPLC–MS method. Moreover, the accuracy of the biosensor's measurements is demonstrated even in spiked samples, meeting the expected concentrations.

Table 4 Comparison between the theoretical concentration of fipronil and that determined experimentally in real samples of honey, analyzed as they are and spiked with known quantities of fipronil

Fipronil ($\mu\text{g kg}^{-1}$)	Expected fipronil concentration ($\mu\text{g kg}^{-1}$)	Biosensor quantification ($\mu\text{g kg}^{-1}$)
Organic farming honey		
0.00	<LOD	/
5.00	5.00	5.84 ± 0.23
7.00	7.00	7.64 ± 0.82
Non-organic farming honey		
0.00	2.35 ± 0.35	3.24 ± 0.90
5.00	7.35 ± 0.35	7.11 ± 0.34
7.00	9.35 ± 0.35	9.11 ± 1.25

Table 3 Comparison of the analytical performance of the developed aptasensor with that of other aptasensors for the determination of fipronil

Transduction method	Range	LOD	Sample	Reference
Fluorescence	5–500 nM	105 nM	Water	[42]
Fluorescence	25–300 $\mu\text{g kg}^{-1}$	53.8 $\mu\text{g kg}^{-1}$ (123 nM)	Eggs	[46]
Fluorescence	10–100 nM	3 nM	Corn, honey, water	[47]
Electrochemistry	0.1 ng/mL–10 $\mu\text{g/mL}$	0.17 nM	Water	[48]
Fluorescence	(5–70) nM	3.4 nM	Cabbage, cucumber	[49]
Electrochemistry (smartphone-based)	2–10 $\mu\text{g kg}^{-1}$	1.07 $\mu\text{g kg}^{-1}$ (2.45 nM)	Honey	This work

Conclusions

This study demonstrates that a competitive replacement assay, based on aptamers, is a suitable approach for quantifying fipronil in food matrices. This assay format addresses the challenges associated with detecting small molecules. By utilizing a simple and cost-effective electrochemical platform that can be used with a smartphone, the proposed assay becomes readily accessible, making it a user-friendly and portable detection system for detecting fipronil.

With a limit of detection (LOD) of $1.07 \mu\text{g kg}^{-1}$, the assay proves to be highly sensitive. To confirm its applicability in real food samples, honey samples were tested. The successful results provide confidence in the reliability of the biosensor for fipronil detection in various food matrices. This indicates that this aptasensor could be a valuable tool for screening and monitoring fipronil contamination in food products, contributing to food safety efforts. Further research and validation on a wider range of food samples would help strengthen its applicability and broaden its potential impact in food safety monitoring.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00216-023-05026-6>.

Author contribution RS: conceptualization; investigation; methodology; writing, original draft; visualization. ND: investigation; methodology; writing, review and editing; visualization. AG: investigation, methodology, writing—review and editing. RT: supervision, conceptualization, methodology, writing—review and editing.

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Declarations

Conflict of interest The authors declare no competing interests.

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