

43 1. Introduction

44 Tetracycline (TC) is a broad-spectrum antibiotic used as a veterinary drug to prevent
45 and to treat diseases caused by Gram-positive and Gram-negative bacteria, atypical organisms
46 such as Chlamydiae, Mycoplasmas, Rickettsia, and protozoan parasites (Eliopoulos et al.,
47 2003). The favorable antimicrobial properties of this drug and the absence of significant
48 adverse side effects have led to their extensive use in the therapy of both human and animal
49 infections (Chopra and Roberts, 2001). Furthermore, it has been reported as the most
50 intensely growth-promoting agent widely used in animal husbandry (Economou and Gousia,
51 2015). Consequently, despite their proven efficacy, the intensive use of this drug is no longer
52 encouraged since its remain residues can be accumulated in the animal bones, as well as in
53 fish, meat, eggs, and milk, on account of the complexation of TC with Ca^{2+} (Nelson et al.,
54 2010). To ensure human food safety, the European Union (EU) has established the maximum
55 residue limits (MRLs) for TC in edible products and milk at $100 \mu\text{g}\cdot\text{kg}^{-1}$, in eggs at
56 $200 \mu\text{g}\cdot\text{kg}^{-1}$ and in the liver at $300 \mu\text{g}\cdot\text{kg}^{-1}$ (European Commission, 1999). However, the
57 MRLs for TCs in honey has not yet been established by Codex, since the treatment of
58 honeybee diseases with antibiotics is unallowable in the EU (Kivrak et al., 2016). Despite
59 banning, some countries, such as Switzerland, UK, and Belgium still use TC treatment for
60 hive diseases and have established, therefore, the MRLs for TC ranging from 10 to $50 \mu\text{g}\cdot\text{kg}^{-1}$
61 (Bargańska et al., 2011).

62 However, TC residues can be successfully determined in various food products
63 requiring highly performing sample preparation steps. Several analytical methods currently
64 exist for TC detection and quantification. High performance liquid chromatography coupled
65 to mass spectrometry (Pokrant et al., 2018), capillary electrophoresis coupled with
66 electrochemiluminescence (Deng et al., 2012), enzyme immunoassay (Gaurav et al., 2014),
67 liquid chromatography-mass spectroscopy (LC-MS) (Desmarchelier et al., 2018) have been
68 described for confirmatory analysis. Other methodologies based on spectroscopy analysis are
69 described (Qin et al., 2016); unfortunately, they suffer from a lack of sensitivity compared to
70 chromatographic techniques (Mitra, 2004). Subsequently, Most of these approaches often
71 require the use of extraction techniques, such as liquid-liquid (Desmarchelier et al., 2018) and
72 solid phase extraction (Shi et al., 2011), in order to accomplish both the analytes pre-
73 concentration and sample cleanup (O'Connor and Aga, 2007). However, the principal
74 limitations of these steps lie in the high costs, the long time of achievement and the
75 requirement of advanced technical skills (Joshi and Anderson, 2012). For this reason, simple,
76 rapid and accurate methods are recommended for the on-site screening of low TC residues
77 without needing extraction or clean-up steps. Due to their advantages of high selectivity, rapid
78 detection, and *in-situ* applications, several optical and electrochemical techniques based on
79 biosensors have been investigated (Lan et al., 2017). Therefore, aptamer-based sensing
80 techniques were widely used for the food safety determination. Notably, there is a growing
81 rise in the aptasensors fabrication for the TC detection (Han et al., 2018; Tang et al., 2017)
82 with a few applications in honey samples (Wang et al., 2015, 2014). **Nevertheless, the main**
83 **limitations of these systems are related to their relative low detection signals** (Yi et al., 2014).
84 Furthermore, in some recent works, the molecularly imprinted sensors had been successfully
85 applied to the analysis of antibiotic residues in honey samples (Bougrini et al., 2016; Lv et al.,
86 2014). This type of sensors, however, often show relatively low sensitivity and specificity
87 when the imprinted membrane is fragile. Hence, on the last few decades, the development of
88 immunosensors grew tremendously to the detection of drug residues in food. **The use of these**
89 **analytical systems has attracted considerable attention** because of the low detection limits and
90 the high selectivity for analyzing complex samples (Majdinasab et al., 2017). In this regard,
91 some immunosensors were performed for TC detection in milk samples (Conzuelo et al.,

92 2013; Liu et al., 2016) with the limits of detection being 0.032, 3.9, and 0.85 ng.mL⁻¹,
93 respectively.

94 Herein, a novel and highly sensitive approach is proposed for the detection of
95 remaining TC residues in honey samples. This immunosensor was conducted on bio-micro-
96 electro-mechanical systems (Bio-MEMS) transducers based on gold micro working electrodes
97 (μ WEs) with fully integrated, reference and counter electrodes. The novelty of this work is
98 related to the functionalization process of μ WEs, which was achieved through two- and three-
99 dimensional (2D and 3D) shapes. These functionalizations were conducted, in one hand, by
100 electroaddressing diazonium salt and, in the other hand, by electrodepositing of a new
101 structure of magnetic nanoparticles coated poly(pyrrole-co-carboxylic acid) (Py/Py-
102 COOH/MNPs), while combining the two methods with a preconcentration technique. The
103 Py/Py-COOH/MNPs as solid supports of biomolecules, is the pioneering aspect of this work
104 dedicated to the combination of submicron, magnetic conducting particles. The quantification
105 of this analyte was performed through competitive detection procedure with TC molecules
106 immobilized on the μ WEs surface toward polyclonal TC antibody. The prepared
107 immunosensor was successfully applied to enhance the detection limit of TC in complex
108 matrices such as honey.

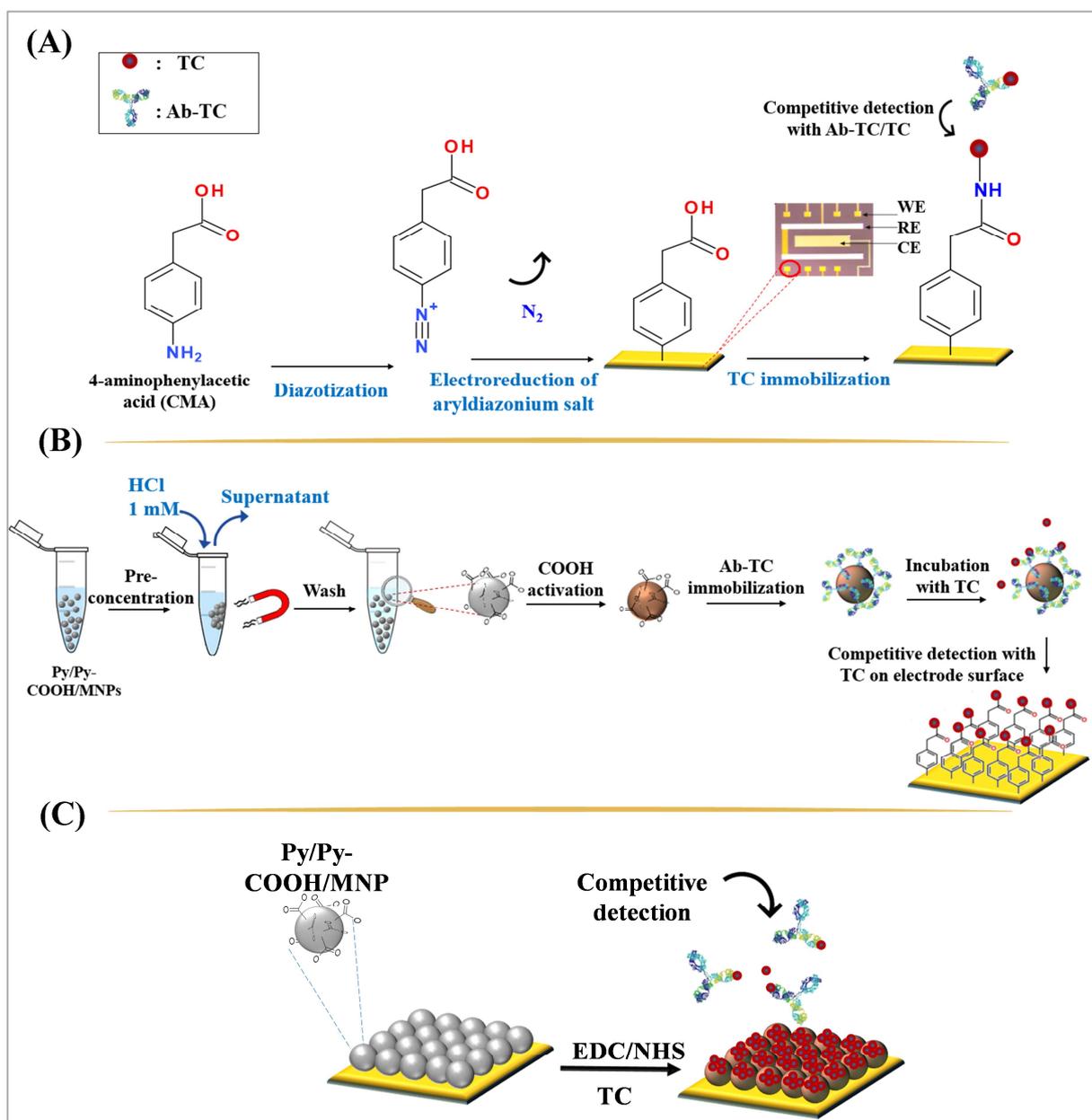
109 2. Experimental

110 2.1. Reagents and solutions

111 Tetracycline (TC), Doxycycline (DX), Oxytetracycline (OX), Chlortetracycline (CT),
112 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), Ethanolamine, Sulfuric acid
113 (H₂SO₄) (30 wt.% in H₂O), 4-aminophenylacetic acid (CMA), sodium nitrite (NaNO₂),
114 Sodium cyanoborohydride (95%), hydrochloric acid (37%, HCl), Carbon tetrachloride,
115 heptane, octadecyltrichlorosilane (OTS), Pure ethanol (99.8%), Acetone (99.9%), Potassium
116 hexacyanoferrate II, Potassium hexacyanoferrate III, Phosphate buffer saline (PBS) with pH
117 7.4 were all purchased from Sigma-Aldrich, France. N-Hydroxysuccinimide (NHS) and
118 Hydrogen peroxide (H₂O₂) (35%, v/v) were purchased from Acros Organics, France. 11-
119 (triethoxysilyl)undecanal (TESUD) was purchased from abcr GmbH, Germany.
120 Polydimethylsiloxane (PDMS: Sylgard 184) was purchased from Dow Corning, France. The
121 sheep polyclonal antibody raised against TC (Ab-TC) was supplied by Abcam, France. A
122 fluorescent labeled antibody was generated by conjugation of Ab-TC with rhodamine using
123 the rhodamine fast conjugation kit (ab188286) by Abcam, France. Rosemary honey sample,
124 guaranteed tetracycline-free was supplied by Secrets d'Apiculteur, Lyon, France. Honey
125 sample with an unknown concentration of TC was commercially provided. Milli-Q ultrapure
126 water was used in all experiments.

127 2.2 Procedure for immunosensor elaboration

128 The development of this immunosensor was conducted by three methods (Fig. 1).
129 Before any functionalization, the Bio-MEMS device surface was cleaned with ethanol in an
130 ultrasonic bath for 10 min, dried under nitrogen flow, then placed under UV-Ozone Cleaner-
131 ProCleaner™ Plus from BioForce for 30 min to remove all organic contaminants.



132

133 **Fig.1.** Schematic illustrations of competitive immunoassay of TC on modified μ WEs by
 134 functionalization with: (A) CMA electroaddressing, (B) CMA electroaddressing followed by
 135 preconcentration of immunomagnetic nanoparticles cross-linked with Ab-TC antibody, (C)
 136 Py/Py-COOH/MNPs.

137 2.2.1 Diazonium immobilization onto gold microelectrodes

138 The cleaned microelectrodes (μ WEs) were functionalized by CMA solution (3 mM)
 139 previously diazotized in an aqueous solution of HCl (20 mM) and NaNO_2 (20 mM) for 15
 140 min at 4 °C. **Subsequently**, nine-repetitive cyclic voltammograms were applied from 0.3 V to
 141 -1.0 V with a scan rate of $80 \text{ mV}\cdot\text{s}^{-1}$. This was done sequentially until all desired μ WEs were
 142 modified with CMA. The carboxylic groups formed on the electrode surfaces were activated
 143 **by** a mixture of 0.4 M EDC and 0.1 M NHS, prepared in ethanol for one hour at room
 144 temperature. Afterwards, **the activated electrode surfaces were** incubated in $40 \mu\text{L}$ of TC at
 145 $100 \mu\text{g}\cdot\text{mL}^{-1}$ for 30 min at 4 °C. Then, the immunosensor was treated with ethanolamine (1%
 146 v/v) in PBS buffer (pH 7.4) for 10 min at room temperature. **This step is crucial to prevent**
 147 **nonspecific bonding phenomenon at the detection stage of TC** (Fig. 1A).

2.2.2 Preconcentrating technique

148
149 The core-shell magnetic nanoparticles coated with poly(pyrrole-co-pyrrole carboxylic
150 acid) (Py/Py-COOH/MNPs) were exclusively synthesized by (Tenório-Neto et al., 2016)
151 using a seeded-polymerization technique. The morphology of these nanoparticles was already
152 presented in our previous work (Hassani et al., 2017) using transmission electron microscopy
153 (TEM). Here, the magnetic nanoparticles stock solution (100 μL) was washed three times
154 with PBS buffer (pH 7.4) in a magnetic field to separate the nanoparticles from the storage
155 solution. EDC and NHS solutions (each at a concentration of 100 mM) were prepared
156 immediately before use. A volume of 250 μL each of EDC and NHS solutions were added to
157 the washed magnetic nanoparticles and incubated with slow rotation at room temperature for
158 90 min. Then, the reaction mixture was rinsed three times with HCl (1 mM) at 4 $^{\circ}\text{C}$ in a
159 magnetic field. Subsequently, 100 μL of purified Ab-TC polyclonal antibody (100 $\mu\text{g}\cdot\text{mL}^{-1}$)
160 was added to the activated nanoparticles and incubated for 2 h at 4 $^{\circ}\text{C}$. The antibody-coated
161 nanoparticles were washed three times with PBS buffer (pH 7.4). The non-reacted activated
162 carboxylic acid groups were blocked with ethanolamine (1% v/v) in PBS buffer for 10 min.
163 The antibody-coated magnetic nanoparticles were then separated from the mixture,
164 resuspended in 500 μL of PBS buffer and incubated with different concentrations of TC (Fig.
165 1B). Simultaneously, the gold μWEs were functionalized by CMA and TC as described in
166 paragraph 2.2.1.

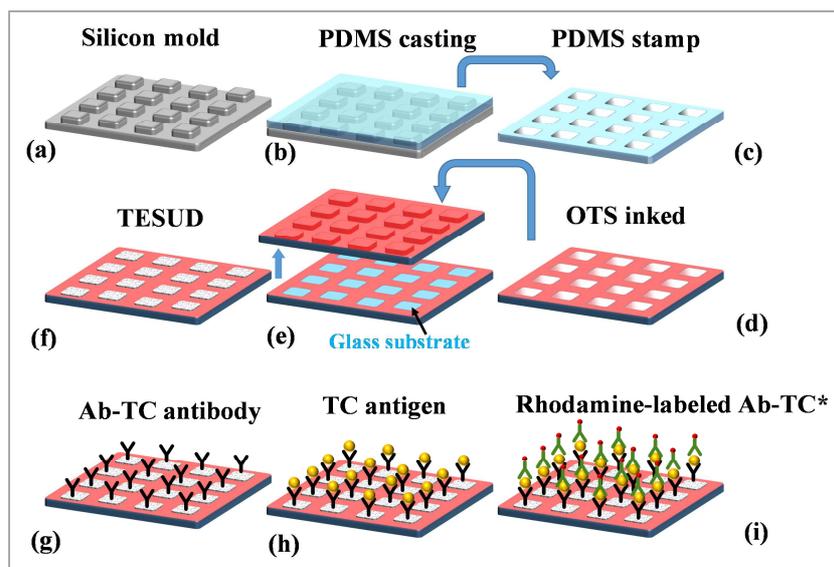
2.2.3 Functionalization with Py/Py-COOH/MNPs

167
168 This functionalization was performed by electrodepositing Py/Py-COOH/MNPs onto
169 microelectrodes by using pulsed chronoamperometric technique (PCA). Briefly, two
170 continuous repetition potentials were applied: the first potential $E_1=-0.2$ V for $t_1=0.1$ s,
171 induces a cathodic electrodeposition process, while the second potential $E_2=0.2$ V for $t_2=0.1$ s
172 oxidizes the electrode surface, and in particular, cleans it from eventual extraneous deposited
173 species. The cycling process was iterated 30 times. Then, the gold μWEs were rinsed with
174 distilled water. Besides, it is interesting to point out that the amplitude of the charge-discharge
175 process remains practically unchanged during the prolonged cycling process, indicating that
176 the electrode surface exhibits a homogenous Py/Py-COOH/MNPs deposition. The activation
177 of carboxylic groups, present in the Py/Py-COOH/MNPs, was carried out by the mixture of
178 EDC/NHS (0.4 M/0.1 M) prepared as described above. The biofunctionalization and blocking
179 steps were performed by incubating the sensor in TC solution (100 $\mu\text{g}\cdot\text{mL}^{-1}$) and
180 ethanolamine/PBS (1% v/v), respectively. Finally, the immunosensor was rinsed with PBS
181 and used for TC detection (Fig. 1C). The homogeneity and the roughness of the resulted
182 μWEs surface were already examined in our preceding study (Hassani et al., 2017). This was
183 conducted by two-, and three-dimensional surface topography using atomic force microscopy
184 (AFM). The interest of using this technique is to ensure that the μWE surfaces were entirely
185 homogeneous with the presence of spherical shapes indicating the presence of MNPs coated
186 with Py/Py-COOH.

2.3. Micro-contact printing

187
188 The soft-lithographical technique called microcontact printing (μCP) of self-
189 assembled monolayers (SAMs) was employed to check the immune TC/Ab-TC reactivity. For
190 this purpose, an elastomeric stamp based on polydimethylsiloxane (PDMS) was fabricated by
191 replica molding (RM) as already described in previous work (Baraket et al., 2013). A mixture
192 of pre-polymer PDMS and curing agent (10:1 w/w) was poured onto a silanized silicon-mold
193 which contains micro-pillars on relief of its surface (Fig. 2a). Subsequently, complete
194 degassing of PDMS/silanized silicon-mold was carried out to ensure that all air bubbles which
195 may create defects on the PDMS stamp surface have been removed (Fig. 2b). After the
196 polymerization step at 52 $^{\circ}\text{C}$ for 1 hour, the PDMS stamp was peeled-off from the silicon

197 mold bearing the micro holes on its surface (Fig. 2c). The stamp was then inked by immersion
 198 for 1 min in heptane solution containing octadecyltrichlorosilane (OTS) (0.1%, v/v) and
 199 carbon tetrachloride (2%, v/v), then dried with a stream of nitrogen (Fig. 2d). Simultaneously,
 200 glass substrates were cleaned with acetone then ethanol, rinsed with distilled water and then
 201 dried with nitrogen. The substrates were activated by submersion in piranha solution (3:1 v/v
 202 of H₂SO₄: H₂O₂) for 30 min then thoroughly rinsed with distilled water and dried with
 203 nitrogen. Afterwards, the μ CP was realized by putting the prepared PDMS stamp in
 204 immediate and conformal contact with a freshly activated glass substrate (Fig. 2e). The
 205 PDMS stamp was peeled-off from the substrate, leaving then the SAMs of OTS on the glass
 206 surface which was incubated afterwards at 100 °C for 45 min to enhance OTS adhesion.
 207 The freshly prepared substrates were submerged in an ethanol solution containing 1% of
 208 TESUD, rinsed with ethanol, dried with a stream of nitrogen and then heated in a vacuum
 209 stove at 100 °C for 60 min to complete the silanization process (Fig. 2f). Subsequently, the
 210 functionalized substrate surface (FSS) was incubated in 250 μ L of Ab-TC antibody (10
 211 μ g.mL⁻¹) already diluted in 4 mM of sodium cyanoborohydride. The use of this reagent was
 212 conducted to avoid adversely reducing aldehydes to nonreactive hydroxyls, allowing covalent
 213 bonding of Ab-TC with the amine of TESUD (Fig. 2g). After that, the FSS were incubated in
 214 TC solution (0.1 pg.mL⁻¹) for 1 h to allow the antibody-antigen interaction followed by rinsing
 215 in PBS and drying under nitrogen (Fig. 2h). Finally, the rhodamine-labeled polyclonal Ab-
 216 TC* (10 μ g.mL⁻¹) were dropped on the FSS for 1 h (Fig. 2i). The samples were then rinsed in
 217 PBS, dried under nitrogen and observed by fluorescence microscopy.



218
 219 **Fig. 2.** Microcontact printing process to immobilize Ab-TC onto TESUD patterns and
 220 sandwich immunoassay with TC and rhodamine-labeled Ab-TC.

221 2.4. Fluorescence microscopy characterization

222 Fluorescence images, as a rapid tool showing the recognition of Ab-TC for its
 223 corresponding antigen (TC), were taken using a fluorescence microscope (Zeiss Axioplan 2
 224 Imaging), equipped with 10 \times and 40 \times lenses and a monochrome camera. Samples were
 225 observed by fluorescent light: TC sample was excited with a 550 (\pm 25) nm band-pass filter
 226 and fluorescence from the sample was observed with a 605 (\pm 70) nm band-pass filter.

227 2.5. Electrochemical measurements

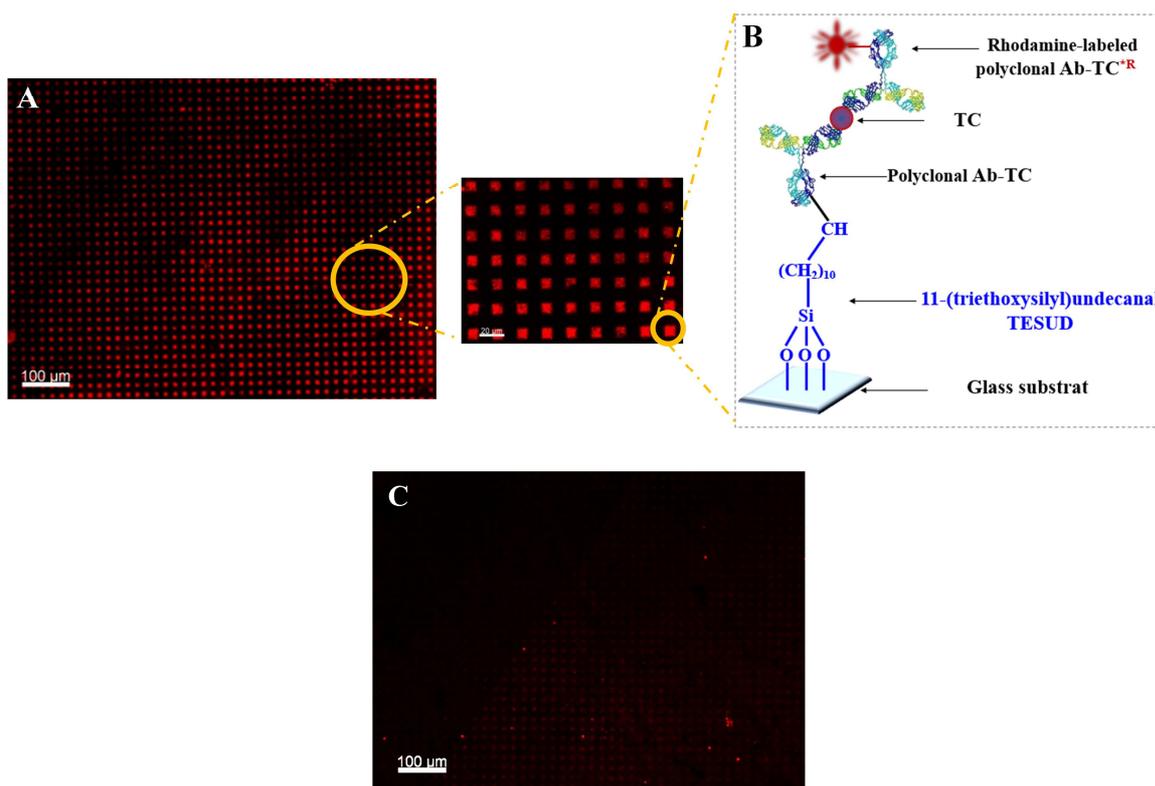
228 All steps of gold μ WEs modification were characterized by cyclic voltammetry (CV)
 229 and electrochemical impedance spectroscopy (EIS) techniques using a multi-channel

230 potentiostat (VMP3 Bio-logic-Science Instrumentation, France). The electrochemical
 231 configuration was performed by using integrated μ WEs, RE and CE of the Bio-MEMS. All
 232 the measurements were performed in a solution of 5.0 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ prepared in PBS
 233 buffer (pH 7.4) and in a Faraday cage. For the cyclic voltammetric measurements, the
 234 potential was scanned from 0.3 V to 0.6 V with a scan rate of $100 \text{ mV}\cdot\text{s}^{-1}$. While the
 235 electrochemical impedance was measured in the 150 mHz-200 kHz frequency range with a
 236 DC potential of -0.3 V/ref and an AC potential of 90 mV.

237 3. Results and discussion

238 3.1 Fluorescence analysis

239 Fluorescent imaging is considered as an effective tool for bio-functionalization
 240 analysis. It's used to certify that the corresponding immune detection process is taking place
 241 based upon the recognition of the antibody for its corresponding antigen. In this study,
 242 fluorescent antibodies were immobilized (chemically) onto a glass substrate for the detection
 243 of TC antigens. Fig. 3A-B shows the fluorescent pattern of Ab-TC^{R} . The red fluorescent
 244 regions indicate tags in which the antibodies are specifically immobilized, while the extinct
 245 areas are those blocked with OTS. In the Fig.3C, we notice an absence of fluorescence due to
 246 the non-cross-reactivity of the doxycycline (DX), as interfering molecules, towards Ab-TC .
 247 This immuno-fluorescent test has reported the strong reactivity of Ab-TC against TC which
 248 can, therefore, be applied for immunosensor development.



249 **Fig. 3.** Fluorescent images of $(\text{Ab-TC}^{\text{R}})$ chemically immobilized on glass substrates after
 250 recognition of TC, (A) Positive homogenous pattern with the blackened regions
 251 corresponding to the OTS blocked glass surface. (B) Magnification of the positive pattern
 252 showing specific detection between Ab-TC^{R} and TC. (C) Fluorescent image of the
 253 biorecognition assay between doxycycline and Ab-TC^{R} .
 254

255 3.2 Electrochemical characterization of modified gold μ WEs

256 The behaviors of CMA and Py/Py-COOH/MNPs films were checked for each step of the
257 Bio-MEMS functionalization by CV analysis. The process of CMA electro-addressing is
258 shown in Fig. S1A (Supplementary information). The initial cycle shows a large and
259 irreversible cathodic wave with a peak potential at -0.8 V revealing diazotated CMA onto the
260 gold μ WEs surface by diazonium salt reduction. However, the reduction wave has been kept
261 unchanged upon the deposition of CMA. This functionalization was checked by variation of
262 the CV before and after gold μ WEs modification (Fig. S1B) (Supplementary information). In
263 this figure, a significant decrease in peak-to-peak of the CV cycles was noticed after CMA
264 electro-deposition against the bare gold μ WEs. This was referred to the broad passivation
265 region of this microelectrodes.

266 While Fig. S1C (Supplementary information) shows the chrono-amperometric process for
267 Py/Py-COOH/MNPs electrodeposition. During this step it was interesting to specify that the
268 amplitude of the charge-discharge process remains nearly unchanged, indicating that the
269 electrode surface displays homogenous Py/Py-COOH/MNPs layer. CV analysis were also
270 investigated to examine the behavior of the μ WEs surfaces after Py/Py-COOH/MNPs
271 electrodeposition. Fig. S1D (Supplementary information) shows the considerable increase in
272 the peak current of cyclic voltammograms after Py/Py-COOH/MNPs deposition compared to
273 bare gold. This was explained by the conductive behavior of Py/Py-COOH coated on MNPs.
274 At these steps, the modified gold μ WEs were ready for bio-functionalization with the target
275 analyte.

276 3.3 Optimization of conditions for detection

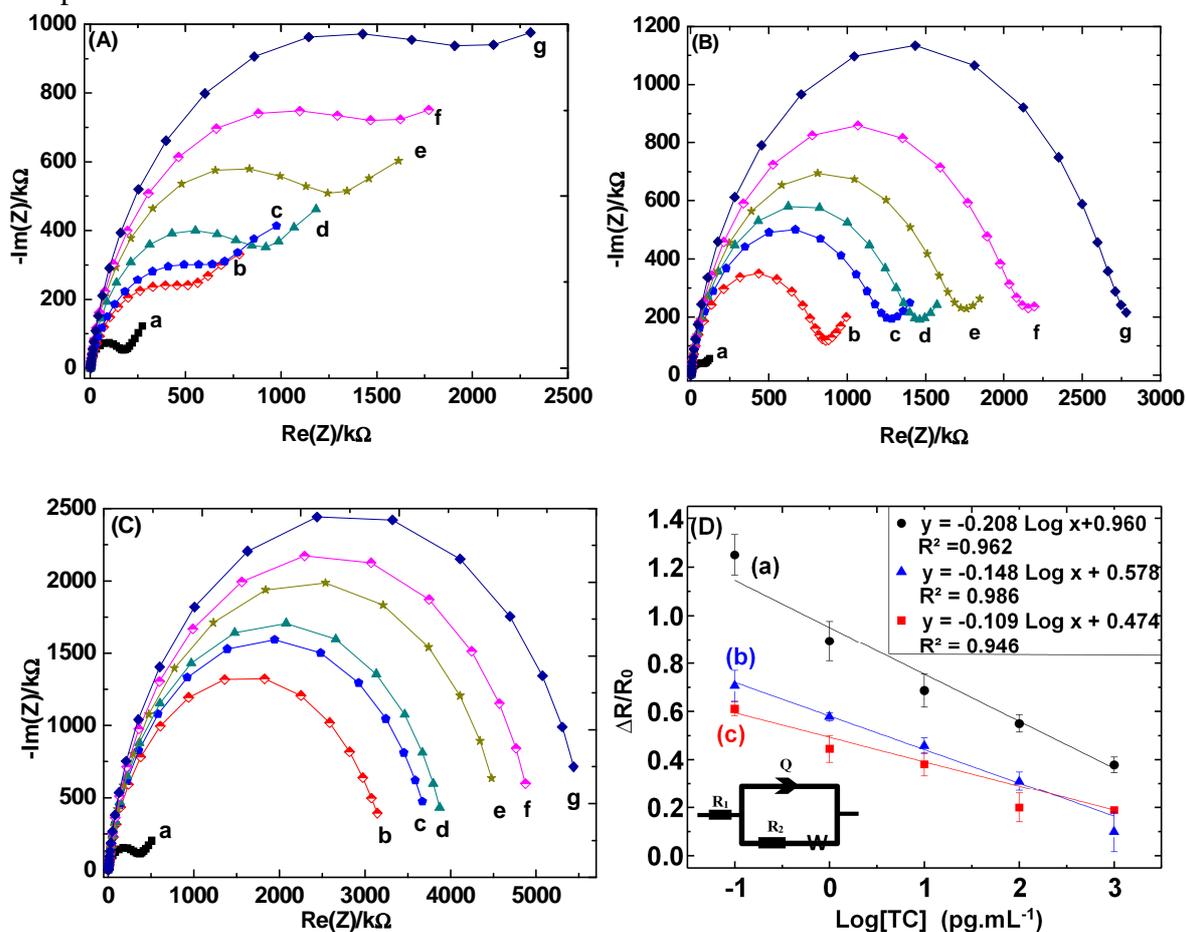
277 The amount of TC immobilized on the surface of gold μ WEs as well as the antibody
278 concentration were subject to optimization described in (Supplementary information).

279 3.4 Competitive detection of TC

280 In this study, the quantification of the analyte has been made by the competitive
281 approach to enhance the detection signal of TC with a small molecular weight (444 Da)
282 compared to Ab-TC (160 kDa). Furthermore, this approach was advantaged in the present
283 work to make pre-concentration allowing to detect only TC and eliminate other irrelevant
284 molecules. This step was performed by incubating the μ WE surfaces of the Bio-MEMS in a
285 mixture of a fixed concentration of Ab-TC previously optimized ($10 \mu\text{g}\cdot\text{mL}^{-1}$) and decreasing
286 levels of standard solutions of TC from $1000 \text{ pg}\cdot\text{mL}^{-1}$ to $0.1 \text{ pg}\cdot\text{mL}^{-1}$ during 30 min at 4°C for
287 each concentration. At least three independent replicated experiments were carried out.
288 Ultimately, the electrode surface was rinsed with PBST (10 mM PBS with 0.05% Tween 20)
289 and analyzed with EIS. Fig. 4A-C show the obtained Nyquist plot semi-circles for the three
290 functionalizations of μ WE surfaces. It can be noticed that for the three approaches, the
291 Nyquist plot semi-circles increase by decreasing TC concentrations. As awaited, this level
292 decreases produce an increase in the charge transfer resistance owed to the presence of free
293 antibodies reacting with TC immobilized on the μ WEs surface.

294 An excellent fitting between the simulated and experimental spectra was obtained for
295 each TC concentration by using the Randles equivalent circuit shown in the inset of Fig. 4D
296 (where R_s is the solution resistance, R_{ct} is the charge-transfer resistance, W is the Warburg
297 impedance and Q is the constant phase element). For each TC concentration, the value of
298 $\Delta R/R_0$ was calculated when R is the charge transfer resistance. The normalized data show
299 three linear equations: $y_a = -0.208 x + 0.960$, $y_b = -0.148 x + 0.578$ and $y_c = -0.109 x + 0.474$
300 with the determination coefficients of 0.962, 0.986 and 0.946, respectively. As can be seen
301 from this figure, the detection sensitivity was highly improved for the Py/Py-COOH/MNPs
302 gold surface modification. For this approach, the configured impedimetric immunosensor
303 have provided the more sensitivity of $0.208 \text{ mL}\cdot\text{pg}^{-1}$ with a limit of detection (LOD) of 1.2
304 $\text{pg}\cdot\text{mL}^{-1}$. It is appealing to notice that this LOD value is nearly 83000 times lower than the

305 maximum residue limits of TC in honey fixed by European regulation. As listed in Table 1,
 306 the proposed system provides relatively wide linear ranges and low LODs in comparison with
 307 other published studies.



308
 309

310

311 **Fig. 4.** Nyquist impedance plots in 5 mM of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ for competitive detection of TC on
 312 the modified μWEs by: (A) Functionalization with CMA, (B) Functionalization with CMA
 313 followed by preconcentration step, (C) Functionalization with Py/Py-COOH/MNPs, where (a)
 314 Bare gold, (b) After TC immobilization at $100 \mu\text{g}\cdot\text{mL}^{-1}$, (c-g) After competitive detection of
 315 TC at 1000, 100, 10, 1 and $0,1 \text{ pg}\cdot\text{mL}^{-1}$, respectively. (D) The sensitivity of the immunosensor
 316 by normalization of Nyquist plot data, inset: the equivalent circuit used for EIS fitting.

317 **Table 1.** Comparison of different techniques for TC detection.

Methods	Linear range ($\text{ng}\cdot\text{mL}^{-1}$)	LOD ($\text{ng}\cdot\text{mL}^{-1}$)	Real sample	References
High-performance liquid-chromatography with fluorescence detection	15-5000	5	Milk	(Kargin et al., 2016)
Photoelectrochemical aptasensor	0.2-1000.0	0.01	Drug sample	(Han et al., 2018)
Terahertz spectroscopy	0-20 \times 10 ⁶	0.45	Water and	(Qin et al., 2017)

			milk	
Enzyme-linked immunosorbent assay and immunochromatographic assay	0.26–2.00	15	Milk and honey	(Chen et al., 2016)
Electrochemical immunoassay	0.05-100	0.006	Honey milk and peanuts	(Que et al., 2013)
Molecularly imprinted polymer mixed with solid-phase extraction	20-600	20	Milk	(Xie et al., 2018)
Electrochemical immunosensor based on the chitosan-magnetic nanoparticles	0.08-1.00	0.03	Milk	(Liu et al., 2016)
Amperometric immunosensor	0.0005 -500	0.86	Milk	(Conzuelo et al., 2013)
This work	0.0001-1	0.0012	Honey	-

318 3.5 Interferences study

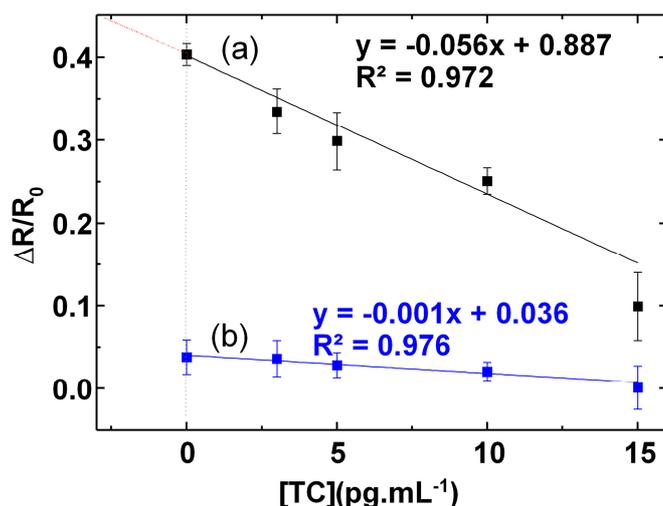
319 The selectivity of the proposed system was investigated in the presence of various
 320 antibiotics from the same family of cyclines that can have cross-reactivity with Ab-TC
 321 antibody. This study was performed by comparing the responses for TC detection with three
 322 analogous molecules namely: chlortetracycline (CT), doxycycline (DX), and oxytetracycline
 323 (OX) (Fig. S3) (Supplementary information). Consequently, this immunosensor was found
 324 to be very selective for TC and can provide reliable results regardless of the presence of the
 325 interfering molecules.

326 3.6 Analysis of spiked honey samples

327 The TC determination in real samples was performed by spiking analyte in blank
 328 honey matrices which indicate that the calibration curve is suitable to determine the TC
 329 content in real unknown samples (Supplementary information). The results of the added
 330 spiked samples are shown in Table S1 (Supplementary information). Good recoveries from
 331 80% to 98% were obtained with the RSD ranging between 1% and 6%, which indicate that
 332 the calibration curve is suitable to determine the TC content in real unknown samples.

333 3.7 Detection of TC in unknown honey sample

334 The standard addition method was further employed to determine the TC
 335 concentration in unknown honey sample. The competitive detection of TC was performed
 336 by a mixture of different standard solutions (3 pg.mL⁻¹, 5 pg.mL⁻¹, 10 pg.mL⁻¹, 15 pg.mL⁻¹)
 337 with the presence of Ab-TC (10 µg.mL⁻¹). For all TC concentrations, the normalized charge
 338 transfer resistance $\Delta R/R_0$ were measured. The extrapolation of regression line predicts the
 339 TC concentration in the diluted honey sample at 2.52 pg.mL⁻¹. Consequently, the
 340 concentration of TC in the unknown sample was 25.2 pg.mL⁻¹ according to the dilution
 341 factor of 10 times (Fig. 5). This level of contamination remains lower than the MRLs of 10
 342 000 pg.mL⁻¹ established by Switzerland, UK, and Belgium.



343

344 **Fig. 5.** Standard addition plots of different TC spiking in diluted unknown honey sample,
 345 using (a) Ab-TC and (b) Ab-155 antibodies.

346 4. Conclusions

347 In the present work, we have discussed the development process of a novel sensitive
 348 and highly selective immunosensor based on 2D and 3D network of immobilization with the
 349 diazonium salt, and a new structure of MNPs coated with Py-COOH for specific detection of
 350 TC. Moreover, the electrochemical analysis performed by these materials allow high
 351 reproducibility and rapid response. The high sensitivity of this immunosensor was evaluated
 352 for the three functionalization methods and was highly improved for the Py/Py-
 353 COOH/MNPs gold surface modification. For this approach, the configured impedimetric
 354 immunosensor have provided a sensitivity of 0.208 mL.pg⁻¹ with a limit of detection of 1.2
 355 pg.mL⁻¹. The selectivity of the present immunosensor platform toward TC was higher when
 356 compared to analogous molecules, namely DX, CT and OX. We have presented a novel and
 357 less costly fabrication process to develop a highly sensing device able to detect low levels of
 358 TC residues in honey samples without any complicated pretreatment. The miniaturization of
 359 this immunosensor will be very beneficial to control the quality of food products on an
 360 industrial scale. However, there are still some limitations of this work related to the
 361 construction time which is a little long. Therefore, our further investigations in this direction
 362 are to shorten the development time of this immunosensor, to meet the requirements of a
 363 large scientific community which can extend its use for other applications. Future works will
 364 also be done, to assess the multi-functionalization of the same device allowing a
 365 simultaneous detection of several analytes.

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371 References

372 Baraket, A., Lee, M., Zine, N., Sigaud, M., Yaakoubi, N., Trivella, M.G., Zabala, M.,
 373 Bausells, J., Jaffrezic-Renault, N., Errachid, A., 2013. Diazonium modified gold
 374 microelectrodes onto polyimide substrates for impedimetric cytokine detection with an
 375 integrated Ag/AgCl reference electrode. Sensors and Actuators B: Chemical 189, 165–
 376 172.

377 Bargańska, Ż., Namieśnik, J., Ślebioda, M., 2011. Determination of antibiotic residues in
378 honey. *TrAC Trends in Analytical Chemistry* 30, 1035–1041.

379 Bougrini, M., Florea, A., Cristea, C., Sandulescu, R., Vocanson, F., Errachid, A., Bouchikhi,
380 B., El Bari, N., Jaffrezic-Renault, N., 2016. Development of a novel sensitive molecularly
381 imprinted polymer sensor based on electropolymerization of a microporous-metal-organic
382 framework for tetracycline detection in honey. *Food Control* 59, 424–429.

383 Chen, Y., Kong, D., Liu, L., Song, S., Kuang, H., Xu, C., 2016. Development of an ELISA
384 and immunochromatographic assay for tetracycline, oxytetracycline, and chlortetracycline
385 residues in milk and honey based on the class-specific monoclonal antibody. *Food*
386 *Analytical Methods* 9, 905–914.

387 Chopra, I., Roberts, M., 2001. Tetracycline antibiotics: mode of action, applications,
388 molecular biology, and epidemiology of bacterial resistance. *Microbiology and molecular*
389 *biology reviews* 65, 232–260.

390 Conzuelo, F., Campuzano, S., Gamella, M., Pinacho, D.G., Reviejo, A.J., Marco, M.P.,
391 Pingarrón, J.M., 2013. Integrated disposable electrochemical immunosensors for the
392 simultaneous determination of sulfonamide and tetracycline antibiotics residues in milk.
393 *Biosensors and Bioelectronics* 50, 100–105.

394 Deng, B., Xu, Q., Lu, H., Ye, L., Wang, Y., 2012. Pharmacokinetics and residues of
395 tetracycline in crucian carp muscle using capillary electrophoresis on-line coupled with
396 electrochemiluminescence detection. *Food Chemistry* 134, 2350–2354.

397 Desmarchelier, A., Anizan, S., Minh Tien, M., Savoy, M.-C., Bion, C., 2018. Determination
398 of five tetracyclines and their epimers by LC-MS/MS based on a liquid-liquid extraction
399 with low temperature partitioning. *Food Additives & Contaminants: Part A*.

400 Economou, V., Gousia, P., 2015. Agriculture and food animals as a source of antimicrobial-
401 resistant bacteria. *Infection and drug resistance* 8, 49.

402 Eliopoulos, G.M., Roberts, M.C., 2003. Tetracycline therapy: update.
403 *Clinical infectious diseases* 36, 462–467.

404 European Commission, 1999. European Commission (1999) *Off J Eur Union* L 60:16–52.

405 Gaurav, A., Gill, J.P.S., Aulakh, R.S., Bedi, J.S., 2014. ELISA based monitoring and analysis
406 of tetracycline residues in cattle milk in various districts of Punjab. *Veterinary World* 7.

407 Han, Q., Wang, R., Xing, B., Chi, H., Wu, D., Wei, Q., 2018. Label-free
408 photoelectrochemical aptasensor for tetracycline detection based on cerium doped CdS
409 sensitized BiYWO 6. *Biosensors and Bioelectronics* 106, 7-13

410 Hassani, N.E.A.E., Baraket, A., Neto, E.T.T., Lee, M., Salvador, J.-P., Marco, M., Bausells,
411 J., Bari, N.E., Bouchikhi, B., Elaissari, A., Errachid, A., Zine, N., 2017. Novel strategy for
412 sulfapyridine detection using a fully integrated electrochemical Bio-MEMS: Application
413 to honey analysis. *Biosensors and Bioelectronics* 93, 282–288.

414 Joshi, M.D., Anderson, J.L., 2012. Recent advances of ionic liquids in separation science and
415 mass spectrometry. *Rsc Advances* 2, 5470–5484.

416 Kargin, I.D., Sokolova, L.S., Pirogov, A.V., Shpigun, O.A., 2016. HPLC determination of
417 tetracycline antibiotics in milk with post-column derivatization and fluorescence
418 detection. *Inorganic Materials* 52, 1365–1369.

419 Kivrak, I., KIVRAK, Ş., Harmandar, M., 2016. Development of a rapid method for the
420 determination of antibiotic residues in honey using UPLC-ESI-MS/MS. *Food Science and*
421 *Technology (Campinas)* 36, 90–96.

422 Lan, L., Yao, Y., Ping, J., Ying, Y., 2017. Recent advances in nanomaterial-based biosensors
423 for antibiotics detection. *Biosensors and Bioelectronics* 91, 504–514.

424 Liu, X., Zheng, S., Hu, Y., Li, Z., Luo, F., He, Z., 2016. Electrochemical immunosensor based
425 on the chitosan-magnetic nanoparticles for detection of tetracycline. *Food Analytical*
426 *Methods* 9, 2972–2978.

427 Lv, Y.-K., Zhang, J.-Q., He, Y.-D., Zhang, J., Sun, H.-W., 2014. Adsorption-controlled
428 preparation of molecularly imprinted hybrid composites for selective extraction of
429 tetracycline residues from honey and milk. *New Journal of Chemistry* 38, 802–808.

430 Majdinasab, M., Yaqub, M., Rahim, A., Catanante, G., Hayat, A., Marty, J.L., 2017. An
431 Overview on Recent Progress in Electrochemical Biosensors for Antimicrobial Drug
432 Residues in Animal-Derived Food. *Sensors* 17, 1947.

433 Mitra, S., 2004. *Sample Preparation Techniques in Analytical Chemistry*. John Wiley & Sons.

434 Nelson, M.L., Dinardo, A., Hochberg, J., Armelagos, G.J., 2010. Brief communication: mass
435 spectroscopic characterization of tetracycline in the skeletal remains of an ancient
436 population from Sudanese Nubia 350–550 CE. *American journal of physical*
437 *anthropology* 143, 151–154.

438 O'Connor, S., Aga, D.S., 2007. Analysis of tetracycline antibiotics in soil: advances in
439 extraction, clean-up, and quantification. *TrAC Trends in Analytical Chemistry* 26, 456–
440 465.

441 Pokrant, E.V., Maddaleno, A.E., Araya, C.E., San Martín, B.V., Cornejo, J., 2018. In-House
442 Validation of HPLC-MS/MS Methods for Detection and Quantification of Tetracyclines
443 in Edible Tissues and Feathers of Broiler Chickens. *J. Braz. Chem. Soc* 29, 659–668.

444 Qin, J., Xie, L., Ying, Y., 2017. Rapid analysis of tetracycline hydrochloride solution by
445 attenuated total reflection terahertz time-domain spectroscopy. *Food chemistry* 224, 262–
446 269.

447 Qin, J., Xie, L., Ying, Y., 2016. A high-sensitivity terahertz spectroscopy technology for
448 tetracycline hydrochloride detection using metamaterials. *Food chemistry* 211, 300–305.

449 Que, X., Chen, X., Fu, L., Lai, W., Zhuang, J., Chen, G., Tang, D., 2013. Platinum-catalyzed
450 hydrogen evolution reaction for sensitive electrochemical immunoassay of tetracycline
451 residues. *Journal of Electroanalytical Chemistry* 704, 111–117.

452 Shi, X., Meng, Y., Liu, J., Sun, A., Li, D., Yao, C., Lu, Y., Chen, J., 2011. Group-selective
453 molecularly imprinted polymer solid-phase extraction for the simultaneous determination
454 of six sulfonamides in aquaculture products. *Journal of Chromatography B* 879, 1071–
455 1076.

456 Tang, Y., Zhang, J., Liu, J.-H., Gapparov, I., Wang, S., Dong, Y., Su, H., Tan, T., 2017. The
457 development of a graphene oxide-based aptasensor used for the detection of tetracycline
458 in honey. *Analytical Methods* 9, 1133–1140.

459 Tenório-Neto, E.T., Baraket, A., Kabbaj, D., Zine, N., Errachid, A., Fessi, H., Kunita, M.H.,
460 Elaissari, A., 2016. Submicron magnetic core conducting polypyrrole polymer shell:
461 Preparation and characterization. *Materials Science and Engineering: C* 61, 688–694.

462 Wang, S., Liu, J., Yong, W., Chen, Q., Zhang, L., Dong, Y., Su, H., Tan, T., 2015. A direct
463 competitive assay-based aptasensor for sensitive determination of tetracycline residue in
464 honey. *Talanta* 131, 562–569.

465 Wang, S., Yong, W., Liu, J., Zhang, L., Chen, Q., Dong, Y., 2014. Development of an
466 indirect competitive assay-based aptasensor for highly sensitive detection of tetracycline
467 residue in honey. *Biosensors and Bioelectronics* 57, 192–198.

468 Xie, Y., Hu, Q., Zhao, M., Cheng, Y., Guo, Y., Qian, H., Yao, W., 2018. Simultaneous
469 Determination of Erythromycin, Tetracycline, and Chloramphenicol Residue in Raw Milk
470 by Molecularly Imprinted Polymer Mixed with Solid-Phase Extraction. *Food Analytical*
471 *Methods* 11, 374–381.

472 Yi, X., Li, L., Peng, Y., Guo, L., 2014. A universal electrochemical sensing system for small
473 biomolecules using target-mediated sticky ends-based ligation-rolling circle
474 amplification. *Biosensors and Bioelectronics* 57, 103–109.

475