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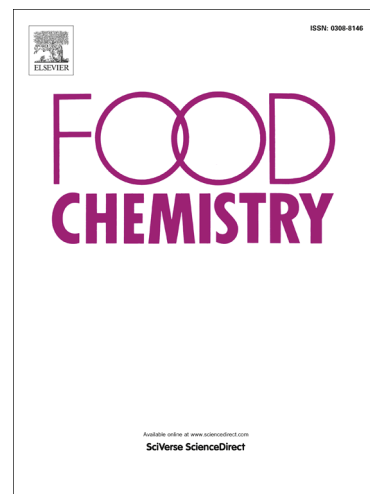
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**Surface plasmon resonance biosensor for the determination of
3-methyl-quinoxaline-2-carboxylic acid, the marker residue of olaquinox,
in swine tissues**

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Abstract

To monitor the illegal use of olaquinox in animals, a monoclonal antibody-based surface plasmon resonance (SPR) biosensor method has been developed to detect 3-methyl-quinoxaline-2-carboxylic acid, the marker residues of olaquinox, in swine tissues. The limit of detection was $1.4 \mu\text{g kg}^{-1}$ in swine muscle and $2.7 \mu\text{g kg}^{-1}$ in swine liver, which are lower than the EU recommended concentration ($10 \mu\text{g kg}^{-1}$). The recoveries were from 82% to 104.6%, with coefficients of variation of less than 12.2%. Good correlations between SPR and HPLC results ($r = 0.9806$, muscle; $r = 0.9698$, liver) and between SPR and ic-ELISA results ($r = 0.9918$, muscle; $r = 0.9873$, liver) were observed in the affected tissues, which demonstrated the reliability of the SPR method. This method would be a rapid and reliable tool for the screening of the residues of olaquinox in the edible tissues of animals.

Keywords: surface plasmon resonance biosensor; 3-methyl-quinoxaline-2-carboxylic acid; olaquinox; residues; swine tissues

15 1. Introduction

16 Olaquinox (OLA, Figure 1), an antibacterial growth-promoting agent in quinoxalines,
17 was widely used in swine for the control of swine dysentery and/or bacterial enteritis in young
18 swine, to improve feed efficiency and increase the rate of weight gain (FAO/WHO, 1990). It
19 was also widely used in poultry and aquatic animals illegally around the world for decades
20 (Carta, Corona, & Loriga. 2005). However, OLA was a mutagen and suspected carcinogen
21 with photosensitive toxicity (Eberlein, Bergner, & Przybilla, 1992; Emmert, Schauder, Palm,
22 Hallier, & Emmert, 2007; FAO/WHO, 1990), renal toxicity (FAO/WHO, 1990), genotoxicity
23 (Chen et al., 2009; FAO/WHO, 1990; Ihsan et al., 2013; Liu et al., 2016; Yang et al., 2015;
24 Zou et al., 2009), cytotoxicity (Huang et al., 2010; Li et al., 2016; Zou et al., 2011). Therefore,
25 the use of OLA in food-producing animals had been banned in some countries and regions
26 (Commission Regulation (EC) No 2788/98, 1998; FAO/WHO, 1995). In China, OLA has
27 been approved as a feed additive for piglets weighing less than 35 kg with a withdrawal
28 period of 35 days (Announcement No. 168, 2001). Recently, The Ministry of Agriculture
29 (MOA) of China stipulated that OLA will be withdrawn from market before 2020
30 (Announcement No. 2638, 2018).

31 Although regulations exist, non-compliance remains a serious issue due to the economic
32 benefits of OLA in food animals and the broad availability of commercial OLA worldwide.
33 Therefore, the development of a rapid and reliable detection method for screening the residues
34 of OLA in edible tissues of food animals is necessary. Traditionally, the use of such
35 compounds should be controlled by the analysis of their respective metabolites in the target
36 tissues. However, because of the lack of sufficient data on metabolism and residue depletion,
37 the acceptable daily intake of OLA has not been recommended, the marker residue (MR) of
38 OLA was not defined, and the maximum residue limits (MRLs) of OLA were not established
39 (FAO/WHO, 1990). Therefore, the safety of edible tissues in the animals administered with

40 OLA could not be guaranteed.

41 In 1995, 3-methylquinoxaline-2-carboxylic acid (MQCA) was tentatively recommended
42 as the MR of OLA by FAO/WHO (1995) for the control of OLA residues in the edible tissues
43 of animals treated with OLA. In 2007, European Reference Laboratory proposed for MQCA
44 in meat a recommended concentration (RC) of $10 \mu\text{g kg}^{-1}$ for analytical method (CRL
45 Guidance Paper, 2007). In China, the MRLs of MQCA, which is in keeping with the report of
46 JECFA, is set at $50 \mu\text{g kg}^{-1}$ in swine liver and $4 \mu\text{g kg}^{-1}$ in swine muscle (Announcement No.
47 235, 2002; FAO/WHO, 1995).

48 During the past decades, several physicochemical methods, including high performance
49 liquid chromatography (HPLC) (Wu et al., 2007; Zhang, Zheng, Zhang, Chen, & Mei, 2011)
50 and liquid chromatography tandem mass spectrometry (LC-MS/MS) (Boison, Lee, & Gedir,
51 2009; Hutchinson, Young, & Kennedy, 2005; Merou, Kaklamanos, & Theodoridis, 2012),
52 have been developed for olaquinox and its marker residue MQCA. However, due to the high
53 cost and the need for skilled scientists, these physicochemical methods are more suitable for
54 confirmatory analysis than screening methods. As an alternative, an antibody-based rapid and
55 sensitive indirect competitive enzyme-linked immunosorbent assay screening method
56 (ic-ELISA) for detecting MQCA residues had also been published (Cheng et al., 2013; Jiang,
57 Beier, Wang, Wu, & Shen, 2013; Yue et al., 2009; Zhang et al., 2015). Although these
58 methods are available, they are acknowledged to be tedious with time-consuming steps such
59 as washing, separation of bound and free antigen, and a lengthy incubation time.

60 The emergence of biosensor-based immunological assays, such as the optical based
61 surface plasmon resonance (SPR) detection which is a label-free technique for the sensitive
62 real-time monitoring of molecular interactions, in the field of food safety testing offers
63 prominent advantages such as high sensitivity, low limit of detection, specificity and
64 robustness (Campbell et al., 2009). In the past few years, the SPR method has been used

65 successfully in the field of food safety detection (Caldow et al., 2005; Devlin et al., 2014;
66 Hirakawa et al., 2018; Olaru, Bala, Jaffrezic-Renault, & Aboul-Enein, 2015; Pan, Li, Wang,
67 Sheng, & Wang, 2017; Yuan, Deng, Lauren, Aguilar, & Wu, 2009). Nevertheless, to our best
68 knowledge, no SPR biosensor method has been developed for detection of MQCA residues.

69 Therefore, the aim of the present study was to develop and validate an SPR biosensor
70 method for the analysis of MQCA residues in edible animal tissue samples without the
71 necessity of a complicated sample preparation procedure.

72 2. Materials and Methods

73 2.1 Chemicals and reagents

74 Ovalbumin (OVA), dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS),
75 N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and ethanolamine
76 hydrochloride (1 M, pH 8.5) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A
77 carboxymethylated dextran CM5 chip was purchased from Biacore AB (Uppsala, Sweden).
78 HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA and 0.005% Surfactant P20, pH
79 7.4) were also purchased from Biacore AB (Uppsala, Sweden). Purified and deionized water
80 was obtained from Millipore reverse osmosis and milli-Q polishing systems. The primary
81 stock solution at 1 mg mL⁻¹ was prepared by dissolving the compound in the purified and
82 deionized water (milli-Q). Serial dilutions were prepared by diluting the primary stock
83 solution in HBS-EP buffer. All other chemicals were also purchased from Sigma-Aldrich (St.
84 Louis, MO, USA) and were of analytical grade.

85 The standard analytes MQCA, NH₂MQCA, and NH₂MQCA-OVA (shown in Figure 1)
86 and the anti-MQCA monoclonal antibody 5B10 (mAb 5B10, 1 mg mL⁻¹) were supplied by the
87 Institute of Veterinary Pharmaceuticals, Huazhong Agricultural University (Wuhan, China), in
88 which their structures and/or their characteristics had been guaranteed (Zhang et al., 2015).
89 Briefly, the standard analyte MQCA was purchased from the Institute of Veterinary Drug

90 Control (Beijing, China). The hapten NH₂MQCA was identified by nuclear magnetic
91 resonance (NMR, Bruker-400 spectrometers, Bruker BioSpin, Switzerland) and ion trap and
92 time-of-flight mass spectrometers coupled with a HPLC system (LC/MS-ITTOF, Shimadzu,
93 Kyoto, Japan), respectively. MS m/z calculated for C₁₀H₉N₃O₂ [M+H]⁺ 204.0768, found
94 204.0763. ¹H NMR (DMSO-d₆) δ_H: 2.73 (s, 3H, C3-CH₃), 6.84, 7.23, 7.39 (each 1H, Ar-H),
95 4.60 (br, 2H, -NH₂), 12.0~13.0 (br, 1H, -COOH). The synthesized coating conjugate
96 NH₂MQCA-OVA was characterized by 8453 UV-Visible spectrophotometer (Agilent 8453,
97 USA), which the estimated incorporation rates of conjugates is 9.7. The obtained specific
98 mAb 5B10 that has isotype IgG1 showed an IC₅₀ value of 17.7 μg L⁻¹ for MQCA and did not
99 exhibit measurable cross-reactivity (CR) with other quinoxalines and their analogues (CR <
100 0.1%) such as OLA, quinocetone, mequindox, cyadox, carbadox, quinoxaline-2-carboxylic
101 acid, desoxymequindox, desoxyquinocetone, and desoxyolaquindox (Zhang et al., 2015).

102 2.2. Immobilization of ligand on CM5 chip

103 As shown in Figure 1, the NH₂MQCA contains a standard amino group. Therefore, it is
104 immobilized covalently on a CM5 chip by an amine coupling procedure in two steps
105 according to Campbell's procedure (Campbell et al., 2009) with modification. Briefly, the
106 chip was allowed to equilibrate to room temperature, and a continuous flow of HBS-EP buffer
107 passing over the sensor surface at a flow rate of 5 μL min⁻¹ was maintained. OVA, as a ligand,
108 was first immobilized on the CM5 chip surface. In this procedure, the carboxyl groups on the
109 CM5 chip surface were activated by 50 μL of a 1:1 (v/v) mixture of 0.4 M
110 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.1 M
111 N-hydroxysuccinimide (NHS) at a flow rate of 7 μL min⁻¹ for 7 min. OVA (10 mg ml⁻¹, pH
112 4.0, 10 mM sodium acetate (NaAc) buffer) was run onto the activated chip at a flow rate of 6
113 μL min⁻¹ for 15 min, with OVA immobilized on the CM5 chip. The remaining un-reacted
114 groups on the sensor surface were deactivated by the injection of ethanolamine-HCl (1 M, pH

115 8.5) at a flow rate of $5 \mu\text{L min}^{-1}$ for 7 min. After the surface was washed with 10 mM sodium
116 hydroxide (NaOH) at a flow rate of $20 \mu\text{L min}^{-1}$ for 1 min (this step was repeated 3 times), 10
117 mM glycine-HCl (pH 3.0) was used to regenerate the chip at a rate of $20 \mu\text{L min}^{-1}$ for 1 min.
118 Second, the same procedure was used to immobilize the ligand NH₂MQCA ($200 \mu\text{g mL}^{-1}$) on
119 the surface of the chip and/or on the carrier protein OVA that had been immobilized on the
120 surface of the chip. Finally, the chip surface was washed with deionized water, then dried
121 under a stream of nitrogen gas, and stored in a desiccated container ($4 \text{ }^\circ\text{C}$)

122 2.3 Optimization of the SPR-biosensor analytical conditions

123 The optical surface plasmon resonance (SPR) biosensor used was a Biacore 3000 (GE
124 Healthcare, Sweden) with Biacore[®] Q control software version 3.0.1, which was used for the
125 sensorgram and data analysis. Because the BIACORE Q Control Software offered a suitable
126 means of developing and running projects automatically under controlled conditions, only
127 limited parameters were necessary for the investigation of the development of the Biacore
128 3000 biosensor assay, including binder dilution, ratio of binder to standard, injection volume,
129 contact time, and the regeneration solution. In this project, studies were conducted at $25 \text{ }^\circ\text{C}$.
130 Briefly, mAb 5B10 (1 mg mL^{-1}) was diluted in HBS-EP buffer (1:100, 1:200, 1:500, 1:800,
131 1:1000) and was tested to determine the optimal concentration of antibody dilution for the
132 inhibition assay in the biosensor (200-300 resonance units (RU)). To test the optimal ratio of
133 binder to standard, injection volume, and contact time, the standard solution was transferred
134 into the wells of a U-bottomed microtitre plate (Greiner Bio One, Gloucestershire, UK) and
135 mixed with antibody solution in different ratios (3:1, 2:1, 1:1, 1:2, 1:3, v/v) by the
136 autosampler via the control software. Then, the mixtures were injected over the sensor chip
137 surface at different flow rates ($15, 20, 25 \mu\text{L min}^{-1}$) for different times (150 s, 240 s, and 300
138 s). In addition, chip surface regeneration was performed to reuse the sensor chip.
139 Regeneration removes bound analyte at the end of each cycle by injecting a pH buffer over

140 the surface but leaves the ligand attached to the chip surface so another sample can be
141 analysed. To test for the optimal regeneration solution, different concentrations of NaOH
142 solution (5 mM, 10 mM, 20 mM) and glycine-HCl (pH 3.0) solution (5 mM, 10 mM, 20 mM)
143 were used.

144 2.4. Preparation of standards for SPR analysis

145 To compare the calibration curves prepared from extracts of the different tissue matrices
146 following extraction with the extracts prepared in HBS-EP buffer, three standard curves were
147 prepared. The standard dose response curve was obtained according to the RU values and
148 concentrations of standards. The standard curves were fitted using the following equation:
149 $y=(A-D)/[1+(x/C)^B]+D$. where A and D are the responses at high and low asymptotes of the
150 curve, respectively, C is the concentration of the targets resulting in 50% inhibition, B is the
151 slope at the inflection point of the sigmoid, and X is the calibration concentration.

152 For standard curve A, a calibration curve consisting of five concentrations was
153 constructed by diluting the MQCA stock standard (1000 $\mu\text{g mL}^{-1}$) in HBS-EP buffer, ranging
154 from 0 to 100 ng mL^{-1} (0, 6.25, 12.5, 25, 50, 100 ng mL^{-1}).

155 To prepare standard curve B, known negative tissue was extracted as described for
156 sample preparation, and aliquots (1 mL) were spiked with MQCA stock standard (1000 μg
157 mL^{-1}) to provide 5 calibration standards (0, 6.25, 12.5, 25, 50, 100 ng mL^{-1}) for the calibration
158 curve.

159 To prepare standard curve C, known negative tissue was spiked with known amounts of
160 MQCA at 0 $\mu\text{g kg}^{-1}$, 6.25 $\mu\text{g kg}^{-1}$, 12.5 $\mu\text{g kg}^{-1}$, 25 $\mu\text{g kg}^{-1}$, 50 $\mu\text{g kg}^{-1}$, and 100 $\mu\text{g kg}^{-1}$ and
161 then extracted as described for sample preparation to obtain 5 calibration standards for the
162 calibration curve.

163 2.5 Sample preparation

164 Samples such as swine muscle and liver were minced and homogenized. Each

165 homogenized sample (2 g) was weighed into a 50-mL polypropylene centrifuge tube. Ethyl
166 acetate (6 mL) and 3.0 mL of 1.25 M HCl were added, and the mixture was vortex-mixed for
167 3 min and then centrifuged for 5 min at $4000 \times g$. The supernatant (4 mL) was transferred into
168 another 50-mL polypropylene centrifuge tube, 4 mL of saturated NaCl solution was added,
169 and the solution was mixed for 1 min. After standing for 5 min, 3 mL of ethyl acetate was
170 dried using nitrogen gas at $50\text{ }^{\circ}\text{C}$. Then, the muscle sample residue was re-dissolved with 1
171 mL of HSB-EP buffer, and the liver sample residue was re-dissolved with 3 mL of HSB-EP
172 buffer and washed with 1 mL of hexane. After being centrifuged for 5 min at $4000 \times g$, the
173 water phase was filtered with a $0.45\text{-}\mu\text{m}$ filter and was then used in the SPR.

174 2.6 Validation of the SPR

175 Because China is the world's largest OLA consumption country, accounting for more
176 than 80% of OLA consumption in the world, the document [2005] No. 17 issued by the
177 Ministry of Agriculture veterinary bureau (2005) was therefore selected for the validation of
178 the SPR method. According to this document, some parameters including limit of
179 determination (LOD), accuracy and precision were determined to validate the SPR method
180 based on the optimized standard curve and the sample preparation procedure. Briefly, 20
181 known negative swine muscle and liver samples, purchased from a local supermarket (Tesco,
182 Marks & Spencer, Sainsbury's) and previously been proven by HPLC analysis (Wu et al.,
183 2007) to be free of OLA and its MR MQCA, were analysed by the SPR method. The
184 determination of the LOD was based on 20 blank samples accepting no false positive rates,
185 with an average + 3 standard deviation (SD). The accuracy and precision of the method were
186 expressed by the recovery and coefficient of variation (CV), respectively. The recovery
187 (percentage) of MQCA was established using five spiked duplicate blanks at levels of $\frac{1}{2}$ MRL
188 ($2\text{ }\mu\text{g kg}^{-1}$ in muscle and $25\text{ }\mu\text{g kg}^{-1}$ in liver), MRL ($4\text{ }\mu\text{g kg}^{-1}$ in muscle and $50\text{ }\mu\text{g kg}^{-1}$ in liver)
189 and 2MRL ($8\text{ }\mu\text{g kg}^{-1}$ in muscle and $100\text{ }\mu\text{g kg}^{-1}$ in liver) obtained from three different

190 analyses and was calculated using the following equation: (concentration
191 measured/concentration spiked) \times 100. CVs were determined by analysing samples spiked
192 with MQCA at the same levels of above from five different analyses. Each concentration level
193 was tested three times in a time span of 2 months.

194 2.7 Comparison of the SPR biosensor with ic-ELISA and HPLC

195 To test the detection capability of the developed SPR biosensor, seven pig samples were
196 supplied by the National Reference Laboratory of Veterinary Drug Residues (Huazhong
197 Agricultural University, HZAU) and MOA Key Laboratory for the Detection of Veterinary
198 Drug Residues in Foods (Wuhan, China). In addition, thirty-five unknown samples, including
199 seventeen swine muscle samples and eighteen swine liver samples that were collected from
200 Northern Ireland by Chen in 2012 were also prepared. These samples were analysed by the
201 current SPR biosensor, the ic-ELISA method and the HPLC analysis.

202 The ic-ELISA method was performed according to the publication (Zhang et al., 2015)
203 that was developed using the same mAb 5B10. The limits of detection ranged from 1.9 μg
204 kg^{-1} to 4.3 $\mu\text{g kg}^{-1}$. The recoveries ranged from 74.2% to 98.9% with a maximum of 17.3%
205 for the CV. The HPLC analyses were performed according to the procedure of Wu et al. (2007)
206 with modifications. Briefly, all HPLC analyses were performed using a Waters HPLC system,
207 comprising a 2695 ternary pump and 2487 UV detection. An Eclipse XDB-C18 (250 mm, 4.6
208 mm I.D.) (Agilent Technology, USA) HPLC column was used for sample separation. The
209 temperature of the HPLC column was set at 30 °C. The mobile phase was acetonitrile/water
210 containing 1% acetic acid (18:82 v/v for the plasma, muscle, liver, and fat samples; 20:80 v/v
211 for the kidney samples). The mobile phase was pumped at a flow rate of 1.0 mL min^{-1} . The
212 spectra of all the samples were obtained from detection at the wavelength of 320 nm.

213 3. Results and Discussion

214 3.1 SPR analysis format design

215 One of the most challenging steps for an SPR-based assay is the design of the analysis
216 format. In this study, two formats (shown in **Figure 2**) were designed to perform the SPR
217 analysis. Format A used NH₂MQCA as the ligand which was immobilized on the CM5 chip
218 surface using an amine coupling method (EDC/NHS was used to activate the
219 carboxymethylated CM5 chip surface before the NH₂MQCA was immobilized). Format B
220 was similar to format A except in using the carrier protein OVA as a linker. For format A,
221 obtaining an obvious change of RU value was easily accomplished. However, in some
222 instances, the small molecular NH₂MQCA directly immobilized on the CM5 chip surface was
223 unstable, resulting in unacceptably low levels of immobilised ligand. For all these reasons,
224 format B was selected to perform the SPR analysis.

225 3.2 The optimization of the SPR-biosensor analytical conditions and procedure

226 An antibody dilution of 1/500 (v/v) was found to give satisfactory results under the assay
227 conditions. The samples (calibrants and spiked samples) were transferred into the wells of a
228 U-bottomed microtitre plate and mixed with antibody solution at a ratio of 1:1 (v/v) by the
229 autosampler via the control software and injected for 240 s over the sensor chip surface at a
230 rate of 20 $\mu\text{L min}^{-1}$. The chip surface was regenerated between cycles using 10 mM sodium
231 hydroxide for 60 s at a flow rate of 20 $\mu\text{L min}^{-1}$ and then using 10 mM glycine-HCl (pH 3.0)
232 for 60 s at a flow rate of 20 $\mu\text{L min}^{-1}$. The binding of the antibody to the chip surface was
233 measured as the change in SPR signal between two reported points before (10 s) and after (20
234 s) each injection. A competitive immunoassay assay format (format B that was described in
235 section 3.1) was used to detect inhibition of antibody binding to the chip surface. The SPR
236 signal was expressed in arbitrary RUs. In this format, as the MQCA concentration increases,
237 more anti-MQCA antibody is bound resulting in fewer antibodies binding to the ligand on the
238 sensor surface. This inhibition of antibody binding to the ligand indicates that the RU readout
239 is inversely related to the MQCA concentration. Standards and samples were analysed in

240 duplicate.

241 The efficiency of immobilization was tested by assessing the R_{\max} value. R_{\max} provides
242 useful information on how effective the immobilized ligand is, in contact with its binding
243 partner. R_{\max} is the maximum binding capacity of the NH₂MQCA (ligand) for the
244 anti-MQCA antibody, as measured in RUs. In this study, the R_{\max} that was obtained was
245 1084.5 RUs achieved by injecting a high concentration (1/10) of anti-MQCA monoclonal
246 antibody (5B10) over the chip surface for an extended time (15 min).

247 3.3 Standard curves

248 In general, matrix matched standard curves are used to reduce potential matrix effects in
249 the analytical procedure (Diblikova, Cooper, Kennedy, & Franek, 2005). However, the
250 preparation of matrix-matched standards from blank samples prior to analysis might be less
251 favourable (when multiple sample tissues are involved) for incorporation into a commercial
252 kit. In order to adapt the test to be commercially viable, three different standard curves were
253 compared in this study to determine if a buffer matrix would suffice. As shown in **Figure 3A**,
254 the HBS-EP buffer-based standard curve (standard curve A) and the matrix-based standard
255 curves (standard curve B and standard curve C) were obtained. Upon comparing these
256 standard curves, good correlations between standard curve A and standard curve B (Figure 3B,
257 $r = 0.9968$) and between the standard curve A and standard curve C (Figure 3C, $r = 0.9922$)
258 were observed. Therefore, standard curve A was selected for this study. Under these
259 optimized conditions, the sensitivity (IC_{50}) of the SPR assay was 12.9 ng mL^{-1} .

260 3.4 SPR method validation and compared with ic-ELISA and HPLC analysis

261 The sample preparation procedures play an important role in the development of the SPR
262 method. In this study, ethyl acetate and HCl were used successfully to extract MQCA
263 compounds from swine muscle and liver samples. Hexane was used to eliminate most of the
264 fat during the extraction procedure. Then, $0.45\text{-}\mu\text{m}$ syringe filters (Whatman, GE Healthcare

265 Life Sciences) were used to remove particulate matter from the extract. This approach is
266 consistent with the study of Zhang et al. (2015) except for the use of the filter.

267 As shown in **Table 1**, based on the results from twenty different blank samples, the
268 LODs of the method were $1.4 \mu\text{g kg}^{-1}$ and $2.7 \mu\text{g kg}^{-1}$ in swine muscle and swine liver,
269 respectively, **which are lower than the RC ($10 \mu\text{g kg}^{-1}$) by EU**. As described in the
270 introduction, OLA is still used in China and the MRLs of the MR which defined as MQCA
271 had been set at $50 \mu\text{g kg}^{-1}$ in swine liver and $4 \mu\text{g kg}^{-1}$ in swine muscle. Therefore, three
272 concentration levels, including $\frac{1}{2}$ MRL, MRL, and 2MRL were selected to evaluate the
273 accuracy and precision of the SRP method in this study. As shown in Table 1, the recoveries
274 of swine muscle and swine liver samples spiked with MQCA at the above three concentration
275 levels were in the range of 82% to 104.6%, with CVs less than 12.2%. These data indicated
276 that the SPR method fill the need for the rapid screening determination of MQCA residues in
277 swine tissues.

278 As shown in **Figure 4**, the calculated concentrations from the standard curve with each
279 technique (ic-ELISA, HPLC, SPR) were compared. Good correlations ($r = 0.9698$, SPR and
280 HPLC; $r = 0.9873$, SPR and ic-ELISA) were observed in the swine liver tissues (Figure 4A).
281 Similarly, good correlations ($r = 0.9806$, SPR and HPLC; $r = 0.9918$, SPR and ic-ELISA)
282 were also observed in the swine muscle tissues (Figure 4B). For the thirty-eight unknown
283 samples, no MQCA could be detected by SPR, ELISA, or HPLC analysis. These data
284 suggested that the SPR method is reliable.

285 In 2015, an ic-ELISA method was developed to determine the residue of MQCA in
286 edible animal tissues using the same mAb 5B10 (Zhang et al., 2015). Comparison between
287 the SPR method and the ic-ELISA method was therefore reasonable. As shown in Table 2,
288 compared with the ic-ELISA method, the SPR method is label-free, more sensitive (IC_{50} , 12.9
289 ng mL^{-1} in SPR $< 17.7 \text{ ng mL}^{-1}$ in ic-ELISA), more precise (CVs, 12.2% in SPR $< 17.3\%$

290 in ic-ELISA).

291 **4. Conclusions**

292 In this study, a mAb-based SPR method for monitoring MQCA in swine muscle and
293 swine liver was developed with label-free, high sensitivity (IC_{50} , 12.9 ng mL⁻¹, LOD, 1.4 μ g
294 kg⁻¹ in swine muscle and 2.7 μ g kg⁻¹ in swine liver), good accuracy (recovery ranged from
295 82% to 104.6%) and precision (CVs, 12.2%). Obviously, the LODs are lower than both the
296 EU RC (10 μ g kg⁻¹) and the Chinese MRL (50 μ g kg⁻¹ in swine liver and 4 μ g kg⁻¹ in swine
297 muscle). The HBS-EP buffer-based standard curve make it more convenient to incorporate
298 into the kit format that can be fully transferable to other laboratories. In summary, the SPR
299 method for MQCA, although no longer in its infancy as a new technology, offers the ability
300 for rapid and reliable detection of trace amounts of MQCA, the marker residues of OLA, in
301 food of animal origin and its implementation will enable timely corrective actions to be taken
302 to prevent unsafe food from entering into the food supply chain.

303

304 **Declaration of interest**

305 We declare that we have no financial and personal relationships with other people or
306 organizations that can inappropriately influence our work, there is no professional or other
307 personal interest of any nature or kind in any product, service and/or company that could be
308 construed as influencing the position presented in, or the review of, the manuscript entitled
309 “Surface plasmon resonance biosensor for the determination of
310 3-methyl-quinoxaline-2-carboxylic acid, the marker residue of olaquinox, in swine tissues”.

311

312

313

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434 **Figure legends**435 **Figure 1** The chemical structure of olaquinox and its metabolite436 **Figure 2** The design of the format of the SPR analysis

437 **Figure 3** Standard curves for the quantification of MCQA. (A) three standard curves
438 based on different matrices. ■ standard curve A, the HBS-EP buffer matrix; ● standard
439 curve B, the blank extract-based matrix; ▲ standard curve C, the spiked extract based matrix
440 (B) comparison of standard curves A and B, $y = 0.9296x + 12.066$, $r = 0.9968$; (C),
441 comparison of standard curves A and C, $y = 0.8979x + 27.753$, $r = 0.9922$

442 **Figure 4** Comparison with SPR, ic-ELISA and HPLC analysis in edible swine tissues.

443 (A) comparison with SPR, ic-ELISA and HPLC analysis in swine liver samples; (B)

444 comparison with SPR, ic-ELISA and HPLC analysis in swine muscle samples.

445

446 **Table Titles**447 **Table 1** The LOD, recoveries and coefficients of variation (CVs) of the SPR448 **Table 2** Comparison between the SPR method and the ic-ELISA method

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450 **Tables**451 **Table 1 The LOD, recoveries and coefficients of variation (CVs) of the SPR**

tissues	LOD ($\mu\text{g kg}^{-1}$)	spiked level ($\mu\text{g kg}^{-1}$)	mean recovery (%)	CV (%)
swine muscle	1.4	2	104.6 ± 12.0	11.5
		4	89.9 ± 9.8	10.9
		8	82.0 ± 6.7	8.2
swine liver	2.7	25	85.8 ± 10.5	12.2
		50	87.1 ± 7.7	8.8
		100	96.1 ± 4.2	4.3

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453

454

Table 2 Comparison between the SPR method and the ic-ELISA method

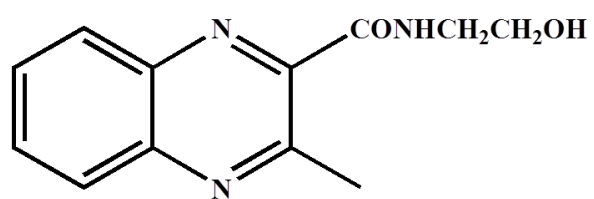
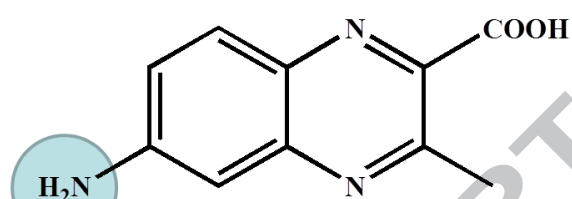
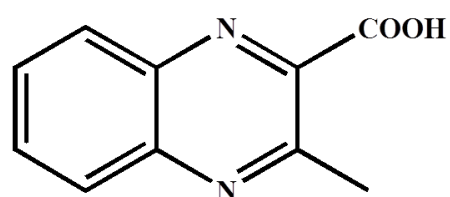
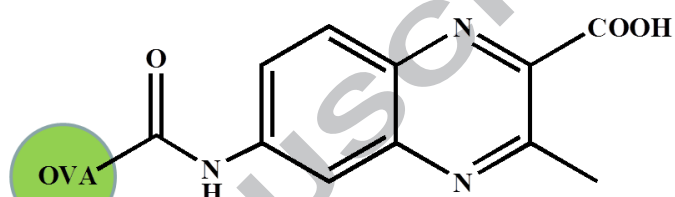
items	SPR method	ic-ELISA method ^a
antibody	Monoclonal antibody 5B10	Monoclonal antibody 5B10
IC ₅₀ (ng mL ⁻¹)	12.9	17.7
LOD (μg kg ⁻¹)	1.4 μg kg ⁻¹ in swine muscle 2.7 μg kg ⁻¹ in swine liver	1.9 μg kg ⁻¹ in swine muscle 4.3 μg kg ⁻¹ in swine liver
recovery	from 82% to 104.6%	from 74.2% to 98.9%
coefficient of variation	<12.2%	<17.3%
the sample preparation	ethyl acetate, HCl, saturated NaCl solution, HSB-EP buffer, hexane, 0.45-μm filter	ethyl acetate, HCl saturation NaCl solution, phosphate buffer, hexane
detection time (does not include the sample preparation time)	5 min sample ⁻¹	90 min kit ⁻¹ (2~3 min sample ⁻¹)
regeneration	yes (at least 200 cycles)	no
label or not	no	yes, horseradish peroxidase labeled is necessary

455

Note: ^athe data obtained from the publication Zhang et al., 2015

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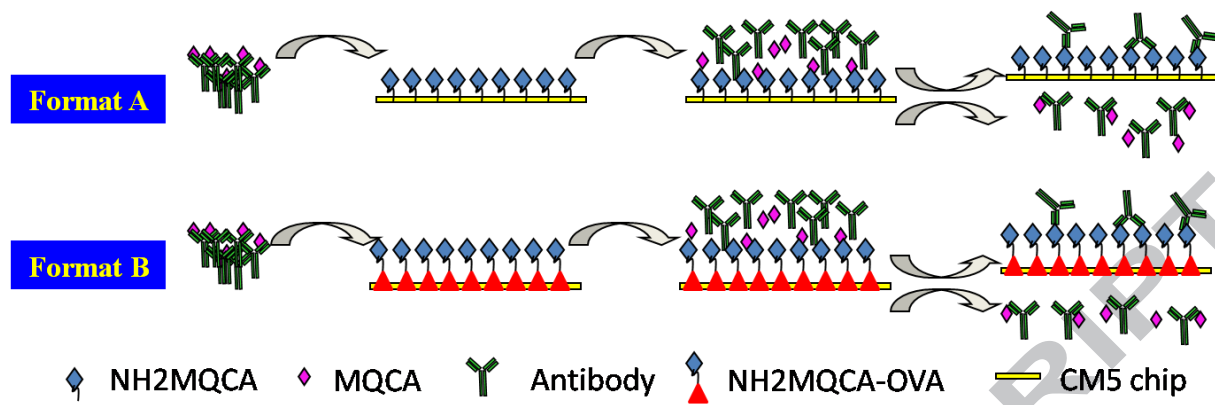
458 **Figures****Olaquinox, OLA****NH₂MQCA****3-methyl-quinoxaline-2-carboxylic acid, MQCA****NH₂MQCA-OVA**

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Figure 1

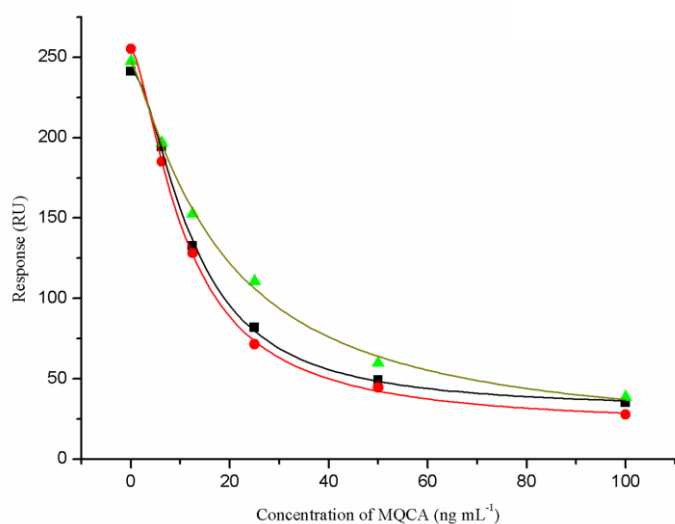


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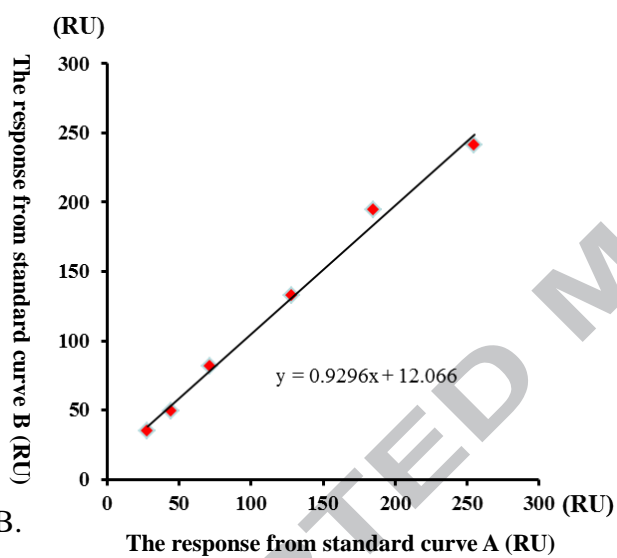
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Figure 2

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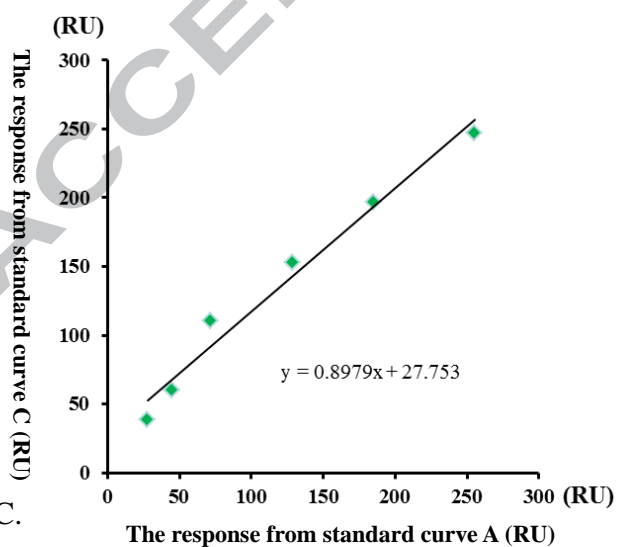


465 A.



466 B.

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468 C.

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Figure 3

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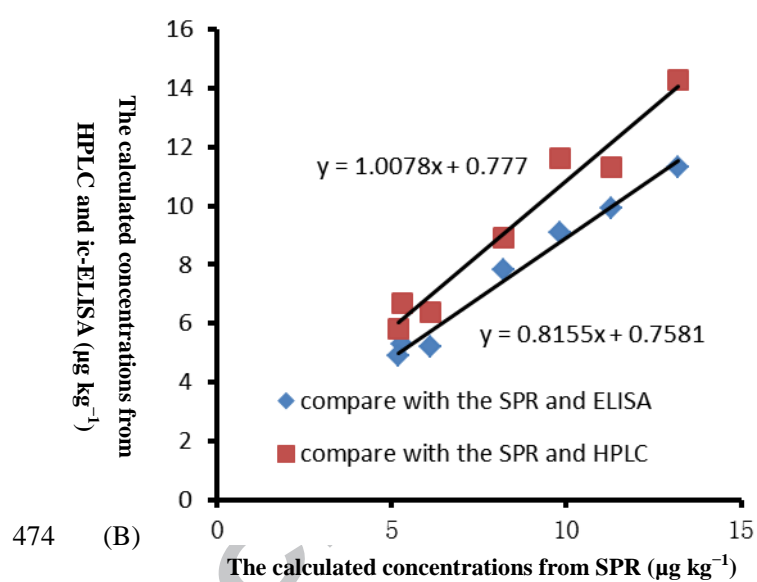
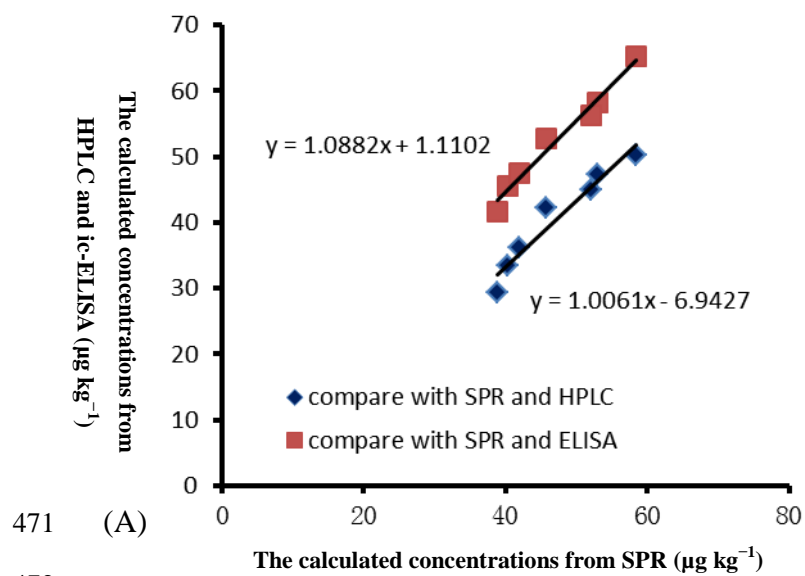


Figure 4

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Research highlights

481

482 ● A new SPR method for the determination of MQCA had been reported.

483 ● The developed SPR method is the first report for MQCA in animal tissues.

484 ● The SPR method can serve as an effective screening tool in any routine laboratory.

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