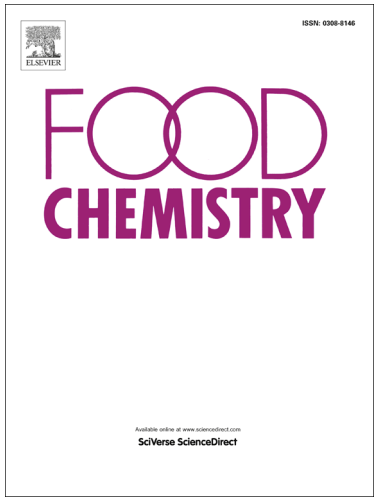


Surface plasmon resonance biosensor for the determination of 3-methyl-quinoxaline-2-carboxylic acid, the marker residue of olaquindox, in swine tissues

Dapeng Peng, Owen Kavanagh, Haijiao Gao, Xiya Zhang, Sijun Deng, Dongmei Chen, Zhenli Liu, Changqing Xie, Chen Situ, Zonghui Yuan

PII: S0308-8146(19)30671-5
DOI: https://doi.org/10.1016/j.foodchem.2019.04.022
Reference: FOCH 24623

To appear in: *Food Chemistry*

Received Date: 5 November 2018
Revised Date: 5 April 2019
Accepted Date: 5 April 2019

Please cite this article as: Peng, D., Kavanagh, O., Gao, H., Zhang, X., Deng, S., Chen, D., Liu, Z., Xie, C., Situ, C., Yuan, Z., Surface plasmon resonance biosensor for the determination of 3-methyl-quinoxaline-2-carboxylic acid, the marker residue of olaquindox, in swine tissues, *Food Chemistry* (2019), doi: https://doi.org/10.1016/j.foodchem.2019.04.022

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

Dapeng Peng[1], Owen Kavanagh[3], Haijiao Gao[1], Xiya Zhang[1], Sijun Deng[2], Dongmei Chen[1], Zhenli Liu[1], Changqing Xie[1], Chen Situ[2*], Zonghui Yuan[1*]

[1] National Reference Laboratory of Veterinary Drug Residues (HZAU) and MOA Key Laboratory for the Detection of Veterinary Drug Residues in Foods

Huazhong Agricultural University, Wuhan, Hubei 430070, China

[2] The Institute for Global Food Security, School of Biological Sciences, Queen's University Belfast, BT9 5AG, Northern Ireland, United Kingdom

[3] School of Health Sciences, Lord Mayor's Walk, York, Y031 7EX, UK.

[*] Corresponding author: Tel.: +86 27 8728 7186; fax: +86 27 8767 2232. E-mail address: c.situ@qub.ac.uk (C. Situ), yuan5802@mail.hzau.edu.cn. (Z. Yuan)

Abstract

To monitor the illegal use of olaquindox 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 olaquindox, in swine tissues. The limit of detection was 1.4 μg kg^{-1} in swine muscle and 2.7 μg kg^{-1} in swine liver, which are lower than the EU recommended concentration (10 μg 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 olaquindox in the edible tissues of animals.

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

1. Introduction

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

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

OLA could not be guaranteed.

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

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

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

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

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

2. Materials and Methods

2.1 Chemicals and reagents

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

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

Control (Beijing, China). The hapten NH2MQCA was identified by nuclear magnetic resonance (NMR, Bruker-400 spectrometers, Bruker BioSpin, Switzerland) and ion trap and time-of-flight mass spectrometers coupled with a HPLC system (LC/MS-ITTOF, Shimadzu, Kyoto, Japan), respectively. MS m/z calculated for $C_{10}H_9N_3O_2$ $[M+H]^+$ 204.0768, found 204.0763. H^1 NMR (DMSO-d6) δ_H: 2.73 (s, 3H, C3-CH_3), 6.84, 7.23, 7.39 (each 1H, Ar-H), 4.60 (br, 2H, -NH_2), 12.0~13.0 (br, 1H, -COOH). The synthesized coating conjugate NH2MQCA-OVA was characterized by 8453 UV-Visible spectrophotometer (Aglient 8453, USA), which the estimated incorporation rates of conjugates is 9.7. The obtained specific mAb 5B10 that has isotype IgG1 showed an IC_{50} value of 17.7 $\mu g\,L^{-1}$ for MQCA and did not exhibit measurable cross-reactivity (CR) with other quinoxalines and their analogues (CR < 0.1%) such as OLA, quinocetone, mequindox, cyadox, carbadox, quinoxaline-2-carboxylic acid, desoxymequindox, desoxyquinocetone, and desoxyolaquindox (Zhang et al., 2015).

2.2. Immobilization of ligand on CM5 chip

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

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

2.3 Optimization of the SPR-biosensor analytical conditions

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

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

2.4. Preparation of standards for SPR analysis

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

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

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

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

2.5 Sample preparation

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

homogenized sample (2 g) was weighed into a 50-mL polypropylene centrifuge tube. Ethyl acetate (6 mL) and 3.0 mL of 1.25 M HCl were added, and the mixture was vortex-mixed for 3 min and then centrifuged for 5 min at 4000 × g. The supernatant (4 mL) was transferred into another 50-mL polypropylene centrifuge tube, 4 mL of saturated NaCl solution was added, and the solution was mixed for 1 min. After standing for 5 min, 3 mL of ethyl acetate was dried using nitrogen gas at 50 °C. Then, the muscle sample residue was re-dissolved with 1 mL of HSB-EP buffer, and the liver sample residue was re-dissolved with 3 mL of HSB-EP buffer and washed with 1 mL of hexane. After being centrifuged for 5 min at 4000 × g, the water phase was filtered with a 0.45-μm filter and was then used in the SPR.

2.6 Validation of the SPR

Because China is the world's largest OLA consumption country, accounting for more than 80% of OLA consumption in the world, the document [2005] No. 17 issued by the Ministry of Agriculture veterinary bureau (2005) was therefore selected for the validation of the SPR method. According to this document, some parameters including limit of determination (LOD), accuracy and precision were determined to validate the SPR method based on the optimized standard curve and the sample preparation procedure. Briefly, 20 known negative swine muscle and liver samples, purchased from a local supermarket (Tesco, Marks & Spencer, Sainsbury's) and previously been proven by HPLC analysis (Wu et al., 2007) to be free of OLA and its MR MQCA, were analysed by the SPR method. The determination of the LOD was based on 20 blank samples accepting no false positive rates, with an average + 3 standard deviation (SD). The accuracy and precision of the method were expressed by the recovery and coefficient of variation (CV), respectively. The recovery (percentage) of MQCA was established using five spiked duplicate blanks at levels of ½ MRL (2 μg kg^{-1} in muscle and 25 μg kg^{-1} in liver), MRL (4 μg kg^{-1} in muscle and 50 μg kg^{-1} in liver) and 2MRL (8 μg kg^{-1} in muscle and 100 μg kg^{-1} in liver) obtained from three different

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

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

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

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

3. Results and Discussion

3.1 SPR analysis format design

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

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

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

duplicate.

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

3.3 Standard curves

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

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

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

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

As shown in **Table 1**, based on the results from twenty different blank samples, the LODs of the method were 1.4 μg kg^{-1} and 2.7 μg kg^{-1} in swine muscle and swine liver, respectively, which are lower than the RC (10 μg kg^{-1}) by EU. As described in the introduction, OLA is still used in China and the MRLs of the MR which defined as MQCA had been set at 50 μg kg^{-1} in swine liver and 4 μg kg^{-1} in swine muscle. Therefore, three concentration levels, including ½ MRL, MRL, and 2MRL were selected to evaluate the accuracy and precision of the SRP method in this study. As shown in Table 1, the recoveries of swine muscle and swine liver samples spiked with MQCA at the above three concentration levels were in the range of 82% to 104.6%, with CVs less than 12.2%. These data indicated that the SPR method fill the need for the rapid screening determination of MQCA residues in swine tissues.

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

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

in ic-ELISA).

4. Conclusions

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

Declaration of interest

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

Acknowledgements

The authors are grateful to the Ministry of Science and Technology of the People's

316 Republic of China (2011DFA32140), the National Natural Science Foundation of China
317 (31772074) and the Fundamental Research Funds for the Central Universities
318 (2662017PY049, 2017BC010) for their financial support.

319 We are also grateful to Katrina Campbell, Christopher Elliott, Nitsara Karoonuthaisiri,
320 Michalina Oplatowska-Stachowiak, Kevin Cooper, and Brett Greer for their warm and sincere
321 help.

322 **References**

323 Announcement No. 168. (2001). Code of practice for the use of feed Drug additives. *Ministry*
324 *of Agriculture of the People's Republic of China*.

325 Announcement No. 235. (2002). Maximum residue limit of veterinary drugs in animal foods.
326 *Ministry of Agriculture of the People's Republic of China*.

327 Announcement No. 2638. (2018). *Ministry of Agriculture of the People's Republic of China*.

328 Boison, J.O., Lee, S.C., Gedir, R.G. (2009). A determinative and confirmatory method for
329 residues of the metabolites of carbadox and olaquindox in porcine tissues. *Analytica*
330 *Chimica Acta, 637,* 128-134.

331 Caldow, M., Stead, S.L., Day, J., Sharman, M., Situ, C., Elliott, C.T. (2005). Development and
332 validation of an optical SPR biosensor assay for tylosin residues in honey. *Journal of*
333 *Agricultural and Food Chemistry,* 53(19), 7367-7370.

334 Campbell, K., Huet, A., Charlier, C., Higgins, C., Delahaut, P., Elliott, C.T. (2009).
335 Comparison of ELISA and SPR biosensor technology for the detection of paralytic
336 shellfish poisoning toxins. *Journal of Chromatography B, 877,* 4079-4089.

337 Carta, A., Corona, P., & Loriga, M. (2005). Quinoxaline 1,4-dioxide: a versatile scaffold
338 endowed with manifold activities. *Current Medicinal Chemistry, 12*(19), 2259-2272.

339 Chen, Q., Tang, S., Jin, X., Zou, J., Chen, K., Zhang, T., Xiao, X. (2009). Investigation of the
340 genotoxicity of quinocetone, carbadox and olaquindox in vitro using Vero cells. *Food*
341 *and Chemical Toxicology,* 47, 328-334.

342 Cheng, L., Shen, J., Wang, Z., Jiang, W., Zhang, S. (2013). A sensitive and specific ELISA for
343 determining a residue marker of three quinoxaline antibiotics in swine liver. *Analytical*

344 *and Bioanalytical Chemistry, 405*, 2653-2659.

345 Commission Regulation (EC) No 2788/98. (1998). Commission Regulation (EC) No 2788/98
346 of 22 December 1998 amending Council Directive 70/524/EEC concerning additives in
347 feedingstuffs as regards the withdrawal of authorisation for certain growth promoters.
348 *Official Journal of the European Communities, L347*, 31-32.

349 CRL Guidance Paper. (2007). CRLs view on state of the art analytical methods for national
350 residue control plans. *CRL Guidance Paper (7 December 2007)*, p7.

351 Devlin, S., Meneely, J.P., Greer, B., Campbell, K., Vasconcelos, V., Elliott, C.T. (2014).
352 Production of a broad specificity antibody for the development and validation of an
353 optical SPR screening method for free and intracellular microcystins and nodularin in
354 cyanobacteria cultures. *Talanta*, 122, 8-15.

355 Diblikova, I., Cooper, K. M., Kennedy, D. G., Franek, M. (2005). Monoclonal
356 antibody-based ELISA for the quantification of nitrofuran metabolite
357 3-amino-2-oxazolidinone in tissues using a simplified sample preparation. *Analytica*
358 *Chimica Acta*. 540, 285-292

359 Document [2005] No.17. (2005). Reference criteria for the recording the reagent (kit) of
360 veterinary drug residue. *The Ministry of Agriculture Veterinary Bureau.*

361 Eberlein, B., Bergner, T., Przybilla, B. (1992). Demonstration of olaquindox phototoxicity in
362 vitro. *Photodermatol Photoimmunol Photomed*, 9 (2), 63-66.

363 Emmert, B., Schauder, S., Palm, H., Hallier, E., Emmert, S. (2007). Disabling work-related
364 persistent photosensitivity following photoallergic contact dermatitis from
365 chlorpromazine and olaquindox in a pig breeder. *Annals of Agricultural and*

Environmental Medicine, 14 (2), 329-333.

FAO/WHO. (1990). Thirty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives: Evaluation of certain veterinary drug residues in food, *World Health Organ Technical Report Series*, NO.799, 23-54.

FAO/WHO. (1995). Fouty-second report of the Joint FAO/WHO Expert Committee on Food Additives: Evaluation of certain veterinary drug residues in food, *World Health Organ Technical Report Series*, NO.851, 19-22.

Hirakawa, Y., Yamasaki, T., Harada, A., Iwasa, S., Narita, H., Miyake, S. (2018). Development of an immunosensor based on surface plasmon resonance for simultaneous residue analysis of three pesticides -boscalid, clothianidin, and nitenpyram- in vegetables. *Analytical Sciences*, 34(5), 533-539.

Huang. X., Zhang, H., Wang, X., Huang, L., Zhang, L., Yan, C., Liu, Y., Yuan, Z. (2010). ROS mediated cytotoxicity of porcine adrenocortical cells induced by QdNOs derivatives in vitro. *Chemico-Biological Interactions*, 185 (3), 227-234.

Hutchinson, M.J., Young, P.B., Kennedy, D.G. (2005). Confirmation of carbadox and olaquindox metabolites in porcine liver using liquid chromatography–electrospray, tandem mass spectrometry. *Journal of Chromatography B, 816*, 15-20.

Ihsan, A., Wang, X., Zhang, W., Tu, H., Wang, Y., Huang, L., Iqbal, Z., Cheng, G., Pan, Y., Liu, Z., Tan, Z., Zhang, Y., Yuan, Z. (2013). Genotoxicity of quinocetone, cyadox and olaquindox in vitro and in vivo. *Food and Chemical Toxicology*, 59, 207-214.

Jiang, W., Beier, R.C., Wang, Z., Wu, Y., Shen, J. (2013). Simultaneous screening analysis of 3-methyl-quinoxaline-2-carboxylic acid and quinoxaline-2-carboxylic acid residues in

edible animal tissues by a competitive indirect immunoassay. *Journal of Agricultural and Food Chemistry, 61,* 10018-10025.

Li, D., Dai, C., Zhou, Y., Yang, X., Zhao, K., Xiao, X., Tang, S. (2016). Effect of GADD45a on olaquindox-induced apoptosis in human hepatoma G2 cells: Involvement of mitochondrial dysfunction. *Environmental Toxicology and Pharmacology,* 46, 140-146.

Liu, Q., Zhang, J., Luo, X., Ihsan, A., Liu, X., Dai, M., Cheng, G., Hao, H., Wang, X., Yuan, Z. (2016). Further investigations into the genotoxicity of quinoxaline-di-N-oxides and their primary metabolites. *Food and Chemical Toxicology,* 93, 145-157.

Merou, A., Kaklamanos, G., Theodoridis, G. (2012). Determination of carbadox and metabolites of carbadox and olaquindox in muscle tissue using high performance liquid chromatography–tandem mass spectrometry. *Journal of Chromatography B, 881-882,* 90-95.

Olaru, A., Bala, C., Jaffrezic-Renault, N., Aboul-Enein, H.Y. (2015). Surface plasmon resonance (SPR) biosensors in pharmaceutical analysis. *Critical Reviews in Analytical Chemistry,* 45(2): 97-105.

Pan, M., Li, S., Wang, J., Sheng, W., Wang, S. (2017). Development and validation of a reproducible and label-free surface plasmon resonance immunosensor for enrofloxacin detection in animal-derived foods. *Sensors (Basel).* 17(9). pii: E1984.

Wu, Y., Yu, H., Wang, Y., Huang, L., Tao, Y., Chen, D., Peng, D., Liu, Z., Yuan, Z. (2007). Development of a high-performance liquid chromatography method for the simultaneous quantification of quinoxaline-2-carboxylic acid and methyl-3-quinoxaline-2-carboxylic acid in animal tissues. *Journal of Chromatography A, 1146,* 1-7.

Yang, Y., Jiang, L., She, Y., Chen, M., Li, Q., Yang, G., Geng, C., Tang, L., Zhong, L., Jiang, L., Liu, X. (2015). Olaquindox induces DNA damage via the lysosomal and mitochondrial pathway involving ROS production and p53 activation in HEK293 cells. *Environmental Toxicology and Pharmacology,* 2015, 40 (3), 792-799.

Yuan, J., Deng, D., Lauren, D.R., Aguilar, M., Wu, Y. (2009). Surface plasmon resonance biosensor for the detection of ochratoxin A in cereals and beverages. *Analytica Chimica Acta*, 656, 63-71.

Yue, N., Ji, B., Liu, L., Tao, G., Eremin, S.A., Wu, L. (2009). Synthesis of olaquindox metabolite, methyl-3-quinoxaline-2-carboxylic acid for development of an immunoassay. *Food and Agricultural Immunology, 20,* 173-183.

Zhang, X., Peng, D., Pan, Y., Wang, Y., Chen, D., Zhou, Q., Liu, Z., Yuan, Z. (2015). A novel hapten and monoclonal-based enzyme-linked immunosorbent assay for 3-methyl-quinoxaline-2-carboxylic acid in edible animal tissues. *Analytical Methods*, *7*, 6588-6594.

Zhang, X., Zheng, B., Zhang, H., Chen, X., Mei, G. (2011). Determination of marker residue of Olaquindox in fish tissue by ultra performance liquid chromatography-tandem mass spectrometry. *Journal of Separation Science*, *34*, 469-474.

Zou, J., Chen, Q., Jin, X., Tang, S., Chen, K., Zhang, T., Xiao, X. (2011). Olaquindox induces apoptosis through the mitochondrial pathway in HepG2 cells. *Toxicology,* 285 (3), 104-113.

Zou, J., Chen, Q., Tang, S., Jin, X., Chen, K., Zhang, T., Xiao, X. (2009). Olaquindox-induced genotoxicity and oxidative DNA damage in human hepatoma G2 (HepG2) cells.

Mutation Research, 2009, 676 (1-2), 27-33.

434 **Figure legends**

435 **Figure 1** The chemical structure of olaquindox and its metabolite

436 **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 SPR

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

449

450 **Tables**

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

tissues	LOD ($\mu g\ kg^{-1}$)	spiked level ($\mu g\ kg^{-1}$)	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

452

453

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

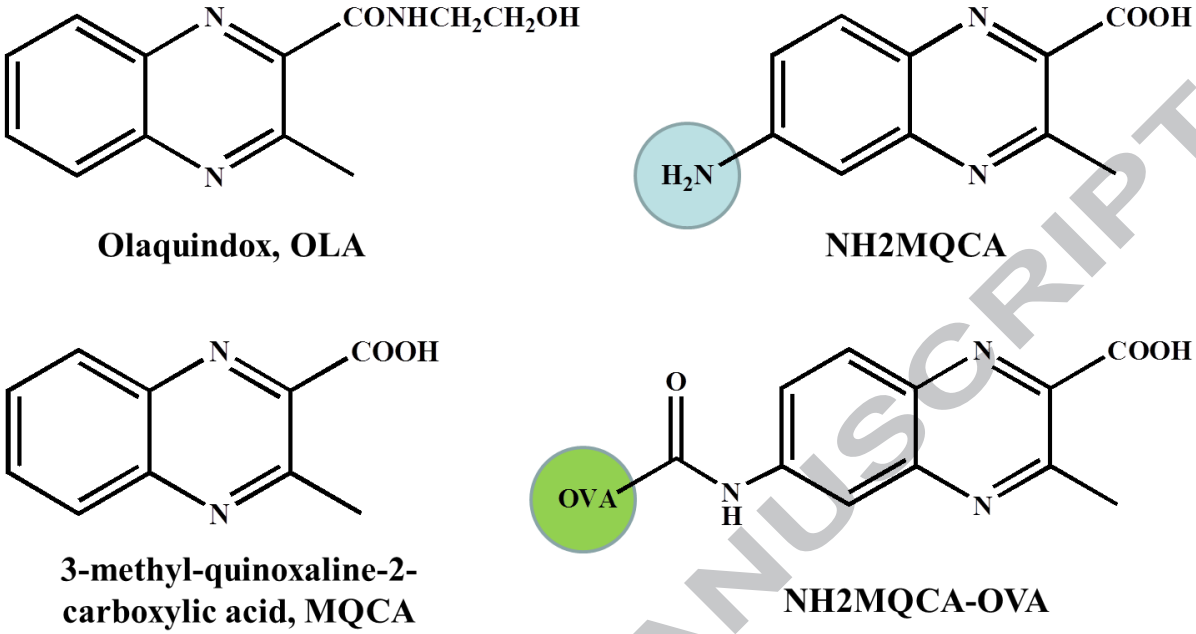
items	SPR method	ic-ELISA method[a]
antibody	Monoclonal antibody 5B10	Monoclonal antibody 5B10
IC_{50} (ng mL^{-1})	12.9	17.7
LOD (μg kg^{-1})	1.4 μg kg^{-1} in swine muscle 2.7 μg kg^{-1} in swine liver	1.9 μg kg^{-1} in swine muscle 4.3 μg kg^{-1} in swine liver
recovery	from 82% to 104.6%	from 74.2% 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: [a]the data obtained from the publication Zhang et al., 2015

456

457

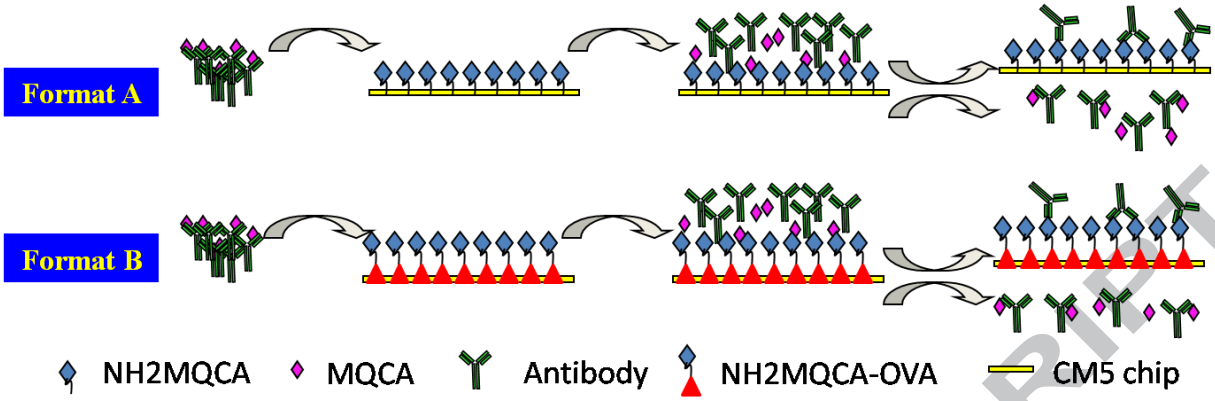
458 **Figures**



459

460 Figure 1

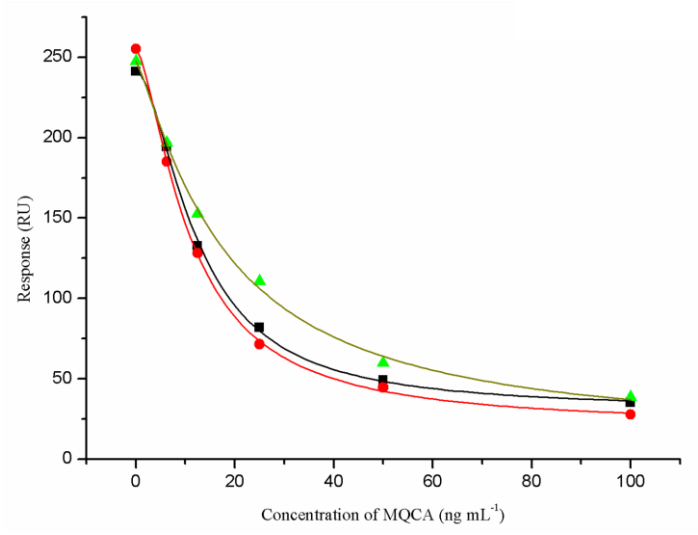
461



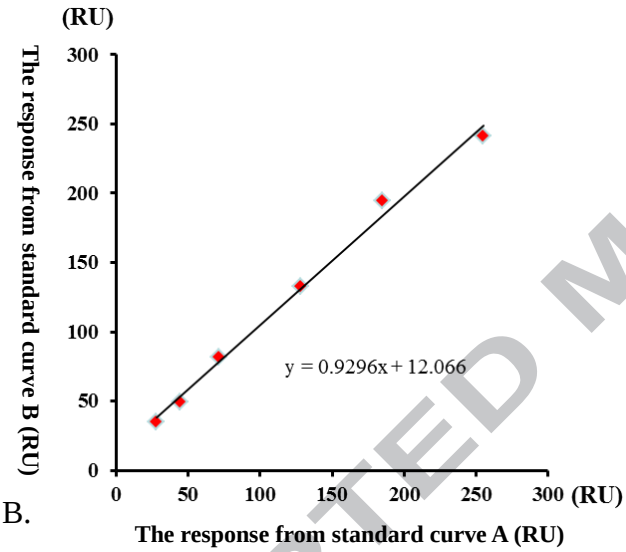
462

463 Figure 2

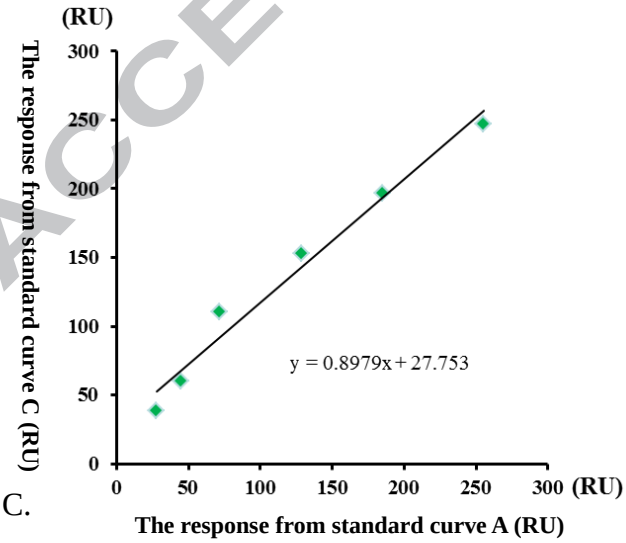
464



A.

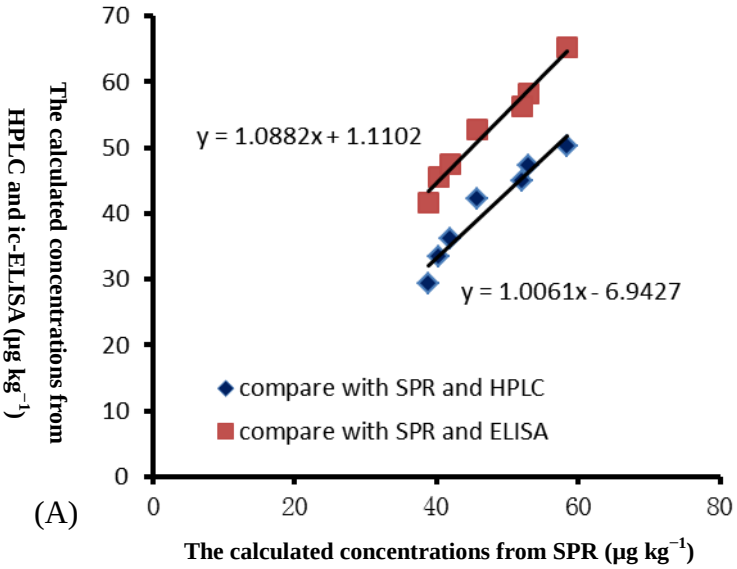


B.



C.

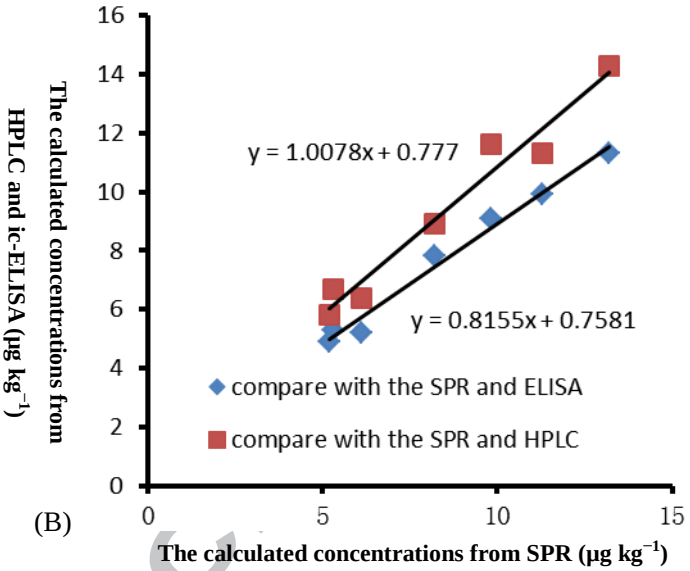
Figure 3



471 (A)

472

473



474 (B)

475

476 Figure 4

477

478

479

Research highlights

- A new SPR method for the determination of MQCA had been reported.
- The developed SPR method is the first report for MQCA in animal tissues.
- The SPR method can serve as an effective screening tool in any routine laboratory.