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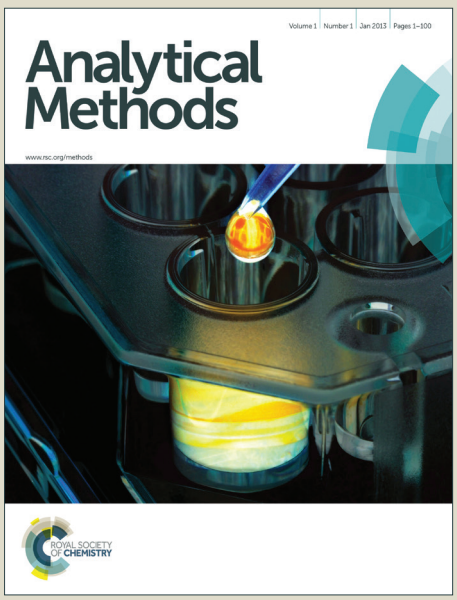

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A novel hapten and monoclonal-based enzyme-linked immunosorbent assay for 3-methyl-quinoxaline-2-carboxylic acid in edible animal tissues

Xiya Zhang, Dapeng Peng[#], Yuanhu Pan, Yulian Wang, Dongmei Chen, Qi Zhou, Zhenli Liu, Zonghui Yuan [*]

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

[*] Corresponding author: Tel.: +86 27 8728 7186; fax: +86 27 8767 2232. E-mail address: yuan5802@mail.hzau.edu.cn.
[#]Co-first author

Abstract

To monitor the illegal use of olaquindox (OLA) in animals, a novel hapten and monoclonal antibody for 3-methyl-quinoxaline-2-carboxylic acid (MQCA), the marker residue of OLA, had been produced. And then, the monoclonal-based indirect competitive enzyme linked immunosorbent assay (ic-ELISA) has been established with simple sample preparation and clean-up. The obtained antibody 5B10 that has isotype IgG1 showed an IC_{50} value of 17.7 μg L^{-1} for MQCA and did not exhibit measurable cross-reactivity with other antibiotics. The limits of detection ranged from 1.9 μg kg^{-1} to 4.3 μg kg^{-1}. The recoveries were from 74.2% to 98.9% with a maximum of 17.3% of the coefficient of variation. Good correlation between ELISA and HPLC results in the incurred tissues demonstrated the reliability of the developed ic-ELISA. It would be a useful tool for the screening of the residues of MQCA in the edible tissues of animals.

Keywords: 3-methyl-quinoxaline-2-carboxylic acid; hapten, monoclonal antibody; indirect competitive enzyme-linked immunosorbent assay; edible animal tissues

1. Introduction

Olaquindox (OLA, **Figure 1**) is a chemically synthetic animal-specific antimicrobial growth promoter which has been extensively used worldwide since 1970s [1]. However, it has been banned since 1999 in the EU due to concerns about its toxic properties (genotoxic and tumorigen in rodent), human health risk and animal welfare issues [2]. OLA is also forbidden in the United States [1, 3], Brazil [4] and Mexico [5]. In China, OLA is approved for use in feed for swine up to 35 kg but prohibited for the poultry and fish [6].

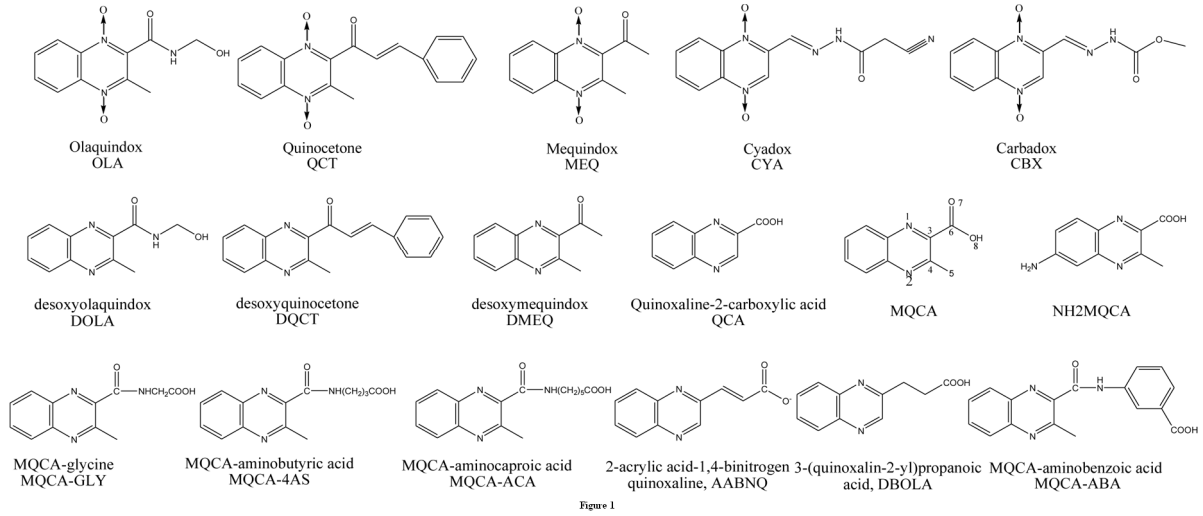

Figure 1 the structure of MQCA and their analogues and derivatives

Methyl-3-quinoxalin-2-carboxylic acid (MQCA, **Figure 1**), the major remaining detectable metabolite of the drug, was defined as the marker substance for OLA by the WHO/FAO Joint Expert Committee on Feed Additives (JECFA) in 1990 [1] and 1995 [3]. The maximum residue limits (MRLs) of MQCA was set as 4 μg kg^{-1} and 50 μg kg^{-1} in swine muscle and swine liver, respectively [1, 3]. Although regulations exist, illegal uses of OLA have been found in food animals, even in aquaculture (1000~2000 mg kg^{-1} feed) for the pursuit of commercial interests in recent decades [7]. Therefore, the need for an effective residue monitoring programme in place for OLA is clear.

Several physic-chemical methods, such as HPLC and LC-MS/MS with the limit of

detection as low as 0.10 μg kg^{-1} [8-12], have been developed for OLA and its marker residue MQCA. These methods are, however, only suitable for confirmatory analysis on 'suspect samples' as they are time consuming, labor intensive and requires specialized and expensive instrumentation and highly trained and skilled personnel to perform the test. Such a method is not useful for screening purpose in which a large number of samples are needed to be examined within a short time frame. Immunoassays, especially indirect competitive enzyme-linked immunosorbent assay (ic-ELISA), on the other hand have been used widely in food safety monitoring as they are rapid, robust, reliable, user-friendly and portable, and, due to the rapid generation of a result, will allow a greater proportion of food samples to be tested thus facilitating more effective control of regulated substances in the food chain.

Several groups had claimed that ELISA methods had been developed for analysing MQCA residues in edible animal tissues since 2005 [13-16]. In these studies, more than 7 different haptens (MQCA, MQCA-aminobenzoic acid (MQCA-ABA), MQCA-aminobutyric acid (MQCA-4AS), 2-acrylic-1,4-binitrogen-quinoline (AABNQ), MQCA-aminocaproic acid (MQCA-ACA), MQCA-glycine (MQCA-GLY), and 3-(quinoxalin-2-yl)propanoic acid (BDOLA), Figure 1) have been designed and synthesized. However, only 2 haptens, including MQCA itself [13-14] and MQCA-4AS [15-16], were successful used to produce MQCA specific antibodies. Even using the same hapten, the obtained antibodies are different. For example, using MQCA as hapten, which were directly conjugated to bovine serum albumin (BSA) through its carboxylic acid group, the specific antibodies against MQCA were respectively produced [13-14], whereas no antibody was produced by Gao et al. [17] and Jiang et al. [16]. These results make us confusing, which motivated us to develop a novel hapten to produce antibodies against MQCA.

In the present study, to obtain the specificity monoclonal antibodies (Mabs) against MQCA, a novel hapten, 6-amino-3-methyl-quinoxaline-2-carboxylic acid (NH_2MQCA), was

designed and synthesized. Then, the most sensitive hapten and Mabs were applied to develop a high sensitive and low cost ic-ELISA for determination of MQCA in various biological matrices without complicated sample preparation and clean-up.

2. Materials and Methods

2.1 Chemicals and reagents

Bovine serum albumin (BSA), ovalbumin (OVA), dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), poly-oxyethylenesorbitan monolaurate (Tween-20), urea hydrogen peroxide, RPMI-1640,glutaraldehyde (GA), N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 3,3',5,5'-tetramethylbenzidine (TMB), polyethylene glycol 1500 (PEG 1500, 50%), hypoxanthine-thymidine-aminopterin, dimethylsulfoxide, hypoxanthine-thymidine, Freund's adjuvant (complete and incomplete), and horseradish peroxidase labeled goat anti-rabbit IgG (HRP-IgG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The standard analytes, such as MQCA, quinoxaline-2-carboxylic acid (QCA), OLA, cyadox (CYA), carbadox (CBX), quinocetone (QCT) and mequindox (MEQ), tetracycline, benzylpenicillin, furazolidone, sulfadiazine, streptomycin, erythromycin and enrofloxacin, were purchased from Institute of Veterinary Drug Control (Beijing, China). Desoxyquinocetone (DQCT), desoxymequindox (DMEQ), desoxyolaquindox (DOLA), and 3-methyl-6-nitroquinoxaline-2-carboxylic acid were supplied by the Institute of Veterinary Pharmaceuticals, Huazhong Agricultural University (Wuhan, China). Fetal calf serum was purchased from Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). All other chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and were all of analytical grade.

2.2 Synthesis of hapten and antigens

The novel hapten NH_2MQCA was prepared following the modified procedure of the previous studies [18-21]. Briefly, 200 mg 3-methyl-6-nitroquinoxaline-2-carboxylic acid, 10

mg 5% Pb/C, and 30 mL methanol were stirred under H_2 for 2 h at room temperature (RT). After Pb/C was filtrated, the solvent was evaporated under reduced pressure. The hapten NH_2MQCA was obtained after the residue was recrystallized in methanol. The structure of NH_2MQCA was identified by the 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 (shown in **Figure 2A**). 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) (shown in **Figure 2B**).

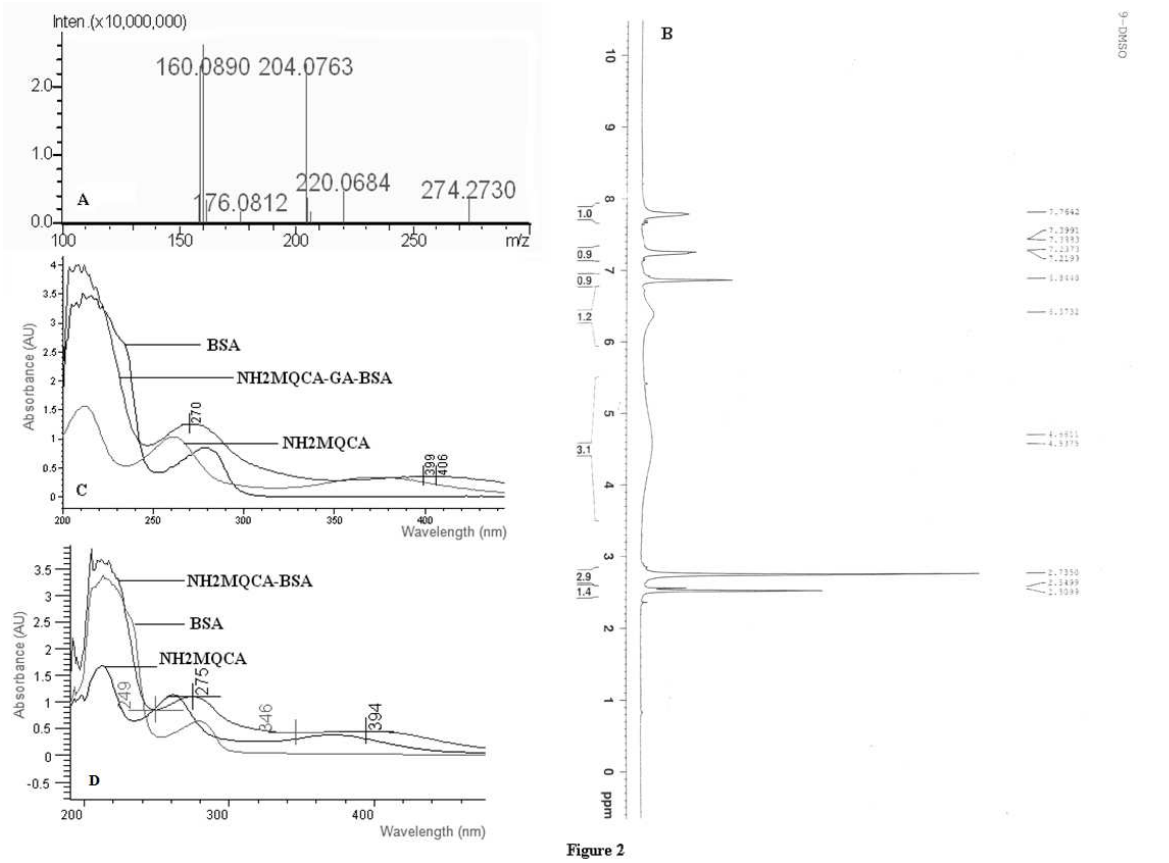

Figure 2

Figure 2 Characterization of hapten and antigen

Note: A, Full scan mass spectra of hapten NH_2MQCA; B, NMR spectra of hapten NH_2MQCA; C, UV absorbance spectrum of NH_2MQCA-GA-BSA, BSA, and NH_2MQCA; D, UV absorbance spectrum of NH_2MQCA-BSA, BSA, and NH_2MQCA)

The hapten NH_2MQCA was conjugated to BSA via linker GA following the modified procedure [22] to prepare NH_2MQCA-GA-BSA (immunogen I). Briefly, 36 mg hapten NH_2MQCA was dissolved in 2 mL of N-dimethyformamide (DMF). The solution was added slowly to 100 mg of BSA with 10 mL phosphate buffered saline (PBS) under stirring. Then, 0.1 mL 25% GA was added dropwise to the mixture. After incubation for 4 h at RT, the solution was centrifuged and supernatant was exhaustive dialysis against PBS and then stored at –20°C.

The hapten NH_2MQCA on the other hand was conjugated to BSA following the modified procedure of the previous study [23] to prepare NH_2MQCA-BSA (immunogen II). Briefly, 34 mg BSA, 40 mg EDC and 25 mg NHS were dissolved in 10 mL PBS (10 mmol L^{-1}) and stirred for 1 h at RT. Then, a solution of 20 mg hapten NH_2MQCA in 1 mL DMF was added dropwise. The reaction mixture was mixed for 3 h at RT. Then, the solution was centrifuged and supernatant was exhaustive dialysis against PBS and then stored at –20°C.

The coating conjugates were synthesized and characterized essentially as described for the immunizing conjugate, except that OVA was used as carrier protein instead of BSA. Verification of conjugate synthesis and estimation of the hapten/protein ratio was performed by the 8453 UV-Visible Spectrophotometer (Aglient 8453, USA).

2.3 Preparation of monoclonal antibodies

All animal experiments that described in the present study were performed in adherence to Huazhong Agricultural University animal experiment center guidelines and approved by Animal Ethics Committee. Female Balb/c mice (6 - 8 weeks old), which had been purchased from Hubei Center for Disease Control and Prevention (Wuhan, China), were inoculated with the immunizing conjugates (immunogen I, II). Firstly, the immunogens were prepared for injection by emulsification of the conjugates in 500 μL of sterile isotonic saline and 500 μL of Freund's adjuvant. This cocktail was mixed vigorously until a homogeneous suspension was

obtained. Complete adjuvant was used for the first injection and incomplete adjuvant was used for the subsequent injections. The immunogen emulsion was injected subcutaneously into multiple sites on the back of each mouse. Blood was collected and titres of antisera were determined by indirect ELISA. The mice giving the best dose–response curve were selected for fusion.

Spleen cells of the immunized mice were fused with myeloma cells Sp2/0 at a ratio of 10:1 according to the standard procedure [24]. Hybridoma from wells having a positive response in the ELISA described below were cloned twice by limiting dilution and expanded to guarantee its monoclonal origin. After cell culture, the cultured hybridoma was intraperitoneally injected into mice to produce ascites. The class and subclass of the isotypes of the secreted antibody were determined by using a mouse monoclonal antibody isotyping kit (Proteintech Group, Inc, Chicago, IL, USA).

The Mabs raised against each of the immunizing conjugates were screened against each of the corresponding coating antigens in a checkerboard for the best dilution of the coating conjugate and antiserum. The extent of cross-reactivity (CR) was assessed by determining the IC_{50} values in the ic-ELISA. Several quinoxaline compounds, their metabolites and some antibiotics, such as OLA, CYA, CBX, QCT, MEQ, DOLA, DQCT, DMEQ, tetracycline, benzylpenicillin, furazolidone, sulfadiazine, streptomycin, erythromycin and enrofloxacin, were selected to test for CR. The concentrations of the standard solutions of the compounds covered the range from 0.1 to 1000 μg L^{-1}. The CR values were calculated as follows: CR = (IC_{50} of MQCA / IC_{50} of competitor) × 100%. The detailed procedure of ELISA was described in the section of indirect ELISA and ic-ELISA. The one with the lowest IC_{50} to MQCA would be selected for the rest of this study.

2.4 Indirect ELISA and ic-ELISA

The protocol used for the indirect ELISA was similar to that described previously [25].

Briefly, 96-well Maxisorp microtitre plates (Nunc, Roskilde, Denmark) were coated with coating conjugates in 100 μL of coating buffer (0.05 mol L^{-1} carbonate buffers, pH 9.6) overnight at 4ºC. The plates were washed three times with PBS containing 0.1% Tween-20 (PBST) and were incubated with 200 μL of 1% OVA in PBS at 37°C for 0.5 h. The plates were then washed with PBST, followed by the addition of 100 μL of antiserum in each well. After 0.5 h of incubation at 37ºC, the plates were washed with PBST and then incubated with 100 μL of HRP-IgG (1:5,000) at 37ºC for 0.5 h. After washing the plate with PBST, 100 μL of TMB substrate solution were added to each well. The samples were incubated for 15 min at RT in the dark, followed by the addition of the stop solution (2 M H_2SO_4). The absorbance at 450 nm was measured on a Tecan Sunrise 2.5 Microplate Reader (SUNRISE, Austria).

The protocol used for the ic-ELISA was similar to that described previously [25]. Briefly, 96-well Maxisorp microtitre plates were coated with 100 μL of coating conjugates. After being washed and blocked, 50 μL of the antibody and 50 μL of varying concentrations of standard analyte or the samples were added to each well. The plates were then incubated, washed, and measured with a Microplate Reader as described above. A linear standard dose response curve was obtained by plotting log [analyte] versus percentage of binding.

2.5 Sample preparation

Samples, such as muscle (swine, chicken, fish, and shrimp) and liver (swine), were minced and homogenized. Each homogenized samples (2 g) were weighed into a 50 mL polypropylene centrifuge tube. 8 mL ethyl acetate and 4 mL HCl (1.5 mol L^{-1}) was added, and the mixture was vortex mixed for 3 min and then centrifuged for 5 min at 4000 r min^{-1}. The supernatant was transferred into another 50 mL polypropylene centrifuge tube and 5 mL of saturation NaCl was added, and mixed for 1 min. After standing 5 min, 4 mL of ethyl acetate was dried using nitrogen gas at 50 °C. Then the muscle samples residue was re-dissolved with 0.5 mL of 10 mmol L^{-1} phosphate buffer and the liver sample residue was re-dissolved with 2

mL of 10 mmol L^{-1} phosphate buffer and washed with 1 mL hexane, and the phosphate buffer was used in the ELISA.

2.6 Validation of the ic-ELISA

The ic-ELISA validation was carried out using 20 different frozen tissue samples (including the muscle of swine, chicken, fish, shrimp, and the liver of swine,) purchased from local retail outlets. All the samples had previously been proven by HPLC analysis [11] to be free of OLA and its marker residue MQCA. The standard solution (MQCA) was diluted in PBS to obtain a five-point standard curve (4, 8, 16, 32, and 64 μg L^{-1}). Each tissue was assayed using ic-ELISA to demonstrate the range of blank matrix effects and to determine the limit of detection (LOD). The determination of 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 represented by recovery and coefficient of variation (CV), respectively. Blank muscle (swine, chicken, fish, and shrimp) in five replicates spiked with known amounts of MQCA at 4 μg kg^{-1}, 8 μg kg^{-1}, 16 μg kg^{-1} and liver (swine) at 15 μg kg^{-1}, 25 μg kg^{-1} and 50 μg kg^{-1}. The recovery (percent) of the spiked MQCA was established using five spiked duplicate blanks for three different analyses and was calculated using the following equation: (concentration measured / concentration spiked) × 100. CVs were determined by analyzing the above samples spiked with MQCA for five different analyses. Each concentration level was tested three times in a time span of 2 months.

2.7 Comparison of the ic-ELISA with HPLC

To test the detection capability and the accuracy of the developed ic-ELISA for incurred tissues, a comparison of ic-ELISA and HPLC was carried out. Seven swines were treated with OLA-containing feed (100 mg kg^{-1}) for 5 consecutive days, followed by a withdrawal period of 1 day, then, animals were sacrificed and organs were removed. Edible tissues such as muscle and liver were collected and homogenized, and subsequently placed in

labeled plastic bags. All samples (liver and muscle) were subjected to this ic-ELISA and HPLC analysis according to the methods described by Wu et al [11].

3. Results and Discussions

3.1 Hapten design and synthesis

The basic chemical structure of MQCA is a quinoxaline ring to which carboxyl and methyl groups are attached. Based on the hypothesis that the quinoxaline ring portion was the antigenic determinant, the hapten MQCA [13-15, 17] and, MQCA-4AS [14, 15, 17] were designed (shown in **Figure 1**). It would place the quinoxaline ring portion of the conjugates exposed on the outside surface of the carrier protein, available for interaction with receptors of the immune system. The antibody against MQCA was successfully produced [13-16], whereas were not obtained as predicted by Gao [17].

The obtained antibodies by Gao showed low sensitivity to MQCA, but showed stronger CRs with MQCA-4AS, DOLA (MQCA-4AS, 3961.3%; DOLA, 4417.3%; DQCT, 367.0%; OLA, 95.4%; QCT, 93.8%) [17]. Comparing the structure of MQCA with these chemicals (**Figure 1**), it was found that except for the common structure of these chemicals consisting in quinoxaline ring, both MQCA-4AS and DOLA also contain the special structure of the acyl amido bond. It suggests that the acyl amido bond may play more important role than quinoxaline ring in the epitope determination. On the other hand, though QCT doesn't contain the acyl amido bond, the CRs data with OLA (95.4%) and QCT (93.8%) were close. The reason is not clear. We therefore developed a new hypothesis that the carboxyl group in the 2-position had the predominant influence on antibody binding.

According to the new hypothesis, it should therefore be avoided that a direct conjugation with the carboxyl group of MQCA and amino groups of protein. Obviously, the hapten should be conjugated to the carrier protein via other-position substituents, except 2-position substituents. To obtain the novel hapten, the molecular modeling was carried out by

HyperChem Release 8.0 software according to the previous studies [26-28]. The electrostatic potential 2D contours that displayed the minimum energy conformations of these analytes are shown in **Figure 3** and the charge distribution of these analytes are shown in **Table 1**. It was found that the hapten NH_2MQCA mimicked well the target analyte MQCA either the charge distribution or the electrostatic potential 2D contours. We thereby choose the NH_2MQCA as hapten in this study.

Table 1 comparison of charge distribution for MQCA and their analogues and derivatives

compound	1	2	3	4	5	6	7	8	sum
MQCA	0.146	-1.804	1.914	2.023	-1.257	-0.748	-0.833	-1.097	-1.656
NH_2MQCA	-0.806	-0.903	0.259	0.523	-0.087	1.277	-1.179	-0.756	-1.672
MQCA-4AS	-0.781	-0.831	0.424	0.523	-0.091	1.043	-1.236		-0.949
DOLA	-0.781	-0.831	0.424	0.523	-0.091	1.045	-1.237		-0.948

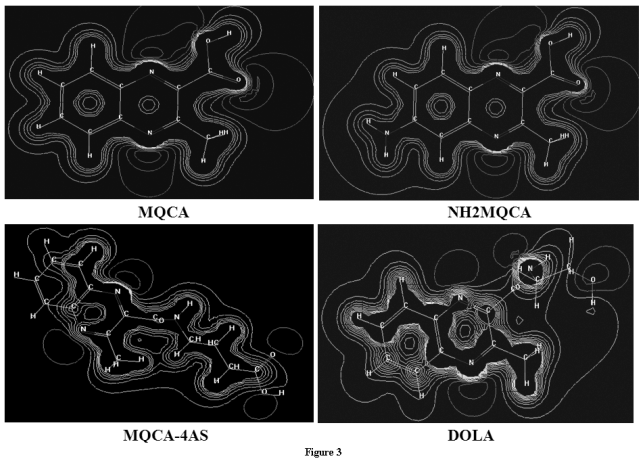

Figure 3 the electrostatic potential 2D contours of MQCA and their analogues and derivatives

3.2 Characterization of the conjugates and monoclonal antibodies

As shown in Figure 2C-2D, the ultraviolet absorbance spectrum of NH_2MQCA-BSA (λmax, 275 nm and 394 nm) and NH_2MQCA-GA-BSA (λmax, 270 nm and 403 nm) were

different from ultraviolet absorbance spectrum of BSA (λmax, 279 nm), and NH_2MQCA (λmax, 261 nm and 371), which prove the synthesis of conjugates successfully. As well, the ultraviolet absorbance spectrum of NH_2MQCA-OVA and NH_2MQCA-GA-OVA are similar with the above conjugates. The estimated incorporation rates of conjugates are 15 (9.7) and 13 (8.8), respectively (shown in **Table 2**). Then, two immunogens (NH_2MQCA–GA–BSA and NH_2MQCA–BSA) were used to elicit the specific antibodies against MQCA. As shown in **Table 2**, the result of the titers of antisera clearly illustrate that the long-spacer-arm GA containing five carbons showed a higher titer than the short-spacer-arm containing one carbon. A possible explanation cited for this is that when a hapten is linked to the carrier protein by a short linker group, it is masked or lost within the protein tertiary structure, but if the linker is sufficiently long, it can project the hapten above the carrier protein surface.

Table 2 the ratio of haptens and carrier proteins and titer of antisera produced with different immunogen

conjugates	ratio	Titer ($1:X\times10^3$)			
		1	2	3	4
Immunogen I (Coating conjugate I)	15 (9.7)	25.6	51.2	51.2	25.6
Immunogen II (Coating conjugate II)	13 (8.8)	16	2	16	16

Therefore, the spleen cells from the mice immunized with NH_2MQCA–GA–BSA were used for the fusion experiment. After several cell fusion and culture, the hybridoma cells 5B10 and 4C6 were rescreened and sub-cloned three times. The titers and specificity of Mabs were determined by indirect ELISA. As shown in **Table 3**, the Mab 5B10 that has isotype IgG1 showed CR towards MQCA (CR = 100%) and did not exhibit measurable CR (CR＜ 0.1%) with other antibiotics such as OLA, QCT, MEQ, DMEQ, DQCT, DOLA, CYA, and CBX, which was selected for the rest of this study. The result confirms our hypothesis and clearly illustrates that the substituent in the 2-position had the predominant influence on

antibody binding. Furthermore, the 3-position methyl substituents had great effect on binding.

3.3 The standard curve for the ic-ELISA

In the present study, coating conjugate I and II were used as homogeneous and heterogeneous coating antigens, respectively. The results have been presented in **Figure 4**, which the IC_{50} with homogeneous format was found to be 17.7 μg L^{-1} compared to 29.3 μg L^{-1} with the heterogeneous format. These findings are different with the study of Kim et al [25] that the coating conjugate heterology may improve the sensitivity of ELISA. The reason is not clear. Therefore, coating conjugate I was selected as coating antigen in the present study.

Table 3 comparisons of the cross-reactivity of antibody from hybridoma cells 5B10 and 4C6

compound	immunogen Ⅰ, 5B10		immunogen Ⅱ, 4C6	
	IC_{50} (μg L^{-1})	CR (%)	IC_{50} (μg L^{-1})	CR (%)
MQCA	17.7	100	95.7	100
NH_2MQCA	15.8	112.2	90.0	106.3
QCA	2380.6	0.7	$>10^7$	<0.01
OLA	$>10^7$	<0.01	$>10^7$	<0.01
DOLA	$>10^7$	<0.01	$>10^7$	<0.01
QCT	$>10^7$	<0.01	$>10^7$	<0.01
DQCT	$>10^7$	<0.01	$>10^7$	<0.01
MEQ	$>10^7$	<0.01	$>10^7$	<0.01
DMEQ	$>10^7$	<0.01	$>10^7$	<0.01
CYA	$>10^7$	<0.01	$>10^7$	<0.01

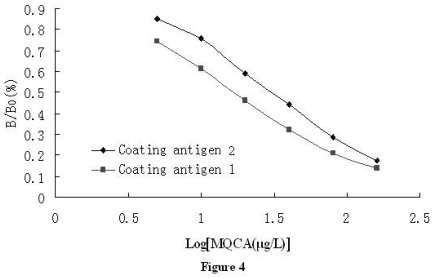

Figure 4

Figure 4 comparison of homogeneous and heterogeneous icELISA

The optimum ic-ELISA conditions were determined as 1 μg mL^{-1} of coating conjugate I concentration, a 1:3.2×10^4 ratio of antibody (5B10) dilution. These were the best conditions and were fixed for the rest of the experiment. As shown in **Figure 5**, the standard curve based on the MQCA matrix calibration range from 4 to 64 μg L^{-1}. The IC_{50} value of the method for MQCA was 17.7 μg L^{-1}.

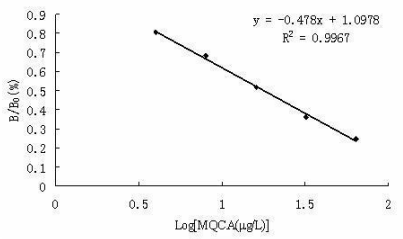

Figure 5

Figure 5 standard curves of the method

3.4 Validation of the ic-ELISA method

Based on the results from twenty different blank samples, the LODs of the method for MQCA matrix calibration were 1.9 μg kg^{-1}, 4.3 μg kg^{-1}, 1.5 μg kg^{-1}, 2.2 μg kg^{-1},1.7 μg kg^{-1} in swine muscle, swine liver, fish muscle, chicken muscle, and shrimp muscle, respectively. As shown in **Table** 4, **t**he recoveries of the above samples spiked with MQCA were in the range from 74.2% to 98.9% and the CVs were less than 17.3%.

Table 4 the LOD, recoveries and coefficients of variation (CVs) of the ic-ELISA

tissues	LOD (μg kg^{-1})	spiked level (μg kg^{-1})	mean recovery (%)	CV (%)
swine muscle	1.9	4	90.2	17.3

		8	98.9	14.6
		16	94.3	15.8
swine liver	4.3	15	75	9.2
		25	74.2	8.7
		50	78.7	10.9
fish muscle	1.5	4	74.5	7.4
		8	82.1	11.8
		16	85.3	13.9
chicken muscle	2.2	4	80.1	7.8
		8	77.3	10.6
		16	75.5	12.7
shrimp muscle	1.7	4	83.0	14.6
		8	80.5	12.6
		16	78.4	11.7

MQCA could be extracted from the swine liver matrix with the aid of acid or alkaline hydrolysis or enzymatic digestion as described in previous experiments [8-9]. However, this method has low recovery due to inadequate tissue de-proteination by the acid, which leads to re-adsorption of MQCA. Therefore, Wu et al. [11] applied a mixture of different concentrations of metaphosphoric acid in 20% methanol to induce greater tissue de-proteination, which resulted in a significant improvement in the absolute recovery in their study. In addition, Hutchinson et al. [9] used a solid-phase extraction (SPE) cartridge for sample clean-up along with non-endcapped benzenesulfonic acid. Wu et al. [11] utilized the anionic property of MQCA and subjected the analytes to a final clean-up using mixed mode anion-exchange SPE column under neutral pH. However, SPE is usually time consuming and causes an increase in analytical cost.

In the present study, metaphosphoric acid, HCl, methanol, and ethyl acetate, were selected to optimize the extraction method. Based on the relative extraction ratio (recovery) of the swine liver sample spiked with MQCA at 50 μg kg^{-1} level, several combinations, metaphosphoric acid/ethyl acetate, metaphosphoric acid/methanol, ethyl acetate, methanol, HCl/ethyl acetate, and HCl/methanol, were used to optimize the sample preparation. As a result, the optimized extraction solvent was the mixture of ethyl acetate and HCl (2:1, v/v). In additional, SPE was avoided during sample preparation, which lowered the analytical cost. Using this sample preparation procedure, the obtained results showed that the developed ic-ELISA could detect MQCA in various biological matrices (swine muscle, swine liver, fish muscle, chicken muscle, and shrimp muscle), of which the average recoveries and CVs in all biological matrices were meet the requirements of the European Decision 2002/657/EC [29].

Furthermore, a good correlation between ELISA and HPLC results was observed in the incurred tissues (shown in **Table 5)**. It suggested that the ic-ELISA is a reliable tool for the detection of MQCA residues in animal edible tissues.

Table 5 Comparison of the results between the HPLC and the ELISA method in swine muscle and liver samples

NO.	swine liver (μg kg^{-1})		swine muscle (μg kg^{-1})	
	HPLC	ELISA	HPLC	ELISA
pig 1	52.8	42.2	11.6	9.1
pig 2	65.2	50.3	14.3	11.3
pig 3	51.6	29.4	6.7	5.3
pig 4	45.5	33.4	5.8	4.9
pig 5	58.2	47.2	11.3	9.9
pig 6	56.3	44.9	8.9	7.8
pig 7	47.5	36.2	6.4	5.2

4. Conclusion

In the present study, we have designed and synthesized a novel hapten and succeeded in the preparation of a highly specific monoclonal antibody against MQCA and development of an ELISA and its kit for monitoring MQCA in tissue samples of swines, chickens, fishes, and shrimps with an IC_{50} of 17.7 ng ml^{-1}. It fills the urgent need for analytical methods for strict monitoring of OLA and its marker residues in food of animal origin.

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References

[1] 36th JECFA Report, WHO Technical Series, 799 (1990) 50-54.

[2] Commission Regulation (EC) No 2788/98, Official J. Eur. Commun., L347 (1998) 31-32.

[3] 42th JECFA Report, WHO Technical Series, 851 (1995) 19-21.

[4] NOM-064-ZOO-2000. (2004) Lineamientos para la clasificación y prescripción de productos farmacéuticos veterinarios por el nivel de riesgo de sus ingredientes activos.

[5] Ministério da Agricultura. Normativa nº 11, assinada no dia 24 de novembro de 2004 e publicada no dia 25 de novembro de 2004. [Acessed jan. 10, 2005]. Avaliable from: www.agricultura.com.br

[6] http://jckspaqj.aqsiq.gov.cn/dwyxspjyjy/gnxgbz/200610/t20061027_9809.htm

[7] K. Wang, Y. Geng. Acta Veterinaria et Zootechnica Sinica, 6 (2002) 565-569.

[8] J.O. Boison, S.C. Lee, R.G. Gedir. Anal. Chim. Acta., 637 (2009) 128-134.

[9] M.J. Hutchinson, P.B. Young, D.G. Kennedy. J Chromatogr. B, 816 (2005) 15-20.

[10] A. Merou, G. Kaklamanos, G. Theodoridis. J Chromatogr. B, 881-882 (2012) 90-95.

[11] Y. Wu, H. Yu, Y. Wang, L. Huang, Y. Tao, D. Chen, D. Peng, Z. Liu, Z. Yuan. J Chromatogr. A, 1146 (2007) 1-7.

[12] X. Zhang, B. Zheng, H. Zhang, X. Chen, G. Mei. J Sep. Sci., 34 (2011) 469-474.

[13] W. Chen, Y. Jiang, B. Ji, C. Zhu, L. Liu, C. Peng, M.K. Jin, R. Qiao, Z. Jin, L. Wang, S. Zhu, C. Xu. Biosens Bioelectron, 24 (2009) 2858-2863.

[14] L. Cheng, J. Shen, Z. Wang, W. Jiang, S. Zhang. Anal. Bioanal. Chem., 405 (2013) 2653–2659.

[15] N. Yue, B. Ji, L. Liu, G. Tao, S.A. Eremin, L. Wu. Food Agric. Immunol., 20 (2009)

173-183.

[16] W. Jiang, R.C. Beier, Z. Wang, Y. Wu, J. Shen. J. Agric. Food Chem., 61 (2013) 10018-10025.

[17] Gao, A. (2005), Master Thesis, Huazhong Agricultural University, Wuhan, China.

[18] J. Francis, J.K. Landquist, A.A. Levi, J.A. Silk, J.M. Thorp. Biochem. J., 63 (1956) 455-457.

[19] K. Mustafa. Monatsh. Chem., 120 (1989) 127-130.

[20] M. Seyhan. Chem. Ber., 84 (1951) 477.

[21] H. Xu, D. Julie, B. Christian, M.L. Philippe, F. Xavier, H. Reynald, F. Bruno. Bioorg. Med. Chem. Lett., 16 (2006) 815-820.

[22] D. Peng, S. Ye, Y. Wang, D. Chen, Y. Tao, L. Huang, Z. Liu, M. Dai, X. Wang, Z. Yuan. J. Agric. Food Chem., 60 (2012) 44-51.

[23] H.T. Lei, Y.D. Shen, L.J. Song, J.Y. Yang, P.C. Olivier, A.H. Simon, H. Wang, Y.M. Sun, T.E. Christopher, Anal. Chim. Acta., 665 (2010) 84-90.

[24] G. Köhler, C. Milstein. Nature, 256 (1975) 495-497.

[25] Y.J. Kim, Y.A. Cho, H.S. Lee, Y.T. Lee, S.J. Gee, B.D. Hammock. Anal. Chim. Acta., 475 (2003) 85-96.

[26] M. Franek, I. Diblikova, I. Cernoch, M. Vass, K. Hruska. Anal. Chem., 78 (2006) 1559-1567.

[27] C.A. Spinks, G.M. Wyatt, H.A. Lee, M.R.A. Morgan. Bioconjugate Chem., 10 (1999) 583-588.

[28] Z.H. Wang, Y. Zhu, S.Y. Ding, F.Y. He, C.B. Ross, J.C. Li, H.Y. Jiang, C.W. Feng, Y.P.

Wan, S.X. Zhang, Z.P. Kai, X.L. Yang, J.Z. Shen. Anal. Chem., 79 (2007) 4471-4483.

[29] Commission 2002/657/EC, Official J. Eur. Commun., L221 (2002) 8-36.