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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

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ABSTRACT: Immunoassays contribute greatly to food safety. Yet there are no reported immunoassays that simultaneously detect MQCA and QCA, the marker residues for olaquindox and carbadox, respectively. Here, a broad-specificity mAb was successfully produced, and the mAb showed good cross-reactivity with both MQCA and QCA, having IC_{50} values in buffer of 4.8 and 9.6 ng/mL, respectively. The calibration curves ranged from 0.3 to 81 μg/kg. The average recoveries ranged from 76% to 108% at different spiked levels (2, 4, and 8 μg/kg for MQCA; and 4, 10, and 20 μg/kg for QCA), and the intra-/interday coefficients of variation were 4.2−13.3%. The limits of detection of MQCA and QCA in chicken, fish, pork, and shrimp were 1.76, 1.32, 1.90, and 1.18 μg/kg, respectively. This method was verified by LC−MS/MS, with a correlation coefficient above 0.98. The immunoassay was rapid and reliable for simultaneous screening analysis of MQCA and QCA residues.

KEYWORDS: *3-methyl-quinoxaline-2-carboxylic acid, quinoxaline-2-carboxylic acid, ELISA, food safety, immunoassay*

INTRODUCTION

Carbadox (methyl-3-(2-quinoxalinylmethylene)carbazate-*N*,*N*′-dioxide) (CBX) and olaquindox (*N*-(2-hydroxyethyl)-3-methyl-2-quinoxaline-carboxamide-1,4-dioxide) (OLA) are well-known synthetic antibacterial drugs of the quinoxaline-1,4-dioxide family, and they are widely used as antimicrobial and growth-promoting agents to improve feed efficiency and to increase the rate of weight gain in aquaculture and animal husbandry.[1] CBX and OLA can be rapidly metabolized in vivo into mono- and desoxy-compounds and corresponding carboxylic acid derivatives.[2,3] Quinoxaline-2-carboxylic acid (QCA) is the major detectable metabolite in animal tissues and is designated as the marker residue for CBX.[4] The 3-methyl-quinoxaline-2-carboxylic acid (MQCA) is the major detectable metabolite of OLA, and it was designated to be the marker residue for monitoring the illegal use of OLA in livestock animal production.[5,6] The chemical structures of CBX, OLA, MQCA, and QCA are shown in Figure 1.

Besides CBX and OLA, China has approved three other quinoxaline-1,4-dioxides, cyadox (CYA), mequindox (MEQ), and quinocetone (QCT), to be used as growth promoters due to their lower toxicity,[7,8] and to their excellent antitumor[9] and antituberculosis[10] effects. The three new growth promoters have metabolic pathways similar to those of CBX and OLA in vivo, and produce many metabolites including MQCA and QCA.[11,12] The chemical structures of the three new quinoxaline-1,4-dioxides, CYA, MEQ, and QCT, and their main metabolites, (*E*)-2-cyano-*N*′-(quinoxalin-2-ylmethylene)-acetohydrazide (BDCYA), 1-(3-methylquinoxalin-2-yl)-ethanone (BDMEQ), and (*E*)-1-(3-methylquinoxalin-2-yl)-3-phenylprop-2-en-1-one (BDQCT), respectively, are shown in Figure 1.

The extensive use of veterinary drugs in animal husbandry has raised worldwide concerns regarding the emergence and spread of antibiotic resistant bacteria in the environment.[13] Quinoxaline-1,4-dioxides and their metabolites also have shown diverse human toxicity, such as carcinogenicity, mutagenicity, and photoallergenic effects.[14,15] Because of these negative effects on health, CBX and OLA were banned by the European Union.[16] All of these concerns helped many countries ban or limit the use of CBX and OLA in food producing animals. In China, there have been many cases of illegal use in animal production.[17] To ensure food safety and protect consumers from these hazards, the European Union, United States, and China have set maximum residue limits (MRLs) and withdrawal periods for CBX and OLA. In China, OLA is approved for use in porcine feed, and the maximum residue limits (MRLs) for MQCA in porcine liver and porcine muscle tissues are set at 50 and 4 μg/kg, respectively.[7] In 1991, the Joint FAO/WHO Expert Committee on Food Additives recommended MRLs for QCA in porcine liver and muscle tissues of 30 and 5 μg/kg, respectively.[16] In 2007, a concentration of 10 μg/kg was recommended as the minimum required performance limit (MRPL) for both MQCA and QCA in meat according to the European Union Reference Laboratory.[18]

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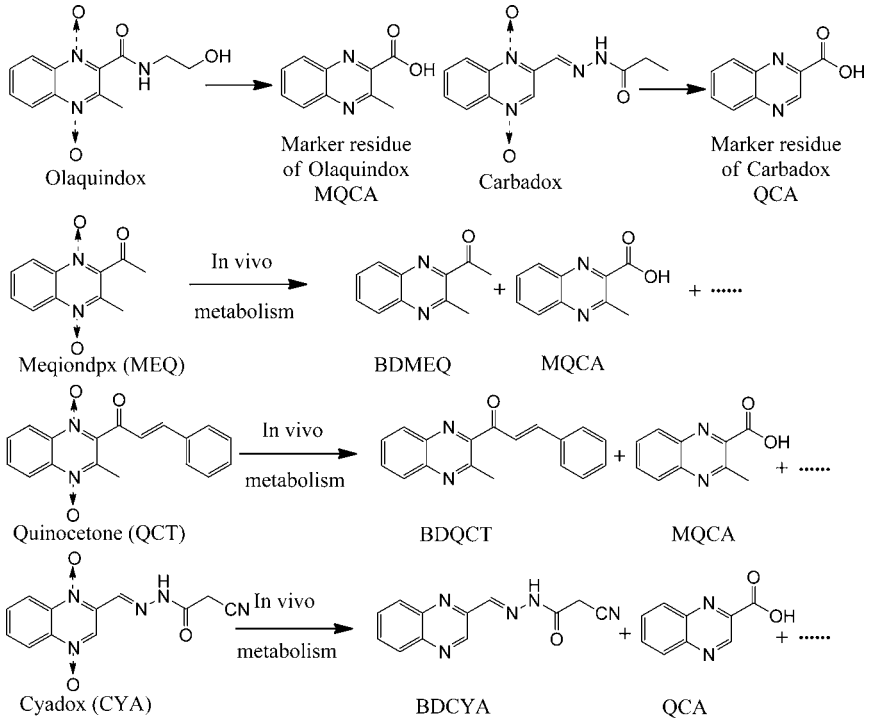

Figure 1. Chemical structures of olaquindox, carbadox, their marker residues, three new quinoxaline-1,4-dioxides, and portions of their metabolites.

Much effort has been devoted to develop sensitive, simple, and reliable analytical methods applicable to determine the marker residues for CBX and OLA. Currently, chromatographic separation combined with mass spectrometric detection is the widely accepted technique for veterinary drug residue analysis in complex food matrixes.[19,20] However, few methods have described the simultaneous analysis of both MQCA and QCA in animal tissues, which include two HPLC[21,22] and three LC–MS/MS[23−25] methods. Nevertheless, these chromatography methods cannot fulfill the demand for a routine screening method for MQCA and QCA, because these methods require expensive equipment, large volumes of solvents, and time-consuming sample cleanup.

The enzyme-linked immunosorbent assay (ELISA) has proven to be a useful, rapid, and sensitive tool for veterinary drug residue analysis.[26,27] In 2011, Peng et al.[28] reported an ELISA method to detect *N*-butylquinoxaline-2-carboxamide, a derivative of QCA. In 2012, Le et al.[29] developed a colloidal gold immunochromatographic assay for the determination of *N*-butylquinoxaline-2-carboxylic acid in edible animal tissues. Both of these methods focused on the detection of one derivative of QCA. Thus, the extracted QCA must be converted into the derivative, *N*-butylquinoxaline-2-carboxamide, to be detected by the ELISA and immunochromatographic assay. The time-consuming derivatization procedure would greatly limit its application as a screening method. Recently, Cheng et al.[30] and Song et al.[31] reported their research on the detection of olaquindox, the parent drug. To our knowledge, there are no published immunoassays describing the simultaneous determination of both MQCA and QCA in animal tissues. To generate high-quality broad-specificity antibodies, one must design a suitable hapten that closely mimics a common portion of the target, maximizing the steric, hydrophobic, and electronic similarity to the target analytes. Besides MQCA and QCA, two novel haptens, 4-(3-methylquinoxaline-2-carboxamido)-butanoic acid (MQCA-4AS) and 3-(quinoxalin-2-yl)propanoic acid (BDOLA), were synthesized on the basis of the common structure of MQCA and QCA, and used for broad-specificity antibody production. This Article discusses the development of a broad-specificity ELISA for the simultaneous detection of MQCA and QCA residues in edible animal muscle tissues.

MATERIALS AND METHODS

Chemicals and Materials. CBX, OLA, MQCA, QCA, and other quinoxaline-1,4-dioxides were obtained from Dr. Ehrenstorfer (Augsburg, Germany). Bovine serum albumin (BSA), ovalbumin (OVA), 1-ethyl-3-(dimethylaminopropyl)-carbodiimide, *N*-hydroxysuccinimide, and Freund's complete and incomplete adjuvants were purchased from Sigma-Aldrich (St. Louis, MO). Horseradish peroxidase conjugated goat antimouse IgG was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Apparatus. The 96-well polystyrene microtiter plates, and 12- and 96-well cell culture plates, were obtained from Costar Inc. (Milpitas, CA). Waters Acquity UPLC system, Acquity BEH C_{18} column, and the Micromass Quattro Premier XE triple-quadrupole mass spectrometer were obtained from Waters (Milford, MA). Spectra Max M5 microplate reader was obtained from Molecular Devices (Sunnyvale, CA).

Buffers. The following buffers were used in this study: (1) coating buffer was 0.05 M carbonate buffer, pH 9.6; (2) blocking buffer consisted of 0.01 M phosphate-buffered saline (PBS), pH 7.4, 0.5% casein, 5.0% sucrose, 0.02% proclin 300, and 5.0% calf serum; (3) washing buffer was PBS with 0.05% Tween 20; (4) standard dilution buffer was 0.01 M PBS, pH 6.5; (5) antibody dilution buffer was PBS containing 0.05% triton 100, and 0.02% proclin 300; (6) enzyme-labeled secondary antibody dilution buffer was the antibody dilution buffer containing 5.0% calf serum; (7) substrate was 0.1% 3,3′,5,5′-tetramethylbenzidine and H_2O_2 in 0.05 M citrate buffer, pH 4.5; and (8) 2 M H_2SO_4 was the stopping reagent.

Synthesis of Immunogen and Coating Antigen. In this study, a novel hapten MQCA-4AS was synthesized by conjugating γ-aminobutyric acid to MQCA. MQCA (0.1 mM) was activated by reaction with 60 μL of oxalyl chloride at 4 °C overnight. The activated MQCA mixture was added dropwise to γ-aminobutyric acid (0.1 mM, dissolved in 0.1 M carbonate–bicarbonate buffer (pH 9.5)), and the

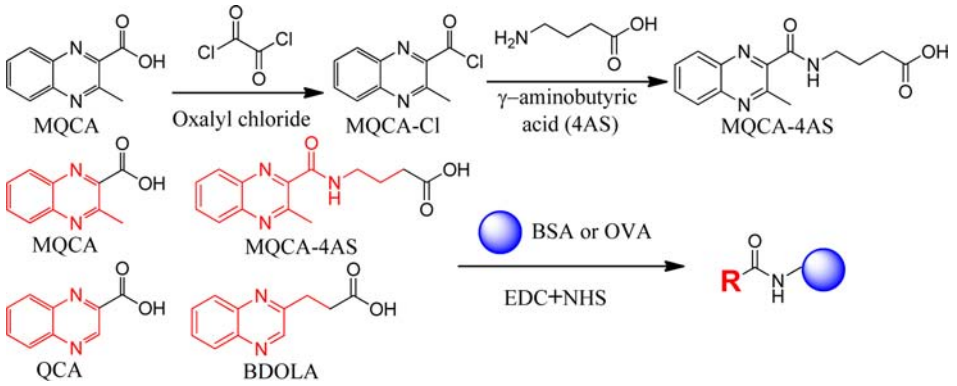

Figure 2. Synthesis scheme for the novel haptens and hapten–protein conjugates.

mixture was gently stirred for 5 h at 4 °C. MQCA, QCA, BDOLA, and MQCA-4AS were conjugated to BSA and OVA by the active ester method for synthesis of immunogens and coating antigens, respectively.[32,33] The haptens (0.1 mM) were activated by 1-ethyl-3-(dimethylaminopropyl)-carbodiimide (0.2 mM) and *N*-hydroxysuccinimide (0.2 mM). The activated mixtures were added dropwise to the carrier proteins (0.02 mM) in 0.1 M carbonate–bicarbonate buffer (pH 9.5). Generation and identification of haptens and hapten–BSA conjugates were carried out by the Beijing WDWK Bio Co. (Beijing, China).

mAb Production. Female BALB/c mice (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China) were immunized for monoclonal antibody (mAb) production. The first injection consisted of immunogen emulsified with Freund's complete adjuvant at a dosage of 50 μg/mouse. Subsequent booster immunizations were administered biweekly with the same dosage of immunogen emulsified with Freund's incomplete adjuvant. After the fifth immunization, the mouse exhibiting the highest antibody titer was sacrificed, and the splenocytes were fused with SP2/0 myeloma cells. The growing hybridoma cells were screened for antibody production by a noncompetitive indirect ELISA. The positive hybridomas were subcloned twice by limiting dilution, and the positive clones were used for ascites production.[34,35] The antibody was purified from ascites using saturated ammonium sulfate, and the purified mAb was stored at −20 °C in 500 μL aliquots.

Optimization of the ELISA Method. Competition experiments were carried out using a checkerboard titration to select the suitable concentrations of immunoreagents. The experimental conditions for the concentrations of coating antigen and antibody, pH and ionic strength of the assay buffer, and the immunoreaction temperature were optimized according to our previous research.[36]

The ELISA approach can be described as follows: microtiter plates were coated with coating antigen (100 μL/well), and incubated at 4 °C overnight. After blocking with 150 μL of blocking solution per well, 50 μL of the standards and 50 μL of the diluted mAb were added to each well, and the plates were incubated at 4 °C for 30 min. After the plates were washed, 100 μL of diluted goat-antimouse IgG-HRP solution was added, and the plates were incubated at 25 °C for 30 min. After the plates were washed, 100 μL of substrate solution was added per well. After incubation at 25 °C for 10 min, the reaction was stopped by addition of 50 μL of 2 M H_2SO_4. The absorbance values were determined by using the microplate reader in the dual-wavelength mode (450 nm for the test and 630 nm for reference).

Curve Fitting and Specificity Determination. Competition curves were graphed by plotting the absorbance values against the logarithm of the analyte concentration. The midpoint of the standard curve was the concentration of competitor that inhibited binding of the antibody by 50% (IC_{50}). The IC_{50} values were calculated by fitting the immunoassay data to a four parameter logistic equation:[37]

$$Y = (A - B)/[1 + (X/X_0)^P] + B$$

where A is the response at high asymptotes of the curve, B is the response at low asymptotes of the curve, P is the slope at the inflection point of the sigmoid, X_0 is the concentration of analyte resulting in 50% inhibition of tracer binding, and X is the calibration concentration.

To determine the specificity of the ELISA, a cross-reactivity study was carried out under optimum conditions. The cross-reactivity of the antibody against MQCA and QCA, and other chemicals (such as quinoxaline-1,4-dioxides and their metabolites) was calculated using the following equation:[35]

$$\text{cross-reactivity (\%)} = (IC_{50}\text{ of MQCA})/(IC_{50}\text{ of other analytes}) \times 100$$

Sample Pretreatment. The edible animal muscle tissues were purchased from local supermarkets and stored in a refrigerator (−20 °C) before use. Prior to the spiking and recovery tests, each sample was demonstrated to be MQCA and QCA free by using LC–MS/MS. Aliquots of blank samples or spiked samples (1.00 ± 0.01 g) were weighed out into polystyrene centrifuge tubes. After 2 M H_2SO_4 (2 mL) and deionized water (4 mL) were added, the samples were vortexed vigorously for 1 min. Next, ethyl acetate (8 mL) was added to each sample, and the mixtures were vortexed and centrifuged at 3000*g* for 10 min at 25 °C. A portion of the supernatants (4 mL) was transferred to centrifuge tubes, and evaporated under nitrogen until dry. Next, hexane (2 mL) and 0.01 M PBS (pH 6.5, 1 mL) were added to the extracts, and the mixtures were vortexed for 1 min. After centrifugation at 4000*g* for 5 min at 25 °C, the hexane supernatants were discarded, and the extracts were used for ELISA analysis.

Method Validation. Validation of the ELISA method was carried out according to the Commission Decision 2002/657/EC[38] and Community Reference Laboratories Residues.[39] The limit of detection (LOD) was based on the mean value of 20 blank samples plus three times the mean standard deviation. The accuracy and precision of the method were represented by recovery and coefficient of variation (CV), respectively. Seven naturally contaminated pork samples were obtained to determine the correlation of the developed ELISA with the LC–MS/MS method. The naturally contaminated samples were confirmed by LC–MS/MS, and evaluated with the developed ELISA method described above, and the two methods were correlated.

Non-Target Metabolite Extraction Method. In this study, an organic solvent extraction procedure was designed to remove the unwanted metabolites from CYA, MEQ, and QCT, but retain the MQCA and QCA metabolites. Because MQCA and QCA are organic acids, they will be in the ionic state in alkaline conditions. BDCYA, BDMEQ, and BDQCT can then be removed by organic solvent extraction. Therefore, methanol, ethyl alcohol, diethyl ether, and ethyl acetate were evaluated to remove the nonionic species. Ethyl acetate produced a low background signal and good recovery and was chosen as the extraction solvent. The organic solvent extraction method was carried out according to the following procedure: Ethyl acetate (10 mL) and 0.1 M PBS (pH 8.0, 5 mL) were added to the samples, and the mixtures were vortexed for 1 min. After centrifugation at 3000*g* for

Table 1. Optimization of Parameters for ELISA Assay Performance

parameters		B_0	IC_{50} (ng/mL)	parameters		B_0	IC_{50} (ng/mL)
competition incubation temp	4 °C, 30 min	1.543	5.4	ionic strength (M)	0.05	1.841	5.8
	25 °C, 30 min	1.764	6.8		0.1	1.756	5.5
	37 °C, 30 min	1.832	8.8		0.2	1.642	5.9
pH	6.0	1.742	5.0		0.4	1.452	6.6
	6.5	1.852	4.8	methanol concn	0%	1.678	5.4
	7.0	1.764	5.2		5%	1.585	5.8
	7.4	1.632	5.5		10%	1.424	7.2
	8.0	1.458	6.4		20%	1.154	10.9

10 min at 25 °C, the ethyl acetate layer was discarded, and the alkaline solution layer was acidified by adding 2 M H_2SO_4 (2 mL) and vortexed for 1 min. Next, ethyl acetate (8 mL) was added to extract MQCA and QCA, and the mixture was vortexed and centrifuged at 3000*g* for 10 min at 25 °C. A portion of the supernatant (4 mL) was transferred to a centrifuge tube, and evaporated to dryness under nitrogen gas. Hexane (2 mL) and 0.01 M PBS (pH 6.5, 1 mL) were added to the residue, vortexed for 1 min, centrifuged at 4000*g* for 5 min at 25 °C, the hexane supernatant was discarded, and the extract was tested using the developed ELISA.

RESULTS AND DISCUSSION

Antigen Synthesis. The selection of the right hapten and the design of the required immunogen are critical in the process of producing broad-specificity antibodies. MQCA and QCA are small molecules without immunogenicity, and as a result they need to be conjugated to a carrier protein to elicit immune responses. The basic chemical structure of MQCA and QCA and other metabolites (Figure 1) is a quinoxaline ring to which carboxyl and methyl groups are attached. For a simple analyte, the hapten design may be predictable, but to produce an antibody that will bind many analytes with similar structures, the required hapten may often be unpredictable. To generate high quality antibodies exhibiting the desired broad-specificity features, one must design a suitable hapten that closely mimics a common portion of all targeted analytes, maximizing the steric, hydrophobic, and electronic similarities.

In this study, MQCA and its derivative MQCA-4AS, and QCA and its derivative BDOLA, were used as haptens and conjugated to the carrier protein for immunogen synthesis. The synthesis routes of the conjugates are shown in Figure 2. MQCA and QCA were directly conjugated to BSA through their carboxylic acid group, but no antibody was produced. To clearly present the structure of MQCA and QCA to the immune system, MQCA-4AS and BDOLA were synthesized and conjugated to the carrier protein with a long spacer arm between the haptens and the carrier protein. MQCA-4AS had the longest spacer arm between the main structure of the hapten and the tertiary structure of the carrier protein, which allowed a clearer presentation of the molecule to the immune system. The immunogens and coating antigens were demonstrated to be successfully synthesized by MALDI-TOF MS analysis. [40] The MQCA-4AS carrier protein conjugate resulted in one mAb.

Characterization of the Antibody. Isotype classification of the mAb was performed with the Pierce Rapid ELISA Mouse mAb Isotyping kit. The isotype of the mAb was determined to be IgG_1 with λ light chains. The affinity of the mAb was measured by the noncompetitive indirect ELISA described by Beatty.[41] The affinity constant of the mAb was determined to be 9.0×10^7 L/mol.

The synthesized coating antigens and the produced mAb were screened and characterized by homologous and heterologous formats to select the best working combinations. From all of the available immunoreagents (four coating antigens and one mAb) tested, the combination of BDOLA-OVA and the mAb showed broad-specificity recognition for both MQCA and QCA, and were used in the following experiments. The titer of the antibody was determined by a checkerboard titration assay using the noncompetitive indirect ELISA. Concentrations ranging from 1 to 0.01 mg/mL for the coating conjugate and from 1/10 000 to 1/80 000 for the mAb were used to carry out the competitive assays. The optimal titer of the mAb used was 1/20 000.

Development of the ELISA. A checkerboard titration was performed to determine the optimal dilution of the coating antigen BDOLA-OVA and mAb. The best working concentration of coating antigen and mAb was determined to be 0.2 and 0.5 μg/mL, respectively. The amount of IgG-HRP used was the recommended dilution of 1:5000.

The aim of this study was to develop a broad-specificity ELISA method for the simultaneous detection of both MQCA and QCA in edible animal tissues. The optimization was performed to obtain the most sensitive assay using MQCA as the competitor analyte. A suitable maximum absorbance value (B_0, ranging from 1.5 to 2.0 units) and a minimum IC_{50} value would benefit from optimization of the ELISA. The influences of several experimental parameters (incubation temperature during competition, pH, ionic strength, and methanol tolerance) on assay characteristics were examined to improve assay performances (B_0 and IC_{50}) of the immunoassay, and the results are given in Table 1.

The incubation temperature is important when the coating antigen and free analyte competitively bind with the mAb. In this study, 4, 25, and 37 °C were tested, and the B_0 values increased as the incubation temperature of the competition was increased from 4 to 37 °C (Table 1). Considering both the B_0 and the IC_{50} values of the assay, 4 °C for 30 min was selected as the optimal incubation parameters for the competition. Significant IC_{50} and B_0 value changes were observed upon changing the pH value from 6.0 to 8.0. The optimum pH of the assay buffer was selected as 6.5, because the B_0 value was the highest and the sensitivity (IC_{50}) was the lowest at this pH. The ionic strength had a strong effect on the immunoassay. Increasing NaCl concentrations from 0.05 to 0.4 M in the assay buffer resulted in lower B_0 values and fluctuating IC_{50} values that were lowest at 0.1 M NaCl. Therefore, the optimal NaCl concentration was selected as 0.1 M. The organic solvent, methanol, which may interfere with antigen−antibody binding, was tested for its effects on the ELISA. The assay sensitivity and B_0 value decreased with an increasing methanol concentration.

Thus, the solution without organic solvent was selected as the assay buffer.

Curve Fitting and Cross-Reactivity Determination. The sensitivity of the ELISA (IC_{50}) was studied by constructing standard curves for MQCA with the concentration range from 0.1 to 324 ng/mL. Representative ELISA curves for MQCA and QCA obtained in this study are shown in Figure 3. IC_{50}, the half maximal inhibitory concentration, represents the concentration of an inhibitor that is required for 50% inhibition binding of the antibody to the coating antigen. The IC_{50} values of 4.8 and 9.6 ng/mL for MQCA and QCA in buffer were calculated using a four parameter logistic equation.

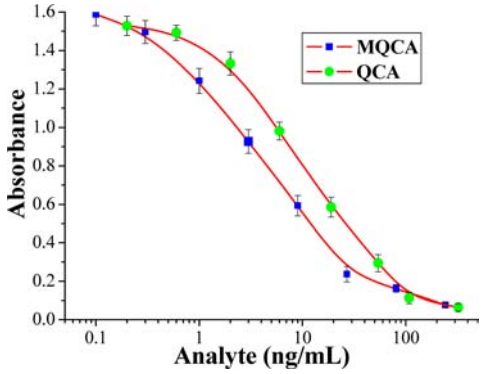

Figure 3. Standard curves from the developed ELSIA for MQCA and QCA. The data are average values of triplicate samples (average ± SD).

In addition to sensitivity, the specificity is also an important parameter for immunoassays. The specificity of the ELISA was evaluated by determining the cross-reactivity with a set of relevant analytes, and the IC_{50}'s and cross-reactivities are summarized in Table 2. The mAb showed good recognition for both MQCA and QCA. As shown in Table 2, the immunoassay also showed good cross-reactivity with BDCYA, BDMEQ, and BDQCT because these three metabolites have a quinoxaline ring moiety the same as MQCA and QCA. However, a false positive would result if one of these three metabolites were in the sample.

Table 2. IC_{50} and Cross-Reactivity Using MQCA as Standard[a]

analyte	IC_{50} (ng/mL)	cross-reactivity (%)	analyte	IC_{50} (ng/mL)	cross-reactivity (%)
MQCA	4.8	100	OLA	155	3
QCA	9.6	50	CBX	>1000	<1
BDQCT	0.9	533	QCT	>1000	<1
BDMEQ	0.2	2400	MEQ	>1000	<1
BDCYA	8.1	59	CYA	122	4

[a]BDCYA, (*E*)-2-cyano-*N*′-(quinoxalin-2-ylmethylene)acetohydrazide; BDMEQ, 1-(3-methylquinoxalin-2-yl)ethanone; BDQCT, (*E*)-1-(3-methylquinoxalin-2-yl)-3-phenylprop-2-en-1-one; CBX, carbadox; CYA, cyadox; MEQ, meequindox; MQCA, 3-methyl-quinoxaline-2-carboxylic acid; OLA, olaquindox; QCA, quinoxaline-2-carboxylic acid; QCT, quinocetone.

Sample Pretreatment. In the European Union and the Unites States, CBX and OLA are used in veterinary medicine. No other quinoxaline-1,4-dioxides are allowed to be used in food producing animals due to the potential carcinogenic, mutagenic, and photoallergenic side effects. Thus, a sensitive method for the extraction and analysis of both MQCA and QCA residues in food animals is required. However, three other quinoxaline-1,4-dioxides (CYA, MEQ, and QCT) have been shown to be potential growth promoters in food animal production due to their excellent antibacterial and growth promoting effects.[7,8] According to our previous research as well as by others,[11,12,14] the three quinoxaline-1,4-dioxides can be metabolized in vivo into many metabolites. The major metabolite of CYA is BDCYA. The major metabolite of MEQ is BDMEQ, and the major metabolite of QCT is BDQCT. There is also a very small amount of MQCA and QCA produced from CYA, MEQ, and QCT. However, the small levels of MQCA and QCA produced as secondary metabolites from CYA, MEQ, and QCT would not be high enough to significantly affect the levels of MQCA and QCA metabolite levels from carbadox and olaquindox. The produced broad-specificity mAb demonstrated good cross-reactivity with BDCYA, BDMEQ, and BDQCT (Table 2). However, the OLA and CBX metabolites MQCA and QCA, respectively, are the only target analytes that should be observed in the European Union and the United States. Thus, in China, the nontarget analytes (BDCYA, BDMEQ, and BDQCT) would contribute to false positives during screening tests for MQCA and QCA by the developed ELISA, if indeed MEQ, QCT, or CYA were used in animal production.

To avoid false positives from these other three quinoxaline-1,4-dioxides, all nontarget metabolites should be removed before analysis. In this study, an organic solvent extraction procedure was designed to remove the unwanted metabolites, but still retain MQCA and QCA. The nontarget metabolites in samples from China were removed by the designed extraction method. As shown in Figure 4, the ethyl acetate extraction procedure is only useful in China because the European Union and the United States do not approve the use of other quinoxaline-1,4-dioxides in animal production.

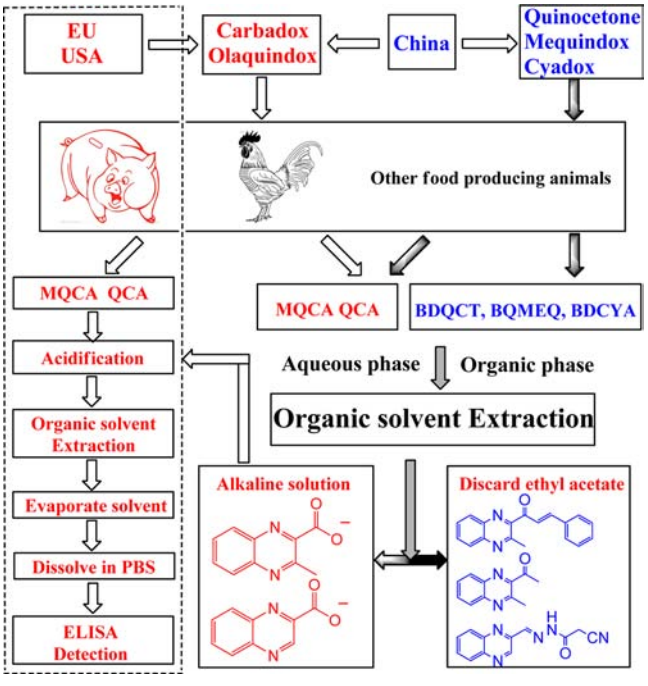

Figure 4. Two different sample preparation procedures, one for China and the other for the EU and U.S.

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Table 3. Mean Recoveries and Coefficients of Variation (CVs) for MQCA[a] and QCA[b] in Four Edible Animal Muscle Tissues (n = 5)

			intra-assay		interassay	
analyte	muscle matrix	spiked (μg/kg)	% recovery ± SD[c]	CVs (%)	% recovery ± SD[c]	CVs (%)
MQCA[a]	fish	2	78 ± 6.8	8.7	85 ± 6.5	7.7
		4	86 ± 6.5	7.6	92 ± 7.4	8.0
		8	92 ± 7.8	8.5	94 ± 4.9	5.2
	shrimp	2	104 ± 5.9	5.7	108 ± 8.4	7.8
		4	95 ± 4.6	5.2	79 ± 8.5	10.8
		8	86 ± 7.1	8.3	89 ± 6.9	7.8
	chicken	2	95 ± 5.6	5.9	82 ± 6.9	8.4
		4	85 ± 5.1	6.0	88 ± 8.2	9.3
		8	91 ± 4.8	5.3	104 ± 9.1	8.8
	pork	2	86 ± 3.6	4.2	77 ± 10.7	12.0
		4	107 ± 6.1	5.7	97 ± 9.7	10.0
		8	86 ± 7.2	8.4	105 ± 8.6	8.2
QCA[b]	fish	4	98 ± 11.5	11.4	79 ± 5.9	7.5
		10	84 ± 9.4	11.2	93 ± 12.4	13.3
		20	88 ± 8.3	9.4	89 ± 6.5	7.3
	shrimp	4	101 ± 7.8	7.7	76 ± 9.4	12.4
		10	87 ± 6.1	7.0	106 ± 8.3	7.8
		20	85 ± 7.0	8.2	93 ± 7.8	8.4
	chicken	4	87 ± 9.5	10.9	101 ± 12.3	12.2
		10	92 ± 7.2	7.8	97 ± 9.1	9.4
		20	87 ± 5.4	6.2	96 ± 11.5	9.3
	pork	4	79 ± 7.2	9.1	84 ± 5.9	7.0
		10	89 ± 6.8	7.6	97 ± 9.7	10.0
		20	102 ± 6.7	6.6	103 ± 7.8	7.6

[a]MQCA, 3-methyl-quinoxaline-2-carboxylic acid. [b]QCA, quinoxaline-2-carboxylic acid. [c]SD, standard deviation.

Assay Validation. The assay validation was carried out by investigating the LOD, accuracy, repeatability, and reproducibility. The LOD is the lowest amount of analyte in a sample that can be detected. On the basis of the determination of 20 different blank samples, the LOD of the developed ELISA in chicken, fish, pork, and shrimp were 1.76, 1.32, 1.90, and 1.18 μg/kg, respectively. To evaluate the accuracy, repeatability, and reproducibility of the ELISA method, a spiked recovery test was conducted. The blank muscle samples were spiked with known amounts of MQCA (2, 4, and 8 μg/kg) or QCA (4, 10, and 20 μg/kg) and then assayed using the proposed ELISA. The mean recovery and CV values were calculated by repeated analysis (n = 5) of the spiked samples and comparison of the intra-assay and interassay CVs. As shown in Table 3, the mean recovery ranged from 76% to 108% for four edible animal muscle tissues, with the interassay CVs in the range of 4.2−11.4% and the intra-assay CVs in the range of 5.2−13.3%. According to the standards of the European Commission (2002),[38] when the MQCA and QCA mass fractions are greater than 1 μg/kg but less than 10 μg/kg, the mean recovery should be in the range from 70% to 110%, and the intra- and interassay CVs should be no more than 15%. Thus, the accuracy and precision are acceptable, and the ELISA method has good repeatability and reproducibility.

Seven naturally contaminated pork samples were used for the final validation of the ELISA method. The MQCA concentrations of seven positive pork samples were simultaneously detected by both the developed ELISA and the reference method (GBT 20746−2006).[42] As can be observed in Figure 5, results measured by the ELISA correlated well with those of the instrumental method, and the coefficient of correlation R^2 was 0.98. The results demonstrate that the developed ELISA can be used for real sample analysis.

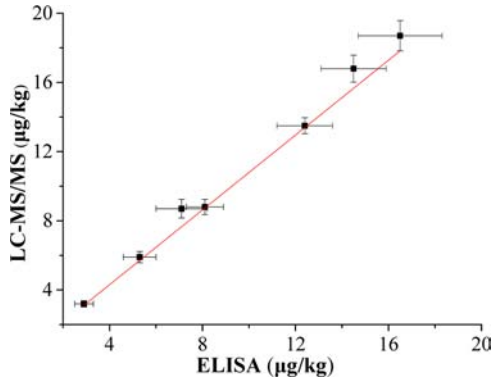

Figure 5. Correlation between the ELISA and LC−MS/MS results of naturally contaminated pork. The data are average values of triplicate samples (average ± SD).

Here, we have produced a broad-specificity ELISA for the simultaneous screening of MQCA and QCA residues in edible animal tissues. The ELISA was verified by an LC−MS/MS method, and its LOD was below the MRL and MRPL set by the European Commission, U.S., and China. The ELISA method was rapid and accurate for screening residues of MQCA and QCA in edible animal muscle tissues, and will contribute greatly for surveillance purposes.

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Notes

The authors declare no competing financial interest.

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