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Developing and Optimizing an Immunoaffinity Cleanup Technique for Determination of Quinolones from Chicken Muscle

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An immunoaffinity chromatographic method was developed using an antibody mediated immunosorbent to selectively extract and purify 10 quinolones (marbofloxacin, norfloxacin, ciprofloxacin, lomefloxacin, danofloxacin, enrofloxacin, difloxacin, sarafloxacin, oxolinic acid, and flumequine) in chicken muscle followed by HPLC. The operating conditions of the immunoaffinity chromatography (IAC) column were optimized, and the IAC has been successfully used for the isolation and purification of 10 quinolones from chicken muscle tissue. The optimized immunoaffinity column sample cleanup procedure combined with HPLC coupling to fluorescence detection afforded low limits of detection (0.1 ng g^{-1} for danfloxacin and 0.15 ng g^{-1} for all other quinolones tested). The method was also applied to determine quinolone residues in commercial muscle samples.

KEYWORDS: Immunoaffinity column; quinolones; monoclonal antibody; multiresidual; chicken muscle

INTRODUCTION

The measurement of veterinary drugs at trace concentrations in biological and environmental matrices requires a reliable and sensitive analytical method. These samples are complex and may contain substantial amounts of interfering substances. Consequently, time- and solvent-consuming sample preparation steps are often necessary prior to analytical determination of the target compounds. In the past decade, the use of solid phase extraction (SPE) has often been an effective cleanup step and a way to reduce the use of organic reagent (especially chlorinated solvent), required for the removal of interfering compounds often seen in conventional liquid−liquid extraction (LLE) (*1*, *2*).

However, most SPE adsorbents nonselectively retain the analyte in the percolated compounds due to hydrophobic interactions. This retention is nonselective and might lead to coextraction of analytes as well as matrix interferences, particularly those featuring the same polarity properties as the analyte, due to the hydrophobic interactions. The advent of the immunoaffinity chromatography (IAC), which is based on molecular recognition and reversible, specific antigen−antibody binding, enables a marked reduction in the amount and number of interfering substances and an almost "pure" analyte for quantitative determination by HPLC or GC. The selective sample enrichment by IAC can improve detection limits for trace analytes and require a smaller volume of sample preparation and less organic solvent usage in comparison to traditional cleanup and concentration methods (*3*, *4*).

IAC can be successfully applied to the isolation of a number of different biological analytes, ranging from the purification of a specific protein from cell culture (*5*) to the determination of pesticides (*6*−*8*), veterinary drugs (*9*−*13*), and chemicals (*14*−*16*) in food and environmental samples. The development of IAC using a monoclonal antibody (mAb) immobilized on sepharose 4B for the determination of quinolones (QNs) is described in this paper. QNs, derivatived from nalidixac acid, have been widely used in veterinary medicine (*17*). However, there is concern associated with potential QN residues in edible animal tissues and also the use of QNs in animals resulting in bacterial resistance to QNs being transferred from animals to humans. To protect consumers' health, many countries, including the People's Republic of China (PRC) and the European Union (EU), have established maximum residue limits (MRLs) for QNs in meat and offals (*18*, *19*). MRLs for several QNs such as danofloxacin, enrofloxacin and its metabolite ciprofloxacin, sarafloxacin, difloxacin, flumequine, and oxolinic acid in chicken muscle range from 10 ng g^{-1} to 500 ng g^{-1}. The U.S. Food and Drug Administration (FDA) had

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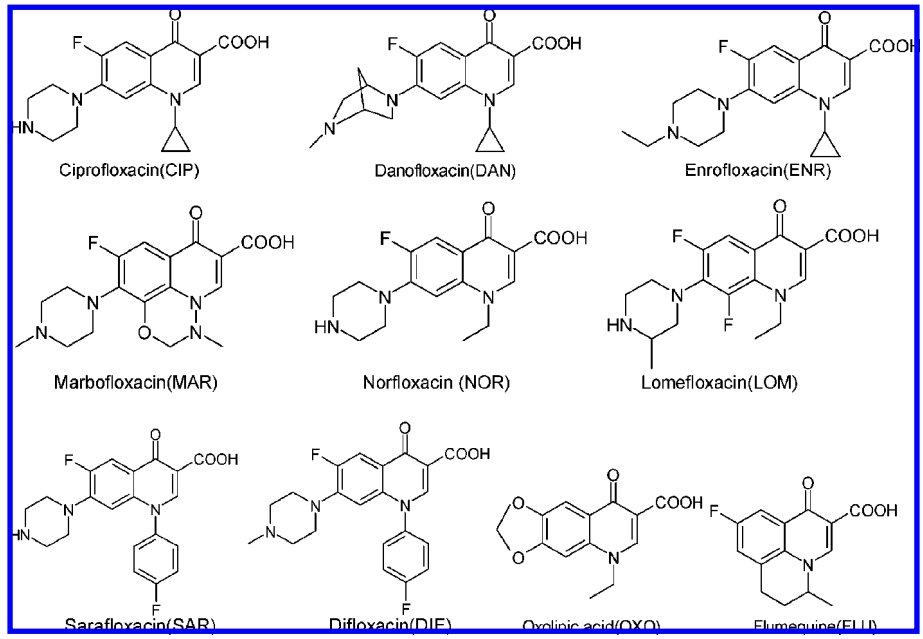

Figure 1. Chemical structures of QNs.

banned the use of enrofloxacin in poultry because of the emergence of fluoroquinolone-resistant *Campylobacter* species among both poultry and humans (*20*).

It is necessary to develop a specific and sensitive technique for the rapid detection and identification of QNs in the food supply. Current methods used for detecting QN residues are primarily chromatographic (*21*−*24*) and immunoassay (*25*−*27*) techniques. Among these methods, HPLC is well recognized as a powerful tool for separation and detection of QNs. Solid phase extraction and liquid−liquid extraction techniques are usually used to clean up samples and to concentrate the target analytes (*21*−*24*). Although satisfactory results were obtained using an online immunoaffinity technique by Holtzapple and co-workers (*11*, *28*), the method still required sophisticated analytical instrumentation and was limited by the detection of only 4 fluoroquinolones (ciprofloxacin, enrofloxacin, difloxacin, and sarafloxacin). Therefore, the objective of this study was to develop an immunoaffinity column based on a broad-specificity monoclonal antibody to selectively extract and purify 10 QNs prior to analysis by HPLC with programmable fluorescence detection (FLD).

MATERIALS AND METHODS

Reagents and Apparatus. Formic acid, glacial acetic acid, potassium monobasic phosphate, sodium hydroxide, sodium dibasic phosphate, and sodium monobasic phosphate were purchased from Beijing Chemical Reagent (Beijing, PRC). Bovine serum albumin (BSA) and ovalbumin (OVA) were purchased from Sigma (St. Louis, MO). Acetonitrile and methanol (HPLC-grade) were obtained from Fisher Scientific (Fair Lawn, NJ). Deionized water was purified with the Milli-Q filtration system (Millipore, Bedford, MA). All other reagents were analytical (or higher) grade. CNBr-activated Sepharose 4B gel was purchased from Amersham Biotech (Sweden).

The reverse phase analytical column was a Symmetry C18 column (250 mm × 4.6 mm, 5 μm) from Waters (Milford, MA). The vortex mixer was from Fischer Scientific (Norcross, GA), and the centrifuge (Hettich ROTOFIX 32A) was purchased from Hettich (Kirchlengern, Germany). The ultraviolet−visible (UV−vis) detector was from Shanghai Analytical Instrument (Shanghai, PRC). The HPLC system consisted of a Waters Alliance 2695 quaternary solvent delivery system with a 2475 fluorescence detector (Waters, Milford, MA). The ELISA plate reader was from TECAN (Durham, NC).

Standards and Solutions. Ciprofloxacin hydrochloride (CIP, 99.8%), norfloxacin (NOR, 100.1%), lomefloxacin (LOM, 99.1%), enrofloxacin (ENR, 99.3%), sarafloxacin hydrochloride (SAR, 99.2%), oxolinic acid (OXO, 99.9%), and flumequine (FLU, 99.5%) were purchased from the National Control Institute of Veterinary Drugs (Beijing, PRC). Difloxacin (DIF, 99.0%) was purchased from Sigma (St. Louis, MO). Danofloxacin (DAN, 99%) was a gift from Dr. Fangyang He (China Agricultural University, Beijing, PRC). Marbofloxacin (MAR) was obtained from Dr. Ehrenstorfer (Augsburg, Germany). Chemical structures of the above QNs are shown in **Figure 1**.

Individual standard stock solutions were prepared in methanol and 0.03 M sodium hydroxide solution (49:1) and stored in brown volumetric flasks at 4 °C for three months. Working standards were prepared daily by diluting the stock solution with methanol.

The following solutions and buffers were used: Phosphate buffered saline (PBS) was prepared by dissolving 0.2 g of KH_2PO_4, 0.2 g of KCl, 2.9 g of $Na_2HPO_4 \cdot 12H_2O$, and 8.8 g of NaCl in 1000 mL of water, and the pH was adjusted to 7.4 with 1 M NaOH. The IAC coupling buffer solution (pH 8.3) consisted of 0.1 M $NaHCO_3$ containing 0.5 M NaCl. The IAC blocking buffer solution (pH 8.0) contained 0.1 M Tris-HCl and 0.5 M NaCl, and the acetate buffer (pH 4.0) was composed of 0.1 M sodium acetate and 0.5 M NaCl. The elution solution was 70% methanol in PBS.

Preparation of Monoclonal Antibodies and Immunosorbent Preparation. First, a six-carbon spacer arm linked the norfloxacin, and hapten was prepared according to the method of Abad et al. (*29*). The hapten was conjugated with a carboxylic group at position C-3 of 6-aminohexanoic acid. A NOR-BSA conjugate was used as immunogen, and NOR-OVA was used as the coating antigen and was synthesized based on the mixed anhydride method of Erlanger and co-workers (*30*). The anti-NOR monoclonal antibodies were produced by the method described by Wang et al. (*31*). Briefly, female BALB/c mice were subcutaneously injected with the immunogen. The mice that showed the highest serum antibody titer were sacrificed. Splenocytes were collected and fused with SP 2/0 myeloma cells. The hybridomas were tested for specific MAbs against NOR-OVA and NOR-BSA by the ELISA described below. Cultures showing the highest inhibition with NOR as the competitor were cloned twice by limiting dilution. The mAb was purified and stored in 50 μL aliquots at −20 °C.

The ELISA as described by Wang et al. (*31*) was used to determine the antibody titer in immunized mice and cross-reactivity of the mAb with 10 QNs. The 50% inhibition value (IC_{50}) for each QN was obtained, and the cross-reactivity of the mAb was calculated as follows:

$$[IC_{50}(\text{hapten, nmol mL}^{-1})/IC_{50}(\text{QN analogs, nmol mL}^{-1})] \times 100\% \quad (1)$$

The anti-NOR mAb was covalently bonded to CNBr-activated Sepharose 4B gel according to the manufacture's instructions and related references (*10, 12*). Briefly, 1 g of CNBr-activated Sepharose 4B was dissolved in 10 mL of 1 mM HCl and the swollen gel was poured into a sintered-glass funnel (40−60 μm). The gel was washed with 400 mL of 1 mM HCl. The mAb (25 mg) was dissolved in 5 mL of the coupling buffer, and the resulting solution was gently stirred overnight at 4 °C with the Sepharose gel. The mixture was washed with 10 gel volumes of IAC coupling solution to remove the unbound mAb. The $NaHCO_3$ eluate was collected and analyzed for the mAb with a UV−vis spectrophotometer to determine the antibody coupling efficiency. Excess binding sites of the gel were blocked using 10 mL of Tris-HCl buffer at 4 °C for 4 h before the antibody-coupled gel was washed with three cycles of alternating acetate buffer and IAC blocking buffer to remove nonspecific material that had been absorbed by the column. Finally, the 1 mL column gel was transferred to a glass column (10 mm × 0.8 mm, i.d.) and stored in PBS at 4 °C until use. The same procedure was performed without the mAb to obtain a control Sepharose 4B column.

Operation of the Immunoaffinity Column. After the IAC was preconditioned with 10 mL of PBS, the analytes, which were dissolved in 20 mL of PBS (or the analyzed sample solutions) were percolated into the IAC at a flow rate of about one drop per second by gravity. The column was washed with 10 mL of PBS followed by 10 mL of water, and then the target drugs were eluted with 4 mL of 70% methanol in PBS. The eluate was evaporated to dryness under a gentle stream of N_2 at 45 °C. The residues were reconstituted with 1 mL of PBS prior to analysis by HPLC. The IAC column was regenerated, by equilibrating with 10 mL of PBS, and stored with PBS at 4 °C before the next use.

HPLC-FLD Conditions. QNs in the eluate were analyzed by HPLC-FLD. The mobile phase consisted of solvent A (0.02% aqueous formic acid solution, pH 2.8) and solvent B (acetonitrile), which were run at a flow rate of 1.0 mL min^{-1} with the following program: 9% solvent B for 8 min, increasing to 40% over 10 min and maintaining for 4 min. During each analysis run, the analytical column was eluted using 90% acetonitrile from 22 to 25 min. All analytes eluted within 24 min, and the column was re-equilibrated, washing with the original solvent, A/B (91/9) over 9 min. The injection volume was 100 μL, and the column temperature was maintained at 35 °C.

The fluorescence excitation/emission wavelengths were programmed at 297/515 nm for MAR from 0.0 to 7.0 min, at 280/450 nm for NOR, CIP, LOM, DAN, and ENR from 7.0 to 17 min, and at 320/365 nm for OXO and FLU after 17 min.

Optimization of Elute Conditions. The QNs elution efficiency from the IAC column was investigated. PBS (10 mL) containing 100 ng of norfloxacin was added to the IAC cleanup column as described above. PBS or 2% acetic acid solutions containing various proportions of methanol (50−100%), and PBS solutions at different pH values (2.0, 11.0) were tested to elute the analyte from the IAC column. The eluates were separately collected before analysis by HPLC-FLD.

Application of the IAC Cleanup to Chicken Muscle Tissue. The control chickens were raised in the central experimental animal facility of the China Agriculture University. The muscle samples were extracted as described previously (*32*). Briefly, 2 g of thawed and minced muscle tissues was weighed and placed in a 50-mL polypropylene centrifuge tube and spiked with the standard working solution. Ten milliliters of phosphate buffer solution (PBS, 0.01 M, pH 7.0) was added to the samples. The samples were allowed to stand for 15 min at room temperature, and then the samples were vortexed for 10 s before centrifugation for 5 min at 2300*g*. The supernatant was collected, and the extraction was repeated. The combined supernatants were percolated slowly into the IAC column by gravity. Subsequent washing, elution, and concentration procedures were as described in the section Operation of the Immunoaffinity Column.

Table 1. Cross-Reactivity of the anti-NOR Antibody Determined by ELISA and the Column Capacity of IAC Determined by HPLC-FLD

			column capacity			
analyte	MW[a]	CR[b] (%)	ng mL^{-1} gel	ng mg^{-1} Ab	nmol mL^{-1} gel	nmol mg^{-1} Ab
NOR	320	100	1589	317.8	6.67	1.33
MAR	363	38	2135	427.0	4.37	0.88
CIP	332	54	1976	395.2	5.95	1.19
LOM	352	20	1738	347.6	4.94	0.99
DAN	358	69	1448	289.6	4.04	0.81
ENR	360	58	1793	358.6	4.98	1.00
SAR	386	17	1645	329.0	4.26	0.85
DIF	400	18	1498	299.6	3.75	0.75
OXO	262	35	1425	285.0	5.43	1.09
FLU	262	29	1632	326.4	6.22	1.24

[a] MW: molecular weight. [b] CR: cross-reactivity.

RESULTS AND DISCUSSION

Antibody and Immunoaffinity Column. The critical step in the production and selection of antibodies against a small molecule drug is the selection of a suitable hapten and appropriate conjugation to a large molecule, such as albumin. In this study, in order to produce a broad-specificity antibody to QNs, the six-carbon spacer arm was coupled to the carboxyl group at position 3 of norfloxacin's structural backbone. The synthesized NOR-BSA conjugate was immunogenic. mAb against NOR was successfully produced using the hybridoma method (*30*). The mAb, which was characterized by the ELISA as described by Wang et al. (*31*), has cross-reactivity with 9 analogues (MAR, CIP, LOM, DAN, ENR, SAR, DIF, OXO, FLU), ranging from 17% to 69% as determined by competitive indirect ELISA (as shown in **Table 1**).

The IAC column prepared from sepharose 4B as the supporting material and the mAb had a coupling efficiency, defined as the percentage of antibody immobilized on the sepharose gel (*10*), of about 98% ($n = 5$). To a 1 mL bed volume of sepharose gel was retained 4.98 mg of mAb. The affinity of the immobilized mAb in the IAC column was evaluated using standard quinolones spiked in chicken muscle samples. As shown in **Figure 2**, 10 QNs (NOR, MAR, CIP, LOM, DAN, ENR, SAR, DIF, OXO, FLU) were all captured by the IAC. No other substances in chicken muscle tissues were retained by the column. In comparison with previous studies (*11, 28*), more QN compounds were captured in this study using broad-specific mAb.

Optimization of Elution Conditions. Appropriate elution solvents are important not only in efficiently uncoupling the captured analyte off the IAC but also in preserving the column reusability (*33*). In the present study, a protocol was specially developed and optimized to elute QNs retained in the IAC column by a small amount of eluent, and account for the reusability of the IAC column.

It was well-known that the bond between the antigen/hapten and the antibody is due to electrostatic forces, Van der Waals forces, hydrogen bonds, and hydrophobic interactions (*4*). To elute the antigen from the antibody−antigen complex in the IAC, the column environment must be changed. A series of elution strategies including changing the pH (*5, 6, 11*), using organic solvents (*10*), and a combination of these techniques (*1, 4, 9, 12, 15*) have been used for disrupting these bonds and eluting the antigen from the IAC column. In this study, the eluent solution strength was investigated by changing the polarity and pH. NOR, which had the highest cross-reactivity with the mAb, was selected to evaluate the elution efficiency.

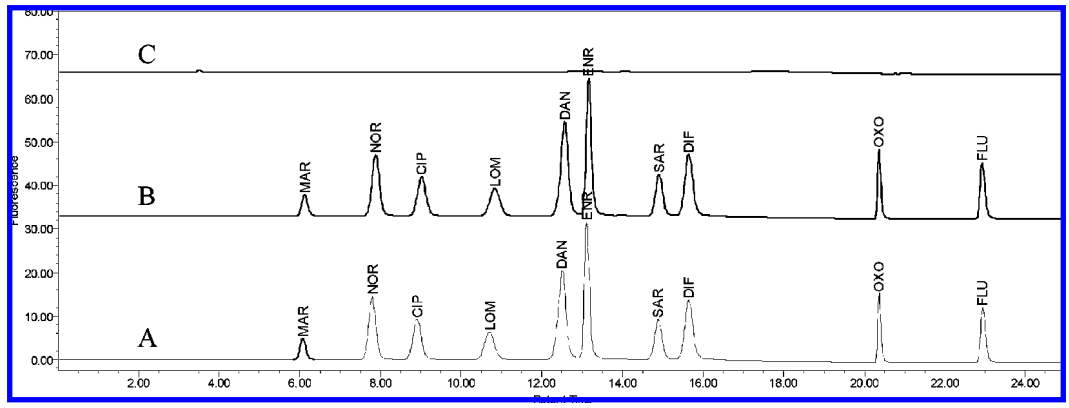

Figure 2. Chromatograms of (A) quinolone standards (100 ng mL^{-1} except for DAN 30 ng mL^{-1}), (B) chicken muscle tissue spiked with 100 ng g^{-1} QNs (except DAN 30 ng g^{-1}), and (C) blank chicken muscle tissue.

Table 2. Recoveries of Norfloxacin (100 ng) at Different Elution Conditions

	recovery/RSD(%)		
elution buffer	elution fraction 1 (3 mL)	elution fraction 2 (3 mL)	total recovery
acetic buffer, pH 2.0	13/9.2	21/8.5	34/7.9
90% methanol in acetic buffer, pH 2.0	18/4.6	19/8.9	37/10.2
70% methanol in acetic buffer, pH 2.0	24/6.9	22/8.7	46/5.7
50% methanol in acetic buffer, pH 2.0	11/7.6	29/11.2	40/10.3
methanol	19/2.1	22/5.9	41/7.0
90% methanol in PBS, pH 7.0	102/8.2	1/5.3	103/4.2
70% methanol in PBS, pH 7.0	99/8.3	3/5.2	102/5.9
50% methanol in PBS, pH 7.0	77/9.3	15/7.5	92/10.2
70% methanol in PBS, pH 2.0	99/11.2	2/7.2	101/9.5
70% methanol in PBS, pH 11.0	100/8.6	3/5.3	103/5.6
PBS, pH 2.0	18/4.5	19/5.2	37/5.8
PBS, pH 11.0	12/3.3	24/5.4	36/7.4

The elution pattern of NOR (100 ng) with the different elution solutions is shown in **Table 2**.

Two percent aqueous acetic acid solution was used to elute quinolones from an online IAC by Holtzapple and co-workers (*11*) using antibodies obtained from SAR as the hapten. However, only 34% of the captured drug in the IAC was desorbed using 6 mL of 2% acetic acid in this study. Recovery was not significantly increased through a larger volume of 2% acetic acid solution (data not shown). It has been suggested by Yarmush et al. (*34*) that antigen−antibody binding may depend more on hydrophobic interactions if acidic or basic pH buffers are ineffective in eluting bound analyte. The discrepancy of our results and the previous study results (*11*) may be due to antigen/antibody interactions at different positions and different steric hindrance obtained with anti-SAR and anti-NOR antibodies, resulting in different desorption conditions.

It has been reported that an acidic solution combined with an organic solvent can improve the elution efficiency of analyte from immunosorbents (*9*). Varying concentrations of methanol (50−90%) in 2% acetic acid at low pH (pH 2.0) as the eluting solution were studied, but poor recoveries (37%−46%) were obtained, which was in agreement with the results of Ding et al. (*12*).

Table 3. Recoveries of Other Quinolones (100 ng) at Optimized Elution Conditions (70% Methanol in PBS, pH 7.0)

	recovery/RSD (%)		
analyte	elution fraction 1 (3 mL)	elution fraction 2 (3 mL)	total recovery
NOR	99/8.3	3/5.2	102/5.9
MAR	98/5.6	1/2.6	99/8.1
CIP	96/5.7	3/2.7	99/5.5
LOM	96/5.8	6/2.1	102/5.8
DAN	89/8.3	9/5.8	98/6.6
ENR	93/5.6	4/2.6	97/2.6
SAR	94/2.9	4/5.9	98/8.8
DIF	91/5.8	5/7.2	96/5.3
OXO	99/8.3	3/5.8	102/9.4
FLU	79/4.5	15/7.2	94/5.1

Organic solvents, which disrupt antigen−antibody interactions by altering polarity, may be more effective and always be as the elution during the IAC analysis, especially with commercial immunosorbents. Pure methanol was also used as elution solution in this research; however, only 41% NOR was eluted from the IAC column. A large amount of methanol can denature antibodies and may cause serious damage to the IAC column and limit the useful life of the column (*8*). Excellent recoveries (99%−102%) were obtained with 70%−90% methanol in PBS at pH ranging from 2 to 11. The observed differences between the pure methanol and methanol in PBS may be explained by the fact that methanol in PBS with appropriate ionic strength can alter the column conditions, which disrupt the ionic bonds, the dielectric constant, and the hydrophobic interactions (*35*) through variation of the ionic strength and polarity. Since large volumes of aqueous buffers lead to prolonged evaporation in subsequent processing, the lower content PBS solution was not employed. Overall, these experiments indicated that elution with >70% methanol in PBS buffer regardless of pH gave excellent recovery (>98%) of bound NOR from the IAC. Although 90% methanol can be evaporated faster, the high methanol concentration may compromise the reusable life of the IAC through irreversible denaturation of the immobilized antibodies. Therefore, 4 mL of 70% methanol in PBS was selected as the eluent, which was conveniently concentrated (without significantly increasing evaporation time) before analysis by HPLC-FLD. Good recoveries were also obtained for other quinolones using this eluent. The elution pattern of the other QNs (100 ng) with the optimized elution solutions is shown in **Table 3**. Using methanol−PBS (7/3, v/v) as the elution solvent, the column capacity decreased 35%−48% after 12 cycles of elution.

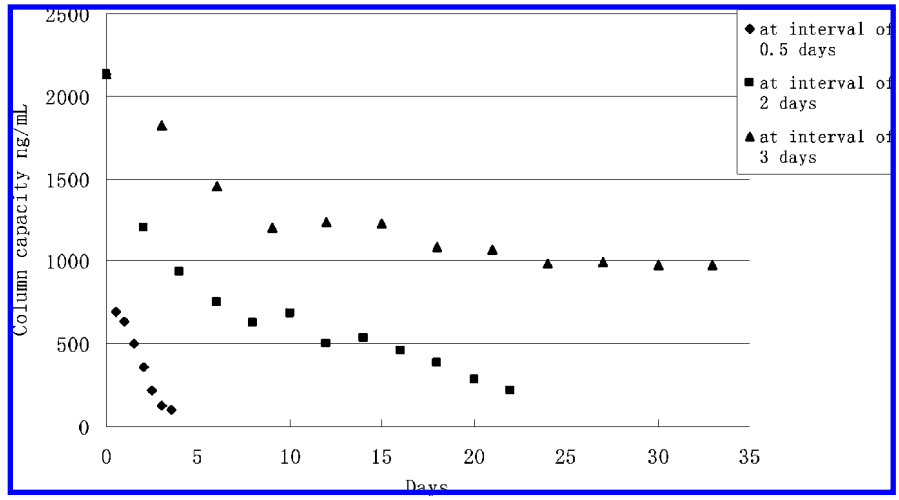

Figure 3. Variation curves of the imunoaffinity column capacity at intervals of 0.5, 2, and 3 days of use.

Table 4. Recoveries and Assay Precision in Fortified Chicken Muscle Samples

	0.5 ng g^{-1}			1.0 ng g^{-1}			10 ng g^{-1}			100 ng g^{-1}		
		RSD[b] (%)			RSD (%)			RSD (%)			RSD (%)	
analyte	R[a] (%)	intra-[d]	Inter-[e]	R (%)	intra-	inter-	R (%)	intra-	inter-	R (%)	intra-	inter-
NOR	89.5	3.2	15.9	92.3	3.4	8.7	88.1	9.2	10.4	96.4	10.6	12.0
MAR	86.6	9.3	8.9	90.1	6.7	8.6	81.6	6.8	9.7	91.0	7.2	7.8
CIP	96.8	4.9	8.7	89.8	11.7	10.6	93.4	10.6	8.4	96.9	5.2	9.9
LOM	93.8	7.3	10.5	91.0	2.5	11.8	93.2	12.5	12.9	95.6	3.8	12.0
DAN[c]	86.4	5.3	15.6	88.0	5.7	17.3	83.1	9.6	13.5	91.3	4.3	10.1
ENR	92.6	6.9	9.9	96.7	4.6	5.9	93.9	11.8	14.8	99.7	8.6	8.9
SAR	83.8	7.8	12.3	89.1	8.9	9.3	88.8	9.9	10.2	96.3	8.9	9.7
DIF	91.2	9.2	16.8	82.1	6.6	7.8	90.8	3.6	8.2	90.1	4.1	8.6
OXO	92.6	7.3	5.7	89.6	6.9	12.3	92.8	12.1	5.6	100.9	2.7	9.2
FLU	93.8	9.9	8.9	96.1	8.5	13.6	88.7	9.7	7.9	91.1	4.2	7.8

[a] R, recovery. [b] RSD, relative standard deviation. [c] The spiked level of DAN was 0.15, 0.3, 3, and 30 ng g^{-1}. [d] Intra:$n = 6$. [e] Inter: $n = 18$.

We also studied the elution efficiency using PBS at different pH values (pH = 2.0 or pH = 11) without organic solvents, which resulted in poor elution (<25%).

The Column Capacity. The capacity is defined as the total number of immobilized active antibodies (specific for the target analyte) relative to the immunosorbent supporting gel (4). The capacity of the IAC column was examined as follows: 20 mL of standard solution of each QN (200 ng mL^{-1}) was loaded into the IAC. The drug-saturated column was washed and eluted using the optimized protocol as described above. The dynamic column capacity was found to be from 3.75 to 6.67 nmol per mL gel (1425−2135 ng mL^{-1}) of target analytes, and the specific capacity was 0.75−1.33 nmol analyte per mg of immobilized antibody (285.0−427.0 ng mg^{-1}), respectively (see **Table 1**).

In the conventional IAC column analysis, the capacity is often evaluated and presented as nanograms of analyte relative to IAC bed volume (ng mL^{-1}) or immobilized antibodies (ng mg^{-1}). In this study, the authors think that the column capacity is better expressed as the moles of analyte per mL of column bed volume (mol mL^{-1} gel) or per mg of antibodies (mol mg^{-1} of antibodies) to reflect molar binding of antigen to the immobilized antibodies. The amount of antibodies immobilized onto the Sepharose solid phase can be measured by the concentration of the antibodies in the binding solution by UV spectrometry. The binding site of the antibody surface could be calculated to account for the binding density, molecular weight of the antibody (about 150 kDa), and two binding sites per antibody. The column capacity was described more effectively using the amount of analytes molecularly binding with the total active site of the antibodies because antibody−antigen binding is related to moles not grams.

The specificity of IAC depended on the specificity of the immunogen used in the production of the antibody. In this study, antibodies produced using NOR-BSA as the immunogen had broad specificity to quinolones. It bound to all quinolones tested. **Table 1** shows the cross-reactivity of the monoclonal antibody with QN determined by ELISA. Though the mAb had low cross-reactivity with some drugs (LOM, DIF, SAR, OXO, FLU), the column capacity of the immunosorbent for these drugs was comparable with that for other quinolones. The capacity of the IAC is exceeded at a lower mass by closely related species. Moreover, no nonquinolone veterinary drugs such as sulfonamides, β-lactam antibiotics, and the anti-NOR antibody were specific to QN drugs, and any interfering compound and tissue matrices were removed by washing with PBS and water.

Since antibody−antigen interactions occur over short distances, steric effects are involved in the coupling reaction. These steric effects are what make antibody−antigen interactions so selective, and only the antigen which produced the immune response, or very closely related molecules, will be able to bind to the antibody. Thus, theoretically, when the sample is running through the immunosorbent, the analytes are selectively retained and subsequently eluted free of the coextractives (*36*).

Column Reusability and Stability. The reusability of the IAC column is important, because each column requires a large quantity of precious antibodies. Column reusability depends on the concentration and activity of the immobilized antibodies and the chemical stability of the support (*4*). As discussed above,

Table 5. Determined Concentrations (ng g^{-1}) of Quinolone Residues in Market Chicken Samples in Qingdao ($n = 3$)

	conc/RSD[b] (ng g^{-1}/%)									
sample code	MAR	NOR	CIP	LOM	DAN	ENR	SAR	DIF	OXO	FLU
QD002	—[a]		2.6/3.6			15.9/3.7				
QD009				26.7/5.8						
QD015		10.8/4.9								
QD019			26.8/5.9			43.6/8.0				
QD037								8.4/8.6		
QD042						2.7/10.5				
QD059								15.3/5.8		
QD060			22.0/7.9			4.9/5.4				
QD078		18.9/10.5		42.7/6.2						
QD088			12.5/7.0			8.5/9.8				
QD102			33.6/8.5			19.8/9.9				
QD112				12.7/8.3						
QD0119					0.7/6.5		8.5/8.6			
QD0120		45.8/8.8								

[a] Below the limit of detection. [b] RSD, relative standard deviation.

the elution solutions have to be selected in order to minimize any detrimental effects on the reusability of the column. And the operation process is also critical to the reusability and performance of the IAC. When the column was used twice a day, the capacity declined rapidly from ca. 2100 ng mL^{-1} to <100 ng mL^{-1} after 8 cycles. When the column was used once every 2 days, the column capacity remained above 500 ng mL^{-1} after 8 cycles of use. The reusability was further improved when it was used once every 3 days (see **Figure 3**), with column capacity fluctuating between 775 and 1026 ng mL^{-1} after 12 uses. The results indicated that the antibody activity could be regenerated after a prolonged storage period. Whether the column capacity can be further improved if the column is stored for a longer period needs to be further investigated.

The capacity of the IAC columns did not significantly deteriorate after 12 months of storage at 4 °C, and the average column capacity ($n = 3$) was 90% of the initial capacity, indicating good storage stability at 4 °C.

Effectiveness of IAC in the Cleanup Chicken Muscle Tissues. Commonly, samples to be cleaned up by IAC should be in an aqueous solution because organic solvents can interfere with the antibody−antigen interaction (*1*, *3*). In this study, chicken muscle samples were simply extracted with PBS (0.01 M, pH 7.0) without protein precipitation before being cleaned-up through an IAC column. A clean baseline of a chromatogram was obtained from all blank samples using the IAC purification technique (see **Figure 2**). Drug-free chicken muscle samples spiked with QNs at each analysis concentration were extracted and analyzed. As shown in **Table 4**, excellent recoveries were obtained for all QNs at all fortification levels, with recoveries ranging from 81.6% to 100.9% at 0.5−100 ng g^{-1} and relative standard deviations from 2.5 to 17.3%. The low relative standard deviation indicated good repeatability of the sample extraction, clean-up, and analysis methods.

The limit of detection (LOD) defined at a signal noise ratio of 3:1 with five replicate blank muscle tissues was 0.15 ng g^{-1} for all analytes except DAN (0.05 ng g^{-1}). The limit of quantification (LOQ) according to the FDA guidelines for residue analysis was 0.5 ng g^{-1} (0.15 ng g^{-1} for danofloxacin) (*37*).

Determination of QN Residues in Market Samples. One hundred and twenty chicken muscle samples randomly collected from the markets of Qingdao were analyzed by the HPLC method. Fourteen of 120 samples (11.7%) were found to contain one or multiple QNs at concentrations from 0.7 to 43.6 ng g^{-1} (**Table 5**). NOR, CIP, ENR, LOM, DAN, SAR, and DIF were determined in chicken samples.

Conclusions. The broad-specificity monoclonal antibody was covalently immobilized on CNBr-activated Sepharose 4B, and the IAC column was characterized and evaluated for selective extraction and cleanup QNs from chicken muscle tissues before analysis by HPLC-FLD. The elution condition was optimized to minimize the use of solvent and improve column reusability. Ten QNs were all captured by the IAC and were efficiently eluted with 4 mL of 70% methanol in PBS. Compared with the other traditional methods, the use of immunoaffinity chromatography in the cleanup step presents a number of advantages including better sensitivity and selectivity, fewer operating steps, lesser hazardous organic solvents usage, and shorter analysis time. Purification and concentration of QNs in muscle matrices could be accomplished in a single step using off-line IAC in place of online. The optimized IAC-HPLC-FLD method was suitable for use in practice.

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