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Effects of Cooking and Storage on Residues of Cyadox in Chicken Muscle

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The aim of this study was to investigate the depletion of residues of cyadox in chicken muscle over time. The heat stabilities of cyadox (CYX) and its two metabolites, 1,4-bisdesoxycyadox (BDCYX) and quinoxaline-2-carboxylic acid (QCA), in water, cooking oil, and as incurred residues in chicken muscle were investigated. CYX was shown to be unstable with a half-life of about 37.7 min in 100 °C water. In hot cooking oil at 180 °C, all three compounds were unstable. CYX decreased quickly and was not able to be detected after heating for 2 min. Diode-array analysis of CYX standard solution in cooking oil indicated that a portion of BDCYX was formed. The residues of CYX and BDCYX deteriorated rapidly in frozen storage, while that of QCA changed slowly. Muscles containing CYX residues were boiled, microwaved, or fried for the specified times. During boiling, CYX and BDCYX were reduced 94% and 81% in 10 min, respectively. During microwave cooking, CYX and BDCYX were reduced 54% and 47% in 2.5 min, respectively. During frying, CYX and BDCYX were reduced 86% and 76%, respectively. No significant reduction of QCA was found for the three cooking methods. The half-lives of CYX residues in cooked chicken muscles were estimated as follows: 2.22 min for CYX and 4.44 min for BDCYX by boiling; 6.66 min for CYX and 9.36 min for BDCYX by microwaving.

KEYWORDS: Cyadox; 1,4-bisdesoxycyadox; quinoxaline-2-carboxylic acid; cooking; stability; chicken

INTRODUCTION

Most data about drug residues in food (meat and animal products) are related to concentrations in raw tissues. Since most of such foods are eaten after cooking, more information about the effects of cooking on drug residues is required to give a more accurate estimation of consumer exposure to the chemicals and their breakdown products. Cyadox (CYX) is a quinoxaline 1,4-dioxide, antimicrobial, growth-promoting agent like carbadox and olaquindox. Its use in food-producing animals could raise the possibility of residues remaining in edible tissues after slaughter. Metabolism research has shown that cyadox is converted into monooxycyadox, bisdesoxycyadox (BDCYX), quinoxaline-2-carboxyl glycine, and quinoxaline-2-carboxylic acid (QCA) in the urine of pig (*1*) (**Figure 1**). QCA was considered to be the last remaining chemical and may serve as a marker residue in chicken and pig on the basis of our works. Methods for analysis of QCA in tissues, such as HPLC with UV detection, GC−MS, LC−MS, have been published (*2*−*6*). The methods for analyzing CYX and BDCYX were rare (*2*, *7*, *8*). To the best of our knowledge, there is not any published information about residues of cyadox and its two main metabolites in cooked chicken tissues. The purpose of this study is to investigate the depletion of residues of cyadox in chicken muscle over time through three cooking methods. In addition, the residual analysis is not always performed in the vicinity of the slaughter and storage of the carcass in frozen conditions for several weeks is sometimes necessary. The stability of the residues in frozen product should be checked to guarantee the reliability of the results. Thus, the storage stability of CYX, BDCYX, and QCA was also one of the objectives in this work.

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MATERIALS AND METHODS

Chemicals. CYX (purity 99%) and BDCYX (purity 98.0%) were obtained from the Institute of Veterinary Pharmaceutics, Huazhong Agricultural University (Wuhan, Hubei, China). QCA (labeled purity 97%) was obtained from Sigma-Aldrich Co. Ltd. The standard stock solutions of 1 mg/mL CYX and BDCYX were prepared in dimethyl sulfoxide, and the standard solution of 1 mg/mL QCA was prepared in methanol. These compounds were stable in solution at 4 °C for at least six months. All reagents were of analytical grade.

Animals. Adult chickens (weight about 1.5 kg) were kept in cages and given cyadox-free basal diet and tap water for at least 1 week. One chicken was kept as a control, receiving the basal diet continuously. Other chickens were gavaged with cyadox suspension at a dosage of 400 mg/kg, and slaughtered 2 h postdosing. The breast muscles were collected, ground, and mixed. Blank chicken muscles for method validation were obtained locally and analyzed to verify the absence of cyadox residues prior to use.

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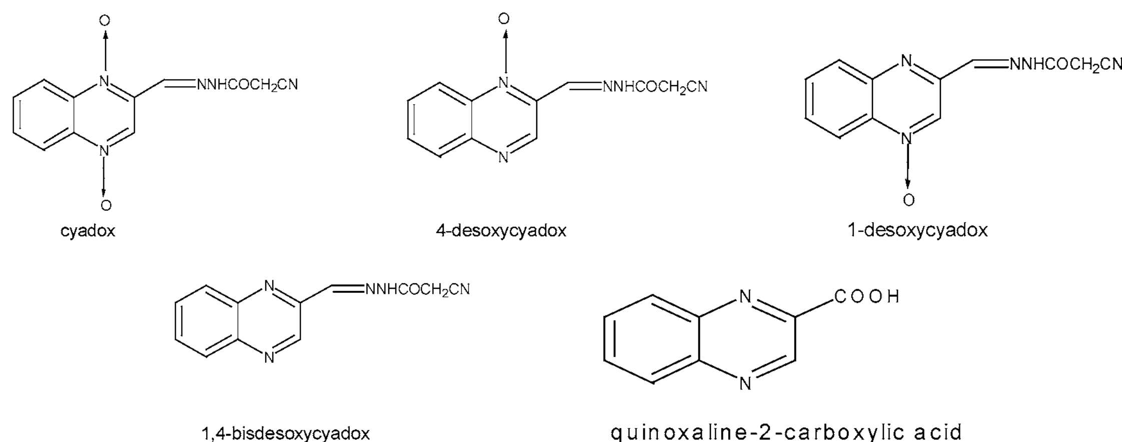


Figure 1. Molecular structures of cyadox and its main metabolites.

Heat Stabilities of CYX, BDCYX, and QCA in Boiling Water. Standard solutions containing 10 μg/mL CYX, BDCYX, and QCA were separately prepared in water from standard stock solutions. These solutions were dispensed into a number of sealed vials, which were immersed into a thermostatically controlled water bath for measurement at 100 °C. The vials were removed at the specified time (0, 2, 5, 10, 15, 20, 25, 30, 40, 50, 60 min for CYX; 0, 5, 10, 15, 20, 30, 45, 60, 90, 120, 180 min for BDCYX and QCA), cooled rapidly, and directly analyzed by HPLC together with controls of unheated CYX, BDCYX, and QCA solutions.

Heat Stabilities of CYX, BDCYX, and QCA in Hot Cooking Oil. Cooking oil (40 mL) was placed in a round-bottom flask containing a magnetic stirring bar and heated to 180 °C. Temperature was monitored using a mercury-in-glass thermometer. 2 mL of standard stock solutions (CYX, BDCYX, and QCA) was separately added into the oil once it had reached temperature. Samples (about 1 mL) were taken at intervals over a period of 1 h (0, 1, 2, 3, 4, 5, 6, 7, 8, 10 min for CYX; 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 15, 20, 25, 40, 50, 60 min for BDCYX and QCA). No oil losses caused by evaporation were observed. These samples were placed in 1.5 mL plastic tubes, which were cooled rapidly by immersion in cold water. The samples were then extracted according to the analytical method for cooking oil.

Stability of Cyadox Residues in the Incurred Muscle Samples. Samples of incurred tissues used for the stability research were ground, dispensed into a number of 50-mL plastic tubes (5 g/tube), and stored frozen at −20 °C.

Cooking Methods Investigated. *(i) Chicken Muscles Boiled in Bags.* Mixed chicken muscles were formed into 5-g "meatballs", which were placed into polythene bags. The neck was sealed. Then the bags were immersed in a water bath preheated to 100 °C, cooked for the specified time (2, 4, 6, 8, 10 min), removed, and allowed to cool.

(ii) Chicken Muscles Microwaved. A 5-g sample was placed on a turnplate. The sample was cooked under full power (700 W) for the specified time (0.5, 1, 1.5, 2, and 2.5 min), removed, and allowed to cool. No juice was collected.

(iii) Chicken Muscles Fried. A piece of chicken muscle (91.77 g) was minced into 2 cm pieces. They were stir-fried in a wok with a small quantity of cooking oil until little juice was seen. The cooking time was about 5 min.

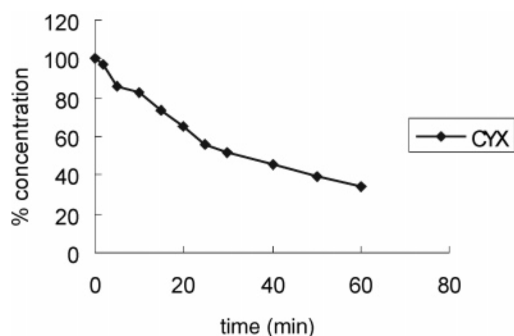
The temperature achieved during each cooking procedure was monitored using a digital thermometer with a sensor (Model TP01, Shenzhen). The model used was suitable for temperatures between 0 and 250 °C with an accuracy of 1.5 °C. All cooking treatments were done in triplicate.

Analytical Methods. *(i) Sample Preparation.* Analyses of cooked or uncooked chicken muscle samples for QCA were based on that developed by Lynch et al. and Qiu et al. (*2*, *3*). The Samples (5 g) were added into 50-mL centrifuge tubes, and 3 mol/L NaOH (10 mL) was added to all tubes, which were then placed into a water bath at 95−100 °C for 30 min. The tubes were removed and allowed to cool to room temperature. Concentrated hydrochloric acid (4 mL) was added into each tube and the tube was mixed for several seconds. Ethyl acetate (10 mL) was added, and the tube was mixed for 1 min and then centrifuged (10 min, 2000*g*). The upper layer (ethyl acetate layer) was transferred into a 50-mL tube, and the extraction was repeated twice with ethyl acetate (20 mL). The three extracts were then combined. Citric acid buffer (5 mL) was added, and the mixture was mixed and centrifuged (2000*g*, 5 min) to let the lower phase clarify. The aqueous phase was transferred to a 50-mL tube. The ethyl acetate phase was reextracted with an additional 5 mL of citric acid buffer, and the aqueous extracts were combined. Concentrated hydrochloric acid (2 mL) was added into each tube and mixed for several seconds. The acidic aqueous extract was applied onto the ion-exclusion column (the resin was washed in sequence with 50 mL of methanol, 50 mL of water, and 50 mL of 1 mol/L HCl before the extract was applied) and washed with 1 mol/L HCl (50 mL). The resin was eluted with 75 mL methanol−water (10:90 v/v) into a 250-mL separatory funnel. Concentrated hydrochloric acid (1 mL) was added to each sample. Chloroform (25 mL) was added and the separatory funnel was inverted several times. The lower organic phase was transferred into a 125 mL round-bottom flask. The extraction was repeated a further two times, and the extracts were combined. The chloroform extract was evaporated to dryness on a rotary evaporator at 45 °C. Residues were dissolved with 2 mL of methanol and centrifuged for 5 min at 5000*g* to clarify the methanol phase. The upper layer was taken for HPLC analysis.

(ii) Analyses of Cooked or Uncooked Chicken Muscle Samples. Analyses of cooked or uncooked chicken muscle samples for CYX and BDCYX were based on the method reported by Qiu et al. (*2*). The sample was homogenized by vortex machine at maximum speed with 10 mL of acetonitrile. The organic phase was collected after centrifugation for 10 min at 2000*g*. The tissue residue was reextracted with 10 mL of acetonitrile, and the organic extract was combined. A 5-mL portion of the combined organic extract was transferred to a 10-mL centrifuge tube and evaporated to dryness in a stream of nitrogen at 40 °C in a water bath. The residue was dissolved in 1 mL of acetonitrile and degreased with *n*-hexane (2 × 1 mL), and *n*-hexane layer was removed with a pipet. The acetonitrile layer was evaporated to dryness and dissolved in 0.5 mL of methanol for HPLC analysis.

(iii) HPLC Analysis. Agilent 1100 Series high performance liquid chromatograph instruments were used for the quantitation of cyadox residues extract. A Hypersil-keystone RP-18 chromatography column (250 × 4.6 mm i.d., 5-μm particle size) was used. The mobile phase containing a 1% formic acid solution and methanol (60:40 v/v) was used for QCA. A fine separation for CYX and BDCYX was achieved by water and acetonitrile with a gradient elution condition. The detailed gradient elution program was as follows: Time (min), water (%), acetonitrile (%); 0, 90, 10; 10, 75, 25. The detection was performed at 305, 280, and 320 nm for CYX, BDCYX, and QCA, respectively

Analytical Method for Cooking Oil. CYX and BDCYX standard solution (2 μg/mL, 100 μL) or QCA standard solution (5 μg/mL, 100 μL) was added to 200 μL of cooking oil. The sample was diluted with



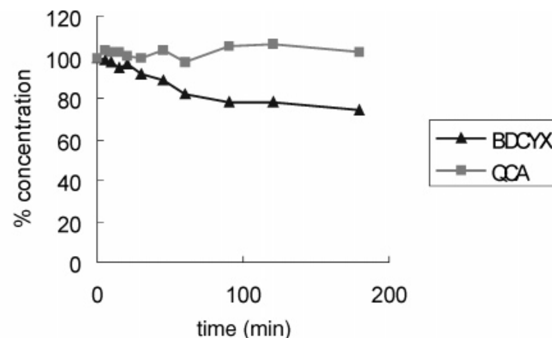


Figure 2. CYX, BDCYX, and QCA stabilities in boiling water.

2 mL of *n*-hexane and extracted into 1 mL of 0.01 mol/L HCl (1 mL of acetonitrile for BDCYX). The extract was measured against a standard of CYX, BDCYX, or QCA with the same solvent using the HPLC conditions described for the standard method.

Method Validation and Calibration Curves. The raw blank samples and cooked blank samples were fortified with CYX, BDCYX, and QCA in order to validate the methods. A standard series in the range of 20−320 μg/kg for CYX and BDCYX and 25−400 μg/kg for QCA in drug-free raw and cooked blank muscle samples was prepared and processed as described in the analytical methods section.

Statistical Analysis. To examine the reduction patterns of cyadox residues in the chicken muscles cooked by boiling and microwaving, the linear regression analysis on the relationship between the natural logarithm of cyadox residues in the muscle and time (min) after the start of the cooking was conducted individually. The pattern was expressed as an equation: $\ln y = \ln A_0 - kx$, where y is the concentration of cyadox residues (μg/kg), x is the time (min) after the start of cooking, and A_0 is the concentration of cyadox in the uncooked chicken muscle (5 g/raw). The analyses were accomplished using SPSS software. For cyadox residues in the muscle cooked by boiling and microwaving, the significance of the difference between cooking times was examined by one-way ANOVA.

RESULTS

Heat Stabilities of CYX, BDCYX, and QCA. Plots of CYX, BDCYX, and QCA concentration in boiling water against time are shown in **Figure 2**. CYX was found to be unstable over a period of 1 h in boiling water and the half-life was about 37.7 min. BDCYX was relatively stable and there was no obvious change after 1 h. QCA was found to be stable over a period of 3 h in boiling water.

The stabilities of three compounds in hot oil at 180 °C were also examined. All three compounds had significant change in the drug concentration among heating times of 0−180 min. CYX decreased quickly and was not able to be detected after heating for 2 min. Two breakdown products appeared, which were not stable under the condition. Plots of BDCYX and QCA concentration in hot oil against time are shown in **Figure 3**.

Stability of the Incurred Muscle Samples. Table 1 showed the concentrations of cyadox residues detected in chicken muscle at different periods of storage. It clearly demonstrated that CYX and BDCYX were not stable. CYX was not detected after 28-day storage. The concentration of BDCYX decreased 55%. The concentration of QCA initially increased and then decreased after 25 days.

Cooking Effect on Residue Concentration. The validation data produced for both raw and cooked blank tissue fortified with three compounds provided an indication of the accuracy of the method for use in the cooking study. The validation data obtained for the method are included in **Table 2**. A series of drug levels were fortified into tissues, and samples were prepared by the described extraction and cleanup procedure.

Figure 3. BDCYX and QCA stabilities in hot cooking oil.

Table 1. Concentrations of Cyadox Residues in Incurred Chicken Muscle after Storage at −20 °C[a]

storage time (days)	CYX concn (μg/kg)	BDCYX concn (μg/kg)	storage time (days)	QCA concn (μg/kg)
0	59	303	0	56
2	56	310	10	58
4	34	286	25	84
8	20	282	50	80
10	<LOQ	211	80	71
16	<LOQ	214	140	61
28	–	136	170	55

[a] LOQ, limit of quantitation; –, not detected.

Table 2. Method Validation Data: Recoveries (Mean, %, $n = 5$) from Raw and Cooked Tissues Fortified with CYX, BDCYX, and QCA

compd	fortified concn (μg/kg)	raw	boiled	microwaved	fried
CYX	20	78(9.2)[a]	71(9.9)	74(14.3)	73(12.2)
BDCYX	20	81(13.8)	76(16.8)	73(9.4)	78(16.5)
QCA	50	70(10.6)	83(9.0)	72(11.5)	73(12.3)

[a] Values in parentheses are relative standard deviations (%).

The lowest level that gave reasonable accuracy and precision was considered to be the limit of quantitation (LOQ). The quantitation limits of 20 μg/kg for CYX and BDCYX and 25 μg/kg for QCA were low enough for cooking experiments. In the evaluation of calibration, the peak area showed a linear relationship with concentration over the range of 20−320 μg/kg for CYX and BDCYX and 25−400 μg/kg for QCA. The correlation coefficients were all above 0.9900. The calibration curves were used to calibrate the levels of three compounds in incurred muscles after cooking.

Figure 4 shows typical HPLC traces of blank and spiked raw chicken muscle. The resulting extracts were free from

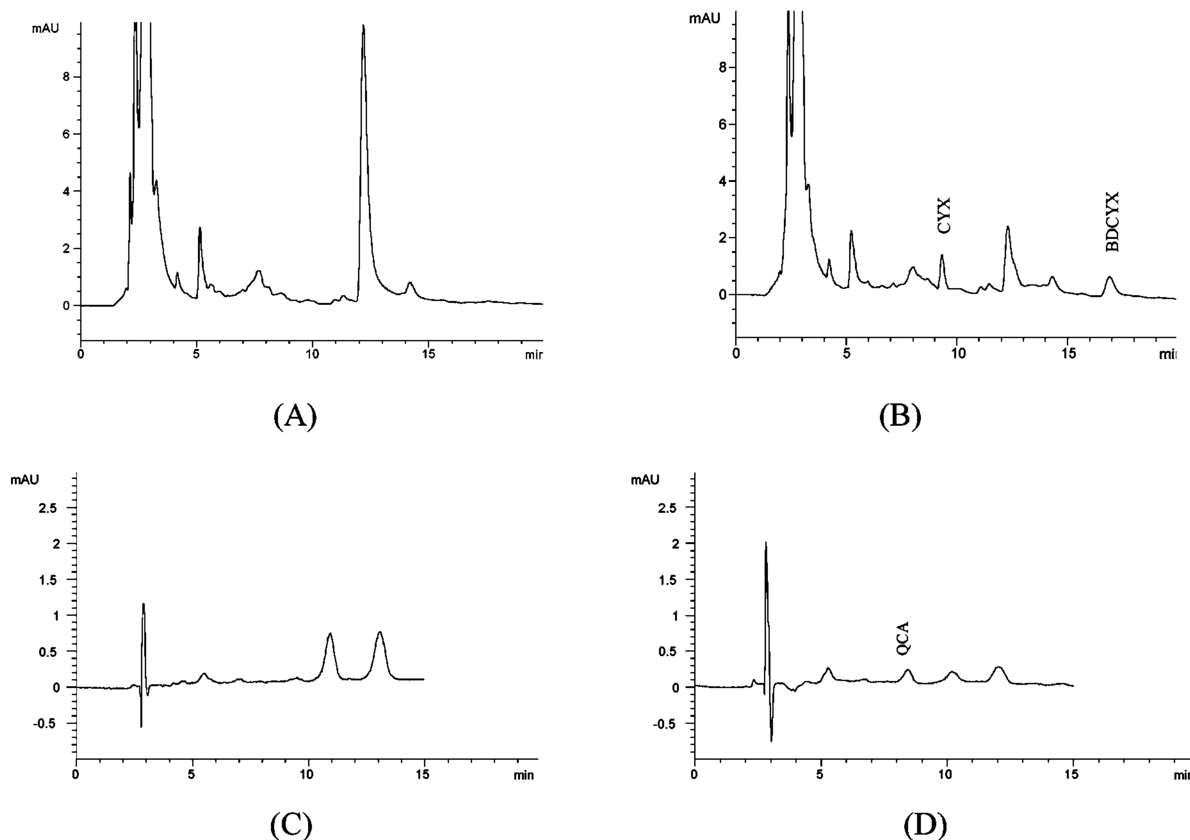


Figure 4. Typical HPLC chromatograms: blank raw chicken sample (**A** and **C**) and spiked (**B**, 20 μg/kg; **D**, 25 μg/kg) raw chicken sample.

Table 3. Results of Boiling Incurred Tissues (μg/kg) (Corrected by Recovery)

boiling time (min)	CYX[b]	BDCYX[b]	QCA[c]
0	320 ± 143	141 ± 75	76 ± 7
2	269 ± 139 (−16)[a]	103 ± 37 (−27)	71 ± 7 (−7)
4	73 ± 49 (−77)	69 ± 25 (−51)	71 ± 12 (−7)
6	31 ± 3 (−90)	54 ± 5 (−62)	69 ± 9 (−9)
8	20 ± 1 (−94)	41 ± 7 (−71)	77 ± 7 (+1)
10	20 ± 4 (−94)	27 ± 6 (−81)	77 ± 9 (+1)
internal cooking temperature (°C)	47.2–57.3	47.2–57.3	45.3–55.7

[a] Values in parentheses are percentages of the difference between uncooked and cooked samples. [b] Difference in the drug levels between cooking times in the same column are significant ($P < 0.01$) by one-way ANOVA. [c] Difference in the drug levels between cooking times in the same column are not significant ($P > 0.05$) by one-way ANOVA.

Table 4. Results of Frying Incurred Tissues (μg/kg) (Corrected by Recovery)

sample	mass (g)	total CYX	total BDCYX	total QCA
raw tissue	91.77	5.41 ± 1.06	25.05 ± 4.51	4.96 ± 0.28
fried tissue	43.70	0.73 ± 0.07 (−86)[a]	5.90 ± 1.25 (−76)	4.18 ± 0.19 (−16)

[a] Values in parentheses are percentages of the difference between uncooked and cooked samples.

interfering compounds for detection in the HPLC traces. Similar chromatograms were obtained from the cooked samples.

The results for the cooking of incurred tissues are given in **Tables 3−5**. Large variations between some replicate analyses were seen in some cases and the reason for this was not clear. In the course of this investigation, CYX and BDCYX were found to be unstable. The loss of CYX varied from 54% for microwaved muscle to 94% for boiled muscle. The loss of BDCYX varied from 47% for microwaved muscle to 81% for boiled muscle. QCA was found to be stable within the precision of the analytical method.

Table 5. Results for Microwaving Incurred Tissues (μg/kg) (Corrected by Recovery)

microwaving time (min)	CYX[b]	BDCYX[b]	QCA[c]
0	63 ± 14	106 ± 12	79 ± 8
0.5	84 ± 28 (+33)[a]	119 ± 17 (+12)	81 ± 9 (+3)
1	81 ± 34 (+29)	93 ± 11 (−12)	80 ± 10 (+1)
1.5	41 ± 11 (−35)	91 ± 12 (−15)	80 ± 8 (+1)
2	31 ± 6 (−51)	64 ± 16 (−40)	83 ± 8 (+5)
2.5	29 ± 2 (−54)	56 ± 17 (−47)	83 ± 9 (+5)
internal cooking temperature (°C)	35.5–44	35.5–44	37.8–41.9

[a] Values in parentheses are percentages of the difference between uncooked and cooked samples. [b] Difference in the drug levels between cooking times in the same column are significant ($P < 0.01$) by one-way ANOVA. [c] Difference in the drug levels between cooking times in the same column are not significant ($P > 0.05$) by one-way ANOVA.

Table 6 lists the linear regression results for the reduction data. Overall, significant linear regressions ($P < 0.01$) were observed between the natural logarithm of levels of CYX and BDCYX and the time (min) after the start of cooking except for QCA. The regression line for QCA could statistically be regarded as horizontal, suggesting that there was no reduction of QCA in the muscle cooked. The half-lives of cyadox in chicken muscles cooked by boiling and microwaving were 2.22 and 6.66 min, respectively. The half-lives of BDCYX in chicken muscles cooked by boiling and microwaving were 4.44 and 9.36 min, respectively.

Table 6. Linear Regression Results of CYX, BDCYX, and QCA from Chickens Cooked by Boiling and Microwaving

drug	ln A_0[a]	k[a]	r
	Boiling		
CYX	5.681	−0.312	−0.916[b]
BDCYX	4.857	−0.156	−0.877[b]
QCA	–[c]	–[c]	0.060[d]
	Microwaving		
CYX	4.416	−0.104	−0.759[b]
BDCYX	4.801	−0.074	−0.800[b]
QCA	–[c]	–[c]	0.454[d]

[a] $\ln y = \ln A_0 - kx$, where y is the concentration of compounds (μg/kg), x is the time (min) after the start of the cooking, and A_0 is the concentration in uncooked chicken muscle (5 g/raw) [b] Statistically significant ($P < 0.01$). [c] Not applicable. [d] Not statistically significant ($P > 0.05$).

DISCUSSION

According to the results of heat stabilities of three compounds, CYX and BDCYX were found to be sensitive to the temperature. The three compounds were not stable at 180 °C in cooking oil, but most oils used in cooking rarely exceed 180 °C, and the internal temperature of the food would be considered lower than this. Diode-array detection using HPLC indicated that CYX was degraded to BDCYX at 180 °C. Temperature is the important factor that affects the stabilities of the three compounds. CYX and BDCYX in incurred tissues were unstable, which indicated that the incurred samples should be analyzed as soon as possible within 2 days. The concentration of QCA initially increased, which mainly resulted from the degradation of CYX and BDCYX during the storage period. But after 25 days, the concentration of QCA decreased, which suggested that QCA itself also degraded over time. This needs to be tested further by fortifying tissues with QCA.

We have studied the metabolism and depletion of cyadox in chicken. The results showed that cyadox was metabolized quickly and was not detected in chicken muscle 2 h after administration, with a continuously fed diet of 100 μg/kg cyadox for 10 days. In this study, the higher dosage than that normally given in animal husbandry was used in order to achieve high residual concentrations. Pretests have been done from 20 min to 4 h after administration for the choice of suitable slaughter time. The chicken was killed 2 h postdosing, which could provide suitable residual concentrations for the three compounds at the same time. Because there exists variation between chickens, in fact, the residual concentrations of the three compounds in chicken muscle were not always suitable for cooking in one chicken. In this study, the data of the three compounds from the boiling method were from three separate chickens.

Before this test, CYX and BDCYX were separately fortified into blank tissues and the tissues cooked. The results showed that CYX could not be changed into BDCYX by the cooking process. CYX and BDCYX also could not be changed into QCA. So in this study, the stability data of the three compounds exactly reflected the effect of cooking on cyadox residues in chicken muscle.

The concentration of CYX in the muscles increased after the start of the cooking by microwaving and the reason is difficult to explain clearly now. Maybe some conjugated form of CYX existed in animal tissues, and CYX was released from the conjugation by microwaving. CYX has a shorter half-life in the boiling procedure compared with that in the microwaving procedure, which suggested that the reduction rate of CYX residues in the muscle during boiling is greater than that by the microwaving cooking method. The half-life may be dependent on the internal temperature in the center of the cooked samples. In the present cooking conditions, the temperatures of samples cooked by boiling were 45−57 °C, and this temperature was higher compared with 36−44 °C in the microwaving procedure.

In conclusion, CYX and BDCYX were found to be unstable in this investigation, and the loss may depend on the cooking time and the internal temperature in the center of cooked samples. QCA was stable in various cooking methods. The reduction of all three compounds observed in the present cooking methods may be explained by (1) conversion into other compounds, (2) transfer from the muscle into the boiling water, or (3) lost with juices, which came from the muscle as it was cooked.

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