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Development and Validation of Reliable Quantitative Methods for Ethoxyquin in Chicken by Fluorescence Detection (LC-FLD) and Isotope Dilution Tandem Mass Spectrometry (LC-IDMS/MS)

Abigail Grace H. Bion*, Aaron C. Dacuya, Alleni T. Junsay, and Benilda S. Ebarvia

Department of Science and Technology, Industrial Technology Development Institute (DOST-ITDI), National Metrology Laboratory of the Philippines, Metrology in Chemistry Section, Bicutan, Taguig City, Philippines
aghbion@itdi.dost.gov.ph; mic@itdi.dost.gov.ph

Abstract **– Ethoxyquin (EQ), a quinoline-based antioxidant, is widely used in food and animal feed, often leading to residues in animal tissues—raising health concerns. This study developed and validated two quantification methods: liquid chromatography with fluorescence detection (LC-FLD) and isotope dilution tandem mass spectrometry (LC-IDMS/MS). Sample preparation employed the QuEChERS technique with enhanced matrix removal-lipid (dSPE EMR-lipid). The LODs/LOQs were 2.4/ 3.6 µg/kg (LC-FLD) and 1.7/ 2.5 µg/kg (LC-IDMS/MS). Both methods showed excellent linearity (R = 0.999) using matrix-matched calibration. Recoveries at three spiking levels ranged from 94% to 106%, while repeatability (RSD ≤ 4.2%, n = 10) and intermediate precision over one, two, and three month period (RSD ≤ 7.6%, n = 3) complied with Horwitz criteria. The methods proved suitable for reliable EQ monitoring in chicken and similar matrices and were fit-for-purpose for homogeneity and stability studies and reference value assignment of a chicken-based reference material.**

I. INTRODUCTION

Poultry, predominantly chicken, is one of the most widely consumed meat in the world and a key source of dietary protein. Consequently, the poultry industry plays a vital role in meeting the growing global demand for high-quality meat products [2]. However, large-scale poultry production exposes chickens to various environmental stressors including heat, handling, transport, and feed quality that can increase the production of reactive oxygen species (ROS) in their body, ultimately causing cellular damage [3].

To minimize these negative effects, antioxidants are routinely incorporated into poultry feed. Ethoxyquin (EQ), a synthetic antioxidant, is widely utilized due to its cost-effectiveness and high antioxidant capacity. Studies have demonstrated that EQ not only stabilizes feed but also improves meat quality and enhances egg yolk pigmentation [4]. Despite these benefits, growing concerns have emerged regarding the safety of EQ, particularly their potential to accumulate in animal tissues and pose human health risks.

In response to health and food safety issues, the European Union has prohibited the use of EQ as an antioxidant [5] while other regulatory bodies have set specific maximum residue limits (MRLs). The U.S. Food and Drug Administration (FDA) established an MRL of 0.5 mg/kg in meat and eggs, 3 mg/kg in chicken liver, and 5 mg/kg in fat [6]. Japan set its MRL to 0.1 mg/kg in poultry, 1 mg/kg in eggs and 0.5 mg/kg in other animal tissues [7].

Various analytical methods have been developed for EQ residue determination. These primarily include chromatographic techniques with fluorescence or electrochemical detection, and a more advanced tandem mass spectrometry. Isotope dilution mass spectrometry (IDMS) is a primary reference method that provides highly accurate and SI-traceable results. This technique enhances analytical performance through isotope-matching, which corrects for signal drift, minimizes matrix effects, and compensates for sample loss [8]. For a cost-effective yet selective detection system, fluorescence detection offers a viable alternative toward compounds with conjugated π-systems, such as EQ [9].

One of the major challenges in EQ analysis in animal-

derived foods is matrix interferences due to high protein and fat content. Reported methods for EQ isolation include liquid-liquid or solid-liquid extraction and the widely adopted QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) approach. To enhance clean-up efficiency, a novel material known as the Enhanced Matrix Removal-lipid (EMR-lipid) has recently been introduced. EMR-lipid selectively removes lipids from high-fat samples through hydrophobic interactions and size-exclusion mechanisms that enable more accurate multi-residue analysis without compromising analyte recovery [10].

While several detection methods have been developed for EQ residue, comprehensive validation studies specific to poultry are limited. Existing literature mainly focused on fish and other seafood matrices. Poultry meat remains less explored in EQ residue monitoring studies due to its matrix complexity. Therefore, there is a need for reliable analytical methods capable of overcoming matrix interferences while maintaining high sensitivity and selectivity.

In this study, two quantification methods: LC-FLD and LC-IDMS/MS were developed and validated with sample preparation involving QuEChERS technique with dSPE EMR-lipid clean-up for accurate and reliable EQ detection in chicken and similar matrices.

II. METHODS

A. Chemicals and Materials

Ethoxyquin hydrochloride (CAS No: 91-53-2) with 93.3 ± 3.0 % purity, was purchased from Dr. Ehrenstorfer (Augsburg, Germany). Isotope labelled Ethoxyquin-D5-hydrochloride (EQ-D5) of 99.9% purity was purchased from Witega (Berlin, Germany). Ammonium acetate was purchased from Sigma Aldrich and analytical grade glacial acetic acid was purchased from Macron Fine Chemicals (USA). HPLC grade methanol and acetonitrile were from J.T. Baker (USA). Ultrapure water (18.2 M Ω) was obtained using a Merck Milli-Q Integral 5 purification system, QuEChERS extraction kit (AOAC 2007.01 method buffered, P/N: 5982-5755), Bond Elut EMR-lipid dispersive SPE (P/N: 5982-1010) were supplied by Agilent Technologies. Chicken fillets purchased from a local supermarket (Parañaque, Philippines) were used as samples for method optimization and validation.

B. Standard solutions

Stock solution (100 mg/kg) was prepared by dissolving 2 mg of ethoxyquin hydrochloride with 20 g methanol in an amber bottle. An intermediate solution (1 mg/kg) was prepared by diluting an appropriate amount of the stock solution with acetonitrile. Solutions were stored at -20 °C until further use.

C. LC-FLD sample preparation and instrumentation

Sample Preparation: The lyophilized chicken muscle (0.5 g) was reconstituted with 8 g of water and extracted using 10 g of acidified acetonitrile (2% acetic acid) and QuEChERS AOAC salt (consisting of 6 g $MgSO_4$ and 1.5 g sodium acetate). 5 mL of the supernatant was transferred into a pre-conditioned EMR-lipid dSPE tube for matrix clean-up. The final extract of 1 mL was filtered using a 0.2 µm nylon syringe filter before analysis.

Calibration solutions: A matrix-matched calibration was employed, where solutions were prepared by spiking a known amount of intermediate solution into an EQ-free, lyophilized chicken matrix. These solutions were then extracted following the above-mentioned procedure. At least 6 working standards with concentration range of 1 – 200 µg/kg were prepared for the calibration curve for LC-FLD.

Instrumentation: Analysis was carried out using the Agilent 1290 Infinity II UHPLC system consisting of an autosampler, quaternary gradient pump, column thermostat, and fluorescence detector. Chromatographic separation was performed by injecting the sample (5 µL) on Agilent Poroshell HPH-C18 (4.6 x 100 mm, 2.7 µm) column with Agilent Poroshell HPH-C18 (4.6 mm) pre-column at 45 °C. With the autosampler set to 10 °C, the analyte was eluted at 0.7 mL/min with a gradient of methanol and 5.0 mM ammonium acetate, pH 5. Briefly, the methanol was kept at 65 % for 7 min, then linearly increased to 90 % by 10 min and held for 15 min. Methanol was restored to 65 % by 16 min and held until 25 min to allow the column to equilibrate before injecting another sample. The excitation and emission wavelengths were 235 and 480 nm, respectively. Quantification was done using matrix-matched calibration. Data acquisition and analysis were performed with Agilent OpenLab software.

D. LC-IDMS sample preparation and instrumentation

Sample preparation: The same extraction procedure described in *Section C* for LC-FLD analysis was applied, with a few additional steps. Specifically, 0.5 g of lyophilized chicken muscle was reconstituted in 8 g of water. The EQ-D5 isotope-labeled internal standard was added, and the mixture was sonicated for 2 hours to allow equilibration. Extraction was then carried out using 10 g of acidified acetonitrile and QuEChERS AOAC extraction salts, followed by matrix clean-up using EMR-lipid dSPE. The final extract (1 mL) was filtered through a 0.2 µm nylon syringe filter prior to analysis.

Calibration solutions: Calibration blends were prepared, in which the EQ standard was mixed with isotopically enriched EQ-D5 to achieve an analyte-to-isotope response ratio of 1. The calibration solutions were sonicated for 2 hours.

Instrumentation: Analysis was carried out using Agilent 6460 Triple Quadrupole Mass Spectrometer with positive electrospray ionization (ESI) source. Chromatographic separation was performed by injecting the sample (10 µL) on Agilent Poroshell HPH-C18 (4.6 x 100 mm, 2.7 µm) column with Agilent Poroshell HPH-C18 (4.6 mm) pre-column at 40 °C using 90-10 acetonitrile-methanol (80 % v/v) and 10 mM ammonium acetate buffer (20 % v/v) at a flow rate of 0.5 mL/min. The autosampler was set to 10 °C. For the MS parameters, a gas temperature of 340 °C with a gas flow rate of 12 L/min, nebulizer pressure of 50 psi, and capillary voltage of 3,000 V were used using multiple reaction monitoring (MRM) mode. *Table 1* provides the MRM transitions and ESI source parameters for EQ and EQ-D5. Quantification was done by exact-matching isotope dilution technique. Data acquisition and analysis were performed with Mass Hunter Workstation.

Table 1. MRM transitions of EQ and EQ-D5

Compound	MRM transitions (m/z)		CE (eV)	FV (V)
	Q1	Q3		
EQ	218.2	160.0 [a]	35	130
		145.9 [b]	35	130
EQ-D5	223.2	161.0 [a]	35	130
		145.9 [b]	35	130

Note: [a] Quantifier, [b] Qualifier

E. Method Validation

The LC-FLD and LC-IDMS methods were validated in accordance with EURACHEM [11] and AOAC [12] guidelines in terms of linearity, repeatability, intermediate precision, trueness, limit of detection (LOD), and limit of quantification (LOQ). Prior to validation, chicken samples were confirmed to be EQ-free. Linearity was assessed using calibration curves prepared in duplicate over a concentration range of 1 to 200 µg/kg (for LC-FLD) and 1 to 100 µg/kg (for LC-IDMS). Repeatability (n=10) and intermediate precision (n=3) were evaluated based on the relative standard deviation (RSD) of spiked chicken samples at three concentration levels: 150, 300, 500 µg/kg. Trueness (n=10) was determined by calculating spike recoveries at three concentration levels using blank chicken samples fortified with EQ. LOD and LOQ were established by analyzing ten replicates of samples containing the lowest detectable concentration of EQ and were calculated based on the resulting area responses.

III. RESULTS AND DISCUSSION

A. LC-FLD Method optimization

The LC conditions from previous studies [13], [14] were adapted and optimized using an Agilent Poroshell HPH-C18 column. Parameters such as mobile phase (elution type, buffer concentration, ratio, and pH), column temperature, flow rate, and detector settings (excitation/emission wavelengths and PMT gain) were systematically varied.

Column temperature was tested at 30 °C, 40 °C, and 45 °C. Higher temperatures produced sharper peaks and shorter elution times, with 45 °C selected as optimal without risking column integrity. Detector optimization revealed that 235/480 nm provided the best response for ethoxyquin (EQ) and reducing interference.

This wavelength combination offered better baseline stability. Sensitivity was further enhanced by increasing the PMT gain, with the best response observed at gain 18.

The 65:35 (v/v) methanol-buffer ratio yielded the best separation, with gradient elution (ramping methanol to 90% from 10–15 min) effectively removing late-eluting contaminants. A buffer concentration of 5 mM ammonium acetate gave the highest area response. Flow rate variation (0.5–0.7 mL/min) showed faster elution and improved peak shape at higher rates. The buffer's pH was evaluated at 4.5, 5.0, and 6.5 (unadjusted pH). Optimal response was observed at pH 5.0, with minimal impact on EQ retention but improved separation from interfering peaks. As EQ is weakly acidic (pKa ~4.5), acidifying the mobile phase helped minimize ionization.

B. LC-IDMS/MS Method Optimization

The LC and MS parameters for IDMS/MS quantification were optimized based on the previous method [15] using an ESI+ mode. Optimization was conducted in terms of fragmentation voltage, collision energy, cell accelerator voltage, gas temperature, gas flow and capillary voltage via multiple reaction monitoring (MRM) segment to obtain the precursor and product ions. Optimized conditions were based on the highest obtained response.

The parent ions of EQ and EQ-D5 were identified to have a mass-to-charge ratio (m/z) of 218.2 and 223.2, respectively, via MS2 scan mode (*Fig. 1*). The corresponding product ions obtained were 145.9 m/z & 160.0 m/z for EQ and 145.9 & 161.0 m/z for EQ-D5 (*Fig. 2*). A reference spectrum from literature [15] confirms the ethoxyquin MS spectra obtained. Fragmentor voltage was optimized at 130 V, and collision energy at 35 eV provided the best MRM response. A nebulizer pressure of 45 psi and capillary voltage of 3000 V yielded the highest ionization efficiency.

C. Validation of the LC-FLD Method

The linearity of the analytical response over the matrix-matched working range of 0.99 to 200.34 µg/kg was satisfactory, with a correlation coefficient (R) > 0.995. The LOD and LOQ were determined by analyzing ten replicates of chicken samples spiked with ethoxyquin

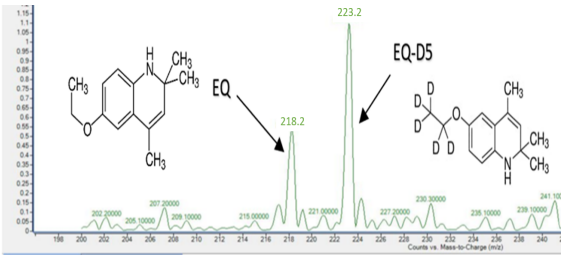

Fig. 1. MS spectrum of a mixed standard solution of EQ and EQ-D5 via MS2 Scan mode

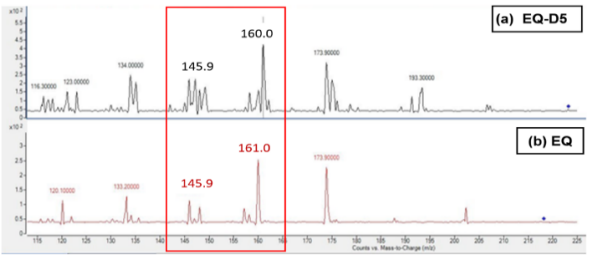

Fig. 2. MS spectra of (a) EQ-D5 and (b) EQ using MS2 Product ion scan mode

at 5 µg/kg. The resulting LOD and LOQ were 2.4 µg/kg and 3.6 µg/kg, respectively, which were substantially lower than the U.S. FDA MRL of 500 µg/kg [6]. Correlation coefficients of the calibration curves, LOD and LOQ results are presented in *Table 2*. At all concentration levels, both repeatability (% RSD ≤ 4.1 %) and intermediate precision (% RSD ≤ 5.8 %) were within acceptable limits (

Table 3). Recoveries ranged from 94 % to 106 % at three fortification levels (200, 400, and 800 µg/kg) complied with the AOAC performance criteria [12], as shown in *Table 4*.

Table 2. Correlation coefficients of the calibration curves, LOD and LOQ using LC-FLD

Parameters	Values
Range, µg/kg (n=11)	0.99 – 200.34
Equation of the line	y = 10.945x - 7.181
correlation coefficient, R	0.999
LOD, µg/kg (n=10)	2.4
LOQ, µg/kg (n=10)	3.6

Table 3. Precision of the LC-FLD method on EQ determination in the lyophilized chicken

Spiked level, µg/kg	RSD (%)				
	Repeatability (n = 10)	Intermediate Precision (n = 3)			
	Month 0	Month			Horwitz
		1	2	3	
150	4.1	1.9	3.7	5.2	< 14
300	1.7	1.5	2.8	5.8	< 12
500	2.5	3.3	0.6	3.6	< 11

Table 4. Trueness by recovery of EQ from lyophilized chicken by LC-FLD

	% Recovery*		
	200 µg/kg	400 µg/kg	800 µg/kg
Range	96-106	95-104	94-105
RSD	3.4	2.9	3.4

*AOAC acceptable criteria: 80-110% [12]

D. Validation of the LC-IDMS/MS Method

The linearity of EQ and its isotopically labeled standard EQ-D5 was evaluated in both solvent and chicken matrix over the range of 1–100 µg/kg (n = 10), with correlation coefficients (r) > 0.995. As shown in *Fig. 3*, EQ responses in solvent and matrix overlapped, indicating negligible matrix effects. This is attributed to the high selectivity of mass spectrometry and the compensatory effect of using isotope-labelled EQ-D5 [8]. Findings support the use of a solvent-based calibration blend for LC-IDMS/MS analysis of EQ in chicken. Furthermore, it was observed that EQ consistently showed higher responses than EQ-D5 at equivalent concentrations in both matrices, indicating that matrix effects did not impact the analyte or internal standard. Accordingly, EQ-D5 concentrations should be adjusted to maintain a 1:1 response ratio. Linearity results are summarized in

.

Table 5. Correlation coefficients of the calibration curves, LOD and LOQ using LC-IDMS

Parameters	EQ	
	solvent	matrix
Range, µg/kg (n=10)	1.2 – 100.2	1.4 – 99.3
Equation of the line	y = 51.60x + 0.6052	y = 55.19x - 52.613
correlation coefficient, R	0.999	0.999
LOD, µg/kg(n=10)	-	1.7
LOQ, µg/kg (n=10)	-	2.5

Repeatability (% RSD ≤ 2.6 %) and intermediate precision (% RSD ≤ 7.6 %) were within acceptable limits at all concentration levels (*Table 6*). Recoveries ranged from 98% to 104% at three fortification levels were within the acceptable performance criteria (*Table 7*). Recovery values from both methods were randomly distributed around 100%, confirming the methods' efficiency in extracting ethoxyquin from the chicken matrix across the tested concentrations.

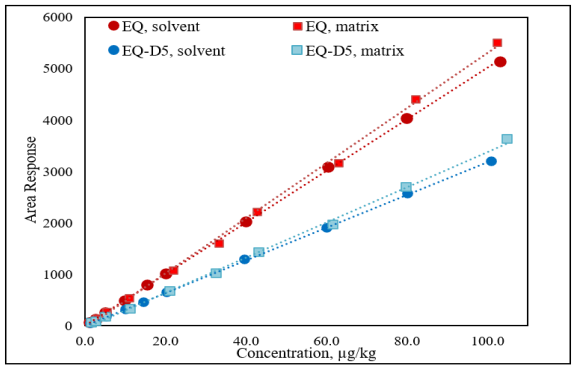

Fig. 3. Comparison of EQ and EQ-D5 response in solvent and matrix.

Table 6. Precision of the LC-IDMS/MS method on EQ determination in the lyophilized chicken

Spiked level, µg/kg	RSD (%)				
	Repeatability (n = 10)	Intermediate Precision (n = 3)			
	Month 0	Month			Horwitz
		1	2	3	
150	2.6	0.03	1.8	7.6	< 14
300	1.3	0.2	1.7	2.9	< 12
500	1.9	4.3	2.1	1.8	< 11

Table 7. Trueness by recovery of EQ from Lyophilized Chicken by LC-IDMS/MS

	% Recovery*		
	170 µg/kg	350 µg/kg	680 µg/kg
Range	100-103	98-104	98-101
RSD	0.8	1.7	1.0

*AOAC acceptable criteria: 80-110% [12]

IV. CONCLUSION

Reliable analytical methods for EQ determination in chicken were successfully developed and validated using LC-FLD and LC-IDMS/MS. Both methods demonstrated excellent performance in terms of linearity, repeatability, intermediate precision, trueness, LOD, and LOQ.

LC-FLD was selected for its accessibility and widespread use in routine testing laboratories. The validated LC-FLD method was then subsequently employed to evaluate the homogeneity and stability of the Philippine Reference Material (PRM 1801) Ethoxyquin in Chicken, developed by the National Metrology Laboratory, DOST-ITDI. In parallel, the higher-order LC-IDMS/MS method that was validated was used for the characterization and assignment of the reference value of the material.

These validated methods support national and international food safety monitoring programs by enabling reliable EQ quantification and ensuring compliance with regulatory requirements.

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