ACTA IMEKO
ISSN: 2221-870X
June 2026, Volume 15, Number 2, 1 - 9

QuEChERS-based determination of ethoxyquin in chicken by LC-FLD and isotope dilution LC-MS/MS

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ABSTRACT
Safety concerns surrounding ethoxyquin (EQ), particularly its potential to accumulate as detectable residues in animal-derived foods, underscore the need for rapid and effective analytical methods for EQ monitoring. A new method was developed and validated for the trace determination of ethoxyquin in chicken. The workflow combines the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) approach with dispersive solid phase extraction Enhanced Matrix Removal–Lipid (dSPE EMR-Lipid) cleanup, followed by quantification using liquid chromatography–fluorescence detection (LC-FLD) and isotope dilution tandem mass spectrometry (LC-IDMS/MS). The limits of detection and quantification were 2.4 and 3.6 µg/kg for LC-FLD, and 1.7 and 2.5 µg/kg for LC-IDMS/MS. Both methods exhibited excellent linearity (R = 0.999) using matrix-matched calibration. Mean recoveries across three fortification levels ranged from 94 % to 106 %, while repeatability ($RSD \leq 4.2$ %, n = 10) and intermediate precision ($RSD \leq 7.6$ %, n = 3) over one-, two-, and three-month periods complied with Horwitz criteria. The developed method was successfully applied to the homogeneity and stability analysis of EQ in a chicken-based reference material.

Section: RESEARCH PAPER

Keywords: ethoxyquin; QuEChERS; isotope dilution mass spectrometry; method validation; EMR-Lipid

Citation: A. G. H. Bion, A. C. Dacuya, A. T. Junsay, B. S. Ebarvia, QuEChERS-based determination of ethoxyquin in chicken by LC-FLD and isotope dilution LC-MS/MS, Acta IMEKO, vol. 15 (2026) no. 2, pp. 1-9. DOI: 10.21014/actaimeko.v15i2.2297

Section Editor: Leonardo Iannucci, Politecnico di Torino, Italy

Received January 15, 2026; **In final form** May 19, 2026; **Published** June 2026

Funding: This work was supported by the Department of Science and Technology (DOST), Philippines.

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1. INTRODUCTION

Poultry meat consumption has increased substantially over the years, positioning chicken as a major contributor to global dietary protein intake. As a result, poultry production plays a key role in supporting the growing demand for quality and nutritionally valuable animal-derived food products [1]. However, intensive poultry production practices subject birds to multiple stress-related factors, including thermal stress, handling, transportation, and variations in feed quality. These conditions can disrupt the normal metabolic balance and promote excessive generation of reactive oxygen species (ROS), leading to oxidative stress and cellular damage [2].

To mitigate oxidative deterioration, antioxidants are routinely incorporated into poultry diets. Ethoxyquin, or EQ, has been extensively used due to its strong antioxidant efficacy and cost-effectiveness.

EQ primarily stabilizes feed by neutralizing free radicals and inhibiting lipid oxidation reactions. Additionally, EQ has been reported to improve certain quality attributes of poultry products, such as enhanced meat color and increased egg yolk pigmentation, contributing to a fresher and more appealing appearance [3], [4].

Growing concerns have emerged regarding the safety of EQ, particularly its potential to accumulate as detectable residues in animal tissues. Insufficient toxicological data on the adverse effects of EQ on animal health environment led to its prohibition as a feed additive in the European Union (Regulation (EU) 2017/962). Nevertheless, EQ continues to be widely used in many countries worldwide, and its long-term health implications remain incompletely understood [5]. Other regulatory authorities have implemented residue-based risk management strategies by establishing maximum residue limits (MRLs). For example, the U.S. Food and Drug Administration (FDA) has set MRLs of 0.5 mg/kg for meat and eggs, 3 mg/kg for chicken liver, and 5 mg/kg for fat [6], while Japan has established limits of 0.1 mg/kg for poultry, 1 mg/kg for eggs, and 0.5 mg/kg for other animal tissues [7]. In line with these regulatory concerns, EQ has been detected at concentrations ranging from 0.14 to

24.20 μg/kg in aquatic products, and from 15 to 238 μg/kg in poultry [8], [9]. Such residues in edible tissues may indirectly compromise human health through the intake of contaminated animal-derived foods, while EQ and its transformation products have been associated with potential mutagenic and carcinogenic effects in animal studies [10].

A range of analytical approaches has been reported for the determination of EQ residues, including chromatographic methods coupled with fluorescence or electrochemical detection, as well as more advanced tandem mass spectrometric techniques. Among these, isotope dilution mass spectrometry (IDMS) is regarded as a higher-order analytical method due to its superior accuracy and robustness. The isotope-matching principle of IDMS effectively compensates for signal variability, matrix effects, and analyte losses during sample preparation [11]. To ensure accurate quantification, the isotopically labeled internal standard must be added prior to extraction, and should closely resemble the target analyte in structure to achieve co-elution and comparable ionization efficiency—requirements readily met by isotopic derivatives [12]. For cost-effective yet selective detection, fluorescence detection remains a suitable alternative for compounds with conjugated π-electron systems, such as EQ [13].

The determination of EQ in animal-derived foods is further complicated by significant matrix interferences arising from high protein and lipid contents. Commonly reported sample preparation strategies include liquid–liquid extraction, solid–liquid extraction, and the widely used QuEChERS approach, which combines salting-out extraction with dispersive solid-phase clean-up to reduce co-extracted matrix components. However, QuEChERS performance depends on analyte properties, matrix composition, and analytical conditions, requiring careful optimization of factors such as solvent composition, pH, sample-to-solvent ratio, and salt selection [14]. To further improve cleanup efficiency, Enhanced Matrix Removal–Lipid (EMR-Lipid) sorbents have been introduced, enabling selective lipid removal via hydrophobic interactions and size-exclusion mechanisms without compromising analyte recovery [13].

Despite the availability of several analytical techniques for EQ determination, comprehensive validation studies focused on poultry matrices remain limited. Most existing studies have concentrated on fish and other seafood products, while poultry meat, despite its complex matrix composition, has received comparatively less attention. Consequently, there is a clear need for reliable analytical methods, capable of effectively addressing matrix interferences while maintaining high sensitivity and selectivity. In the present study, two complementary quantitative methods were developed and fully validated for EQ determination in chicken: liquid chromatography with fluorescence detection (LC-FLD) and isotope dilution tandem mass spectrometry (LC-IDMS/MS). The analytical workflow integrates QuEChERS extraction with dispersive solid-phase extraction, using EMR-Lipid cleanup to achieve accurate and reliable residue analysis.

2. EXPERIMENTS

2.1. Chemicals and materials

Ethoxyquin hydrochloride (purity 93.3 ± 3.0 %) was obtained from Dr. Ehrenstorfer (Augsburg, Germany), and the isotopically labelled internal standard, ethoxyquin-D5 hydrochloride (EQ-D5, purity 99.9 %), was sourced from Witega (Berlin, Germany). Ammonium acetate was supplied by Sigma-Aldrich, while analytical-grade glacial acetic acid was purchased from Macron Fine Chemicals (USA). HPLC-grade methanol and acetonitrile were provided by J.T. Baker (USA). Ultrapure water (18.2 MΩ) was produced using a Merck Milli-Q Integral 5 system. The QuEChERS extraction kit, consisting of 6 g $MgSO_4$ and 1.5 g sodium acetate (AOAC 2007.01, P/N 5982-5755), ceramic homogenizers and Bond Elut EMR-Lipid dispersive SPE tubes (P/N 5982-1010), were obtained from Agilent Technologies. Chicken fillets purchased from a local supermarket in Parañaque, Philippines, were used for method optimization and validation.

2.2. Optimization of extraction procedure

The AOAC 2007.01 standard method for QuEChERS described by S. Lehotay et al. [15] was modified and optimized in terms of sample size, addition of ascorbic acid, acetic acid concentration of the extraction solvent, matrix cleanup, and final diluent of the sample solution.

2.3. LC-FLD analysis

2.3.1. Sample preparation

0.5 g of lyophilized chicken muscle was weighed and placed into a 50 mL polypropylene centrifuge tube. Pre-spiked chicken samples were reconstituted with 8 g of water, followed by the addition of 10 g of acetonitrile containing 2 % acetic acid and two ceramic homogenizers. The mixture was vortexed for 1 minute, then stored at 4 °C for at least 1 h for equilibration. QuEChERS salt was added into the tube and shaken vigorously for 1 minute. Samples were then centrifuged at 5000 rpm for 5 minutes. 5 mL of the supernatant was transferred into an EMR-lipid dSPE, which was pre-conditioned with 1 mL of water and vortex mixed for 10 s. The sample solution was shaken vigorously in a vortex mixer for 1 minute and centrifuged at 5000 rpm for 5 minutes. The final extract of 1 mL was filtered through a 0.2 μm nylon syringe filter before analysis.

2.3.2. Standard and calibration 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.

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 in Section 2.3.1. At least 6 working standards with a concentration range of 1–200 μg/kg were prepared for the calibration curve for LC-FLD.

2.3.3. Instrumentation

Analysis was performed using an Agilent 1290 Infinity II UHPLC system equipped with a quaternary pump, an autosampler, a column oven, and a fluorescence detector. Separation was achieved by injecting 5 μL of sample onto an Agilent Poroshell HPH-C18 column (4.6 × 100 mm², 2.7 μm) fitted with a matching guard column, maintained at 45 °C. The autosampler temperature was set to 10 °C. Elution was carried out at 0.7 mL/min, using a gradient of methanol and 5.0 mM ammonium acetate (pH 5). The methanol fraction was held at 65 % for the first 7 minutes, increased linearly to 90 % by 10 minutes, and maintained for 15 minutes, followed by a return

to 65 % at 16 minutes, and re-equilibration until 25 minutes. Fluorescence detection was conducted at 235 nm (excitation) and 480 nm (emission), and quantification was performed using matrix-matched calibration. Data acquisition and processing were carried out using Agilent OpenLab software.

2.4. LC-IDMS/MS analysis

2.4.1. Sample preparation

The extraction procedure described for LC-FLD analysis (Section 2.3.1) was followed with minor modifications. Briefly, 0.5 g of freeze-dried chicken muscle was reconstituted with 8 g of water, after which the isotopically labelled internal standard (EQ-D5) was added to obtain a sample blend (SB) with a measured isotope response ratio of native EQ to EQ-D5 of approximately 1. The mixture was sonicated for 2 hours to ensure equilibration between native EQ and EQ-D5. Extraction was then performed using 10 g of acidified acetonitrile and QuEChERS salts, followed by EMR-dSPE matrix clean-up. The final extract (1 mL) was filtered through a 0.2 µm nylon syringe filter prior to analysis.

2.4.2. Standard and calibration solutions

Standard solutions were all prepared gravimetrically. A stock solution of EQ (100 mg/kg) and the isotope-labelled standard solution EQ-D5 (20 mg/kg) were prepared separately in methanol. Intermediate solutions (0.2 mg/kg) were prepared by diluting the stock solutions with acetonitrile.

Calibration blend: Quantification was performed using single-point exact-matching double isotope dilution mass spectrometry (EMD-IDMS), which is based on the measured isotope ratio of EQ to EQ-D5 in both the calibration blends (CBs) and sample blends (SBs). An initial UHPLC screening was carried out to approximate the EQ mass fraction in the samples. Using this estimate, equal amounts of EQ and EQ-D5 intermediate solutions were combined and analysed to approach a 1:1 isotope ratio between the native and labelled standards. Four native standard solutions (A–D) were prepared, and from each, two calibration blends (A1–D2) were generated, resulting in eight CBs containing native and labelled internal standards at roughly equal proportions. The CBs were prepared in acetonitrile and sonicated for 2 hours, then subjected to the same extraction protocol as the SBs described in Section 2.4.1. LC-MS/MS analysis was used to compare the CBs for consistency and reproducibility, and the blend with an isotope ratio closest to unity was selected for subsequent sample measurements.

2.4.3. 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 × 100 mm², 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	FV
	Q1	Q3	(eV)	(V)
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

[a] Quantifier ion; [b] Qualifier ion

2.5. Method validation

The LC-FLD and LC-IDMS methods were validated in accordance with EURACHEM [16] and AOAC [17] 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 evaluated using both solvent-based and matrix-matched calibration curves, prepared in duplicate. Calibration standards were prepared over concentration ranges of 1–200 µg/kg for LC-FLD and 1–100 µg/kg for LC-IDMS/MS. Calibration levels were initially expressed in terms of solution concentrations, to enable direct comparison between solvent-based and matrix-matched calibration curves. This approach facilitated the assessment of matrix effects and the selection of the most appropriate calibration strategy for quantitative analysis. 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 determined following the EURACHEM approach, and were established by analysing ten replicates of samples containing the lowest detectable concentration (5 µg/kg) of EQ, and were calculated based on the resulting area responses. The LOD and LOQ were estimated from the standard deviation (SD) of these replicates using equations (1) and (2), respectively [16]:

$$LOD = \frac{AR_{\text{ave}} + 3\,S}{a}\, x\, \frac{m_{\text{solution}}}{m_{\text{sample}}}, \tag{1}$$

$$LOQ = \frac{AR_{\text{ave}} + 10\,S}{a}\, x\, \frac{m_{\text{solution}}}{m_{\text{sample}}}, \tag{2}$$

where A_{ave} is the average peak area response, a is the slope of the calibration curve, s is the standard deviation of the peak area response, and m_{solution} and m_{sample} are the masses of the solution and the sample, respectively. To ensure sample homogeneity at this low concentration level, a bulk sample was prepared by spiking ethoxyquin into powdered chicken at 5 µg/kg. The sample was then rehydrated with water to form a slurry, thoroughly mixed, and subsequently freeze-dried prior to analysis.

3. RESULTS AND DISCUSSION

3.1. Optimization of sample preparation

In this study, freeze-dried chicken powder was used instead of fresh chicken muscle, to enhance sample stability over extended periods. Freeze-drying reduces moisture content, thereby limiting enzymatic activity and microbial growth that could lead to degradation of both the matrix and EQ. This

improved stability is particularly important for intermediate precision studies and long-term analyses, ensuring consistent sample composition and analyte integrity. The freeze-drying conditions and preparation of the powdered material were adopted from the literature [18].

The AOAC 2007.01 QuEChERS method, described by Lehotay et al. [15], was systematically optimized by evaluating several key parameters, including sample size, addition of ascorbic acid (AA), acetic acid (HAc) concentration in the extraction solvent, matrix cleanup, and the final diluent of the extract. The influence of the sample size (0.5, 1, and 2 g) of freeze-dried chicken on extraction efficiency was first examined. A 0.5 g sample yielded the highest recoveries (Figure 1). The improved performance at lower sample size is likely due to reduced matrix interference and more efficient phase separation between the aqueous and organic layers (Figure 2). However, sample size must remain representative of the matrix. Despite the improved performance, recoveries remained below the acceptable range (80–110 %), requiring further optimization of the extraction procedure.

The effect of incorporating dispersive SPE EMR-Lipid cleanup following QuEChERS extraction was then assessed. As shown in Figure 3, the signal response for ethoxyquin increased markedly with EMR-Lipid treatment, accompanied by a cleaner baseline and improved chromatographic profiles (Figure 4). This sorbent effectively removes co-extractive components responsible for signal suppression or enhancement. These findings align with Han et al.'s report, demonstrating the effectiveness of EMR-Lipid in producing clean extracts for GC-MS/MS analysis of pork, salmon, avocado, and kale [19]. EMR-Lipid not only retains lipid fractions, but also differentiates them from other hydrophobic constituents, enhancing selectivity and minimizing loss of hydrophobic analytes, thereby improving recovery [20].

The influence of HAc concentration (0 %, 1 %, and 2 %) in acetonitrile on extraction performance was also investigated.

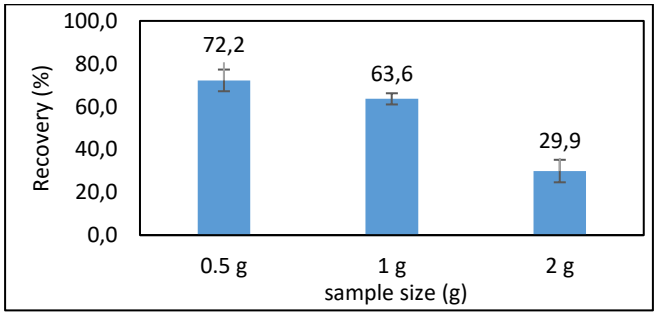

Figure 1. Effect of sample size. Error bars represent standard deviation (n = 3).

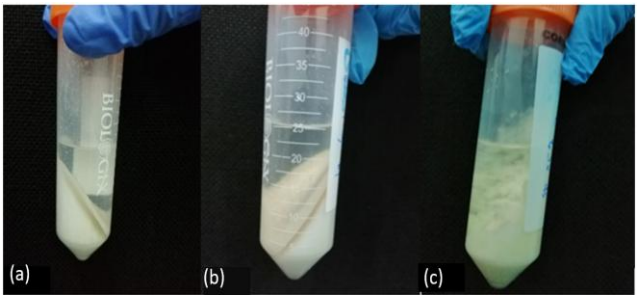

Figure 2. Comparison of sample extracts using (a) 0.5 g, (b) 1 g, and (c) 2 g after QuEChERS extraction.

Acid is usually added to the extraction solvent to facilitate protein precipitation efficiency [20]. As illustrated in Figure 5, EQ responses increased with higher acid content. The 2 % HAc–acetonitrile mixture was selected for the final method, owing to its good repeatability (n = 3, RSD = 3.42 %) compared to 1 % HAc (n = 3, RSD = 25.95 %). Consistent with previous optimization studies, concentrations above 2 % HAc resulted in EQ degradation, whereas 2 % HAc eliminated solution turbidity and improved extraction performance [21].

The effect of the final sample diluent was evaluated using two strategies: (1) direct injection of the filtered extract and (2) air-drying followed by reconstitution in 20:80 acetonitrile–water. The composition of the injected solution can influence analytical

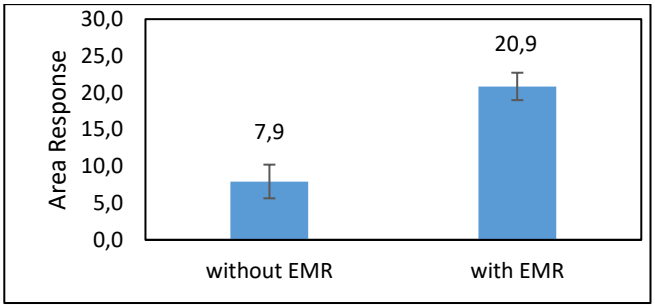

Figure 3. Effect of EMR-lipid clean up. Error bars represent standard deviation (n = 3).

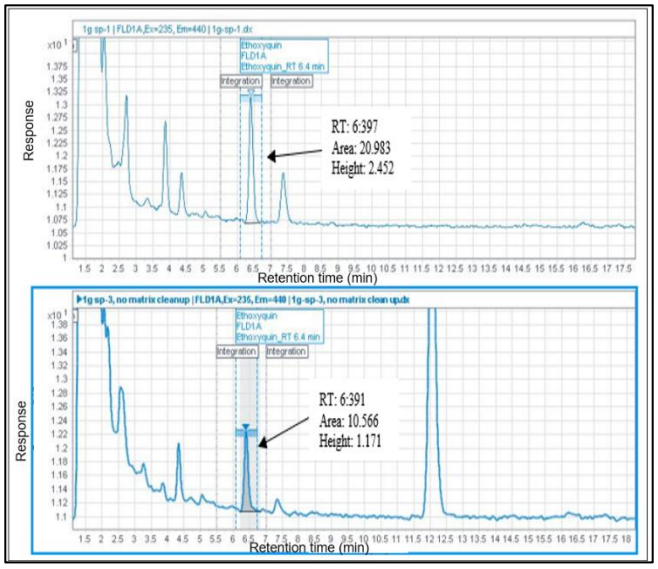

Figure 4. 0.025 mg/kg spiked sample chromatograms with (top) and without (bottom) EMR-lipid cleanup.

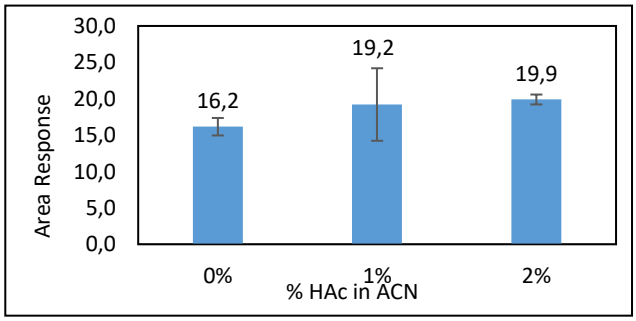

Figure 5. Effect of acetic acid concentration in the extraction solvent. Error bars represent standard deviation (n = 3).

performance because the sample solvent affects peak shape, retention, and ionization efficiency in LC-FLD or LC-MS/MS. A previous report indicates that direct injection of acetonitrile-rich extracts can reduce EQ sensitivity due to mismatch with the initial mobile phase composition, whereas diluting the extract with a small proportion of water (< 20 %) can improve peak shape and reduce matrix suppression [21]. In this study, however, the highest analytical response was obtained with the direct-injection approach (Figure 6). Lower responses for diluted samples likely resulted from oxidative degradation of EQ during air-drying, as no inert gas was used. Direct injection preserved analyte integrity and provided the best sensitivity under the conditions tested.

A comparative study of external and matrix-matched standard calibration was conducted to identify the most suitable approach for ethoxyquin analysis in chicken. Re-analysis using schemes 1 (no drying) and 2 (with drying) showed that matrix-matched calibration effectively compensated for matrix effects. Scheme 1 produced acceptable recoveries with matrix-matched calibration and good repeatability, whereas external standard calibration yielded recoveries below the acceptance range. Scheme 2 resulted in poor repeatability and recoveries outside the acceptable range with both calibration approaches (Figure 6). Accordingly, scheme 1 combined with matrix-matched calibration was selected for the final method.

To assess potential oxidation of ethoxyquin, the effect of adding ascorbic acid prior to spiking was evaluated, based on previous studies in pears [22] and fishery products [23], showing reduced oxidative losses and improved recoveries. With external calibration, spike recoveries improved slightly in the presence of ascorbic acid, but remained below the acceptable range (80–110%). Using matrix-matched calibration, recoveries were satisfactory without ascorbic acid, whereas adding ascorbic acid resulted in recoveries above the acceptable range (Figure 7). Consequently, the ascorbic acid step was omitted from the extraction procedure, and matrix-matched calibration was adopted for the final method.

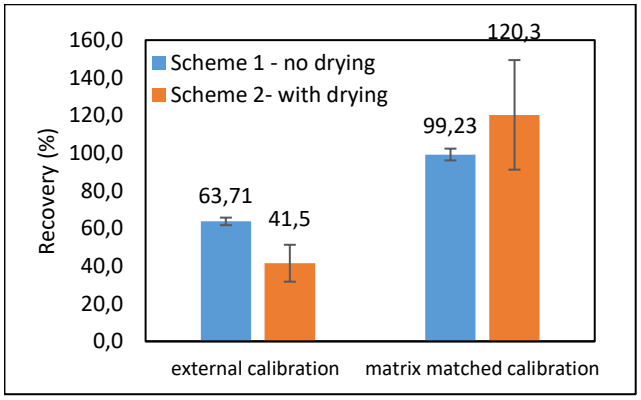

Figure 6. Recovery comparison of Scheme 1 and 2 using external and matrix-matched calibration. Error bars represent standard deviation (n = 3).

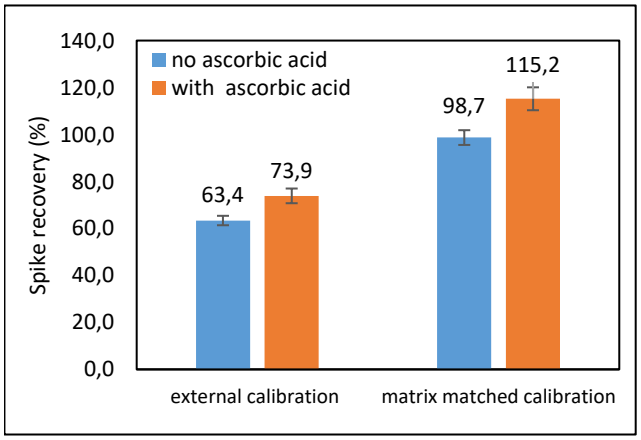

Figure 7. Effect of ascorbic acid. Error bars represent standard deviation (n = 3).

3.2. Optimization of UHPLC-FLD

LC conditions from previous studies [24], [25] were adapted and optimized, using an Agilent Poroshell HPH-C18 column. Mobile phase composition, buffer concentration and pH, column temperature, flow rate, and detector settings (excitation/emission wavelengths and PMT gain) were systematically evaluated. A gradient elution of methanol and 5 mM ammonium acetate buffer provided optimal sensitivity and resolution, with a 65:35 (v/v) methanol–buffer ratio and methanol ramped to 90 % between 10–15 minutes to remove late-eluting contaminants, resulting in a total run time of 25 minutes. Buffer pH was tested at 4.5, 5.0, and 6.5 (unadjusted pH), with pH 5.0 offering improved separation from interfering peaks while maintaining EQ retention. A slight acidification minimized ionization of the weakly acidic EQ (pKa ~4.5). Column temperature optimization showed that 45 °C produced sharper peaks and shorter elution without compromising column integrity. Detector settings of 235/480 nm and PMT gain of 18 provided the best signal, baseline stability, and reduced interference.

3.3. Optimization of LC-IDMS/MS conditions

LC–MS/MS is widely regarded as the most suitable technique for residue analysis, due to its high sensitivity, selectivity, and robustness in complex matrices, as well as its ability to resolve co-eluting compounds based on molecular mass [26]. Using the chromatographic conditions optimized for UHPLC–FLD, mass spectrometric parameters were further optimized in positive electrospray ionization (ESI+) mode to maximize analyte response. Key parameters, including fragmentor voltage, collision energy, cell accelerator voltage, gas temperature and flow, and capillary voltage, were optimized under multiple reaction monitoring (MRM) conditions to identify precursor and product ions. EQ and its isotopically labelled analogue EQ-D5 were confirmed by MS2 scans, with precursor ions observed at m/z 218.2 and 223.2, respectively. Characteristic product ions were m/z 145.9 and 160.0 for EQ, and m/z 145.9 and 161.0 for EQ-D5. Optimal ionization efficiency was achieved using a fragmentor voltage of 130 V, collision energy of 35 eV, nebulizer pressure of 45 psi, and capillary voltage of 3000 V. The resulting mass spectra were consistent with previously reported reference data [21].

3.4. Validation of the LC-FLD method

Method linearity was evaluated using solvent-based and matrix-matched calibration curves for LC-FLD over the concentration range of 1 to 200 µg/kg (n = 11). In addition to correlation coefficient assessment (R > 0.995) (Table 2), analysis of variance (ANOVA) was performed to evaluate the significance of the calibration regressions [27]. The ANOVA results confirmed that all calibration regressions were statistically significant within the evaluated concentration range (Table 3). A lack-of-fit test was not performed, because only one measurement was obtained for each calibration level, which did

Table 2. Calibration curves, LOD and LOQ of LC-FLD and LC-IDMS/MS.

Parameters	LC-FLD		LC-IDMS/MS	
	solvent	matrix	solvent	matrix
Range, µg/kg	1.13-201.01	0.99 – 200.34	1.2 – 100.2	1.4 – 99.3
Equation of the line	$y = 15.041\,x - 0.254$	$y = 10.945\,x - 7.181$	$y = 51.60\,x + 0.6052$	$y = 55.19\,x - 52.613$
Correlation coefficient, R	0.999	0.999	0.999	0.999
LOD, µg/kg (n = 10)	-	2.4	-	1.7
LOQ, µg/kg (n = 10)	-	3.6	-	2.5
Matrix effect, %	-27.2		7.0	

Table 3. Analysis of variance (ANOVA) of the solvent-based and matrix-matched calibration models for ethoxyquin by LC-FLD

Source	LC-FLD				LC-IDMS/MS			
	Deg. of freedom	Sum of square	Mean square	*F*	Deg. of freedom	Sum of square	Mean square	*F*
A. Solvent-based								
Regression	1	10150809	10150809	918975	1	29234130	29234130	50826
Residual	9	99	11		8	4601	575	
Total	10	10150908			9	29238731		
B. Matrix								
Regression	1	5269963	5269963	8887	1	32608989	32608989	4780
Residual	9	5337	593		8	54577	6822	
Total	10	5275300			9	32663567		

LC-FLD: $F_{crit\,(0.05)} = 5.1$; LC-IDMS/MS: $F_{crit\,(0.05)} = 5.3$

Table 4. Precision results of LC-FLD and LC-IDMS/MS methods using spiked freeze-dried chicken powder.

Spiked level, µg/kg	LC-FLD					LC-IDMS/MS					Acceptable % RSD [a]
	Repeatability	Intermediate precision				Repeatability	Intermediate precision				
		Month			s_{pooled} [b]		Month			s_{pooled} [b]	
		1	2	3			1	2	3		
150	4.1	1.9	3.7	5.2	3.9	2.6	0.1	1.8	7.6	4.5	< 14
300	1.7	1.5	2.8	5.8	3.8	1.3	0.2	1.7	2.9	1.9	< 12
500	2.5	3.3	0.6	3.6	2.8	1.9	4.3	2.1	1.8	3.0	< 11

[a] Acceptable % RSD calculated from Horwitz [29]

[b] s_{pooled} is the pooled standard deviation calculated from measurements obtained on different analytical days (after 1, 2, and 3 months of repeatability study)

*AOAC acceptable criteria: 80–110 % [17]

not allow the estimation of the pure error required for a lack-of-fit evaluation.

The matrix effect (ME) was assessed by comparing the slopes of matrix-matched and solvent-based calibration curves using Equation (3) [28]:

$$ME\ (\%) = \left(\frac{m_{\mathrm{B}}}{m_{\mathrm{A}}} - 1\right) \times 100, \tag{3}$$

where m_{B} and m_{A} represent the slopes of the matrix-matched and solvent-based calibration curves, respectively. The calculated matrix effect was −27.2 %, indicating signal suppression due to matrix components. This result suggests a smoderate matrix influence for LC-FLD. Nevertheless, the application of matrix-matched calibration effectively compensates for this suppression, ensuring reliable quantification of EQ in chicken samples.

The LOD and LOQ were determined from ten replicate analyses of chicken samples fortified at 5 µg/kg, yielding values of 2.4 µg/kg and 3.6 µg/kg (RSD = 9.56 %), respectively. These limits are well below the U.S. FDA MRL of 500 µg/ kg [6]. Calibration parameters, including linearity, LOD, and LOQ, are summarized in Table 2.

The concentration levels selected for precision and trueness evaluation were based on the MRL for ethoxyquin in fresh chicken (0.1 mg/kg). Considering the approximate 75 % water content of fresh chicken, freeze-drying results in a concentration factor of about four, corresponding to 0.4 mg/kg in the dried matrix. Accordingly, validation levels were selected to represent approximately 0.5 ×, 1 ×, and 2 × the MRL. These levels were chosen to evaluate method performance around the regulatory limit. Repeatability (intra-day precision) was evaluated from ten replicate analyses (n = 10) performed under the same conditions within a single day, while intermediate precision (inter-day precision) was assessed from three replicate analyses (n = 3) conducted on different days. The repeatability RSD values were ≤ 4.1 %, and intermediate precision RSD values were ≤ 5.8 %, meeting established acceptance criteria from Horwitz equation [29] (Table 4). To further compare intra-day and inter-day variability, a pooled standard deviation (s_{pooled}) was calculated from measurements obtained on different days using equation (4):

$$s_{\mathrm{pooled}} = \sqrt{\frac{(n_1 - 1)\,s_1^2 + (n_2 - 1)\,s_2^2 + \cdots + (n_k - 1)\,s_k^2}{n_1 + n_2 + \cdots + n_k - k}}, \tag{4}$$

where n_1 is the sample size of the first data set, s_1 is the standard deviation of the first data set, n_2 and s_2 are the sample size and standard deviation of the second data set, respectively, and n_k and s_k are the sample size and standard deviation of the k^{th} data set. k represents the total number of data sets included in the pooled standard deviation calculation. The resulting s_{pooled} was higher than the repeatability standard deviation, confirming that inter-day variability was greater when data from multiple time points were collectively considered. Mean recoveries ranged from 94 % to 106 % at three fortification levels of 200, 400, and 800 µg/kg, in compliance with AOAC performance criteria [17], as shown in Table 5.

Table 5. Trueness by recovery of LC-FLD and LC-IDMS/MS methods using spiked samples of blank freeze-dried chicken powder.

Concentration level	Spike recovery (RSD), % *	
	LC-FLD	LC-IDMS/MS
200 µg/kg	96-106 (3.4)	100-103 (0.8)
400 µg/kg	95-104 (2.9)	98-104 (1.7)
800 µg/kg	94-105 (3.4)	98-101 (1.0)

3.5. Validation of the LC-IDMS/MS method

The linearity of ethoxyquin (EQ) and the isotopically labeled internal standard (EQ-D5) was investigated using both solvent-based and matrix-matched calibration standards across the concentration range of 1–100 µg/kg (n = 10). Excellent linear responses were obtained for both analytes, with correlation coefficients (r) exceeding 0.995 (Table 2). The close similarity between the solvent-based and matrix-matched calibration curves (Figure 8) suggests that matrix-related signal variation was minimal.

To further verify calibration performance, the regression models were statistically evaluated using ANOVA (Table 3). The results demonstrated significant regression across the studied concentration range, supporting the linear behavior of the calibration curves. A lack-of-fit assessment was not carried out because single measurements were used for each calibration level, which prevented the estimation of pure error necessary for the test.

Using equation (3), the calculated matrix effect was 7.0 %, which is considered negligible according to SANTE guidelines, where signal suppression or enhancement below 20 % does not require correction [30]. This minimal matrix influence is attributed to the high selectivity of tandem mass spectrometry and the compensatory effect of the isotopically labeled internal standard EQ-D5 [11].

This finding supports the use of a solvent-based calibration blend for LC-IDMS/MS quantification of EQ in chicken. Although EQ exhibited consistently higher signal responses than EQ-D5 at equivalent concentrations in both matrices, this did not affect quantification accuracy; therefore, EQ-D5 levels were adjusted to maintain a 1:1 isotope response ratio. The LOD and LOQ, determined from ten replicate analyses of chicken samples fortified at 5 µg/k, were 1.7 µg/kg and 2.5 µg/kg (RSD = 7.43 %), respectively. The values are below the U.S. FDA MRL, demonstrating the method's suitability for detecting trace levels of ethoxyquin. The results are summarized in Table 2.

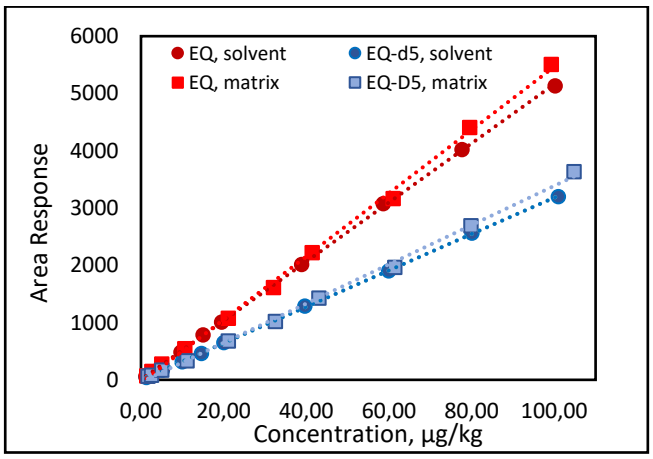

Figure 8. Comparison of eq and eq-d5 response in solvent and matrix

Repeatability (% RSD ≤ 2.6 %) and intermediate precision (% RSD ≤ 7.6 %) were within acceptable limits at all concentration levels. To account for between-day variation, standard deviations from analyses performed on different days were pooled to calculate the s_{pooled} using equation (4). The resulting s_{pooled} values were higher than the repeatability standard deviations, indicating greater inter-day than intra-day variability (Table 4).

Recoveries ranged from 94 % to 106 % at three fortification levels (200, 400, and 800 µg/kg) complied with the AOAC performance criteria [17], as shown in Table 5. Moreover, the recovery values were randomly distributed around 100 %, confirming the method's efficiency in extracting ethoxyquin from the chicken matrix across the tested concentrations.

Overall, both the LC-FLD and the LC-IDMS/MS method exhibited satisfactory validation performance in terms of precision and trueness. However, the validation was conducted within a single laboratory. Further inter-laboratory and ruggedness studies are recommended to confirm the broader applicability and transferability of the method.

3.6. Analysis of samples

To further evaluate the applicability of the method beyond the validation matrix, fresh chicken tissues (liver, wings, legs, and breast) purchased from a local supermarket were analysed in triplicate (n = 3). Ethoxyquin was only detected in liver at a concentration of 2.4 µg/kg, which is below the method LOQ for LC-FLD (3.6 µg/kg) and LC-IDMS/MS (2.4 µg/kg), while no detectable levels were observed in the other tissues.

To assess method performance at low concentration levels, simulated incurred samples (0.020 and 0.050 mg/kg) were analysed using the LC-FLD method. The native ethoxyquin concentration was first determined, and samples were then spiked with 150 µg/kg and 100 µg/kg, respectively, during sample processing. Recoveries were calculated by subtracting the native concentration from the total measured concentration after spiking. Satisfactory recoveries at both levels confirm the method's capability for accurate quantification at trace concentrations (Table 6).

The method has been applied in ongoing work, involving reference material characterization and value assignment, further supporting its practical applicability. Detailed results from this application are not presented here, due to their intended use in proficiency testing schemes.

4. CONCLUSION

A simple and reliable analytical method based on a modified QuEChERS extraction, combined with EMR-Lipid dispersive SPE clean-up, was successfully developed for the determination of ethoxyquin in chicken, using LC-FLD and isotope dilution LC-MS/MS. Key extraction parameters influencing method

Table 6. Spike recovery of 'incurred' sample.

Sample concentration*, µg/kg	Spike concentration, µg/kg	% Spike recovery	% RSD
20	150	97.5	2.4
50	100	94.7	3.3

*Simulated incurred sample-containing EQ (n = 3)

performance were optimized, and instrumental conditions for both techniques were refined to achieve high sensitivity, reduced matrix effects, and adequate chromatographic separation. The validated methods demonstrated satisfactory linearity, precision, and trueness. Although moderate signal suppression (−27 %) was observed for LC-FLD, the application of matrix-matched calibration effectively compensated for matrix effects, ensuring accurate quantification. In contrast, LC-IDMS/MS showed a negligible matrix effect (7.0 %), indicating high reliability against matrix-related variation.

Owing to its extraction efficiency and compliance with established performance criteria, the method provides reliable quantification of ethoxyquin, and is suitable for routine application in food testing laboratories for monitoring residues in chicken and related matrices. However, the validation was conducted within a single laboratory, using one set of instrumentation, and no detailed assessment of cost, throughput, or environmental impact was performed. Further cost–benefit evaluations, inter-laboratory and ruggedness studies (e.g., across different instruments and operators) are recommended to confirm the broader applicability and practical implementation of the method.

ACKNOWLEDGEMENT

The author would like to thank the Department of Science and Technology (DOST) and the DOST–Philippine Council for Industry, Energy, and Emerging Technology Research and Development (DOST-PCIEERD) for supporting this research.

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