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Establishment of analysis method for the quantification of residues of halquinol and its metabolites in livestock and fishery products using liquid chromatography–tandem mass spectrometry

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Abstract

In this study, an analysis method was established for the quantification of residues of halquinol and its metabolites in livestock and fishery products using liquid chromatography–tandem mass spectrometry (LC–MS/MS). We selected beef, pork (muscle and fat), chicken, egg, milk, flat fish, eel, and shrimp as target samples for validation of the method owing to them being typical livestock and fishery products. Validation of the developed analysis method was performed using liquid chromatography–tandem mass spectrometry (LC–MS/MS) at three concentration levels (0.5, 1, and 2 × the maximum residue limits) following the Codex Alimentarius (CODEX) guidelines (CAC/GL 71–2009). For all samples, correlation coefficients (R^2) exceeded 0.99, recoveries ranged between 75.59 and 119.36%, and coefficients of variation (CV) ranged between 1.39 and 28.66%, thus satisfying CODEX guidelines. In addition, inter-laboratory validation was conducted, and the resulting recoveries and CVs satisfied the CODEX guidelines; LOQ was established as 10 μg kg^{-1} for pig muscle and 5 μg kg^{-1} for the other samples. Therefore, the analysis method developed in this study can accurately and precisely screen for and quantify halquinol and its metabolites in livestock and fishery products.

Keywords Halquinol, Analysis method, LC–MS/MS, Livestock products, Veterinary drug residues

Introduction

Annual consumption of livestock and fishery products has been on the rise due to their role as crucial sources of essential nutrients, including proteins and minerals, in human diets [1, 2]. To enhance production efficiency and prevent diseases in these products, administering veterinary drugs is essential. The usage of veterinary antibiotics is also increasing with the growing consumption of these products, projected to reach 105,596 tons in 2030, compared to the 63,151 tons used in 2010 [3]. However, excessive use of veterinary drugs can lead to the presence of their residues in livestock and fishery products, which can adversely affect human health [4, 5]. Prolonged exposure to these residues may cause allergies, dysbiosis, increased antibiotic resistance, and other related issues [6]. Antibiotic resistance, in particular, is a significant concern, as the use of veterinary drugs has been linked to a consistent increase in antibiotic-resistant pathogenic bacteria, thereby reducing therapeutic efficacy and limiting available treatment options [7, 8]. The World Health Organization (WHO) has also issued warnings about the risks

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of increased antibiotic resistance in major pathogenic bacteria [9]. The emergence of super-bacteria, such as methicillin-resistant and vancomycin-resistant Staphylococcus aureus, has drawn significant attention from international organizations and governments due to the increase in antibiotic resistance resulting from the misuse of veterinary medicine [10].

In light of the fact that 60% of human pathogenic bacteria originate from animals, the use of veterinary antimicrobials is crucial to prevent infections in livestock and fishery products, which in turn safeguards human health upon consumption [11]. Moreover, the occurrence of diseases in these products reduces their viability and ultimately leads to decreased production. Hence, the use of veterinary drugs is imperative to ensure adequate production and to prevent disease. However, the administration of veterinary drugs must be regulated. Consequently, international organizations, as well as nutrition and food safety organizations in most countries, have established maximum residue limits (MRLs) for veterinary drugs in livestock and fishery products to safeguard public health.

Halquinol is a mixture of quinoline derivatives that contains 5,7-dichloro-8-hydroxyquinoline (5,7-DCHQ) at 57–74% w/w, 5-chloro-8-hydroxyquinoline (5-CHQ) at 23–40% w/w, and 7-chloro-8-quinoline (7-CHQ) at 0–4% w/w. It serves as a broad-spectrum antimicrobial agent and is utilized for its antibacterial, antifungal, and antiprotozoal properties. It is also used as a growth promoter in swine and poultry. For example, it is administered to swine to treat and prevent diarrhea caused by Escherichia coli and Salmonella spp. A previous pharmacokinetic study was conducted in pigs to confirm the presence of halquinol metabolites through oral administration. According to the study, the primary halquinol metabolites identified were in the form of glucuronides, specifically 5-chloro-8-hydroxyquinoline glucuronide (5-CHQG) and 5,7-dichloro-8-hydroxyquinoline glucuronide (5,7-DCHQG), while sulfate form was detected in trace amounts [12, 13]. The recommended maximum residue limits (MRLs) for halquinol in swine muscle, fat with skin, liver, and kidney are 40, 350, 500, and 9000 μg kg^{-1}, respectively [14]. Notably, the Codex Alimentarius (CODEX) has recommended that the definition of residues for quantification should include not only 5-CHQ and 5,7-DCHQ but also their metabolites, 5,7-CHQG and 5,7-DCHQG [15]. Several methods for analyzing halquinol have been developed, including the use of diatomaceous earth as a dispersing agent or use of QuEChERS kit [16–18]. However, these methods have limitations, such as not including metabolites in the target analyte and not accounting for pork fat in the target samples [16]. Other analysis methods for halquinol had been reported, but they either used an internal standard [17] or did not focus on livestock and fishery products [18].

Thus, in this study, we aimed to establish an analysis method that could quantify residues of halquinol and its metabolites in livestock and fishery products.

Materials and methods

Chemicals and reagents

The 5-CHQ (95.0%) and 5,7-DCHQ (99.0%) standards were purchased from Sigma Aldrich (St. Louis, USA). 5-CHQG (99.0%) and 5,7-DCHQG (99.0%) were purchased from Biosynth-Carbosynth (Compton, UK), methanol (MeOH) and acetonitrile (MeCN) from Merck Inc. (Darmstadt, Germany), and formic acid and ethylenedinitrilotetraacetic acid disodium salt (Na_2-EDTA) from Sigma-Aldrich.

Sample preparation

In this study, eight types of livestock and fishery products—beef, pork (muscle and fat), chicken, egg, milk, flat fish, eel, and shrimp—were used to evaluate the analysis method. The sample collection quantity and sample pre-treatment method were selected following the Korean Food Code, as beef, pork. Pre-treated samples were homogenized and stored at −20 ℃ for later use in the evaluation of the analysis method. Each homogenized sample (2 g) was extracted with 10 mL of 80% MeCN containing 100 ppm of Na_2-EDTA by shaking for 10 min, following which they were kept in a freezer at −20 ℃ for 30 min. Then, the samples were centrifuged at 4700 *G* and 0 ℃ for 10 min, and the obtained supernatant was used for analysis.

LC–MS/MS instruments and analytical conditions

Separation and analysis were conducted on a Shimadzu Nexera X2/LC-30AD system (Shimadzu, Osaka, Japan) using a Shimadzu LCMS-8060 triple quadrupole mass spectrometer with a Waters XBridge™ C_{18} column (2.1 mm × 150 mm, 3.5 μm particle size, Waters, Dublin, Ireland). Gradient conditions were set to binary gradient, and the mobile phases comprised water containing 0.1% formic acid (v/v, mobile phase A) and MeCN containing 0.1% formic acid (v/v, mobile phase B). The gradient elution was performed utilizing the subsequent conditions: at time 0 min, 2% B; at time 1.00 min, 2% B; at time 7.00 min, 70% B; at time 7.30 min, 80% B; at time 10.20 min, 98% B; at time 13.20 min, 98% B; at time 13.21 min, 2% B; and at time 16.00 min, 2% B. The LC–MS/MS analysis was carried out under the following operating parameters: flow rate of 0.25 mL min^{-1}, injection volume of 5 μL, auto-sampler temperature set to 15 ℃, column oven temperature maintained at 40 ℃, capillary voltages set to 4.0 kV in positive polarity, capillary

temperature set at 300 ℃, and argon used as the collision gas. Ionization of samples was conducted using an electrospray ionization (ESI) mode with positive and negative switching modes.

Method validation

The linearity, accuracy, precision, limit of quantification (LOQ), and matrix effect (ME) of the developed method were evaluated following the guidelines outlined by CODEX [19] and the National Institute of Food and Drug Safety Evaluation (NIFDS) of Korea [20]. Evaluation of the analysis method was conducted using matrix-matched calibration.

The accuracy and precision of the analysis method were evaluated at three concentrations: 0.5, 1, and 2×the MRL for each sample. Except the pork muscle, the MRLs for the other samples were established as 0.01 mg kg^{-1} temporarily for introduction of positive list system (PLS) for veterinary drug in livestock and fishery products, followed by evaluation at the following MRLs: 0.005 mg kg^{-1}, 0.01 mg kg^{-1}, and 0.02 mg kg^{-1}. It is to be noted that while the CODEX-specified MRL was existed for pork fat, for the application of method to the other livestock fat samples, the MRL for fat was set to 0.01 mg kg^{-1}. The matrix-matched calibration curves were prepared using the following six target compound concentrations: 0.25, 0.5, 1, 2, 4, and 8×the MRL. The LOD and LOQ were defined as 3×and 10×of the signal-to-noise ratio for the lowest calibration concentrations, respectively.

The ME was determined by substituting the peak areas obtained from analyzing standard spiked samples before extraction with peak areas obtained from analyzing the standard compound dissolved in the same solvent as the extraction solvent, into the following equation. For this purpose, the standard solution was prepared at the same concentration level as the final test solution of the standard spiked sample [21]. Each sample was analyzed by conducting three replicate experiments at a single concentration (1×MRL).

$$\mathrm{ME}(\%) = \frac{(\mathrm{A} - \mathrm{B}) \times 100}{\mathrm{B}}$$

where A is the peak area of the standard spiked samples, and B is the peak area of the standard solution.

Furthermore, we performed inter-laboratory (inter-lab) validation with Seoul Regional Food and Drug Administration (SRFDA, Seoul, Korea) and Société Générale de Surveillance Korea (SGS, Seoul, Korea) to confirm the reproducibility of the developed analysis method by evaluating and comparing its accuracy, precision, and LOQ. LOQ was calculated as 10 times the standard error that was divided by the slope of the calibration curve. The highest value obtained from the calculation for each sample was established as the LOQ, and was rounded off to the fourth decimal place.

Results and discussion

MS optimization

The optimized multiple reaction monitoring (MRM) conditions under which the target compounds exhibited the highest sensitivity were determined. Mixture solution was directly injected into the LC–MS/MS in order to search to the optimized analysis conditions of 5-CHQ, 5,7-DCHQ and its metabolites (5-CHQG and 5,7-DCHQG). Table 1 shown that the optimized retention time, ionization, precursor ion, product ion, and collision energy of target analytes. The product ion that the highest intensity was used for quantification, and the second and third highest intensity product ion were used for

Table 1 Multiple reaction monitoring conditions for the target compounds

Compounds	Retention time (min)	Ionization	Exact mass (*m/z*)	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)
5-CHQ	7.43	$[M+H]^+$	179.01	180.1	**117.2***	27
					145.1	22
					100.1	44
5,7-DCHQ	5.62	$[M+H]^+$	355.05	356.1	**180.1***	17
					145.1	48
					117.2	55
5-CHQG	9.03	$[M+H]^+$	212.98	214.0	**150.3***	27
					179.2	24
					123.1	38
5,7-DCHQG	6.17	$[M+H]^+$	389.01	389.8	**214.2***	18
					150.1	53
					179.2	46

*Quantitative ion

Table 2 Comparison of linearity, accuracy, and precision of analysis method by extraction solution(n = 5)

Sample	Compound	80% MeCN containing 100 ppm of Na_2-EDTA				80% MeCN			
		R^2	Spiking levels (μg kg^{-1})	Average recovery (%)	CV (%)	R^2	Spiking levels (μg kg^{-1})	Average recovery (%)	CV (%)
Beef	5-CHQ	0.9989	5	96.73	8.15	0.9814	5	120.37	12.72
			10	97.18	3.73		10	110.37	19.44
			20	100.41	3.86		20	81.92	16.39
	5,7-DCHQ	0.9989	5	103.00	7.32	0.9923	5	110.78	6.39
			10	105.77	7.90		10	91.62	4.83
			20	111.14	9.29		20	80.17	6.14
	5-CHQG	0.9972	5	115.92	4.57	0.9998	5	94.49	12.42
			10	92.50	9.56		10	83.61	5.67
			20	76.40	5.40		20	85.80	9.31
	5,7-DCHQG	0.9968	5	118.65	5.34	0.9988	5	104.63	12.05
			10	96.32	6.56		10	92.02	7.50
			20	77.70	5.31		20	90.41	8.48
Pork muscle	5-CHQ	0.9981	20	94.80	7.75	0.9521	20	101.60	12.34
			40	103.40	4.24		40	110.40	39.60
			80	106.56	3.05		80	64.32	18.10
	5,7-DCHQ	0.9996	20	106.86	4.12	0.9867	20	111.48	8.33
			40	104.54	4.28		40	104.32	14.59
			80	100.42	7.25		80	85.30	5.36
	5-CHQG	0.9996	20	97.77	16.03	0.9990	20	92.16	20.63
			40	87.18	14.22		40	81.68	11.86
			80	90.48	13.52		80	102.51	14.30
	5,7-DCHQG	0.9991	20	94.14	15.62	0.9941	20	81.94	21.87
			40	87.90	14.20		40	80.84	9.35
			80	93.09	12.10		80	99.52	11.73
Chicken	5-CHQ	0.9998	5	95.80	9.05	0.9125	5	67.06	26.98
			10	99.68	5.38		10	69.86	84.03
			20	97.94	3.79		20	131.63	28.13
	5,7-DCHQ	0.9988	5	95.99	8.57	0.9867	5	70.33	16.33
			10	99.78	6.47		10	77.80	42.85
			20	98.56	3.32		20	123.57	18.00
	5-CHQG	0.9969	5	112.92	11.11	0.9975	5	104.62	8.05
			10	99.08	3.24		10	95.51	11.56
			20	78.44	3.58		20	103.75	10.34
	5,7-DCHQG	0.9975	5	119.34	2.74	0.9982	5	99.57	5.58
			10	98.98	3.48		10	91.78	8.66
			20	77.32	2.70		20	99.28	10.77

Table 2 (continued)

Sample	Compound	80% MeCN containing 100 ppm of Na_2-EDTA				80% MeCN			
		R^2	Spiking levels (μg kg^{-1})	Average recovery (%)	CV (%)	R^2	Spiking levels (μg kg^{-1})	Average recovery (%)	CV (%)
Egg	5-CHQ	0.9996	5	97.60	8.93	0.9716	5	152.67	24.82
			10	106.12	2.99		10	153.92	10.29
			20	96.89	6.68		20	102.44	21.39
	5,7-DCHQ	0.9995	5	104.60	2.95	0.9987	5	101.29	5.91
			10	104.47	5.50		10	89.44	3.22
			20	105.98	2.77		20	78.68	7.30
	5-CHQG	0.9982	5	90.37	27.98	0.9997	5	114.49	4.77
			10	103.11	16.11		10	110.98	5.92
			20	101.77	13.63		20	90.21	6.76
	5,7-DCHQG	0.9971	5	96.90	28.66	0.9992	5	112.84	2.74
			10	99.98	12.05		10	114.72	3.95
			20	99.19	9.80		20	94.39	7.63
Milk	5-CHQ	0.9995	5	91.30	4.95	0.9902	5	78.31	29.95
			10	95.59	4.89		10	42.82	23.69
			20	98.57	3.53		20	46.66	39.86
	5,7-DCHQ	0.9987	5	105.81	8.50	0.9921	5	96.57	32.62
			10	106.06	10.55		10	63.27	18.34
			20	107.00	5.15		20	68.18	16.72
	5-CHQG	0.9944	5	120.22	28.07	0.9881	5	149.63	30.45
			10	90.45	27.83		10	129.39	11.99
			20	109.82	25.48		20	200.95	21.51
	5,7-DCHQG	0.9987	5	88.65	27.17	0.9919	5	106.48	49.77
			10	81.65	24.94		10	128.60	8.86
			20	102.48	25.75		20	169.59	25.00

confirmation of halquinol. As results, 5-CHQ was shown that the highest intensity at *m/z* 180 and *m/z* 117 for precursor ion and product ion each other. In 5,7-DCHQ, the highest intensities of precursor ion and product ion were shown at *m/z* 356 and *m/z* 180. Their metabolites, 5-CHQG and 5,7-DCHQG were shown that the highest intensity of precursor ion at *m/z* 214 and *m/z* 390, product ion at *m/z* 150 and *m/z* 214 each other. Also, we separated the analytes to each different retention tigher sensitivity of analysis method under the gradient conditions.

Sample extraction

In this study, we established the halquinol extraction method based on a multi-veterinary drug residue determination method [22], with the following modifications: a cool-down step was added before the centrifugation step of the existing extraction method; for the pork fat sample extraction method in particular, centrifugation was conducted twice after the cool-down step. By adding these steps, we expected to increase the coagulation and precipitation of the fat in the livestock and fishery product samples, which results in a higher efficiency of fat elimination [23]. Also, we added to EDTA in extraction solution for improving the extraction efficiency [16]. In this study, we evaluated the recovery of five livestock products using two types of 80% MeCN extraction solutions: one containing Na_2-EDTA and the other without Na_2-EDTA. The variation in recovery outcomes between the two extraction solutions was presented in Table 2. As results, the use of Na2-EDTA-containing extraction solution was found to satisfy the CODEX guidelines in terms of linearity and recovery.

Table 3 Linearity, accuracy, and precision of analysis method(n = 5)

Sample	Compound	R^2	Spiking levels (μg kg^{-1})	Average recovery (%)	CV (%)
Beef	5-CHQ	0.9989	5	96.73	8.15
			10	97.18	3.73
			20	100.41	3.86
	5,7-DCHQ	0.9989	5	103.00	7.32
			10	105.77	7.90
			20	111.14	9.29
	5-CHQG	0.9972	5	115.92	4.57
			10	92.50	9.56
			20	76.40	5.40
	5,7-DCHQG	0.9968	5	118.65	5.34
			10	96.32	6.56
			20	77.70	5.31
Pork muscle	5-CHQ	0.9981	20	94.80	7.75
			40	103.40	4.24
			80	106.56	3.05
	5,7-DCHQ	0.9996	20	106.86	4.12
			40	104.54	4.28
			80	100.42	7.25
	5-CHQG	0.9996	20	97.77	16.03
			40	87.18	14.22
			80	90.48	13.52
	5,7-DCHQG	0.9991	20	94.14	15.62
			40	87.90	14.20
			80	93.09	12.10
Pork fat	5-CHQ	0.9959	5	101.13	4.96
			10	102.16	2.83
			20	98.71	6.65
	5,7-DCHQ	0.9969	5	107.57	10.32
			10	94.77	4.62
			20	95.70	8.72
	5-CHQG	0.9959	5	103.86	9.92
			10	94.68	6.50
			20	85.09	1.52
	5,7-DCHQG	0.9982	5	83.63	8.72
			10	81.62	10.18
			20	76.61	13.99
Chicken	5-CHQ	0.9998	5	95.80	9.05
			10	99.68	5.38
			20	97.94	3.79
	5,7-DCHQ	0.9988	5	95.99	8.57
			10	99.78	6.47
			20	98.56	3.32
	5-CHQG	0.9969	5	112.92	11.11
			10	99.08	3.24
			20	78.44	3.58
	5,7-DCHQG	0.9975	5	119.34	2.74
			10	98.98	3.48
			20	77.32	2.70

Table 3 (continued)

Sample	Compound	R^2	Spiking levels ($\mu g\ kg^{-1}$)	Average recovery (%)	CV (%)
Egg	5-CHQ	0.9996	5	97.60	8.93
			10	106.12	2.99
			20	96.89	6.68
	5,7-DCHQ	0.9995	5	104.60	2.95
			10	104.47	5.50
			20	105.98	2.77
	5-CHQG	0.9982	5	90.37	27.98
			10	103.11	16.11
			20	101.77	13.63
	5,7-DCHQG	0.9971	5	96.90	28.66
			10	99.98	12.05
			20	99.19	9.80
Milk	5-CHQ	0.9995	5	91.30	4.95
			10	95.59	4.89
			20	98.57	3.53
	5,7-DCHQ	0.9987	5	105.81	8.50
			10	106.06	10.55
			20	107.00	5.15
	5-CHQG	0.9944	5	120.22	28.07
			10	90.45	27.83
			20	109.82	25.48
	5,7-DCHQG	0.9987	5	88.65	27.17
			10	81.65	24.94
			20	102.48	25.75
Flat fish	5-CHQ	0.9994	5	100.89	1.56
			10	101.96	3.55
			20	97.39	1.39
	5,7-DCHQ	0.9986	5	85.34	9.44
			10	93.25	2.64
			20	103.92	7.48
	5-CHQG	0.9951	5	119.36	7.18
			10	109.94	14.02
			20	112.14	11.47
	5,7-DCHQG	0.9967	5	116.04	17.62
			10	110.29	9.88
			20	108.96	14.50

Table 3 (continued)

Sample	Compound	R^2	Spiking levels (μg kg^{-1})	Average recovery (%)	CV (%)
Eel	5-CHQ	0.9996	5	97.52	3.15
			10	94.20	4.29
			20	93.98	1.63
	5,7-DCHQ	0.9976	5	100.82	4.30
			10	96.07	4.21
			20	95.04	4.83
	5-CHQG	0.9967	5	93.42	27.51
			10	105.79	26.06
			20	112.34	18.81
	5,7-DCHQG	0.9931	5	92.51	25.50
			10	104.93	24.39
			20	111.81	18.04
Shrimp	5-CHQ	0.9982	5	102.17	5.85
			10	102.83	2.92
			20	105.69	3.58
	5,7-DCHQ	0.9993	5	102.45	4.01
			10	107.33	7.04
			20	108.90	7.04
	5-CHQG	0.9972	5	103.93	8.58
			10	80.15	15.42
			20	81.01	16.65
	5,7-DCHQG	0.9981	5	96.94	5.67
			10	76.73	15.70
			20	75.59	14.52

Table 4 Matrix effect of compounds by sample

Compound	Matrix effect (%)								
	Beef	Pork muscle	Pork fat	Chicken	Egg	Milk	Flat fish	Eel	Shrimp
5-CHQ	− 31.09	− 37.87	− 65.11	− 21.63	− 19.52	− 14.98	− 1.56	− 24.32	− 0.21
5,7-DCHQ	32.29	50.70	− 49.02	52.18	− 19.39	88.33	56.67	− 1.66	62.72
5-CHQG	− 53.24	− 56.73	− 1.09	− 30.30	− 61.78	− 82.69	2.85	27.59	− 20.05
5,7-DCHQG	− 64.52	− 71.39	− 60.83	− 44.62	− 73.40	− 77.07	− 8.40	− 8.01	− 37.18

Table 5 Inter-lab validation results of the accuracy and precision

Sample	Compound	Spiking levels (μg kg^{-1})	Average recovery (%)				CV (%)
			Institution A[a]	Institution B[b]	Institution C[c]	Total	
Beef	5-CHQ	5	96.73	92.73	115.40	101.62	7.12
		10	97.18	88.80	114.13	100.04	5.51
		20	100.41	88.83	102.69	97.31	6.95
	5,7-DCHQ	5	103.00	97.67	118.87	106.51	6.36
		10	105.77	98.03	109.33	104.38	7.52
		20	111.14	98.02	102.11	103.76	9.84
	5-CHQG	5	115.92	99.20	119.93	111.68	9.33
		10	92.50	97.33	113.43	101.09	10.29
		20	76.40	87.85	103.03	89.09	10.15
	5,7-DCHQG	5	118.65	101.93	119.60	113.39	8.91
		10	96.32	95.47	111.33	101.04	7.08
		20	77.70	84.73	102.64	88.36	7.78
Pork muscle	5-CHQ	20	94.80	100.23	106.13	100.39	6.66
		40	103.40	98.08	103.03	101.50	4.30
		80	106.56	93.43	99.99	99.99	7.36
	5,7-DCHQ	20	106.86	102.30	118.67	109.28	4.20
		40	104.54	96.00	109.27	103.27	5.55
		80	100.42	101.35	99.31	100.36	5.74
	5-CHQG	20	97.77	86.40	106.13	96.77	14.47
		40	87.18	107.93	103.03	99.38	15.06
		80	90.48	117.61	99.99	102.69	18.81
	5,7-DCHQG	20	94.14	87.25	118.20	99.86	13.02
		40	87.90	108.65	109.10	101.88	15.02
		80	93.09	117.55	99.87	103.50	16.24
Pork fat	5-CHQ	5	101.13	105.87	119.07	108.69	4.39
		10	102.16	104.13	114.73	107.01	4.86
		20	98.71	95.57	104.98	99.75	5.68
	5,7-DCHQ	5	107.57	99.53	108.87	105.32	8.98
		10	94.77	104.97	104.07	101.27	7.27
		20	95.70	98.70	104.33	99.58	6.97
	5-CHQG	5	103.86	107.47	115.00	108.78	7.62
		10	94.68	103.90	106.27	101.62	7.03
		20	85.09	98.75	106.41	96.75	7.96
	5,7-DCHQG	5	83.63	106.80	116.47	102.30	14.41
		10	81.62	102.57	109.13	97.77	14.14
		20	76.61	98.82	105.98	93.80	16.60
Chicken	5-CHQ	5	95.80	101.60	107.80	101.73	7.38
		10	99.68	104.53	106.90	103.70	5.39
		20	97.94	112.78	103.89	104.87	7.94
	5,7-DCHQ	5	95.99	106.60	118.80	107.13	8.40
		10	99.78	102.57	109.23	103.86	5.10
		20	98.56	107.15	102.02	102.58	5.12
	5-CHQG	5	112.92	93.87	105.73	104.17	13.24
		10	99.08	82.87	104.77	95.57	10.33
		20	78.44	93.37	101.65	91.15	12.59
	5,7-DCHQG	5	119.34	96.53	113.33	109.73	10.93
		10	98.98	79.30	104.93	94.40	11.84
		20	77.32	87.32	102.55	89.06	10.59

Table 5 (continued)

Sample	Compound	Spiking levels (μg kg^{-1})	Average recovery (%)				CV (%)
			Institution A[a]	Institution B[b]	Institution C[c]	Total	
Egg	5-CHQ	5	97.60	102.47	107.27	102.45	7.14
		10	106.12	98.80	113.07	106.00	4.75
		20	96.89	98.77	106.72	100.79	6.28
	5,7-DCHQ	5	104.60	106.73	109.33	106.89	2.52
		10	104.47	101.50	110.60	105.52	4.53
		20	105.98	98.15	108.95	104.36	4.62
	5-CHQG	5	90.37	103.60	116.93	103.63	21.32
		10	103.11	100.13	117.80	107.01	12.41
		20	101.77	99.90	109.39	103.69	10.43
	5,7-DCHQG	5	96.90	104.67	108.40	103.32	21.45
		10	99.98	98.80	113.60	104.13	9.35
		20	99.19	98.65	112.49	103.44	7.48
Milk	5-CHQ	5	91.30	103.40	106.47	100.39	8.07
		10	95.59	105.50	100.57	100.55	6.30
		20	98.57	101.17	94.90	98.21	5.33
	5,7-DCHQ	5	105.81	99.67	102.40	102.63	7.50
		10	106.06	103.10	95.50	101.55	8.33
		20	107.00	108.57	96.43	104.00	4.08
	5-CHQG	5	120.22	105.20	106.47	110.63	23.27
		10	90.45	100.93	100.57	97.32	20.97
		20	109.82	101.32	94.90	102.01	20.28
	5,7-DCHQG	5	88.65	98.13	91.40	92.73	20.60
		10	81.65	99.73	90.20	90.53	25.59
		20	102.48	100.42	92.62	98.51	19.65
Flat fish	5-CHQ	5	100.89	81.60	112.27	98.25	10.92
		10	101.96	97.63	108.33	102.64	4.76
		20	97.39	97.62	102.66	99.22	1.08
	5,7-DCHQ	5	85.34	97.47	86.67	89.83	9.77
		10	93.25	101.47	96.97	97.23	4.89
		20	103.92	99.85	108.41	104.06	6.46
	5-CHQG	5	119.36	78.00	85.20	94.19	21.54
		10	109.94	85.80	98.83	98.19	17.04
		20	112.14	94.98	106.99	104.70	13.50
	5,7-DCHQG	5	116.04	83.53	113.73	104.43	22.05
		10	110.29	98.67	104.23	104.40	9.83
		20	108.96	100.32	102.47	103.92	13.15

Table 5 (continued)

Sample	Compound	Spiking levels (μg kg^{-1})	Average recovery (%)				CV (%)
			Institution A[a]	Institution B[b]	Institution C[c]	Total	
Eel	5-CHQ	5	97.52	101.93	101.07	100.17	4.32
		10	94.20	109.47	110.70	104.79	8.50
		20	93.98	105.98	111.12	103.69	6.58
	5,7-DCHQ	5	100.82	106.47	117.20	108.16	4.95
		10	96.07	103.17	113.23	104.16	4.91
		20	95.04	105.18	109.93	103.38	6.47
	5-CHQG	5	93.42	100.20	114.93	102.85	20.69
		10	105.79	104.80	117.73	109.44	19.94
		20	112.34	103.12	109.61	108.88	15.49
	5,7-DCHQG	5	92.51	94.60	118.13	93.29	19.41
		10	104.93	100.40	113.20	103.23	18.94
		20	111.81	104.28	110.21	108.99	14.76
Shrimp	5-CHQ	5	102.17	94.73	103.00	99.38	6.86
		10	102.83	100.73	95.67	102.04	3.05
		20	105.69	104.65	98.09	105.30	4.24
	5,7-DCHQ	5	102.45	97.60	96.13	100.63	4.07
		10	107.33	105.00	93.57	106.46	5.50
		20	108.90	105.75	98.45	107.72	5.72
	5-CHQG	5	103.93	83.00	118.07	96.08	14.12
		10	80.15	93.90	107.60	85.31	14.61
		20	81.01	79.83	99.94	80.57	14.64
	5,7-DCHQG	5	96.94	85.53	108.00	92.66	8.70
		10	76.73	100.80	100.33	85.75	18.64
		20	75.59	87.95	98.98	80.23	13.97

[a] NIFDS; [b]SRFDA, [c]SGS

Results of validation of the analytical method

Method validation was conducted by confirming the linearity, accuracy, precision, LOD, and LOQ of the analysis results. Linearity was evaluated using the coefficient of determination (R^2), which exceeded 0.99 for all of the target compounds for every sample, thus satisfying CODEX guidelines. The accuracy was determined through the halquinol recovery values and ranged between 75.59 and 119.36%, while the precision was determined through the coefficients of variation (CV) and ranged between 1.39 and 28.66% therefore, the results for all the samples satisfied the CODEX guideline at all three concentrations. Table 3 shows the correlation coefficients, average recoveries, and CVs of all the samples at all three concentrations.

Matrix effect

The ME was classified into three categories including positive and negative values (21): no ME (ME < 20% or > − 20%); medium ME (20% to 50% or − 20% to − 50%); and strong ME (ME > 50% or < − 50%). The MEs showed diversity, with values ranging between − 77.70 to 88.33% by sample and compound (Table 4). Furthermore, the MEs of livestock product samples were confirmed to overall be stronger than the MEs of fishery product samples: four livestock product samples exhibited no MEs (16.7%), six exhibited medium MEs (25.0%), and fourteen exhibited strong MEs (58.3%), while six fishery product samples exhibited no MEs (50.0%), four exhibited medium MEs (33.3%), and two exhibited strong MEs (16.7%).

Inter-lab validation results of developed analytical method

In the inter-lab validation, the recoveries ranged between 80.23 and 113.39% and the CVs between 1.08 and 25.59% (Table 5); these results satisfied the CODEX guideline CAC/GL-71 2009. LOQs for all the target compounds were evaluated as 5 μg kg^{-1} for every sample except pork muscle, for which the LOQ was evaluated as 10 μg kg^{-1} (Table 6). The LOQs of the pork muscle sample were evaluated as less than half-value of the CODEX-recommended MRL

Table 6 LOQ of the developed analytical method

Compound	Sample	LOQ (mg kg^{-1})				
		Institution A[a]	Institution B[b]	Institution C[c]	Inter-lab	Final
Beef	5-CHQ	0.0040	0.0027	0.0041	0.0041	0.005
	5,7-DCHQ	0.0035	0.0013	0.0044	0.0044	
	5-CHQG	0.0050	0.0039	0.0048	0.0050	
	5,7-DCHQG	0.0033	0.0023	0.0043	0.0043	
Pork muscle	5-CHQ	0.0048	0.0089	0.0091	0.0091	0.01
	5,7-DCHQ	0.0078	0.0061	0.0080	0.0080	
	5-CHQG	0.0036	0.0046	0.0044	0.0046	
	5,7-DCHQG	0.0049	0.0091	0.0031	0.0091	
Pork fat	5-CHQ	0.0016	0.0035	0.0031	0.0035	0.005
	5,7-DCHQ	0.0046	0.0050	0.0038	0.0050	
	5-CHQG	0.0038	0.0047	0.0041	0.0047	
	5,7-DCHQG	0.0038	0.0022	0.0031	0.0038	
Chicken	5-CHQ	0.0046	0.0013	0.0011	0.0046	0.005
	5,7-DCHQ	0.0025	0.0010	0.0021	0.0025	
	5-CHQG	0.0035	0.0027	0.0022	0.0035	
	5,7-DCHQG	0.0046	0.0018	0.0031	0.0046	
Egg	5-CHQ	0.0048	0.0032	0.0043	0.0048	0.005
	5,7-DCHQ	0.0036	0.0039	0.0029	0.0039	
	5-CHQG	0.0033	0.0026	0.0030	0.0033	
	5,7-DCHQG	0.0047	0.0025	0.0039	0.0047	
Milk	5-CHQ	0.0050	0.0035	0.0034	0.0050	0.005
	5,7-DCHQ	0.0050	0.0017	0.0028	0.0050	
	5-CHQG	0.0036	0.0041	0.0032	0.0041	
	5,7-DCHQG	0.0050	0.0044	0.0049	0.0050	
Flat fish	5-CHQ	0.0033	0.0013	0.0031	0.0033	0.005
	5,7-DCHQ	0.0050	0.0041	0.0044	0.0050	
	5-CHQG	0.0037	0.0020	0.0031	0.0037	
	5,7-DCHQG	0.0050	0.0044	0.0031	0.0050	
Eel	5-CHQ	0.0029	0.0039	0.0038	0.0039	0.005
	5,7-DCHQ	0.0050	0.0045	0.0048	0.0050	
	5-CHQG	0.0032	0.0031	0.0019	0.0032	
	5,7-DCHQG	0.0046	0.0050	0.0044	0.0050	
Shrimp	5-CHQ	0.0033	0.0026	0.0029	0.0033	0.005
	5,7-DCHQ	0.0042	0.0044	0.0039	0.0044	
	5-CHQG	0.0033	0.0017	0.0011	0.0033	
	5,7-DCHQG	0.0039	0.0046	0.0041	0.0046	

[a] NIFDS; [b]SRFDA, [c]SGS

(40 μg kg^{-1}), while the LOQs for the other samples were evaluated as below the half-value of the MRL (10 μg kg^{-1}) for introducing the PLS system. Therefore, from these inter-lab validation results, the reproducibility and applicability of the developed analytical method were confirmed.

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Author contributions
THL and JYK conceived and designed the experiments. THL reviewed the literature and elucidated the compound structures. THL performed the LC–MS/MS measurements and wrote the manuscript. LTH, SYP and JYK analyzed the experimental data. JDC and MG provided guidance and supervised the study. All authors helped prepare the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests
The authors declare that they have no competing interests.

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