

Monitoring of Diquat and Paraquat in Livestock Products by LC-MS/MS Combined with Modified QuEChERS Methods

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Abstract: The continued growth in domestic meat consumption has prompted the development of pesticide analytical methods for quality control of livestock products. Among the pesticides managed by national safety programs, diquat and paraquat pose a significant challenge for conventional multi-residual analysis due to their strong polarity and high solubility in water. The objective of this study was to develop analytical methods for diquat and paraquat to monitor their residues in livestock products distributed in domestic markets. Five livestock products such as beef, pork, chicken, milk and eggs were selected as typical test samples. The analytical methods of diquat and paraquat were established by optimizing all the steps to meet with the criteria of CODEX guidelines. The optimized extraction methods were achieved by using the mixtures of water and acetonitrile containing 0.5% formic acid as the extraction solvent and acetonitrile containing 0.5% formic acid as the subsequent extraction solvent. C₁₈ dispersive solid-phase extraction was found to be effective for sample clean-up prior to LC-MS/MS analysis. The standard calibration curves of diquat and paraquat showed good linearity with the coefficients of determination (R^2) ranging from 0.9902 to 0.9986 for diquat and from 0.9968 to 0.9997 for paraquat. The established methods achieved recoveries ranging from 66.5 to 100.4% for diquat and ranging from 73.5 to 95.6% for paraquat. A total of 409 livestock samples from domestic markets were investigated for monitoring of diquat and paraquat by the established methods coupled with LC-MS/MS. Diquat and paraquat were found in the livestock samples at levels below the limit of quantitation (0.01 mg/kg). The analytical methods developed in this study could be successfully applied for monitoring diquat and paraquat in real livestock samples from domestic markets.

Keywords: Diquat, Livestock product, Paraquat, Pesticide, QuEChERS

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Introduction

The livestock industry is rapidly growing sector of agriculture due to factors such as population growth, increased livestock production and animal industrial technology. Since the 1980s, there has been a gradual increase in global demand for livestock products, and meat consumption is expected to rise until 2050 [1,2]. In South Korea, per capita meat consumption increased by 2.87% per year from 2000 to 2019. This trend is expected to continue in the future due to population

growth, rising incomes and increasing online demand [3]. As the consumption and distribution of livestock products continue to grow, it is important to ensure the safety of livestock products from potential risks.

Pesticides can accumulate in the bodies of animals through unpredictable routes such as ingestion of contaminated feed, drinking water and exposure to pesticides on farms. Pesticides in livestock products have the potential to enter the human body through the food chain [4,5]. To regulate pesticide residues in livestock products, the Korea Ministry of Food and Drug Safety (KMFDS) establishes maximum residue levels (MRLs) for pesticides that may be found in livestock products. The KMFDS issues official guidelines for pesticide analytical methods to manage residues in agricultural products. In addition, the Positive List System (PLS) has been implemented to ensure that chemicals such as pesticides and antibiotics for which MRLs have not been established are detected below 0.01 mg/kg in livestock products. However, unintentional exposure to chemicals and the growing demand for livestock products require the development of analytical methods to update existing technologies.

Diquat and paraquat are of nonselective bipyridylium herbicides that inhibit electron transfer system in the plant photosystem 1, generating large amounts of reactive oxygen species (ROS) to kill plant cells [6,7]. Diquat and paraquat have been widely used in over 90 countries due to their potent and rapid herbicidal effects. Considering the global demand for safe agricultural products, the human toxicity of diquat and paraquat has been the subject of extensive research. The exposure to paraquat has been known to result in significant toxicity to various body organs, including the lungs and skin, and diquat has been shown to exert potent cytotoxicity [8,9]. Consequently, the use of paraquat is presently banned in countries such as the European Union and China, and it has been prohibited from use and distribution in Korea as well, thus no related pesticides are currently registered. However, paraquat and diquat are strongly adsorbed to soil and are highly soluble in water, suggesting that they remain in the environment for a long time after use [10]. There is a possibility that the residues may be unexpectedly ingested by livestock or contaminated during distribution and persist in livestock products [11]. Consequently, Korea has established tolerance levels for diquat and paraquat in livestock products for monitoring pesticide residues in livestock products distributed within the country.

The analytical methods used for the detection of pesticide residues in agricultural commodities and foodstuffs are notified by the KMFDS through the MFDS Food Gazette. The analytical method can be classified into two categories: multi-residue test and single-residue test. The multi-residue test is an efficient method for simultaneous analysis of a number of pesticides simultaneously. However, its sensitivity, precision and reliability are limited due to its suboptimal analysis of individual components. Single-residue methods analyze individual components under optimal conditions, thereby ensuring greater sensitivity, precision and reliability. The KMFDS has recommended single-residue methods for the analysis of substances that are challenging to multi-residue analysis [12].

Diquat and paraquat are highly polar and have a high solubility in water (700 g/L and 620 g/L, respectively) compared to other pesticide components. As a result, multi-residue methods such as the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) approach, which are often used for the analysis of pesticide residues in foodstuffs, are not suitable for the analysis of diquat and paraquat [13]. Instead, they are typical challenging targets that require optimized single component methods [14,15]. Existing single residue analytical methods for diquat and paraquat are based on solid phase extraction and column methods, which are currently under development and in use. However, these methods are time-consuming due to the need for complex sample preparation, such as heating, are expensive depending on the method [16,17]. Therefore, there is a need to develop and newly update analytical methods for diquat and paraquat.

In this study, we optimized analytical methods for diquat and paraquat in livestock products using LC-MS/MS coupled with modified QuEChERS approaches and applied to monitor their residues in real livestock products from domestic markets. The proposed methods can be used to monitor livestock products in domestic distribution as a basis for livestock product safety.

Results and Discussion

Optimization of Sample Preparation Methods

The methods for the determination of diquat and paraquat in livestock products were optimized by fortifying diquat at the limit of quantitation (LOQ, 0.01 mg/kg) and 10 times the LOQ in the high fat beef and milk samples. A preliminary study was first carried out to investigate previously reported methods [17] that might be applicable to our study. The methods were time consuming (approximately 4 h per sample) as they used a stream of N₂ gas stream to remove organic solvents from the sample extracts. The HLB cartridge column failed to clean the high fat beef and milk samples, resulting in an ion ratio tolerance of more than $\pm 30\%$, which is within the acceptable range of the CODEX [18]. The recovery values ranged from 16.9% to 158.3% depending on the sample, which was not within the acceptable range of 60% to 120% of the CODEX and the KMFDS. In addition, the coefficient of determination of the linearity of the matrix-matched calibration curve with known methods was less than 0.99, depending on the sample. These suggest that the previously reported methods were not appropriate for our study. Therefore, the sample preparation and clean-up methods were developed by modifying the previous methods, omitting the N₂ gas stream drying step and using C₁₈ or PSA as a sorbent instead of HLB.

The methods developed in our study showed the recoveries ranging from 56.8% to 74.0% for high fat beef samples (Table 1). Double extraction improved recoveries more, from a minimum of 58.4% to a minimum of 59.5%. Acetonitrile with 0.5% formic acid was found to be better than 75% acetonitrile in 0.5% aqueous formic acid as a second extraction solvent for the sample precipitates, improving recovery from a minimum of 59.5% to 65.7%. For sample clean-up, C₁₈ was found to be more effective than PSA, with recoveries of 65.9% and 74.0% better than those of 56.8% and 66.1% for PSA. PSA has a strong affinity for polar substances present in the sample, allowing the adsorption and removal of polar substances including organic acids and polar pigments, while C₁₈ has the ability to adsorb and remove non-polar substances such as fats [19]. It was therefore suggested that the relatively low recovery with PSA compared to C₁₈ was due to the adsorption of diquat, which has a pronounced polar character, on PSA. C₁₈ is known to effectively remove non-polar interfering molecules, such as fats, present in livestock samples, resulting in a higher recovery than that observed in samples without an adsorbent or those treated with PSA [20,21].

Sample preparation methods were established as above and applied to milk samples as an additional test sample. The established methods for the milk sample spiked with diquat at the LOQ and 10 times the LOQ gave recoveries ranging from 67.1%

Table 1. Results of the method optimization study by recovery of diquat from fortified high-fat beef and milk samples

Chemicals	Samples	Extraction methods	Recovery (%) ^{a)}	
			Fortified levels (mg/kg)	
			0.01 (LOQ)	10 x LOQ
Diquat	Beef	1 ^{b)}	58.4 $\pm$ 3.2	58.4 $\pm$ 7.2
		2 ^{c)}	59.5 $\pm$ 1.4	65.0 $\pm$ 2.4
		3 ^{d)}	65.7 $\pm$ 7.9	70.5 $\pm$ 8.4
		4 ^{e)}	65.9 $\pm$ 4.5	74.0 $\pm$ 5.5
		5 ^{f)}	56.8 $\pm$ 2.6	66.1 $\pm$ 8.4
	Milk	3	67.8 $\pm$ 11.4	79.2 $\pm$ 15.5
		4	69.5 $\pm$ 2.1	93.8 $\pm$ 1.6
		5	67.1 $\pm$ 4.4	81.8 $\pm$ 5.0

^{a)}Data are means $\pm$ standard deviation of 5 replicates.

^{b)}Single extraction with 75% (v/v) acetonitrile in 0.5% (v/v) aqueous formic acid.

^{c)}Double extraction twice with 75% (v/v) acetonitrile in 0.5% (v/v) aqueous formic acid.

^{d)}Double extraction twice with 75% (v/v) acetonitrile and 100% acetonitrile in 0.5% (v/v) aqueous formic acid.

^{e)}C₁₈ SPE clean-up after double extraction d.

^{f)}PSA SPE clean-up after double extraction d.

to 93.8% (Table 1). Double extraction resulted in the recoveries of 67.8% at the LOQ level and 79.2% at the 10LOQ level. Much higher recoveries were observed for C₁₈ clean-up than for PSA clean-up, which is in good agreement with the results as above. Therefore, the established methods would be applicable to all livestock samples tested in this study. Thus, all livestock samples were prepared and purified as described above for the monitoring of diquat and paraquat.

Method Validation

Method validation for diquat and paraquat in livestock product samples was performed based on selectivity, linearity, matrix effect, and ion ratio tolerance, according to the CODEX [18] and the KMFDS guidelines. LC-MS/MS analysis detected diquat and paraquat at retention times of 2.26 and 0.57 minutes, respectively, with no interfering peaks on the chromatogram in control samples at these retention times (Fig. 1), indicating that the established methods have selectivity between samples.

Matrix-matched calibration curves showed that the coefficient of determination (R^2) of the regression equation ranged from 0.9902 to 0.9986 for diquat and from 0.9968 to 0.9997 for paraquat, demonstrating a good linearity with the coefficient greater than 0.99 (Table 2). Sample matrix effects on the calibration ranged from an average of -49.21 to 150.37% for diquat and from an average of -0.19 to 29.96% for paraquat, giving ion ratio tolerances from an average of -9.66 to 11.23% for diquat and from an average of -2.82 to 4.08% for paraquat within the acceptable range of $\pm 30\%$ (Table 3). These results confirm that the estab-

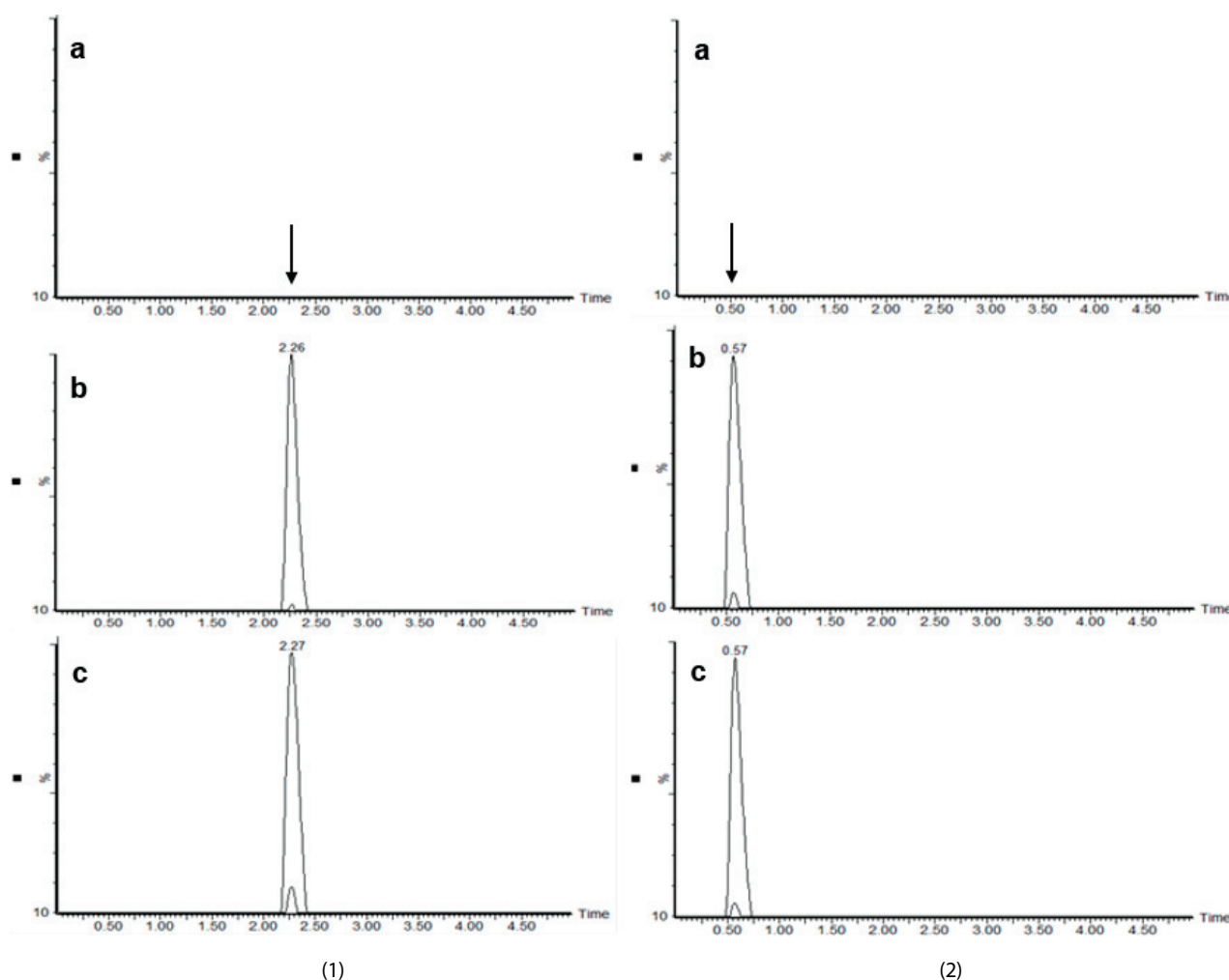


Fig. 1. Typical LC/MS/MS chromatograms of diquat (1) and paraquat (2) fortified in high fat beef samples at 0.01 mg/kg (LOQ): a. control samples, b. standard 0.01 mg/kg, c. samples fortified at 0.01 mg/kg. The arrow symbols represent the retention times of diquat and paraquat, respectively.

Table 2. Correlation factors of determination (R^2) of matrix-matched calibration curves of diquat and paraquat

Chemicals	R^2 in livestock sample matrices				
	Milk	Egg	Pork	Beef	Chicken
Diquat	0.9962	0.9986	0.9981	0.9979	0.9902
Paraquat	0.9983	0.9996	0.9968	0.9990	0.9997

Table 3. Sample matrix effects on the calibration linearity of diquat and paraquat

Chemicals	Sample matrix effects, % ^{a)} (Ion ratio tolerances, %) ^{b)}				
	Milk	Egg	Pork	Beef	Chicken
Diquat	-49.21 (-9.21)	83.70 (-5.88)	150.37 (11.23)	44.91 (-9.96)	82.15 (-7.70)
Paraquat	-90.19 (-2.28)	29.96 (4.08)	-27.79 (-2.82)	-67.64 (-2.66)	-56.66 (-2.82)

^{a)}[(Slope of linearity curve in matrix - slope of linearity curve in solvent only)/(slope of linearity curve in solvent only)] × 100%.

^{b)}Automatically calculated based on instrument sequence analysis.

lished methods comply with the required standard guidelines and are considered suitable for the analysis of diquat and paraquat in livestock products.

Accuracy, Precision and Reliability of Established Methods

The accuracy and precision of the established methods described above were verified using the recovery test criteria set out in the CODEX and the KMFDS guidelines in four levels of testing and five replicates. Each sample was subjected to a recovery test at 0.01 mg/kg (LOQ) and 5, 10, and 50 times the LOQ. The recovery values and percentages of coefficients of variation (% of CVs) for diquat were found to be 66.5~100.4% and 2.5~17.3%, respectively, while those for paraquat were 73.5~96.5% and 2.4~12.1%, respectively (Table 4). In particular, the % of CV in our study was much lower than that of previously reported methods [17], suggesting that the established methods were newly developed. These results were in accordance with the CODEX guideline [18], indicating that the method developed in our study is suitable for the determination of diquat and paraquat in livestock product samples.

For cross-validation by another laboratories to ensure the reliability of the methods, the established methods were also tested

Table 4. Recovery results of diquat and paraquat for the method accuracy and precision studies in the fortified livestock samples

Chemicals	Samples	Recovery (%) [*]			
		Fortified levels (mg/kg)			
		0.01 (% of CV)	0.05 (% of CV)	0.1 (% of CV)	0.5 (% of CV)
Diquat	Milk	73.5±5.2 (7.0)	82.6±8.1 (9.8)	98.0±8.4 (8.5)	80.7±10.2 (12.6)
	Egg	68.6±6.7 (9.7)	74.8±3.3 (4.4)	79.7±6.2 (7.8)	75.9±1.9 (2.5)
	Pork	70.8±12.2 (12.2)	80.3±5.6 (6.9)	77.0±5.3 (6.9)	73.8±4.1 (5.6)
	Beef	66.5±4.5 (6.8)	81.9±7.2 (8.7)	76.7±3.7 (4.8)	82.6±6.9 (8.3)
	Chicken	81.9±9.1 (11.1)	81.6±6.2 (7.6)	100.4±17.4 (17.3)	74.9±6.1 (6.1)
Paraquat	Milk	91.8±7.8 (8.4)	78.9±5.7 (7.3)	77.8±5.5 (7.0)	75.2±1.9 (2.5)
	Egg	73.5±4.8 (9.7)	79.3±6.3 (8.0)	75.7±1.9 (2.5)	78.2±2.6 (3.3)
	Pork	77.0±7.9 (10.2)	73.9±2.8 (3.8)	74.1±2.3 (3.1)	74.7±2.0 (2.6)
	Beef	87.7±6.4 (7.3)	96.5±5.3 (5.5)	84.7±4.6 (5.4)	92.0±3.3 (3.6)
	Chicken	80.6±9.7 (12.1)	86.8±10.2 (11.7)	77.1±1.9 (2.4)	77.4±3.0 (3.8)

^{*} Data are means±standard deviation of 5 replicates.

for inter- and intra-institutional reliability. The recovery values and % of CVs for diquat were found to be 75.0~103.8% and 1.2~11.2% in the intra-institutional test and 60.5~100.4% and 1.7~15.6% in the inter-institutional test, respectively (Table 5). These values and percentages for paraquat were 71.2~93.2% and 0.7~17.1% in the intra-institutional test and 73.5~92.0% and 1.7~8.7% in the inter-institutional test, respectively (Table 6). The developed methods met the above CODEX guidelines, and the cross-validation by other laboratories showed the method to be highly reliable. Thus, the methods developed in this study could be used institutionally for the determination of diquat and paraquat in livestock product samples.

Monitoring of Diquat and Paraquat in Livestock Products from Domestic Markets

As demonstrated above, the established methods were found to be newly developed methods applicable to the investigation of diquat and paraquat in real samples of livestock products. Thus, the developed methods were used to monitor the residue levels of diquat and paraquat in a total of 409 samples of seven types of livestock products (beef, pork, chicken, milk, eggs, mammal by-products and poultry by-products) distributed in domestic markets in Korea. All samples were prepared as described

Table 5. Intra-institutional recovery results of diquat and paraquat in the fortified livestock samples

Chemicals	Samples	Recovery (%)*		
		Fortified levels (mg/kg)		
		0.01 (% of CV)	0.1 (% of CV)	0.5 (% of CV)
Diquat	Milk	75.8±5.8 (7.7)	75.3±4.4 (5.8)	77.2±6.0 (7.8)
	Egg	89.1±4.1 (4.6)	82.5±7.3 (8.8)	96.1±6.3 (7.0)
	Pork	76.2±5.9 (7.8)	75.0±2.6 (3.4)	76.7±2.0 (2.6)
	Beef	78.7±8.8 (11.2)	77.5±1.7 (2.2)	79.0±2.0 (2.5)
	Chicken	103.8±2.1 (2.0)	75.2±0.9 (1.2)	75.4±3.9 (5.2)
Paraquat	Milk	71.2±1.4 (1.6)	76.1±3.0 (4.0)	71.2±1.4 (1.6)
	Egg	93.1±12.8 (13.8)	80.9±6.4 (7.9)	78.9±5.4 (6.9)
	Pork	79.7±13.6 (1.9)	86.6±1.6 (1.9)	91.67±6.7 (7.3)
	Beef	74.5±4.8 (6.5)	72.1±1.8 (2.5)	71.5±0.5 (0.7)
	Chicken	93.2±4.8 (5.1)	74.4±1.9 (2.1)	72.3±1.5 (2.1)

* Data are means±standard deviation of 5 replicates.

Table 6. Inter-institutional recovery results of diquat and paraquat in the fortified livestock samples

Chemicals	Samples	Recovery (%)*		
		Fortified levels (mg/kg)		
		0.01 (% of CV)	0.1 (% of CV)	0.5 (% of CV)
Diquat	Milk	73.5±3.4 (4.6)	98.0±7.4 (7.5)	80.7±7.3 (9.1)
	Egg	68.6±4.1 (6.0)	79.7±4.4 (5.5)	75.9±1.3 (1.7)
	Pork	70.8±5.5 (7.7)	77.0±3.7 (4.8)	73.8±2.7 (3.7)
	Beef	66.5±2.7 (4.0)	76.7±2.5 (3.3)	82.6±5.0 (6.1)
	Chicken	81.9±6.6 (8.1)	100.4±15.7 (15.6)	74.9±3.1 (4.1)
Paraquat	Milk	91.8±6.3 (6.9)	77.8±3.8 (4.9)	75.2±1.3 (1.7)
	Egg	73.5±3.2 (4.3)	75.7±1.3 (1.7)	78.2±1.8 (2.3)
	Pork	77.0±5.5 (7.1)	74.1±1.6 (2.1)	74.7±1.3 (1.8)
	Beef	87.8±5.0 (5.7)	84.7±3.5 (4.1)	92.0±2.7 (2.9)
	Chicken	80.6±7.0 (8.7)	77.1±1.3 (1.7)	77.4±2.0 (2.6)

* Data are means±standard deviation of 5 replicates.

Table 7. Monitoring results for residues of diquat and paraquat in the livestock samples from domestic markets

Chemicals	Residues (mg/kg)*				
	Milk	Egg	Pork	Beef	Chicken
Diquat	<0.01	<0.01	<0.01	<0.01	<0.01
Paraquat	<0.01	<0.01	<0.01	<0.01	<0.01

* Data are obtained from analyses in triplicate.

above and subjected to LC-MS/MS analysis. Diquat and paraquat were found to be less than 0.01 mg/kg in livestock products (Table 7), which is the residue limit level set by the Korea Ministry of Food and Drug Safety (KMFDS).

Pesticide residues in livestock products can come from agricultural by-products that are the raw materials for livestock feed. It is important to ensure the safety of pesticide residues in livestock products as considering that the consumption of meat and milk products is increasing with rising national incomes. In this study, the residues of diquat and paraquat were evaluated to investigate the safety of livestock products distributed in Korea. The residues of diquat and paraquat in livestock products were found to be below the limit of quantitation in all samples. Therefore, the results of the present study indirectly suggest that the residues of diquat and paraquat in livestock products distributed in Korea are managed at safe levels, and these results may help strengthen the competitiveness of Korean livestock products in domestic and international markets.

The safety assessment of pesticides in livestock products distributed to consumers can be used as a predictor of pesticide poisoning incidents in non-target livestock and wildlife [22,23]. Pesticide safety assessment in livestock products provides basic data for the risk assessment of target pesticides by correlating qualitative data on behavioral and psychological changes in animals due to pesticide poisoning with quantitative data on pesticide types and residue levels [24,25]. Monitoring of pesticides in livestock products can be used to predict the potential for pesticide contamination of livestock food products due to the use of pesticide ingredients permitted only for crops in livestock, such as the social issue of pesticide eggs that occurred in Korea in the past [26]. Thus, our findings may provide a basis for future periodic assessment of diquat and paraquat residues in livestock products distributed in Korea. The monitoring of pesticide residues in livestock products should be performed by performing multi-residue analysis for pesticides registered for use on crops [27,28]. In this study, the residues of diquat and paraquat in livestock products could not be analyzed simultaneously due to their physicochemical characteristics. In the future, it is necessary to develop an analytical method that can simultaneously analyze diquat and paraquat with other pesticides in order to more rapidly assess all pesticides in circulating livestock products.

Materials and Methods

Chemicals

The stock solution of diquat was purchased from Kemidas (Gunpo, Gyeonggi, Korea) at a concentration of 1,000 mg/L dissolved in acetonitrile, while the stock solution of paraquat was purchased from HPC Standards (Cunnersdorf, Germany) at a concentration of 1,000 mg/L dissolved in acetonitrile. All solvents used for sample preparation and instrumental analysis were of HPLC grade purchased from J.T. Baker (Pennsylvania, USA). Formic acid (purity 99%) was purchased from Sigma-Aldrich (St. Louis, USA).

Livestock Products

The CODEX classification of livestock products was taken into account for the development of analytical methods. Five representative livestock species, namely cattle, pigs, chickens, milk and eggs, were selected on the basis of the 'Guidelines for

Standard Procedures for the Development of Methods for Food and Other Products' of the KMFDS. The high-fat meat cuts (sirloin, pork belly, and chicken leg) were selected as typical meat samples to ensure the validity of the analysis of samples with high fat content.

Samples were purchased from domestic markets in 24 of the most populous cities in seven metropolitan cities and six provinces. The samples were cut into small pieces, frozen in a deep freezer and then mixed with dry ice. The mixed samples were placed in sample bags for analysis. For eggs, the shells were removed and mixed, and the milk was used immediately after purchase. Mammalian poultry by-products were collected including edible parts such as liver and proximal organs. A total of 409 samples were purchased nationwide, including 64 beef, 71 pork, 70 chicken, 70 milk, 70 eggs, 32 mammalian by-products and 32 poultry. The samples were then analyzed for diquat and paraquat using developed analytical methods.

Sample Preparation

Sample preparation methods for diquat and paraquat were investigated according to the CODEX and the KMFDS guidelines. Previously reported methods [17] were first tested in order to investigate methods suitable for our study. Diquat in high fat beef and milk were used as representative samples to optimize sample preparation methods.

To optimize the extraction method, 5.0 g of homogenized sample was weighed in triplicate into 50 ml conical centrifuge tubes and fortified with diquat at concentrations of 0.01 mg/kg and 0.1 mg/kg. The samples were added to 75% (v/v) acetonitrile in 0.5% (v/v) aqueous formic acid solution and extracted by vortexing for 2 min. The samples were then centrifuged at 4°C for 5 min at 3,500 rpm and frozen at -80°C for 5 min to separate supernatant and precipitates. The supernatant obtained in this way was transferred to new 50 mL conical centrifuge tubes. The precipitates were re-extracted with 9 mL of acetonitrile containing 0.5% formic acid or 9 mL of 75% (v/v) acetonitrile in 0.5% (v/v) aqueous formic acid solution, followed by extraction and centrifugation as described above. The resulting supernatant was transferred to the 50 mL conical centrifuge tube containing the first supernatant and subjected to sample clean-up. Sample clean-up was performed using dispersive solid phase extraction (dSPE) based on the QuEChERS method. PSA and C₁₈ were used as sorbents in dSPE, by adding 25 mg of PSA or 25 mg of C₁₈ to 1 mL of the final extracted aliquot obtained from above. The aliquot was mixed for 2 min and centrifuged at 8,000 rpm for 3 min. The resulting supernatant was filtered through a 0.2 µm PTFE-D syringe filter and used for LC-MS/MS.

Method Validation

The analytical methods for diquat and paraquat were validated on the basis of the calibration efficiency of their standard solutions, taking into account the linearity of the standard curve, coefficient of determination (R^2), the limit of quantitation, the recovery test, the ion ratio and the matrix effect.

The calibration curves of diquat and paraquat were obtained from their standard solutions prepared in solvent or sample matrix. For the calibration curves in solvent, diquat and paraquat were dissolved, respectively, in acetonitrile to prepare their 1,000 mg/kg stock solutions. Their working solutions were obtained by subsequent dilution of the stock solutions with acetonitrile to give 0.001, 0.002, 0.005, 0.01, 0.02, 0.05 mg/kg. For matrix-matched calibration curves, the stock solutions were subsequently diluted with acetonitrile containing control sample extracts to obtain the working solutions at the above concentrations. The limit of quantitation (LOQ) was calculated as follows: $\text{LOQ (mg/kg)} = [\text{minimum detectable amount (ng)/injection volume (}\mu\text{L)}] \times [\text{final sample volume (mL)/sample amount (g)}]$, taking into account the signal-to-noise ratio of 10 on the chromatogram analyzed by LC-MS/MS. The accuracy and precision of the methods in this study were evaluated using the recovery test criteria forth as outlined in the CODEX and the KMFDS guidelines (Table 8). Recovery tests for diquat and paraquat were performed in five replicates at 0.01 mg/kg (LOQ) and 5, 10, and 50 times the LOQ according to the KMFDS guidelines.

Table 8. CODEX recovery test criteria for accuracy and precision of the analytical methods for diquat and paraquat

Concentrations (µg/kg)	Recovery range (%)	% of coefficient variation (% of CV)	
		Intra-lab	Inter-lab
≤1	50~120	36	54
>1~≤10	60~120	32	46
>10~≤100	70~120	22	34
>100~≤1000	70~110	18	25
>1000	70~110	14	19

Table 9. Multiple reaction monitoring conditions of LC-MS/MS for the analysis of diquat and paraquat in livestock samples

Chemicals	Precursor ions (m/z)	Product ions (m/z)	
		Quantitative (CE, eV)*	Qualitative (CE, eV)*
Diquat	183.2	157.2(22)	130.1(38)
Paraquat	186.2	171.2(27)	77.2(40)

* Collision energy

Instrumentals

LC-MS/MS was a Waters model Xevo TQD-MS triple stage quadrupole mass spectrometer coupled with a SCHERZO SM-C₁₈ stainless column (50 × 2 mm, 2.7 µm). The mobile phase for diquat analysis consisted of water (A) with 0.5% formic acid and 50 mM ammonium formate and 95% acetonitrile (B) with 0.5% formic acid and 5.0 mM ammonium formate. The mobile phase was flowed at 0.5 mL/min as follows: 5% B at isocratic for 1.0 min, 90% B with linear gradient for 2.5 min, 70% B with linear gradient for 4.0 min and 70% B at isocratic for 4.1 min. The 70% B after the 90% B in the solvent gradient was used to wash the C₁₈ column before the next sample analysis. The mobile phase for paraquat analysis was a mixture of water (A) with 0.5% formic acid and 50 mM ammonium formate and 84% acetonitrile (B) with 0.5% formic acid and 50 mM ammonium formate at a flow rate of 0.4 mL/min as: 40% B at isocratic for 1.0 min, 90% B with linear gradient for 2.5 min and 90% B at isocratic for 4.0 min. The LC-MS/MS conditions were optimized as follows: desolvation N₂ flow 650 L/hr, cone gas flow 50 L/h, capillary voltage 2.7 Kv, ion source temperature 150°C and desolvation temperature 600°C. The positive ion mode of the electron spray ionization method was used for LC/MS/MS analysis. The multiple reaction monitoring conditions of LC-MS/MS for the analyses of diquat and paraquat are shown in Table 9.

Data Availability: All data are available in the main text or in the Supplementary Information.

Author Contributions: Lee SW and Kim JY: conducted the experiments, Kim SH: wrote original draft, Jo HW and Moon JK: investigation and data curation, Kim IS: designed, supervised, edited all draft, and financed the research.

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Additional Information:

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