



Target and non-target analytical method for potential hazardous substances in livestock and pet hair using liquid- and gas chromatography quadrupole time-of-flight mass spectrometry

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ABSTRACT

Extraction using acetonitrile and water and quadrupole time-of-flight mass spectrometry (LC and GC-QTOF/MS) techniques were used to screen for potential hazardous substances in livestock and pet hair. In addition, LC-MS/MS and GC-MS/MS techniques were used for verification of the analytical method and quantitative analysis of pesticides, veterinary drugs, mycotoxins and antioxidants in hair. Optimized sample preparation involves extracting 0.05 g of sample with 0.6 mL of ACN and 0.4 mL of distilled water. In addition, the two layers were separated by adding 0.1 g of NaCl. Then, both the ACN and water layers were analyzed by LC-TOF/MS, and the ACN layer was analyzed by GC-TOF/MS. Most of the matrix effects of livestock and pet hair were less than 50%, but some matrices and components showed high results, so matrix matching correction was applied for more precise quantification. Method validation was performed for 394 constituents (293 pesticides, 93 veterinary drugs, 6 mycotoxins and 2 preservatives) in dog, cat, cow and pig hair and chicken and duck feathers. All components showed good linearity ($r^2 \geq 0.98$) in the developed assay. The quantification limit of all compounds was set at 0.02 mg/kg, which is the lowest level that satisfies the recovery rate standard. The recovery experiment was repeated 8 times at 3 concentrations. Most of the components were extracted with the ACN layer, and the recovery rate was 63.35–119.98%. In order to confirm the efficiency of extracting harmful substances from actual samples, 30 hairs of livestock and pets were screened.

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1. Introduction

Hair analysis is used in human forensics and for the detection of prohibited drugs in sports as it is convenient, quick, and tamper-proof compared to conventional blood or urine. In addition, as hair continues to grow by ~1 cm every month, checking for long-term drug abuse using hair analysis is easier [1–3]. Drugs injected into the human body move from the circulatory system to the hair through passive transport processes and consequently transdermal secretions (sweat and sebum). The dosage, physicochemical properties, and pharmacokinetics of the drug affect the concentration accumulated in the hair [4–6]. Although human hair

has been widely analyzed for drug detection, studies on livestock or pet hair have not been performed thus far.

In South Korea, residual substances in livestock products are verified during production in eco-friendly and non-antibiotic-certified production farms. Unlike agricultural products, livestock products cannot be directly analyzed during the production stage. Hence, urine, feces, or blood tests are performed. However, collecting urine and feces is difficult, and the concentration of the corresponding drug may be reduced with the addition of other matrices to the sample. Moreover, the drug may be excreted in urine and feces within one day of administration. Hence, the drug might not even remain in the sample at the time of testing depending on its physicochemical properties. Hair is mainly composed of keratin protein, has a pH of ~6, and does not carry a net charge. Therefore, undissociated basic drugs might remain in the hair [1,6]. In addition, drugs are more likely to remain in hair because they are relatively non-polar than metabolites. However, metabolites will likely be excreted via urine in different concentrations [7]. And hair samples are easier to collect and administer than urine, feces,

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blood, and tissue, and drugs present in hair are more chemically stable to detect. Additionally, there is less matrix effect on the hair compared to other samples, resulting in simplified extraction and purification.

In recent years, the number of households raising pets has increased significantly. As pets are considered like family, interest in their health is also increasing. One of the ways to directly check the health of a pet is a hair test. This can identify hazardous substances that can be directly exposed to the body. However, in order to identify potential hazardous substances exposed to the pet's body through various routes, existing target analysis for specific substances has limitations. To solve this problem, this study utilized quadrupole time-of-flight mass spectrometry (QTOF-MS). Unlike conventional mass spectrometers, TOF-MS is capable of qualitative analysis of substances by using its own library or analysis equipment manufacturer's library. Accordingly, unlike conventional targeted analysis, non-targeted analysis that can identify a substance without having a standard substance is possible. In addition, although quantitative analysis is possible by itself, it has specifications of high resolution and fast ion scan speed, so it is widely used in monitoring research or investigating new hazardous substances. Based on these advantages, many studies using TOF-MS have been reported. In related research, TOF-MS has been used to analyze more than 2,500 illegal drugs and metabolites in urine and plasma, analysis of micro pollutants in marine organisms, and analysis of pharmaceuticals in water quality [8–13]. The analysis of these potential hazardous substances is also very important in terms of risk assessment. Recently, as mentioned above, the perception of pets as a family is increasing, and accordingly, many opinions on reexamination of the standards of hazardous substances previously set are presented, and interest and concern about the health of pets are also increasing. Therefore, it is thought that the analysis method and system construction that we are trying to approach can be an important stepping stone for conducting integrated risk assessment, which is a new direction of risk assessment. This is a method to predict the external exposure from the number of hazardous substances in animals by calculating the internal exposure through biomonitoring, not the deterministic or probabilistic exposure assessment method, which is the existing risk assessment method.

The use of this TOF-MS makes it possible to analyze various potential hazardous substances, but there are important parts that need to be reviewed beforehand. The detector itself can identify many substances, but there must be a way to extract such substances from the matrix we are targeting. Of course, using TOF-MS, we want to analyze even unspecified substances, rather than analyzing specific substances, but this is because it is impossible to extract all substances with one or two pretreatment methods. Therefore, it is important which materials we want to see in which samples and matrices. This study aims to analyze potential harmful substances for livestock and pets. The main route through which livestock and pets can be exposed to hazardous substances is eventually feed, and therefore it is very important to identify the characteristics of hazardous substances that may remain in feed. The range of feed is largely classified into ingredient feed, supplementary feed, and compound feed. Ingredient feed is a material of vegetable, animal, or mineral origin, which is used directly as feed or as a raw material for compound feed. Supplementary feeds consist of feed additives, preservatives, and flavoring agents. Compound feeds are classified from formulated feed for livestock such as dairy cows, pigs, and chickens to feeds for pets and aquatic animals, which are manufactured by mixing various ingredient feeds and supplementary feeds. As such, feed is exposed to a wide range of hazardous substances when it is composed of very diverse raw materials, and in the case of compound feed in particular, it can be seen that the possibility of exposure to hazardous substances is

higher because it is mixed with many raw materials [14]. Because of this, there is a possibility that various types of hazardous substances may be retained or formed in the feed, notably pesticide residues, veterinary drug residues, and mycotoxins. As a method for extracting such hazardous substances, QuEChERS analysis has been most commonly used [15–18]. This solid-liquid-liquid extraction method based on the partition ratio of the organic and water layers and a subsequent purification step using a small amount of sorbent such as C₁₈ and primary secondary amine (PSA) affords a small amount of purified sample. In addition, the extraction and purification efficiency for the target can be optimized by employing a buffer as the extraction solvent, which enables extraction by changing the polarity of the organic solvent and using various purification sorbents. However, the QuEChERS method is only efficient in extracting relatively non-polar substances as extraction requires a non-polar organic solvent layer. Therefore, to analyze polar residual pesticides, EURL developed a QuPPe analysis method based on methanol and buffer extraction [19,20]. However, analyzing highly polar substances or water-soluble compounds even via QuPPe analysis remains challenging. Different classes of veterinary drugs have different physicochemical properties and require different pretreatment methods. Some ingredients have stronger polarity than those of residual pesticides, which require methanol and distilled water for extraction and subsequent TOF-MS non-targeted analysis. In contrast, extraction of non-polar substances and effective resolution of the substances via column purification is challenging. This indicates that the simultaneous extraction of compounds of different classes and characteristics is difficult.

Therefore, in this study, a method for extracting hydrophobic and hydrophilic substances such as residual pesticides, residual veterinary drugs, mycotoxins, and preservatives contained in livestock and pet hair was developed. The organic and water layers were separated using NaCl, and each layer was analyzed by LC- and GC-TOF/MS. Method verification was performed for 394 hazardous compounds LC- and GC-MS/MS. We combined our extraction method with MS/MS to evaluate the quantitative analytical performance and with TOF-MS to evaluate the non-targeted analysis.

2. Materials and methods

2.1. Chemicals and reagents

Standard materials were used as products of Sigma Aldrich (St. Louis, MO, USA) or Cfm Oskar Tropitzsch GmbH (Marktredwitz, Germany). Acetonitrile (ACN) from Merck (Darmstadt, Germany) was purchased, and purified water was obtained through a Milli-Q system (Millipore, Bedford, MA, USA). Sorbitol, sodium chloride, D-(+)-gluconic acid- δ -lactone, ammonium formate (HCOONH₄) and 3-ethoxy-1,2-propanediol were purchased from Sigma-Aldrich (St. Louis, MO, USA). did Shikimic acid and formic acid (HCOOH) were from Fisher Scientific (Pittsburgh, PA, USA). Analyte protecting agents (APs) are used to reduce analyte degradation and adsorption in GC-MS/MS systems. AP mixture preparation method was written in supplementary material.

2.2. LC-MS/MS and GC-MS/MS conditions

The LC-MS/MS instrument was a QTRAP 5500 triple-quadrupole mass spectrometer (AB SCIEX, Concord, Canada) equipped with a Nanospace SI-2 liquid chromatography system (Osaka soda Co., Ltd., Tokyo, Japan). An Imtakt Unison UK-C18 UP column (150 mm $\times$ 3.0 mm, 3.0 μ m) was used for chromatographic separations. The mobile phase was (A) water containing 0.1% HCOOH and 5 mM HCOONH₄; (B) Methanol containing 5 mM HCOONH₄ and 0.1% HCOOH was used. Detailed gradient conditions are described in the

supplementary material. Ion mode was performed using electrospray ionization in positive ion mode at 4,500 V or negative ion mode at -4,500 V. The temperature was set at 450°C and the curtain gas pressure at 30 psi. Multiple reaction monitoring (MRM) was used for quantification of each hazardous substance [16,21]. Details have been written in the supplementary material.

GC-MS/MS instrumentation was performed on a 7000D triple-quadrupole mass spectrometer (Agilent, Santa Clara, USA) equipped with a 7890B gas chromatograph. A DB-5MS capillary column (30 m × 0.25 mm, 0.25 μm) was used. The temperature of the injector was 250°C. The flow rate of helium was set to 1.5 mL/min. Oven conditions were as follows: initial temperature 90°C (held for 3 min), 20°C/min to 120°C, and 8°C/min to 300°C (held for 3 min). The MRM conditions of each material are attached to the supplementary data.

2.3. LC-TOF/MS and GC-TOF/MS conditions

LC-TOF/MS instrumentation was performed on a triple TOF 6600 (AB SCIEX, Concord, Canada) equipped with a Nexera X2 liquid chromatography system (Shimadzu, Kyoto, Japan). Column, mobile phase composition, and gradient conditions are the same as those for the mass spectrometer above. Electrospray ionization mode was used and analyzed in positive ion mode at 5,500V or negative ion mode at -5,500V. The temperature was set to 600°C and the curtain gas pressure was set to 25 psi. GC-TOF/MS instrumentation was performed on a 7890B Gas Chromatograph equipped with GC x GC-TOF-MS (LECO, St. Joseph, MI, USA). An HP-5MS 5% phenyl methyl silox capillary column (30 m × 0.25 mm, 0.25 μm) was used. The temperature of the injector was set to 250°C and the helium flow rate was set to 1.5 mL/min. Oven conditions are the same as mass spectrometer conditions above. Details are provided in the supplementary material.

2.4. Sample preparation

Homogenized hair samples (powder, 0.05 g) were weighed in a 2 mL centrifuge tube, and 0.4 mL of distilled water and 0.6 mL of ACN were added. It was then shaken vigorously for 1 minute. NaCl (0.1 g) was added to the centrifuge tube above and shaken for 1 minute on a shaker. Then, ACN and water layer were separated by centrifugation at 13,000 G for 10 minutes. The ACN and water layers were each filtered through a 0.2 μm membrane filter and analyzed by LC-MS/MS and LC-TOF/MS analysis, respectively [22]. GC-TOF/MS and GC-MS/MS analysis were performed after adding AP to the ACN layer above. The overall sample preparation flow chart is shown in Fig. 1.

2.5. Method validation

Method validation was performed to confirm the extraction efficiency and evaluate the compatibility of our extraction method with MS for quantitative analysis of the hazardous substances in livestock and pet hair. In order to validate the method, we followed the SANTE guidelines [23] and assessed various parameters including linearity, limit of quantitation (LOQ), accuracy, precision, and matrix effect. Linearity was evaluated by the coefficient of determination (r^2). The LOQ of the analytical method was determined as the lowest spike level that met the verification criteria as a result of the recovery test. After adding three concentrations of pesticides, veterinary drugs, and mycotoxins to the blank sample, the recovery rate was measured and the relative standard deviation (RSD) was calculated to evaluate the accuracy. For each analyte, the slope of the calibration curve for the pure solvent and the slope of the matrix-matched calibration curve were calculated, and then

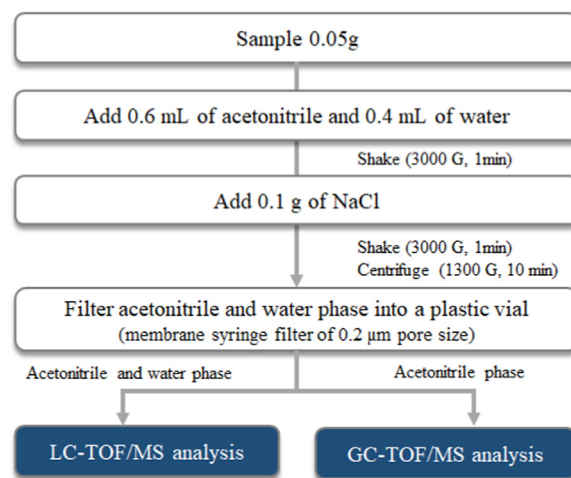


Fig. 1. Flow chart of the sample preparation of livestock and pet hair for LC-TOF/MS and GC-TOF/MS analysis.

the matrix effect was evaluated using the equation:

$$\text{Matrix effect (\%)} = 100 \left(\frac{\text{Slope of calibration curve in matrix}}{\text{Slope of calibration curve in solvent}} - 1 \right).$$

3. Results and discussion

3.1. Optimization of instrument conditions

To quantify a total of 394 hazardous compounds, including 293 pesticides, 93 veterinary drugs, 6 mycotoxins, and 2 preservatives, we utilized the MRM mode. MRM offers highly selective quantification of analytes within complicated mixtures [16,21]. We compared the ionization efficiency of each target compound in positive and negative modes for optimal MRM conditions. We selected the precursor and product ions based on the ones demonstrating the highest sensitivity, and the collision energy (CE) value providing the best sensitivity was employed. GC-MS/MS MRM conditions performed a full scan of target compounds and selected precursor ions. For optimization of the precursor ion conditions, an appropriate CE value was set and a scan was performed for the product ion. These precursor ions were identified using the recommended method by the Ministry of Food and Drug Safety (MFDS) for residual pesticide and veterinary drug analysis, as well as the standard analytical method for feed suggested by the Ministry of Agriculture, Food and Rural Affairs [24,25]. To ensure suitable separation of target and non-target analytes in both LC and GC-TOF-MS analysis, we set the time period to 30 min [16,21].

The LC-TOF-MS spectra were acquired in full scan mode and data-dependent MS/MS (ddMS2) mode. In full scan mode, the parameters were set as follows: resolution of 70,000, scan range of 50–1000, and maximum injection time of 100 ms. The resolution in ddMS2 mode is 30,000 and the maximum injection time is 100 ms. In the ddMS2 mode, spectra were acquired for the three most intense peaks per cycle. The data acquisition was performed using Sciex OS 4.1 software, which was equipped with the US National Institutes of Standards and Technology (NIST) MS database (NIST MS Search 2.0, NIST/EPA/NIH Mass Spectral Library; NIST 2002) for data processing and peak matching. For GC-TOF-MS, the detector voltage was set to 1,600 V, and the filament bias was -70 V. And the EI spectrum was collected from 50 to 550 m/z at a speed of 60 spectra/s. The data processing was performed using Leco ChromaTOF software version 3.25 equipped with the US NIST MS database.

Table 1
Optimization of the extraction process to obtain an equal volume of ACN and water layers (n=5).

% of ACN	Ammonium formate						Sodium chloride					
	1 g		2 g		5 g		1 g		2 g		5 g	
	ACN (μL)	Water (μL)	ACN (μL)	Water (μL)	ACN (μL)	Water (μL)	ACN (μL)	Water (μL)	ACN (μL)	Water (μL)	ACN (μL)	Water (μL)
50	3533	5667	3250	5950	3500	5700	4267	5033	4333	4967	4067	5200
60	4667	4600	4500	4800	4500	4800	4733	4600	4800	4567	4767	4600
70	6233	3333	6000	3500	6300	3250	6500	3067	6467	3133	6533	3100
80	7300	2333	7500	2200	7500	2200	7533	2167	7600	0	7533	0

3.2. Optimizing sample preparation

We developed a rapid and eco-friendly analysis method to extract various non-polar and polar hazardous substances in hair by minimizing the use of organic solvents and reagents. As ACN and water are generally used for extraction, we optimized them using HCOONH₄ and NaCl to properly separate the upper (ACN) and lower (water) layers. The optimization of the separation of the ACN and water layers is shown in Table 1.

3.2.1. Optimization of the separation of ACN and water layers

As the volumes of the ACN and Water layers affect the estimated concentration of the substance, they must be accurately measured. The effectiveness of HCOONH₄ and NaCl in separating the ACN and water layers was compared. In this case, the volumes of the separated non-polar (ACN) and polar (water) layers may be different from those before separation based on the separation reagent used [22,26–28]. Therefore, the ratio of the upper and lower layers was determined based on the added separation reagent.

For accurate quantification, it is very important that equal volumes of the ACN and water layers are obtained. 10 mL of 50, 60, 70, and 80% ACN was added to 1 g, 2 g, and 5 g of HCOONH₄ and NaCl, and the volumes recovered from the upper and lower layers were measured using a 100 μL micropipette. We observed that the volumes of each layer for both separation reagents were not significantly different, and the separation of the layers was apparent to the naked eye. However, the volume of the recovered upper and lower layers varied considerably depending on the ACN:water ratio. When 60% ACN was added, both the layers had similar volumes; 1 mL of 60% ACN containing a mixture of standards was added to 0.05 g of a cow hair sample, and 0.1 g, 0.2 g, and 0.5 g of HCOONH₄ and NaCl were added separately to prepare various samples. The volumes recovered from the upper and lower layers were measured. A thick emulsion was formed in the sample containing HCOONH₄, indicating the absence of a clear separation of layers. In addition, the volume ratio of the ACN and water layers was not constant. In contrast, a clear separation of layers was observed when 0.1 g of NaCl was used and the layers were recovered in consistent volumes. Therefore, 0.05 g of hair, 0.1 g of NaCl, and 1 mL of 60% ACN were selected as the optimal sample preparation conditions.

3.2.2. Selection of the extraction solvent

Extraction depends on the physicochemical properties of the target compounds and the extraction solvents. The pH of the solvent affects the extraction efficiency [16,21]. The prerequisite of this study is to identify widely applicable extraction conditions. Therefore, two sets of extraction conditions were designed to evaluate the effect of pH on the recovery of target compounds. In our previous study, pesticides and veterinary drugs were confirmed to be stable at solvent pH 4–5 [16,21]. We used two sets of conditions, (1) 0.01% HCOOH in 60% ACN and (2) 60% ACN, to optimize the validation of target compounds in a blank cow hair sample. An extraction efficiency test was performed for 313 compounds using

LC-MS/MS. The extraction efficiency did not change in the presence or absence of 0.01% HCOOH. Moreover, a 10-fold dilution and the consequent pH change did not affect extraction efficiency. Therefore, 60% ACN was selected as the extraction solvent for method validation.

3.3. Method validation

The linearity, LOQ, accuracy, precision and matrix effect of the method were investigated and the results are listed in Table 2.

3.3.1. Selectivity and linearity

MRM conditions were established for 394 compounds (293 pesticides, 93 veterinary drugs, 6 mycotoxins and 2 preservatives). Seven concentrations ranging from 1 to 100 μg/L were used to evaluate linearity in GC and LC-MS/MS analysis. The calibration curves for most substances exhibited excellent linearity with an r^2 value of ≥ 0.98 .

3.3.2. LOQ

The quantification limit of the 394 hazardous compounds was selected as the lowest concentration that satisfies the recovery rate according to the SANTE guidelines. This method of calculating the limit of quantification is commonly used for MS/MS analysis [29–31]. As a results, the LOQ value for the 394 hazardous compounds was found to be 0.02 mg/kg.

3.3.3. Matrix effects

The matrix effect arises from competition between matrix compounds eluted with compounds formed at the interface of the mass spectrometer. This competition can lead to either negative or positive influences on the responses. A matrix effect value of 0% indicates the absence of any matrix effect. In general, a matrix effect of lower than ± 50 is considered negligible. In all hair samples except cat hair, about 80% of the total compounds had low or negligible matrix effects. Out of a total of 394 compounds, in chicken and duck feather and cow, pig, and dog hair, 309, 333, 351, 332 and 322 compounds, respectively, showed weak substrate effects of lower than $\pm 50\%$ (Fig. 2). However, in cat hair, about 70% of the total compounds showed a strong matrix effect of more than $\pm 50\%$. All of the tested matrices showed a strong matrix effect on the detection of chloramphenicol, diazinon, diclazuril, florfenicol, kitasamycin, maduramycin, sarafloxacin, and, toltrazuril. Additionally, except in cow hair, a strong matrix effect was observed on chlorsulfuron, cynazine, ethaboxam, forchlorfenuron, halosulfuron-methyl, imazosulfuron, inabenfide, metazosulfuron, penoxsulam, piperonyl butoxide, sulfamonomethoxine, and uniconazole, ranging from - 76.16 to 208.61% in chicken and duck feathers and pig, dog, and cat hair. Therefore, we used matrix matched calibration to accurately quantify all the compounds. For those cases where we confirmed maximum residue limit (MRL) that exceeded or was comparable to the result, we applied the standard material addition method for more precise analysis. Linearity and matrix effects of matrix matched calibration curve are described in the supplementary material.

Table 2

Summary of LOQ, linearity, matrix effect, recovery and RSD for each class of hazardous substances obtained from the upper layer and lower layer.

Type	Class	No.	LOQ (mg/kg)	r^2	Matrix effect (%)	Recovery (%)	RSD (%)
Upper layer (ACN)							
Mycotoxin	Aspergillus	4	0.02	0.9811 ~ 0.9998	-27.26 ~ 169.32	65.35 ~ 116.30	2.25 ~ 16.77
	Fusariums	2	0.02	0.9808 ~ 0.9984	-52.01 ~ 112.33	90.57 ~ 108/53	5.88 ~ 18.86
Pesticide	Benzimidazole	3	0.02	0.9825 ~ 0.9998	-78.22 ~ 161.02	65.22 ~ 108.54	3.11 ~ 17.37
	Benzoylurea	5	0.02	0.9836 ~ 0.9997	-72.77 ~ 222.14	64.06 ~ 114.21	3.48 ~ 18.40
	Carbamate	24	0.02	0.9802 ~ 0.9998	-45.58 ~ 218.99	64.97 ~ 117.43	1.91 ~ 19.11
	Heterocyclic	3	0.02	0.9898 ~ 0.9994	-38.72 ~ 141.67	66.84 ~ 117.94	4.13 ~ 19.92
	Imidazole	2	0.02	0.9858 ~ 0.9984	-34.51 ~ 131.10	66.51 ~ 110.78	3.87 ~ 15.58
	Organobromous	5	0.02	0.9856 ~ 0.9999	-45.22 ~ 220.06	65.30 ~ 112.07	1.60 ~ 17.25
	Organochlorine	43	0.02	0.9800 ~ 0.9999	-76.16 ~ 208.46	64.45 ~ 119.63	1.25 ~ 19.95
	Organofluorous	23	0.02	0.9804 ~ 0.9999	-68.64 ~ 215.03	65.37 ~ 119.98	1.47 ~ 19.62
	Organophosphorous	52	0.02	0.9804 ~ 0.9999	-90.15 ~ 205.95	63.80 ~ 119.96	1.07 ~ 19.89
	Pyrethroids	2	0.02	0.9935 ~ 0.9990	-33.10 ~ 140.02	67.88 ~ 108.57	2.63 ~ 19.27
	Spinosad	5	0.02	0.9825 ~ 0.9994	-41.40 ~ 154.40	66.03 ~ 117.26	4.28 ~ 19.99
	Sulfonamide	2	0.02	0.9821 ~ 0.9986	-42.24 ~ 87.92	63.85 ~ 106.78	4.58 ~ 17.53
	Triazine	7	0.02	0.9810 ~ 0.9999	-72.55 ~ 224.44	65.60 ~ 119.62	3.09 ~ 19.82
	Triazole	20	0.02	0.9809 ~ 0.9999	-71.82 ~ 148.25	65.32 ~ 117.74	1.73 ~ 19.62
	Other	97	0.02	0.9802 ~ 0.9998	-75.78 ~ 224.38	63.68 ~ 119.90	1.38 ~ 19.96
Preservative	BHA, BHT	2	0.02	0.9871 ~ 0.9994	-27.66 ~ 8.53	75.37 ~ 115.98	3.38 ~ 19.72
Veterinary drug	Aminocoumarine	1	0.02	0.9912 ~ 1.9992	-7.82 ~ 145.69	81.87 ~ 110.72	6.74 ~ 17.55
	Amphenicol	1	0.02	0.9822 ~ 0.9991	15.58 ~ 161.39	81.84 ~ 105.28	2.30 ~ 17.82
	Anabolic steroid	1	0.02	0.9821 ~ 0.9986	-30.53 ~ 157.58	70.84 ~ 107.98	5.94 ~ 18.91
	Anticholinergic	1	0.02	0.9859 ~ 0.9992	-6.17 ~ 169.30	66.99 ~ 110.46	2.14 ~ 18.86
	Avermectin	3	0.02	0.9845 ~ 0.9990	-34.34 ~ 207.23	71.81 ~ 113.03	3.46 ~ 19.42
	Beta-agonist	2	0.02	0.9887 ~ 0.9992	4.37 ~ 204.29	65.78 ~ 107.06	5.66 ~ 19.92
	Cocciostat	3	0.02	0.9856 ~ 0.9994	-98.56 ~ 175.76	70.14 ~ 110.37	2.99 ~ 17.35
	Diaminopyrimidine	1	0.02	0.9810 ~ 0.9980	0.91 ~ 177.47	67.28 ~ 109.63	5.12 ~ 17.03
	Fluoroquinolone	2	0.02	0.9917 ~ 0.9996	27.07 ~ 168.23	65.29 ~ 112.43	1.27 ~ 16.47
	Imidazole	11	0.02	0.9816 ~ 0.9997	-96.00 ~ 200.17	64.84 ~ 117.61	2.41 ~ 17.99
	Lincosamide	1	0.02	0.9860 ~ 0.9997	-52.43 ~ 182.12	82.95 ~ 111.61	3.50 ~ 19.49
	Macrolide	6	0.02	0.9817 ~ 0.9991	-32.51 ~ 291.18	64.59 ~ 113.30	3.47 ~ 19.66
	Nitrofurantoin	1	0.02	0.9808 ~ 0.9980	-67.35 ~ 161.55	70.62 ~ 113.77	6.31 ~ 16.95
	Nitroimidazole	2	0.02	0.9861 ~ 0.9987	-37.77 ~ 168.02	63.35 ~ 106.22	3.11 ~ 17.63
	Phenicol	2	0.02	0.9841 ~ 0.9999	-89.41 ~ 202.39	69.40 ~ 109.52	5.70 ~ 18.16
	Pleuromutilin	2	0.02	0.9803 ~ 0.9998	-60.77 ~ 209.21	67.88 ~ 111.56	4.06 ~ 18.45
	Polypeptide	1	0.02	0.9872 ~ 0.9991	45.34 ~ 181.71	83.20 ~ 111.07	3.16 ~ 16.50
	Quinolone	8	0.02	0.9819 ~ 0.9997	-84.96 ~ 195.41	26.26 ~ 113.45	2.51 ~ 19.90
	Sulfonamide	23	0.02	0.9801 ~ 0.9999	-83.73 ~ 213.51	65.60 ~ 111.64	2.37 ~ 19.97
	Tricyclic	1	0.02	0.9949 ~ 0.9991	-6.45 ~ 152.17	66.80 ~ 104.49	5.34 ~ 16.63
Lower layer (Water)							
Additive	Melamine	1	0.02	0.9819 ~ 0.9999	-78.86 ~ -11.2.	77.32 ~ 92.64	2.55 ~ 15.42
Mycotoxin	Ochratoxin A	1	0.02	0.9872 ~ 0.9989	-87.31 ~ -24.72	75.92 ~ 92.88	3.18 ~ 10.26
Pesticide	Propamocarb	1	0.02	0.9867 ~ 0.9993	-74.26 ~ 25.87	30.81 ~ 38.56	3.52 ~ 19.45
	Quinmerac	1	0.02	0.9822 ~ 0.9990	-85.11 ~ -11.38	27.96 ~ 38.12	4.17 ~ 19.71
Veterinary drug	Clopidol	1	0.02	0.9850 ~ 0.9985	-84.17 ~ -32.49	29.52 ~ 37.42	6.87 ~ 19.16
	Danofloxacin	1	0.02	0.9869 ~ 0.9989	-82.08 ~ -20.48	29.53 ~ 38.32	4.00 ~ 17.02
	Difloxacin	1	0.02	0.9808 ~ 0.9999	13.74 ~ 175.69	70.75 ~ 111.40	5.09 ~ 17.45
	Enrofloxacin	1	0.02	0.9819 ~ 0.9948	-82.10 ~ -14.04	28.57 ~ 39.18	3.68 ~ 19.9
	Marbofloxacin	1	0.02	0.9835 ~ 0.9997	-84.57 ~ -14.52	26.26 ~ 39.94	4.08 ~ 19.40
	Nalidixic acid	1	0.02	0.9868 ~ 0.9990	-87.76 ~ -17.11	22.68 ~ 40.45	3.86 ~ 18.45
	Nicosulfuron	1	0.02	0.9866 ~ 0.9978	-83.73 ~ -9.61	29.41 ~ 40.63	4.32 ~ 19.56
	Ofloxacin	1	0.02	0.9868 ~ 0.9972	-84.96 ~ -14.80	29.25 ~ 39.78	4.40 ~ 18.81
	Orbifloxacin	1	0.02	0.9911 ~ 0.9996	-84.16 ~ -19.94	29.86 ~ 39.11	4.18 ~ 18.65
	Pefloxacin	1	0.02	0.9518 ~ 0.9990	-86.52 ~ -10.95	24.50 ~ 38.37	6.16 ~ 19.25
	Sarafloxacin	1	0.02	0.9867 ~ 0.9974	-89.52 ~ -42.71	19.98 ~ 40.30	4.19 ~ 18.99
	Sulfacetamide	1	0.02	0.9806 ~ 0.9998	-87.20 ~ -20.64	27.40 ~ 37.52	3.93 ~ 19.22
	Sulfaguanidine	1	0.02	0.9831 ~ 0.9949	-85.34 ~ -16.45	28.93 ~ 40.56	7.11 ~ 19.67
	Sulfamethoxazole	1	0.02	0.9801 ~ 0.9987	-81.68 ~ -8.13	24.62 ~ 40.44	2.50 ~ 17.96
	Sulfamonomethoxine	1	0.02	0.9870 ~ 0.9993	-84.83 ~ -3.78	24.06 ~ 38.38	4.85 ~ 18.14
	Sulfasomidine	1	0.02	0.9829 ~ 0.9952	-86.00 ~ -17.70	20.66 ~ 39.64	3.72 ~ 19.92
	Sulfisoxazole	1	0.02	0.9823 ~ 0.9981	-83.72 ~ -14.74	24.73 ~ 37.21	5.16 ~ 19.49

3.3.4. Accuracy and precision

Accuracy and precision were evaluated by measuring the recovery rate and RSD according to the SANTE guidelines [23]. The recovery rate was performed at the LOQ, LOQ 2-fold level, and LOQ 5-fold concentration level for each target substance in the hair and feathers of 6 species, including dogs, cats, cows, pigs, chick-

ens, and ducks. And the experiment was repeated 8 times. Accuracy and precision were confirmed for both the ACN and Water layers. A total of 21 compounds were detected in the water layer. Among them, only 10 compounds (clopidol, danofloxacin, enrofloxacin, marbofloxacin, melamine, nicosulfuron, ochratoxin A, ofloxacin, orbifloxacin, and quinmerac) were recovered from the

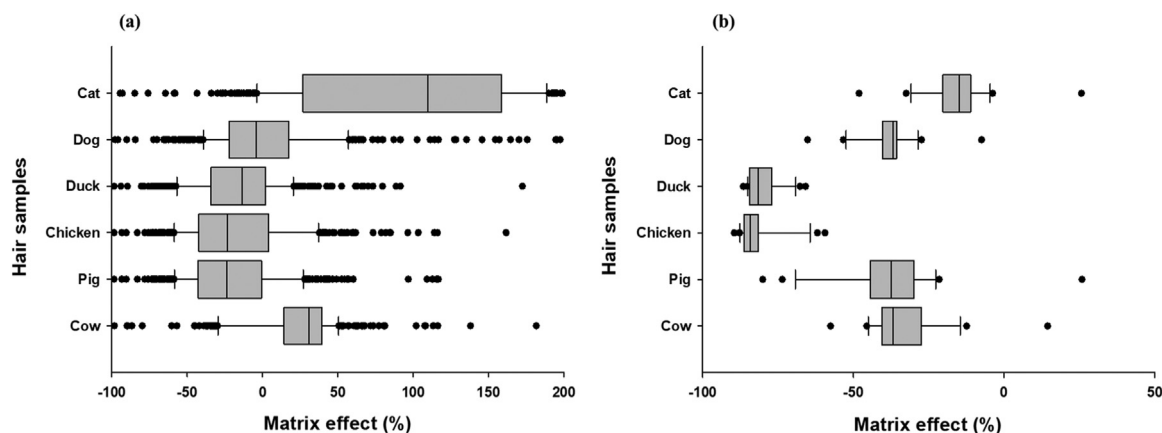


Fig. 2. Matrix effects of 394 hazardous compounds at (a) ACN and (b) water layer in hair samples.

Table 3

ToF/MS results of the hazardous compounds identified at ACN layer in 30 hair samples.

Instrument	Sample name	Resolution	Compound name	Library score	Formula
GC-ToF/MS	Dog 4	1,000	Tramadol	91.6	$C_{16}H_{25}NO_2$
	Dog 6		Aspirin	92.7	$C_9H_8O_4$
	Cow 1		Bifenthrin	88.0	$C_{23}H_{22}ClF_3O_2$
LC-ToF/MS	Cat 8	15,000	Icaridin	96.8	$C_{12}H_{23}NO_3$
	Dog 4	15,000	Tramadol	100.0	$C_{16}H_{25}NO_2$
	Dog 10	17,500	Climbazole	97.3	$C_{15}H_{17}ClN_2O_2$
	Dog 10	25,000	Praziquantel	91.8	$C_{19}H_{24}N_2O_2$
	Duck 3	30,000	Tiamulin	99.2	$C_{28}H_{47}NO_4S$

water layer. Also, 11 compounds (difloxacin, nalidixic acid, pefloxacin, propamprcarb, sarafloxacin, sulfacetamide, sulfaguanidine, sulfamethoxazole, sulfamonomethoxine, sulfasomidine, and sulfoxazole) were identified in both the water layer and the ACN layer. Most of the compounds showed recoveries ranging from 70% to 120% in all matrices. Looking at the results of some components, diethylcarbamazine showed rather low recovery results in most matrices. In particular, those that had the lowest recovery in pig hair (74.40%), but satisfied the >70% level specified in MFDS guidelines. Additionally, low average recoveries (< 80%) were observed for isometamidium and metronidazole-OH in pig hair. By contrast, fenothiocarb showed high recoveries in most matrices, and the values did not exceed the SANTE guideline level of 120% [23]. However, the average fenothiocarb recovery was lower in cor hair (91.33%) compared with other matrices. In addition, a high average recovery (>110%) was observed for spiromesifen in pig hair. Also, the average recovery rate for pig hair was the highest, while chicken feathers was the lowest. Additionally, the average recoveries of all compounds were 96.55, 87.75, 93.39, 97.22, 93.22, 89.40, and 92.92% in chicken and duck feathers and cow, pig, dog, and cat hair, respectively. The average recovery rate for pig hair was the highest, while chicken feathers was the lowest. Accuracy and precision are described in the supplementary material.

Previous studies have analyzed veterinary drugs in cow and pig hair. In 2006, Cubarsi et al. established a method to analyze sulfamethazine in cow and pig hair using LC-DAD [32], and in 2007, they developed a method for the analysis of enrofloxacin and ciprofloxacin in cow and pig hair using LC-FLD [33]. In 2009, Castellari et al. developed a method to analyze tetracycline and oxytetracycline in livestock hair using LC-MS/MS [34]. However, these studies were limited to specific drugs in livestock hair. Our method overcomes this issue, where many potentially hazardous substances such as pesticides and veterinary drugs have been identified from the hair samples of various animals.

3.4. Analysis of the hair samples using GC and LC-TOF-MS

TOF-MS can investigate non-target compounds in addition to the target compounds. A standard material-based targeted analysis is required for quantitative analysis. However, prior to this analysis, non-targeted profiling for pesticides, veterinary drugs, and other potentially hazardous compounds (common ingredients used during sample preparation) was conducted [35]. GC- and LC-TOF-MS were used for the non-targeted profiling of 30 hair samples (10 livestock, 10 cat, and 10 dog). Chroma TOF and SCIEX OS 1.4 software were used to process the GC- and LC-TOF-MS data, respectively, where the precursor mass and fragment ion patterns of the compounds were compared with the data in the libraries. Tramadol, which is an analgesic prescribed to dogs that relieves acute and chronic pain caused by surgery or arthritis [36], was identified in dog hair by both GC- and LC-TOF-MS (Tables 2 and 3). The molecular ion of tramadol was observed at m/z 264.1949 (mass error < 6 ppm) in LC-TOF-MS. Aspirin, which is a common analgesic and antipyretic, was identified in dog hair by GC-TOF-MS (Table 3). Additionally, bifenthrin was identified in cow hair. Bifenthrin, an insecticide in the pyrethroid family, is used on various agricultural crops such as rice straw, which is widely used as livestock feed [37]. In addition, antifungal drugs such as climbazole, icaridin, and praziquantel have been identified in dog and cat hair by LC-TOF-MS (Table 3). This can be explained by the fact that dogs are frequently exposed to grass fields and consequently antifungal agents [38,39]. The protonated molecular ions of climbazole, icaridin, and praziquantel were observed at m/z 293.1046, 230.1744, and 313.1905, respectively (mass error < 6 ppm). The fragment ion patterns of compounds identified by both GC- and LC-TOF-MS showed high library concordance (>88%) with a mass error of less than 6 ppm. Representative chromatograms and MS/MS spectra of the hazardous compounds are shown in Fig. 3.

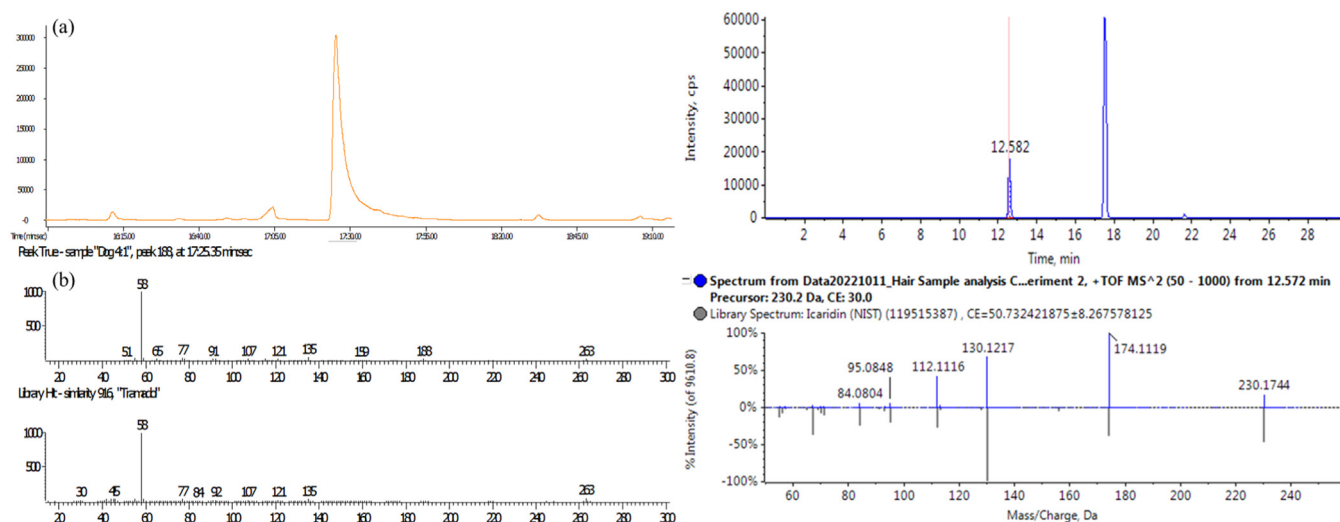


Fig. 3. Representative GC and LC-TOF-MS (a) chromatogram and (b) mass spectrum of tramadol and icaridin identified at ACN layer in dog and cat hair.

4. Conclusion

We developed a quantitative and qualitative analysis method for 394 polar and non-polar hazardous compounds (293 pesticides, 93 veterinary drugs, 6 mycotoxins, and 2 preservatives) found in pet and livestock hair. All the hazardous substances showed a LOQ of 0.02 mg/kg and recoveries of 63.35–119.98% (except the water layer). The accuracy and precision were suitable for all matrices. This analytical method meets the analytical quality control standards of the SANTE guidelines [23]. Hazardous compounds were detected upon analysis of 30 hair samples using GC- and LC-TOF-MS, where the target compounds bifenthrin, praziquantel, and tiamulin as well as the non-target compounds aspirin, climbazole, icaridin, and tramadol were detected. Therefore, our non-targeted profiling method successfully identified various hazardous substances in pet and livestock hair.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRedit authorship contribution statement

Hyung-Ju Seo: Validation, Writing – original draft. **Tae Woong Na:** Methodology, Conceptualization, Writing – original draft, Writing – review & editing. **Seung Hwa Lee:** Conceptualization, Investigation, Visualization. **Ho Jin Kim:** Investigation, Visualization, Writing – review & editing. **Sunghie Hong:** Methodology, Supervision. **Hyunjeong Cho:** Project administration, Writing – review & editing.

Data availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.chroma.2023.464170](https://doi.org/10.1016/j.chroma.2023.464170).

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