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Simultaneous determination of multi-class veterinary drugs in fishery products with liquid chromatography–tandem mass spectrometry

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Abstract

The objective of this study was to optimize the analytical method for multi-class veterinary drug residues of 64 compounds in fishery products. Several compounds from veterinary drugs are banned or unauthorized in fishery products according to the Korean Food Code. Samples were extracted using acetonitrile/water (4:1, v/v) and the clean-up step was carried out by adding octadecylsilane and acetonitrile-saturated hexane. The target compounds were confirmed and quantified using liquid chromatography–tandem mass spectrometry (LC–MS/MS). The proposed method was validated according to the CODEX guidelines (CAC/GL-71), and most target compounds were found to be in acceptable quantities under the requirements of the validation guidelines. The recovery of analytes was typically in the 60–120% range, and precision, expressed as the coefficient of variation was less than 31% at all levels of concentration. The limit of quantification ranged from 0.03 to 3 μg kg^{-1} in the fishery products. Moreover, the application of the proposed method to 96 real samples demonstrated that no drug residues exceeded the Korean maximum residue limits (MRLs). This evaluation method provides reliable identification and quantification of multi-class veterinary drugs in fishery products and can be an efficient means to inspect drugs currently banned or not approved for aquaculture in Korea.

Keywords: Analytical methods, Fishery product, LC–MS/MS, Multi-class analysis, Veterinary drugs

Introduction

Global apparent food fish consumption has increased at an average annual rate of 3.1%, from 1961 to 2017, higher than the corresponding increase in other animal protein foods (meat, dairy, milk, etc.) that increased by an average of 2.1%. Fishes have become an import source of proteins and are supplied by capture fisheries and aquaculture production. The latter accounts for 46% of total production and 52% of human consumption [1]. In order to achieve greater productivity, intensive cultivation systems are employed, which increases susceptibility to diseases caused by parasitic, bacterial, viral, and fungal infections [2]. Therefore, the use of veterinary drugs in aquaculture is necessary for the prevention and treatment of infectious diseases. However, even with careful use, residues of these drugs can remain in fishery products and affect public health [3]. In addition, there are instances of unapproved or prohibited drugs used in aquaculture [4]. This practice carries potential risks related to the development of antibiotic resistance, which can lead to serious human health problems worldwide [5–7]. To restrict human exposure to veterinary drug residues, several governmental authorities have established maximum residue limits (MRLs) to ensure the quality and safety of consumer products. In Korea, the Ministry of Food and Drug Safety (MFDS) regulates the use of veterinary drugs in food-producing animals, sets MRLs for approved veterinary drugs based

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on acceptable daily intakes (ADIs) and develops standard analytical methods for veterinary drug residues in foods of animal origin. The MFDS, as a regulatory agency, also has the primary responsibility for ensuring food safety by inspecting imported and domestic fishery products. Our previous study showed that veterinary drugs were detected in fish samples in 217 of 958 samples (22.7%), and in 12 samples (1.3%) the drug content exceeded the Korean MRLs. Various compounds such as enrofloxacin, oxytetracycline, and trimethoprim were detected in fishery products; enrofloxacin was the chemical most frequently present in quantities exceeding the Korean MRLs [8]. In the Korean Food Code, analytical methods based on liquid chromatography–tandem mass spectrometry (LC–MS/MS) have been used to detect multi-class residues in fish [9]. Although veterinary drugs for which MRLs have been established can be regulated, there is no way to control the distribution of food containing unapproved drugs in fishery products. In addition, the overuse and improper application of veterinary drugs without observing the required withdrawal period can result in high residue levels in fish and, as a result, increase unintentional human exposure leading to health risks [10]. In Korea, the percentage of imported fishery products is approximately 31% and has increased from 2011 to 2018 [11]. Varying degrees of drug MRLs are imposed on fishery products imported from different countries, and these products are not inspected for drug residues outside these established MRLs or residue tolerances. In efforts to ensure food safety in Korea, the MFDS is preparing to introduce a positive list system (PLS). The PLS program for veterinary drugs that will be implemented in 2024 or after. Five major livestock (beef and derivatives, pork and derivatives, poultry and derivatives, milk and eggs) and fishery products will be subject to the PLS first. Thus far, in the absence of established MRLs in Korea, the current default policy has been to apply CODEX standards or, failing that, the lowest MRL set for similar products. However, with the implementation of the PLS, a default tolerance of 10 μg kg^{-1} will apply to drugs with no established Korean MRLs. Following the full implementation of the new system, residual drug substances without established MRLs or residue tolerances will be subjected to law enforcement [12]. Accordingly, to ensure implementation of the PLS, the development of fast and reliable analytical methods that allow facile inspection of these unregulated veterinary drugs.

Several countries and institutions have adopted multiclass simultaneous analysis to determine veterinary drug residues in animal products using LC–MS/MS. LC–MS/MS is the most significant quantitative analytical technique developed in recent years [13–16]. This method can be employed for developing a method for multiple analytes with a single sample preparation and the ability to determine target analytes in one run. The aim of this study was to develop a screening and confirmatory method suitable for analyzing a wide range of 64 compounds in fish using LC–MS/MS with an optimized extraction method. The selected analytes include drugs approved for use in food-producing animals other than fish (e.g., cefazoline) and drugs that are completely banned from use in all food-producing animals (e.g., clenbuterol). The method was validated in terms of its quantitative performance characteristics based on CODEX guidelines [17]. In addition, this method was applied to analyze commercial samples obtained from domestic markets to determine the residue levels of various veterinary drugs. The evaluated method provides reliable identification and quantification of 64 compounds in fishery products successfully demonstrated in real samples.

Materials and methods

Chemicals and reagents

All high-purity (> 90%) chemical standards were purchased from Sigma-Aldrich (St. Louis, MO, USA and Steinheim, Germany): acetanilide, azaperol, caffeine, carbendazim, chlorpromazine, clopidol, closantel, diminazene, diphenhydramine hydrochloride, loperamide, oxyclozanide, ractopamine, thiacloprid, tinidazole, toltrazuril sulfone, tripelennamine, and valnemulin. Arprinocid, halofuginone, isometamidium, monoacetyl dapsone, pirlimycin, yohimbine, and zilpaterol were purchased from Toronto Research Chemicals (Toronto, Canada). Antipyrine, berberine, cefazoline, cyproheptadine, and naloxone were purchased from USP (Rockville, MD, USA). The other compounds used were purchased from Dr. Ehrenstorfer (Augsburg, Germany). LC–MS grade acetonitrile (MeCN), methanol (MeOH), and n-hexane were purchased from Merck Inc. (Darmstadt, Germany). Formic acid (≥ 95%) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Octadecylsilane (C_{18}) (55–105 μm, 125 Å) was purchased from Waters (Milford, MA, USA) and a syringe filter from Teknokroma (Barcellona, Spain) was used in PTFE membrane filters (0.2 μm). Standard stock solutions (1000 μg/mL) of the investigated drugs were prepared in MeCN, MeOH, MeOH/water (50:50, v/v), water, and MeOH/DMSO (50:50, v/v), respectively. All stock solutions were stored in the dark at − 20 °C.

Sample collection and preparation

To evaluate the applicability of the proposed analytical method, we collected 10 kinds of fish species (n = 96): abalone (n = 11), catfish (n = 8), eel (n = 15), flat fish (n = 11), rockfish (n = 11) manila clam (n = 5), mudfish

(n = 10), salmon (n = 9), sea bream (n = 5) and shrimp (n = 11) from fish markets and websites. The specimens were randomly collected between April and September 2020 in the Republic of Korea. The edible tissues (over 500 g) of each sample were homogenized and stored in a freezer (− 20 °C) until further analysis.

Samples (2 g) were extracted with 10 mL of MeCN/water (4:1, v/v) by shaking (5 min) and centrifugation at 4500×*g* for 10 min. The supernatants were then transferred to a tube containing C_{18} powder. Then, 10 mL of MeCN saturated in hexane was added, and the mixture was shaken for 1 min. The sample was centrifuged at 4500×*g* for 5 min, and the bottom solution (5 mL) below the hexane layer was transferred to a new centrifuge tube. The sample was dried with nitrogen gas below 40 °C. The residue was dissolved in 1 mL of MeOH/water (1:1, v/v) and then filtered with a 0.2 μm PTFE filter before analysis.

LC–MS/MS method

For LC–MS/MS experiments, a Shimadzu LCMS 8060 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) was used with a Waters X-SELECT HSS C_{18} (2.1 mm × 150 mm, 3.5 μm particle size) chromatographic column. The mobile phases were used water with aqueous 0.1% (v/v) formic acid (designated A), and MeCN with aqueous 0.1% (v/v) formic acid (designated B). The chromatographic separation was performed in gradient mode: 0–0.5 min, increase to 5% B, 0.5–5.5 min increase to 60% B, 5.5–6.0 min increase to 100% B, 6.0–10.0 min maintained at 100% B, 10.0–10.2 min decrease to 5% B, and 10.2–12.0 min maintained at 5% B. A flow rate of 0.3 mL/min was used, and the injection volume was set at 5 μL. Mass analysis was performed using an electrospray ionization (ESI) source in both positive and negative switching modes. The capillary, column, and auto-sampler temperatures were set at 350, 40, and 15 °C, respectively. Additionally, the capillary voltage was both 3.6 kV (positive) and − 2.8 kV (negative), the cone voltage was 30 kV in all compounds and argon gas was used.

Method validation

The proposed method was validated according to CODEX guidelines (CAC/GL-71). All concentration levels used in the validation are listed in Table 2. The performance parameters were selectivity, linearity, accuracy, precision, limit of detection (LOD), and limit of quantitation (LOQ). Linearity was demonstrated for all 64 compounds by preparing a six-point matrix-matched calibration curve in the range of target concentrations (0.5–80 μg kg^{-1}). Recovery and precision were determined by analyzing blank samples (flatfish, eel, and shrimp) at three different concentrations and estimated from five replicates for each concentration: 1, 2, and 10 μg kg^{-1} for analytes with zero tolerance or 5, 10, and 20 μg kg^{-1} for other analytes. Banned compounds and metabolites with zero tolerance were: dapsone, monoacetyl dapsone, dimetridazole, metronidazole, metronidazole-OH, ipronidazole, 2-methyl quinoxaline-2-carboxylic acid (MQCA), colchicine, and clenbuterol. Moreover, the following compounds were analyzed according to the marker residue given in parentheses: olaquindox (MQCA), and toltrazuril (toltrazuril sulfone). Recoveries were calculated by comparing the concentrations of the extracted samples with those from the matrix-matched calibration curve. Matrix-matched standards for calibration checks were used regularly during the analysis. The LOD and LOQ were defined with a signal-to-noise (S/N) ratio of ≥ 3 and ≥ 10, respectively. The LOQ were calculated analysing blank samples spiked at the lowest concentration of the analyte for which signal-to-noise ratio was 10. The peak to peak signal-to-noise ratios were used and they were calculated using LabSolutions software.

Results and discussion

LC–MS/MS analysis

This study was conducted to develop a quantitative analytical method for multi-class veterinary drugs that were not approved or banned in fishery products. The MS parameters were optimized by the direct infusion of individual veterinary drug solutions at 1000 μg mL^{-1} in MeOH/water (1:1, v/v) in the mass spectrometer. The electrospray source was used in positive or negative ionization mode to provide the highest signals. For each compound, the multiple reaction monitoring (MRM) transition with the highest intensity was used as the quantifier, while the other transition was used as the qualifier. The mass parameters for all analytes, such as collision energy and cone voltage, were optimized automatically by the software of the instrument. Table 1 lists the specific MS/MS parameters and retention times of all target drugs in this study. For the majority of analytes, the $[M+H]^+$ ion was sufficiently intensive that it could be selected as precursor ion for MS/MS. The rest of compounds appeared as negative hydride ions $[M-H]^-$ (closantel, clorsulon, diclazuril, efrotomycin, oxyclozanide, roxarsone, toltrazuril sufone). Others, like berberine, and isometamidium showed peaks corresponding to $[M]^+$; peaks for acriflavine corresponded to $[M-Cl]^+$, while those for bacitracin corresponded to $[M+3H]^{3+}$. Chromatographic analysis was based on a previously employed method for multi-class drug separation [18]. The separation was performed in a reversed-phase X-SELECT HSS C_{18} (2.1 mm × 150 mm, 3.5 μm, Waters) column, and the mobile phases used were water with aqueous 0.1% (v/v) formic acid (designated A) and

Table 1 LC–MS/MS parameters of 64 compounds

Class	Compounds	ESI (+/−)	Molecular weight (m/z)	Precursor ion (m/z)	[a]Product ion (m/z)	Collision energy (eV)	Retention time (min)
Analgesics/antipyrectics	Acetanilide	+	135.1	135.9	43.0	31	4.92
					51.0	50	
					77.0	30	
	Phenacetin	+	179.1	179.9	**65.1**	37	5.54
					93.0	28	
					110.0	22	
Antidiarrheals	Berberine	+	336.1	336.0	**263.0**	53	5.32
					278.0	43	
					304.0	33	
	Loperamide	+	476.2	477.0	72.0	51	6.50
					210.0	51	
					266.0	25	
Antiemetics	Metoclopramide	+	299.1	299.8	**140.9**	49	4.27
					184.0	31	
					226.9	20	
Antifungals	Fluconazole	+	306.1	307.0	169.1	22	4.64
					220.1	18	
					238.2	17	
Antihistamine	Cyproheptadine	+	287.2	288.2	**96.1**	25	5.86
					191.1	30	
					215.1	48	
	Diphenhydramine	+	255.2	256.1	115.0	55	5.48
					152.0	35	
					167.1	13	
	DL-Methylephedrine HCl	+	179.1	179.9	57.0	19	3.70
					117.0	21	
					147.1	22	
	Tripelennamine	+	255.2	256.1	**72.1**	32	4.83
					91.0	36	
					119.0	34	
Anti-inflammatory	Antipyrine	+	188.1	188.9	**56.0**	34	4.58
					58.5	34	
					104.0	25	
	Colchicine	+	399.2	400.1	310.2	27	5.29
					326.2	24	
					358.2	23	
	Ketoprofen	+	254.1	255.1	77.1	45	6.99
					105.0	22	
					209.1	15	

Table 1 (continued)

Class	Compounds	ESI (+/−)	Molecular weight (m/z)	Precursor ion (m/z)	[a]Product ion (m/z)	Collision energy (eV)	Retention time (min)
Benzimidazoles	Carbendazim	+	191.1	192.0	105.1	35	3.67
					132.2	29	
					160.1	18	
	Clorsulon	−	378.9	378.0	277.1	22	5.60
					242.2	22	
					342.1	12	
	Closantel	−	661.9	660.8	**127.0**	50	8.55
					315.1	34	
					345.0	36	
	Diethylcarbamazine	+	199.2	200.1	**72.0**	24	3.34
					100.1	16	
					127.0	15	
	Emamectin benzoate	+	885.5	886.2	82.1	55	7.38
					126.2	43	
					158.0	35	
	Morantel	+	220.1	221.2	**111.1**	25	4.64
					123.0	25	
					164.0	25	
	Oxyclozanide	−	400.9	399.9	202.1	22	7.60
					363.9	17	
					381.9	22	
Cefalosporines	Cefazolin	+	454.0	454.7	**155.9**	18	4.39
					294.9	17	
					323.0	13	
	Cefoperazone	+	645.1	645.8	**143.0**	36	4.78
					148.0	52	
					530.0	12	

Table 1 (continued)

Class	Compounds	ESI (+/−)	Molecular weight (m/z)	Precursor ion (m/z)	[a]Product ion (m/z)	Collision energy (eV)	Retention time (min)
Coccidiostat	Clopidol	+	191.0	192.0	51.1	44	3.73
					87.1	30	
					101.1	26	
	Diclazuril	−	406.0	405.0	299.0	28	7.46
					334.0	19	
					335.1	19	
	Diminazene	+	281.1	282.0	102.1	37	3.05
					119.1	17	
					254.2	9	
	Halofuginone	+	413.0	414.0	100.1	24	4.90
					120.1	21	
					138.1	20	
	Imidocarb	+	348.2	349.0	145.1	55	3.26
					162.2	25	
					188.1	29	
	Isometamidium	+	460.2	460.2	269.1	50	4.45
					298.1	25	
					313.1	20	
	Robenidine	+	333.1	333.9	111.0	50	6.50
					138.0	25	
					155.1	20	
	Roxarsone	−	262.9	262.0	123.1	25	3.33
					153.1	20	
					244.0	11	
	Toltrazuril	−	457.1	456.0	**42.2**	22	7.31
					399.1	12	
Diaminopyrimidines	Pyrmethamine	+	248.1	249.1	177.0	30	4.95
					198.1	45	
					233.1	28	
Growth supplement	Clenbuterol	+	276.1	277.0	**140.0**	46	4.50
					168.0	31	
					203.0	17	
	Nandrolone	+	274.2	275.0	**109.1**	26	6.80
					257.2	16	
					239.2	17	
	Ractopamine	+	301.17	302.0	**107.0**	34	4.10
					121.0	23	
					284.1	13	
	Zilpaterol	+	261.15	262	**185.1**	25	3.10
					202.1	19	
					244.2	14	

Table 1 (continued)

Class	Compounds	ESI (+/−)	Molecular weight (m/z)	Precursor ion (m/z)	[a]Product ion (m/z)	Collision energy (eV)	Retention time (min)
Lincosamides	Pirlimycin	+	410.2	411.1	56.1	52	4.62
					112.1	30	
					363.1	19	
Nitroimidazoles	Dimetridazole	+	141.1	142.1	81.1	26	3.88
					95.1	23	
					96.1	15	
	Ipronidazole	+	169.1	170.1	**109.1**	26	5.59
					123.1	25	
					124.1	19	
	Metronidazole	+	171.1	171.8	**82.0**	25	3.47
					98.0	22	
					111.0	23	
	Metronidazole-OH	+	187.1	188.0	68.1	22	3.14
					123.1	13	
					126.0	17	
	Tinidazole	+	247.1	248.0	82.1	34	4.40
					121.1	16	
					128.0	21	
Pesticides	Thiacloprid	+	252.0	253.0	90.1	38	5.67
					99.1	41	
					126.2	21	
Polypeptides	Baciitracin	+	1421.7	475.2	**86.1**	22	4.74
					110.1	49	
					199.1	28	
Quinoxalines	Olaquindox	+	188.1	189.0	102.0	32	4.63
					143.0	16	
					145.0	16	
Sedative	Arprinocid	+	277.1	278.1	107.1	55	4.75
					108.1	52	
					143.0	25	
	Azaperone	+	327.2	328.2	95.1	64	4.35
					121.1	19	
					123.1	44	
	Azaperol	+	329.2	330.2	109.1	57	4.05
					121.1	20	
					149.1	30	
	Carazolol	+	298.2	299.2	**116.1**	21	4.91
					194.1	30	
					222.1	20	
	Chlorpromazine	+	318.1	319.1	58.1	30	6.18
					86.1	20	
					246.0	24	
	Scopolamine	+	303.1	303.8	**103.0**	38	3.85
					138.0	22	
					156.0	17	

Table 1 (continued)

Class	Compounds	ESI (+/−)	Molecular weight (m/z)	Precursor ion (m/z)	[a]Product ion (m/z)	Collision energy (eV)	Retention time (min)
Sulfonamides	Dapsone	+	248.1	249.0	**92.0**	25	4.91
					108.1	22	
					156.0	14	
	Monoacetyl dapsone	+	290.1	291.0	**92.0**	30	5.06
					108.1	23	
					156.1	17	
	Succinyl-sulfathiazole	+	355.0	356.0	108.1	27	4.18
					192.1	24	
					256.1	17	
	Sulfabendzamide	+	276.1	277.1	92.1	25	5.66
					108.1	21	
					156.0	15	
	Sulfameter	+	280.1	281.1	92.1	30	4.62
					108.1	25	
					156.1	19	
	Sulfamoxol	+	267.1	268.1	92.1	25	4.37
					108.1	25	
					156.0	15	
	Sulfapyridine	+	249.1	250.1	92.1	26	4.11
					108.1	25	
					156.0	16	
	Sulfisomidine	+	278.1	279.1	92.1	30	3.56
					124.1	20	
					186.1	20	
Others	Caffeine	+	194.1	195.1	42.1	33	3.88
					110.1	23	
					138.1	20	
	Efrotomycin	−	1144.6	1143.6	274.2	49	7.10
					773.4	34	
					791.3	27	
	Naloxone	+	327.1	328.0	**212.0**	40	3.53
					253.1	27	
					268.0	27	
	Valnemulin	+	564.4	565.0	**147.0**	39	6.11
					164.0	32	
					263.1	19	
	Yohimbine	+	354.2	355.1	**117.0**	52	4.69
					144.0	31	

[a] The bold text expressed as quantification ion

Table 2 Validation results for the analytical method of 64 compounds in 3 kinds of food matrices

Compounds	Spiking level (μg/kg)	Flatfish (n = 5)		Eel (n = 5)		Shrimp (n = 5)	
		Rec. (%)	CV (%)	Rec. (%)	CV (%)	Rec. (%)	CV (%)
Acetanilide	5	117.4	10.6	73.7	19.0	107.1	10.9
	10	93.4	12.8	80.5	6.9	98.5	14.4
	20	75.0	6.2	72.8	4.1	90.5	13.9
Antipyrine	5	88.0	11.6	67.4	24.8	100.5	13.0
	10	79.0	2.2	76.7	4.1	100.6	8.6
	20	71.3	6.2	74.5	9.1	95.0	11.4
Arprinocid	5	112.1	3.4	67.2	1.4	100.3	5.6
	10	85.8	7.1	80.7	5.4	93.8	9.7
	20	70.9	4.9	76.5	3.5	89.0	7.1
Azaperone	5	113.9	9.9	98.4	7.7	88.0	9.8
	10	98.0	11.6	87.1	11.2	101.8	29.6
	20	70.0	10.8	77.6	8.7	84.1	18.0
Azaperol	5	103.0	6.4	60.6	12.6	92.1	6.5
	10	91.2	7.9	79.2	10.1	97.5	11.8
	20	73.8	4.9	84.2	6.8	89.1	7.5
Bactitracin	5	93.4	13.3	82.6	24.6	115.8	11.4
	10	65.3	29.3	74.7	3.5	89.8	12.7
	20	78.4	8.8	81.3	10.6	78.9	6.8
Berberine	5	116.4	14.0	65.4	7.5	97.6	13.1
	10	88.5	4.8	78.1	4.0	98.4	14.9
	20	71.6	8.0	74.1	2.0	90.0	13.0
Caffeine	5	89.7	14.2	74.7	18.7	115.3	8.4
	10	73.9	10.5	77.9	14.0	98.6	5.3
	20	68.4	7.3	70.6	11.1	97.2	5.5
Carazolol	5	97.5	12.8	68.7	9.1	95.5	8.4
	10	84.2	9.1	78.2	5.6	87.2	11.8
	20	70.5	6.0	74.7	2.6	84.5	5.2
Carbendazim	5	120.4	3.9	104.4	6.4	108.8	9.4
	10	88.9	5.9	88.9	2.2	93.2	8.2
	20	68.1	9.1	65.4	9.9	83.3	7.3
Cefazolin	5	110.1	20.9	104.2	26.2	118.8	26.0
	10	79.5	9.2	88.1	22.3	91.0	24.5
	20	70.7	9.9	72.3	13.9	92.7	22.0
Cefoperazone	5	79.3	13.1	62.2	26.9	110.0	21.2
	10	81.1	17.2	84.2	22.0	96.6	18.3
	20	73.9	16.5	78.8	5.7	109.8	7.7
Chlorpromazine	1	118.0	11.4	119.5	6.1	81.6	24.4
	2	86.0	9.4	80.5	17.1	114.5	14.9
	10	63.1	9.6	78.2	22.3	87.6	23.0
Clenbuterol	1	69.1	26.2	72.6	28.9	112.9	25.9
	2	88.6	22.6	80.7	9.1	90.6	19.5
	10	75.2	9.5	78.7	6.0	83.8	9.2
Clopidol	5	98.4	11.7	60.4	26.9	95.3	16.1
	10	110.1	20.9	74.3	22.2	98.5	11.0
	20	79.5	9.7	71.5	5.6	99.5	11.4
Clorsulon	5	70.7	9.9	71.5	8.5	113.7	6.9
	10	77.3	11.3	94.8	8.3	99.0	11.5
	20	78.3	5.7	81.4	20.5	103.8	9.1

Table 2 (continued)

Compounds	Spiking level (μg/kg)	Flatfish (n = 5)		Eel (n = 5)		Shrimp (n = 5)	
		Rec. (%)	CV (%)	Rec. (%)	CV (%)	Rec. (%)	CV (%)
Closantel	5	98.4	12.7	75.1	19.5	105.4	10.3
	10	92.1	4.7	90.9	5.9	101.9	9.6
	20	82.8	18.2	92.9	12.6	98.9	14.0
Colchicine	1	61.5	30.8	99.7	13.6	98.0	5.5
	2	70.5	12.0	81.8	4.7	95.8	15.2
	10	68.1	7.2	77.5	6.0	92.6	9.8
Cyproheptadine	5	100.8	17.4	85.1	13.4	79.2	17.6
	10	91.8	11.4	76.3	22.8	96.1	30.4
	20	73.9	7.1	78.8	17.1	78.8	16.9
Dapsone	1	65.7	27.6	75.0	31.1	113.6	14.2
	2	61.5	9.0	77.7	9.0	96.7	6.7
	10	67.1	14.0	68.8	12.9	85.9	11.1
Monoacetyl dapsone	1	60.1	18.4	60.3	24.2	72.6	29.2
	2	68.4	17.2	80.7	22.1	91.1	18.5
	10	87.9	6.5	92.7	3.9	93.1	7.6
Diclazuril	5	98.1	12.1	67.7	6.4	119.8	2.7
	10	90.5	3.8	94.0	10.5	119.3	4.4
	20	91.8	9.9	96.0	4.4	116.0	2.5
Diethylcarbamazine	5	105.3	10.9	118.9	5.8	106.0	13.8
	10	84.2	10.8	98.6	6.2	105.1	19.6
	20	61.8	8.8	78.2	7.5	92.7	12.1
Dimetridazole	1	103.1	24.6	115.1	8.7	117.7	22.9
	2	77.7	16.2	80.8	9.6	117.2	14.1
	10	60.5	6.2	63.1	13.3	92.1	7.4
Diminazene	5	106.9	10.6	73.5	19.6	105.0	10.2
	10	84.1	10.2	99.8	8.0	89.9	5.4
	20	71.0	4.8	97.4	13.8	86.4	13.2
Diphenhydramine	5	96.2	8.0	80.1	13.4	81.2	23.7
	10	92.3	14.2	73.5	19.5	117.8	2.7
	20	72.7	8.1	61.9	9.2	99.1	29.2
DL-Methylephedrine HCl	5	82.1	9.3	117.0	9.1	96.6	9.5
	10	70.8	5.9	94.9	6.0	88.9	14.2
	20	60.3	6.0	75.8	10.9	89.5	10.7
Efrotomycin	5	116.9	23.4	116.7	21.0	104.9	14.5
	10	101.6	11.3	107.1	17.7	104.0	24.3
	20	98.3	10.8	112.8	7.9	108.6	7.5
Emamectin benzoate	5	94.7	10.8	67.6	5.9	109.5	4.2
	10	86.6	5.9	80.0	8.7	98.9	7.2
	20	83.4	6.3	83.2	8.4	93.3	7.5
Fluconazole	5	113.9	8.3	72.5	7.5	108.6	6.9
	10	89.0	5.0	79.0	4.2	99.9	9.2
	20	70.2	5.1	73.4	4.5	95.9	8.6
Halofuginone	5	101.5	16.7	73.1	16.9	108.5	11.8
	10	78.1	14.3	87.1	9.7	90.8	11.0
	20	72.0	10.5	79.0	9.7	90.5	4.5
Imidocarb	5	83.9	19.9	115.3	11.4	104.5	19.0
	10	85.1	7.8	111.6	27.0	104.5	17.2
	20	80.1	5.9	118.1	7.3	90.4	30.5

Table 2 (continued)

Compounds	Spiking level (μg/kg)	Flatfish (n = 5)		Eel (n = 5)		Shrimp (n = 5)	
		Rec. (%)	CV (%)	Rec. (%)	CV (%)	Rec. (%)	CV (%)
Ipronidazole	1	107.1	18.3	75.0	22.1	85.0	10.0
	2	95.2	10.3	64.0	4.7	107.3	22.7
	10	62.6	6.0	62.0	5.9	88.1	13.0
Isometamidium	5	97.2	8.3	89.0	10.8	98.2	7.8
	10	88.9	6.8	104.2	18.8	100.5	11.3
	20	87.9	15.2	105.6	2.9	108.2	8.5
Ketoprofen	5	96.9	4.3	72.8	15.1	119.3	3.2
	10	88.8	8.5	87.9	9.9	110.7	9.3
	20	77.9	9.1	81.0	9.7	106.5	8.1
Loperamide	5	96.7	6.8	69.0	7.2	98.3	3.8
	10	82.6	3.9	78.2	8.8	90.2	6.7
	20	75.3	5.5	79.2	7.9	84.7	5.0
Metoclopramide	5	114.5	26.1	75.4	12.1	97.9	12.3
	10	81.6	12.0	80.8	23.4	90.9	9.3
	20	70.3	6.6	71.7	8.1	92.0	13.3
Metronidazole	1	89.3	12.3	107.2	7.8	120.3	13.2
	2	70.9	18.3	66.9	9.0	105.1	8.9
	10	60.9	7.7	63.3	15.3	90.0	8.8
Metronidazole-OH	1	68.3	6.6	111.1	11.1	97.2	12.6
	2	60.0	9.2	103.3	6.6	91.9	8.3
	10	63.6	6.3	104.2	10.2	85.1	11.4
Morantel	5	95.8	6.3	73.9	6.2	108.4	5.7
	10	85.5	9.7	83.9	3.6	107.6	16.0
	20	71.5	5.2	79.1	4.6	102.9	8.6
Naloxone	5	102.4	11.1	68.6	23.2	101.3	6.6
	10	80.6	4.9	79.6	7.0	100.8	8.7
	20	70.9	4.2	79.7	7.4	89.1	10.4
Nandrolone	5	99.1	9.1	75.5	10.3	106.6	7.9
	10	88.3	4.5	82.5	9.5	104.6	10.6
	20	75.3	5.7	83.8	6.5	94.3	5.1
Olaquindox	1	92.5	6.2	72.1	12.1	75.7	10.7
	2	86.5	6.9	82.6	10.4	89.2	7.3
	10	70.5	7.5	64.7	5.1	62.7	14.0
Oxyclozanide	5	104.3	12.9	64.2	14.9	97.8	10.7
	10	95.3	4.9	97.5	15.8	117.7	13.4
	20	86.9	16.4	104.3	16.0	114.9	6.6
Phenacetin	5	117.9	9.6	110.6	9.5	101.4	13.9
	10	88.8	8.1	95.8	6.1	99.3	13.2
	20	72.0	9.7	74.6	8.3	93.1	8.8
Pirlimycin	5	115.2	5.5	70.8	10.2	105.3	6.5
	10	82.9	6.4	79.7	3.9	94.9	5.7
	20	72.9	5.2	74.7	3.8	90.7	5.5
Pyrmethamine	1	79.9	5.5	67.9	12.0	87.8	4.0
	2	80.6	7.9	69.5	8.6	85.5	9.5
	10	69.8	4.6	75.3	8.6	77.5	6.0
Ractopamine	5	87.8	7.6	60.5	12.3	94.4	2.9
	10	81.9	5.6	79.6	3.4	91.8	7.5
	20	72.6	6.6	75.0	4.1	88.6	6.5

Table 2 (continued)

Compounds	Spiking level (μg/kg)	Flatfish (n = 5)		Eel (n = 5)		Shrimp (n = 5)	
		Rec. (%)	CV (%)	Rec. (%)	CV (%)	Rec. (%)	CV (%)
Robenidine	5	98.0	13.9	67.9	17.8	80.1	12.5
	10	71.7	12.7	74.7	4.8	77.3	16.1
	20	82.3	20.3	70.2	15.1	72.2	17.8
Roxarsone	5	118.0	22.4	111.2	18.9	111.5	8.5
	10	82.5	7.0	76.4	10.9	103.9	29.6
	20	61.7	6.1	72.4	4.2	113.8	14.9
Scopolamine	5	97.1	6.9	61.6	11.6	111.8	11.7
	10	82.1	4.9	74.1	11.5	97.1	11.6
	20	70.5	10.5	79.4	7.8	89.0	6.9
Succinyl-sulfathiazole	5	75.8	17.1	78.3	20.2	89.2	24.3
	10	77.4	10.0	77.5	6.2	80.3	15.3
	20	70.6	13.3	80.5	8.9	81.5	9.7
Sulfabendzamide	5	112.3	4.6	66.1	10.7	111.5	4.3
	10	87.0	9.1	74.8	8.5	101.6	11.9
	20	70.1	7.2	71.2	9.2	97.4	8.6
Sulfameter	5	105.5	11.8	62.6	11.2	111.5	4.3
	10	81.8	11.5	72.7	8.7	101.6	11.9
	20	70.4	14.2	69.8	9.3	97.4	8.6
Sulfamoxol	5	112.2	8.0	73.6	18.4	101.5	6.8
	10	78.9	6.8	80.8	6.1	102.8	3.6
	20	70.7	7.5	78.1	17.0	98.8	7.1
Sulfapyridine	5	115.3	7.9	113.5	4.6	110.9	7.1
	10	91.7	9.5	94.1	9.1	100.3	7.4
	20	71.4	4.6	74.1	6.7	93.8	7.7
Sulfisomidine	5	86.6	15.2	113.7	6.6	115.9	3.7
	10	73.8	12.1	94.0	3.6	100.1	13.7
	20	63.1	5.3	72.9	10.2	96.5	5.4
Thiacloprid	5	96.6	4.7	71.8	8.3	108.1	5.1
	10	83.5	6.2	78.3	6.4	102.2	10.3
	20	70.4	5.3	72.1	8.0	98.8	9.9
Tinidazole	5	117.0	8.6	72.2	23.0	104.6	7.1
	10	92.2	8.8	83.0	8.5	98.3	7.0
	20	70.8	9.9	73.0	3.0	92.2	10.5
Toltrazuril	5	78.8	11.2	79.1	17.5	99.2	14.6
	10	91.4	5.6	107.6	3.1	111.8	11.6
	20	90.3	7.5	114.3	4.9	118.9	7.2
Tripelennamine	5	110.5	20.9	81.9	21.1	110.0	11.9
	10	115.7	9.6	69.9	12.8	115.9	21.4
	20	83.0	14.0	73.6	14.3	97.2	18.0
Valnemulin	5	87.9	9.7	60.9	8.4	94.3	5.8
	10	79.7	5.1	72.6	7.3	84.1	7.4
	20	75.3	5.6	74.0	7.0	80.0	5.1
Yohimbine	5	111.9	8.3	71.4	19.9	88.8	4.3
	10	81.3	6.9	78.8	4.8	87.7	11.7
	20	70.2	8.7	77.3	9.9	89.2	8.9
Zilpaterol	5	96.1	6.8	73.8	9.2	113.5	6.5
	10	80.4	8.2	83.2	7.9	105.6	10.3
	20	70.0	7.0	75.2	5.9	98.9	10.3

Rec. recovery, *CV* coefficient validation

MeCN with 0.1% (v/v) formic acid (designated B). Several different mobile phases consisting of water, methanol, and MeCN as the organic phase with different mobile additives, such as ammonium formate, were tested to achieve optimal conditions. It was determined that the combination of mobile phases consisting of water with aqueous 0.1% (v/v) formic acid and MeCN with aqueous 0.1% (v/v) formic acid provided better peak shape and intensity for the majority of the target compounds. The optimal mobile gradient program was set to last 12 min to improve the selectivity and resolution of the target compounds.

Sample preparation methods

Various studies of multi-residue analytical methods have used a combination of water and organic solvents to extract target compounds from the matrix [19, 20]. In this study, sample extraction was optimized based on a previously employed method for multi-class drug analysis of fishery products [18]. MeCN/water (4:1, v/v) was selected as the extraction solvent to afford high-extraction recovery and precipitate proteins in the sample. For better clean-up of the samples, C_{18} absorbent was used to remove fats and non-polar interference compounds in the matrix [21], and MeCN-saturated in hexane was added to eliminate potential interference during analysis and provide satisfactory recoveries for most compounds [22].

Validation of the analytical method

Validation was achieved based on the criteria outlined in the CODEX guidelines (CAC/GL 71-2009). Six different concentration levels were applied (2.5, 5, 10, 20, 40, and 80 μg kg^{-1} for analytes with no specific limits; 0.5, 1, 2, 10, 20, and 40 μg kg^{-1}; for analytes with zero tolerances), and matrix-matched calibration curves showed good linearity with a coefficient of determination (r^2) greater than 0.98. The chromatogram peaks of the representative compounds are presented in Additional file 1: Figure S1. The results of the recovery and precision experiments are listed in Table 2. The majority of analytes met CODEX requirements with recovery values typically in the range of 60–120% and a coefficient of variation (CV) less than 31%, but relative few analytes were slightly outside the acceptable limits (carbendazim, diethylcarbamazine, DL-methylephedrine HCl, roxarsone, sulfasomidine in flatfish, carbendazim, diphenhydramine, and sulfameter in eel). Some losses occurred during sample preparation, although a slightly lower recovery with satisfactory repeatability (CV less than 10%) is not a significant problem. Although these results do not completely meet the requirements of the CODEX guideline for a quantitative confirmation method, such periodic outliers are expected in multi-class, multi-residue analyses [23]. A difference was observed in the results obtained for the three matrices, which may be due to matrix effects. The LOQ ranged from 0.03 μg kg^{-1} (pyrmethamine) to 3 μg kg^{-1} (clorsulon, toltrazuril sulfone and tripelennamine) in the three matrices (Table 3). The LOQs were in all cases less than 3 μg kg^{-1} and satisfied the criteria less than 10 μg kg^{-1}, which is a default LOQ for PLS. The LOQ values in this study are similar or lower than those from previous studies. Saxena et al. [22] reported that LOQs of 24 veterinary drugs were ranged from 5 to 10 μg kg^{-1} in aquaculture shrimps and Dasenaki et al. [24] reported that LOQs of 115 veterinary drugs were in all cases below 5 μg kg^{-1} in fish tissue.

This analytical method allows the simultaneous extraction of veterinary drugs with vastly different physicochemical properties from various matrices by employing a simple extraction solvent. Thus far, studies have been conducted on substances that have already been approved or frequently used in fish animals [18, 25]. Therefore, this study is particularly significant as it is the first report of the successful analysis of several drugs not currently approved in fish animals, approved for use in fish animals by other governments, and without any published tolerances. However, further work is required to improve the established multi-class residue method and achieve better results for those compounds analyzed with insufficient accuracy.

Matrix effect

For complex samples such as fishery products, matrix effects (MEs) occur, especially when analyzing in ESI mode, wherein the signal is enhanced if the value is positive and suppressed if the value is negative. The matrix effect can be classified as (1) soft ($-20\% < ME < 20\%$), (2) medium ($-50\% < ME < -20\%$ or $20\% < ME < 50\%$), and (3) strong($ME < -50\%$ or $ME > 50\%$) [26]. Matrix-matched and solvent standard curves were compared to evaluate the ME, which were calculated as follows:

$$ME(\%) = \left(\frac{Slope_{matrix\ matched\ standard\ curve}}{Slope_{solvent\ standard\ curve}} - 1 \right) \times 100. \quad (1)$$

The ME calculated for all compounds in each matrix are listed in Table 3. As shown in Table 3, a significant matrix effect is observed in this study. Most of the compounds in fish tissue were subjected to signal suppression, whereas few compounds were subjected to signal enhancement. According to matrices, the matrix effects were mostly soft and medium in flat fish, but strong effects in eel and shrimp (strong effects were 12 compounds in flat fish, 52 compounds in eel and 48 compounds in shrimp). As observed from the results, the

Table 3 Matrix effects and LOQ of 64 compounds

Compounds	Flat fish		Eel		Shrimp	
	% matrix effect	LOQs (μg/kg)	% matrix effect	LOQs (μg/kg)	% matrix effect	LOQs (μg/kg)
Acetanilide	−28	1	−6	1	−23	0.4
Antipyrine	−26	1	−67	1	−71	0.2
Arprinocid	20	0.4	−93	0.4	−93	0.1
Azaperone	−67	0.3	−83	0.3	−85	0.1
Azaperol	−62	2	−94	1	−94	1
Bacitracin	−48	1	−57	1.3	−51	0.3
Berberine	−4	0.5	−89	0.5	−88	0.2
Caffeine	−5	0.5	−53	1	−62	0.2
Carazolol	−7	0.4	−88	0.5	−88	0.2
Carbendazim	−1	0.4	−84	0.5	−94	0.1
Cefazolin	3	1	302	1	312	0.5
Cefoperazone	15	1	179	1	137	0.3
Chlorpromazine	−85	0.4	−94	0.2	−95	0.1
Clenbuterol	−20	0.3	115	0.4	138	0.1
Clopidol	−9	1	−59	1	−64	0.3
Clorsulon	−20	0.3	−22	3	−11	0.1
Closantel	−36	0.5	−90	0.5	−86	0.2
Colchicine	16	0.5	16	0.5	3	0.5
Cyproheptadine	−78	0.3	−94	0.4	−94	0.1
Dapsone	14	0.2	26	0.2	−8	0.3
Monoacetyl dapsone	−2	0.5	46	0.2	−19	0.3
Diclazuril	−21	0.2	−94	0.3	−92	0.3
Diethylcarbamazine	−62	1	−82	2	−90	2
Dimetridazole	−35	0.5	−59	0.5	−80	0.5
Diminazene	−75	0.4	−62	1	−46	0.4
Diphenhydramine	−55	0.3	−75	0.04	−84	0.2
DL-Methylephedrine HCl	−55	0.3	−58	1	−78	0.1
Efrotomycin	−35	1	15	0.3	48	0.5
Emamectin benzoate	−27	0.2	−88	0.4	−84	0.1
Fluconazole	28	0.2	−83	0.2	-82	0.1
Halofuginone	−23	0.1	−54	1	−53	0.1
Imidocarb	19	0.3	−92	0.3	−84	0.1
Ipronidazole	−73	0.5	−72	0.5	−57	0.5
Isometamidium	122	0.4	−82	0.4	−61	0.1
Ketoprofen	−12	1	−89	0.5	−89	0.2
Loperamide	−31	0.2	−97	0.3	−96	0.1
Metoclopramide	−27	1	−32	1	−29	0.4
Metronidazole	27	0.5	132	0.5	−37	0.5
Metronidazole-OH	48	0.5	423	0.5	−37	0.5
Morantel	35	1	−87	1	−88	0.4
Naloxone	−35	0.4	−68	0.5	−62	1
Nandrolone	−16	2	−34	0.4	−4	0.3
Olaquindox	40	0.3	41	0.2	−27	0.1
Oxyclozanide	−26	0.3	−94	0.2	−93	0.1
Phenacetin	−12	1	−49	1	−64	0.3
Pirlimycin	−6	0.3	−93	0.3	−93	0.1
Pyrmethamine	−27	0.1	−78	0.2	2	0.03
Ractopamine	−23	0.2	−91	0.4	−90	0.3

Table 3 (continued)

Compounds	Flat fish		Eel		Shrimp	
	% matrix effect	LOQs (μg/kg)	% matrix effect	LOQs (μg/kg)	% matrix effect	LOQs (μg/kg)
Robenidine	−91	2	−89	1	−87	2
Roxarsone	−33	2	106	2	−32	2
Scopolamine	−20	0.2	−70	0.4	−67	0.1
Succinyl-sulfathiazole	17	1	−65	1	−64	0.3
Sulfabendzamide	23	1	−60	0.3	−69	0.3
Sulfameter	14	0.8	18	0.3	−5	0.3
Sulfamoxol	58	1	−73	1	−58	0.3
Sulfapyridine	7	0.4	−71	0.3	−81	0.1
Sulfisomidine	−19	0.4	−84	0.5	−87	0.1
Thiacloprid	23	0.4	−89	0.5	−90	0.1
Tinidazole	13	0.4	−51	0.4	−65	0.1
Toltrazuril	−26	2	−48	3	−42	1
Tripelennamine	−44	3	153	2	87	1
Valnemulin	−27	0.3	−76	0.3	−75	0.1
Yohimbine	8	0.5	−53	0.3	−53	0.2
Zilpaterol	3	0.4	−83	0.3	−86	0.2

ME% was very varying it means that significant MEs were observed for most analytes and it is consistent with other studies [23, 24]. The best way to overcome the matrix effect is the use of internal standards [24]. However, some internal standards are expensive, and finding appropriate internal standards for each analyte is difficult and unfeasible. Therefore, the matrix effect was compensated for by carrying out a matrix-matched calibration or through the standard addition method. These methods can be used for the correct quantification of target compounds in the analysis of real samples.

Application to real samples

In this study, we analyzed the levels of veterinary drug residues in domestic fishery products. We collected a total of 96 fishery samples from diverse provinces in Korea. Analysis of the samples showed that sulfisomidine was detected in salmon at 2 μg kg^{-1} and sulfameter was detected in catfish at 40 μg kg^{-1}. Sulfonamides, including sulfisomidine and sulfameter are widely used in veterinary medicine and aquaculture for the prevention and treatment of microbial infections. Because of the low cost and high effectiveness of sulfonamides, they are the most frequently used antibiotics [27]. In previous studies, sulfamethoxazole was found at relatively low concentrations of 0.54–68.0 μg kg^{-1} in fish muscles, while high concentrations of sulfadiazine at 6.5–143.3 μg kg^{-1} were reported in mollusks [28, 29]. Caffeine was detected in abalone, catfish, and mudfish at 1, 2, and 5 μg kg^{-1}, respectively. Caffeine is considered one of the most widely used pharmacologically active compounds (PhACs) pollutant because of its high abundance in the environment [30]. Pharmaceuticals are usually highly water-soluble, and when released into marine environments, fish and other aquatic organisms accumulate these PhACs [31]. In addition, previous studies have demonstrated the potent antimicrobial pharmacological properties of caffeine against bacterial fish pathogens in vitro [32]. However, there is no MRL imposed on caffeine in animal products in various countries. Therefore, few studies have reported caffeine residues in animal and fishery products. In other studies, caffeine was found at concentrations of 1.07, 1.00, and 1.37 μg kg^{-1} in black rockfish, gray mullet, and red sea beam, respectively [33]. Our study detected 3 veterinary drugs (sulfisomidine, sulfameter, and caffeine) from the 5 fishery samples (Table 4); however, the Korean MRLs were not exceeded in any of the tested samples. According to the monitoring results, this proposed method can be applied to determine the concentration of veterinary drug residues in fishery products. When the PLS system comes into effect, we expect that the established method will show successful analytical performance in fish inspection programs.

Table 4 Veterinary drug residues in fishery products

Species	Sample number	Detected number	Compounds	Concentration (μg kg^{-1})	MRLs [9] (μg kg^{-1})
Abalone	11	1	Caffeine	1	–[a]
Catfish	8	1	Sulfameter	40	100[b]
		1	Caffeine	2	–[a]
Eel	15	–	–	–	–
Flatfish	11	–	–	–	–
Rockfish	11	–	–	–	–
Manila clam	5	–	–	–	–
Mudfish	10	1	Caffeine	5	–[a]
Salmon	9	1	Sulfisomidine	2	100[b]
Sea bream	5	–	–	–	–
Shrimp	11	–	–	–	–

[a] Exempted substances (no MRL required)

[b] Sum of sulfonamides

Abbreviations

LC–MS/MS: Liquid chromatography–tandem mass spectrometry; MRL: Maximum residue limit; MFDS: Ministry of Food and Drug Safety; ADI: Acceptable daily intake; PLS: Positive List System; MeCN: Acetonitrile; MeOH: Methanol; DMSO: Dimethyl sulfoxide; LOD: Limit of detection; LOQ: Limit of quantitation; MQCA: 2-Methyl quinoxaline-2-carboxylic acid; MQCA: Olaquindox; MRM: Multiple reaction monitoring; CV: Coefficient of variation; ME: Matrix effect; PhAC: Pharmaceutically active compound.

Supplementary Information

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Additional file 1: Figure S1. Representative chromatograms in flatfish at the spiking levels of 10 μg kg^{-1}.

Authors' contributions

EJ conceived and designed the experiment, interpreted the data, and wrote the paper; HP performed the experiments, analyzed and interpreted the data and wrote the paper; SP performed the experiments, analyzed and interpreted the data; JC performed the revising of the manuscript; HJY provided ideas for the paper and financial support; JHK supervised the project and revised the final manuscript. All authors have agreed to the published version of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declarations

Competing interests

The authors declare that they have no competing interests.

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