Note

Screening Assay of Residual Antibiotics in Livestock Samples by LC-MS/MS

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A LC-MS/MS screening assay of multi-class antibiotics was developed for 19 residual antibiotics in livestock samples. Sample preparation employed the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) approach using 0.5% formic acid in acetonitrile–methanol (8 : 2), with salting-out using magnesium sulfate, trisodium citrate and sodium chloride. Recovery values from 5 different livestock samples ranged from 45.5 to 121.6%, and the RSDs were under 18% at two concentration levels. The limit of quantification values of 19 analytes were under 10 μg/kg in all livestock samples, and the procedure can detect almost all analytes under the MRL. Screening capability was confirmed by employing spiked samples. This new screening assay for residual antibiotics in livestock samples is expected to be useful for routine laboratory tests.

Key words: antibiotic; livestock sample; LC-MS/MS; QuEChERS approach; screening assay

Introduction

Antibiotics are widely used for the treatment and prevention of many kinds of infectious diseases in animals, and lead to increased productivity in farms. Tetracyclines and penicillins are often administered to animals because they have broad spectra and/or strong antibacterial activities, but residues may remain in the animal tissues. There is a lot of information about residual antibiotics detected at inspection institutes, because livestock products such as beef, pork and chicken are imported and widely sold in markets. Therefore it is necessary to develop a convenient screening assay which can analyze as many samples as possible at once.

Even though many reports have described analysis of antibiotics in livestock samples, there are few methods to analyze multi-class antibiotics including penicillins, tetracyclines and macrolides[1)–5)], because different classes of antibiotics have quite different chemical and physical properties. In addition, complicated clean-up has been necessary to analyze multi-class antibiotics by LC-MS/MS. Therefore the objective of this study is to develop an easy screening method for routine assay of antibiotics in livestock samples. We focused on the QuEChERS approach[6)–9)] for LC-MS/MS analysis of residual antibiotics in livestock samples.

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Materials and Methods

Chemicals and reagents

Ampicillin (ABPC, purity: 98.0%), benzylpenicillin potassium (PCG, 98.0%), cephalexin (CEX, 90.0%), chlortetracycline hydrochloride (CTC, 98.0%), erythromycin (EM, 90.0%), kitasamycin (KT, 90.0%), oxytetracycline hydrochloride (OTC, 99.0%), phenoxymethylpenicillin (PCV, 95.0%), tetracycline hydrochloride (TC, 99.0%) and tylosin (TS, 93.0%) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Cloxacillin sodium (MCIPC, 98.6%), dicloxacillin sodium (MDIPC, 98.2%), doxycycline hyclate (DC, 98.2%), nafcillin sodium (NFPC, 99.9%), oleandomycin (OM, 89.6%), oxacillin sodium (MPIPC, 99.0%) and spiramycin (SPM, 97.5%) were purchased from Hayashi Pure Chemical Industries (Osaka, Japan). Tilmicosin (TMS, 98.5%) was purchased from Eli Lilly Japan (Hyogo, Japan) and mirosamycin (MRM, 95.9%) was purchased from Kyoritsu Pharmaceutical Company (Tokyo, Japan).

ABPC, PCG, CEX, MCIPC, MDIPC, NFPC, MPIPC and PCV were accurately weighed in 10 mg portions, then diluted with distilled water to 10 mL and used as stock standard solutions (1,000 μg/mL). Other analytes were weighed in 5 mg portions, then diluted with methanol to 50 mL and used as stock standard solutions (100 μg/mL). Stock standard solutions were stored at −20℃ for up to one month.

Working standard solution for calibration curves was prepared by mixing all analytes and diluting with 0.5% formic acid in acetonitrile–methanol (8 : 2) at the level

Table 1. Multiple reaction monitoring conditions

Group	Analytes	Molecular weight	Precursor ion	Quantitative product ion	Collision energy (eV)	Qualitative product ion	Collision energy (eV)	Polarity
Tetracyclines	OTC	460.4	461	426	19	443	12	+
	TC	444.4	445	410	18	427	11	+
	DC	444.4	445	428	16	154	32	+
	CTC	478.9	479	444	19	154	27	+
Cephalosporins	CEX	347.4	348	158	6	140	25	+
Penicillins	ABPC	349.4	350	106	21	79	38	+
	PCG	334.4	333	192	15	74	26	−
	PCV	350.4	349	93	46	114	21	−
	MPIPC	401.4	400	259	16	356	11	−
	MCIPC	435.9	434	293	15	390	9	−
	NFPC	414.5	413	272	16	243	27	−
	MDIPC	470.3	468	327	14	424	12	−
Macrolides	SPM	843.1	844	174	34	101	46	+
	TMS	869.1	870	174	41	88	62	+
	MRM	727.9	729	158	26	116	33	+
	OM	687.9	689	158	26	544	13	+
	EM	733.9	735	158	29	577	19	+
	TS	916.1	917	174	37	156	41	+
	KT	771.9	773	109	34	174	29	+

of 1 μg/mL.

Working standard solution for spiking was prepared by mixing analytes with methanol at the level of 100 times fortification (PCG 0.4 and 1 μg/mL, NFPC 0.5 and 1 μg/mL and others 1 and 10 μg/mL). 500 μL of this solution was added to each livestock sample at 30 minutes before sample preparation.

Acetonitrile, distilled water and methanol (both HPLC grade), formic acid and ammonium formate (both LC/MS grade), EDTA-2Na (ethylenediaminetetraacetic acid disodium salt), magnesium sulfate and sodium chloride were purchased from Wako Pure Chemical Industries. Trisodium citrate dehydrate was purchased from Kanto Chemical Company (Tokyo, Japan).

Samples

The livestock samples (cattle muscle, swine muscle, chicken muscle, egg and milk) were purchased from local supermarkets in Tokyo and confirmed to be free from the targeted analytes. Each of them except milk was homogenized and stored at −20℃. Milk was stored at 4℃.

LC-MS/MS conditions

The analysis was carried out using a Prominence series (Shimadzu Corporation, Kyoto, Japan) HPLC system and L-column 2 ODS (2.1 mm i.d.×150 mm, 5 μm, Chemicals Evaluation Research Institute, Tokyo, Japan) at 40℃. The gradient was applied with 0.1% formic acid in 10 mmol/L ammonium formate (pH 4.0, A) and acetonitrile (B). The initial condition (A : B=95 : 5) was maintained for 3 min, and then the acetonitrile concentration was increased to 90% over 7 min, and maintained for 5 min. Then, the mobile phases were re-equilibrated to the initial condition for 5 min. The flow rate was 0.2 mL/min.

A TSQ Quantum Access MAX (Thermo Scientific Japan, Kanagawa, Japan) mass spectrometer, was used and operated in positive and negative electrospray ionization modes, with voltages of 3.0 kV and 2.5 kV, respectively. Vaporizer temperature was 465℃ and capillary temperature was 220℃. Tuning was performed by direct infusion of 1 μg/mL of each standard solution, and the optimized conditions of multiple reaction monitoring are presented in Table 1.

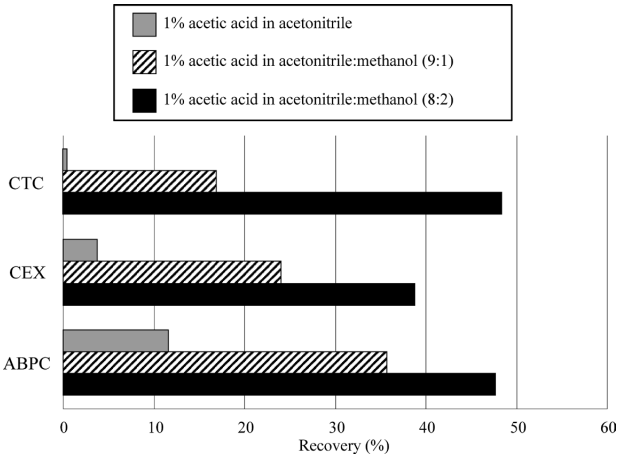

Fig. 1. Comparison of extraction solvents and recoveries of analytes spiked into cattle muscle at the level of 100 μg/kg

Sample preparation

A 5 g aliquot of each sample was weighed and added into a 50 mL polypropylene centrifuge tube. 2.5 mL of 0.1 mol/L EDTA-2Na in distilled water and 15 mL of 0.5% formic acid in acetonitrile–methanol (8 : 2) were added. After homogenizing, 4 g of magnesium sulfate, 1.5 g of trisodium citrate dehydrate and 1 g of sodium

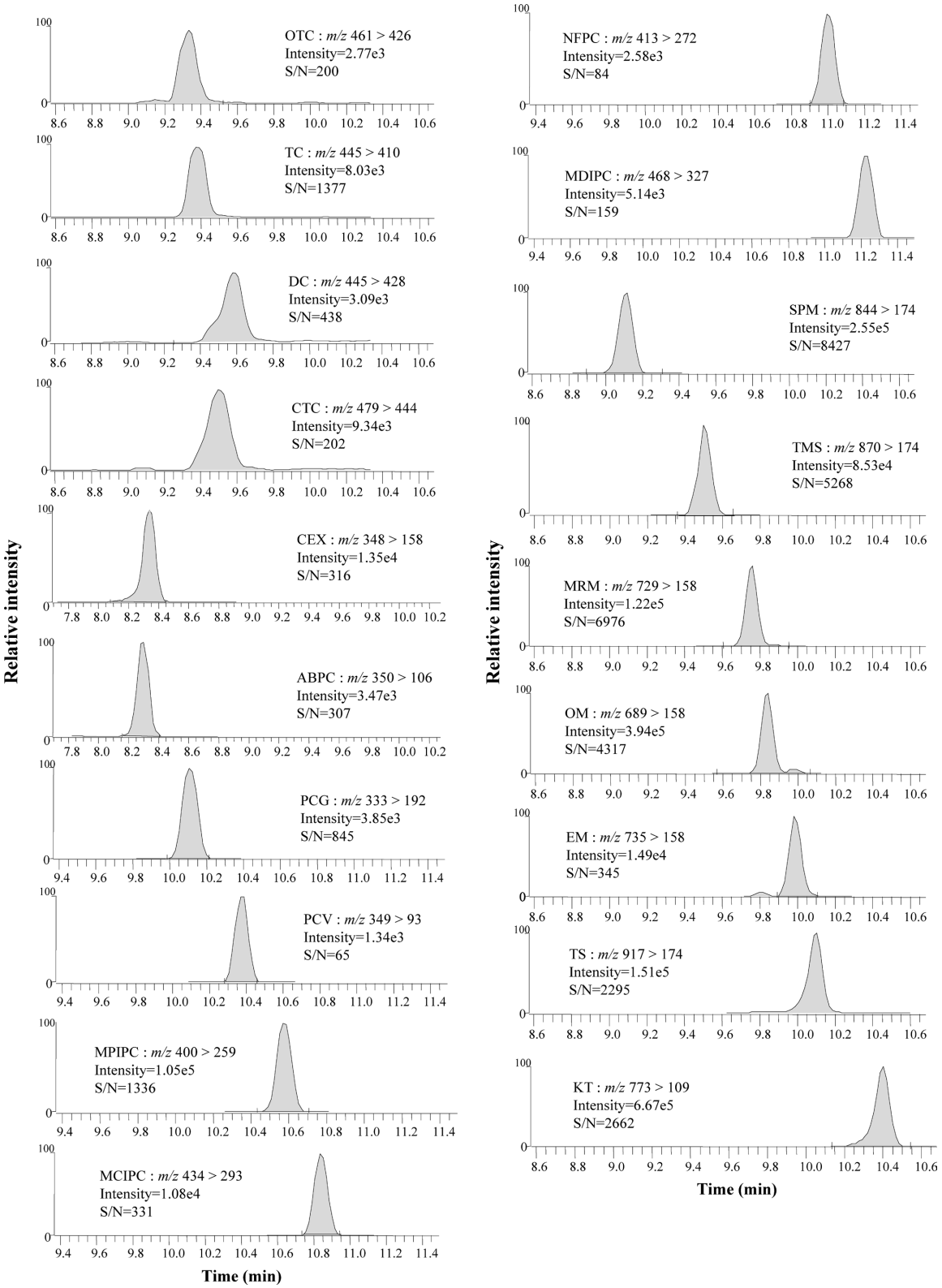

Fig. 2. MRL chromatograms of all analytes in cattle muscle for recovery test

Concentration levels: OTC (50 μg/kg), TC (50 μg/kg), DC (100 μg/kg), CTC (50 μg/kg), CEX (200 μg/kg), ABPC (30 μg/kg), PCG (50 μg/kg), PCV (10 μg/kg), MPIPC (300 μg/kg), MCIPC (40 μg/kg), NFPC (5 μg/kg), MDIPC (30 μg/kg), SPM (200 μg/kg), TMS (100 μg/kg), MRM (10 μg/kg), OM (50 μg/kg), EM (50 μg/kg), TS (50 μg/kg), KT (10 μg/kg).

Table 2. Recoveries and RSDs of antibiotics from 5 kinds of samples

Analytes	Spiked level (μg/kg)	Cattle muscle		Swine muscle		Chicken muscle		Egg		Milk	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
OTC	10	61.5	5.6	63.1	2.2	69.0	3.0	66.3	5.5	107.3	4.4
	100	66.8	5.1	62.4	1.0	61.4	3.0	67.6	5.1	70.5	1.5
TC	10	70.1	3.8	65.8	5.1	78.3	5.7	73.5	4.8	113.3	1.5
	100	75.0	3.5	68.3	2.1	69.6	3.6	71.7	5.9	82.1	1.5
DC	10	84.3	9.7	76.3	3.1	84.4	6.1	78.2	7.4	121.6	4.9
	100	75.4	4.4	77.7	2.4	86.8	0.8	68.4	2.9	78.0	1.4
CTC	10	73.1	3.4	70.6	8.8	82.4	5.4	71.3	6.5	105.8	2.9
	100	80.6	3.7	69.8	2.1	83.2	1.2	67.7	3.2	77.6	0.5
CEX	10	56.1	9.3	51.4	9.1	64.9	8.4	45.5	5.5	81.3	2.7
	100	65.0	3.6	52.9	4.7	73.1	5.7	74.8	4.4	78.3	3.5
ABPC	10	67.3	5.3	60.0	8.1	71.0	5.3	50.2	8.6	83.1	3.2
	100	73.5	4.4	75.3	3.9	81.1	2.6	79.7	1.9	78.8	6.6
PCG	4	100.5	3.7	78.2	4.7	74.9	3.2	64.3	3.2	73.5	2.0
	10	86.0	14.8	73.5	9.1	98.3	4.1	88.6	6.3	108.0	4.0
PCV	10	77.3	10.6	86.2	5.8	108.8	4.3	90.1	17.9	115.2	3.5
	100	80.2	4.0	75.1	4.9	87.7	2.3	83.6	3.7	89.2	2.4
MPIPC	10	85.1	6.0	82.1	5.1	94.7	1.2	81.2	5.9	100.1	4.5
	100	81.2	3.1	77.2	2.1	82.2	1.8	78.2	2.2	85.8	3.1
MCIPC	10	88.8	8.7	94.7	4.2	101.5	4.8	82.9	6.6	106.5	3.8
	100	86.6	8.0	77.9	3.6	79.9	1.9	87.3	6.7	78.8	1.3
NFPC	5	92.5	4.2	80.6	4.1	81.8	1.8	76.8	3.3	75.8	2.9
	10	88.2	3.4	86.6	8.1	86.9	4.7	84.7	7.9	101.4	5.6
MDIPC	10	104.1	2.4	90.8	5.8	98.3	1.4	95.2	6.9	112.8	6.2
	100	88.7	10.1	76.8	2.4	69.2	2.1	99.4	6.5	85.2	1.9
SPM	10	84.6	8.7	71.0	5.1	84.5	6.4	76.4	6.6	88.1	5.4
	100	99.4	2.9	77.1	2.6	85.5	2.6	87.0	3.9	99.5	1.2
TMS	10	105.5	14.6	94.4	6.2	98.5	5.9	82.6	5.5	115.8	13.4
	100	106.0	3.6	99.6	4.6	87.5	1.9	89.5	5.0	84.6	0.8
MRM	10	88.0	0.6	78.1	1.9	74.1	5.6	67.0	3.5	87.1	2.5
	100	100.0	3.5	74.0	3.1	77.7	2.5	65.2	7.8	72.4	1.4
OM	10	92.9	0.8	85.5	2.5	89.3	1.6	70.9	4.7	89.5	2.7
	100	104.1	2.4	82.7	3.5	80.1	1.9	80.4	7.6	80.1	0.9
EM	10	89.8	10.5	52.7	10.0	95.1	6.8	95.4	4.4	110.9	10.6
	100	100.4	5.9	65.3	2.0	98.6	5.8	95.6	5.3	76.4	4.8
TS	10	78.8	6.3	61.8	3.6	65.5	10.3	63.8	4.6	94.1	4.2
	100	77.9	7.5	66.7	2.5	73.5	3.5	67.5	4.3	82.8	0.9
KT	10	82.9	3.5	66.6	4.2	73.7	3.9	70.4	3.5	89.9	4.2
	100	102.6	7.1	65.5	1.6	73.3	2.0	69.7	1.3	81.0	1.0

$n = 5$

Table 3. MRL, LOD and LOQ values for each drug

Analytes	Cattle muscle			Swine muscle			Chicken muscle			Egg			Milk		
	MRL (μg/kg)	LOD (μg/kg)	LOQ (μg/kg)	MRL (μg/kg)	LOD (μg/kg)	LOQ (μg/kg)	MRL (μg/kg)	LOD (μg/kg)	LOQ (μg/kg)	MRL (μg/kg)	LOD (μg/kg)	LOQ (μg/kg)	MRL (μg/kg)	LOD (μg/kg)	LOQ (μg/kg)
OTC	200[a)]	2	4	200[a)]	1	2	200[a)]	2	4	400[a)]	1	4	100[a)]	1	4
TC	200[a)]	2	4	200[a)]	1	2	200[a)]	1	2	400[a)]	1	2	100[a)]	1	2
DC	100	2	4	50	2	4	50	1	2	—[b)]	1	4	—[b)]	2	4
CTC	200[a)]	2	4	200[a)]	1	4	200[a)]	1	2	400[a)]	1	2	100[a)]	1	2
CEX	200	2	4	10	2	4	—[b)]	2	4	—[b)]	4	10	100	2	4
ABPC	30	2	4	60	2	4	20	1	4	10	1	4	20	2	4
PCG	50	1	4	50	1	4	50	4	10	4	2	4	4	2	4
PCV	—[b)]	1	4	30	1	4	—[b)]	2	4	—[b)]	1	4	—[b)]	2	4
MPIPC	300	1	2	300	1	2	300	1	2	—[b)]	1	2	30	1	2
MCIPC	40	1	2	300	1	2	300	1	2	—[b)]	1	2	20	1	2
NFPC	5	1	2	5	1	2	5	1	2	5	1	2	5	1	2
MDIPC	30	1	2	300	1	2	300	1	2	—[b)]	1	2	10	1	2
SPM	200	0.1	0.5	200	0.5	2	200	0.1	0.5	—[b)]	0.1	0.5	200	0.1	0.5
TMS	100	1	4	100	1	4	70	0.1	0.5	—[b)]	0.5	2	50	0.1	0.5
MRM	—[b)]	0.1	0.5	50	0.1	0.5	40	0.1	0.5	—[b)]	0.1	0.5	—[b)]	0.1	0.5
OM	50	0.1	0.5	100	0.1	0.5	200	0.1	0.5	—[b)]	0.1	0.5	50	0.1	0.5
EM	50	0.5	2	50	0.5	2	50	1	4	90	1	4	40	0.5	2
TS	50	0.1	0.5	50	0.1	0.5	50	0.1	0.5	200	0.1	0.5	50	0.1	0.5
KT	—[b)]	0.1	0.5	200	0.1	0.5	200	0.1	0.5	200	0.5	2	—[b)]	0.1	0.5

$n = 3$

[a)] MRLs for oxytetracycline, tetracycline and chlortetracycline are established for the sum of residues of these three drugs.

[b)] MRL is not defined.

chloride were added to the tube, which was then vortexed immediately for 1 min. The sample was centrifuged at 3,000 rpm for 10 min, and the supernatant was poured into a 20 mL volumetric flask. After dilution with 0.5% formic acid in acetonitrile–methanol (8 : 2) to exactly 20 mL, a portion of the solution was transferred to a 1.5 mL microtube and centrifuged at 13,000 rpm for 5 min. 10 μL of supernatant was injected into the LC-MS/MS.

Quantification

Calibration curves were obtained from matrix-matched calibration curves, *i.e.,* calibration curves were calculated from peak areas of each chromatogram obtained from blank samples spiked with working standard solution to the level of 1, 2.5, 5, 10, 20, 50 μg/L. Five different concentrations among them were used.

Results and Discussion

LC-MS/MS analysis of analytes

MRM transitions of each analyte were determined by using 0.1% formic acid in 10 mmol/L ammonium formate and acetonitrile as the mobile phase; this is used in our routine laboratory tests. Full scans showed that $[M+H]^+$ or $[M-H]^-$ ions had the highest abundance for each analyte. Using those ions as the precursor ion, product ion scan was performed. The most abundant product ion was selected as the quantitative ion, and the second most abundant as the qualitative ion.

For LC separation, we examined two columns, CAPCELL PAK C18 MG3 and L-column 2 ODS. Since DC and CTC were not eluted from the former column, the L-column 2 ODS was adopted. It was confirmed that all analytes could be detected.

Extraction process

Based on previous reports[6)–9)], an extraction process was developed using CTC, CEX and ABPC as indicators, because these analytes are amphoteric compounds and are expected to be hard to extract with acetonitrile only. All studies below were performed after spiking 50 μL of 1 μg/mL standard mixture into cattle muscle.

First, buffering effects were compared between sodium acetate buffer and sodium citrate buffer. With sodium acetate buffer, the muscle sample formed a thick mass when reagents were added, and mixing was difficult. Recoveries were also poor. Therefore citrate buffer was adopted.

Second, extraction solvents consisting of mixtutes of acetonitrile and methanol with 1% acetic acid were compared. The result is shown in Fig. 1. Although recoveries improved as the ratio of methanol was increased, matrices derived from samples were increased and peak of matrices overlapped with analyte peaks. Therefore, we adopted acetonitrile–methanol (8 : 2) mixed solution.

Third, various acidic conditions were compared, *i.e.*, not adding or adding 0.1, 0.5 and 1% acetic acid or formic acid to the extraction solvent. Recoveries increased as the acidity was increased, but there was no difference between 0.5% and 1% formic acid. Hence 0.5% formic acid was added to the extraction solvent because matrix levels were lower than with 1%.

Fourth, various amounts of water were compared, *i.e.*, addition of 0, 2.5, 5 and 10 mL of water. When no water was added, recoveries were much lower for example CEX recovery was under 30% *versus* over 50% when water was added. There were no significant differences from 2.5 mL to 10 mL, so 2.5 mL of water was adopted.

SPE clean-up was skipped, because multi-class antibiotics have wide-ranging properties and absorption might lead to decreased recoveries[8), 9)]. Instead, high-speed centrifugation was carried out to remove particulates.

Recovery tests

In this study, recovery tests were conducted at two concentration levels (10 μg/kg for PCG and NFPC, and 10 and 100 μg/kg for others). Typical MRL chromatograms of all analytes in cattle muscle are shown Fig. 2.

Quantification values were obtained from matrix-matched calibration curves. Although the ionization efficiencies of PCG, PCV, MPIPC, MCIPC, NFPC, MDIPC, MRM, OM, EM, TS and KT were influenced slightly by matrices, others were greatly enhanced. Therefore matrix-matched calibration curves were used, and each calibration curve was exhibited good linearity (r^2>0.999). Results of recovery tests are shown in Table 2. Although recoveries of OTC, CEX and ABPC were slightly low and the precision, was insufficient, other analytes could be detected at the MRL. Therefore, this method is suitable for screening these targeted analytes.

Detection capacity as a screening method

The LOD and LOQ values were calculated at the *S*/*N* ratio of 3 and 10 for spiked samples. These results are shown in Table 3.

Application

This method was applied to samples purchased at

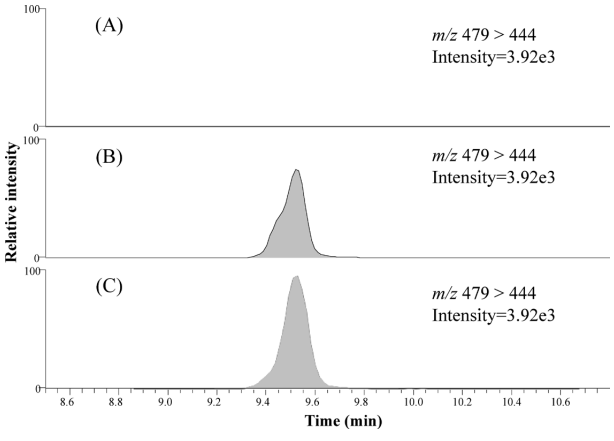

Fig. 3. Chromatograms showing the result of application

(A) negative sample of swine muscle (B) positive sample of swine sample (C) 5 μg/L standard solution of CTC.

markets in Tokyo. Ten samples each of muscles, egg and milk were analyzed, and no analytes were detected except in one sample of swine muscle, in which the MS/MS peak of CTC was detected and the quantification value was about 14 μg/kg. The chromatograms are shown in Fig. 3.

Conclusion

We have developed a screening assay for residues of 19 antibiotics (4 tetracyclines, 9 β-lactams and 7 macrolides) in livestock samples using LC-MS/MS and QuEChERS. This method is suitabe for monitoring these analytes in muscles, egg and milk, and should be applicable to routine laboratory testing for residual antibiotics in livestock samples.

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