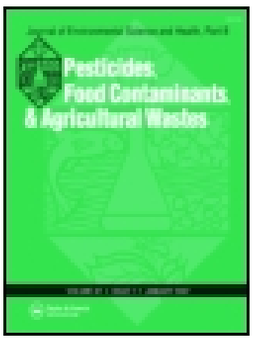

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Screening of antibiotic residues in fresh milk of Kathmandu Valley, Nepal

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ABSTRACT

The prevalence of two groups of antibiotics; namely penicillin and sulfonamides was studied in fresh milk available in Kathmandu Valley of Nepal. The milk samples (n = 140) were collected from three different sources; individual farmers, cottage dairies and organized dairies of Kathmandu valley. Qualitative and semi-quantitative analysis with rapid screening kits revealed that 23% samples were positive for antibiotic residues in the fresh milk for penicillin and sulfonamide groups (1–256 μg/kg). High performance liquid chromatography (HPLC) analyses detected 81% samples positive for amoxicillin (68–802 μg/kg), 41% for sulfadimethoxine (31–69 μg/kg), 27% for penicillin G (13–353 μg/kg), and 12% for ampicillin (0.5–92 μg/kg). Due to the precision and accuracy of liquid chromatography method, it detected more positive samples and consequently presented higher prevalence than the rapid screening kits. The antibiotic residues were found above the maximum residue limits that presented serious threat to consumer health and raised a serious concern regarding the implementation and monitoring of international regulations in developing countries.

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Antibiotic residues; fresh milk; high performance liquid chromatography (HPLC); Nepal; rapid screening test kits

Introduction

The antimicrobial agents have frequently been used in animal feed for prophylactic and therapeutic purposes and as growth promoting agents in subtherapeutic concentrations in livestock and poultry. Veterinary drug residues are the metabolites or degradation products of pharmacological compounds remaining in the food obtained from animals undergoing drug treatments and/ or used as growth promoters. Maximum residue limit (MRL) is the highest amount of residue remaining after the utilization of a veterinary and medicinal product (given in mg/kg or μg/kg on a fresh mass basis) which can be accepted by the public to be lawfully tolerable or acceptable in a food.[1] The overuse of veterinary antimicrobial drugs may lead to the presence of drug residues in animal derived foods that may directly affect the health of consumers.[2]

The quality of milk is impaired by the frequent use of veterinary antibiotics in animal husbandry.[3] The presence of antimicrobial residues in milk affects the dairy industry as the bacteria used for fermentation are sensitive to even subtherapeutic levels of frequently used antibiotics that results in impaired coagulation, maturation and development of desired organoleptic characteristics in the final dairy product.[4] This can be best exemplified by the fact that presence of β-lactam residues at MRLs or even below the limit can delay the coagulation of sheep milk yogurt by 40 min that affects the quality of final product.[5] The consumption of antibiotic residues through milk induces the allergic reactions and other chronic health problems in humans.[6] Furthermore, consumption of small doses of antibiotic can cause selective growth of resistant bacteria in the intestinal tracts resulting in their overgrowth.

The most common types of antimicrobials used in dairy animals in developing countries are sulfonamides, aminoglycosides, β-lactam, tetracyclines, macrolides, and quinolones.[7] β-Lactam antibiotics are extensively used in food animal practices against the bovine mastitis, pneumonia, bacterial arthritis and diarrhea.[8] Penicillin G, ampicillin, amoxicillin, cephalosporin, and cloxacillin are commonly used β-lactam antibiotics.[9] The antibiotic residues are likely to increase the public health concern due to the development of drug resistance in intestinal bacterial populations and among the opportunistic pathogens.[10]

In this study, the presence of antibiotic residues was evaluated by rapid screening kits followed by quantification of antibiotic residues by high performance liquid chromatography (HPLC) analyses of milk samples. The MRLs of penicillin and sulphonamides in milk, established by European Union, United States, and Canada are summarized in Table 1.[11,12] Most of the developing countries are lacking in the regulations of veterinary/antibiotic drug use in animal farming. This study aims to generate the awareness among the concerned agencies, farmers, and consumers regarding the proper use of antibacterial agents and such compounds.

Materials and methods

Sample collection

Cow milk samples (n = 140; 50 mL per sample) were obtained from three different sources: individual farmers (n = 69), cottage dairies (n = 38) and organized dairies (n = 33) (dairies with milk production capacity > 2000 L/day) of Kathmandu Valley of

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Table 1. Maximum residue limits (MRL) for penicillin and sulfonamides in milk established by European Union, United Sates, and Canada.

Antibiotic	Concentration (μg/kg)		
	Canada	European Union	United states
Amoxicillin	—	4	10
Ampicillin	10	4	10
Penicillin G	6	4	0
Sulfadimethoxine	10	—	10
Sulfonamides	—	100	—

Nepal. Samples were collected at the early morning and random sampling method was followed. The milk samples were collected in sterile bottles followed by coding (supplementary material, Table S1) and kept in refrigerator (4°C) for further analysis. The milk samples were stored at −40°C for HPLC analysis.

Chemicals and reagents

Penicillin G sodium salt (Sigma Aldrich, USA) [Sodium (2S,5R,6R)-3,3-dimethyl-7-oxo-6-[(phenylacetyl)amino]-4-thia-1-azabicyclo [3.2.0] heptane-2-carboxylate], sulfadimethoxine (Fluka) [4-Amino-N-(2,6-dimethoxy-4-pyrimidinyl) benzene sulfonamide], para amino benzoic acids (PABA) and penicillinase enzyme (Penase, Difco, USA: 100 mL x 1 unit, potency = 20,000 L.U./mL/min) were purchased from Rodejanarug Pharmaceutical Ltd., Thailand. Ampicillin trihydrate [(2S,5R,6R) -6-{[(2R)-2-Amino-2-phenylacetyl] amino}-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo [3.2.0] heptane-2-carboxylic acid trihydrate] and amoxicillin trihydrate [(2S,5R,6R)-6-{[(2R)-2-Amino-2-(4-hydroxyphenyl) acetyl] amino}-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo [3.2.0] heptane-2-carboxylic acid trihydrate] were received from National Medicine Laboratory of Department of Drug Administration, Nepal. All other chemicals used were of analytical grade.

Qualitative and semi-quantitative analysis of antibiotic residues

Rapid screening test

Rapid screening test is an agar diffusion test based on the inhibition of growth of *Bacillus stearothermophilus* var. *calidolactis* ATCC 10149. This test was performed by using rapid antibiotic residues screening kits according to the protocol mentioned by Rodejanarug Pharmaceutical Ltd. Thailand. The milk samples were heated in water bath at 82 ± 2°C for 2 min to denature the heat labile natural inhibitors and eliminate the microbial growth from the raw milk. The heated milk sample (100 μL) was added to the test kit. Negative control was the antibiotic free milk, supplied by the kit manufacturer and the positive control was prepared by spiking the antibiotics in the milk. All samples were then incubated for 2 h 45 min in water bath at 64 ± 2°C. The change in medium color was observed every 15 min.

Semi-quantitative test for residues of penicillin and sulfonamide group

The quantities of penicillin and sulfonamide residues were determined by using antibiotic residue kits and estimated by comparing the intensity of purple color (0–256 μg/kg) with the standard color chart provided by the manufacturer. Different concentrations of antibiotics with standard color chart were used for standardization of test kit for β-lactam group (penicillin) and sulfonamides.

Penicillin group

Penicillinase enzyme (0.05 mL) was added to positive milk sample (2 mL) and mixed by shaking for 1 min. The enzyme mixed sample (0.1 mL) was added to the test kits, followed by incubation at 64°C in water bath for 2 h 45 min. Intensity of purple color was measured by scale and compared with standard color chart to calculate the concentration of penicillin in the sample.

Sulfonamide group

A known amount (10 mg) of para-amino benzoic acid (PABA) was dissolved in 10 mL sterile distilled water. The resultant solution (7.5 μL) was introduced to test kit (for positive samples only) followed by the milk samples (100 μL). Same test was carried with the milk sample without PABA. All the test kits were incubated at 64°C for 2 h 30 min. The results were obtained by comparing the color intensity with the standard color chart.

Quantitative analysis of milk samples by high performance liquid chromatography

Extraction method and quantitative HPLC analysis for penicillin group

Sample for penicillin group was prepared by following the method described by Khaskheli et al.[13] with some modifications. A known amount (2.5 mL) of sample was taken in 10 mL sterilized pyrex screw capped centrifuge tubes and vortexed for 30 s with 200 μL of aqueous solution of acetic acid (10%, v/v). The mixture solution was transferred to small 2 mL sterile vials followed by centrifugation (3500 rpm) for 10 min at 4°C. The supernatant was removed by disposable syringe (while upper fat layer was left intact) and was filtered through 0.45 μm nylon filter. The filtrate was transferred into 1.5 mL HPLC sterile vials. Aliquots (10 μL) were injected into the Agilent HPLC 1100 system (Agilent, Germany). The separation was performed on LiChroCART RP-18 column (250× 4.6 mm, 5 μm, Purospher STAR Merk, USA). A binary solvent system was used as mobile phase consisting of 0.01 M potassium phosphate (monobasic) and methanol (60:40, v/v). The column temperature was set at 40°C with a solvent flow rate of 1 mL/min. The UV detector was monitored at 250 nm for penicillin group. The β-lactam antibiotic residues in each sample were identified by standard calibration curve method. The quantification of antibiotic residues in each sample was performed based on peak area comparison with standard calibration curve.

Extraction method and quantitative HPLC analysis for sulfonamides group

The samples for quantification of sulfonamides were prepared accroding to the method described by Chung et al.[7] with some modifications. A known amount of milk sample (500 mg) was vortexed for 2 min with 0.5 mL of potassium phosphate

solution (0.1%, w/v). Acetonitrile (5 mL) was added in the mixture solution, followed by centrigugation at 4000 rpm for 15 min. The supernatant was then mixed with 5 mL of hexane and further centrifuged for 5 min at 3500 rpm. The lower layer of solution was concentrated in water bath at 60°C for 1.5 h. The concentrate was dissolved in 250 μL of 50% acetonitrile (v/v) and 250 μL serile HPLC grade water. It was placed in ultrasonic bath for 10 min and centrifuged at 6000 rpm for 30 min. The extract was then passed through 0.2 μm nylon filter and injected (10 μL) to HPLC system. A binary solvent system was used as mobile phase consisting of (A) acetonitrile: 0.1% and (B) potassium phosphate (dibasic) solution (16:84, v/v). The column temperature was set at 40°C with a solvent flow rate of 1.2 mL/min. The UV detector was monitored at 270 nm for sulphonamides and residues were quantified by standard calibration curve method.

HPLC method validation

Different concentrations (5, 10, 20, 50, 100, and 200 μg/kg) of standards; penicillin G sodium salt, amoxicillin tri-hydrate, ampicillin tri-hydrate, and sulfadimethoxine were injected into HPLC and standard curves were made. Limit of detection (LOD), Limit of quantification (LOQ) and recovery rates were calculated for reliability and accuracy of the results.[7,14] LOD and LOQ were calculated by following Equations (1) and (2).[15]

$$\text{LOD(Limit of Detection)} : 3\times\text{SD/slope} \quad (1)$$

$$\text{LOQ(Limit of Quantification)} : 10\times\text{SD/slope} \quad (2)$$

To determine the recovery rates, 100 μg/kg of amoxicillin, ampicillin, penicillin G, and sulfadimethoxine standards were separately added to the milk samples and recovery rates were calculated by following Equation (3).[15]

$$\%\ \text{Recovery} = \frac{(\text{Concentration of spiked sample} - \text{Concentration of un-spiked sample})}{(\text{Concentration of added antibiotic})} \quad (3)$$

Statistical analysis

One-way analysis of variance (ANOVA) and Tukey tests were carried out to determine significant group differences ($p < 0.05$) between means by using SPSS statistical software package (SPSS, version 16.0).

Results and discussion

Antibiotic residue in fresh milk analysed by rapid screening test kits

Out of 140 milk samples tested by rapid screening kits, 23% samples were found positive for the antibiotic residues. Among the positive samples, 38% samples were positive for penicillin group and 78% were positive for sulfonamide group. The results indicated that 27% samples were positive for antibiotic residues in organized dairy 1 and was significantly higher ($p < 0.05$) than other origins of milk samples whereas, 23% and 21% milk samples were positive from individual farmers and cottage dairies, respectively. In organized dairy 2, 18% milk samples were positive for antibiotic residues which was significantly ($p < 0.05$) lower among all sample sources.

Semi-quantitative tests revealed that all positive samples contained antibiotic residues in the concentration range of 1–256 μg/kg for penicillin and sulfonamides (Table 2). Due to semi-quantitative nature of the test, the exact concentration of antibiotic residues could not be detected. The values were calculated according to the color chart provided by the manufacturer in a specific concentration range for each group.

Table 2. Semi-quantitative analysis of penicillin and sulfonamides residues.

		Number of positive samples			
Antibiotics	Concentration (μg/kg)	Individual farmer	Cottage dairy	Organized dairy 1	Organized dairy 2
Penicillin	0	6	10	3	—
	0–1	1	2	3	2
	8–16	—	—	1	—
	128–256	2	—	—	—
Sulfonamide (with PABA)	0	1	2	2	1
	0–1	2	—	2	—
	1–2	1	—	2	—
	8–16	1	—	—	—
	16–32	—	2	—	—
	32–64	—	5	—	—
	128–256	4	3	1	1
Sulfonamide (without PABA)	0	3	9	5	1
	0–1	1	—	1	—
	2–4	1	1	—	—
	64–128	—	—	1	—
	128–256	4	2	—	1

Where (—) indicates not detected. Total number of samples; n = 140 (Individual farmer = 69, cottage dairy = 38, organized dairy 1 = 22, and organized dairy 2 = 11).

The average level of the residue was in the range of 0–16 μg/kg for penicillin (except 2 samples that were in the range of 128–256 μg/kg) and 0–64 μg/kg for sulfonamides (except for 9 samples, that were in the range of 128–256 μg/kg). The prevalence of antibiotic residues was found to be higher than previously reported by Veterinary Standard and Drug Administration Office of Nepal,[16] which reported the antibiotic residues in the range of 1–2 μg/kg for penicillin in milk samples. Higher levels of antibiotic residue in this study might be due to excessive use of antibiotics during late rainy or early autumn season (time when the samples were collected), when the incidence of mastitis and other diseases is comparatively higher. Thapaliya et al.[17] reported lower levels of antibiotic residues (milk samples collected in winter) compared to the current study that might be due to less use of antibiotics during winter season. The results of current investigation corroborate with the previous study reported by Yamaki et al.[18] who described the seasonal factor affecting the prevalence of the antibiotics in summer—early autumn.

Due to lack of robustness for precise assessment of antibiotic residues with the rapid screening kit method, the milk samples were further subjected to HPLC analysis for the quantitative analysis of antibiotics residues.

Antibiotic residue analysed by HPLC method

HPLC analyses showed that 81% of milk samples were found to be positive for amoxicillin, 41% for sulfadimethoxine, 27% for penicillin G, and 12% for ampicillin residues. Amoxicillin residues were detected in the highest concentration while the presence of ampicillin residues was the lowest among the four types of antibiotics analyzed.

The result based on the origins of milk revealed that the highest percentage of amoxicillin (87%) and penicillin G (45%) were found in milk samples from cottage dairies. Whereas, 27% of milk samples from organized dairy 2 were found positive for ampicillin and 55% for sulfadimethoxine residues. The prevalence of ampicillin (9%) and penicillin G (17%) was significantly lower ($p < 0.05$) in the milk samples from individual farmers and prevalence of sulfadimethoxine (32%) was lowest ($p < 0.05$) in the samples from organized dairy 1 (Figure 1).

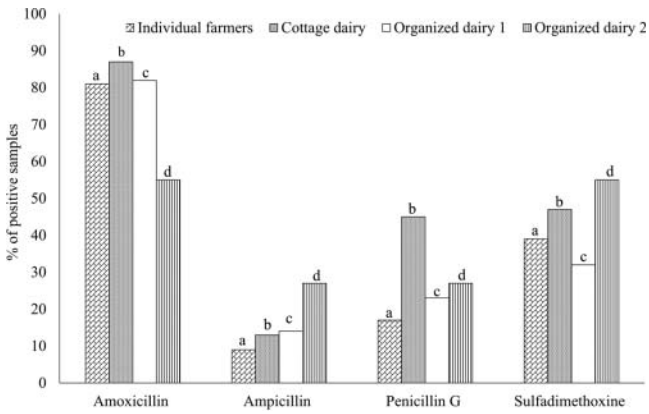

Figure 1. Percentage of positive samples for antibiotic residue on the basis of the source (individual farmers, cottage dairy, organized dairy 1 and 2) of milk samples detected by HPLC. Different superscript letters (a–d) present significant difference between the groups.

Quantification of antibiotic residues was done by HPLC analyses of milk samples and the samples were categorized according to concentration range of antibiotic residues (Table 3); amoxicillin (68–802 μg/kg), sulfadimethoxine (31–69 μg/kg), penicillin G (13–353 μg/kg), and ampicillin (0.5–92 μg/kg).

The standard calibration equations, recovery rates, LOD and LOQ of tested antibiotic standards were presented in Table 4. The regression coefficient (R^2) of standard curves showed superior linearity. Recovery rates were in the range of 75% to 106%, which were in the array of good recovery. LOD and LOQ of penicillin group were in the range of 0.85–1.07 μg/kg and 2.83–3.57 μg/kg, respectively, whereas, LOD and LOQ of sulfadimethoxine were 1.14 and 3.80 μg/kg, respectively.

Recent reports are revealing about the public health concerns due to the presence of antibiotic residues in various animal-based foods. Allergic reactions of antibiotics likely to occur when a pre-sensitized individual is challenged by exposure to antibiotics.[19] Allergic reactions due to β-lactam antibiotic residues in milk have been characterized by dermatitis, pruritus and urticaria in pre-sensitized individuals.[20] The increased risk of immuno-allergic reactions has been reported due to β-lactams and macrolides residues in food products.[21] Consumption of subtherapeutic dose of antibiotics for prolong period might results in the emergence of antibiotic resistant strains of bacteria. Besides this, it has also negative impact on dairy processing industries such as yoghurt and cheese manufacturing industries.[22]

In this study, the residue levels of amoxicillin and penicillin G in all the positive milk samples were above the MRLs (4 μg/kg) according to limits established by European Union (EU),[12] however, for ampicillin, 56.25% of positive samples were above MRLs. The residue levels of sulfonamide (sulfadimethoxine) in milk samples were below the MRLs according to the regulations established by EU (sum of all sulfonamides <100 μg/kg per sample).[7] However, the sulfadimethoxine residue levels in all the positive samples were above the MRLs (10 μg/kg), according to limits established by Canada, Republic of Korea and United States for sulfadimethoxine.[23] Occurrence of more than one type of antibiotic residues in one sample might be due to the use of medicated feed or simultaneous use of different types of antibiotics intravenously/systemically or locally at the udder.[8] The main reason behind the use of more than one type of antibiotic is due to the malpractice of animal health technicians and poor or no enforcement of veterinary drugs regulations. More use of the antibiotics in lactating animals would result in elevated secretion of antibiotics in the milk.[24] The high prevalence of β-lactam antibiotics (penicillin G, amoxicillin, and ampicillin) in this study is attributed to their frequent use in the treatment of mastitis and other systemic diseases.[25]

In Nepal, quantitative analysis for antibiotic residues in milk has not yet been extensively conducted for broad range of antibiotics with precise analytical methods and hence there is lack of such data and awareness among the concerned agencies, dairy farmers and consumers. Only limited numbers of research reports are available for qualitative and semi-quantitative analyses.

Table 3. Quantitative analysis of antibiotic residues in milk samples by HPLC.

	Concentration of antibiotic residues (μg/ kg)			
Sample No.	Amoxicillin	Ampicillin	Penicillin G	Sulfadimethoxine
1	459 ± 0.03	—	—	31 ± 0.00*
2	126 ± 0.02	—	14 ± 0.3	61 ± 0.04*
3	68 ± 0.03	—	13 ± 0.4	31 ± 0.05*
4	152 ± 0.03	—	15 ± 0.6	—
5	242 ± 0.02	—	19 ± 0.4	31 ± 0.03*
6	237 ± 0.02	—	—	—
7	365 ± 0.02	—	—	—
8	479 ± 0.01	—	13 ± 0.2	37 ± 0.20*
9	297 ± 0.02	—	—	—
10	378 ± 0.03	—	—	50 ± 0.02*
21	435 ± 0.03	—	14 ± 0.4	31 ± 0.30*
22	490 ± 0.02	—	—	—
23	272 ± 0.01	—	—	—
24	240 ± 0.05	—	—	—
25	231 ± 0.02	81 ± 0.2	—	—
26	359 ± 0.01	—	—	—
27	292 ± 0.04	—	—	—
29	266 ± 0.04	—	—	—
30	431 ± 0.02	—	—	—
31	357 ± 0.03	—	—	—
32	282 ± 0.04	—	—	—
34	297 ± 0.1	7 ± 0.1	—	61 ± 0.00*
35	319 ± 0.04	—	—	—
36	362 ± 0.03	—	14 ± 0.3	—
37	259 ± 0.03	—	—	—
38	213 ± 0.03	—	72 ± 0.3	—
39	561 ± 0.04	—	—	—
46	376 ± 0.02	3 ± 0.2**	—	32 ± 0.01*
47	82 ± 0.03	3 ± 0.0**	—	33 ± 0.03*
48	162 ± 0.03	—	—	31 ± 0.00*
49	123 ± 0.04	—	100 ± 0.4	—
50	233 ± 0.01	—	55 ± 0.9	—
51	138 ± 0.04	17 ± 1.0	—	—
52	134 ± 0.03	—	60 ± 0.2	—
53	437 ± 0.07	—	—	33 ± 0.00*
54	228 ± 0.04	—	—	—
55	459 ± 0.02	—	—	35 ± 2.00*
56	492 ± 0.04	0.7 ± 0.2**	—	39 ± 0.06*
57	195 ± 0.03	—	—	60 ± 0.05*
58	294 ± 0.04	—	—	—
59	156 ± 0.04	—	89 ± 0.4	—
60	246 ± 0.04	—	—	—
61	182 ± 0.02	—	—	59 ± 0.00*
62	229 ± 0.02	—	—	31 ± 0.03*
63	232 ± 0.05	—	—	67 ± 0.01*
65	561 ± 0.04	—	—	56 ± 0.01*
66	237 ± 0.70	—	—	56 ± 0.01*
67	303 ± 0.03	—	—	63 ± 0.02*
68	188 ± 0.04	—	—	31 ± 0.04*
69	367 ± 0.05	—	—	69 ± 0.06*
70	136 ± 0.05	0.5 ± 0.1**	—	37 ± 0.50*
71	322 ± 0.04	—	—	65 ± 0.02*
72	316 ± 0.02	—	—	—
73	199± 0.07	—	—	—
74	355 ± 0.04	—	—	—
75	248 ± 0.03	—	120 ± 0.6	32 ± 0.06*
76	304 ± 0.03	4 ± 0.0	—	62 ± 0.01*
77	271 ± 0.04	—	84 ± 0.1	31 ± 0.04*
78	194 ± 0.04	—	—	55 ± 0.01*
79	549 ± 0.03	—	205 ± 0.4	—
80	429 ± 0.02	—	—	33 ± 0.03*
81	343 ± 0.02	—	98 ± 0.4	—
82	548 ± 0.03	—	—	33 ± 0.04*
83	344 ± 0.03	—	83 ± 0.3	39 ± 0.05*
84	302 ± 0.02	—	—	—
85	518 ± 0.03	—	353 ± 0.2	—
86	342 ± 0.03	—	—	—
87	413 ± 0.03	—	—	33 ± 0.02*
88	407 ± 0.03	—	—	—
89	79 ± 0.01	—	49 ± 0.5	31 ± 0.04*

(*Continued on next page*)

Table 3. (Continued).

Sample No.	Concentration of antibiotic residues (μg/ kg)			
	Amoxicillin	Ampicillin	Penicillin G	Sulfadimethoxine
90	371 ± 0.04	—	—	31 ± 0.00*
91	201 ± 0.03	—	—	33 ± 0.02*
92	373 ± 0.02	—	—	59 ± 0.01*
93	370 ± 0.02	—	—	—
94	204 ± 0.04	—	—	—
95	779 ± 0.03	51 ± 0.1	180 ± 0.5	—
96	174 ± 0.01	—	—	37 ± 0.10*
97	234 ± 0.03	2 ± 0.1**	26 ± 0.4	31 ± 0.02*
98	802 ± 0.03	—	30 ± 0.3	—
99	100 ± 0.02	—	13 ± 0.3	—
100	750 ± 0.00	58 ± 0.1	—	—
101	247 ± 0.02	—	47 ± 0.1	31 ± 0.04*
102	499 ± 0.02	—	—	—
103	370 ± 0.10	—	—	32 ± 0.03*
104	397 ± 0.04	—	24 ± 0.5	32 ± 0.02*
105	235 ± 0.04	2 ± 0.2**	—	37 ± 0.50*
106	120 ± 0.04	—	—	—
107	220 ± 0.01	2 ± 0.0**	—	—
108	253 ± 0.04	—	—	—
110	296 ± 0.02	—	—	—
111	173 ± 0.05	—	107 ± 0.2	32 ± 0.04*
112	253 ± 0.04	—	—	—
113	657 ± 0.02	24 ± 0.2	58 ± 0.1	—
114	326 ± 0.01	—	51 ± 0.3	—
115	234 ± 0.03	—	—	—
116	267 ± 0.03	—	98 ± 0.5	32 ± 0.01*
117	223 ± 0.01	—	55 ± 0.5	34 ± 0.00*
118	256 ± 0.04	—	—	32 ± 0.01*
120	151 ± 0.05	—	—	—
121	253 ± 0.01	—	14 ± 0.1	32 ± 0.01*
122	267 ± 0.04	—	61 ± 0.2	31 ± 0.03*
123	—	—	—	37 ± 0.30*
126	433 ± 0.08	—	—	—
127	266 ± 0.03	—	15 ± 0.5	—
128	183 ± 0.05	—	—	56 ± 0.04*
129	—	6 ± 0.2	—	36 ± 2.00*
130	159 ± 0.02	—	—	—
131	193 ± 0.07	—	—	—
132	292 ± 0.05	—	—	62 ± 0.07*
133	190 ± 0.02	—	—	32 ± 0.60*
135	212 ± 0.03	—	—	33 ± 0.03*
136	604 ± 0.03	—	—	—
137	632 ± 0.03	—	—	—
138	182 ± 0.05	—	—	—
139	—	—	—	34 ± 0.02*
140	246 ± 0.02	92 ± 0.5	—	34 ± 0.20*

Where (—) indicates not detected. Total number of samples; n = 140.
*Below MRLs established by EU.
**Below MRLs established by EU but above MRLs established by Canada, Republic of Korea and United States.
All other samples were above MRLs.

Table 4. Parameters of calibration curves, recovery rates, limit of detection and limit of quantification of antibiotics.

Antibiotics	Standard calibration equation (y = ax + b)			Recovery rates (%)	LOD (μg/kg)	LOQ (μg/kg)
	a	b	R^2			
Amoxicillin	199.17	769.39	0.9977	106	0.97	3.22
Ampicillin	792.37	−1211.3	0.9949	96	1.07	3.57
Penicillin G	241.91	−769.95	0.9936	75	0.85	2.83
Sulfadimethoxine	56722	51929	0.9967	92	1.14	3.80

Conclusion

The wide range of antibiotics residues detected in the milk samples from Kathmandu valley reflected the haphazard use of antibiotics for the treatment of infections and practice of milk withdrawal without following the regulations. Prevalence of high antibiotic residues is also related to the lack of education and lack of awareness among the people. In

developing country like Nepal, there is no strict legislative standard for antibiotic residues in food due to lack of surveillance and monitoring system from the concerned authorities. This study, which is first of its kind, would serve as a base line data on the prevalence of antibiotic residue in milk. This could be useful for the formulation and development of the programs and plans to minimize the use of antibiotics in dairy food chain. However, further comprehensive study is warranted to know the residual effects of detected antibiotics in human health.

Acknowledgments

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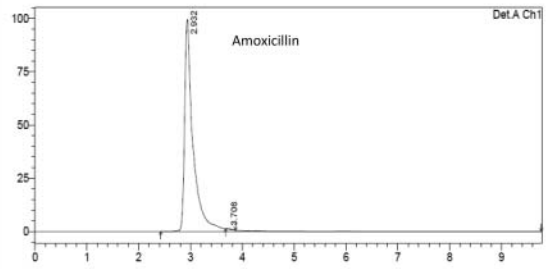
Supplementary Materials

Table S1. Sample coding.

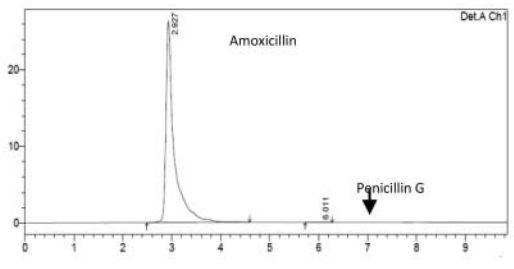
Sample Number	Source of Milk	Sample Number	Source of Milk
1	Cottage Dairy	**71**	Individual farmers
2	Cottage Dairy	**72**	Individual farmers
3	Cottage Dairy	**73**	Individual farmers
4	Cottage Dairy	**74**	Individual farmers
5	Cottage Dairy	**75**	Cottage dairies
6	Cottage Dairy	**76**	Individual farmers
7	Cottage Dairy	**77**	Individual farmers
8	Cottage Dairy	**78**	Individual farmers
9	Cottage Dairy	**79**	Individual farmers
10	Cottage Dairy	**80**	Individual farmers
11	Cottage Dairy	**81**	Individual farmers
12	Cottage Dairy	**82**	Individual farmers
13	Individual farmer	**83**	Individual farmers
14	Individual farmer	**84**	Individual farmers
15	Individual farmer	**85**	Individual farmers
16	Individual farmer	**86**	Individual farmers
17	Individual farmer	**87**	Individual farmers
18	Individual farmer	**88**	Individual farmers
19	Individual farmer	**89**	Cottage Dairies
20	Individual farmer	**90**	Cottage Dairies
21	Individual farmer	**91**	Cottage Dairies
22	Individual farmer	**92**	Cottage Dairies
23	Individual farmer	**93**	Organized Dairy 1
24	Individual farmer	**94**	Organized Dairy 1
25	Individual farmer	**95**	Organized Dairy 2
26	Individual farmer	**96**	Organized Dairy 2
27	Individual farmer	**97**	Cottage Dairies
28	Individual farmer	**98**	Cottage Dairies
29	Individual farmer	**99**	Cottage Dairies
30	Individual farmer	**100**	Cottage Dairies
31	Individual farmer	**101**	Cottage Dairies
32	Individual farmer	**102**	Cottage Dairies
33	Individual farmer	**103**	Cottage Dairies
34	Individual farmer	**104**	Individual farmer
35	Individual farmer	**105**	Organized Dairy 1
36	Cottage Dairy	**106**	Organized Dairy 1
37	Cottage Dairy	**107**	Organized Dairy 1
38	Cottage Dairy	**108**	Organized Dairy 1
39	Cottage Dairy	**109**	Organized Dairy 1
40	Cottage Dairy	**110**	Organized Dairy 1
41	Cottage Dairy	**111**	Organized Dairy 1
42	Cottage Dairy	**112**	Organized Dairy 1
43	Organized Dairy 1	**113**	Organized Dairy 1
44	Organized Dairy 1	**114**	Individual farmers
45	Organized Dairy 1	**115**	Individual farmers
46	Cottage dairy	**116**	Organized Dairy 1
47	Cottage dairy	**117**	Organized Dairy 1
48	Cottage dairy	**118**	Organized Dairy 1
49	Cottage dairy	**119**	Individual farmers
50	Individual farmer	**120**	Individual farmers
51	Cottage dairy	**121**	Individual farmers
52	Cottage dairy	**122**	Individual farmers
53	Cottage dairy	**123**	Organized Dairy 2
54	Individual farmer	**124**	Organized Dairy 2
55	Individual farmer	**125**	Organized Dairy 2
56	Individual farmer	**126**	Organized Dairy 2
57	Individual farmer	**127**	Organized Dairy 2
58	Individual farmer	**128**	Organized Dairy 2
59	Individual farmer	**129**	Organized Dairy 2
60	Organized Dairy 1	**130**	Organized Dairy 1
61	Organized Dairy 1	**131**	Organized Dairy 1
62	Organized Dairy 1	**132**	Individual farmers
63	Individual farmer	**133**	Individual farmers
64	Individual farmer	**134**	Individual farmers
65	Individual farmer	**135**	Individual farmers
66	Individual farmer	**136**	Individual farmers
67	Individual farmer	**137**	Individual farmers
68	Individual farmer	**138**	Organized Dairy 2
69	Individual farmer	**139**	Organized Dairy 2
70	Individual farmer	**140**	Individual farmers

Table S2. Chromatograms of penicillin (amoxicillin, ampicillin and penicillin G).

Chromatogram of sample 1



Chromatogram of sample 2



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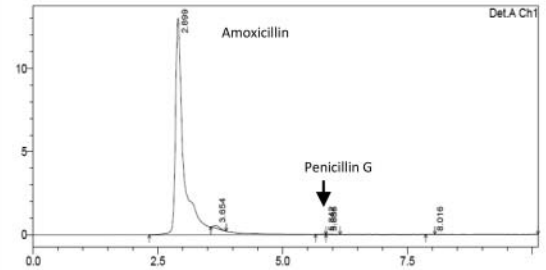
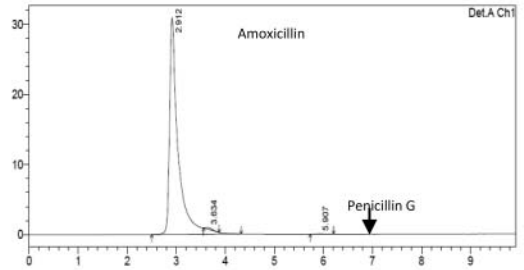
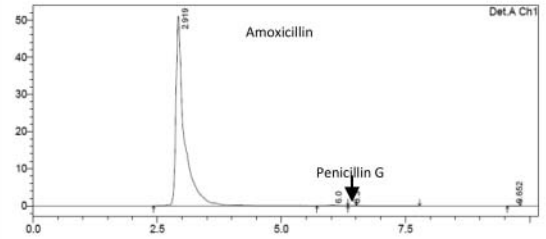


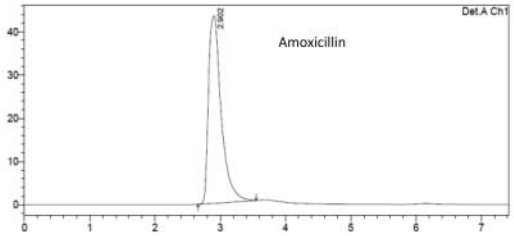
Chromatogram of sample 4



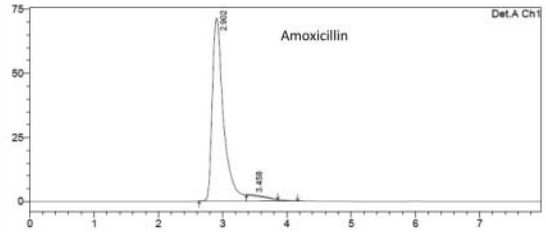
Chromatogram of sample 5



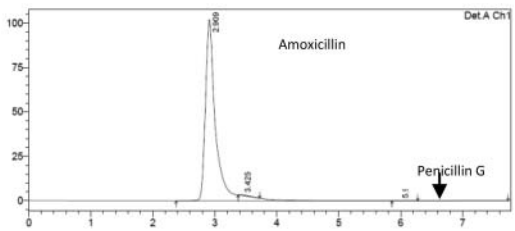
Chromatogram of sample 6



Chromatogram of sample 7



Chromatogram of sample 8



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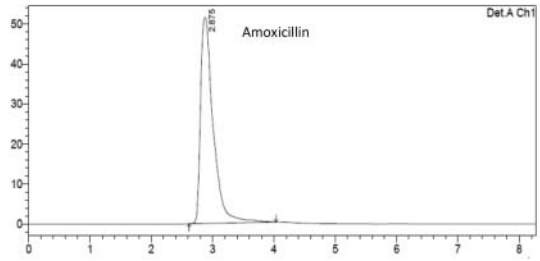
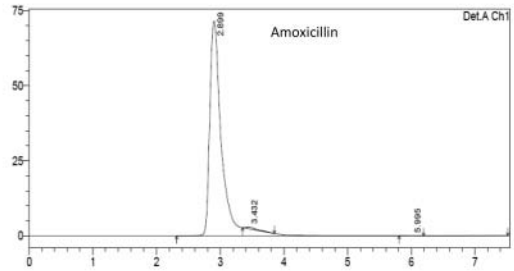


Chromatogram of sample 10



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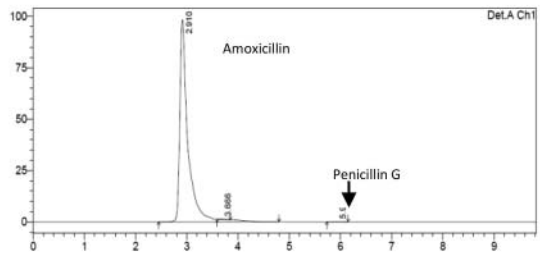
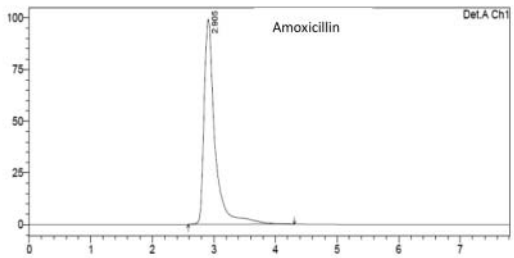


Chromatogram of Sample 22



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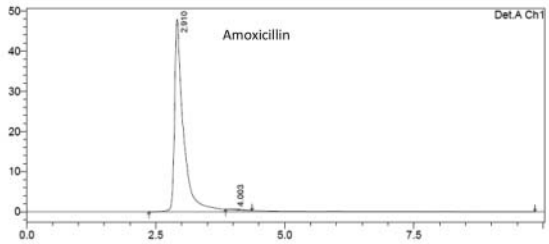
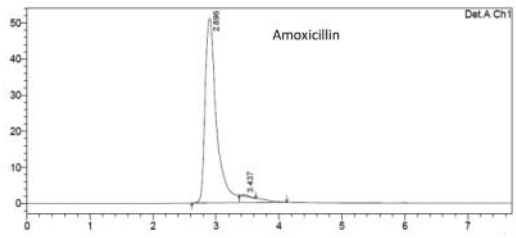


Chromatogram of Sample 24



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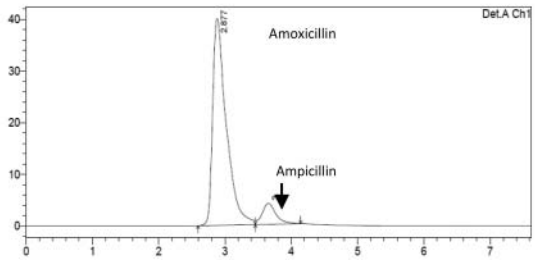
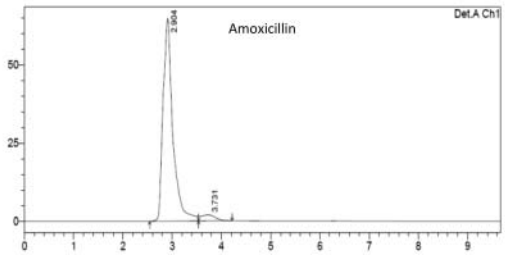


Chromatogram of Sample 26



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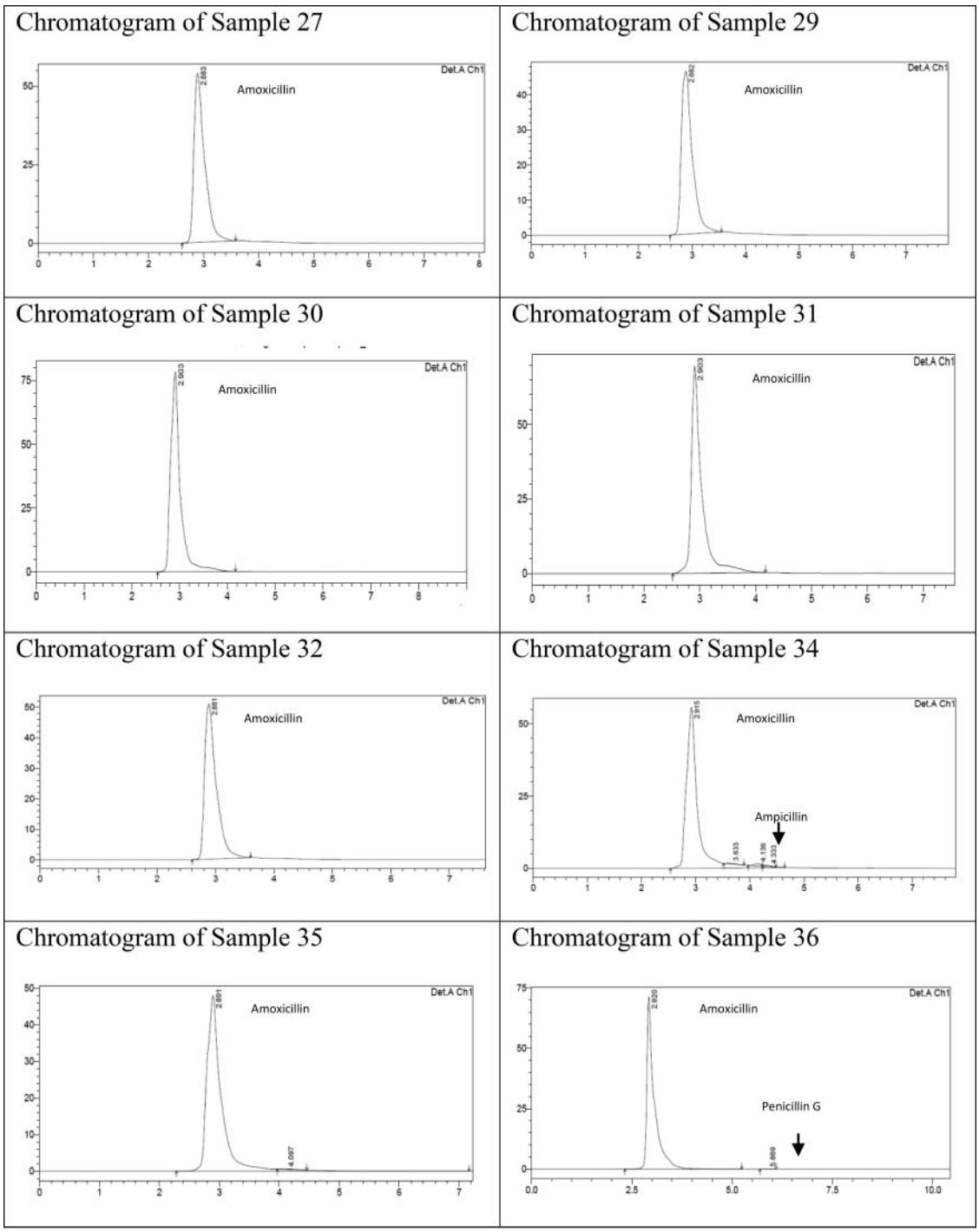

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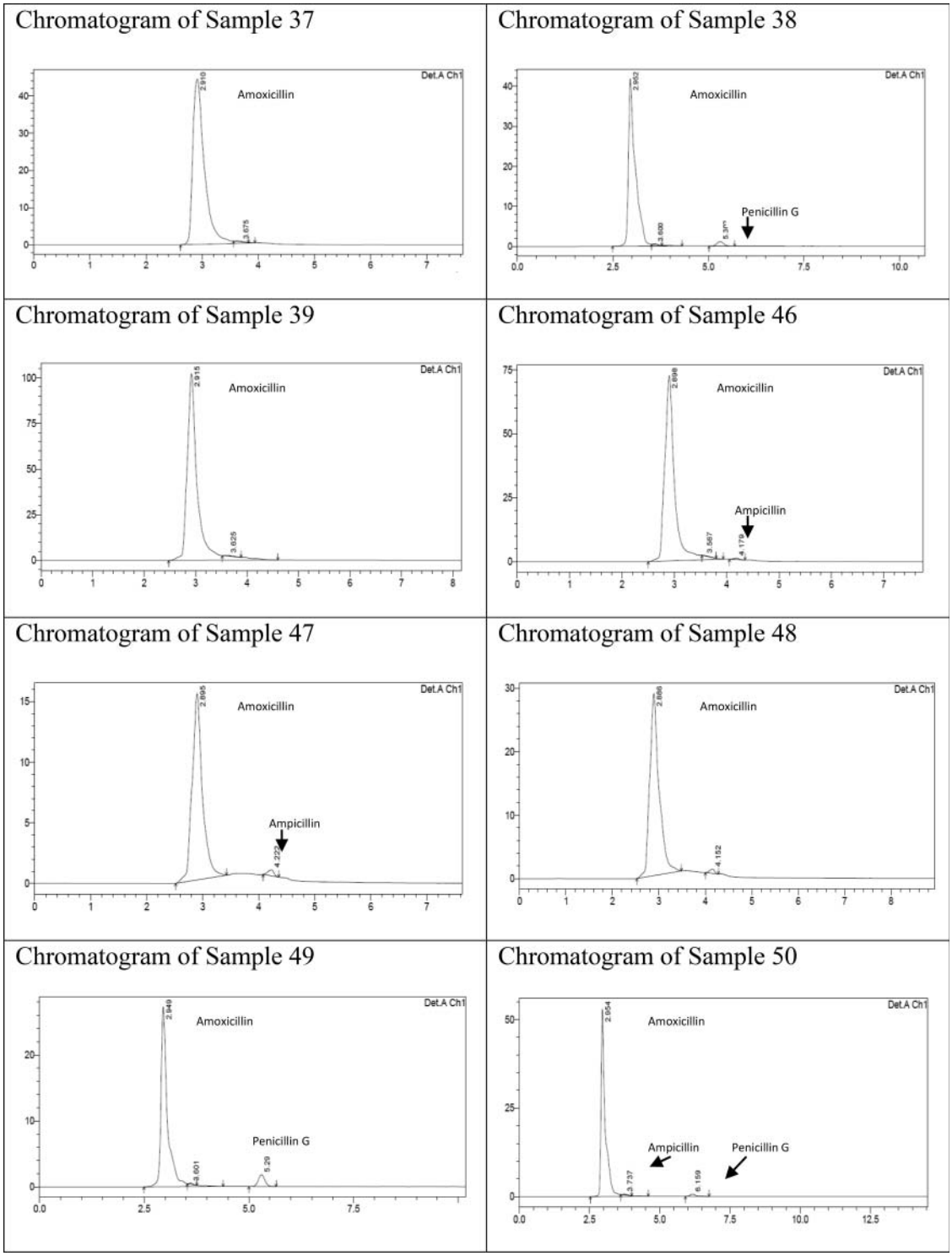

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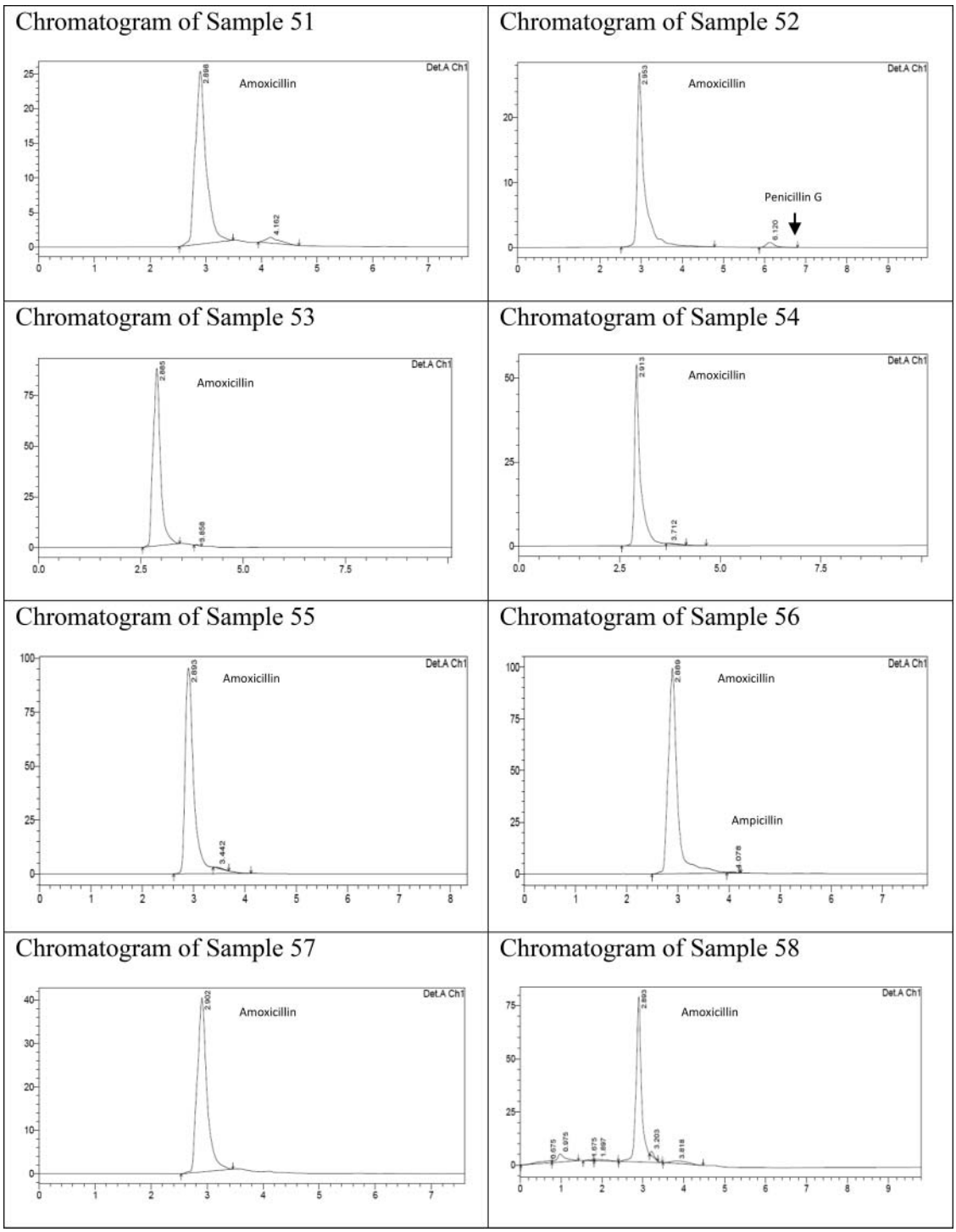

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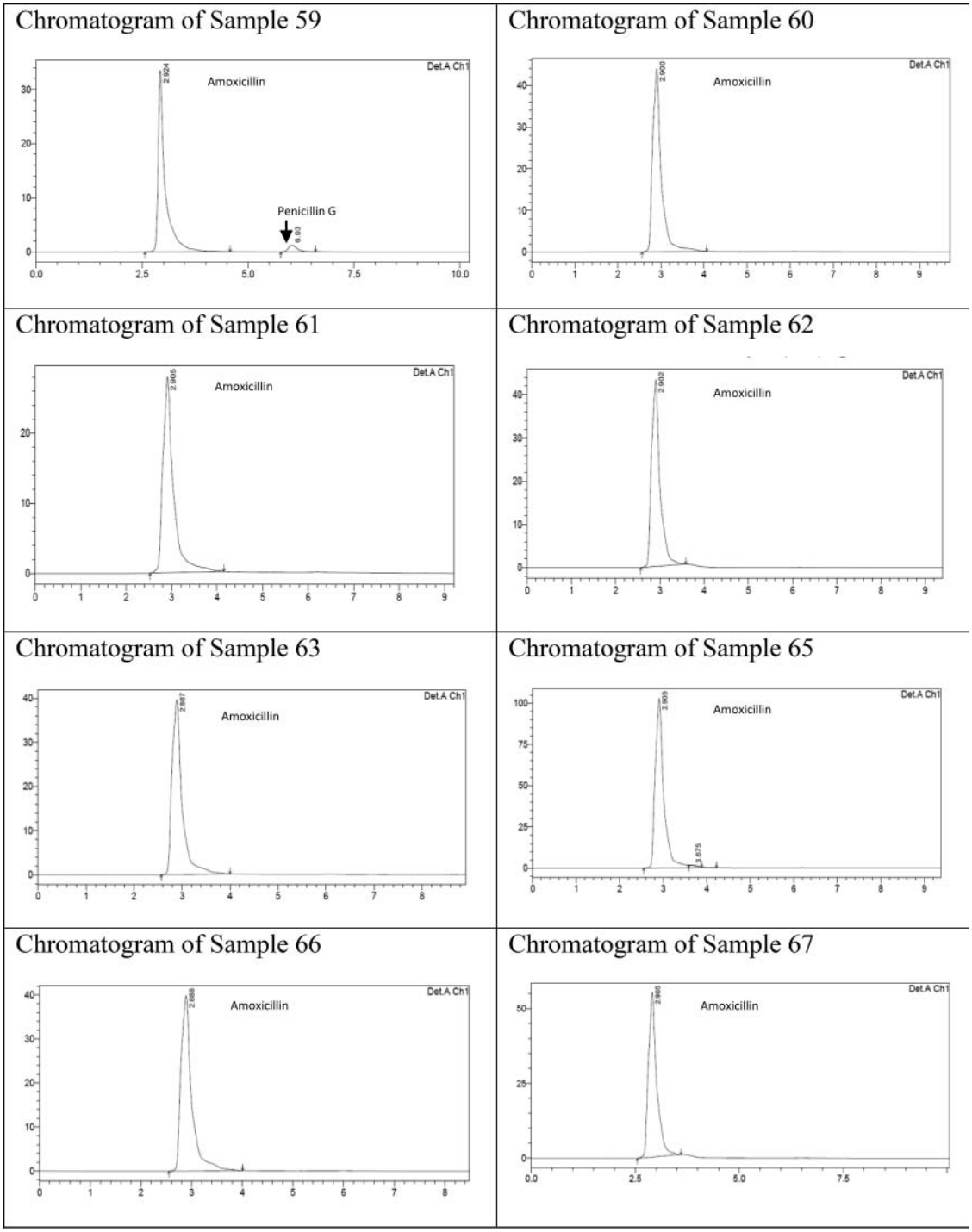

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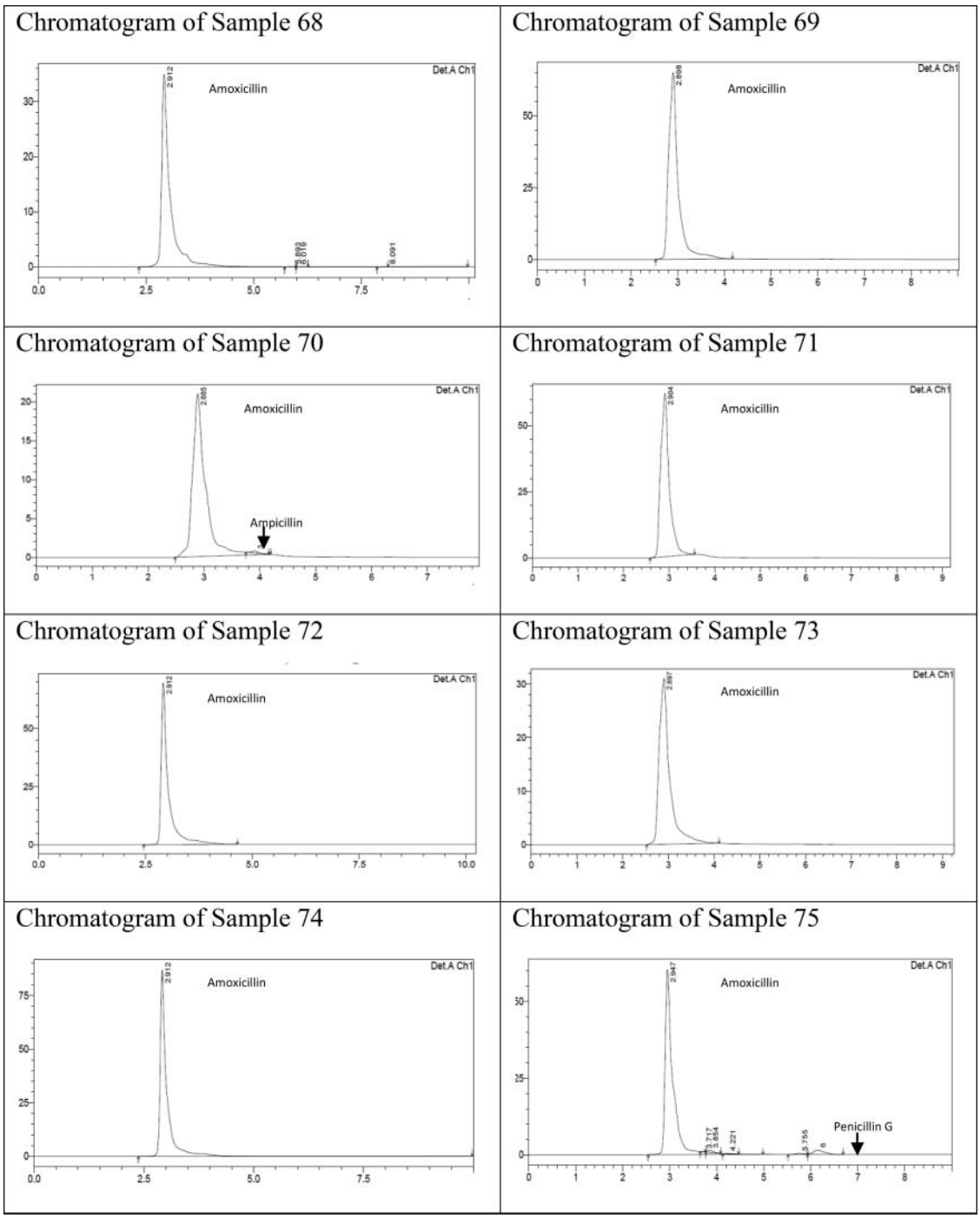

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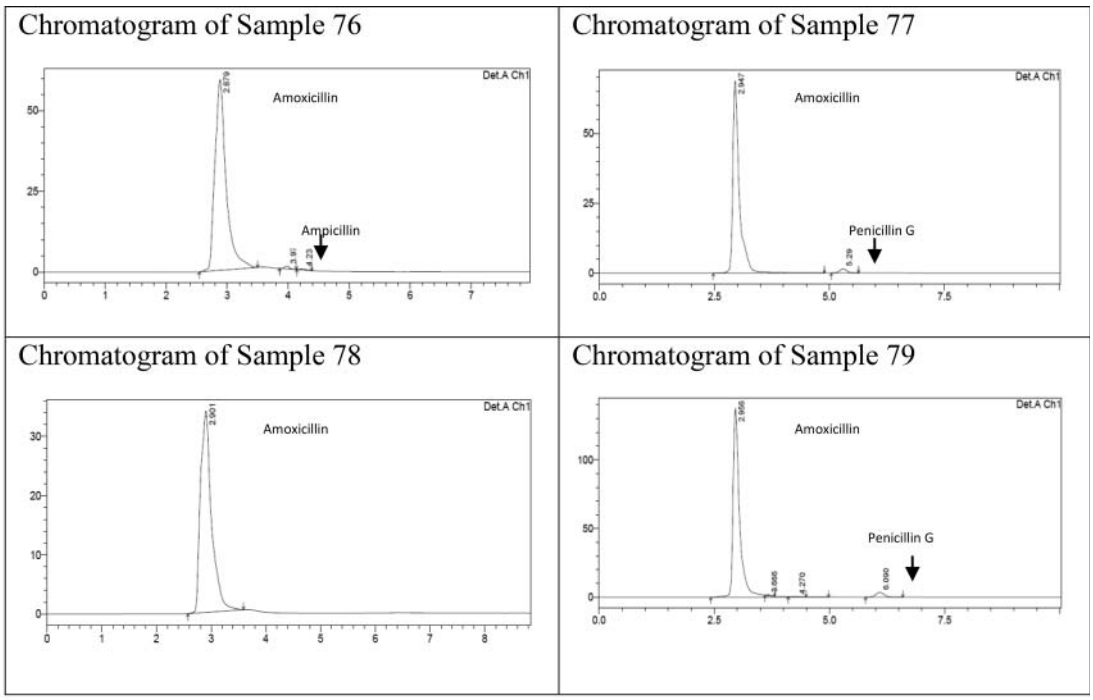

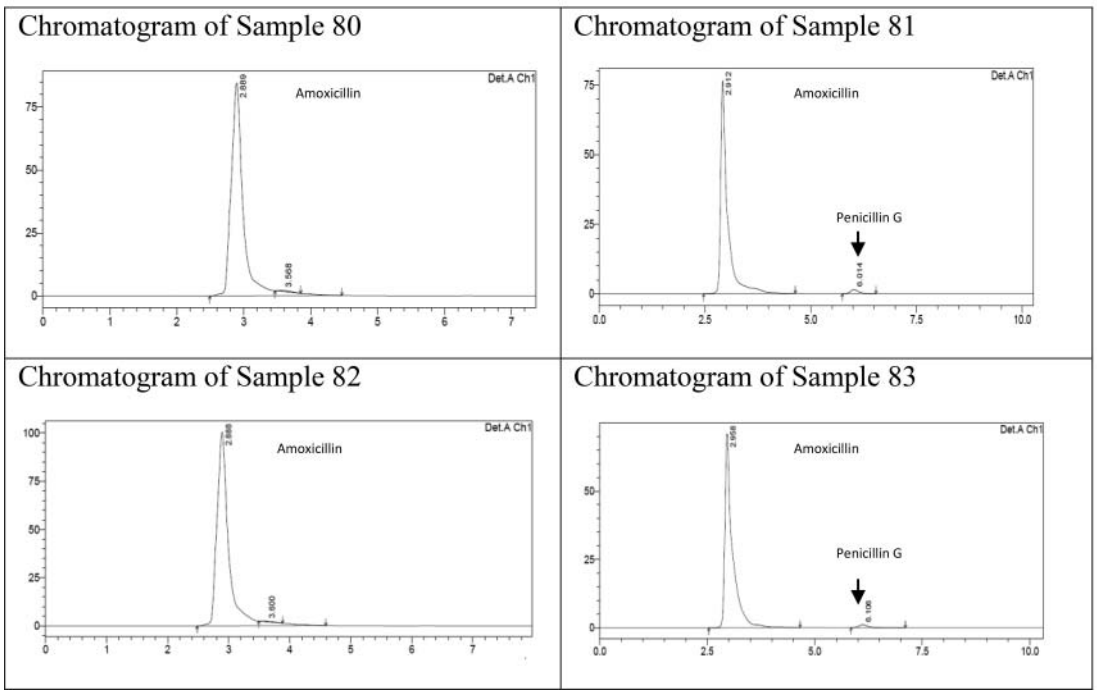

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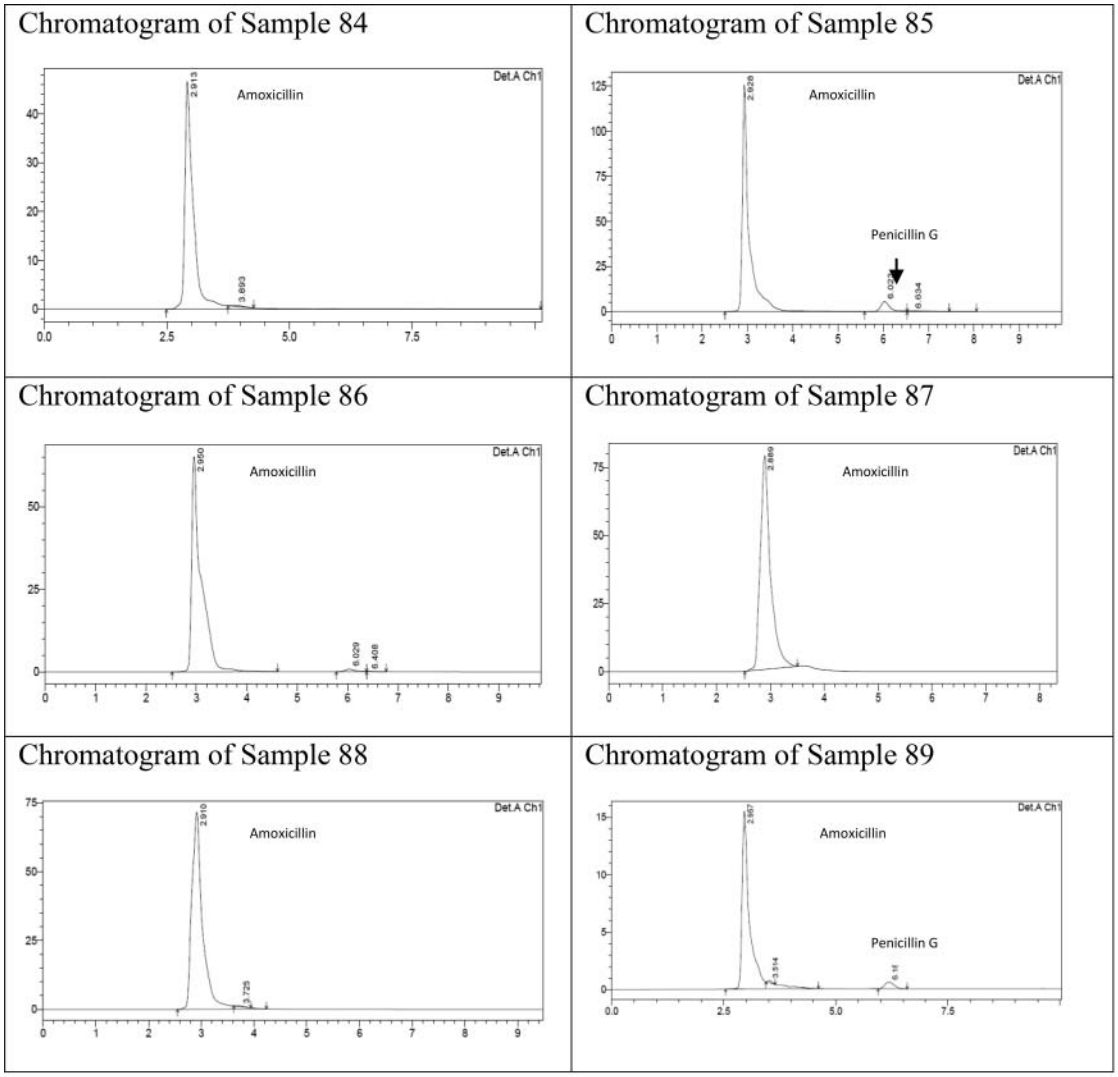

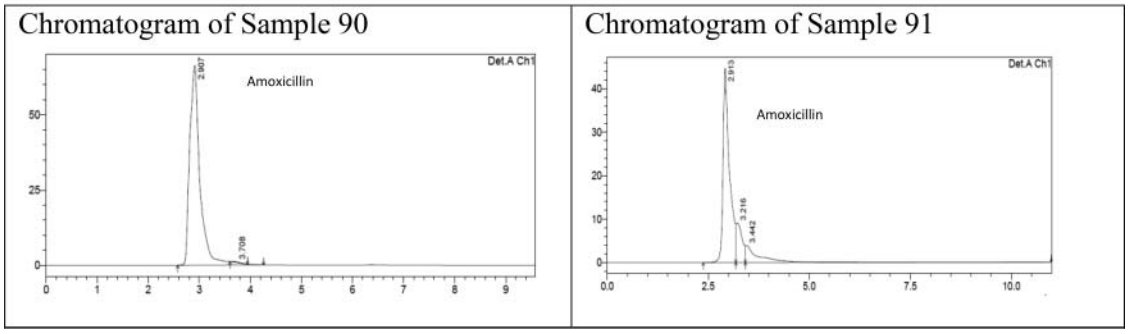

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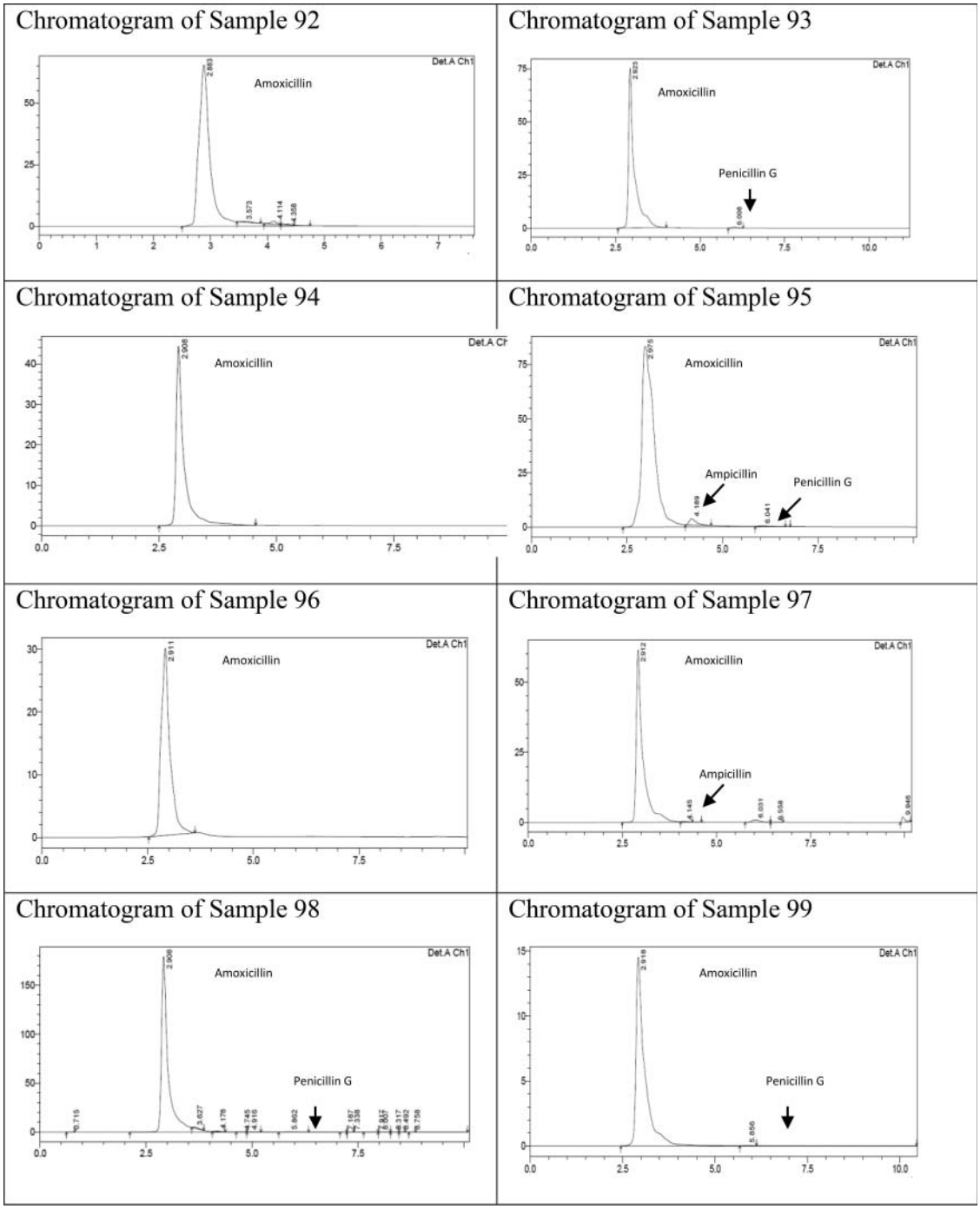

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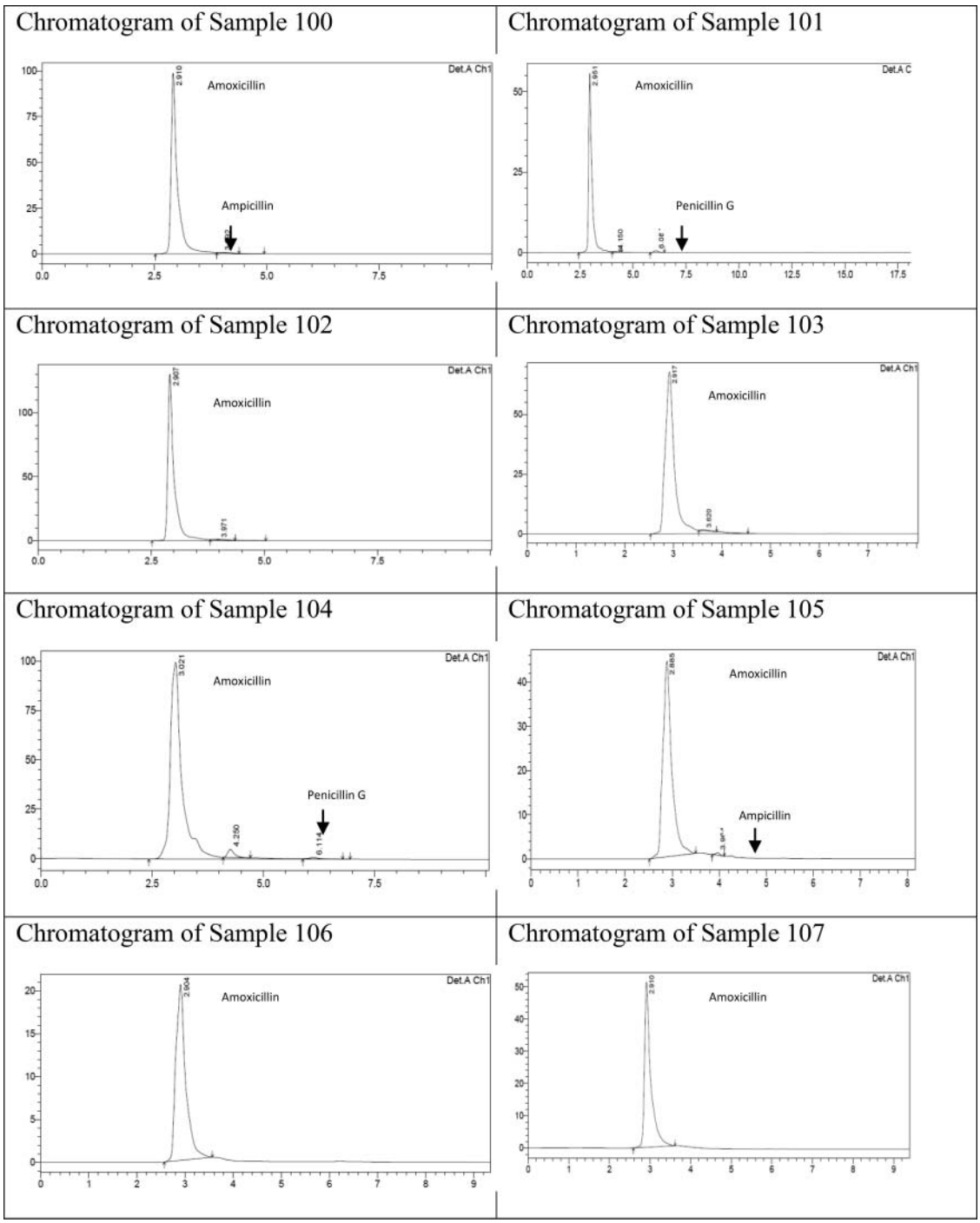

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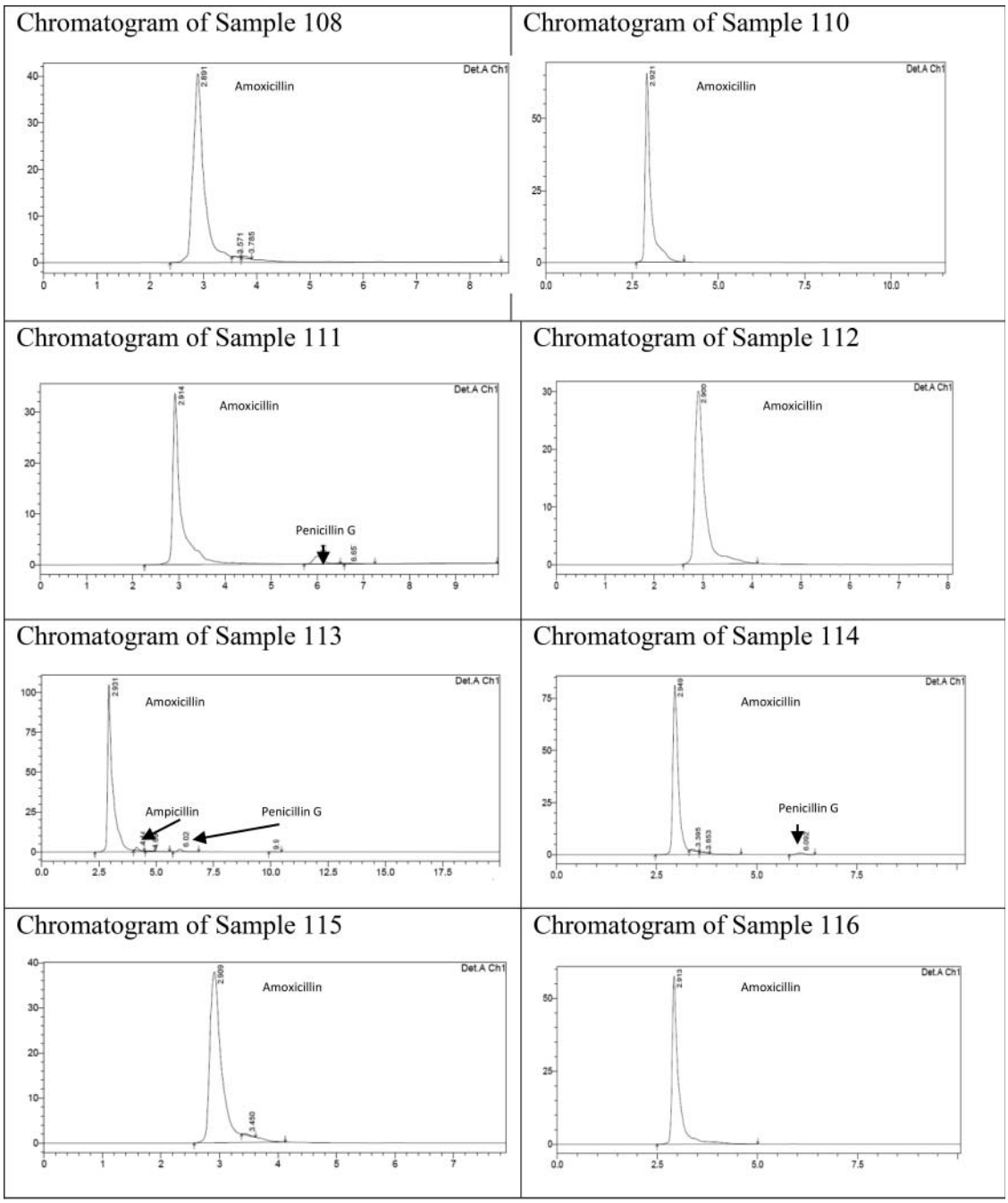

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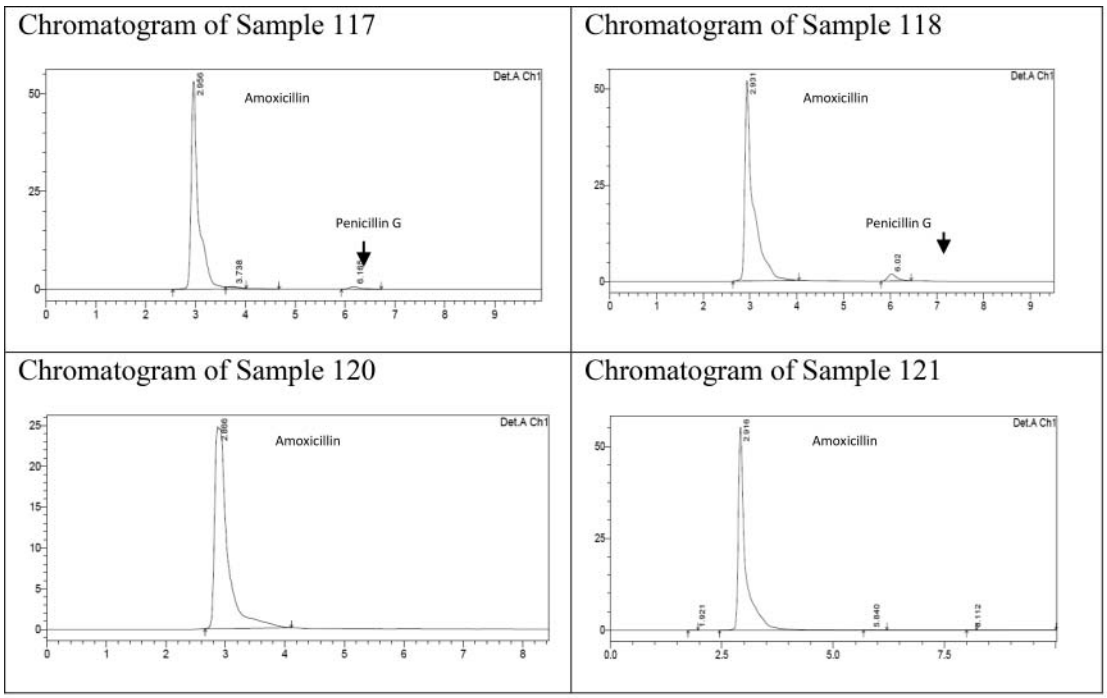

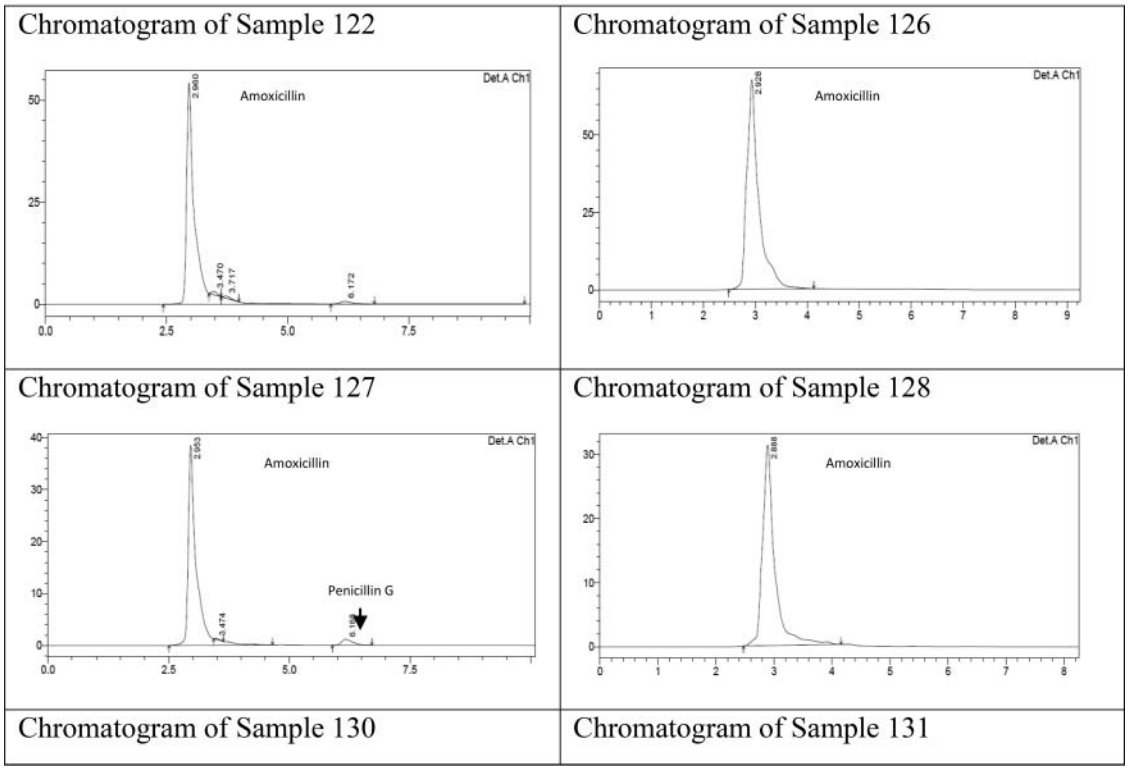

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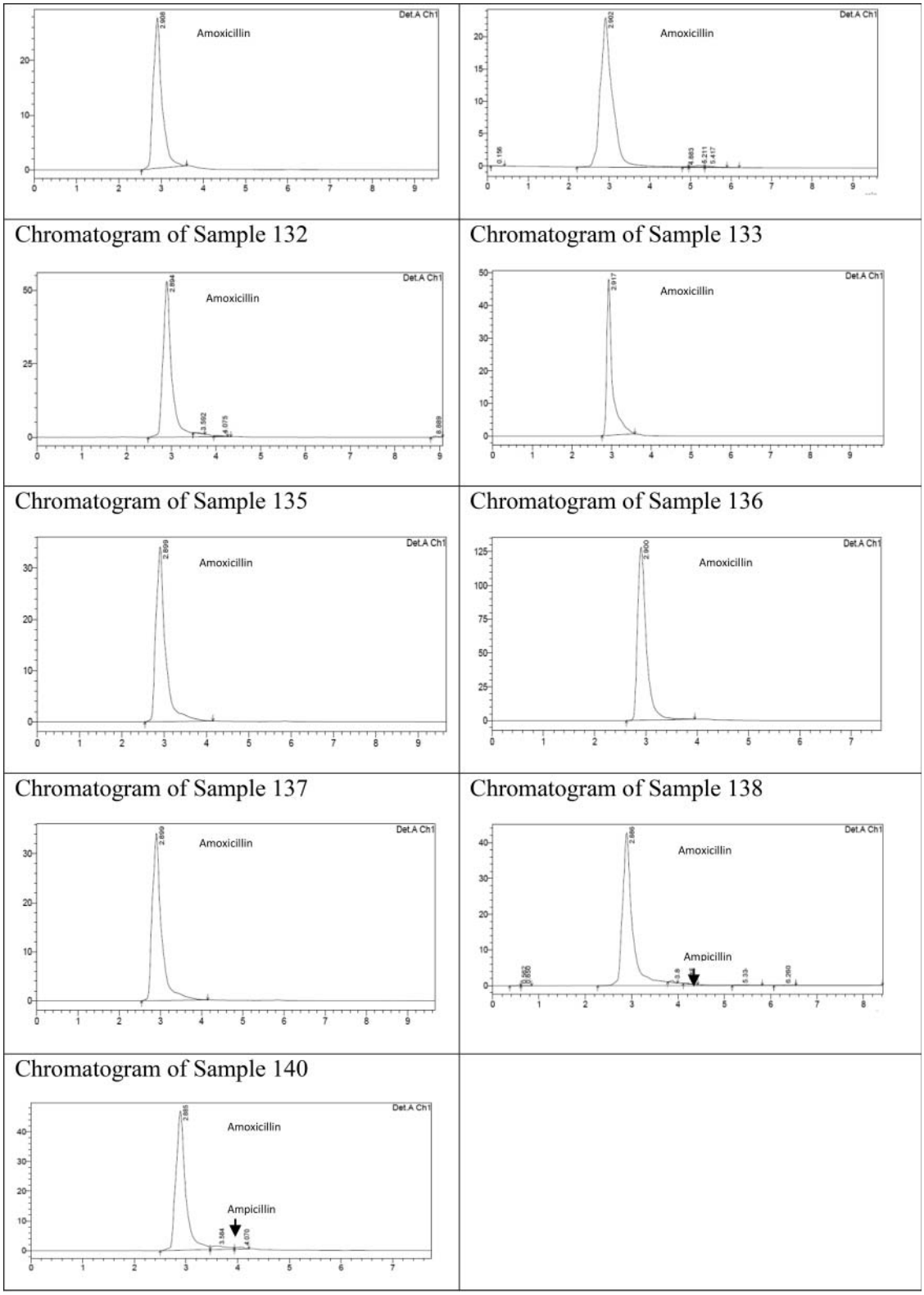
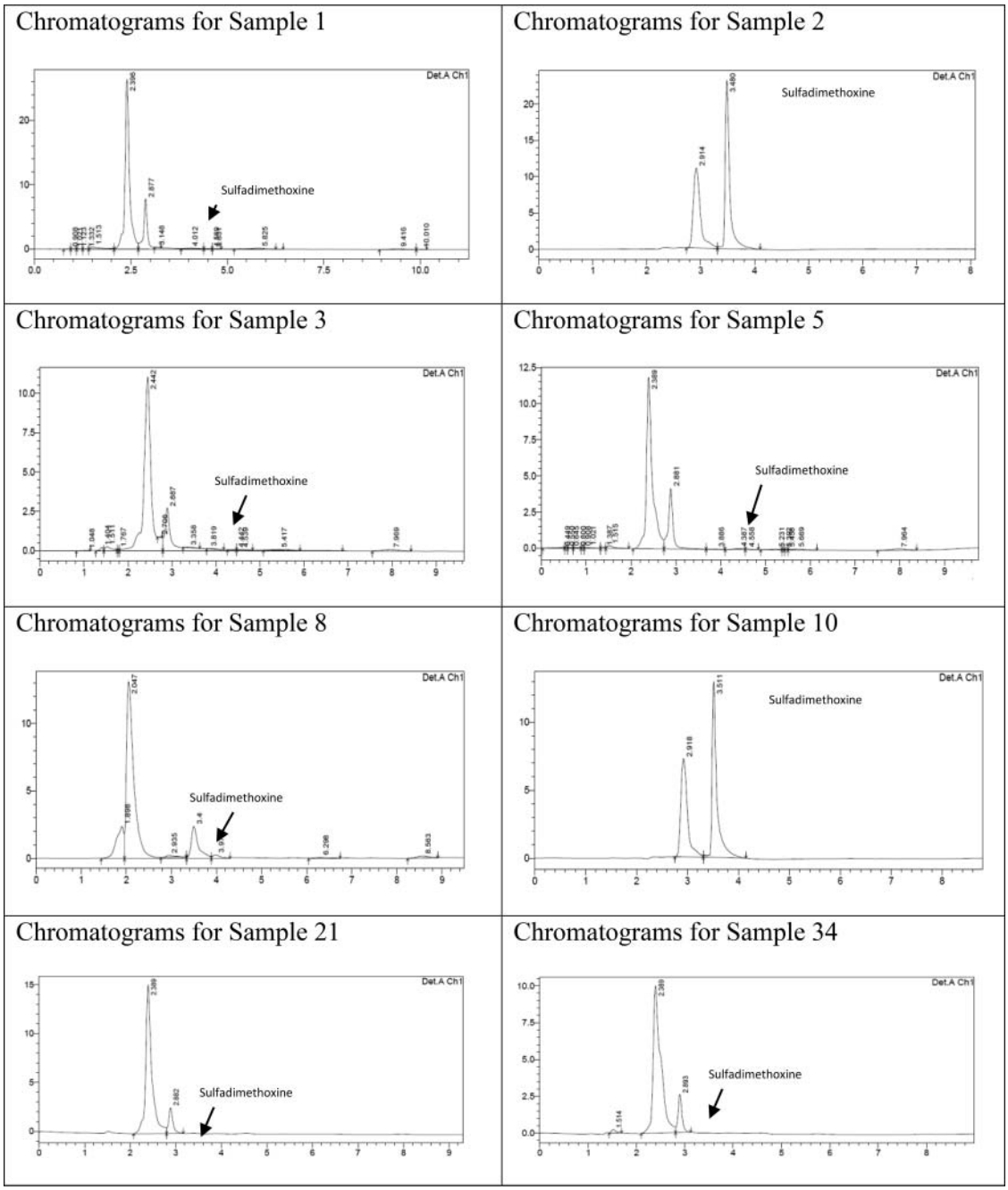
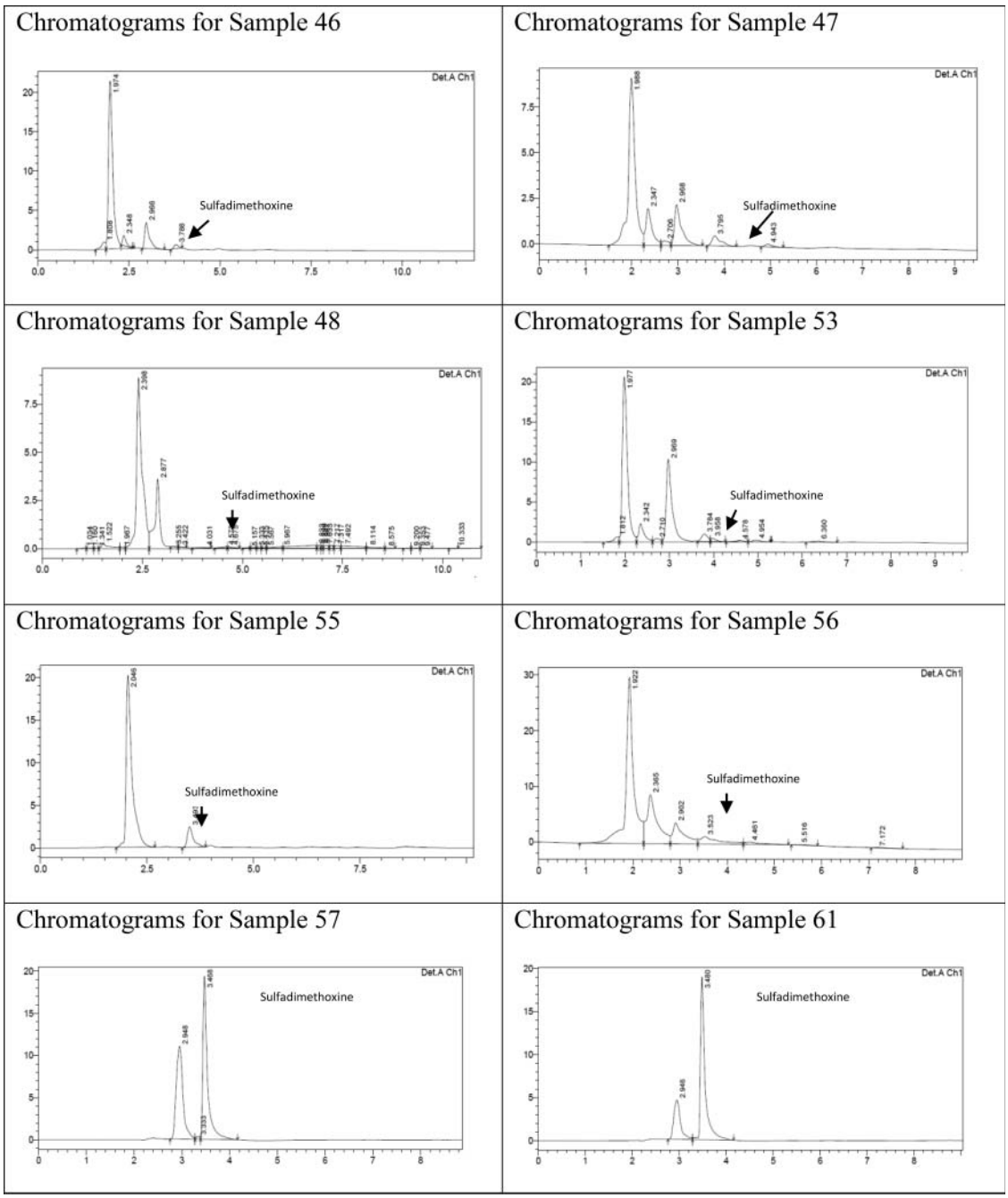


Table S3. Chromatograms for Sulfadimethoxine.



(*Continued on next page*)

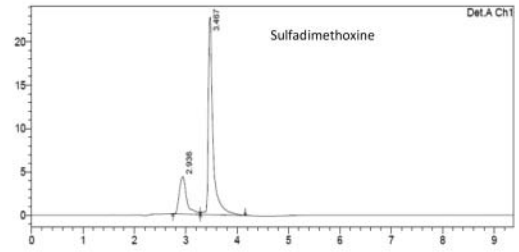
Table S3. (*Contined*).



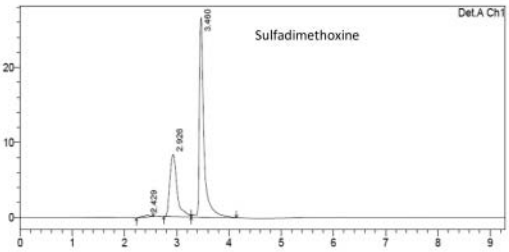
(*Continued on next page*)

Table S3. (*Contined*).

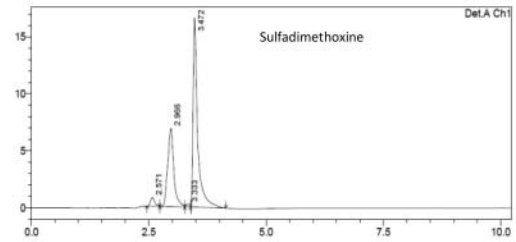
Chromatograms for Sample 62



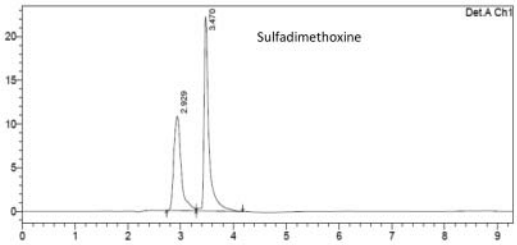
Chromatograms for Sample 63



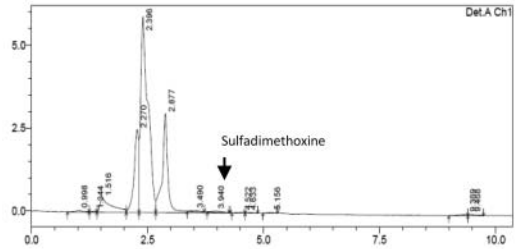
Chromatograms for Sample 66



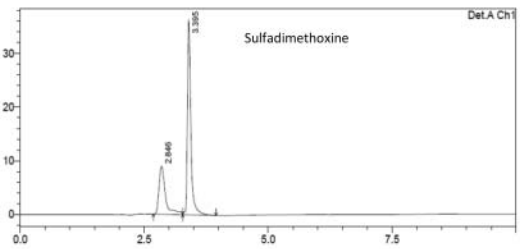
Chromatograms for Sample 67



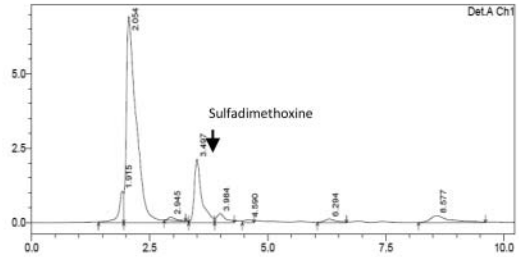
Chromatograms for Sample 68



Chromatograms for Sample 69



Chromatograms for Sample 70



Chromatograms for Sample 71

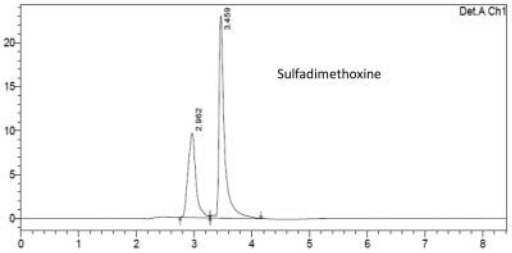


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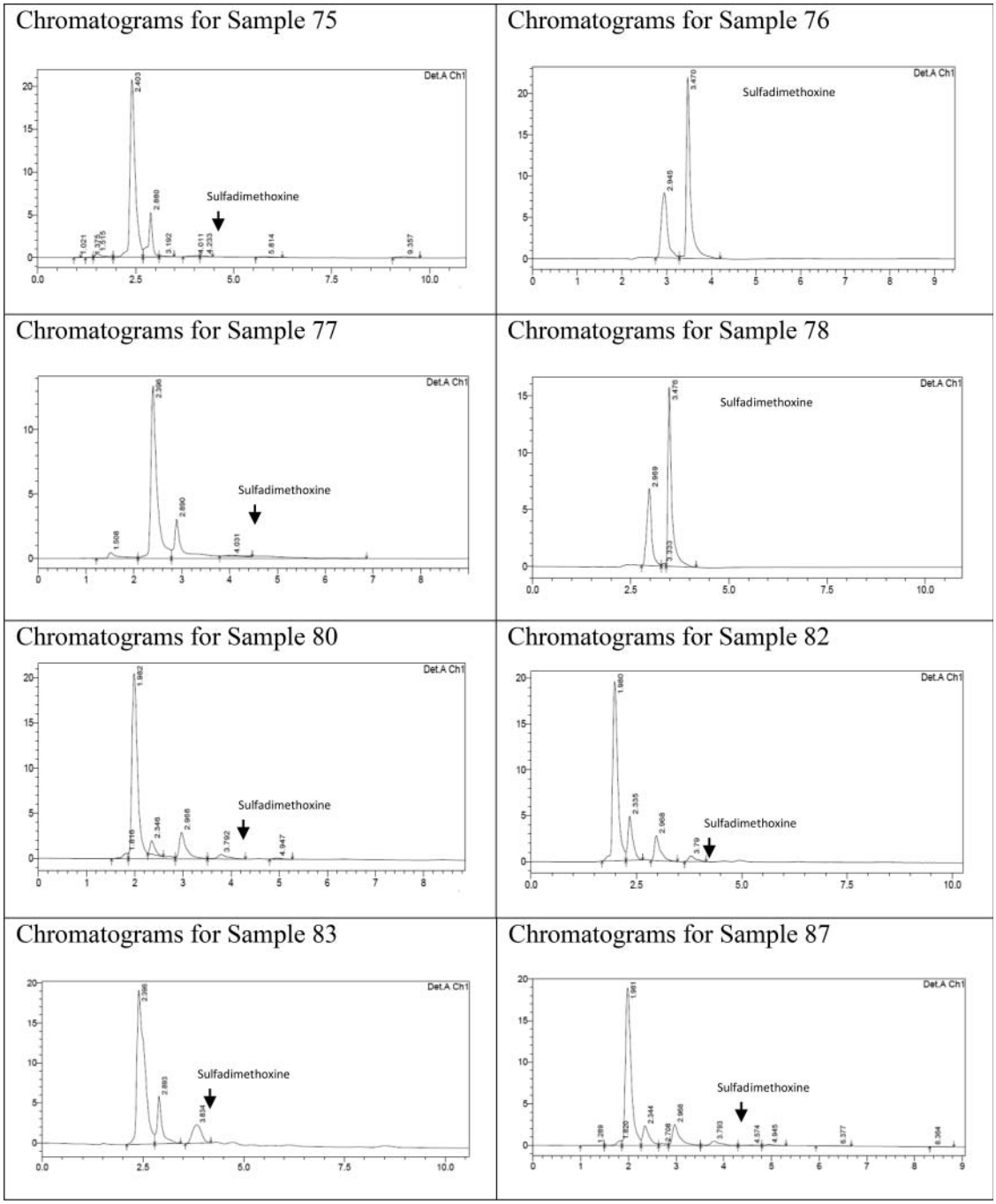
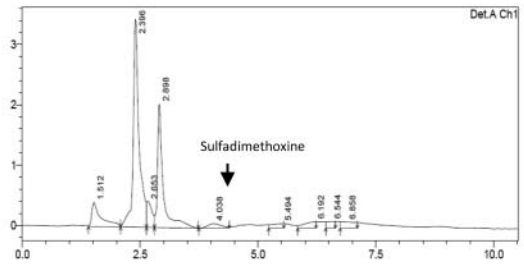
Table S3. (*Contined*).



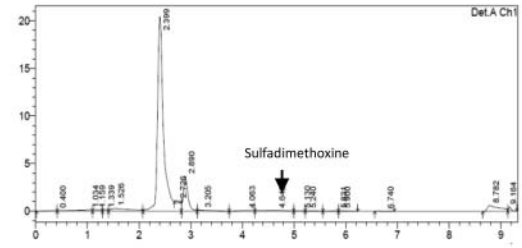
(*Continued on next page*)

Table S3. (*Contined*).

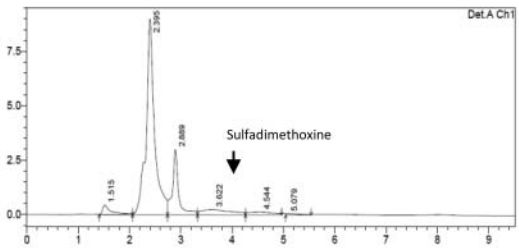
Chromatograms for Sample 89



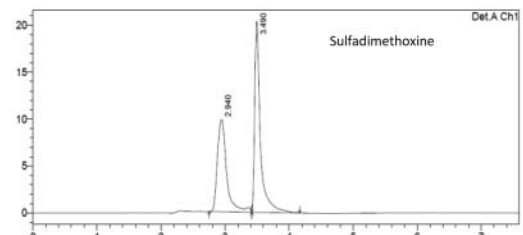
Chromatograms for Sample 90



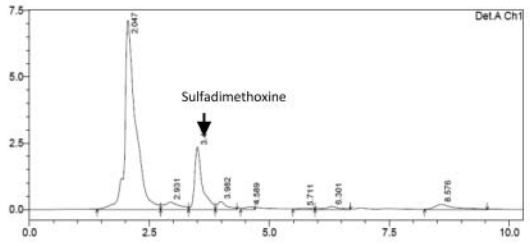
Chromatograms for Sample 91



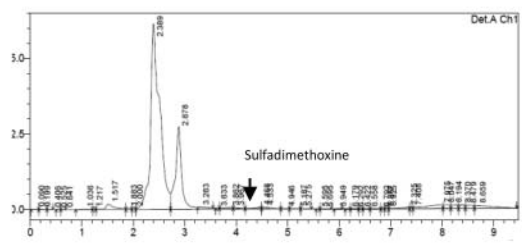
Chromatograms for Sample 92



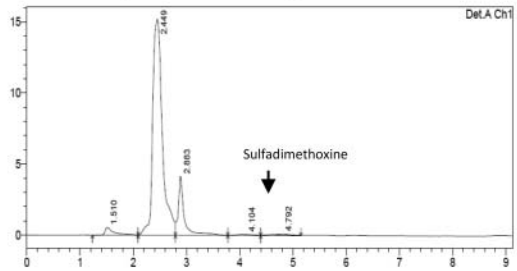
Chromatograms for Sample 96



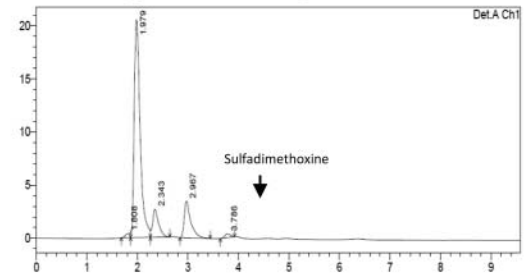
Chromatograms for Sample 97



Chromatograms for Sample 101



Chromatograms for Sample 103



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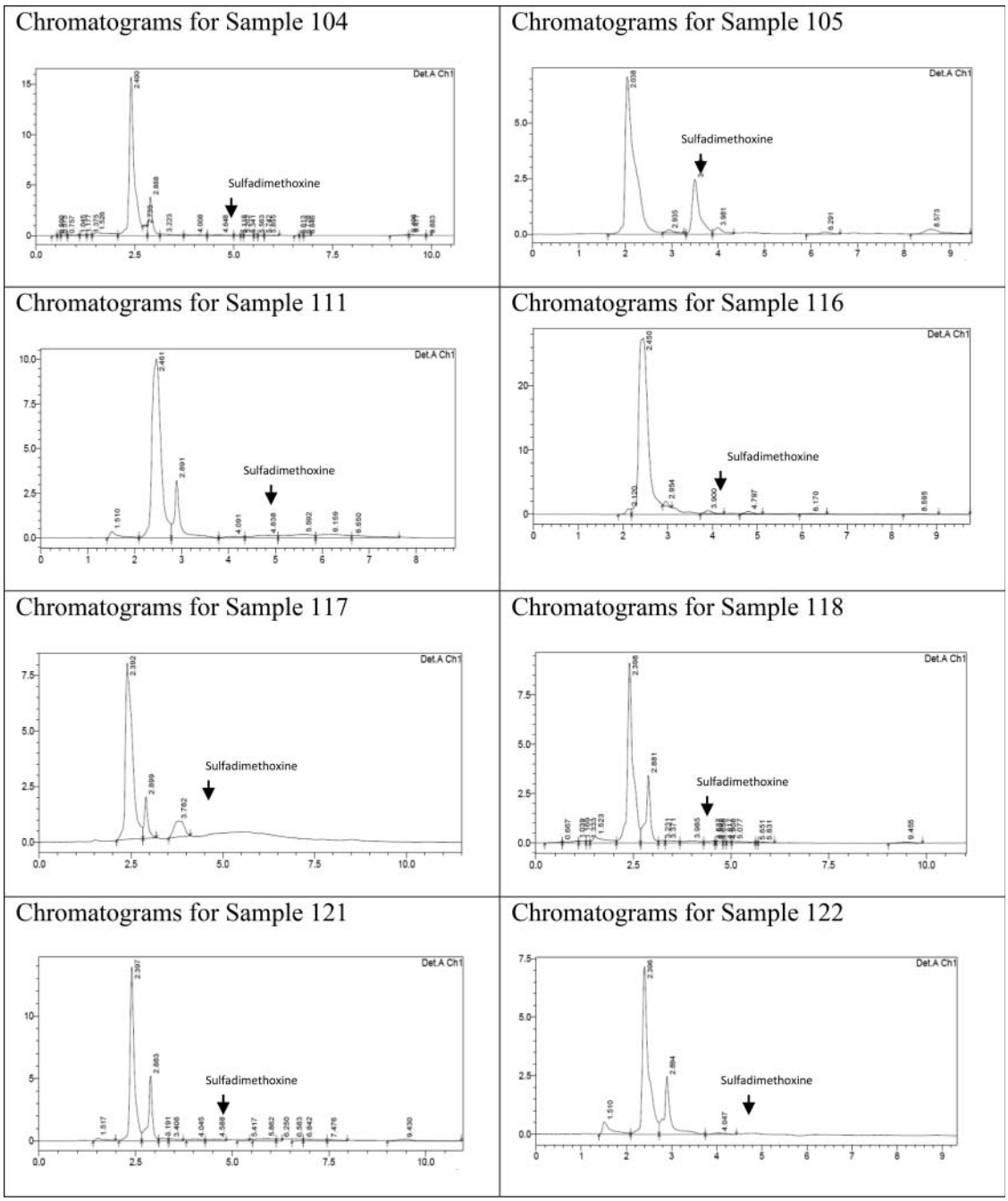
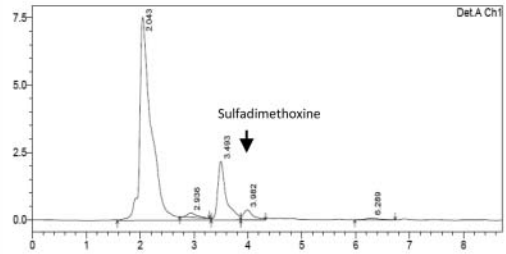


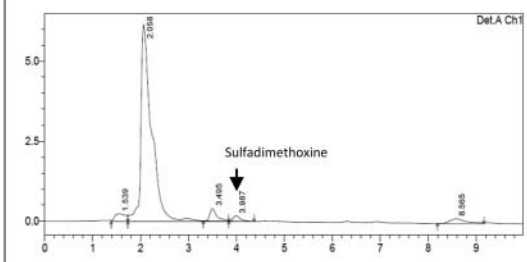
(*Continued on next page*)

Table S3. (*Contined*).

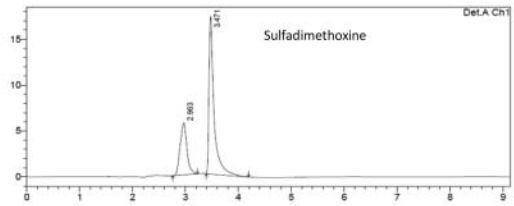
Chromatograms for Sample 123



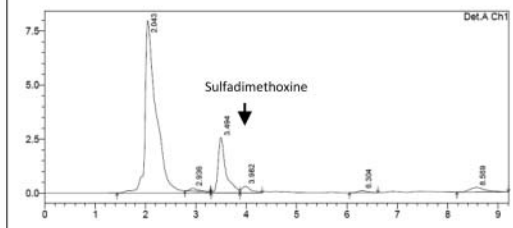
Chromatograms for Sample 127



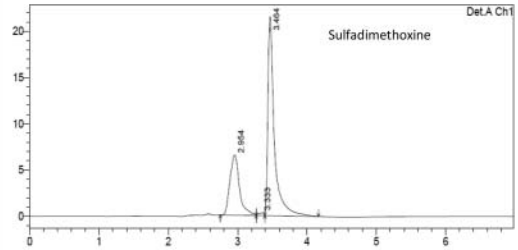
Chromatograms for Sample 128



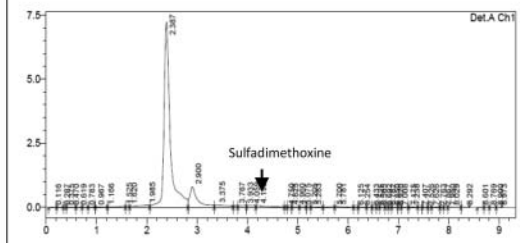
Chromatograms for Sample 129



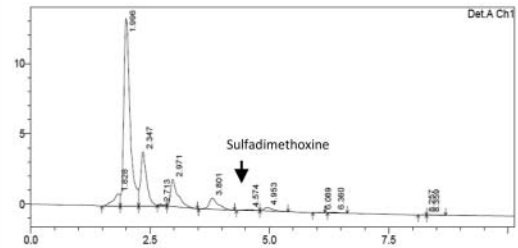
Chromatograms for Sample 132



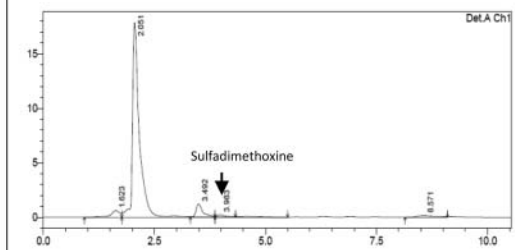
Chromatograms for Sample 133



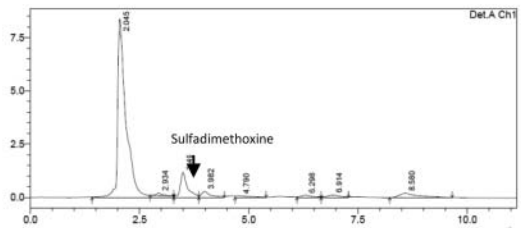
Chromatograms for Sample 135



Chromatograms for Sample 139



Chromatograms for Sample 140



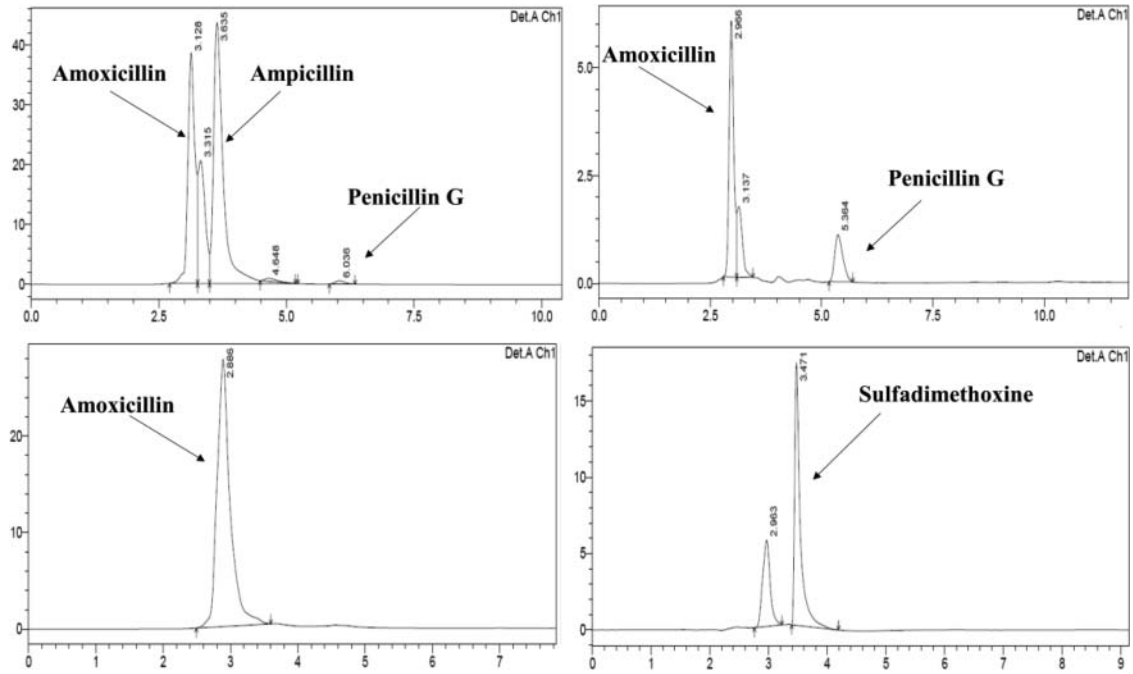


Figure S1. Chromatograms of pure antibiotic standards.