Article

Migration of Cefquinome Antibiotic Residues from Milk to Dairy Products

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Abstract: The aim of this study was to investigate the distribution of cefquinome in different dairy products during the processing of naturally contaminated milk or spiked milk. The analysis of cefquinome residues in milk, skimmed milk, buttermilk, whey, cream, butter, curd, and cheese samples was performed using a water:acetonitrile solvent extraction and C_{18} dispersive solid-phase extraction (d-SPE) clean-up, followed by ultrahigh-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC–MS/MS) determination. The target concentration of cefquinome was achieved in the spiked milk (100 μg kg^{-1}). During its processing, the antibiotic migrated primarily with the skimmed milk as opposed to cream (ratios of 3.6:1 and 2.8:1 for experiments A and B, respectively), and with the buttermilk during butter manufacture (ratios of 6.9:1 and 4.6:1), but was equal in the curd and whey during the manufacture of cheese. In the milk collected from treated animals, the measured concentration of cefquinome was considerably high (approx. 5000 μg kg^{-1}). The results obtained from the dairy products were similar to those obtained in the spiked study (ratios of 8.2:1 and 3.1:1 for experiments A and B, respectively, during the separation of skimmed milk and cream; 6.0:1 and 5.0:1 for A and B, respectively, during the separation of buttermilk and butter). However, during cheesemaking, cefquinome migrated with the whey after cutting the curd, with ratios of 0.54:1 and 0.44:1 for experiments A and B, respectively. The difference in the migration of cefquinome between curd and whey in spiked and animal studies is probably due to the different concentration levels in the two different experiments. The results of this study showed that, in dairy products manufactured from milk containing cefquinome residues, the drug migrated primarily with the high-water-containing fractions.

Keywords: antibiotic residues; cefquinome; milk; dairy products; UHPLC–MS/MS analysis

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1. Introduction

Cephalosporins belong to the β-lactam group of antibiotics, and are important antimicrobials used in both human and veterinary medicine [1]. These drugs act primarily by inhibiting the penicillin-binding proteins (PBPs), which are enzymes involved in the biosynthesis of peptidoglycan. Peptidoglycan is a polymer consisting of sugars and amino acids that represents the primary component of the bacterial cell wall. The β-lactam nucleus irreversibly binds to the PBPs because its chemical structure is similar to that of the sugar–amino acid backbone that forms peptidoglycan. This leads to the inactivation of the

enzymes and the impairment of cell wall formation, resulting in the inhibition of bacterial cell growth [2].

Cephalosporins are particularly resistant to the action of β-lactamases, and can be categorized into four different generations based on their spectrum of activity [3]. In particular, their effectiveness against Gram-positive bacteria decreases between the first and third generations, while their activity against Gram-negative bacteria increases. The fourth-generation cephalosporins are particularly important, because they are broad-spectrum antimicrobials active against both Gram-positive and Gram-negative organisms [4]. Therefore, the European Medicinal Agency (EMA) advises that they should only be administered systemically to animals in the event that clinical conditions respond poorly to narrow-spectrum antibiotics [5]; this is because resistant bacteria might transfer resistance genes to human pathogens. This concern is supported by the increased incidence of resistance to cephalosporins in human *Klebsiella pneumoniae* [6] and *Escherichia coli* infections [5,7,8]. These publications suggest that, although resistance can be mostly attributed to the inadequate use of antibiotics in humans, the spread from animal reservoirs via food or the environment can also contribute to the dissemination of resistance in the community [5,8]. In 2012, the Food and Drug Administration (FDA) banned all extra-label use of cephalosporins in cattle, pigs, and poultry, due to an increasing number of incidences of residue detection in milk and meat [9]. Other studies also raised concerns over the extra-label usage of ceftiofur in poultry [10].

Cefquinome is the only fourth-generation cephalosporin approved for use in food-producing animals; this drug has good activity against most Gram-negative bacilli—especially *Enterobacteriaceae*—and is currently licensed in Europe for use in cattle, pigs, and horses to treat respiratory diseases, arthritis, meningitis, or dermatitis. Absorption of orally administered cefquinome is poor, while the absorption following intramuscular and subcutaneous administrations proceeds relatively quickly, with a maximal serum concentration achieved within 0.5–2 h [11]. Cefquinome ointment can also be administered intramammarily for the treatment of *E. coli* mastitis in dairy cattle [12], showing a plasma elimination half-life of 1.5–3 h. Excretion is predominantly renal when parenterally administered, while the drug is excreted mainly in milk following intramammary treatments. Moreover, the use of radiolabelled cefquinome showed that this drug is metabolised to a small extent, as the only compound identified in treated animals was unchanged cefquinome [11]. Cefquinome withdrawal periods in milk depend on the formulation of the drug and the route of administration, and can vary from 24 h [13] to 5 days [14]. In order to monitor the presence of cefquinome residues in food products, maximum residue limits (MRLs) have been established for different species and target tissues [15].

The veterinary drug residues that may be found in milk can distribute into different dairy products during their manufacture, based on the residues' physicochemical properties [16], and can degrade depending on the different factors that affect their stability [17,18]. For the β-lactams, temperature is one of the most important factors, which can lead to inactivation of the drugs. In particular, greater thermal degradation has been observed in milk when heating time is prolonged and temperature increased [19].

Considering the importance of cefquinome as a very effective antibiotic in human medicine, the aim of this study was to investigate the effects of processing technologies on the distribution of its residues into different dairy products during manufacture.

2. Materials and Methods

2.1. Milk for Naturally Contaminated and Spiked Studies

All milk samples were collected from cows at the Teagasc Animal & Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland.

To obtain milk naturally contaminated with cefquinome (natural study), two cows (A, a Jersey [361 kg] and B, a Friesian [568 kg]) requiring treatment for acute interdigital necrobacillosis were selected from a herd being treated, or due for treatment, for non-udder-related infections. Both cows were treated with Cobactan 2.5% *w/v* suspension for injection

(Intervet Ireland Ltd., Dublin 24, Ireland) at a rate of 1 mg cefquinome kg^{-1} body weight or 2 mL 50 kg^{-1} body weight. Cobactan 2.5% was administered at 10 a.m., following milking, on the first day of treatment.

All of the milk from the cows was collected at the next milking—i.e., 7 h following injection (thus containing residues of the drug if it was excreted in milk)—and used for dairy product manufacture (natural study).

The milk used as a control was collected from the same cows two weeks after the end of the treatment, when all of the injected antibiotic was already excreted.

For the control experiments (spiked study), the residue-free milk was spiked with Cobactan 2.5% to achieve final levels of 0.1 mg kg^{-1}.

The physicochemical parameters of the milk used regarding fat, protein, lactose, and total solids (%) were 4.8 ± 1.1, 3.4 ± 0.09, 4.8 ± 0.09, and 13.6 ± 1.1, respectively, and 4.8 ± 0.56, 3.65 ± 0.18, 4.68 ± 0.15, and 13.8 ± 0.68, respectively, for the Jersey and Friesian cows, respectively.

All milk samples were frozen at −80 °C until UHPLC–MS/MS analysis.

2.2. Manufacture of Dairy Products

The milk from cows A and B from both natural and spiked studies was divided into three aliquots, left unpasteurized, and used for product manufacture is shown in Figure 1.

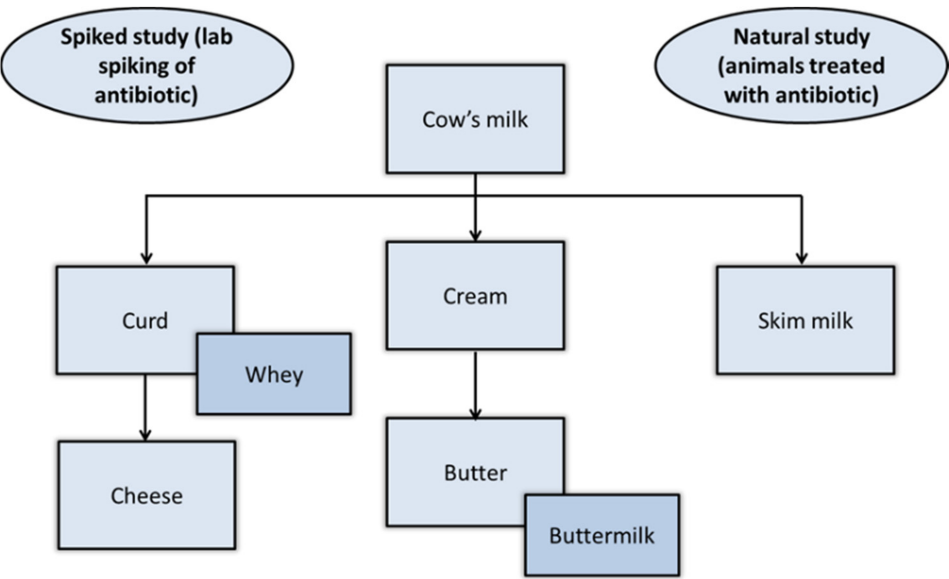

Figure 1. Experimental design used for the manufacture of dairy products from spiked milk and milk collected from treated animals. This protocol was undertaken in independent duplicate, with triplicate analyses on each occasion for both the spiked study and the natural study.

For cheesemaking, 10 L of milk with an initial pH of 6.7 was heated to 32 °C, with constant agitation. When the temperature reached 20 °C, 0.5 g of starter culture (DVS, A2005 containing *Lactococcus lactis* ssp. lactis, *Lactococcus lactis* ssp. cremoris, and *Streptococcus thermophiles* (Chr. Hansen, Cork, Ireland) was added. When the pH of the milk was below 6.55 (20–45 min), rennet (CHY-MAX Plus, Chr. Hansen; 2 mL diluted in 10 mL of sterile water) was added, and the milk was allowed to set for ~75 min, after which the curd was cut. After 5 min, the temperature was increased at a rate of 1 °C every 5 min (while stirring continuously), to a final temperature of 36 °C. When the final temperature was reached, about one-third of the whey was drained, and the curd was put into cheese moulds (9 cm diameter × 8 cm high) that allowed the remaining whey to drain. Whey and curd samples were collected at this point. The cheese in the moulds was turned every 30 min for 3 h, and subsequently every 90 min until brining, which occurred at pH 5.25–5.3 (8–10 h post moulding). During that time, the cheese was covered in foil to maintain the temperature.

The separation of whole milk to skimmed milk and cream was carried out by heating the milk to 50 °C and separating it using a disc-bowl centrifuge (Armfield, Hampshire, UK).

For butter manufacture, the cream (1 L) was chilled to 4 °C and whisked in a food blender until it separated into buttermilk and butter. The buttermilk was subsequently decanted through a spout so that the butter could be collected and rinsed with water to prevent souring.

After manufacture, aliquots of all the different products were placed in a 50 mL polypropylene container and frozen at −80 °C until analysis, while the remaining parts were kept in large plastic bags and stored at the same temperature.

2.3. Cefquinome Detection by Ultrahigh-Performance Liquid Chromatography Coupled with Tandem Mass Spectrometry (UHPLC–MS/MS)

2.3.1. Materials and Reagents

Cefquinome sulphate and the internal standard cefquinome-d_7 hydroiodide were purchased from LGC Standards (Teddington, Middlesex, UK) and Toronto Research Chemicals (Toronto, ON, Canada), respectively. Ultrapure water (18.2 MΩ cm^{-1}) was obtained in-house using a Millipore water purification system (Cork, Ireland). Ultrahigh-performance liquid chromatography (UHPLC)-grade acetonitrile (MeCN) was purchased from ROMIL Ltd. (Cambridge, UK). Dimethyl sulfoxide (DMSO), 98–100% formic acid (HCOOH), and ammonium acetate were purchased from Sigma-Aldrich (Dublin, Ireland).

Dispersive solid-phase extraction (d-SPE) centrifuge tubes containing 500 mg of end-capped C_{18} sorbent were purchased from UCT (Wexford, Ireland), and membrane filters (Captiva Econofilter, PTFE, 13 mm, 0.2 µm) were purchased from Agilent Technologies Ltd. (Cork, Ireland).

2.3.2. Preparation of Standard Solutions

Primary stock solutions of cefquinome and cefquinome-d_7 were prepared at concentrations of 1 mg mL^{-1} in DMSO. Cefquinome intermediate standard solutions were prepared in water at concentrations of 200 µg mL^{-1} and 4 µg mL^{-1}. Six working calibration standard solutions (cal 1–6) were subsequently prepared at concentrations of 0.04, 0.08, 0.4, 2, 5, and 10 µg mL^{-1} by diluting the intermediate solutions in water. A working internal standard solution was prepared by diluting 100 µL of the cefquinome-d_7 stock solution in 50 mL of water to obtain a final concentration of 2 µg mL^{-1}. All solutions were prepared monthly and stored in 2.5 mL aliquots in 15 mL polypropylene tubes at −80 °C.

2.3.3. UHPLC–MS/MS Quality Control and Calibration

To obtain negative control samples, whole milk, skimmed milk, buttermilk, and cream were purchased from a local supermarket. Whey, curd, cheese, and butter negative control samples were obtained in-house from laboratory-scale milk processing and cheesemaking at Teagasc, Moorepark. All negative control samples were verified to be free of cefquinome residues prior to validation and test sample analysis.

Positive controls were obtained by spiking four negative samples post-extraction, in order to monitor for loss of analytes during the sample preparation. Two negative samples were spiked with 100 µL of the cal 2 working calibration solution at 0.08 µg mL^{-1} to obtain samples spiked at 2 µg kg^{-1}, while the other two controls were spiked with 100 µL of the cal 5 working calibration solution at 5 µg mL^{-1} to give a final concentration of 250 µg kg^{-1}.

Matrix-matched calibrants were spiked by adding 100 µL of six working standard solutions with concentrations between 0.04 and 10 µg mL^{-1}, in order to obtain a calibration curve in the range of 2.0–500 µg kg^{-1}.

2.3.4. Sample Preparation for UHPLC–MS/MS Analysis

The samples were removed from the freezer and allowed to thaw at room temperature. To ensure sample homogeneity, whole milk, skimmed milk, buttermilk, cream, and whey were manually and gently mixed end over end. A portion of each sample (2 g ± 0.01 g)

was weighed into a 50 mL polypropylene tube. The samples from the animal treatment study were diluted in order to obtain results within the calibration range of the method; therefore, 0.1 g (for the whole milk, skimmed milk, buttermilk, and whey samples) and 0.2 g (for the cream, butter, curd, and cheese samples) were weighed into the 50 mL tube, and the weight was subsequently made up to 2 g with a corresponding negative control. A 100 µL volume of the working internal standard solution was added to all calibrants, controls, and test samples to obtain a final concentration of 0.1 µg kg^{-1}. Whole milk, skimmed milk, buttermilk, whey, and cream samples were shaken gently for 1 min, and all tubes were allowed to stand for 15 min. A 7.5 mL volume of MeCN was added to all samples. A 0.9 mL volume of water was added to the matrix calibrants, while 1 mL of water was added to all of the other tubes. The liquid samples (whole milk, skimmed milk, buttermilk, whey, and cream) were vortexed for 1 min using a multi-tube vortexer (Nova-Tech International Talboys 945008, Kingwood TX, USA). The solid samples (curd, cheese, and butter) were homogenised over ice for 15 s using an Ultra-Turrax probe blender (IKA, Staufen, Germany). All samples were centrifuged at 2842× *g* (4 °C) for 15 min using an MSE Mistral 3000i centrifuge (Davidson and Hardy, Dublin, Ireland), and the resulting supernatant was poured into a d-SPE centrifuge tube containing 500 mg of endcapped C18 sorbent. The tubes were vortexed for 40 s and centrifuged at 2842× *g* (4 °C) for 15 min. The entire supernatant was transferred into a 15 mL polypropylene tube, and the MeCN was evaporated under nitrogen using a TurboVap LV evaporator (Biotage, Uppsala, Sweden) at 40 °C, until only the aqueous part remained (volume < 1 mL). The volume was then made up to 1 mL with water, and the extracts were vortexed for 10 s. Samples were subsequently centrifuged at 2842× *g* (4 °C) for 15 min, and the final extracts were filtered through 0.2 µm PTFE filter units directly into UHPLC vials.

2.3.5. UHPLC–MS/MS Conditions

The UHPLC–MS/MS method previously developed by Di Rocco et al. (2017) [20] was adapted and used for this study. The gradient conditions were adjusted as follows: (1) 0–1.5 min, 100% A; (2) 1.5–3.5 min, 80% A; (3) 3.5–4.30 min, 70% A; (4) 4.30–5.30 min, 50% A; (5) 5.30–6.30 min, 0% A; (6) 6.30–8.20 min, 0% A; (7) 8.20–8.30 min, 100% A; (8) 8.30–15 min, 100% A. The injection volume was 1 µL in partial loop with needle overfill mode.

Cefquinome-d_7 was implemented into the method to improve accuracy; therefore, tuning was performed by teed infusion of 1 µg mL^{-1} internal standard aqueous solution with mobile phase A:B (50:50, *v/v*). The precursor ion was $[M + H]^+$, and the monitored transition was 535.7→141.2 *m/z*. The optimal cone voltage and collision energy were 26 V and 14 eV, respectively. The acquisition window was 3.70–4.40 min (retention time (RT) for cefquinome = 4.04 min; RT for cefquinome-d_7 = 4.03 min). The dwell time was set at 0.110 s for both analyte and internal standard.

2.3.6. UHPLC–MS/MS Method Validation

The validation was based on the criteria established by Commission Decision 2002/657/EC [21]. In order to verify the selectivity of the method, the absence of interferences between the analyte and the internal standard was investigated by injecting each compound individually. Moreover, a total of 36 blank samples were analysed during the proposed study and checked for any matrix interferences. Retention time and ion ratio were examined for identification purposes. The linearity of the calibration curve was considered satisfactory if $R^2 \geq 0.98$ and if individual residuals did not deviate by more than ±20% from the calibration curve. Trueness and interday precision (calculated as relative standard deviation (RSD%)) were assessed for each matrix by spiking 11 blank samples at 2× the lowest calibration level (4.0 µg kg^{-1}, corresponding to the second calibration level) and 0.5× the highest calibration level (250 µg kg^{-1}, corresponding to the second-to-last calibration level). For the liquid samples, skimmed milk was used as a negative control for the matrix-matched calibration curve, and the validation was carried out over a period of five days (three replicates on day 1 and two replicates on all other days). For the solid samples, the

matrix-matched calibration curve was built using a blank cheese sample, and validation was performed over a period of three days (three replicates on day 1 and four replicates on all other days). The results were considered satisfactory if in agreement with 2002/657/EC guidelines [21].

2.4. Statistical Analysis

The statistical analysis was performed using OriginPro 2019 version 9.6.0.172 software. ANOVA assumptions were tested using the Kolmogorov–Smirnov test to check for normality, and Levene's (squared deviations) test to check for homogeneity of variance. ANOVA post hoc analysis was carried out with the Bonferroni test for all comparisons except for the cream/butter/buttermilk in the natural study, for which the non-parametric Kruskal–Wallis H test was performed, followed by post hoc analysis using Dunn's test. The comparison between experiment A and experiment B for each of the products, for both the spiked and natural studies, was conducted with the Mann–Whitney U test. The level of significance chosen was $\alpha = 0.05$.

2.5. Ethics Statement

No ethical approval licence was required for this study, as all of the milk used in the animal studies was taken from cows that were naturally on cefquinome treatment for mild clinical mastitis.

3. Results

3.1. UHPLC–MS/MS Method Validation

Satisfactory results were obtained for all matrices from the validation study (Table 1). For the liquid samples, trueness ranged from 85% to 103%, and interday precision ranged between 2.6% and 9.3%. For the solid samples, trueness and interday precision were in the ranges 90–104% and 2.3–8.6%, respectively. No interferences were observed from the selectivity study. All retention times and ion ratios were within the permitted limits, as outlined in 2002/657/EC [21]. The calibration curves were linear in the concentration range of interest, as R^2 was greater than 0.99 after fitting a linear equation, and individual residuals did not deviate by more than ±15% from the calibration curve.

Table 1. Inter-assay validation results obtained from the UHPLC–MS/MS method developed for the analysis of cefquinome in dairy products.

Sample Type	Matrix	Cefquinome Spiked Levels (μg kg^{-1})	Mean ± SD (μg kg^{-1})	RSD (%)	Trueness (%)
Liquid samples (n = 11) (days = 5)	Whole milk	4.0	4.1 ± 0.15	3.8	101
		250	258 ± 9.8	3.8	103
	Skimmed milk	4.0	4.0 ± 0.26	6.4	100
		250	251 ± 8.8	3.5	100
	Buttermilk	4.0	3.5 ± 0.33	9.3	87
		250	226 ± 7.9	3.5	91
	Whey	4.0	3.5 ± 0.20	5.8	86
		250	214 ± 5.6	2.6	85
	Cream	4.0	4.0 ± 0.20	5.1	99
		250	256 ± 9.2	3.6	102
Solid samples (n = 11) (days = 3)	Curd	4.0	3.6 ± 0.25	6.8	90
		250	237 ± 5.5	2.3	95
	Cheese	4.0	3.9 ± 0.34	8.6	98
		250	244 ± 7.5	3.1	97
	Butter	4.0	3.9 ± 0.16	4.1	99
		250	259 ± 7.2	2.8	104

SD = standard deviation; RSD = relative standard deviation.

3.2. *Sample Analysis*

3.2.1. Spiked Study

As shown in Table 2, all of the replicates were similar in most cases within each duplicate, although between the duplicates some of the results varied (e.g., butter and buttermilk), but the trends were similar, and after statistical analysis it was not possible to identify statistically significant differences between the duplicates for the same type of product. The target concentration of cefquinome in the milk (100 µg kg^{-1}) was achieved, although it was slightly higher in the second duplicate. When the whole milk was separated into skimmed milk and cream, the antibiotic migrated primarily with the skimmed milk. When butter was manufactured from the cream (Table 2 comparison β), cefquinome migrated with the buttermilk, with each of the fractions becoming significantly different from the others, while during cheesemaking (Table 2 comparison γ) the concentration of cefquinome was similar in the whey and the curd.

Table 2. Concentrations (µg kg^{-1}) of cefquinome residues in the samples obtained from the spiked studies.

	Experiment A				Experiment B			
	Replicate			Mean ± SD	Replicate			Mean ± SD
	1	2	3		1	2	3	
Whole milk $^{\alpha\,\gamma}$	114	109	109	111 ± 2.9	118	129	119	122 ± 6.1
Skimmed milk $^{\alpha}$	103	94	99	99± 4.5	117	116	119	117 ± 1.5
Cream $^{\alpha\,\beta}$	26.9	26.7	28.4	27.3 ± 0.93	42.8	39.6	45.2	42.5 ± 2.8
Butter $^{\beta}$	11.3	14.3	12.4	12.7 ± 1.5	17.9	29.4	28.6	25.3 ± 6.4
Buttermilk $^{\beta}$	89	85	88	87± 2.1	108	127	115	116 ± 9.6
Whey $^{\gamma}$	108	102	103	104 ± 3.2	113	116	114	114 ± 1.5
Curd $^{\gamma}$	112	120	119	117 ± 4.4	118	123	123	121 ± 2.9
Cheese $^{\gamma}$	104	104	104	104 ± 0	117	119	116	117 ± 1.5

$^{\alpha}$—Comparison of whole milk, skimmed milk, and cream: **whole milk/skimmed milk** (p = 0.01014), **whole milk/cream** ($p = 1.68 \times 10^{-7}$), **skimmed milk/cream** ($p = 4.26 \times 10^{-7}$). $^{\beta}$—Comparison of cream, butter, and buttermilk: **cream/butter** ($p = 8.36 \times 10^{-5}$), **cream/buttermilk** ($p = 1.99 \times 10^{-8}$), **butter/buttermilk** ($p = 5.38 \times 10^{-9}$). $^{\gamma}$—Comparison of whole milk, whey, curd, and cheese: whole milk/whey (p = 0.21228), whole milk/curd (p = 0.21228), whole milk/cheese (p = 0.17263), **curd/whey** (p = 0.0059), **curd/cheese** (p = 0.005), cheese/whey (p = 1). Comparisons in bold are statistically significant.

3.2.2. Natural Studies

Table 3 shows the results of the natural study experiments. The concentration of cefquinome was considerably high in the milk from treated animals (approximately 5000 µg kg^{-1}). In most cases, the triplicate values were similar within each independent duplicate, although one of the triplicate values was low for the cream in duplicate A. When the whole milk was separated into skimmed milk and cream (Table 3: comparison α), the antibiotic migrated with the skimmed milk. During butter manufacture (Table 3 comparison β), cefquinome migrated with the buttermilk, while during cheesemaking (Table 3 comparison γ), cefquinome migrated with the whey.

3.2.3. Comparison between Spiked and Natural Studies

During both the separation of the whole milk and the buttermaking, there was little difference between the experiments with spiked milk and experiments with treated animals (natural study), although some variation in the results was observed in the experiments on milk from treated animals (Figure 2). There was a difference between the spiked and natural studies in the migration of cefquinome between curd and whey during cheesemaking: in the spiked experiments, cefquinome migrated equally with the curd and whey, while in the experiments with milk from treated animals, cefquinome migrated with the whey (Figure 2).

Table 3. Concentrations (μg kg^{-1}) of cefquinome residues in the samples obtained from the natural studies.

	Experiment A				Experiment B			
	Replicate			Mean ± SD	Replicate			Mean ± SD
	1	2	3		1	2	3	
Whole milk $^{\alpha\,\gamma}$	114	109	109	111 ± 2.9	118	129	119	122 ± 6.1
Skimmed milk $^{\alpha}$	103	94	99	99± 4.5	117	116	119	117 ± 1.5
Cream $^{\alpha\,\beta}$	26.9	26.7	28.4	27.3 ± 0.93	42.8	39.6	45.2	42.5 ± 2.8
Butter $^{\beta}$	11.3	14.3	12.4	12.7 ± 1.5	17.9	29.4	28.6	25.3 ± 6.4
Buttermilk $^{\beta}$	89	85	88	87± 2.1	108	127	115	116 ± 9.6
Whey $^{\gamma}$	108	102	103	104 ± 3.2	113	116	114	114 ± 1.5
Curd $^{\gamma}$	112	120	119	117 ± 4.4	118	123	123	121 ± 2.9
Cheese $^{\gamma}$	104	104	104	104 ± 0	117	119	116	117 ± 1.5

$^{\alpha}$—Comparison of whole milk, skimmed milk, and cream: **cream/whole milk** ($p = 5.19 \times 10^{-8}$), **cream/skimmed milk** ($p = 1.08 \times 10^{-7}$), whole milk/skimmed milk ($p = 1$). $^{\beta}$—Comparison of cream, butter, and buttermilk: cream/butter ($p = 0.7456$), **cream/buttermilk** ($p = 0.0058$), **butter/buttermilk** ($p = 0.0021$). $^{\gamma}$—Comparison of whole milk, whey, curd, and cheese: **whole milk/whey** ($p = 0.00342$), **whole milk/curd** ($p = 2.85727 \times 10^{-8}$), **whole milk/cheese** ($p = 2.35813 \times 10^{-6}$), **curd/whey** ($p = 9.05296 \times 10^{-5}$), curd/cheese ($p = 0.16598$), **cheese/whey** ($p = 0.02178$). Comparisons in bold are statistically significant.

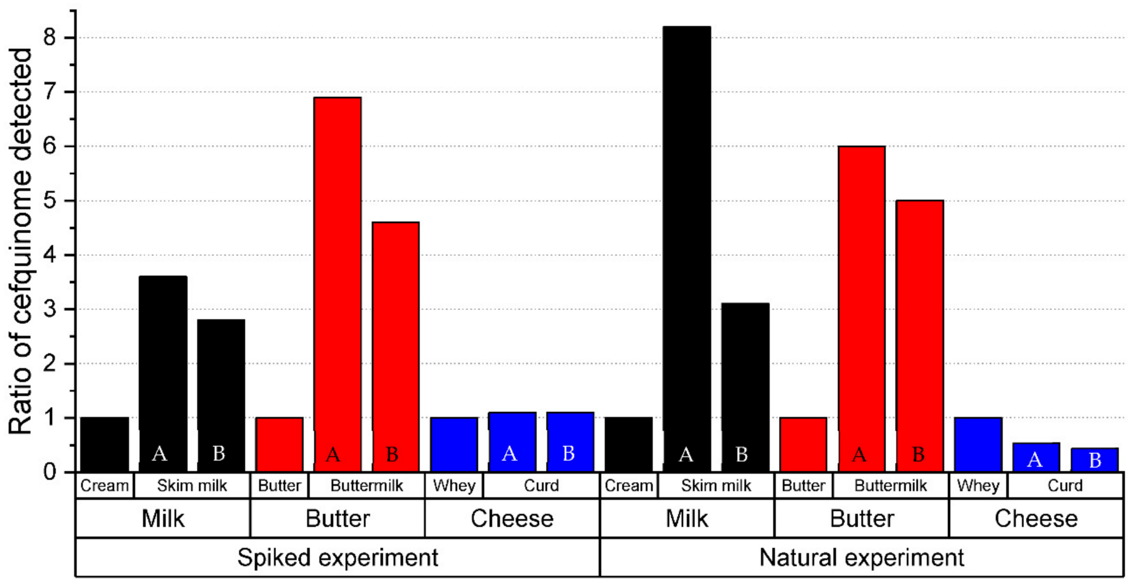

Figure 2. Migration of cefquinome during dairy product manufacture. The ratio of cefquinome is calculated for each of the related products. Both replicas (A and B) are shown for spiked and natural experiments.

4. Discussion

As reported in Table 3, high concentrations of cefquinome residues were measured in bovine milk samples collected ~7 h post-treatment. The recommended withdrawal period for milk for the drug used in this study is 24 h. However, further work would be required to assess the persistence of cefquinome beyond the recommended interval. Our results showed that cefquinome residues are excreted in the milk of dairy cows following administration by injection. Furthermore, it was observed that the drug migrated from milk to dairy products, and that it was not degraded during product manufacture.

The published studies reporting the investigation of the migration of β-lactam residues into different dairy products employed spiked milk, which can be easily obtained or produced in the laboratory. However, differences between spiked and naturally contaminated milk—possibly due to animal metabolism—should be taken into account when performing such investigations. The research presented in this work is unique, because it addresses ambiguities related to the use of spiked milk by also comparing the distribution of the drug residues into a number of dairy products manufactured from milk obtained from naturally

treated cows. In addition, it must be stated that very limited research on the migration of drug residues in dairy derivatives, and their degradation during manufacture, is available in the literature—particularly in relation to β-lactams. This is mostly due to the fact that these compounds can be very difficult to analyse, and are unstable to a number of factors, such as temperature and pH [19].

Horton et al. (2015) [22] investigated the degradation of cefquinome in milk via heat treatment, pH adjustment, fermentation, and enzymatic hydrolysis as a bioremediation strategy for waste milk containing cefquinome residues. Cefquinome concentrations were stable at 4 and 10 °C for 144 h, while the concentrations declined at 37 °C. Degradation was more rapid at 50 °C, with a cefquinome half-life of approximately 31 h. Roca et al. (2011) [19] previously reported a half-life of approximately 100 min at 60 °C in spiked skimmed milk. The differing results are probably due to the different experimental conditions used, such as type of milk, spiking levels, heating methods, and incubation temperatures. At pH 1 and 4, cefquinome was relatively stable for the experimental time period of 168 h, although degradation was observed at pH 10, and concentrations significantly decreased after only 8 h. While pH 4 would be reasonably relevant in cheesemaking, further studies would be required to determine the degradation at the pH of milk.

The stability of drugs such as triclabendazole and other flukicides during dairy product manufacture has been demonstrated [16,23,24], while the effects of yoghurt production on penicillin drugs (e.g., penicillin G, cloxacillin, oxacillin, dicloxacillin, ampicillin, and nafcillin) in spiked milk have been investigated by Grunwald and Petz (2003) [17]. In the latter work, the main factors causing degradation of the penicillins were the temperature (90 °C for 15 min) during heat treatment of the milk before the yoghurt cultures were added, the binding of the penicillins to milk proteins, and the fermentation time (8 h at 43 °C). Degradation products of penicillin G (penillic, penicilloic, and penilloic acids) were identified via LC–MS/MS.

Roca et al. (2011) [19] analysed the effects of different temperatures and times of heat treatment during conventional milk processing on the concentration of 10 β-lactam antibiotics (penicillins and cephalosporins). Heat treatment of 120 °C for 20 min led to high degradation of β-lactams and cephalosporins, although among the cephalosporins investigated (cefoperazone, cefquinome, cephalexin, cefalonium, cephapirin, and cefuroxime), cefquinome showed the lowest degradation rate. Ultrahigh heat treatment (140 °C for 4 s) resulted in lower degradation percentages for both penicillins and cephalosporins. Good stability of nine β-lactam antibiotics, including cefquinome, had been previously reported when applying milder heat treatments, such as 40 °C for 10 min, or 60 °C for 60 min [25].

In this study, unpasteurized milk was used; therefore, severe heat treatments were not applied, and the cheese was made using a maximum temperature of 36 °C. The main purpose of our research was to investigate the migration of cefquinome residues into different dairy products if the former were present in the milk used for their manufacture. As a consequence, we decided to avoid any heat treatments that could cause degradation of the drug. In addition, raw milk is often employed for cheese manufacture at Irish farmhouses [26]; therefore, the current study contributed to assessment of the risk of the presence of antibiotic residues in dairy products when unpasteurized milk is used.

In relation to the drug distribution, this study demonstrated that cefquinome residues present in the milk migrated primarily with skimmed milk and buttermilk during product manufacture, and partially with whey during cheesemaking. Among the published papers reported in the literature, Giraldo et al. (2017) [27] showed that the coagulation of goat milk spiked with cephalosporin drugs led to variation in the transfer rate of the antibiotics from milk to whey, although cefquinome migrated mainly with the whey. In that study, only rennet was added to the milk to separate the curds and whey, while in the present study cheese was manufactured by also using starter cultures to promote acidification and aid in the formation of the curd. This difference could explain the different results. Hakk et al. (2016) [28] investigated the distribution of penicillin G, sulfadimethoxine, oxytetracycline, erythromycin, ketoprofen, thiabendazole, and ivermectin between skimmed milk and

milk fat derived from spiked bovine whole milk using radiochemical analysis. The results showed that more than 90% of the residues migrated with the skimmed milk for most of the drugs, depending on their lipophilicity. A subsequent study by Shappell et al. (2017) [29] focused on the distribution of these compounds between curd, whey, and milk protein fractions, and confirmed that the migration of the residues is correlated with their physicochemical properties, and that it is concentration-dependent. The research published by Lupton et al. (2018) [30] extended the investigation to 15 compounds by including 8 more drugs (acetaminophen, acetylsalicylic acid/salicylic acid, ciprofloxacin, clarithromycin, flunixin, phenylbutazone, praziquantel, and thiamphenicol). Those studies also showed that some of the drugs preferentially associated with whey proteins, while others associated with casein.

Cefquinome is an organic acid with pKa values of 2.51 and 2.91, and low fat solubility [11]. The hydrophilic/lipophilic properties of cefquinome sulphate at different pH values (pH 6.5, 7.4 and 8.5) have previously been reported, showing it to be a very hydrophilic compound, with a very low partition coefficient (Pow) of 0.01–0.02 [31]. The present study confirmed the hydrophilic properties of cefquinome, as it mostly partitioned into high-water-containing products. When milk spiked with cefquinome was separated, >90% of the residues migrated with the skimmed milk, and the levels of residues found in buttermilk were approximately 4–5 times higher than those found in butter. Similar results were obtained from the animal experiments, where >90% of the residues migrated with the skimmed milk, >70% with the whey, and the levels of residues found in buttermilk were approximately six times higher than the levels found in butter.

During cheesemaking, a difference was observed in the distribution of the antibiotic between curd and whey when using spiked milk or milk from treated animals. The distribution was approximately equal when using spiked milk, while it was approximately 0.5:1 between curd and whey when using the milk from the animal experiments. This is probably due to the different concentrations used in spiked samples and samples from treated animals, in agreement with the findings of Shappell et al. (2017) [29]. In addition, the hydrophilic nature and low pK_a values of cefquinome could result in limited distribution of the antibiotic to tissues [32] and promote its excretion with the milk. However, a number of conditions can cause differences in milk composition [33]. For example, variation of pH values and albumin, casein, and fat levels could affect the ability of cefquinome residues to bind with the milk proteins. The lower levels of casein allow the residues to bind more with the whey proteins, thus increasing the fraction of residues migrating with the whey in the natural treatment studies. The migration and protein binding of the drug residues could also be influenced by the fact that the milk used in this study did not undergo pasteurization, which could result in changes in the content of all protein fractions and their structural loss [34]; further studies would be required in order to investigate this fully.

With regard to sample analysis, this study was based on the method previously developed by Di Rocco et al. (2017) [20] for the determination of 30 β-lactam antibiotic residues in bovine muscle, with some modifications. The water fraction of the extraction solvent, which increased the polarity of the solution, was found to be fundamental for the efficient extraction of cefquinome residues from the matrices. While the liquid samples were vortexed for 1 min following the addition of the water/acetonitrile solution, a homogenisation step employing a probe blender was used for dissolving the solid samples, in order to reduce the matrix components to small particles and facilitate the extractability of the analyte. In addition, the implementation of a cefquinome-d_7 internal standard into the method enabled us to overcome the undesirable effects of the different sample matrices, providing a high degree of accuracy in the quantitation of cefquinome residues.

The calibration curve was established following a series of experiments aimed to verify the linearity of the method over an extended range of concentrations. The results showed some carry-over after injections of samples spiked with cefquinome at concentrations higher than 750–1000 μg kg^{-1}. The carry-over was still present when injecting blank samples on a brand new analytical column, following zero-volume injections, and after

replacing the sample loop and the line connecting the UHPLC to the MS system, while it disappeared after a source cleaning, confirming the contamination of the MS as the main problem. Therefore, it was decided to set the calibration range of the method to 2–500 μg kg^{-1}, and to analyse samples containing higher concentrations of the drug, using dilution with negative controls.

During this study, the samples were maintained at −80 °C prior to analysis. While some degradation of β-lactam antibiotics had been previously observed in samples of different matrices stored at 4 °C or −20 °C, no degradation was reported for samples stored at −76 °C [35].

5. Conclusions

The results of this study show that residues of cefquinome migrate with the skimmed milk as opposed to cream, buttermilk as opposed to butter, and equally between curd and whey during dairy product manufacture from spiked milk. In the case of naturally contaminated milk collected following animal treatment, the residues mostly migrated with the whey, due to different concentrations investigated during the study, or due to the binding mechanisms of the antibiotic during animal metabolism. This would have food safety implications if residues were present in the milk used for manufacture. Moreover, a change in the conditions used for dairy product manufacture—such as temperature, time, and pH—could alter the level of contamination from cefquinome residues. In relation to the withdrawal period, daily milk samples should be collected, and further studies would be required in order to investigate the persistence of cefquinome residues in milk.

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