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Short communication

Transfer of antibiotics from goat's milk to rennet curd and whey fractions during cheese-making

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Abstract

The transfer of 35 antibiotics from milk to curd and whey was evaluated. Cheeses were produced at laboratory scale, from antibiotic-free goat's milk spiked with different antibiotic concentrations between 0.25 and 4 times the Maximum Residue Limits established in milk. Drug concentrations in milk, curd and whey were analysed by UHPLC-HRMS. Results indicated that most antibiotics were mainly transferred from milk to whey (up to 85.9 %), with retention percentages in the curd lower than 50%, except for ceftiofur (59.7%) and dicloxacillin (52.8%). In most cases, drug distribution was unaffected by the antibiotic concentration in milk and correlated significantly to the drug lipophilicity (Log P) for β-lactams (R^2= 0.54) and sulfonamides (R^2= 0.62). When drug ionization was considered (Log D), improved correlation coefficients were obtained for macrolides (R^2 = 0.98). However, other factors besides the drug solubility should be considered to explain and predict the partitioning of antibiotics during cheese-making.

Keywords: rennet curd; whey; antibiotics; partitioning; UHPLC-HRMS

1. Introduction

Antibiotics are commonly used to treat and prevent mastitis and other infectious diseases in dairy livestock. However, improperly applied, antibiotic therapy could lead to the presence of drug residues in milk, posing a risk to consumer health mainly related to the development of multi-drug resistant bacteria (World Health Organization, 2019).

To protect consumers, the European Union established Maximum Residues Limits (MRLs) for pharmacologically active substances in foodstuffs of animal origin, including milk (Commission Regulation (EU) No 37/2010) and the implementation of national residue monitoring plans (Council Directive 96/23/EC). However, no limits have been set for dairy products and consumers might be exposed to significant amounts of antibiotics, even higher than those indicated for milk, in concentrated products like cheeses (Cabizza et al., 2017; Cabizza et al., 2018; Gajda, Nowacka-Kozak, Gbylik-Sikorska, & Posyniak, 2018; Quintanilla, Beltrán, Molina, Escriche, & Molina, 2019a).

Additionally, antibiotics present in milk are also released into the whey fraction during cheese-making (Giraldo, Althaus, Beltrán, & Molina, 2017) leading to negative effects on humans, animals, and the environment, given the food and agricultural applications of this by-product (Fresno, Darmanin, López, Camacho, & Álvarez, 2015; Prazeres, Carvalho, & Rivas, 2012). Scientific literature data on the partitioning of antibiotics during milk processing is limited, and mainly focused on a reduced number of veterinary drugs. Some of these research suggest that drug lipophilicity could explain the behaviour of diverse veterinary substances and be used to predict their distribution into the different milk matrices (Hakk et al., 2016; Lupton, Shappell, Shelver, & Hakk, 2018; Shappell, et al., 2017). However, to understand better the partitioning of antibiotics in curd and milk whey, and to evaluate its potential impact on consumer health, a higher number of substances belonging to different antibiotic groups should be considered. Therefore, the

aim of this study was to evaluate the transfer of numerous antibiotics from goat's milk to rennet curd and whey during cheese-making, and their connection to the lipophilicity of such substances.

2. Material and methods

2.1. Experimental procedure

Experimental cheeses were produced at laboratory scale, in triplicate, using antibiotic-free goat's milk spiked with five different concentrations of 35 antibiotics ranging from 0.25 to 4 times the MRL established for such substances in milk. Milk, rennet curd and whey fractions were analysed using Orbitrap Exactive^{TM} analyser to investigate the partitioning of antibiotics in different dairy matrices, and their relation to the lipophilicity of the neutral (Log P) and ionizable forms (Log D) of such substances.

2.2. Antibiotics and spiked milk samples

Table 1 presents the commercial references and the range of concentrations of the antibiotics, as well as the 4 internal standards used in this study. For each of them, a stock solution was prepared in methanol at a concentration ranging from 250 to 1500 µg/mL, which was stored at -20°C for further use.

Goat's milk for cheese production was spiked at different drug concentrations from working solutions containing simultaneously the considered antibiotic substances, which were daily prepared by diluting conveniently the standard stock solutions that had been made previously.

2.3. Cheese-making process

Raw milk was daily obtained from the experimental herd of Murciano-Granadina goats of the Universitat Politècnica de València (Valencia, Spain) and analysed for chemical composition by MilkoScan 6000 (Foss, Hillerød, Denmark), somatic cell count by

Fossomatic 5000 (Foss), total bacterial count by Bactoscan FC (Foss) and pH by a conventional pH-meter (Basic 20, Crison, Barcelona, Spain).

The chemical composition (g/100 g) of goat's milk used for cheese-making had an average (mean ± SD) total solids content of 8.98 ± 0.06, fat 5.32 ± 0.37, lactose 4.63 ± 0.04 and protein 3.62 ± 0.06. The somatic cell count and total bacterial count reached 5.86 log cells/mL and 4.34 log cfu/mL, respectively, and the average pH value was 6.86 ± 0.05.

Curd and whey samples were obtained from a laboratory scale cheese-making procedure according to Giraldo et al. (2017). Thus, raw milk (40 ± 0.5 g) was heated at 33 ± 1ºC in a water bath and curdled using animal rennet (1:10000. Suministros Arroyo, Santander, Spain) in 50 mL conical centrifuge tubes. After coagulation (30 min at 33ºC), the curd was cut and heated for 15 min at 35 ± 1ºC, being mixed with a scraper. Then, the tubes were centrifuged at 3000 rpm for 10 min, and the whey separated using a metallic tea strainer.

Milk, curd, and whey fractions were accurately weighed to apply a mass balance to calculate the partitioning of antibiotics through a cheese-making procedure.

2.4. Analysis of antibiotic residues in dairy matrices

Antibiotic concentrations in milk, curd and whey samples were measured in duplicate, by UHPLC-HRMS, according to Igualada, Giraldo, Font, & Yusà (2021). Antibiotics were quantified by matrix-matched calibration curves using isotopic internal standards (Table 1), except for macrolides and lincosamides, which were quantified by external calibration. Sample treatment involved a liquid-liquid extraction (LLE) using acetate buffer 0.2 mol/L at pH 5.2 and acetonitrile (20/80, v/v), followed by C18 dispersive Solid Phase Extraction (dSPE).

For a chromatographic analysis, an Accela liquid chromatography UHPLC system (Thermofisher Scientific, Bremen, Germany) equipped with a Kinetex C18 XB column (50 x 3.00 mm, 2.6 µm) (Phenomenex, Madrid, Spain) was applied. The chromatographic conditions were the following: an injection volume of 10 µL, a flow rate of 400 µL/min and the temperature of column reaching 25°C. Separations carried out using a binary gradient that combined 0.1% formic acid aqueous solution and methanol containing 0.1 % formic acid as mobile phase.

The Orbitrap Exactive^{TM} analyser (Thermofisher Scientific, Bremen, Germany) was equipped with a heated electrospray ionization interface (HESI-II) and operated in positive and negative mode within the mass range of 80-1,200 m/z. The data acquisition was executed in full scan mode (65-500 Da) at a resolving power of 50000 FWHM with 5 ppm of mass tolerance, by using the Thermofisher Scientific's Xcalibur 2.1.0 software.

2.5. Statistical analysis

Drug concentration ratios between curd and whey fractions ([curd]/[whey]) were calculated to evaluate the partitioning of antibiotics during cheese-making. Normalized drug distribution rates, expressed as percentage, were also determined by applying a mass balance.

Experimental data were analysed using Statgraphics Centurion XVII software (StatPoint Technologies, Inc., Warrenton, VA). To investigate the effect of the antibiotic concentration and the experimental replicate on drugs distribution, a one-way ANOVA test was performed. Tukey's multiple-comparison test was used for paired comparison of average treatments and the level of significance was determined at $p< 0.05$.

The partition (Log P) and the distribution (Log D) coefficients of the antibiotics were considered to evaluate the relation between drug lipophilicity and drug partitioning (Log [curd]/[whey]) during cheese-making by applying a lineal regression model. Log D

values at goat's milk pH used in this study (pH= 6.86) were calculated using Log P and pKa values reported in Table 1, according to the equations specified by Hakk et al. (2016) for acidic and basic substances: $Log\ D_{acid} = Log\ P + Log\ [1/(1 + 10^{pH-pKa})]$ and $Log\ D_{base} = Log\ P + Log\ [1/(1 + 10^{pKa-pH})]$.

As the percentage of moisture (or included whey) can vary between cheeses, the antibiotic existence in the dry curd fraction (0% moisture) was used for the calculation of the logarithm of the concentration ratios (Log 0% [curd]/[whey]). Antibiotic concentration in the dry curd fraction was calculated according to Shappell et al. (2017), by subtracting the whey-entrained drug amounts in the wet curd, considering that the interstitial whey of the wet curd and the separated whey fraction had the same antibiotic concentration.

A Principal Components Analysis (PCA) was carried out to detect potential connection among variables and the antibiotic groups considered.

3. Results and discussion

3.1. Drug distribution between curd and whey

As shown in Fig. 1., antibiotics found in milk were mainly released into the whey fraction (up to 85.9%) during the drainage of the experimental cheeses. Thus, in general, the percentage of antibiotics retained in the curd fraction was lower than 50% in all cases, except for ceftiofur (59.7%) and dicloxacillin (52.8%) and very variable between drugs.

Similar curd retention percentages to those obtained in this study, were reported by Shapell et al. (2017) for oxytetracycline (15%), erythromycin (22%) and sulfadimethoxine (28%), when assessing the transfer of different veterinary drugs from skim milk to whey and curd fractions. Only in the case of benzylpenicillin (12%) was the result half of that shown in this experiment. In a similar study, Lupton et al. (2018) reported a higher retention rate close to 50% for ciprofloxacin.

156 In general, the [curd]/[whey] ratios (Table 2) were drug-dose independent ($p > 0.05$) and
157 lower than one for most of the antibiotics considered, as such substances were mainly
158 released into the whey, reaching higher concentrations than those found in the curd
159 fraction. However, some antibiotics including most β-lactams, tilmicosin, danofloxacin,
160 ciprofloxacin, sulfadimethoxine, sulfaquinoxaline and tetracyclines were more
161 concentrated in the rennet curd matrix, showing concentrations higher than those obtained
162 for the whey fraction. And, in some cases (oxacillin, cefoperazone, cloxacillin, nafcillin,
163 dicloxacillin, desfuroylceftiofur, and ceftiofur) being between 1.7 and 3.3 times higher
164 than drug concentration initially present in milk.
165 Fig. 2A. shows the PCA-biplot of the correlation coefficients among the variables
166 considered as arranged by the position of antibiotics on each principal component (PC)
167 axis. The two PCs accounted for 49.48 % and 36.47 % of the variance, respectively. As
168 shown in Fig. 2A., Log P and Log 0% [curd]/[whey] were the most important variables
169 for the formation of PC1, that was negatively correlated to the pKa of the antibiotics.
170 Instead, pKa was the most important variable for PC2, being negatively correlated to the
171 Log 0% [curd]/[whey].
172 Regarding to antibiotic groups (Fig. 2B.), macrolides and quinolones were correlated to
173 the drug lipophilicity (Log D and Log P) while sulfonamides and tetracyclines were more
174 correlated to the variable pKa.

175 *3.2. Empirical modelling of drug distribution between curd and whey fractions*

176 To explain the partitioning of the different antibiotic groups the Log 0% [curd]/[whey]
177 ratio was calculated for plotting with the drug lipophilicity (Log P) and lipophilicity plus
178 ionization (Log D). All data were used (fifteen distribution ratios per drug obtained from
179 three replicates for each of the five concentrations assessed) in the lineal regression
180 analysis for those antibiotics with available Log P and pKa values ($n = 30$). As drug

distribution was dose independent for most antibiotics, the average Log 0% [curd]/[whey] was employed in the statistical analyses. For the exceptions (Table 2), the slope resulting from plotting the antibiotic concentration retained in the curd with respect to that released into the whey for each of the 5 concentrations that were considered.

As shown in Fig. 3, drug distribution between curd and whey fractions during cheese-making was significantly correlated to the drug lipophilicity (Log P) for β-lactams (R^2= 0.54, p= 0.0245) and sulfonamides (R^2= 0.62, p= 0.0117). When ionization of the molecules at the pH of the medium (6.86) was considered (Log D), an improved correlation was obtained for macrolides and lincosamides (R^2= 0.98, p= 0.0074). However, for quinolones (R^2= 0.80, p= 0.1076) and tetracyclines (R^2= 0.89, p= 0.0570) correlations did not become statistically significant and the results were inconclusive. Moreover, quinolones and ionized forms of tetracyclines showed an inverse tendency to that observed in the other drug families (Fig. 3.), with ciprofloxacin and chlortetracycline, having the lowest lipophilicity values in their respective groups of antibiotics, being among the drugs more concentrated in the curd matrix (Table 2), with retention percentages of 31.9 % and 37.2 %, respectively (Fig. 1.). The high affinity of ciprofloxacin (Lupton et al., 2018; Pápai et al., 2010) and chlortetracycline (Dantas et al., 2020) to binding to curd proteins and to form insoluble quelates with metal ions like calcium present in milk could explain the different behaviour of these antibiotics.

These results suggest that factors other than lipophilicity of the antibiotics as well as milk composition, or the cheese-making process itself (heat treatments, pH, maturation time, etc.) should be considered to better explain and predict the transfer of antibiotics from milk to rennet-curd cheeses. Thus, as reported by Quintanilla et al. (2019a, 2019b) when assessing the transfer of antibiotic from goat's milk to rennet curd cheeses, the percentage of β-lactams retained in the ripened cheese manufacture (8.4-16.4 %) differs significantly

206 to those obtained for the same substances in the fresh cheese production (58.4– 75.2 %).
207 On the contrary, the retention of oxytetracycline was higher in the ripened cheese (68 %)
208 than in the fresh cheese (37.5 %).

209 **4. Conclusions**

210 Results herein indicate that antibiotics present in milk are transferred mostly from milk
211 to whey during cheese-making, which could carry damaging implications for humans,
212 animals, and the environment. In addition, the lower amounts of antibiotics transferred
213 from milk to curd could achieve, in some cases, higher concentrations than those indicated
214 for milk, with negative consequences for public health. In general, drug distribution was
215 not affected by the antibiotic concentration initially present in milk, and significantly
216 related to the drug lipophilicity for some antibiotic groups, for which the resulting
217 distribution models could be a useful tool to predict the partitioning of such substances
218 during cheese-making.
219 However, it would be of great interest to include other aspects related to the physico-
220 chemical properties of the veterinary drugs, the milk nature, or the different cheese-
221 making conditions, in order to achieve a more accurate predicting equations allowing to
222 define the potential risk of finding certain antibiotics in curd and whey fractions, and thus,
223 evaluating the associated consequences for public and animal health, and the
224 environment.

Credit authorship contribution statement

Jennifer Giraldo: data curation, formal analysis, investigation, methodology, resources, software, validation, writing original draft preparation, writing-review and editing. **Carmen Igualada:** methodology, software, supervision, validation. **Roberto Cabizza:** formal analysis, investigation, resources, writing original draft preparation, writing-review and editing. **Rafael Althaus:** conceptualization, data curation, supervision. **María Carmen Beltrán:** conceptualization, data curation, investigation, supervision, writing original draft preparation, writing-review and editing.

Declaration of Competing Interest

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

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Table 1.

Antibiotics used to evaluate the partitioning of antibiotics during the cheese-making process.

Antibiotics	Reference	Log P	pKa	EU-MRL (µg/kg)	Concentration ranges (µg/kg)
β-lactams					
Ampicillin	59349[a]	1.35	3.24	4	1, 2, 4, 8, 16
Benzylpenicillin	46609[a]	1.67	3.53	4	1, 2, 4, 8, 16
Cloxacillin	46140[a]	2.53	3.75	30	7.5, 15, 30, 60, 120
Dicloxacillin	46182[a]	3.02	3.75	30	7.5, 15, 30, 60, 120
Nafcillin	32071[a]	3.52	3.31	30	7.5, 15, 30, 60, 120
Oxacillin	46589[a]	2.05	3.75	30	7.5, 15, 30, 60, 120
Cefalexin	33989[a]	0.65	3.26	100	25, 50, 100, 200, 400
Cefoperazone	32426[a]	1.43	3.19	50	12.5, 25, 50, 100, 200
Ceftiofur	34001[a]	2.05	2.83	100	25, 50, 100, 200, 400
Desfuroylceftiofur	D289980[b]	-	-	100	25, 50, 100, 200, 400
Penicillin G-D7[*]	32985[a]	-	-		100
Macrolides[**]					
Erythromycin	46256[a]	2.83	8.38	40	10, 20, 40, 80, 160
Spiramycin	46745[a]	3.06	9.33	200	50, 100, 200, 400, 800
Neo Spiramycin	N390040[b]	-	-	200	50, 100, 200, 400, 800
Tilmicosin	33864[a]	4.95	10.16	50	12.5, 25, 50, 100, 200
Lincosamides[**]					
Lincomycin	15443869[c]	0.91	7.97	150	37.5, 75, 150, 300,
Quinolones					
Danofloxacin	33700[a]	1.20	5.65	30	7.5, 15, 30, 60, 120
Enrofloxacin	33699[a]	1.88	5.69	100	25, 50, 100, 200, 400
Ciprofloxacin	33434[a]	0.65	5.76	100	25, 50, 100, 200, 400
Flumequine	45735[a]	2.41	6.00	50	12.5, 25, 50, 100, 200
Norfloxacin-D5[*]	CH001[d]	-	-		100
Sulfonamides					
Sulfacetamide	46770[a]	0.07	4.30	100	25, 50, 100, 200, 400
Sulfadiazine	35033[a]	-0.12	6.99	100	25, 50, 100, 200, 400
Sulfadimethoxine	46794[a]	1.48	6.91	100	25, 50, 100, 200, 400
Sulfamerazine	46826[a]	0.34	6.99	100	25, 50, 100, 200, 400
Sulfamethazine	46802[a]	0.80	6.99	100	25, 50, 100, 200, 400
Sulfamethoxypyridazine	46858[a]	0.32	6.84	100	25, 50, 100, 200, 400
Sulfapyridine	31738[a]	0.03	6.24	100	25, 50, 100, 200, 400
Sulfaquinoxaline	45662[a]	1.30	6.79	100	25, 50, 100, 200, 400
Sulfathiazole	46902[a]	0.05	6.93	100	25, 50, 100, 200, 400
Sulfadimethoxine-D6[*]	SA001[d]	-	-		100
Tetracyclines					
Chlortetracycline	C4881[a]	-0.53	9.04	100	25, 50, 100, 200, 400
4-epi-Chlortetracycline	268231000[e]	-	-	100	25, 50, 100, 200, 400
Doxycycline	33429[a]	-0.54	8.33	100	25, 50, 100, 200, 400
Oxytetracycline	46598[a]	-1.50	7.41	100	25, 50, 100, 200, 400
4-epi-Oxytetracycline	257711000[e]	-	-	100	25, 50, 100, 200, 400
Tetracycline	31741[a]	-0.62	8.24	100	25, 50, 100, 200, 400
4-epi-Tetracycline	233121000[e]	-	-	100	25, 50, 100, 200, 400
Demeclocycline[*]	46161[a]	-	-		100

Log P: partition coefficient from www.chemspider.com, using the ADC Lab-predicted values, and pKa values from www.drugbank.ca, accessed on October 2021; EU-MRL: European Union-Maximum Residue Limit fixed in milk (European Union, 2010). [*]Isotopically labelled Internal Standard (IS). [**]External calibration (without IS). [a]Sigma-Aldrich Química, S.L. (Madrid, Spain); [b]Toronto Research Chemicals, Inc. (Toronto, Canada); [c]Honeywel Riedel-de-Haën, A.G. (Seelze, Germany); [d]WITEGA Laboratorien Berlin-Adlershof GmbH. (Berlin, Germany); [e]Acros Organics B.V.B.A. (Geel, Belgium). Data missing (-): not found in the literature.

316 **Table 2.**

317 Antibiotic concentration ratios between rennet curd and whey fractions according to
318 the drug levels in milk used for cheese production.

Antibiotics	Equivalent drug concentration in raw milk					SE
	0.25 EU-MRL	0.50 EU-MRL	1 EU-MRL	2 EU-MRL	4 EU-MRL	
β-lactams						
Ampicillin	0.6340	0.6499	0.6579	0.8380	0.8640	0.1082
Benzylpenicillin	-	0.6974[a]	0.8364[ab]	1.0593[bc]	1.1460[c]	0.0607
Cloxacillin	3.1627	1.6863	1.8479	1.7777	1.6846	0.6066
Dicloxacillin	-	2.5481	2.9804	2.7347	2.9666	0.3770
Nafcillin	-	1.6329	2.2341	1.8727	1.9095	0.2525
Oxacillin	1.4387	1.1847	1.4818	1.3743	1.3889	0.1596
Cefalexin	0.7704	0.9543	0.7373	0.8573	0.7221	0.1304
Cefoperazone	-	1.5672	1.6351	1.8369	1.4601	0.3251
Ceftiofur	3.8501	3.2923	3.3410	3.6693	3.5616	0.5586
Desfuroylceftiofur	-	1.6020	2.0521	2.7207	2.7902	0.5348
Macrolides						
Erythromycin	-	0.8097	1.1361	1.2325	0.9941	0.1088
Spiramycin	0.8422	0.8148	0.9012	0.9147	0.9480	0.0589
Neo Spiramycin	0.7184	0.7840	0.9213	0.9043	0.9721	0.0595
Tilmicosin	1.1266	1.1830	1.2521	1.3548	1.1803	0.1909
Lincosamides						
Lincomycin	0.6766	0.6680	0.7381	0.7918	0.7910	0.0639
Quinolones						
Danofloxacin	2.5954[b]	1.2345[a]	1.2068[a]	1.1124[a]	1.1553[a]	0.2465
Enrofloxacin	0.8959	0.8527	1.0855	1.0248	1.1022	0.2066
Ciprofloxacin	1.2683	1.0753	1.1289	1.0148	1.0692	0.0856
Flumequine	0.3696[a]	0.7651[ab]	1.0662[b]	0.8552[ab]	0.9024[ab]	0.1554
Sulfonamides						
Sulfacetamide	-	0.4520	0.4128	0.4422	0.3648	0.1157
Sulfadiazine	0.5404	0.6128	0.6780	0.6203	0.5927	0.0740
Sulfadimethoxine	1.7401	1.2173	1.1633	1.0826	1.0199	0.2259
Sulfamerazine	0.4975	0.6832	0.7816	0.7308	0.6572	0.1218
Sulfamethazine	0.9658	0.9318	0.9264	0.8483	0.8507	0.0791
Sulfamethoxypyridazine	0.6928	0.8510	0.9242	0.8086	0.7942	0.1222
Sulfapyridine	0.8481	0.7887	0.8623	0.8501	0.7817	0.1081
Sulfaquinoxaline	2.2027	1.7707	1.7819	1.6665	1.5317	0.3497
Sulfathiazole	-	0.5976	1.2442	1.2599	1.1072	0.2029
Tetracyclines						
Chlortetracycline	-	1.6501	1.6978	1.3602	1.0789	0.2581
4-epi-Chlortetracycline	0.7270	0.5099	0.5121	0.4279	0.3425	0.2094
Doxycycline	0.4109	0.4258	0.4477	0.4173	0.3146	0.0977
Oxytetracycline	0.3539	0.3730	0.3628	0.3145	0.2312	0.0548
4-epi-Oxytetracycline	-	1.0272	1.2235	1.2023	0.8467	0.2299
Tetracycline	0.7623	0.7121	0.7774	0.6970	0.6341	0.1636
4-epi-Tetracycline	-	0.7735	0.7784	0.7044	0.5581	0.1016

319 EU-MRL: European Union-Maximum Residue Limit fixed in milk (European Union, 2010). SE: Standard Error.
320 Data missing: (-): drugs with CCβ out of evaluated concentration range for some of the three matrices (milk, cheese,
321 whey) considered. [a, b, c]: different letters in the same row indicate significant differences ($p < 0.05$).

322 **Figure captions**

323 **Fig. 1.** Normalized percentages of antibiotics retained in the rennet curd fraction and
324 released into the whey during cheese-making.

325 **Fig. 2. (a)** PCA-biplot of the main components from the PCA analysis; **(b)** Diagram of
326 dispersion for antibiotics (n= 30) according to PCA components.

327 **Fig. 3.** Relation between the logarithm of antibiotic concentration in curd (0% moisture)
328 to whey ratio and partition (Log P) and distribution coefficients (Log D) for β-lactams **(a,**
329 **b)**; macrolides and lincosamides **(c, d)**; quinolones **(e, f)**; sulfonamides **(g, h)**; and
330 tetracyclines **(i, j)**.