Distribution of Chemical Residues among Fat, Skim, Curd, Whey, and Protein Fractions in Fortified, Pasteurized Milk

Weilin L. Shelver,* Sara J. Lupton,† Nancy W. Shappell,‡ David J. Smith,* and Heldur Hakk

USDA-ARS, Red River Valley Agricultural Research Center, Biosciences Research Laboratory, 1605 Albrecht Boulevard, Fargo, North Dakota 58102-2765, United States

Supporting Information

ABSTRACT: The distribution of 12 environmental contaminants or metabolites with diverse polarities (2,2′,4,4′,5-pentabromodiphenyl ether; bisphenol A; estrone; glyphosate; β-hexabromocyclododecane; imidacloprid; 2,3',4,4′,5-pentachlorobiphenyl; 3′-methylsulfone 2,2′,4,5,5′-pentachlorobiphenyl; 1,2,7,8-tetrachlorodibenzofuran; 2-hydroxy-1,3,7,8-tetrachlorodibenzo-p-dioxin; tetrabromobisphenol A; and triclocarban) among skim milk, fat, curd, whey, whey retentate, and whey permeate was characterized. Analysis of these compounds along with 15 drugs previously studied provided a robust linear model predicting the distribution between skim and fat and the chemical’s lipophilicity (log P, ρ^2 = 0.71; log D, ρ^2 = 0.79). Similarly, distribution between curd and whey was correlated with lipophilicity (log P, ρ^2 = 0.63; log D, ρ^2 = 0.73). Phenolic compounds had less predictable distribution patterns based on their lipophilicities. Within the whey fraction, chemicals with greater lipophilicity are associated with whey proteins more than hydrophilic chemicals. The resultant model could help predict the potential distribution of chemical contaminants among milk products in cow milk, if present.

INTRODUCTION

Pesticides or environmental contaminants of milk or milk products may occur after the consumption of contaminated feed, exposure to contaminated facilities, and/or contamination during milk handling, transport, and/or processing. Reported levels of pesticides and/or persistent organic pollutants in milk vary depending on geographic region, year of survey, and chemical classes analyzed.1–6 The distribution of a chemical among a variety of milk products is dependent on its physicochemical properties, the milk processing methods used, and the final milk products produced.

Previously, we tested the hypothesis that drug distribution among milk fractions could be predicted based on lipophilicity (log D, distribution coefficients or log P, partition coefficients).7–9 A mixed linear model was proposed for the distribution between milk fat and skim milk based on the study of seven veterinary drugs (Table S1).7 The seven drugs included four antibiotics [erythromycin (ERY), penicillin G (PENG), oxytetracycline (OTET), and sulfadimethoxine (SDMX)], two anthelmintics [ivermectin (IVR) and thiabendazole (THLA)], and one analgesic [ketoprofen (KETO)]. A linear model of the same drugs was also described for the distribution between curd and whey, as well as whey protein associations.8 The model was then expanded to include four additional antibiotics [ciprofloxacin (CIPR), clarithromycin (CLA), thiamphenicol (TAP), and phenylbutazone (PBZ)], three analgesics/antipyretics [acetylsalicylic acid (ASP)/salicylic acid, acetaminophen (TYL), and flunixin (FNX)], and one anthelmintic [praziquantel (PZQ)] (Table S2).9 The distribution model fit (ρ^2) decreased when the data set was expanded from 7 to 15 drugs; however, modeling based on log D values still provided a better fit than those based on log P.

In this report, we consider 12 environmental contaminants to better understand the possible impacts of a broader range of log D and log P values on xenobiotic distribution in milk. Specifically, we studied the herbicide glyphosate (GLY); an insecticide, imidacloprid (IMI); a common component of plastics and canned food liners, bisphenol A (BPA); an antibacterial, triclocarban (TCC); a steroid hormone, estrone (E1); several brominated flame retardants, β-hexabromocyclododecane (β-HBCD), tetrabromobisphenol A (TBBPA), and 2,2′,4,4′,5-pentabromodiphenyl ether (BDE-99); and a number of persistent organic pollutants and their metabolites [1,2,7,8-tetrachlorodibenzo-p-dioxin (1,2,7,8-TCDD); 2-hydroxy-1,3,7,8-tetrachlorodibenzo-p-dioxin (2-OH-1378-TCDD); 2,3′,4,4′,5′-pentachlorobiphenyl (PCB-118); and 3′-methylsulfone-2,2′,4,5,5′-pentachlorobiphenyl (3′-MeSO2-PCB-101)]. To the best of our knowledge, the fate/distribution of these chemicals in milk processing has not
been reported. The chemicals studied in this report expands the polarity range of the previous studies (now spanning log $D$ from $-4.2$ to $7.3$) and fills in the knowledge gap for chemicals with log $D$ values between $3.4$ and $6.6$. Data obtained from this study have been combined with data from our previous reports, allowing us to expand distribution modeling to include a total of 27 chemicals.

## RESULTS AND DISCUSSION

### Chemical Distribution from Whole Milk into Milk Fat and Skim Milk

Milk partitioning into lipid was highly reproducible, with typical coefficient of variance (CV) values of $\leq 5\%$; exception was GLY with CV up to $19\%$ (Tables S5–S16). The high CV of GLY was due to its low partitioning into milk fat (Table S8). Similarly, CV of partitioning into skim milk was $\leq 5\%$; exceptions were BDE, $\beta$-HBCD, 3′-MeSO$_2$-PCB-101, PCB, and TCC because of low amounts in the skim milk. Recoveries (sum of total radioactivity in skim milk and milk fat) to the initial drug concentration in whole milk were $\leq 20$ for highly nonpolar persistent environmental contaminants (BDE-99, $\beta$-HBCD, 3′-MeSO$_2$-PCB-101, PCB-118, TCC, and 1278-TCDD; Figure 1). Also as expected, polar chemicals partitioned to a large degree into skim milk, resulting in milk fat/whole milk concentration ratios of $<1$ (GLY was 0.2, and IMI was 0.5; Figure 1). For the phenolic compound BPA, substitution of four phenyl hydrogens with bromines to form TBBPA (Table 1) increased lipophilicity (log $D = 3.60$ vs $6.69$) and was reflected by TBBPA’s milk fat/whole milk concentration ratio of $10.5$ compared to that of $8.2$ for BPA (Figure 1). Hydroxylation of a molecule decreases its relative lipophilicity with respect to its nonhydroxylated analogue, as is commonly observed during oxidative metabolism. Although 1278-TCDD and 2-OH-1378-TCDD have very similar log $D$ values (6.15 and 6.22, respectively) hydroxylation resulted in reduced lipid solubility and a $\sim 30\%$ reduction in milk fat distribution. However, the addition of a more polar functional group onto a pentachloro biphenyl molecule to form 3′-MeSO$_2$-PCB-101 did not shift the milk fat distribution pattern when compared to PCB-118. One possible explanation may be due to the change of chlorine substitution pattern.

As would be anticipated, the data indicated that nonpolar chemicals concentrate into high lipid milk fractions. The concentration ratios in milk fat relative to whole milk for moderately polar phenolic compounds were about $10$ (BPA, 8.2; TBBPA, 10.5; 2-OH-1378-TCDD, 11.2; and E1, 15.8) and were $\sim 18$–$20$ for highly nonpolar persistent environmental contaminants (BDE-99, $\beta$-HBCD, 3′-MeSO$_2$-PCB-101, PCB-118, TCC, and 1278-TCDD; Figure 1). Also as expected, polar chemicals partitioned to a large degree into skim milk, resulting in milk fat/whole milk concentration ratios of $<1$ (GLY was 0.2, and IMI was 0.5; Figure 1). For the phenolic compound BPA, substitution of four phenyl hydrogens with bromines to form TBBPA (Table 1) increased lipophilicity (log $D = 3.60$ vs $6.69$) and was reflected by TBBPA’s milk fat/whole milk concentration ratio of $10.5$ compared to that of $8.2$ for BPA (Figure 1). Hydroxylation of a molecule decreases its relative lipophilicity with respect to its nonhydroxylated analogue, as is commonly observed during oxidative metabolism. Although 1278-TCDD and 2-OH-1378-TCDD have very similar log $D$ values (6.15 and 6.22, respectively) hydroxylation resulted in reduced lipid solubility and a $\sim 30\%$ reduction in milk fat distribution. However, the addition of a more polar functional group onto a pentachloro biphenyl molecule to form 3′-MeSO$_2$-PCB-101 did not shift the milk fat distribution pattern when compared to PCB-118. One possible explanation may be due to the change of chlorine substitution pattern.

Although literature describing the milk partitioning of the exact compounds studied here has not been found, there are several relevant studies available for comparison. For example, Jensen and Hummel$^{10}$ administered 2,4,5-trichlorophenoxy-acetic acid containing 2,3,7,8-TCDD to lactating dairy cows and found that 2,3,7,8-TCDD residues in cream exceeded those in milk by a factor of about 10. Although this is much lower than our reported ratio of $\sim 19$ for 1,2,7,8-TCDD (Figure 1), the difference could originate from the “medium heavy cream” used in the Jensen and Hummel study$^{10}$ which would have a fat content $<36\%$. On the basis of our previous

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![Figure 1](https://example.com/figure1.png)  
**Figure 1.** Chemical distribution and relative concentration ratios from whole milk into skim milk and milk fat fractions. Bars represent percent mean of all concentrations (n = 3 concentrations, 3 replicates per concentration, replicate exceptions are n = 2 replicates each for 1278-TCDD 20 and 200 nM and n = 2 replicates for BDE-99 2000 nM) ± SD of the three dose means based on disintegrations per minute (dpm) of skim milk and milk fat fractions compared to whole milk dpm. Values on graph represent the mean ratio of the drug concentration in the fraction (milk fat or skim milk) to the initial drug concentration in whole milk ± SD of means between doses (n = 3 mean dose ratios). Sum of stack plot represents total chemical recovery. log $D$ values given for each compound at bottom of plot.
reports by Hakk et al. and Lupton et al., our milk fat had an average fat content of 82%. Regardless, our data confirmed those of Jensen and Hummel in that the majority of dioxin residues would be associated with milk fat.

Compounds with a log $D$ or $P$ value of about 6 consistently concentrated in milk fat (or cream as cited in references). Concentrations of dichlorodiphenyltrichloroethane (DDT, Table S4) (log $D$ 6.22 and log $P = 5.92$) in raw whole milk (5% lipid), skim milk, and cream (70% lipid) were reported as 7.5, 0.2, and 67.2 ppm, respectively, with a cream/whole milk ratio of 9.0. Pasteurization produced a slight increase of the cream/whole milk distribution ratio, as pasteurized whole milk

__Table 1. Drug Structures and Physicochemical Properties__

| Compounda | Class/Use | M.W. S.A. (mL/mol)b | log $P$ | pKac | log $D$ |
|-----------|-----------|---------------------|--------|------|--------|
| $^{14}$C-Glyphosate (GLY) | Herbicide/pesticide | 169.07 g/mol 50 (20 nM/200 nM) 5.0 (2000 nM) | $-$3.26 ± 1.53 | 5.89 ± 0.40 | $-$4.24 ± 1.49 |
| $^{14}$C-Imidacloprid (IMI) unknown label | Insecticide/pesticide | 255.66 g/mol 25.3 (20 nM/200 nM) 2.5 (2000 nM) | 0.39 ± 0.59 | 5.28 ± 0.59 | $-$0.38 ± 0.59 |
| $^{14}$C-Bisphenol A (BPA) | Plasticizer | 228.28 g/mol 53.5 (20 nM/200 nM) 6.0 (2000 nM) | 3.60 ± 0.27 | 3.60 ± 0.27 | 3.60 ± 0.27 |
| $^{14}$C-Estrone (E1) | Hormone | 270.57 g/mol 51.3 (20 nM/200 nM) 5.7 (2000 nM) | 3.62 ± 0.45 | 3.62 ± 0.45 | 3.62 ± 0.45 |
| $^{14}$C-3’-methylsulfone-2,2’,4,4’,5,5’-pentachlorobiphenyl (3-MeSO-PCB-101) | PCB Metabolite | 404.52 g/mol 53 (20/100/500 nM) | 4.62 | 4.62 | 4.62 |
| $^{14}$C-Trilocarban (TCC) | Antibacterial/disinfectant | 315.58 g/mol 30 (20 nM/200 nM) 3.0 (2000 nM) | 5.39 ± 0.45 | 5.39 ± 0.45 | 5.39 ± 0.45 |
| $^{14}$C-2-hydroxy-1,3,7,8-tetrachlorodibenzo- $p$-dioxin (2-OH-1378-TCDD) | TCDD Metabolite | 337.97 g/mol 64.6 (20 nM/100 nM) 12.8 (500 nM) | 6.15 ± 0.32 | 6.15 ± 0.32 | 6.15 ± 0.32 |
| $^{14}$C-2,3’,4’,5’-pentachlorobiphenyl (PCB-118) | Coolants/plasticizers/hydraulic fluids/pesticides/ flame retardant | 326.43 g/mol 10.3 (50 nM/200 nM) 2.5 (2000 nM) | 6.78 ± 0.35 | 6.78 ± 0.35 | 6.78 ± 0.35 |
| $^{14}$C-$\beta$-hexachlorocyclooctadecane ($\beta$-HBCD) unknown label | Flame Retardant | 641.69 g/mol 2 (200/500/2000 nM) | 7.22 ± 0.65 | 7.22 ± 0.65 | 7.22 ± 0.65 |
| $^{14}$C-1,2,7,8-tetrachlorodibenzo- $p$-dioxin (1278-TCDD) | Industrial and incineration byproduct | 321.97 g/mol 67.8 (20 nM/200 nM) 6.8 (2000 nM) | 6.22 ± 0.72 | 6.22 ± 0.72 | 6.22 ± 0.72 |
| $^{14}$C-Tetrabromo Bisphenol A (TBBPA) | Flame Retardant | 543.87 g/mol 25 (20 nM/200 nM) 3.7 (2000 nM) | 6.69 ± 0.58 | 6.69 ± 0.58 | 6.69 ± 0.58 |
| $^{14}$C-2,4’,5,5’-pentabromo diphenyl ether (BDE-99) | Flame Retardant | 564.69 g/mol 49 (20 nM) 8.76 (200 nM) 0.98 (2000 nM) | 7.31 ± 0.62 | 7.31 ± 0.62 | 7.31 ± 0.62 |

“Compound radioactively labeled with a directed label and specified on the structure with a red asterisk. An asterisk within a ring indicates a uniform label on the ring. Exceptions: IMI and $\beta$-HBCD carbon labels are unknown. $^b$SAs were adjusted depending on dose, as indicated. Values in parentheses are nominal concentrations for initial fortification. $^c$Average log $P$ calculated from literature log $P$ values accessed from www.chemspider.com, www.drugbank.ca, www.ebi.ac.uk/chembl/, and pubchem.ncbi.nlm.nih.gov/ on 7/14/2017 using the predicted and experimental values were available. $^d$Values for log $D$ at pH 6.8 were calculated using log $P$ values from above sources and $pK_a$’s from www.drugbank.ca, www.ebi.ac.uk/chembl/, www.druginfosys.com, pubchem.ncbi.nlm.nih.gov/, Johansson and Anle accessed on 7/14/2017.

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contained 6.0 ppm and cream contained 70.2 ppm DDT resulting in a cream/whole milk ratio of 12.12 Langlois et al.13 reported the identical ratio of cream/whole milk for DDT in spite of a fat content for cream of only 37%. Relative to the Mann12 and Langlois et al.13 reports, higher milk fat/whole milk concentration ratios were found in this study for compounds having log P ~ 6 (TCC, log P = 5.39, ratio 17.6; 1278-TCDD, log P = 6.22, ratio 18.7; PCB-118, log P = 6.78, ratio 19.5; Figure 1), which is also consistent with IVR (log P = 6.61, ratio 18.4) as reported by Hakk et al. The exception was 2-OH-1378-TCDD (log P = 6.15) which had a milk fat/whole milk concentration ratio of 11.2 in this study (Figure 1). These lower concentration ratios reported in the literature versus the current findings may be a reflection of differences in composition of the milk fat prepared here and the cream prepared in the cited reports.

A compound with a log P value similar to that of BPA (log P = 3.60) is the organophosphate cruformate (log P = 3.33, Table S4), which was fed to cows.14 Similar to BPA, which concentrated eightfold in fat relative to whole milk, cruformate concentrated about fivefold into cream.14 If values were adjusted to reflect lipid mass yield (15% of whole milk in their study, 10% in ours) the fivefold concentration would increase to ~7.6-fold, in close agreement with the eightfold concentration found for BPA. For fenithion (log P = 3.21, Table S4), an organothiophosphate insecticide, the concentration ratio of fat/whole milk was ~5, with 80–90% of the fenithion found in the fat fractions. In the current work, the E1 (log P = 3.62) milk fat/whole milk concentration ratio was ~16 and 3′-MeSO2-PCB (log P = 4.62, calculated) was ~18. Thus, the present results and those of O’Keeffe et al.14,15 suggested that factors in addition to log P also govern chemical disposition in milk.

Similar to the studies done by Hakk et al.7 and Lupton et al.,9 GLY and IMI (this study) distributed predominantly into the skim milk; thus, the concentration ratio between skim milk/whole milk was ~1, whereas the ratio of milk fat/whole milk was ~0.2 (Figure 1). Hakk et al.7 observed similar distributions for compounds with low log D values, such as OTET, PEN, and ERY, as did Lupton et al.9 for ASP, CIPR, TAP, and TYL despite the diversity of chemical structures.

Using literature values of log P and pKw for each chemical (Tables 1, S1, and S2), and standard deviation (SD) log D values were calculated for ionizable compounds.16 Relationships between log D or log P values and log [milk fat]/[skim milk] distributions, including 99% confidence interval (CI) and prediction interval, are shown in Figure 2A (log D) and 2B (log P). There are apparent uncertainties with respect to log D or log P for many of the studied compounds (Figure 2A,B). In general, distribution uncertainties with regard to log D or log P are much greater than the error associated with measurements of milk fat or skim partitioning. By combining the log [milk fat]/[skim milk] data of the current set with results obtained from those of Hakk et al.7 and Lupton et al.,9 the linear regression with log D had a regression coefficient of 0.79 and with log P, the resulting linear regression had an r2 = 0.71 (Figure 2A,B). The slightly better regression using log D data reinforces the conclusions of Hakk et al.7 and Lupton et al. that log D was a better predictor of the distribution between milk fat and skim milk than log P. Nevertheless, Figure 2A indicates that based on the 99% CI for log D, numerous outliers were present when all 27 compounds were modeled. Outliers with respect to the 99% CI for the log D plot (Figure 2A) included ERY, FNX, TAP, TBBA, 2-OH-1378-TCDD, and TYL compounds which distributed more toward milk fat than predicted. 2-OH-1378-TCDD likely would fall within the 99% CI based on the SD of the calculated log D. Conversely, E1, 3′-MeSO2-PCB-101, OTET, PBZ, and PZQ distributed more toward milk fat than predicted. Overall, the greatest limitation to predicting the behavior of any one chemical contaminant in milk seems to be the uncertainty associated with literature log P and pKw values used to calculate log D values in the model derivation.

Slopes of the linear log D and log P models were not 1, but 0.33 and 0.39, respectively (Figure 2). There was no reason to expect a 1:1 relationship between log D or P values of a chemical and its distribution between milk fat and skim milk. The lower slopes do indicate modeled chemicals that typically distribute to a greater extent into skim milk than merely reflected by their log D or P values. Distribution data were not affected by the presence of degradates because none were detected by thin-layer chromatography (TLC) (Table S3). The model slopes highlight the differences between the simple, ideal, octanol/water partition system and the complex milk matrix which consists of water, lipid, protein, sugar, minerals, and micelles. We hypothesize that the presence of these additional milk components could account for the enhanced
Numerical values on the graph represent the mean ratio (concentration in skim milk ± SD of all three dose mean percentages based on dpm of whey and curd (at 70% moisture) fractions compared to fortin = 2 replicates each for is, TBBPA, 3′-MeSO₂-PCB-101, 2-OH-1378-TCDD, and TCC, were more evenly distributed into both curd (40–60%) and whey (35–60%). Highly polar compounds had the lowest affinity for curd, for example, GLY (16.5%) followed by IMI (23.7%; Figure 3, Tables S17–S28).

When curd data (normally 70% moisture) were expressed on a dry matter basis, the concentration ratios of 0% moisture curd to whey (Tables S17–S28) were >100 for the most lipophilic compounds, that is, 1278-TCDD (115), BDE-99 (327), β-HBCD (152), and PCB-118 (136), and for two of the phenolics, BPA (111) and E1 (104). Other phenolic compounds, that is, TBBPA and 2-OH-1378-TCDD, had much lower concentration ratios of 32 and 18, respectively, whereas 3′-MeSO₂-PCB-101 (56) and TCC (46) were also lower than the most lipophilic compounds. The 0% moisture curd/whey concentration ratios for the most polar compounds ranged from 9.2 for IMI to 2.5 for GLY (Tables S17–S28).

Results for TBBPA were unexpected based on its structural similarity to BPA. The fire-retardant TBBPA is identical in the base structure to the plasticizer BPA with the exception that the 4-ortho hydrogens, with respect to the phenolic hydroxyls, are replaced by bromines. Bromination of the ortho-protons enhanced lipophilicity (log P) of TBBPA compared to BPA. In the 0% moisture curd/whey, however, the concentration ratio decreased from 111 for BPA to 32 for TBBPA (Tables S17–S28). Based solely on lipophilicity (log P), the curd/whey concentration ratio would have been expected to increase for TBBPA relative to BPA. One possibility for the lower concentration ratio for TBBPA is that the much larger atomic radius of bromine (compared to hydrogen) resulted in steric hindrances for potential casein–TBBPA interactions.

Hydroxylation and methylsulfonation of chemicals altered distribution patterns in milk. Aromatic hydroxylation decreased lipophilicity slightly and thus increased distribution into skim milk for phase 1 and into whey for phase 2. For example, 2-OH-1378-TCDD had a greater distribution into skim milk compared to 2-TCDD. Comparison of PCB-118 and 3′-MeSO₂-PCB-101 also indicated that a methyl sulfone group decreased lipophilicity compared to the parent compound.
Hydrophilic compounds distributed more evenly between curd and whey. For example, the curd/whey concentration ratio for GLY (log $D = -4.24$) was 1.4 and for IMI (log $D = -0.38$) was 2.4, similar to SDMX (ratio 3.2), PENG (ratio 1.2), OTET (ratio 1.4), ERY (ratio 2.4), and KETO (ratio 2.4) as previously reported. Given the diversity of chemical structures tested, the log $D$ value of hydrophilic compounds does provide some predictive measure for curd and whey distribution. Similarly, TAP and TYL possessed fairly low curd/whey concentration ratios, that is, 1.3 and 1.5, respectively.

Figure 4A (log $D$) and 4B (log $P$) shows the relationships between log $D$ or log $P$ values and log[0% moisture curd]/

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Chemical Distribution from Whey into Retentate and Permeate. In order to assess the percent of drug associated with the whey proteins, ultrafiltration in conjunction with centrifugation was performed (phase 3, Figure 5). The expected volume of retentate was 33% of the applied sample volume based on centrifugation time and speed, with the actual measured mean for all compounds being 37 ± 3.3%. Mean recovery of radioactivity across all compounds was 100 ± 4.5%. Mean nonspecific binding of compounds to filters ranged from 0.2% for GLY to 22.5% for E1. Compounds with >3% filter binding include PCB-118 (6.4%), BDE-99 (7.1%), β-HBCD (8.2%), BPA (13.4%), and E1 (22.5%) (Tables S29−S40). Although compounds with high log D values could be expected to be “sticky” in the aqueous medium, four compounds with high log D values [TCC (log D = 5.39), 2-OH-1378-TCDD (log D = 6.15), 1278-TCDD (log D = 6.22), and TBBPA (log D = 6.69)] had filter binding of ≤2.4%.

The associations of the 12 xenobiotics with whey protein, as determined by the percentage of compound measured in the retentate, revealed three groupings (Figure 6). The first was represented by GLY and IMI that have negative log D values (−4.24 and −0.38, respectively), where there was essentially no association with the whey protein (<5%) occurred (Tables S29 and S30). The second grouping was composed of BPA, E1, 3’-MeSO2-PCB-101, 2-OH-1378-TCDD, and 1278-TCDD, which had moderate associations with whey protein, ranging from 33 to 76% (Tables S31−S33, S35, and S38). Similar to our findings of ~64% association of E1 with whey protein, Wolford and Argoudelis22 reported 48 and 53% of 17β-estradiol and E1, respectively, associated with whey protein. The third grouping was composed of those compounds that were almost totally associated with retentate whey proteins (84−98%, one outlier of 107% for PCB-118 due extremely low starting radiocarbon in the whey). Chemicals in this grouping included BDE-99, β-HBCD, PCB-118, TBBPA, and TCC (Tables S34, S36, S37, S39, and S40). If present in whey, these compounds would concentrate in whey-derived protein products.
### Table 2. Compound Associated with Casein or Whey Protein (nmol/mg Protein and Percent Association Based on Whole Milk)

| compound     | nominal conc. of whole milk (actual) nM | nmol/mg casein protein | nmol/mg whey protein | conc. in casein/whey protein | mean % casein association based on whole milk | mean % whey association based on whole milk |
|--------------|----------------------------------------|------------------------|----------------------|-----------------------------|---------------------------------------------|---------------------------------------------|
| GLY          | 20 (22)                                | 0.08                   | 0.15                 | 0.53                        | 7.92                                        | 3.68                                        |
|              | 200 (217)                              | 0.71                   | 1.40                 | 0.51                        |                                             |                                             |
|              | 2000 (2059)                            | 6.52                   | 14.09                | 0.46                        |                                             |                                             |
| IMI          | 20 (20)                                | 0.21                   | 0.11                 | 1.91                        | 15.43                                       | 3.19                                        |
|              | 200 (201)                              | 2.20                   | 1.13                 | 1.95                        |                                             |                                             |
|              | 2000 (2066)                            | 22.33                  | 12.29                | 1.82                        |                                             |                                             |
| BPA          | 20 (22)                                | 0.44                   | 0.23                 | 1.91                        | 45.76                                       | 6.68                                        |
|              | 200 (216)                              | 4.54                   | 2.25                 | 2.02                        |                                             |                                             |
|              | 2000 (1992)                            | 42.43                  | 20.88                | 2.03                        |                                             |                                             |
| E1           | 20 (20)                                | 0.21                   | 0.10                 | 2.10                        | 17.86                                       | 3.52                                        |
|              | 200 (200)                              | 1.66                   | 0.96                 | 1.73                        |                                             |                                             |
|              | 2000 (1796)                            | 15.89                  | 8.70                 | 1.83                        |                                             |                                             |
| 3-MeSO₂-PCB-101 | 20 (24)                              | 0.06                   | 0.04                 | 1.50                        | 4.25                                        | 0.99                                        |
|               | 100 (70)                               | 0.18                   | 0.12                 | 1.50                        |                                             |                                             |
|               | 500 (628)                              | 1.60                   | 1.08                 | 1.48                        |                                             |                                             |
| TCC          | 20 (19)                                | 0.05                   | 0.10                 | 0.50                        | 5.92                                        | 3.62                                        |
|              | 200 (193)                              | 0.54                   | 0.95                 | 0.57                        |                                             |                                             |
|              | 2000 (1938)                            | 5.68                   | 9.72                 | 0.58                        |                                             |                                             |
| 2-OH-1378-TCDD | 20 (22)                              | 0.16                   | 0.64                 | 0.25                        | 17.85                                       | 16.79                                       |
|               | 100 (107)                              | 0.77                   | 3.26                 | 0.24                        |                                             |                                             |
|               | 500 (556)                              | 4.71                   | 16.16                | 0.29                        |                                             |                                             |
| PCB-118      | 50 (60)                                | 0.07                   | <LOQ                 |                             | 3.42                                        | 0.70                                        |
|              | 200 (221)                              | 0.35                   | 0.24                 | 1.46                        |                                             |                                             |
|              | 2000 (2203)                            | 2.90                   | 2.37                 | 1.22                        |                                             |                                             |
| β-HBCD       | 200 (229)                              | 0.38                   | <LOQ                 |                             | 2.95                                        | 0.59                                        |
|               | 500 (656)                              | 1.09                   | 0.59                 | 1.85                        |                                             |                                             |
|               | 2000 (2067)                            | 3.21                   | 2.11                 | 1.52                        |                                             |                                             |
| 1278-TCDD    | 20 (27)                                | 0.11                   | 0.06                 | 1.83                        | 4.14                                        | 1.29                                        |
|              | 200 (184)                              | 0.46                   | 0.34                 | 1.35                        |                                             |                                             |
|              | 2000 (1784)                            | 5.07                   | 3.58                 | 1.42                        |                                             |                                             |
| TBBPA        | 20                                    | 0.27                   | 0.79                 | 0.34                        | 18.01                                       | 22.96                                       |
|              | 200 (234)                              | 3.53                   | 9.36                 | 0.38                        |                                             |                                             |
|              | 2000 (1815)                            | 21.63                  | 76.47                | 0.28                        |                                             |                                             |
| BDE-99       | 20 (20)                                | 0.12                   | 0.03                 | 4.0                         | 6.66                                        | 1.20                                        |
|              | 200 (178)                              | 1.05                   | 0.38                 | 2.76                        |                                             |                                             |
|              | 2000 (1890)                            | 18.33                  | 3.35                 | 5.47                        |                                             |                                             |

*SA of some compounds required different doses, as indicated by bold text. Each fortified level contains three replicates. bThese data were derived from phase 2 data and have whey associated drug subtracted, using "0% moisture curd" as described in text. cThese data were derived from phase 3 data as described in the text. dMean of all doses. eLess than limit of quantitation (<LOQ). LOQ for PCB-118 is 1.92 nmol/L and for β-HBCD was 9.87 nmol/L. fInconsistent with other doses. No explanation.

The percent of whole milk dose associated with either casein or whey proteins is reported in Table 2. About 25% of TBBPA and 2-OH-1378-TCDD from whole milk distributed to whey, yet ∼90 and 70% (TBBPA and 2-OH-1378-TCDD, respectively) of that were associated with whey protein.
proteins can be calculated (Table 2). Chemical saturation of casein or whey protein was not observed because the mass of chemical per milligram protein increased as the concentration increased. In some instances, the initial expected fortification concentrations in whole milk differed from measured concentrations, as seen with 3′-MeSO2-PCB-101 and β-HBCD. Whey protein association values for the lowest dose of BDE-99 are questionable because the starting skim milk contained <2 nM and whey 0.3 nM. However, confidence in casein/whey protein association results is enhanced by the agreement found across doses (Table 2), exception was BDE-99, where ratios ranged from 2.8 to 5.5.

For the majority of chemicals tested (BDE-99, BPA, E1, β-HBCD, IMI, 3′-MeSO2-PCB-101, PCB-118, and 1278-TCDD), the association with caseins was greater than that for whey proteins (ratio > 1, Table 2). The importance of methodology is evident when comparing our findings to those of Wolford and Argoudelis22 that used equilibrium dialysis with E1 and the slightly more hydrophilic compound E2. They reported that E1 and E2 were largely (>84%) bound to protein when incubated in skim milk, and >50% of the bound estrogens was associated with whey proteins. These data are in contrast to our findings for E1, in which the association (nmol/mg protein) ratio was approximately 2 for casein/whey. The difference between the results of the two studies was most likely the precipitation of curd caseins in the present work versus the presence of soluble caseins used for dialysis by Wolford and Argoudelis22 (1979).

Other chemicals that preferentially associated with caseins relative to whey protein (ratio > 1) include THIA (2.5), IVD (2.0),8 TYL (1.4), CIPR (2.0), and PZQ (1.5).7 Although the current work used a majority of chemicals with log D greater than 3.4, our previous reports described only one such chemical (IVR). The casein/whey protein association ratio of IVR was more similar to BPA (2.0), E1 (1.9), and IMI (1.9) (Table 2).

In spite of higher distribution of GLY into whey than curd (Figure 3), there was in fact very little preferential retention of GLY associated with whey protein (Figure 6). Similarly, TCC, 2′OH-1378-TCDD, and TBBPA also had casein/whey protein ratios <1. Although most of the total TCC dose was partitioned with milk fat (mean 85%), the remainder distributed almost equally between whey and 0% moisture curd (57% curd, Table S22). TCC remaining in the whey was concentrated almost exclusively in the retentate (98%) during ultracentrifugation (Table S34). The log D values of 2′OH-1378-TCDD (6.15) and TBBPA (6.69) did not predict the respective mean casein/whey protein ratios of 0.26 and 0.33. Both chemicals also distributed to a lesser extent than predicted into milk fat. The common feature of both compounds is a hydroxyl moiety between two halogens (chlorines for 2′OH-1378-TCDD and bromines for TBBPA).

Previously studied chemicals that had higher association for whey proteins versus caseins were PENG (casein/whey ratio = 0.2), ERY (0.5), KETO (0.4), SDMX (0.8);8 TAP (0.5), CLA (0.4), and FNX (0.25).8 Although the distribution between lipid and aqueous phases was markedly dependent on the property of proteins, namely lipophilicity, small-molecule binding to proteins seems to be more dependent on specific functional groups within the protein. Identifying the specific functional groups and binding domains that can associate with studied chemicals within a plethora of whey and casein proteins lies outside the scope of the present research.

Relation to Consumer Products. To determine how the distributions of these compounds, if detected in whole milk, related to consumer products, the percent distributions into the milk end products of milk fat, curd, permeate, and retentate based on data generated from the current studies as well as those reported in Hakk et al.,7 Shappell et al.,4 and Lupton et al.9 The PZQ bar has additional information on which milk end products comprise whole milk, skim milk, curd, low-fat curd, and whey, as a guide to where drug may partition during commercial milk processing. For percentage of chemical associated with whey protein see supplemental information tables S29–S40.
milk fat, curd, retentate, and permeate were calculated in relation to the starting concentration in whole milk. Figure 7 includes the experimentally derived percentages of each compound in high-fat products which would include butter, cream, and cheese; low-fat products would include skim milk, low-fat cheese, yogurt, and low-fat derived whey protein products such as whey protein powders and baby formulas. Comparable to compounds previously tested,6,8 higher log $D_6$ compounds (i.e., E1, 3′-MeSO$_2$-PCB-101, TCC, PCB-118, $\beta$-HBCD, 1278-TCDD, and BDE-99) generally distributed to high-fat products such as butter and cream. High-fat products that contain protein (i.e., cheese) will concentrate both mid- to high-range log $D$ molecules such as BPA, 2-OH-1378-TCDD, and TBBPA along with the higher log $D$ compounds. Two compounds with low log $D$’s, that is, GLY and IMI, will primarily distribute into aqueous products, such as skim milk and whey.

Determining where a compound would concentrate in consumer products will also depend on the processing steps involved and what specific end product is being manufactured. For example, whole milk processed into skim milk and cream would generally have compounds with high log $D$ values concentrated in butter and cream, whereas compounds with low log $D$ values will be in skim milk. Compounds with mid-range log $D$ values will be split between the higher fat products and skim milk. However, if whole milk is processed directly into cheese, then the mid-range and high-range log $D$ value compounds will mainly concentrate in the cheese.

## CONCLUSIONS

The partitioning of 12 environmental contaminants or metabolites into milk fractions was assessed. Partitioning between milk fat and skim milk and between 0% moisture curd and whey was usually governed by the compound’s lipophility. If a chemical was found in whey, the more nonpolar the compound the more likely it would be found in whey protein products. Phenolic compounds were the main chemicals that fell outside of the 99% CIs of the models’ regression analyses. These models provide a tool using log $D$ as the primary chemical property to predict the distribution of chemicals into various milk products.

## EXPERIMENTAL SECTION

### Safety.
Nuclear Regulatory Commission and USDA regulations were followed for handling all radiolabeled chemicals.

#### Selection of Drugs and Concentrations.
Chemicals selected for study had to be potential environmental contaminants, encompass a wide range of lipophilicities, and be available with radiolabel ($^3$H or $^{14}$C) incorporation. The chemicals selected had a log $P$ range of −3.3 to 7.3. Chemical structures, site of radiolabel, specific activity (SA), and physiochemical properties are provided in Table 1.

To detect potential concentration-dependent distribution, chemical concentrations spanning 3 orders of magnitude (i.e., 20–2000 nM) were generally used. The lowest concentration (usually 20 nM) was typically relevant to possible contamination scenarios with sufficient activity to allow radiochemical detection. Higher concentrations were used to determine whether concentration influenced overall xenobiotic distribution. In some instances, concentrations were adjusted because of limited solubility or if the SA of the radiolabeled compound was inadequate for the sensitivity of the analysis (Table 1). As a result of adding unlabeled chemical (typically 9:1 parts) for the highest dose, SA was lowered, relative to low concentration.

#### Chemicals, Supplies, and Equipment.
Raw (unpasteurized, nonhomogenized) cow milk was obtained from the bulk milk tank located at the North Dakota State University (Fargo, ND) Dairy farm within 48 h of milking. Non-radiolabeled chemicals and solvents were obtained from Sigma-Aldrich (St. Louis, MO), U.S. Pharmacopeia (Rockville, MD), or other common vendors. Radiolabeled E1, GLY, PCB-118, and $\beta$-HBCD were procured through American Radiolabeled Chemicals, Inc. (ARC, St. Louis, MO). A mixture of the $\beta$- and $\gamma$-diastereoisomers of $^{14}$C-1278-TCDD was identified in the ARC product. Flash chromatography on a silica gel column eluted with hexane containing increasing amounts of methylene chloride (0–50%) was used to isolate $^{14}$C-1278-TCDD. $^{14}$C-BPA and $^{14}$C-TCC were purchased from Moravek Inc. (Brea, CA). $^{14}$C-IMI was a gift from Bayer Crop Science (Research Triangle Park, NC). $^{14}$C-7,8-ring $^{14}$C-1278-TCDD was purchased from ChemSyn Science Laboratories (Lenexa, KS). $^{14}$C-2,2′,4,4′,5-pentabromodiphenyl ether (BDE-99) was synthesized using published methods.23 2-OH-1378-TCDD was prepared in-house from [UL-7,8-ring $^{14}$C]-1278-TCDD by in vitro oxidation with human CYP1A1R Baculosomes (Cypex Ltd., Dundee, UK) and a glucose-6-phosphate dehydrogenase regeneration system according to manufacturer’s instructions. $^{14}$C-2,2-bis(4-hydroxy-3,5-dibromophenyl)propane (TBBPA) was synthesized by brominating bis$^{[14]$C}-phenol A with 4.2 equivalents of bromine in 1:1 methanol/water; bis$^{[14}$C]-phenol A was prepared in-house from [UL-16$^{14}$C]-phenol (2.0 mCi, 25 mCi/ mmol) and acetone according to a published method.24 3′-$^{14}$C-MeSO$_2$-PCB-101 was synthesized de novo by Cadogan coupling as described in Haraguchi et al.25 using sodium $^{[14}$C]-methyl thiolate for label introduction.

Silica gel plates were purchased from Analtech (Newark, DE). Scintillation cocktails were purchased from MP Biomedicals, LLC, (Ecolite; Solon, OH) or PerkinElmer (Waltham, MA; Carbosorb, and Permafluor). Amicon Ultra-15 centrifugal filters were purchased from Millipore (Billerica, MA). An Allegra X-14R centrifuge was obtained from Beckman-Coulter (Brea, CA). Liquid milk product fractions were mixed with scintillation fluid and assayed using a Tri-Carb 1900 liquid scintillation counter (LSC, Packard, Meriden, CT). Solid milk product samples were combusted using a Packard model 307 tissue oxidizer (Meriden, CT), trapped into Carbosorb, diluted with Permafluor, and then assayed by LSC. Sample purity was assessed by TLC and radioassay using a Bioscan AR-2000 Imaging Scanner for TLC (Washington, DC).

#### Determination of Chemical Purity and Confirmation of Test Article Stability.
TLC analyses were used to assess chemical purities before and after the experiments, although for GLY, high-performance liquid chromatography instead of TLC was employed. Initial analyses were used to evaluate dose purity, whereas postincubation analyses were used to evaluate whether chemical degradation occurred during milk processing. TLC conditions and results are included in Table S3. GLY radiochemical purity (98.0 ± 0.4%, $n = 4$) was determined based on Nagatomi et al.26 using a Waters 2695 HPLC, a radiochromatographic detector (Packard LFA 515TR, PerkinElmer, Waltham, MA), and a Dionex IonPac AS12 column (4 ×
200 mm, 9 μm, Dionex Company, Sunnyvale, CA). The mobile phase was isocratic 0.2% aqueous formic acid/acetonitrile (5/95 v/v), and the flow rate was 1 mL/min.

**Milk Processing and Radiochemical Analysis.** The milk processing experiments consisted of three sequential phases. Specific details pertaining to preparation of phases are reported in Hakk et al. and Shappell et al. Briefly, 12 tubes of raw milk (50 mL) were pasteurized at 63 °C for 30 min. Triplicate tubes were fortified with each level of radiolabeled chemicals using three working solutions or with the appropriate solvent for blank milk, as described in Table 2. In phase 1, the fortified, pasteurized, whole milk samples were separated into milk fat and skim milk by centrifugation after equilibration; the partitioning of chemical between these phases was then determined by radiochemical detection methods. In phase 2, the skim milk originating from phase 1 was partitioned into curd and whey (enzymatically with rennet) and the skim milk originating from phase 1 was partitioned into curd and whey (enzymatically with rennet) and the distribution of the target chemical between these phases was determined by radiochemical detection methods. In phase 3, the residual whey (15 mL) from phase 2 was separated into a protein-enriched fraction (>10 kD), retentate (residual whey (15 mL) from phase 2 was separated into a protein-enriched fraction (>10 kD), retentate (residual whey (15 mL) from phase 2 was separated into a protein-enriched fraction (>10 kD), retentate (residual whey (15 mL) from phase 2 was separated into a protein-enriched fraction (>10 kD), retentate (residual whey (15 mL) from phase 2 was separated into a protein-enriched fraction (>10 kD), retentate (residual whey (15 mL) from phase 2 was separated into a protein-enriched fraction (>10 kD), retentate). The amount of free chemical measured in permeate (calculated by concentration and volume) was subtracted from the total amount of chemical present in retentate. The difference was assumed to be the amount of chemical associated with whey protein. Residual radioactivity on ultrafilters (measured by combustion analysis) was considered nonspecific binding and was subtracted from the fortified whey results; however, radioactivity present in filter washes was included with retentate radioactivity. Averaged Kjeldahl protein concentrations in curd from Shappell et al. and Lupton et al. and the resultant 0% moisture curd radioactivity (see below) along with its SA were used to calculate nanomole per milligram casein protein association. Similarly, averaged Kjeldahl protein concentration in retentate from Shappell et al. and Lupton et al. and the protein associated radioactivity and its SA in retentate was used to calculate nanomole per milligram whey protein association.

**Calculation of Chemical Associated with Casein and Whey Protein.** The percentage of chemical associated with whey proteins was calculated according to Shappell et al. Briefly, the amount of free chemical measured in permeate (calculated by concentration and volume) was subtracted from the total amount of chemical present in retentate. The difference was assumed to be the amount of chemical associated with whey protein. Residual radioactivity on ultrafilters (measured by combustion analysis) was considered nonspecific binding and was subtracted from the fortified whey results; however, radioactivity present in filter washes was included with retentate radioactivity. Averaged Kjeldahl protein concentrations in curd from Shappell et al. and Lupton et al. and the resultant 0% moisture curd radioactivity (see below) along with its SA were used to calculate nanomole per milligram casein protein association. Similarly, averaged Kjeldahl protein concentration in retentate from Shappell et al. and Lupton et al. and the protein associated radioactivity and its SA in retentate was used to calculate nanomole per milligram whey protein association.

**Statistical Analyses.** Standard statistical methods were used to calculate means and variability and make inferences with respect to the significance of differences between means. Linear regression was used to assess dose dependence of the observed drug distribution log ratio of [chemical]skin milk/[chemical]skim milk or 0% moisture [chemical]curd/[chemical]whey. Dose dependency was based on instances when the slope differed (P < 0.05) from zero. Because curd is 70% moisture and contains a small quantity of entrained whey, a 0% moisture curd radioactivity value was calculated by subtracting entrained whey-associated radioactivity (calculated based on the percent moisture) from curd. The value representing entrained whey was added back to the whey fraction. Coefficient of variation with respect to measured partition values across doses was typically much less than 10%, whereas literature values for log P for a given chemical could sometimes differ by an order of magnitude or greater. Therefore, distribution data were modeled using mean log P values ± SD for each chemical. Mean log P values were calculated from predicted and measured entries included in Chemsipider, DrugBank, ChemBL, and Pubchem databases. For 3′-MeSO2-PCB-101, the log P value was derived from using conversion of chlorohexocriptiatriene into p-chlorophenyl methyl sulfone as a model, which has log differences of 1.76. By using PCB-101 log P of 6.38 and subtracting 1.76, the log P of 3′-MeSO2-PCB-101 was derived as 4.62. Log D values were calculated as described by Scherrer and Howard using a pH of 6.8 (reflecting the pH of milk); to obtain a theoretical range of log D values for each compound, the range of log P values derived from the above sources was used in conjunction with the range of pKa values obtained from the same sources; log D values were averaged and SDs calculated. Relationships between the log distribution ratios and lipophilicity (log D and log P) were performed using linear function and included the 99% CI and prediction interval by GraphPad Prism Version 7.03 (GraphPad Software, La Jolla, CA).

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b00762.

TLC conditions, data table summaries for individual drugs for phases 1, 2, and 3, and tables of compound structures for previous compounds and discussion compounds (PDF)

**AUTHOR INFORMATION**

**Corresponding Author**

*E-mail: Weilin.Shelver@ars.usda.gov. Phone: +1-701-239-1425. Fax: +1-701-239-1430 (W.L.S.).

**ORCID**

Weilin L. Shelver: 0000-0002-4721-9945

Sara J. Lupton: 0000-0002-4566-595X

Nancy W. Shappell: 0000-0003-4080-4372

David J. Smith: 0000-0001-8883-4744

**Notes**

The authors declare no competing financial interest.

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