Transfer of melamine from feed to milk and from milk to cheese and whey in lactating dairy cows fed single oral doses

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ABSTRACT

A study was conducted to evaluate the excretion pattern, after a single oral dose, of melamine from feed into milk, and the subsequent transfer to cheese and whey. The transfer of cyanuric acid was also investigated. Twenty-four lactating Holstein cows were randomly allocated to 4 treatments and received single doses of melamine as follows: 0.05, 0.50, 5.00, and 50.00 g/cow for groups D1, D2, D3, and D4, respectively. Individual milk samples were collected for melamine and cyanuric acid analyses on d 1, 2, 3, 4, 5, and 7. Milk collected individually from the second milking after melamine ingestion was used to make cheese on a laboratory scale. Melamine and cyanuric acid were extracted using a solid-phase extraction cartridge, and analyses were carried out by liquid chromatography–mass spectrometry/mass spectrometry. Maximal melamine concentrations occurred between 6 and 18 h after treatment and increased with log dose (linear and quadratic), ranging from 0.019 to 35.105 mg/kg. More than 60% of the melamine that was transferred to the milk was observed within 30 h after melamine ingestion. Melamine was not detected (limit of detection was 0.002 mg/kg) in milk 5 d after treatment in group D1, and 7 d after treatment in groups D2, D3, and D4. Blood urea nitrogen was not influenced by melamine ingestion. During cheese making, melamine was transferred mainly to the whey fraction. Cyanuric acid was not detected in any of the samples (milk, cheese, or whey). The excretion pattern of melamine in milk and whey may represent a health concern when cows ingest more than 0.50 g of melamine/d. However, only at intake levels of 5 and 50 g/d did cheese exceed the limits as set forth by the European Union. The results confirmed that melamine contamination of milk and milk products may be related not only to direct contamination, but also to adulteration of animal feeds.

Key words: cheese, cyanuric acid, melamine, milk

INTRODUCTION

Melamine (1,3,5-triazine-2,4,6-triamine) is a stable chemical intermediate used to manufacture resin and plastic, such as adhesive components and polymers commonly used in food packaging and plastic ware. The US Food and Drug Administration includes melamine in its list of indirect additives used in food contact substances (code number 000108-78-1). The risk of migration from packaging material to food is remote because melamine is only hydrolyzed in strong alkaline or acid solutions. According to Ishiwata et al. (1987), some acidic foods (e.g., coffee, fermented milk, lemon and orange juice) packaged in melamine-formaldehyde containers may be contaminated at levels ranging from 0.54 to 2.21 mg/kg when exposed to 95°C for 30 min. For this reason, when melamine is found in milk, milk products, feeds, or foods, an intentional addition is usually suspected. Pure melamine contains 66.7% nitrogen by weight compared with 46.6% in urea and 40.8% in biuret. The high nitrogen content therefore makes melamine attractive as a nitrogen adulterant in high-protein feedstuffs.

In recent years, various national authorities have reported health-related incidents from ingestion of triazine-contaminated feeds or foods. Early in 2007, US authorities were alerted to various reported cases of renal failure and deaths in dogs and cats exposed to melamine-contaminated pet foods (FDA, 2007). This was concomitant with the introduction of a batch of triazine-contaminated wheat gluten imported from China (Dobson et al., 2008). During 2008, more than 54,000 Chinese children were affected by renal disease when they ingested melamine-tainted infant formula. Of these, about 12,900 babies were hospitalized, 4 of whom died (Lam et al., 2009). From September 2008 to March 2010, the European Rapid Alert System for Food and Feed (**RASFF**) had recorded 72 notifications (excluding the category “food contact material”) concerning melamine contaminated food and feeds (data collected from RASFF’s online researchable database on March 2010; <https://webgate.ec.europa.eu/rasff-window/portal>). Following documented cases of kidney stones and renal failure in Chinese patients, as well as pets around the world, the melamine-tolerable daily

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intake (**TDI**) was updated by the FDA (2007) and the European Food Safety Authority (EFSA, 2008) to 0.63 and 0.50 mg/kg of BW, respectively.

Only a few published works evaluated the effect of melamine ingestion in livestock. Among these, Clark (1966) reported that a daily ingestion of 10 g of melamine by sheep weighing approximately 35 kg resulted in crystalluria and subsequent death, whereas intakes below 7 g/d were considered safe. Newton and Utley (1978) evaluated melamine as an alternative to urea as a non-protein nitrogen source to optimize rumen protein synthesis and suggested that melamine was not acceptable for ruminants due to incomplete hydrolysis in the rumen. However, these authors did not report adverse effects due to melamine ingestion. Despite these data, until 2008, no reports considered the possible transfer of dietary melamine from contaminated feed to milk. Only recently have 2 studies shown the existence of a pathway for melamine transmission from feed to milk. Cruywagen et al. (2009) found that the transfer efficiency of melamine in milk from cows fed 15 g of melamine/d was about 2%; Shen et al. (2010), who fed cows with 90, 270, and 450 mg of melamine/d, reported a transfer efficiency of 0.95, 0.70, and 0.66%, respectively.

Another aspect to be considered pertains to the associative effect of melamine and cyanuric acid. Dobson et al. (2008) conducted an epidemiological study on cats in the United States and an experiment on rats that showed the simultaneous ingestion of melamine and cyanuric acid is more dangerous for the renal system than melamine alone, probably because of the higher consistency and insolubility of melamine-cyanurate crystals. The metabolism of triazine in vitro, and in particular the hydrolysis of melamine into cyanuric acid, was established in aerobic microorganisms, such as *Arthrobacter* spp. (Strong et al., 2002), *Klebsiella terrigena* (Shelton et al., 1997), and *Pseudomonas* spp. (Jutzi et al., 1982). *Arthrobacter* and *Klebsiella* spp. were not isolated from rumen liquor, whereas *Pseudomonas aeruginosa* was found in the rumen of sheep (Duncan et al., 1999). Although Jutzi et al. (1982) showed that melamine was hydrolyzed anaerobically by 1 of the 3 investigated *Pseudomonas* strains, it is not clear whether this strain occurs in the rumen. Newton and Utley (1978) indicated that melamine resulted in a slight increase in rumen NH_3 concentrations in steers, but they concluded that if melamine was degraded in the rumen, it could not be regarded as a satisfactory nitrogen source for ruminants. The extent of melamine degradation in the rumen therefore remains unknown, but based on current knowledge, the possibility of cyanuric synthesis from melamine in ruminants cannot be excluded.

To date, no direct contamination of dairy products with cyanuric acid has been registered in Europe (data from RASFF), whereas 3 cases of low contamination of infant formula (200–400 μg of cyanuric acid/kg) were reported in the United States (FDA, 2009). Compared with the other triazines, as well as urea and biuret, cyanuric acid has the lowest nitrogen content (25%).

The objective of this current study was to investigate the effect of increasing oral doses of melamine on milk melamine concentration, transfer efficiency and clearance rate of melamine from milk, and partitioning of milk melamine into whey and cheese. The possible presence of cyanuric acid in milk was also investigated because of a potential hydrolytic melamine pathway in the gastrointestinal tract.

MATERIALS AND METHODS

Experimental Procedures

Twenty-four lactating Holstein cows, 42 ± 4.1 (SE) DIM, with an average daily milk production of 36.9 ± 0.6 (SE) kg, weighing 601 ± 45 (SE) kg, were used in the trial. All of the cows were housed at the CER-ZOO Research and Experimental Centre (San Bonico, Piacenza, Italy) in the same freestall barn. The experiment was conducted in accordance with the EC Council Directive guidelines for animals used for experimental and other scientific purposes (EC, 1986).

Diet was formulated according to the Nutrient Requirements of Dairy Cattle guidelines (NRC, 2001) for a cow weighing 600 kg, 60 DIM, with a 38-kg milk yield (3.8% fat and 3.35% protein). The diet (Table 1) was composed of maize silage (31.2% DM), dehydrated alfalfa hay (16.7% DM), grass hay (4.1% DM), and a concentrate supplement (48% DM). The diet was fed ad libitum (5% orts expected) as a TMR once daily at 0800 h. The TMR and orts were weighed on d 1 and 5 for each group, and the average weekly intake was calculated. Samples of the TMR were taken on d 1 and 5 of the experimental period for chemical analyses. Samples were dried at 55°C in a ventilated oven until a constant weight was reached; the samples were then ground through a 1-mm sieve (Thomas-Wiley laboratory mill, Arthur H. Thomas Co., Philadelphia, PA) and stored at -20°C . Analyses were performed in duplicate according to the Association of Official Analytical Chemists methods (AOAC, 1990) for DM (method 930.15), CP (method 975.06), ash (method 942.05), crude lipids (method 954.02), and ADF without residual ash (method 973.18). The NDF was analyzed with the addition of a heat-stable amylase and corrected for residual ash (Mertens, 1997) without sodium sulfite with the aid of an Ankom 2200 fiber analyzer (Ankom Technology, Macedon, NY).

Table 1. Ingredients and chemical composition of the basal diet used in the trial

Item	% of DM
Ingredient	
Maize silage	31.2
Alfalfa hay	16.7
Grass hay	4.1
Cotton seed, whole with lint	8.5
Corn meal	18.3
Barley meal	6.6
Protein and mineral supplement ¹	10.3
Calcium soap (Megalac ²)	0.9
Soybean meal	3.4
Chemical composition	
CP	16.2
Crude fat (ether extract)	4.9
ADF _{OM} ³	20.4
aNDF _{OM} ³	34.0

¹Contains per kilogram: 600 g of soybean meal, 300 g of sunflower meal, 120,000 IU of vitamin A, 9,000 IU of vitamin D₃, 90 mg of vitamin E, 3.6 mg of Co, 19.2 mg of I, 1.44 mg of Se, 600 mg of Mn, 62.4 mg of Cu, 2,240 mg of Zn, 360 mg of Fe.

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³ADF_{om} = ADF expressed exclusive of residual ash; aNDF_{om} = NDF assayed with a heat-stable amylase and expressed exclusive of residual ash.

After a 1-wk adaptation period to the diet, the animals were allotted in a completely randomized design to 4 groups of 6 cows each and treated with a single dose of melamine on d 1 of the experimental period (d 8). Four doses of melamine (melamine 99%, Sigma-Aldrich, St. Louis, MO) were used in the respective treatments: 0.05 g (**D1**), 0.50 g (**D2**), 5.00 g (**D3**), or 50.00 g (**D4**) of melamine per cow. Single doses of melamine were prepared for each cow by mixing the melamine into 100 g of soybean meal and suspending in 300 g of water. The mixture was then administered to the cows before TMR distribution (at 0800 h) in the form of an oral bolus.

The cows were milked twice daily at 0230 and 1430 h. Milk samples were collected individually at the p.m. milking on d 1, 2 (including the a.m. milking; i.e., 18 h after treatment), 3, 4, 5, and 7, and stored at -20°C until analyzed for melamine and cyanuric acid. Milk samples from d 1 and 4 were also analyzed for fat, protein, and lactose contents (infrared analysis, Milko-scan model FT120, Foss Electric, Hillerød, Denmark). Blood samples were collected on d 1 at 1500 into Li-heparinized (17 U of heparin/mL of blood) test tubes (B.D. Vacutainer System, Becton, Dickinson and Co., Plymouth, UK). Plasma was subsequently obtained by 15 min centrifugation at 4°C and $3,500 \times g$ and stored at -20°C until analyzed for BUN content (autoanalyzer, ILAB 600, Instrumentation Laboratory, Lexington, MA).

Milk from the second milking posttreatment (18 h) was collected individually from each of the 24 cows

and processed into 24 semi-soft cheeses on a laboratory scale. Eight liters of the total milk yield per cow was heated to 40°C , followed by the addition of 2.4 mL of rennet solution and 160 mL of whey starter (containing *Streptococcus thermophilus*). The milk was allowed to coagulate for 15 min before cutting the curd, followed by a second cutting after a 15-min interval. The curds were kept in strainers at 42°C for 1 h, and subsequently stored at 37°C for 2 d. Whey samples were collected by placing containers beneath a strainer before incubating the cheese. Samples were stored at -20°C before analyzing for melamine and cyanuric acid. The surface of the cheese was treated with sodium chloride (50 g/kg of curd) and finally conditioned on the shelf at 5°C for 2 wk. The cheeses were subsequently stored at -20°C before analyzing for fat, protein, water, melamine, and cyanuric acid content.

For the duration of the trial, milk produced by the experimental cows was collected separately and discarded in accordance with Italian regulations.

Analytical Procedures

Melamine and cyanuric acid were purchased from Sigma-Aldrich. A stock standard melamine solution (400 mg/L) was prepared by dissolving 20 mg of melamine in 50 mL of a 2% aqueous formic acid:methanol (50:50 vol/vol) solution. Working standard solutions (from 0.2 to 10 mg/L) were prepared by further dilution with 25 mM ammonium formate (pH 3.2):methanol (10:90 vol/vol, HPLC mobile phase). Aliquots of these standards were diluted with a purified extract of melamine-free milk to obtain 12 calibrant solutions at a melamine concentration of between 0.005 and 1.2 mg/L. For cyanuric acid, a stock standard solution was prepared by dissolving 20 mg of cyanuric acid in 50 mL of a 5% ammonia:methanol solution (50:50 vol/vol), and working standard solutions (2 and 10 mg/L) were prepared in the HPLC mobile phase.

Extraction of Melamine and Cyanuric Acid

The melamine content of milk and whey was measured according to Shia et al. (2008), with some modifications. An aliquot of 10 mL of milk or whey was diluted with 10 mL of 0.2 M HClO₄; after centrifugation at $11,000 \times g$ for 10 min at 4°C , the supernatant was filtered through a folded filter paper. An aliquot of 5 mL was purified through an Oasis MCX cartridge (6 mL, 500 mg, Waters Corp., Milford, MA) previously conditioned with 5% NH₄OH in methanol, followed by 1% HCl in methanol (3 mL of each) and equilibrated with methanol, followed by distilled water (3 mL of each). After washing the column with distilled water

and then methanol (3 mL of each), melamine was slowly eluted (0.5 mL/min) with 5% NH_4OH in methanol (4 mL) into a graduated glass vial; subsequently, the eluate was concentrated to 1 mL under a gentle stream of nitrogen.

Regarding cyanuric acid, an aliquot of 10 mL of milk or whey was diluted with 0.2 M HClO_4 as described for melamine. Subsequently, a 5-mL aliquot was purified through an Oasis MAX cartridge (3 mL, 60 mg, Waters Corp.) previously conditioned with 0.1 M NaOH , and then equilibrated with methanol followed by distilled water (3 mL of each). After washing the column with 5% NH_4OH in methanol followed by methanol (3 mL of each), cyanuric acid was slowly eluted (0.5 mL/min) with 5% formic acid in methanol (5 mL) into a graduated glass vial; subsequently, the eluate was concentrated to 1 mL under a gentle stream of nitrogen.

In cheese samples, melamine and cyanuric acid were extracted from 10 g of grated cheese with 100 mL 0.2 of M HCl :methanol (75:25 vol/vol) using a rotary-shaking stirrer for 45 min. After filtration through folded filter paper, aliquots of the filtrate (5 mL) were purified through Oasis MCX and MAX cartridges as with the melamine and cyanuric acid extraction in milk.

Liquid Chromatography–Mass Spectrometry/Mass Spectrometry Analysis

Analysis was carried out using a liquid chromatography–tandem MS system, consisting of a LC 1.4 Surveyor pump (Thermo-Fisher Scientific, San Jose, CA), a PAL 1.3.1 sampling system (CTC Analytics AG, Zwingen, Switzerland), and a Quantum Discovery Max triple-quadrupole mass spectrometer (Thermo-Fisher Scientific); the system was controlled by Excalibur 1.4 software (Thermo-Fisher Scientific). Melamine and cyanuric acid were separated on a Luna hydrophilic interaction liquid chromatography (HILIC) column (3 μm particle size, 150 $\times$ 2.0 mm, Phenomenex, Torrance, CA,) with a mobile-phase of 25 mM ammonium formate (pH 3.2):methanol (10:90 vol/vol); the flow rate was 0.15 mL/min. For melamine, ionization was carried out with an electrospray ionization interface (Thermo-Fisher Scientific) in positive mode as follows: spray capillary voltage, 4.0 kV; sheath and auxiliary gas, 8 and 1 psi, respectively; temperature of the heated capillary, 270°C. The mass spectrometric analysis was operated in selected reaction monitoring. For fragmentation of $[\text{M}+\text{H}]^+$ ion 127 m/z , the argon collision pressure was set to 1.3 mTorr; the selected fragment ions were 85 and 68 m/z with collision energy of 19 and 32 V, respectively. Before analysis, the purified extract of the sample was diluted with the mobile phase; the dilution factor (between 3 and 100) depended on the melamine

content. The calibrant standards were diluted with the melamine-free milk extract as for the samples. The calibration was linear in the range of 0.005 to 0.30 mg/L and 0.20 to 1.2 mg/L. Quantitative determination was performed using LC-Quan 2.0 software (Thermo-Fisher Scientific). For cyanuric acid, ionization was carried out with an electrospray ionization interface in negative mode using the same parameters as for melamine analysis. For fragmentation of $[\text{M}-\text{H}]^+$ ion 128 m/z , the argon collision pressure was set to 1.3 mTorr; the selected fragment ions were 85 and 42 m/z with collision energy of 15 V.

Statistical Analyses

Data were tested for normality with the Shapiro-Wilk test. Nonnormal variables were log-normal transformed before statistical analysis (Petrie and Watson, 2006). The rate of clearance of melamine from milk (mg/L per h) for each experimental unit was fitted by linear regression (REG procedure; SAS Institute, 2003) of the log-normal transformed melamine concentration (mg/L) over time (h) using data from maximum to minimum melamine concentrations.

Data measured or calculated once during the trial (i.e., output of melamine at 18 and 30 h) or at the end (i.e., maximal melamine concentration, total melamine output, efficiency of melamine transfer to milk, rate of melamine clearance, BUN, cheese and whey parameters) were analyzed as a completely randomized design using the GLM procedure of SAS according to the model described below:

$$Y_{ij} = \mu + \alpha_i + e_{ij},$$

where Y_{ij} is the dependent variable on the j th subject assigned to treatment i , μ is the overall mean, α_i is the fixed effect of melamine log dose ($i = 1$ to 4), and e_{ij} is the residual error.

Data measured over time (i.e., milk yield, fat, protein, and lactose of milk) were analyzed as repeated measurements in a completely randomized design using the MIXED procedure of SAS Institute (2003), according to the model described below (Littell et al., 1998):

$$Y_{ijk} = \mu + \alpha_i + \beta_{ij} + \gamma_k + (\alpha \times \gamma)_{ik} + e_{ijk},$$

where Y_{ijk} is the dependent variable at time k on the j th subject assigned to treatment i ; μ is the overall mean; α_i is the fixed effect of melamine log dose ($i = 1$ to 4); β_{ij} is the random effect for the subject j assigned to treatment i ; γ_k is the fixed effect of time; $(\alpha \times \gamma)_{ik}$ is the interaction between melamine log doses and time; and e_{ijk} is the residual error. Each variable was subjected

to 2 covariance structures: compound symmetry and unstructured. Using the Akaike information criterion and the Schwarz Bayesian criterion, compound symmetry was the covariance structure that best fitted the model. The linear and quadratic effects (Lowry, 1992) of melamine ingestion on a logarithmic scale were evaluated, and significance was declared at $P < 0.05$.

RESULTS AND DISCUSSIONS

Performance of the Method

Melamine recovery rates were determined by spiking known blank samples at levels of 1 and 10 mg/kg for melamine and of 10 mg/kg for cyanuric acid. Average recoveries were (mean $\pm$ SE) $93.8 \pm 2.6\%$ and $89.8 \pm 3.4\%$ for melamine and cyanuric acid, respectively. Simultaneous chromatographic separation of melamine and cyanuric acid can be performed using different types of analytical columns, in particular HILIC and anion exchange columns (Varelis and Jeskelis, 2008; Chan et al., 2009). With the HILIC column, good separation was obtained using high acetonitrile content ($>70\%$) in the mobile phase (Grumbach et al., 2004; Heller and Nochetto, 2008). Because of the limited supply of acetonitrile during 2008, we replaced acetonitrile with methanol. Unexpectedly, the signal $[M+H]^+$ of melamine increased approximately 5-fold compared with acetonitrile, probably due to better ionization, which improved the instrumental limit of detection. To avoid any possible overpressure due to the high methanol content in the mobile phase (90%), the flow rate was set to 0.15 mL/min. For melamine, the limit of detection (signal-to-noise ratio of 3:1) and the limits of quantification (LOQ, signal-to-noise ratio of 10:1) in milk and whey were 0.002 and 0.006 mg/kg, whereas in cheese they were 0.005 and 0.015 mg/kg, respectively. The limits of detection and quantification for cyanuric acid were 30-fold higher than for melamine (0.06 and 0.18 mg/kg for milk and whey; 0.15 and 0.45 mg/kg for cheese).

Melamine Excretion Pattern in Milk

Recently, the European Union (EU, 2008) fixed the maximum allowable level of melamine for food and feeds at 2.5 mg/kg, a mid-point between the unavoidable background presence of melamine (OECD, 2002) and unacceptable adulteration. The limit was considered by authorities to ensure a large margin of safety.

The lowest dose of melamine used in our trial was 0.05 g per cow (D1), which was calculated according to the European allowed melamine limit in feed for a

cow ingesting 20 kg of DM per day. The other doses of melamine were 10-, 100-, and 1,000-fold higher than that of D1 (i.e., 0.50, 5.00, and 50.00 g/cow for D2, D3, and D4, respectively). The dosages were log-transformed (referred to as log dose) over 4 log cycles (one dose each) to obtain equally spaced dose levels (Littell et al., 2006). Dry matter intakes for the D1, D2, D3, and D4 groups were (kg/d, mean $\pm$ SE) 22.1 ± 2.8 , 23.2 ± 4.2 , 22.8 ± 3.7 , and 22.6 ± 3.2 , respectively.

Melamine excretion levels in milk following the single oral dose of the D1, D2, D3, and D4 groups, are presented in Figure 1. No melamine was detected in the milk of any cow the day before melamine administration. Melamine was already detected in the milk as early as 6 h after ingestion. A similar trend was observed in the experiment by Cruywagen et al. (2009), where melamine was detected in milk 8 h after first ingestion. In our trial, mean maximal milk melamine concentrations were observed 18 h after ingestion, except for treatment D4 (highest dose), where the maximal concentration was already observed 6 h after ingestion. It thus appears that the milk melamine excretion pattern could be dose dependent. Melamine dose also had a clear effect on the time at which milk melamine concentration declined below the limit of detection (0.002 mg/kg), namely 102 h for D1 and 174 h for D2, D3, and D4 (Figure 1).

A log dose effect (linear and quadratic $P < 0.001$) was observed for total milk melamine excretion and maximal milk melamine concentration (Table 2), the latter ranging from 0.019 to 35.105 mg/kg for the D1 and D4 groups, respectively. The total melamine output into milk (log dose) at 18 and 30 h increased linearly ($P < 0.001$); it ranged from 46.4 (D1) to 72.5% (D4) at 18 h, and from 65.7 (D1) to 84.6% (D4) at 30 h from melamine ingestion (Table 2). More than 60% of the total melamine was excreted within 30 h. The efficiency of melamine excretion into milk ranged between 2.3 and 3.3% of ingested melamine for the D1, D3, and D4 groups, which was in line with the 2% reported by Cruywagen et al. (2009). Shen et al. (2010) found no relation between melamine doses and transfer efficiencies in milk, which were 0.95, 0.69, and 0.65% in cows fed 90, 270, and 450 mg/d of melamine, respectively. Within the melamine fed range, the authors proposed a linear equation for predicting transfer efficiency in milk. When applying the Shen et al. (2010) equation to the D1 and D2 groups, the transfer efficiency in our experiment was 1.56%, quite similar to that measured in the D1 group and lower than the 9.1% observed in the D2 group (Table 2). When we looked at the individual data of the D2 group, 3 animals showed a transfer efficiency that ranged from 5.5 to 8%, and that of the other 3 ranged from 9 to 15%. The variability of this group was

Figure 1. Melamine excretion pattern via milk in Holstein cows that received different single oral doses of melamine (mean mg/kg $\pm$ SE). Treatment doses (g of melamine/cow): 0.05 (D1), 0.5 (D2), 5 (D3), and 50 (D4). Horizontal dashed (D2, D3, and D4) and dotted (D3 and D4) lines indicate the World Health Organization's maximal proposed limit of melamine in food for infants (1 mg/kg; WHO, 2008), and the European Union maximal allowed limit for feeds and foods (2.5 mg/kg; EU, 2008), respectively.

higher, but its minimal value was about 2- to 3-fold higher than the maximal values from D1, D3, and D4 groups. The milk melamine concentration of the D2 group was approximately 4 times higher than values observed in the other groups. If a linear effect is considered, then the 10-fold factor between treatments theoretically should have yielded a melamine concentration of around 0.2 to 0.3 mg/kg for the D2 group compared with the observed 0.86 mg/kg. It is therefore apparent that total milk melamine excretion, as well as excretion efficiency and maximal milk melamine concentration, followed a quadratic pattern. Although the data of the D2 group were quite variable, no outliers were detected. Because the percentage of milk melamine output (at 18 and 30 h), as well as the clearance rate of melamine from milk, were linear ($P < 0.001$), it appears that

unknown confounding factors might have affected milk melamine excretion.

The observed slope for the rate of melamine clearance in milk was different among treatments. Rate of clearance increased linearly ($P < 0.001$) with log dose from 0.027 mg/kg per h in treatment D1 to 0.062 mg/kg per h in D4. Melamine is slightly soluble in water (Merck, 1983), but being a small and polar amine, it can readily spread in organic extracellular fluids such as urine and milk (Smith et al., 1994; Baynes et al., 2008). The clearance rate of melamine from milk was similar to that reported by Cruywagen et al. (2009) for cows that ingested 17 g of melamine/d for 8 consecutive days. In their trial, no melamine was detected in milk 156 h after the last melamine ingestion. The shorter clearance period in group D1 of the current trial could

Table 2. Melamine excretion via milk and BUN (mmol/L) in Holstein cows that received different single oral doses of melamine

Item	Treatment ¹				SEM	P-value	Contrast	
	D1	D2	D3	D4			Linear	Quadratic
Total milk melamine excretion, ² mg	1.17	45.31	166.38	1,391.71	0.12	***	***	***
Efficiency of melamine transfer to milk, %	2.35	9.06	3.33	2.78	0.12	***	0.508	***
Maximal melamine concentration, ² mg/kg	0.019	0.860	2.934	35.105	0.152	***	***	***
Output of melamine at 18 h, %	46.43	51.35	62.70	72.47	3.73	***	***	0.523
Output of melamine at 30 h, %	65.65	68.80	75.57	84.60	2.68	***	***	0.973
Rate of clearance, mg/kg per h	0.027	0.037	0.047	0.062	0.002	***	***	0.161
Clearance time, ³ h	85.7	159.0	168.0	171.0	3.0	***	***	***
BUN, mmol/L	4.36	3.55	3.78	3.84	0.28	NS	0.284	0.131

¹Single-dose melamine treatments were D1 = 0.05 g/cow, D2 = 0.5 g/cow, D3 = 5 g/cow, D4 = 50 g/cow.

²Data were lognormal transformed before statistical analyses. The mean estimators reported in the table were calculated according to Serfling (2002).

³Time after melamine ingestion until milk melamine levels reached undetectable levels.

*** $P < 0.001$.

be ascribed to the low level of melamine supplementation in our trial, namely 0.05 mg, as Shen et al. (2010) observed by feeding cows 0.09 mg of melamine/d.

The milk yields of treated cows during the experimental period were not affected by the melamine intake or by dose $\times$ time interaction (Table 3), in agreement with the results on cow performance reported by Cruywagen et al. (2009).

Melamine tolerable daily intakes of 0.63 and 0.50 mg/kg of BW were indicated as safe by the FDA (2007) and EFSA (2008), respectively. These tolerable daily intake values were calculated based on toxicity experiments on rats (OECD, 2002) and took into consideration the “no observable adverse effect level” of 63 mg/kg of BW (FDA, 2007). Data on the toxicity of melamine in ruminants is limited. No effect on performance was reported by Newton and Utley (1978) when steers were fed 45 g of melamine/d, corresponding to about 90 mg of melamine/kg of BW. On the other hand, Mackenzie and van Rensburg (1968) reported that sheep fed 100 mg of ammeline/kg of BW per day (the first derivative of melamine after the hydrolytic substitution of an amine with a hydroxyl) for several weeks suffered formation of kidney stones.

At the start of the experimental period, the average BW of the cows were 608 ± 15 , 604 ± 31 , 593 ± 20 ,

and 600 ± 24 kg. Considering animal weight, each cow received (on average) 0.08 ± 0.002 , 0.83 ± 0.04 , 8.44 ± 0.29 , and 83.51 ± 3.31 mg of melamine/kg of BW for D1, D2, D3, and D4, respectively. The amounts of melamine used in the present work were therefore much lower than the acute lethal dose, 50%, of melamine (i.e., 3,160 mg/kg of BW; OECD, 2002).

Melamine intake had no effect on milk CP, fat, or lactose contents (Table 3), or on BUN levels. Considering the close relationship between MUN and BUN (DePeters and Ferguson, 1992), the latter result agreed with that of Cruywagen et al. (2009), who reported no effect of melamine in milk components and MUN.

Transfer from Milk to Cheese

Milk, cheese, and whey analyses data are presented in Table 4. Melamine treatment had no effect on cheese yield, and efficiency ratios varied between 9.15 and 9.85 g of cheese/100 mL of milk. The melamine concentration in milk, cheese, and whey increased as melamine dosage increased. Most of the milk melamine was transferred to the whey fraction (almost 85%, on average), and the transfer efficiency did not appear to be treatment related. On average, only 1.9% of the milk melamine was transferred to cheese. This leaves an apparent unac-

Table 3. Milk yield and milk composition in Holstein cows that received different single oral doses of melamine

Item	Treatment ¹				SEM	P-value		Contrast	
	D1	D2	D3	D4		Log dose	Log dose $\times$ time	Linear	Quadratic
Milk yield, kg/d	37.1	38.0	38.1	38.1	0.04	NS	NS	0.687	0.787
Crude fat, g/kg	38.6	37.9	35.1	34.0	2.5	NS	NS	0.110	0.931
CP, g/kg	32.9	32.2	32.6	33.1	0.4	NS	NS	0.579	0.137
Lactose, g/kg	52.9	52.9	52.7	52.9	0.5	NS	NS	0.984	0.995

¹Single-dose melamine treatments were D1 = 0.05 g/cow, D2 = 0.5 g/cow, D3 = 5 g/cow, D4 = 50 g/cow.

Table 4. Melamine concentration in milk, whey, and cheese; weight efficiency ratios of cheese-making; melamine partition in whey and cheese; mass balance of melamine (sum of total melamine in milk, whey, and cheese); and cheese composition¹

Item	Treatment ²				SEM	P-value	Contrast	
	D1	D2	D3	D4			Linear	Quadratic
Milk melamine, mg/kg	0.016	0.635	2.680	21.731	0.106	***	***	***
Whey melamine, ³ mg/kg	0.014	0.610	2.491	21.293	0.108	***	***	***
Cheese melamine, ³ mg/kg	0.005	0.096	0.276	4.709	0.149	***	***	0.547
DM cheese melamine, ³ mg/kg	0.007	0.135	0.428	7.245	0.149	***	***	0.539
Weight efficiency ratio, kg of milk/kg of cheese	9.22	9.15	9.46	9.85	0.42	NS	0.264	0.587
Whey melamine partition, %	79.74	87.03	83.67	88.30	3.63	NS	0.196	0.738
Cheese melamine partition, %	3.20	1.39	0.96	2.14	0.39	**	*	**
Mass balance, %	83.14	88.43	84.64	90.44	3.71	NS	0.288	0.945
Cheese composition								
Water, %	35.33	35.40	35.35	35.17	0.60	NS	0.850	0.843
Crude fat, %	33.92	34.70	34.67	36.45	1.19	NS	0.171	0.679
CP, %	26.83	26.08	24.58	25.05	0.80	NS	0.069	0.453

¹Cheeses were produced from milk of the second milking of Holstein cows that received different single oral doses of melamine.

²Single-dose melamine treatments were D1 = 0.05 g/cow, D2 = 0.5 g/cow, D3 = 5 g/cow, D4 = 50 g/cow.

³Data were lognormal transformed before statistical analyses. The mean estimators reported in the table were calculated according to Serfling (2002).

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

counted melamine loss of approximately 13%, which is not readily explained. It is postulated that some of the melamine could have been degraded during the 14-d conditioning period. No documented results could be found to support this hypothesis. Treatment had no effect on the water, fat, or protein content of the cheese.

Health Risk of Milk, Whey, and Cheese

Cows fed feed tainted with high levels of melamine could produce milk and milk products exceeding the currently allowed limits of melamine for feed and food. Besides the European allowed limit of 2.5 mg of melamine/kg for feeds and foods, WHO has proposed a limit of 1 mg of melamine/kg of infant formula as providing a sufficient margin of safety for children (WHO, 2008). The maximal milk melamine concentration in group D1 was 0.019 mg/kg, which is about 50-fold lower than the limit of 1 mg of melamine/kg for infant formula. Applying a concentration factor of 8.3 (assuming the TS content of milk to be 120 g/kg), melamine levels in dry milk or infant formula would thus be 7-fold lower than 1 mg/kg. Considering the milk of D2 cows, the health risk for children becomes relevant because the melamine concentration of dried milk, calculated in the same way, would be 7.16 mg/kg. The melamine concentration of milk produced by D3 and D4 cows was similar to that which caused renal failure in Chinese children during 2008. Therefore, allowing cows to ingest 5 to 50 g of melamine/d, whether intentional or via contaminated feed, could present a serious health risk to consumers of dairy products.

Because the ratio between melamine concentrations in milk and whey is about 0.9, arguments regarding exceeding the melamine legal limit would similarly apply for whey. The concentration factor (from liquid to powder) for whey is higher (14 to 17) than that for milk, which increases the risk for whey powder. On the other hand, in the case of cheese, only the highest level used in the current trial (D4) resulted in a melamine content that exceeded the 2.5 mg/kg limit on a DM basis. Due to the partitioning ratios of melamine from milk to whey and cheese, cheese-making should not represent a health concern for humans when cows consume feed that contains less than 5 g of melamine/d (D3).

Cyanuric Acid Excretion in Milk

No cyanuric acid was detected in any of the milk, whey, or cheese samples. Some authors have investigated the microbial pathway metabolism of melamine (Cook and Huetter, 1981; Jutzi et al., 1982; Cook and Huetter, 1984; Shelton et al., 1997). In particular, Jutzi et al. (1982), examining the degradation pathway metabolism in soil/ubiquitous bacteria (i.e., *Pseudomonas* spp. strain A and *Klebsiella pneumoniae*), indicated that melamine could be metabolized by successive deamination of the triazine ring, followed by hydrolysis. Ammeline, ammelide, and cyanuric acid were accumulated by bacteria when melamine was the sole source of nitrogen. Newton and Utley (1978), using melamine as a NPN source for ruminants, stated that melamine poorly contributed to the ruminal ammonia pool as it was slowly hydrolyzed in the rumen. Moreover, cyanu-

ric acid has a low pK_a (6.9) and is probably absorbed in the stomach of monogastric animals (Dobson et al., 2008) and excreted as an unmetabolized compound in the urine (WHO, 2008). A similar absorption could be expected for ruminants (Cruywagen et al., 2009), even if no data are currently available.

Even though the lack of cyanuric acid does not minimize the toxic effect of melamine, it can be assumed that, if a cow ingested a high amount of melamine alone (e.g., feed containing 2.5 g of melamine/kg of DM, as in group D4), the milk should not pose a higher health risk than when both melamine and cyanuric acid were present in the feed. The renal calculi found in triazine-poisoned American pets were more consistent and less soluble with respect to those of melamine-poisoned Chinese children because the pet food was contaminated with melamine and cyanuric acid at a 1:1 ratio (Dobson et al., 2008).

CONCLUSIONS

The study confirmed the pathway for the transmission of melamine from feed to milk, as well as the rapid excretion thereof, even at a very low level of melamine ingestion. The melamine contamination of milk and milk products is therefore not only related to intentional external addition, but also to adulteration of animal feeds. The safety span for the total disappearance of melamine in milk after a single ingestion should be at least 7 d. The cheese-making process showed that melamine is almost totally transferred to whey. Health concerns arising from human intake of milk and its derivatives would be relevant for fluid and dry milk or whey only if cow diets exceed the European allowed limit for feed. In the case of cheese, only the highest tested contamination level (50 g/d) would represent a human health risk.

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