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The assessment of melamine and cyanuric acid residues in eggs from laying hens exposed to contaminated feed

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Abstract

The aim of this study was to monitor the carry-over of melamine and its metabolite cyanuric acid from melamine-contaminated feed administered to layers into eggs. Ten experimental Isa Brown layers (36 week of age) were fed melamine-contaminated feed containing 100 mg of melamine per kg of feed. The duration of the experiment was 6 weeks. Eggs were collected during whole experiment. Analysis of eggs was done by a simple extraction of melamine and cyanuric acid residues, using a water-acetonitrile mixture and analysed by gas chromatography - triple quadrupole mass spectrometry. Melamine and cyanuric acid residues were detected in all eggs collected from the experimental layers, immediately after the first administration of melamine-contaminated feed. The mean concentrations of melamine in the egg yolk and egg white recalculated on a dry matter basis were 1.90 mg·kg^{-1} ± 0.158 and 10.84 mg·kg^{-1} ± 3.951 ($P \leq 0.01$); the cyanuric acid contents were 6.54 mg·kg^{-1} ± 0.2.466 and 4.07 mg·kg^{-1} ± 0.909, respectively. Melamine and cyanuric acid were not detected in eggs from control layers. Concentrations of melamine and cyanuric acid in eggs decreased quickly after melamine feeding was stopped. Our results indicate that melamine undergoes biotransformation to cyanuric acid in the layer's body that also passed into the eggs. The results verified the presence of distribution metabolic pathway of melamine and its easiest transfer into egg yolk. Moreover, the biotransformation of melamine into cyanuric acid in eggs of layers was confirmed.

Contaminated feed, gas chromatography, food safety, layers

Melamine is a chemical substance used in a wide range of industrial applications, for example in the production of glass-reinforced plastics and plastic materials, as a component of organic coatings, or as a coolant. Commercially produced melamine may contain structural analogues such as cyanuric acid, ammelide, ammeline and melamine cyanurate. People can be exposed to the effects of melamine and its analogues from many different sources, including food and the environment (World Health Organization, WHO 2009). In March 2007, it was revealed that wheat gluten, rice protein and maize protein imported from China to the USA for the production of feeds for domestic animals were contaminated with considerable amounts of melamine and cyanuric acid. Products made from these raw materials caused renal failure in dogs and cats (Brown et al. 2007). In July 2008, several cases of kidney stones and renal failure in Chinese children were reported, being attributed to the use of melamine-contaminated baby food. It was also found that raw milk was adulterated with melamine for a period of several months in order to deceive quality control analysis of milk, i.e. to increase the content of proteins (WHO 2008b). After this incident, the problem of melamine-contaminated milk and dairy products was investigated very extensively, revealing a total of 22 baby food manufacturers who used melamine-contaminated starting materials in which the concentrations of melamine exceeded 2.5 mg·kg^{-1}. Subsequently, the Chinese government seized up to 2,000 tonnes of Sanlu milk powder from the warehouse and 9,000 tonnes from its customers. Gossner et al. (2009) have provided detailed information on melamine-contaminated milk and dairy products.

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Accumulation of melamine in eggs was investigated in experiments conducted by Yang et al. (2009). Elevated concentrations of melamine were also detected in eggs imported from continental China to Hong Kong where melamine originated from a feeding mixture designed for the feeding of layers (BBC 2008). Melamine was also detected in fresh eggs and egg powder made in China, at concentrations varying within 0.1–4.0 $mg \cdot kg^{-1}$, and 2.9–4.7 $mg \cdot kg^{-1}$, respectively (International Food Safety Authorities Network, INFOSAN 2008). In addition, melamine was also found in Australia (egg albumin powder, egg powder and fried chickens), Japan (frozen squid), the USA (egg products) and South Korea (egg powder). It should be pointed out that the concentrations of melamine in the Republic of Korea did not exceed the limit of 0.1 $mg \cdot kg^{-1}$ in any of the total of 1,202 samples (WHO 2009).

The aim of this study was to determine the influence of continuous administration of melamine on the metabolic state of layers by haematological and biochemical blood tests and possibility of biotransformation melamine to cyanuric acid in eggs.

Materials and Methods

The experiment was performed in the accredited enclosure of the Institute of Nutrition, Animal Husbandry, and Animal Hygiene, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences in Brno for 6 weeks. Rearing conditions complied fully with technological specifications for the rearing of Isa Brown laying hens. During the experiment, Isa Brown layers (10 control and 10 experimental animals), individually housed using cage technology were fed a commercial feeding mixture (N1) containing the following nutritional components ($g \cdot kg^{-1}$): dry matter 885.3, gross protein 150.9, fat 22.8, fibre 21.9, starch 44.5, ash 82.4, Ca 31.5, P 5.54, Mg 5.2 and metabolizable energy (ME) 10.7 $MJ \cdot kg^{-1}$. Melamine was added to the feeding mixture intended for experimental layers at a concentration of 100 $mg \cdot kg^{-1}$. Melamine in the feed was determined using gas chromatography combined with a triple quadrupole mass spectrometer, in the ISO 17025 accredited laboratory. The extraction yield was 97.2%.

The eggs were collected daily for a period of 6 weeks (5 week feed contained melamine, 1 week feed without addition of melamine). Each egg was weighed followed by separation of the egg white, yolk, and shell. Mixed samples were prepared from the weekly production of eggs, egg yolk and egg white separately, using a homogenizer. Egg shells were not analysed. All samples were freeze-dried at -59 °C, homogenized by grinding and stored in a cold place at 5 °C for subsequent analysis for melamine and cyanuric acid.

Analysis was performed with 0.5 g of homogenized sample separately egg yolk and separately egg white that was extracted using 20 ml of the DEA extraction medium (water/acetonitrile) and agitated vigorously for 1 min. The mixture was centrifuged for 10 min (3,072 × *g*) and then filtered through the nylon filter with a pore size of 0.45 mm. A sample of 200 ml was taken from the solution and evaporated at 70 °C to dryness using liquid nitrogen. The residue was dissolved in 200 ml of pyridine and mixed with 200 ml of a silanizing reagent (SYLON BFT) and 100 ml of the benzoguanamine internal standard (1 $mg \cdot l^{-1}$ in pyridine). The entire mixture was stirred and incubated for 45 min at 70 °C. The resultant sample was analysed by gas chromatography - triple quadrupole mass spectrometry (GC-MS/MS). Successful use of the method based on gas chromatography coupled with a tandem detector in the analysis of egg samples has been reported by Miao (2010). The concentrations of melamine and cyanuric acid in the egg yolk and white are provided in $mg \cdot kg^{-1}$ per dry matter content of the sample.

The results were processed using mathematical and statistical methods implemented in the statistical programme Unistat 5.6 for Excel (2005). The mean values and their differences were evaluated by multiple comparison using the Tukey-HSD test, at levels of significance $P \leq 0.01$ and $P \leq 0.05$. Each indicator is represented by the mean value (x) and standard deviation (± SD).

Results

The results obtained in this study confirmed the carry-over of melamine from feed into eggs and a correlation between exposure time (i.e. the time at which the melamine-contaminated feed was administered) and the measured concentrations of melamine in eggs. Eggs in the control group did not contain melamine at a detectable concentration. In the experimental group, melamine was detected in eggs immediately after exposure to the melamine-contaminated feed. In the course of the experimental period, the mean concentrations of melamine in the egg yolk and egg white recalculated on a dry matter basis were 1.90 $mg \cdot kg^{-1}$ ± 0.158 and 10.84 $mg \cdot kg^{-1}$ ± 3.951, respectively. The difference between the mean concentrations of melamine in the egg yolk and egg white was found

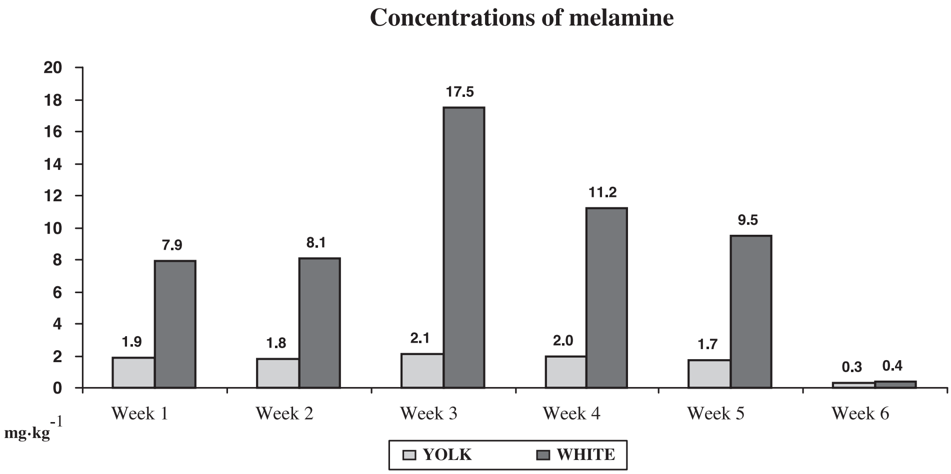

Fig. 1. Concentrations of melamine in the egg yolk and egg white in experimental layers during the experiment (melamine recalculated on a dry matter basis)

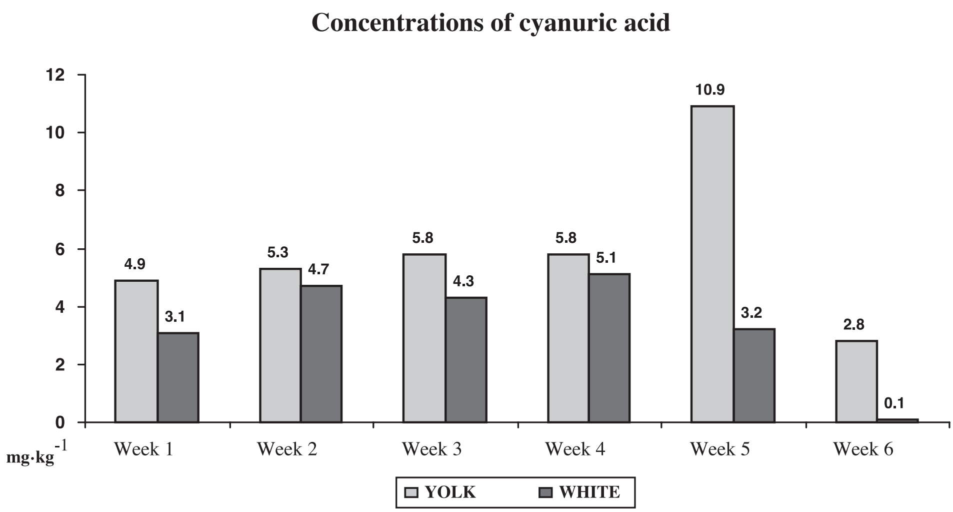

Fig. 2. Concentrations of cyanuric acid in the egg yolk and egg white in layers exposed to feed contaminated by melamine (cyanuric acid recalculated on a dry matter basic)

highly significantly different ($P \leq 0.01$). The highest contents of melamine in the egg yolk and egg white were found in the 3rd week of the experiment (Fig. 1).

In our study, we also measured cyanuric acid as a structural analogue of melamine. In the course of the experiment, the mean concentrations of cyanuric acid in the egg yolk and egg white were 6.54 $mg \cdot kg^{-1} \pm 2.466$ and 4.07 $mg \cdot kg^{-1} \pm 0.909$, respectively, as recalculated on a dry matter basis (Fig. 2). However, the difference between the mean values of cyanuric acid in the egg yolk and egg white was not significant ($P > 0.05$). The highest contents of cyanuric acid were detected in the egg yolk in week 5 of the experiment and in the egg white in week 4 of the experiment (Fig. 2).

Discussion

Concentrations of melamine in eggs did not increase in weeks 4 and 5 but showed a consistent declining trend. Similar findings were reported by Dong et al. (2010) who reported the maximum content of melamine on day 17 after melamine exposure. In our study, the content of melamine measured in egg white exceeded the permissible limit of 2.5 $mg \cdot kg^{-1}$ in food (WHO 2008) for 5 weeks of experiment. During the last week of the experiment, the concentration of melamine in eggs (egg yolk and egg white) dropped rapidly to the limit of detection of the analytical method used. The results of analysis showed that melamine accumulated in the egg white (10.84 $mg \cdot kg^{-1}$) to a significantly higher extent than in the egg yolk (1.90 $mg \cdot kg^{-1}$). This means that the egg white is the primary site for melamine accumulation in eggs. Our results are in agreement with the conclusions made by Dong et al. (2010) who found higher concentrations of melamine in powdered egg white (1.78 $mg \cdot kg^{-1}$) compared to powdered egg yolk (0.73 $mg \cdot kg^{-1}$).

The limit of detection for cyanuric acid was significantly lower (approximately × 50) compared to melamine in all measured matrices. Although modern analytical methods are able to detect these substances, their sensitivities may differ. Dong et al. (2010) who used the LC-MS method (Turnipseed et al. 2008) for the analysis of egg samples did not detect cyanuric acid in analysed samples of eggs and did not even mention the possible biotransformation of melamine into cyanuric acid in layers. However, it follows from our experiments based on gas chromatography that melamine could convert into cyanuric acid in the layer's body. However, the difference between the mean values of cyanuric acid in egg yolk and egg white was not significant ($P > 0.05$). The concentrations of cyanuric acid increased during the experiment, particularly in the egg yolk, showing a similar trend as melamine.

In conclusion, the fate of melanine's metabolites is highly important for determination of "safe periods" – time from administration of melanine containing feed to hens to being sure that no melanine residues are present in the produced eggs.

The transfer of melanine and its metabolite (cyanuric acid) from melamine-contaminated feed into the eggs was monitored in this study. The analytical method was based on extraction (water : acetonitrile mixture), derivatization to silyl derivates and simultaneous determination of both target analytes by simple and fast GC-MS/MS. Within the study, the dynamics of melanine transfer (including its metabolite) from the hen's organism into the eggs was clearly demonstrated.

Although a few studies dealing with the metabolism of melanine and it metabolites can be found in scientific literature, the quantitative registration of metabolite pathways declaring the amount of food safety related compounds in eggs within time is quiet unique. Our study thus contributes to better understanding of the melanine issue worldwide.

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