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Development and validation of a micellar liquid chromatography-based method to quantify melamine in swine kidney

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ABSTRACT

Melamine is a toxic compound illegally added to animal feed to falsely boost protein content. This represents a strong threat to consumer's health, as melamine can reach human through the food chain. An easy and reliable micellar liquid chromatography-method was developed to detect melamine in swine kidney. The analyte was extracted by shaking in methanol. Melamine was eluted from the HPLC column without interferences in <8.0 min. Mobile phase was a 0.11 M sodium dodecyl sulfate – 7.5% propanol at pH 3 solution running under isocratic mode through a C18 column at 1 mL/min and absorbance detection at 210 nm. The method was validated according to the Food and Drug Administration guidelines: sensitivity, calibration range (0.3–30 μg/g), linearity (r^2 > 0.9997), precision (<7.6%), accuracy (−8.3–3.6%), recovery (82.1–92.4%) and robustness (<5.1%). The methodology was applied to swine kidney samples purchased from a local supermarket. The method can be used as a screening method for routine post-mortem diagnose melamine intoxication in animals and detect feeding with poultry containing melamine, in order to withdraw the corresponding flesh. This would be mandatory to prevent consumer intoxication and improve the quality of food.

1. Introduction

Melamine (2,4,6-triamino-1,3,5-triazine; $C_3H_6N_6$; MW = 126 g/mol; pKa = 5.1; Fig. 1) is an inexpensive chemical intermediate widely used in the manufacture of resins and plastics (Marco-Peiró, Beltrán-Martinavarro, Rambla-Alegre, Peris-Vicente, & Esteve-Romero, 2012; Rambla-Alegre, Peris-Vicente, Marco-Peiró, & Beltran-Martinavarro, 2010). Recently, it was found out that melamine was illegally added to animal feed (Karras, Savvas, Patakioutas, Promoni, & Albanis, 2007), as well as in milk and derived products (Rambla-Alegre, Peris-Vicente, Marco-Peiró, & Beltran-Martinavarro, 2010) to apparently increase the protein concentration and then the commercial price. The ingestion of these contaminated foods was related to renal diseases, urolithiasis and bladder cancer, which can even lead to death, in animals (cats, dogs, rats, swine and fish) and humans (Dobson et al., 2008; Puschner, Poppenga, Lowenstine, Filigenzi, & Pesavento, 2007; Reimschuessel et al., 2008; WHO et al., 2009).

One potential source of exposure of humans to melamine is carry-over from contaminated flesh from animals nourished with melamine-spiked feed (EFSA, 2007). A study performed by the US Food and Drugs Administration (FDA) has described the risk to human health associated with eating products from animals that have been fed with melamine and its analogs (FDA, 2007). Health effects from exposure to melamine vary depending on the amount and duration of exposure, but they would represent a long-term human Health Risk (EFSA, 2010). Therefore, the determination of the melamine level of animals previous to its consumption by humans is essential to assess food safety.

China's incident (Zhang et al., 2009), prompted many countries and regions worldwide to establish action limits for the presence of melamine and/or melamine and its analogs in dairy-based foods. World Health Organization (WHO), in 2008, and European Food Safety Authority (EFSA), in 2010, established by independent studies a tolerable daily intake (TDI) of 0.2 mg/kg body weight (EFSA, 2007; EFSA, 2010). The Codex Alimentarius, in the July 2012 regulation, set the safety limit of melamine in 2.5 mg/kg for adults food and animal feed (Codex Alimentarius Commission, 2010; Montague-Jones, 2010). In 2010, Health Canada also set a maximum tolerable amount of melamine of 2.5 μg/g for adult food products (Health Canada, 2010).

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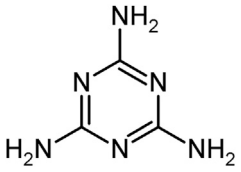

Fig. 1. Chemical structure of melamine.

Previous studies in rats (Wu et al., 2009) and pigs (Baynes et al., 2008) have proven that melamine is rapidly absorbed after being administered orally. Melamine is distributed into the extra cellular fluids, as it cannot pass through the cellular membrane to enter into cells (WHO et al., 2009). Melamine is mainly present in blood, from which is filtered by the kidney and eliminated essentially unchanged through urine (Reimschuessel et al., 2008). Therefore, the level of melamine in urine, blood (Marco-Peiró et al., 2012) and kidney can be related to the degree of intoxication of the animal.

Several analytical techniques have been applied to the detection of melamine in viscera and animal tissues: liquid chromatography coupled to mass spectrometry (LC–MS/MS) (Filigenzi, Puschner, Aston, & Poppenga, 2008; Qin et al., 2010; Wu et al., 2009) and indirect competitive enzyme-linked immunosorbent assay (ELISA) tandem gas chromatography-mass spectrometry (GC–MS) (Wang et al., 2010). These methods require large volumes of toxic solvents, lengthy extraction and tedious derivatization step, improving the probability of handling errors and loss of quality, and mass spectrometry also involves high costs. Although these methods are selective and sensitive, they are not suitable for routine analysis because of the large procedure, needing of many experimental steps and for financial reasons (Peris-Vicente, Simó-Alfonso, Gimeno Adelantado, & Doménech Carbó, 2005).

These problems can be avoided by the use of micellar liquid chromatography (MLC). Melamine has been detected in milk (Rambla-Alegre, Peris-Vicente, Marco-Peiró, & Beltran-Martinavarro, 2010), dietetic supplements (Beltrán-Martinavarro et al., 2012), biological fluids (Marco-Peiró et al., 2012) and water (Beltrán-Martinavarro et al., 2013), using micellar solutions to extract the analyte and using mobile phases under isocratic conditions to resolve it from matrix. The use of micellar solutions allows the solubilization of hydrophobic and hydrophilic compounds permitting the direct injection, after a simple filtration, of aqueous extracts containing suspended apolar compounds, without risk of precipitation into the column (Chin-Chen, Carda-Broch, Peris-Vicente, Esteve Romero, & Marco-Peiró, 2013; Rambla-Alegre et al., 2011). The elution parameters depending on the mobile phase composition can be simulated using a chemometric approach from the results obtained by testing few mobile phases. This reduces the cost and the experimental work performed to optimize the chromatographic conditions. Moreover, micellar solutions are less toxic, non-flammable, biodegradable and relatively inexpensive in comparison to aqueous-organic solvents (Esteve-Romero et al., 2010).

The aim of this work was to develop a fast, simple and inexpensive procedure for the determination of residue levels of melamine in swine kidney. It could be used in the routine analyses to measure the degree of contamination of flesh marketed for human consumption, and to ensure food safety. The analytical method must be validated following the Food and Drug Administration Foods Program Guidelines for Chemical Methods in terms of limit of detection, sensitivity, linearity, precision, accuracy, recovery and robustness (FDA, 2012). The chromatographic procedure should be evaluated by applying to real samples of swine kidney purchased in local supermarkets.

2. Experimental

2.1. Chemical and reagents

Melamine (99% purity) was obtained from Aldrich (St Louis, MO, USA). Sodium dodecyl sulfate (SDS, 99% purity) and sodium hydroxide were from Merck (Darmastadt, Germany). Sodium dihydrogen phosphate 1-hydrate and 1-propanol were purchased from Scharlab (Barcelona, Spain). Methanol, formalin, hydrochloric acid and triethylamine were all purity >99.99% and supplied by J.T. Baker (Deventer, The Netherlands). Ultrapure water was in-laboratory generated from distilled water using an ultrapure water device (Millipore S.A.S., Molsheim, France). Nylon filters were obtained from Sartorius-Stedim (Goettingen, Germany). Swine kidney samples were purchased in a local supermarket.

2.2. Instrumentation and software

The HPLC analyses were carried out in an Agilent Technologies Series 1100 apparatus (Palo Alto, CA, USA) equipped with an isocratic pump, a degasser, an autosampler and a diode array detector (range 190–700 nm). The pH of the solutions was measured with a pH meter equipped with a combined Ag/AgCl/glass electrode (Crison GLP 22, Barcelona). The balance was Mettler-Toledo AX105 Delta-Range (Greifensee, Switzerland). The vortex shaker was a Reax Top (Heidolph, Kelheim, Germany) and the ultrasonic bath was an Ultrasons-H (Selecta, Barcelona, Spain). The centrifuge was a Centronic–BL (Selecta). The mincer was a Taurus Ba-850 (Taurus Group, Oliana, Spain).

2.3. Chromatographic conditions

Stationary phase was in a reversed phase Kromasil C18 column (150 mm × 4.6 mm, 5 μm particle size) (Scharlab). The selected mobile phase was 0.11 M SDS 7.5% 1-propanol pH 3. Flow rate, injection volume and UV wavelength were 1 mL/min, 20 μL and 210 nm respectively. The injected solutions and mobile phases were filtered through 0.45 μm nylon membranes. Michrom software was used for chromatographic data treatment (Rambla-Alegre, Peris-Vicente, Esteve-Romero, & Carda-Broch, 2010).

2.4. Preparation of mobile phase and stock solutions

The micellar mobile phases were prepared by weighing the appropriate amount of SDS and disodium monohydrogenphosphate and solving it in ultrapure water. Then, the pH was adjusted by adding the appropriate amount of NaOH 0.1 M or HCl 0.1 M respectively. After this, 1-propanol or butanol was added, if necessary. Finally the solution was adjusted to the desired volume with ultrapure water, ultrasonic and filtered.

Stock solutions with 1; 100 and 200 μg/g of melamine were prepared by dissolving the appropriate amount in methanol. Working solutions were prepared by diluting the stock solutions in the adequate solvent. Stock solutions were stored at +4 °C.

2.5. Experimental procedure

Swine kidney samples were finely ground using a mincer. Afterward, 1 g of homogenized meat was mixed with 2 mL methanol with the aid of an ultrasonic bath for 30 min at 122 W input power and 50 kHz. In the case of spiked samples, the adequate volume of the melamine-in-methanol solution was injected into the ground meat and stored during one day (Cano-Sancho, Marin, Ramos, Peris-Vicente, & Sanchis, 2010), before ultrasonication. Furthermore, it was centrifuged at 3000 rpm for 10 min. The supernatant