A new method for the quantification of Melamine in milk by Absorption Diode Array Thin-Layer Chromatography

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3.5 A new method for the quantification of Melamine in milk by Absorption Diode Array Thin-Layer Chromatography

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Introduction

Melamine (1,3,5-triazine-2,4,6-triamine or cyanuramide, $C_3H_6N_6$) is a trimer of cyanamide, with a 1,3,5-triazine skeleton (Figure 3.5-1). The molecule contains 66% nitrogen by mass and, if mixed with resins, has fire retardant properties due to its release of nitrogen gas when burned or charred. The word melamine (from German) is a combination of the word melam (which is a distillation derivative of ammonium thiocyanate) and amine [1]. Melamine is also a metabolite of cyromazine, an insecticide in which the proton of an NH_2-group is substituted by a cyclopropyl group.

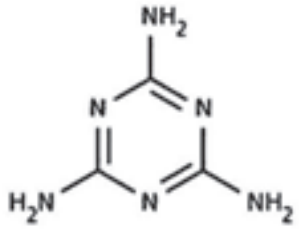

Fig. 3.5-1: *shows the formula of melamine*

In September 2008, several companies were implicated in a scandal involving milk and infant formula which had been adulterated with melamine, leading to kidney stones and other renal failure, especially among young children [2]. Melamine was illegally added to food products in order to increase the apparent protein content. Standard tests such as the Kjeldahl test estimate protein levels by measuring the nitrogen content. Because of melamine's high nitrogen content, it can cause the protein content of food to appear higher than the true value.

For the determination of melamine in milk [3] and food [4] High Performance Liquid Chromatography (HPLC) in combination with mass spectrometry is used. A Thin Layer Chromatography (TLC) method for melamine quantification is not described. The purpose of our publication is to develop a fast and inexpensive method that will work reliably. It should be used for screening and quantification of melamine in milk and milk products.

Experimentals

Milk was diluted with the same amount of water and applied band-wise (7 mm) on a TLC K60 silica gel 60 foil (No. 1.05554.0001 from Merck, Darmstadt, Germany) in the amount of 1 µL. A Desaga AS 30 sample applicator (Desaga, Heidelberg, Germany) was used with a spraying velocity of 80 sec/µL. The plate was developed in a vertical glass chamber (without chamber saturation) to a distance of 55 mm with the solvent mixture of 2-propanole, CH_2Cl_2, water (3+1+1,V/V). The separation took 35 minutes. The solvents were purchased from Roth, Karlsruhe, Germany.

The plate was dried in a gentle stream of air for 5 minutes and placed in a chlorine containing chamber for 5 minutes. Chlorine was produced from 10 mL KMnO4-solution (3 g $KMnO_4$ in 100 mL of water) and 10 mL HCl (25 mL 32% HCl dissolved in 50 mL of water). HCl and $KMnO_4$ were purchased from Merck, Darmstadt, Germany. Five minutes after mixing the chamber was filled with chlorine and the TLC-plate could be placed.

Two staining reagents, Wuster's blue and starch/iodine reagents were found to show sufficient sensitivity. 500 mg of Wuster's blue reagent (N,N,N',N'-tetramethyl-1,4-phenyldiammonium chloride from Alfa Aesar, Karlsruhe, Germany), was dissolved in 100 mL of acetone and is stable for 8 hours. To produce the starch/iodine reagent 250 mg of potassium iodide (from Riedel-de Haën, Seelze, Germany) was dissolved in 25 mL of water. 750 mg starch (according to Zulkowsky from Merck, Darmstadt, Germany) was dissolved in 25 mL of water. Both solutions were mixed and diluted with 30 mL of ethanol (from Roth, Karlsruhe, Germany). The mixture was stable for one day. For spraying 800 mg KI and 800 mg starch were dissolved in 25 mL of water, respectively. Both solutions were mixed and diluted with 10 mL of ethanol.

The chlorinated TLC-plate was dipped for 2 sec in Wuster's blue reagent, forming deep blue zones on a light blue background. Dipped plates were scanned by

use of a diode-array scanner (TIDAS TLC 2010 system J&M company, Aalen, Germany) [5]. Scanning should be done within 6 hours after dipping in the wavelength range from 550 to 615 nm. Blue-brown zones are formed on a light dark background, if the chlorinated plate is dipped for 1 sec in starch/iodine reagent. The colours remain stable for days. The zones were scanned in the wavelength range from 490 to 610 nm. Scanning of a single track was done for 550 spectra in the wavelength range from 200 to 1000 nm in 55 seconds.

Results and Discussion

Figure 3.5-2 shows a starch/iodine stained plate. Pure milk was applied on track 1. Track 2 to 8 show milk samples, spiked with different amounts of melamine. The milk sample on track 2 was spiked with 160 ng and the sample on track 8 with 4 ng melamine. 1 µL of water diluted milk (1+1,V/V) was applied on each track.

In Figure 3.5-3 and 3.5-4, eight milk samples were applied on the TLC-plate. On each track 80 ng of melamine were separated. The plate in Figure 3.5-3 was stained using the starch/iodine reagent. The plate in Figure 3.5-4 was stained using Wuster's blue reagent.

The amount of 80 ng melamine in 0.5 µL milk is 160 ng in 1 µL pure milk, or 160 mg melamine in 1L of milk. The statistically defined quantification limit is 25 ng or 50 mg melamine in 1L of pure milk.

For quantification purposes the light absorption (A) was measured directly on plate, using equation (1).

$$A(\lambda, k=1) = \left(\frac{J_0}{J} - 1\right) \quad (1)$$

In this equation J_0 stands for the reflected light intensity of a clean TLC-plate and J refers to the reflected light of the sample. A single track of the TLC-plate was scanned, measuring 550 spectra in the wavelength range from 200 to 1000 nm. A separation distance of 1 cm was scanned in 100 spectra. The spectral intensity was calculated using equation (1). The intensity distribution of a single track drawn with the separation distance is called "densitogram".

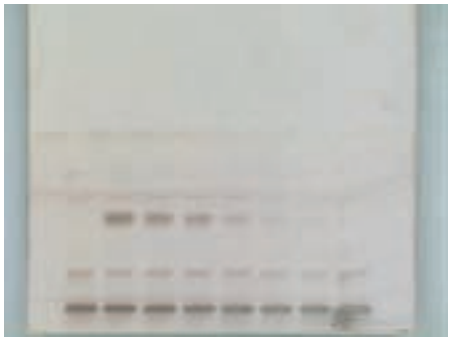

Fig. 3.5-2: *shows a plate stained with starch/iodine reagent. On the first track a milk sample without melamine is shown*

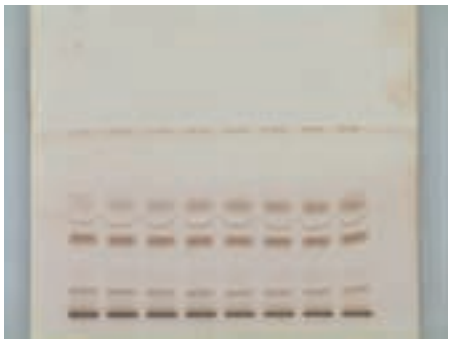

Fig. 3.5-3: *shows eight melamine spiked milk samples (each with 1 µL applied), stained with starch/iodine reagent*

Fig. 3.5-4: *shows eight melamine spiked milk samples (each with 1 µL applied), stained with Wuster's blue reagent*

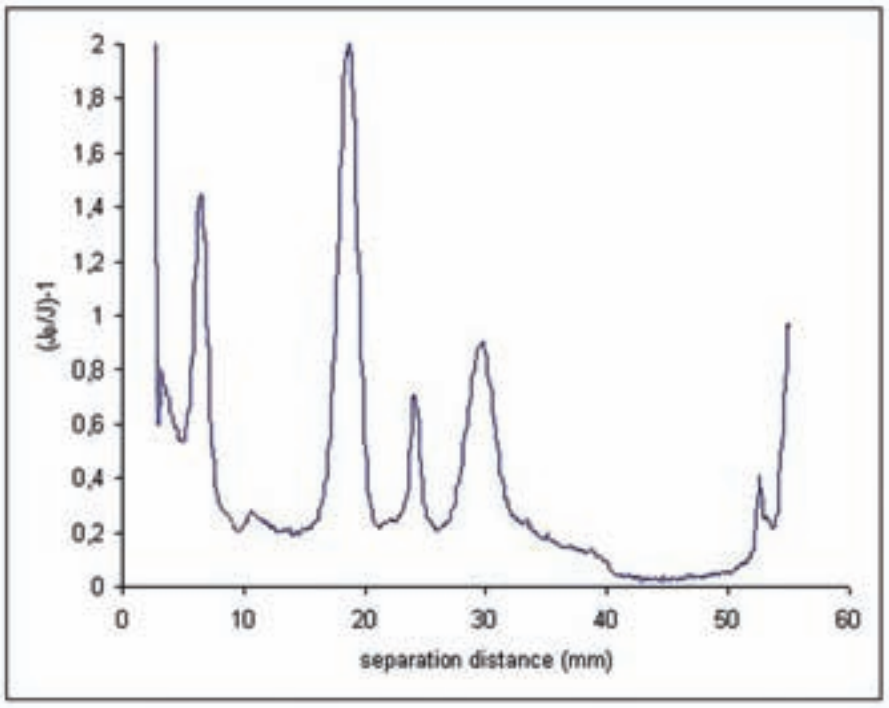

Fig. 3.5-5: *shows the densitogram of melamine spiked milk sample. The melamine signal (containing 80 ng) can be seen at 18.7 mm separation distance. The plate was dipped in Wuster's blue reagent and evaluated in the wavelength range from 550 to 615 nm*

Figure 3.5-5 shows the absorption densitogram of a Wuster's blue stained milk sample, which contains 80 µg/mL of melamine. The melamine peak appears at 18.7 mm separation distance and shows an Rf-value of Rf = 0.30. A huge signal at the point of application can be seen at 3 mm separation distance. The main milk content did not move with the mobile phase and remained at the point of application. The front signal can be seen at 55 mm separation distance. The milk signals were scanned below 10 mm and above 21 mm separation distance.

Accuracy was tested using a spiked milk sample diluted with water to the desired concentrations to compare them with the same spiked milk sample diluted with milk. The same sensitivity of the calibration curve was calculated (either dissolved with milk or water) for both staining methods. This indicates that milk does not interfere with the melamine zone.

The calibration plot Figure 3.5-6 shows linearity for both staining methods in the range from 4 ng to 160 ng melamine, evaluated using the absorption formula [5, 6]. The statistically evaluated limit of detection (LOD) for both methods is 15 ng (30 mg melamine/L milk) [7]. The statistically evaluated limit of quantification (LOQ) for both methods is 25 ng (50 mg melamine/L milk) <[7]. The standard deviation of spiked milk samples over all measurement steps with a melamine level of 20 ng/zone for Wuster's reagent is 8.9% and for the starch/iodine reagent 7.1%.

Eight samples were applied in parallel on a 10 by10 cm TLC-plate. The total measurement time for 8 samples was 65 minutes, including all sample pre-treatment steps. The method is suitable for melamine screening in milk and milk powder down to 50 mg melamine/L milk at a low cost level.

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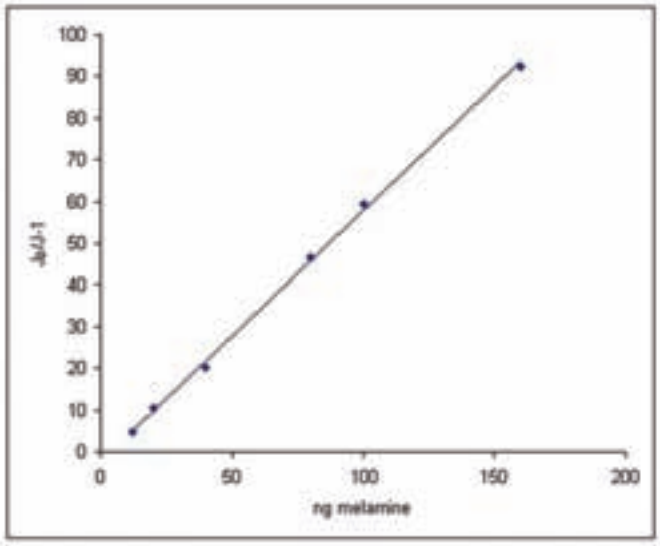

Fig. 3.5-6: *shows the calibration curve of melamine spiked milk, stained with starch reagent and measured in the wavelength range from 490 to 610 nm. Compared were the reflected light intensity (J) and the reflected light intensity of a clean plate surface (J_0)*