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Short communication

A sensitive spectrofluorimetric method for the quantification of melamine residue in milk powder using the Mannich reaction in aqueous solutions

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ABSTRACT

The objective of this study was to develop a spectrofluorimetric method for the quantitative determination of melamine. The method was based on the complexation of melamine with a mixture of formaldehyde and chemicals including a ketone group, as described by the Mannich reaction. The complex was determined by spectrofluorimetric measurement as it is characterized by specific spectroscopic properties that are related to the chromophore of the ketone compounds. 1,3-Diphenylpropane-1,3-dione (DPPD) was tested as a ketone compound. The fluorescence spectrum of the complex presented a maximum of absorption at 325 nm.A quenching of the fluorescence occurred when melamine was added into the solution. The kinetic of fluorescence quenching was followed to determine quantitatively the melamine concentration. An internal standard was added to quantify melamine. The method was tested to determine the level of melamine in contaminated milk powder. The recovery value was 97% and the limit of detection was 0.007 μg $mL^{-1.}$

1. Introduction

Melamine (2,4,6-triamino-1,3,5-triazine) is a chemical used in manufacturing plastics and fertilizer products. Several recent studies reported numerous cases of nephrolithiasis and, in some instances, renal failure in Chinese babies following consumption of melamine-contaminated infant formula (milk powder). Some manufacturers illegally used melamine as an adulterant to increase the food products apparent protein content. For the same purpose, melamine was added to animal feeds and thus the industrial chemical was detected in eggs and in all food categories that use milk powder as an ingredient.

Previously reported methods for the quantitative determination of melamine include enzyme immunoassay (EIA), gas chromatography mass spectrometry (GC–MS), liquid chromatography mass spectrometry (LC–MS), and high-performance liquid chromatography (HPLC) with UV detection [1–4]. Standard methods enacted by the Chinese government for determining melamine in raw milk and dairy products included HPLC–UV, LC–MS, and GC–MS methods [GB/T 22388 2008, GB/T 22400 2008]. However, the high cost of operation and maintenance of GC/LC–MS systems as well as the labor intensive derivatization that GC–MS requires limits their use in milk product factories.

For the quantitative determination of melamine as a chemical contaminant in food such as lard, potato proteins, food-stimulants and beverages, only few methods have been reported, such as spectrophotometry [5], liquid chromatography[6–8] and gas chromatography. Several studies [9–11] used HPLC/MS to determine the melamine in pet food by enzyme immunoassay. GC–MS (gas chromatography–mass spectrometry) has also been used after trimethylsilylation for the determination of melamine and its analogs in wheat gluten and pet food matrices.

This latter method has been recommended by the European Commission to analyze consignments of wheat gluten, corn gluten, corn meal, soy protein, rice bran and rice protein concentrate originating from developing countries, in particular from China. Melamine has been detected using liquid chromatography in beverages at levels of 0.54, 0.72, 1.42 and 2.2 mg kg^{-1} in coffee, orange juice, fermented milk and lemon juice, respectively, with a limit of detection of 0.05 mg L^{-1}. These levels are due to the migration of melamine from the cup, made of melamine–formaldehyde resin, into the beverage under acidic conditions [8].

In our previous work [12] we measured melamine in Chinese fish by a simple

spectrophotometric method using the Mannich reaction resulting from interaction between melamine formaldehyde and a ketone compound.

The objective of this work was to develop a new spectrofluorimetric method, more sensitive than the spectrophotometric one for the quantitative determination of melamine in powdered milk. The

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fluorescence spectroscopy was used to monitor the complex of melamine, formaldehyde and DPPD, as described by the Mannich reaction.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and solvents used were of analytical grade or of a higher grade, when available. Formaldehyde, 1,3-Diphenylpropane-1,3-dione (DPPD), melamine, ethyl acetate, were purchased from Fisher, (MA, USA). Ultra pure water was prepared using a multi-Q filter system (Millipore, MA, USA).

2.2. Instruments

The solutions were monitored by Cary UV spectrophotometer (Varian Inc., CA, USA). The fluorescence was measured by a HITACHI 7000 Spectrofluorimeter. Measurement of pH was done using a Mettler Toledo (OH, USA) pH-meter.

2.3. Standard solutions

Stock aqueous solution of melamine was transferred into a volumetric flask to produce a solution with a concentration of 36 μg mL^{-1}. The solution was shaken for 20–30 min until complete dissolution of the melamine crystals. Samples were prepared for analysis by mixing 1 mL of aqueous DPPD solution 12 μg mL^{-1}, 1 mL of pure formaldehyde and different volumes of melamine stock solutions (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.8 mL). De-ionized water was transferred to each sample to reach a final volume of 5 mL. The final concentration of DPPD was 2.4 μg mL^{-1} and the melamine concentrations varied from 0.72 μg mL^{-1} to 5.76 μg mL^{-1}.

Table 1 presents the composition of samples used for melamine analysis.

2.4. Extraction procedure

About 10 g of milk powder, polluted by 0.15 mg of melamine, were transferred into a 100 mL polypropylene centrifuge tube to which 50 mL of extraction solvent (diethylamine:water:acetonitrile/ 10:40:50) was added. After mixing to thoroughly wet the entire sample, the mixture was centrifuged for 20 min at 5000 rpm. In order to remove fatty acids from the milk sample, 20 mL of the supernatant extraction solvent were shaken with dichloromethane which was then discarded using a separator funnel.

Thus, the aqueous layer containing melamine was ready to be analyzed. To fortify the samples, different concentrations of melamine were added to the same volume of extracted milk powder sample. Table 2 summarizes the volumes of different chemicals used in different mixtures.

Table 1
Composition of the samples used for melamine analysis.

Volume of melamine [36 μg mL^{-1}] (ml)	Volume DDPD [12 μ gmL^{-1}] (ml)	Volume of formaldéhyde (ml)	Volume of water added (ml)
0.1	0	1	3.9
0.1	1	1	2.9
0.2	1	1	2.8
0.3	1	1	2.7
0.4	1	1	2.6
0.6	1	1	2.4
0.8	1	1	2.2

Table 2
summarizes the volumes of different chemicals solutions used in the internal standard addition method for the melamine determination in the polluted milk powder.

Volume of sample extracted from the milk (ml)	Volume of melamine added (ml)	Volume of DDPD (ml)	Volume of formaldehyde (ml)	Volume of water added (ml)
1	0	1	1	2
1	0.5	1	1	1.5
1	1	1	1	1
1	1.5	1	1	0.5
1	2	1	1	0

2.5. Calibration curves and recovery

Stock solutions were used to prepare solutions of lower concentrations to build the calibration curve using fluorescence measurements. Linearity was performed with melamine working solutions within the range 0.72–5.76 μg mL^{-1}.

Recovery experiments were performed by standard addition method with fortifying melamine working solution added to samples. The percentage of recovery (%*R*) was calculated as follows:

$$\%R = [(C_r - C_f)/C_r]$$

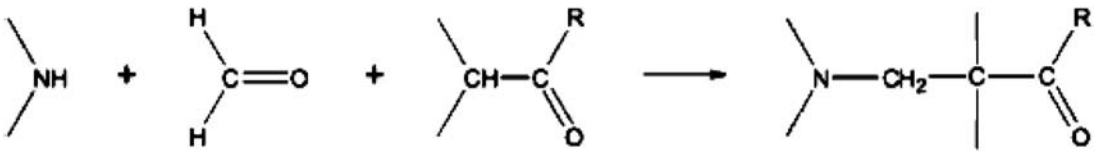
C_r = Real concentration of melamine in the fortified samples;
C_f = Concentration of melamine obtained by the internal standard addition curve.

3. Results and discussion

3.1. Identification of the melamine–formaldehyde–DPPD complex

The Mannich reaction (Eq. 1) consists of an amino alkylation of an acidic proton placed next to a carbonyl functional group with formaldehyde and ammonia or any primary or secondary amine.

The final product is a β-amino-carbonyl compound. Reactions between aldimines and

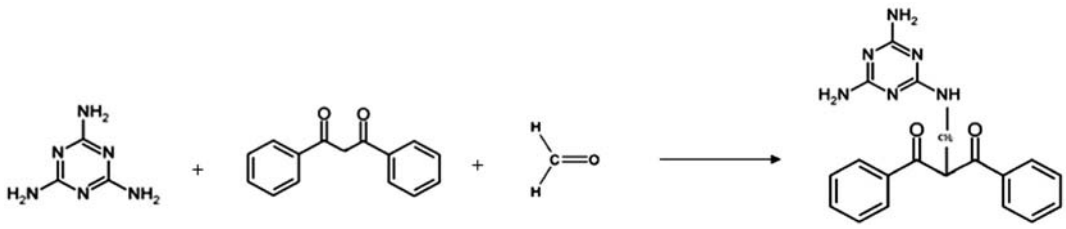


(1)

α-methylene carbonyls are also considered Mannich reactions because these imines form between amines and aldehydes [13].

We hypothesized that the reaction between DPPD, formaldehyde and melamine must be similar to the reaction in Eq. (1).

The DPPD solution does not have any evident band of absorption between 190 nm and 350 nm; however when DPPD was mixed with formaldehyde it presents a characteristic UV spectrum with a maximum at 205 nm as shown in the Fig. 2.

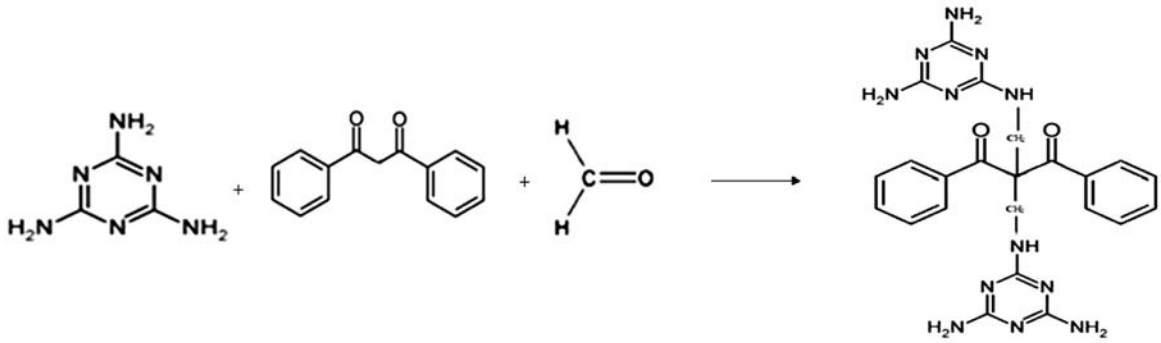


(2)

The mechanism of the reaction is given in Eq. (2).

Or complexed with melamine formaldehyde may substitute with two atoms of hydrogen in the carbon located between two ketone groups as shown in the following equation:

The complex formed by mixing melamine, formaldehyde and 1,3-diphenylpropane1,3-dione present in the UV spectrum at 210 nm a maximum (Fig. 3). A redshift has been observed from 195 nm of the melamine to 210 nm after complex formation.



3.1.1. UV spectra of melamine, DPPD and complexes obtained by mixing melamine, DPPD and formaldehyde according to the Mannich reaction

Melamine compound presents a UV spectrum with a maximum absorbance at 195 nm as shown in (Fig. 1). Formaldehyde does not have any UV spectrum.

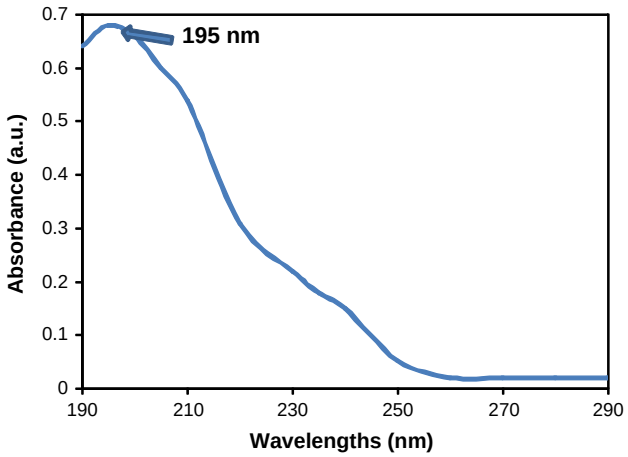


Fig. 1. UV spectrum of melamine in aqueous solution 0.72 mg/l.

3.1.2. Evolution of the complex melamine-formaldehyde–DPPD absorption spectrum with melamine concentration

Fig. 4 illustrates the change of the complex spectra obtained by the Mannich reaction at five different concentrations of melamine, ranging from 0.72 mg L^{-1} to 5.75 mg L^{-1}, and with constant concentration of formaldehyde and 1.3-diphenylpropane-1,3-dione. It has been observed that when the melamine concentration

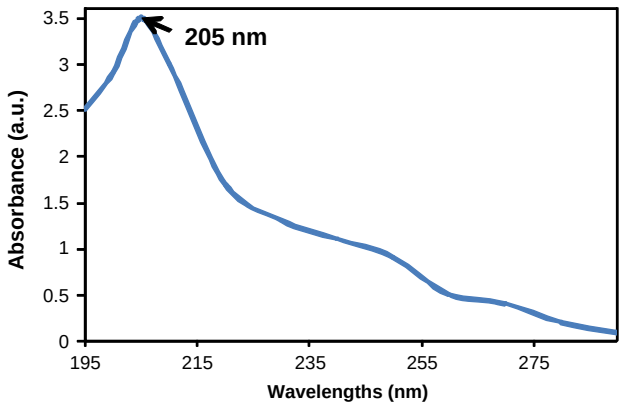


Fig. 2. UV spectrum of DPPD 5 ppm complexed with formaldehyde.

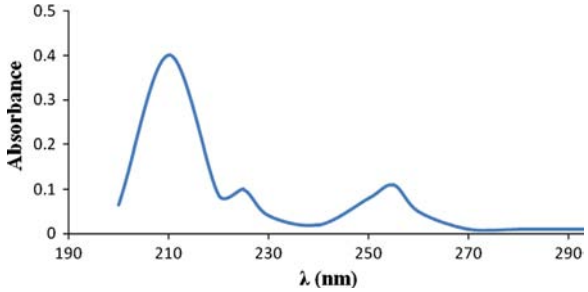


Fig. 3. UV spectrum of the complex obtained according to the Mannich reaction after mixing melamine 12 ppm, 2.4 ppm of DPPD and 1 ml of formaldehyde.

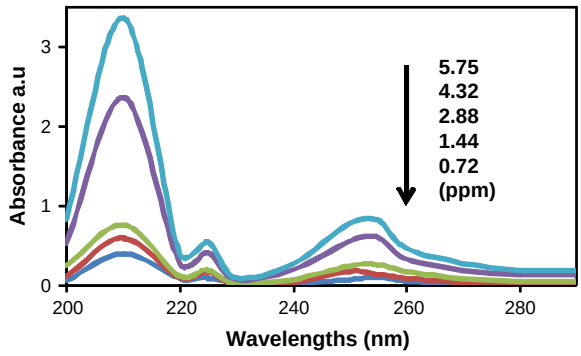


Fig. 4. Evolution of the absorption spectrum of the complex melamine- Formaldehyde–DPPD depending on the concentration of melamine (melamine ppm: 0.72–1.44–2.88–4.32–5.75 down to up), DPPD = 2.4 ppm, Formaldehyde = 1 ml.

increases the intensity of the UV absorption spectrum increases. Data were used for the quantitative measurement of melamine in the contaminated fish [11].

In this work, we extended a previous study by investigating the method sensitivity using the fluorescence evolution of the complex obtained by Mannich reaction.

3.1.3. *Evolution of the complex melamine, formaldehyde and DPPD fluorescence spectrum with melamine concentration*

Solution without addition of melamine has been processed and contained 1 mL of DPPD with 1 mL of formaldehyde. The mixture of DPPD with formaldehyde gave rise to a complex presenting a UV spectrum with a maximum at 205 nm as illustrated in Fig. 2. When the melamine solution was added, the UV absorption spectrum exhibited a maximum at 210 nm. In this case when excited with a wavelength at 210 nm there was a fluorescence spectrum with a maximum at 325 nm shown in Fig. 5.

Quenching phenomena has been observed when melamine was added to the solution. As shown in the fluorescence spectra Fig. 6 the fluorescence intensities decreased progressively with the increasing concentration of melamine.

The limit of detection was determined by the minimum melamine concentration able to inhibit measurably fluorescence of the solution obtained by mixing the DPPD with formaldehyde.

3.2. *Regression curve between the complex formation and the melamine concentration*

A calibration curve was built to examine the linearity of the method. The least squares method was used to calculate the regression equation. A good linear correlation was obtained between the fluorescence evolution of the complex and the additional concentration of melamine. Fig. 7 shows the regression

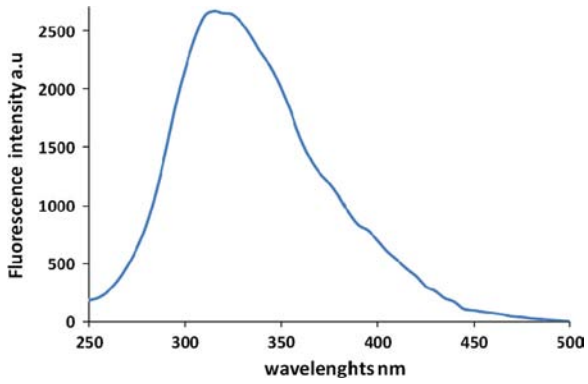


Fig. 5. Fluorescence spectrum of the complex between 1,3-diphenylpropane-1,3-dione and formaldehyde λ_{ex} = 210 nm; λ_{obs} = 325 nm.

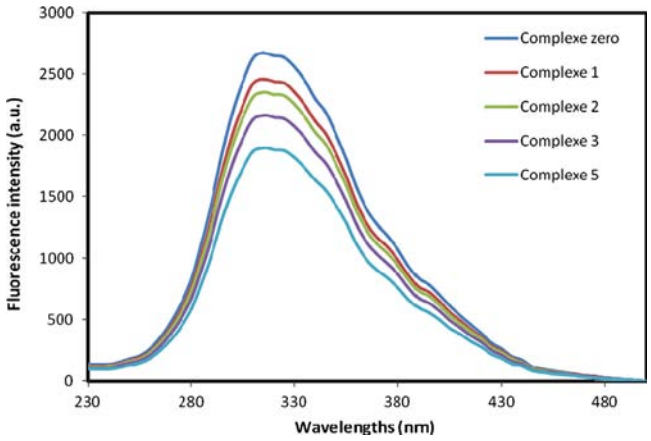


Fig.6. Evolution of the fluorescence spectrum of the complex obtained according to the Mannich reaction using the mixture of Melamine, Formaldehyde and DPPD depending on the concentration of melamine [ppm](0–0.72–1.44–4.32–5.76).

curve of complex fluorescence as a function of melamine concentration.

Correlation coefficients were higher than 0.99 in a concentration range of 0.72 mg L^{-1}–5.76 mg L^{-1}. The precision of the method was evaluated with relative standard deviations (RSD) of melamine determination in five samples. The %RSD was 2.7%.

The detection limit of the method is 0.007 μg mL^{-1} as defined by the signal/ noise ratio = 3 [14], was 10 times more sensitive than the UV absorption.

3.3. *Spectrofluorimetric method for quantification of melamine using the internal standard addition model*

A spectrofluorimetric method using the internal standard addition was examined to quantitatively determine melamine concentrations in samples. A calibration curve was described by the following equation:

$$IF^* = aC \times b$$

$$IF^* = \left(IF_0 \frac{IF_0}{C_0}\right) \times C_{add} + IF_0{}^*$$

where IF* = (IF_0/IF) normalized fluorescence intensity (arbitrary values) is equal to the ratio of the fluorescence intensity before adding the internal standard IF_0 to the absorbance intensity after adding the internal standard (IF), C_0: solute concentration to be estimated. C_0 is determined by the negative intercept of the curve with the abscissa axis [15,16].

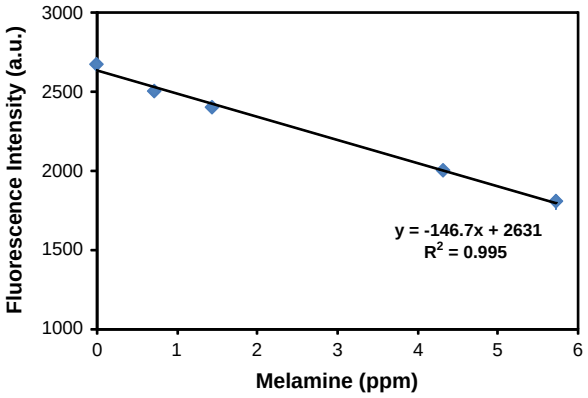


Fig. 7. Regression curve for the complex vs. melamine concentration.

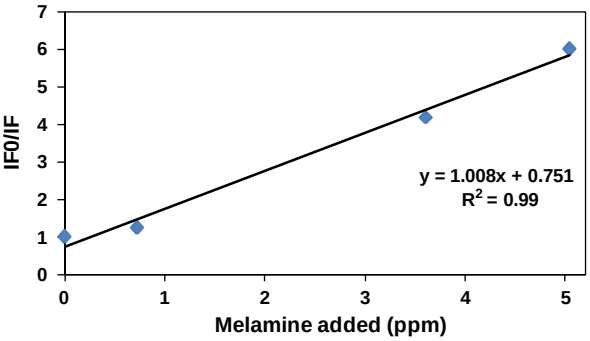


Fig. 8. Calibration curve for the internal standard addition method.

Table 3
Validation parameters.

Parameter	Result
Maximum absorption	210 nm
Linearity range (μg mL⁻1)	0.72–5.76
Standard equation regression	Y=−126.0*x*+2620
Correlation coefficient	R^2=0.99
Accuracy (% Recovery)	97.3%
Precision	99.78% (intra-day precision) and 99.82% (inter-day precision)
Specificity	No interference at the selected wavelength
LOD (μg mL⁻1)	0.007
LOQ (μg mL⁻1)	0.021

IF_0^*: Normalized fluorescence intensity of the starting solution, and C_{add}: known added concentrations. The plot of IF* vs. C_{add} is shown in Fig. 8. The internal standard used in this method was melamine that we would like to determine (C_0). To this initial solution, different known concentrations (C_{add}) were added. The average recovery for five samples spiked with melamine as described above in Table 1 was estimated to be 97% ± 3. Table 3 summarizes the method validation parameters.

3.4. Application of the method on the determination of melamine in contaminated milk

Milk powder selected from the local market was contaminated by adding 0.15 mg of melamine in 10 g of powder. As detailed previously, extraction of melamine was followed. The amount of melamine added to the milk must be completely transferred into the aqueous phase. The expected concentration in the volume of the aqueous phase for analysis was 7.5 mg L^{-1}.

Fluorescence spectra of the solutions described in Table 2 were obtained and the evolution of those spectra was used to build the internal standard curve.

According to the composition of the solutions to be analyzed the calculated melamine concentration after five time dilution was 1.5 μg mL^{-1} (n=5). However the experimental values obtained from the curve of the internal addition method is 1.46 μg mL^{-1} (n=5).

3.5. Validation of the method

The proposed method has been validated according to International Conference on Harmonization guidelines [17] for validation of analytical procedures which include limit of detection, limit of quantitation, linearity, repeatability and intermediate precision, recovery, specificity, and accuracy.

3.5.1. Limit of detection and limit of detection

The limit of detection (LOD) and limit of quantitation (LOQ) of the developed method were determined by analyzing progressively low concentration of the melamine working solution (7.5 μg mL^{-1}). The LOD is defined as the smallest concentration of the analyte that gives a signal to noise ratio of 3. The LOD was found to be 0.007 μg mL^{-1}. The LOQ is the smallest concentration of the analyte that gives a signal to noise ratio of 10. The LOQ and LOD of melamine were found to be 0.007 and 0.021 μg mL^{-1} respectively.

3.5.2. Linearity

Over the selected concentration range, a linear relationship was obtained between the fluorescence intensity and concentration (Fig. 7). Linearity was established using five different concentrations: (0.72, 1.44, 2.16, 2.88 and 5.76 μg mL^{-1}) of melamine working standard solution.

Each concentration level was prepared in five replicates (n=5). Results have shown that this method is linear over the range of 0.72–5.76 mg L^{-1}. The linear regression analysis demonstrates an excellent relationship between the fluorescence intensity and concentration of melamine added with correlation coefficients (R^2) of 0.99.

3.5.3. Precision

The precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was evaluated by analyzing five replicates of the standard solution of melamine (36 μg mL^{-1}) on the same day. The intermediate precision was determined by comparing the results on different days (4 days). The mean values and standard deviations of fluorescence measurement are listed in Table 3.

3.5.4. Recovery

The recovery of the method was determined by the standard addition method on selected samples. A known amount of melamine was added to a solution with an initial concentration C_0 (0.72 μg mL^{-1}), at three levels 50%, 100%, and 150% (n=5). The percentage recovery was calculated according to reference [16] and data are presented in Table 3. The recovery was found to be 97.3%.

3.5.5. Specificity

The amine compounds that may occur in milk are mainly proteins and particularly caseins. The chemical structures of these caseins or other proteins can give rise during the Mannich reaction

to complexes possessing shifted absorption spectra relative to those obtained with melamine.

This allows a selective excitation of the complex obtained with melamine and avoiding possible interferences that might occur with the complex obtained with proteins in milk powder.

4. Conclusion

In summary, the spectrofluorimetric method that was developed based on the Mannich reaction is simple, novel and specific for the quantitative determination of melamine content in contaminated milk. Furthermore, this method can be applied to screen all kinds of foods, such as milk derivatives, eggs, pet food, or wheat gluten, for example. The newly reported method showed higher level of accuracy and sensitivity when compared to the previously developed spectrophotometric method. An important advantage of the fluorescence study is related to the spectroscopic selectivity by exiting the specific band of the complex obtained by the Mannich reaction.

References

[1] M.S. Filigenzi, E.R Tor, R.H. Poppenga, L.A. Aston, B. Puschner, Rapid Commun. Mass Spectrom. 21 (2007) 4027–4032.
[2] T. Ding, J. Xu, J. Li, C. Shen, B. Wu, H. Chen, S. Li, Chin. J. Chromatogr. 26 (2008) 6–9.
[3] B. Kim, L.B. Perkins, R.J. Bushway, S. Nesbit, T. Fan, R. Sheridan, V. Greene, J. AOAC Int. 91 (2008) 408–413.
[4] W. Leo, M.S. Henday, L. Xiaodong, T. Mark, C.W. Schnute, Dionex Corporation LPN-01/10/07, 1991.
[5] R. Hirt, C. Doughman, W.R. Schmitt, J. Agric. Food Chem. 3 (1955) 1044–1046.
[6] R. Bisaz, A. Kummer, Mitt. Geb. Lebensmittelunters. Hyg. 74 (1983) 74–79.
[7] T. Inoue, H. Ishiwata, K. Yoshihira, A. Tamimura, J. Chromatogr 346 (1985) 450–452.
[8] H. Ishiwata, T. Inoue, T. Yamazaki, K. Yoshihira, J. Assoc. Off. Anal. Chem. 70 (1987) 457–460.
[9] H. Ishiwata, T. Inoue, A. Tanimura, Food Add. Contam. 3 (1986) 63–69.
[10] B. Kim, LB. Perkins, RJ. Bushway, S. Nesbit, T. Fan, R. Sheridan, V. Greene, J. AOAC Int. 91 (2008) 408–413.
[11] J.V. Sancho, M. Ibañez, S. Grimalt, O.J. Pozo, Anal. Chimica Acta 530 (2005) 237–243.
[12] J. Rima, M. Abourida, T. Xu, I.L. Cho, S.S Kyriacos, J. Food Compos. Anal. 22 (2009) 689–693.
[13] C. Mannich, W. Krosche Ueber, Arch. Pharmazie 250 (1912) 647–667.
[14] D. MacDougall, W.B. Crummett, Anal. Chem. 52 (1980) 2242–2249.
[15] B. Muel, G. Lacroix, Bull. Soc. Chim. Fr. 11 (1960) 2139.
[16] J. Rima, M. Lamotte, J. joussot-Dubien., Anal. Chem. 54 (1982) 1059–1070.
[17] Proceedings of the International Conference onHarmonisation (ICH), Commission of the European Communities, 1996, http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm265700.htm.