Development of a method for melamine determination in beer and beer-type beverages by GC-MS/MS

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Abstract

Based on the European regulation, the Czech Association of Breweries and Malthouses recommends 2.5 mg/kg as maximum permitted concentration of melamine for beer, barley, malt, hop, and their products. For this reason, the first analytical method for melamine determination in beer and beer-type beverages was developed. The sample preparation included ion-exchange solid-phase extraction and subsequent derivatization with BSTFA. Qualitative and quantitative analysis was performed by gas chromatography with tandem mass spectrometry. Validation parameters of the method, such as limits of detection and quantification were 0.1 mg/L and 0.4 mg/L, respectively. Extended uncertainty of the final method was evaluated to 0.1 mg/L. The method was applied on twenty samples of beer lagers, or other beer styles; all had the level of melamine under the limit of quantification (< 0.4 mg/L).

Key words: beer, gas chromatography, melamine, tandem mass spectrometry

1 Introduction

Melamine (1,3,5-triazine-2,4,6-triamine) is generally used as an additive for plastics, glues or paint colors. In the past years, it was abused by the Asian food industry as a supplement apparently increasing nutritious quality of a product (WHO, 2008). Since one molecule of melamine contains six nitrogen atoms (Figure 1), it falsely rises the total nitrogen determination, which is used for the evaluation of a protein content. Even though the toxicity of isolated melamine has not been proved, its presence in food deceives consumers and could be harmful (WHO, 2008). For instance, it could form insoluble crystals with other triazine derivatives (e.g. by-products of melamine synthesis) that may cause kidney stones or even renal failure (Dobson, 2008; Skinner, 2010).

In connection with suspicious increase in health problems of targeted customers, melamine was detected primarily in pet food (Dobson, 2008) or infant nutrition food (Skinner, 2010). Therefore, several analytical methods for melamine determination in various matrices have been developed. For instance, for dried or fresh dairy products (Cai, 2008; Zhao, 2013; Faraji, 2017), drinking water and wastewater (Beltrán-Martínnavarro, 2013), fish and pork kidney (Nochetto, 2013; Beltrán-Martínnavarro, 2014), or body fluids (Marco-Peiró, 2012). Various sample preparation techniques were used for extraction of melamine from sample matrices, e.g., ion-exchange
solid-phase extraction (SPE) (Cai, 2008; Zhao, 2013), cloud-point extraction (Nascimento, 2015), or disper- 
sive liquid-liquid microextraction (Mirzajani, 2016; 

Faraji, 2017). Qualitative and quantitative analyses were 

usually based on separation techniques, such as liquid 

cromatography with the fluorescence or spectropho-

tometric detection (de Lourdes Mendes Finta, 2014; 

Faraji, 2017), micellar liquid chromatography (Peris-Vi-

cente, 2016), capillary zone electrophoresis (Demirhan, 

2015), and gas or liquid chromatography with tandem 

mass spectrometry (Cai, 2008; Nochetto, 2013; Zhao, 

2013). For gas chromatographic analysis, derivatization 

by \(N,O\)-bis(trimethylsilyl)trifluoroacetamide (BSTFA) 

was used to increase volatility of melamine (Cai, 2008; 

Zhao, 2013). Another derivatization by dapsyl chloride 

was used to increase detection sensitivity of liquid 

cromatography with spectrophotometric detection (Faraji, 

2017). Besides separation techniques, melamine was 

further determined by molecularly imprinted impedi-

metric sensors (Wu, 2012), electrochemical sensors 

(Zhao, 2019), electron fluorescence (Nascimento, 2015), 

and by ion-mobility spectrometry (Mirzajani, 2016). 

Although many analytical techniques were developed, only 

a limited number of them was applied on various food 

and beverages (except milk products), even though the 

European regulation (EU No. 594/2012/ES) from 2012 

stated maximum permitted concentration to 2.5 mg/kg 

for food and beverages in general.

This paper presents the first method for melamine 

determination in beer. It reacts to the recommendation of 

the Czech Association of Breweries and Malthouses 

(CABM), from 2018, which according to European regu-

lation (EU No. 594/2012/ES) lists the same maximum per-

mitted level of melamine in beer, ciders, barley, malt, hop, 

and their products. Based on these criteria, the authors 

of this paper developed an analytical method for the de-

termination of melamine, consisting from the SPE prepa-

ration and subsequent analysis by gas chromatography 

with tandem mass spectrometry (GC-MS/MS). Finally, 

the developed method was applied on twenty beer samples.

2 Material and Methods

2.1 Chemicals and Materials

Ammonium hydroxide solution (25%), pyridine 

(≥ 99.8%), melamine (99.95%), \(N,O\)-bis(trimethylsilyl) 

trifluoroacetamide with trimethylchlorosilane (BSTFA + 

TMCS, 99:1), and SPE columns Discovery DSC-SCX (500 

mg, 3 mL) were obtained by Sigma-Aldrich. Formic acid 

(98–100%) and methanol (≥ 99.9%) were produced by 

Riedel-de Haën and Honeywell, respectively.

2.2 Sample preparation

Given beers were produced and sampled by Czech, Pol-

ish, Hungarian and Romanian breweries, and consisted 

of beer lagers (11 samples) and other beer styles (9 sam-

ples). The samples were prepared according to the 

method described above (Cai, 2008). A beer sample was 
degassed by ultrasonication for 5–10 min, and sub-

sequently acidified by formic acid to concentration 1 mL/L. 

The SPE sorbent was conditioned by 3 mL of methanol and 

then by aqueous formic acid solution (1 mL/L). 

1 mL of the beer sample was passed through the SPE 
column, washed by 3 mL of aqueous formic acid solution 

(1 mL/L), then by 3 mL of methanol, and gently dried by 
vacuum. Analyte was eluted from the sorbent by 2 mL of 
5% ammonium hydroxide in methanol. Eluent was dried 
under a gentle argon flow at 50 °C in heat-block Pierce 
Reacti-Therm (Thermo Scientific, USA). Derivatization of 
dry residuum was performed by 200 µL of pyridine and 
100 µL of BSTFA at 70 °C for 30 min.

2.3 Fortified samples and blanks

Aqueous solution of formic acid (1 mL/L) was used as 
a blank sample. Beer samples, previously degassed and 
acidified (1 mg/L), were fortified by stock solution of 
melamine (516 mg/L in methanol) to concentrations 0.5 or 2.5 mg/L for the purpose of method optimiza-

tion, validation, and latter quantification by a method of 
two-level standard addition. The preparation procedure 
for blank and fortified samples was the same as for the 
test samples.

2.4 Analysis by GC-MS/MS

Analyses were performed by gas chromatograph Agi-

lent 7890B (Agilent Technologies, USA) with column 

HP-5MS-UI (5% phenyldimethylpolysiloxan, 30 m × 

0.25 mm × 0.25 µm). The sample injection of 2 µl was 
made in a split mode (3:1) at 250 °C, with a constant 
flow of helium of 1 mL/min (6.0 purity, Air Products, 
Czech Republic). Septum purge was set to 3 mL/min 
and a gradient temperature program was 75 °C (1 min) 
-10 °C/min - 220 °C (2 min) - 10 °C/min - 260 °C (0 min) 
-25 °C/min - 300 °C (2 min). Trimethylsilyl derivative of 
melamine was detected by the triple quadrupole mass 
spectrometer Agilent 7000D (Agilent Technologies, 
USA), with interface temperature set to 250 °C. Electron 
ionization was carried out at 70 eV at 230 °C, and colli-

sion energy of nitrogen gas was set at 10 eV (6.0 purity, 
Air Products, Czech Republic). Detection in the product 
ion mode of the parent ion \(m/z\) 327 was adapted. Op-

imization, validation parameters and quantification 
results were calculated from the peak area of the quanti-

tification ion \(m/z\) 171.
2.5 Method Development

N,N,N-tris(trimethylsilyl) derivative of melamine (1.7 mmg/L) prepared by derivatization of dry residuum of melamine stock solution by BSTFA at 70 °C for 30 min, was used for the optimization of chromatographic and detection conditions. Detection in the full scan mode was firstly used for the analyte identification, then only detection in the product ion mode of the parent ion $m/z$ 327 was adapted. In order to find the appropriate volume for quantitative extraction of melamine from SPE sorbent, the fortified samples (2.5 mg/L of melamine) were individually eluted by 1, 2, 3, 5, 6, 7, 8, and 9 mL of 5% ammonium hydroxide in methanol. Absolute recovery and matrix effect were calculated according to Caban (2012).

All samples were prepared in triplicate. Finally, the limit of detection (LOD), the limit of quantification (LOQ), and extended uncertainty were calculated from a standard deviation of ten analyses of fortified beer samples with melamine concentration at 0.5 mg/L. Quantification in real beer samples was performed by the methodology of two-level standard addition, with melamine fortified beer sample at 0.5 and 2.5 mg/L.

3 Results and Discussion

3.1 Method Development

Acquired GC-MS full-scan spectrum of trimethylsilyl derivative of melamine standard was used for the confirmation of target analyte eluting at 19.1 min, in accordance with reference NIST library (CAS#: 60585-93-5; NIST#: 384197). Detection of the analyte was further conducted in the product-ion mode to prevent false positive results. The highest response for parent ion ($m/z$ 327) fragmentation was observed at collision energy 10 eV.

Previously reported work documented SPE procedure for the purpose of quantitative determination of melamine (Cai, 2008). However, the procedure was developed for milk, thus it can be assumed that different interferents were retained by SPE sorbent than those from beer, which could further affect the recovery as well as the analyte elution. First, elution volumes of 5% solution of ammonia were compared in order to elute the highest possible amount of analyte from SPE sorbent. Figure 2 shows a comparison of relative responses which depend on individual elution volumes. Relative responses were calculated from areas of the quantitative fragment $m/z$ 171, and, in this case, they reflect an approximate quantity of the analyte. The highest relative response was achieved by using 2 mL of 5% ammonia solution. As can be seen, higher volumes of elution solvent result in lower responses. It is assumed, the higher volume of elution agent is used, the more interferences that possibly suppress the analyte response are eluted. Furthermore, it was experimentally observed that longer air exposition of the elution agent was inversely proportional to its efficiency due to its high volatility. Hence, it is essential to work with freshly prepared ammonia solution and expose it minimally to the laboratory environment. Non-systematic differences among relative recoveries (Figure 2) were most likely the results of combination of these both phenomena. In addition, the volume of 1 mL of ammonia solution led to a minimum response, as the volume seems to be too low to elute enough amount of analyte from the SPE cartridge. An additional parameter of absolute recovery was also calculated (see 2.5 Method Development). First approach was absolute recovery determination by using a sample of melamine external standard. Even though the mean absolute recovery was 83.00% (RSD 0.01%) which was consistent with the model method (Cai, 2008), the estimated matrix effect (< 10.00%) had a high variability (RSD > 60.00%). For this reason, the method of a standard addition for determination of absolute recovery and further quantification was adapted. This second approach appears to be effective in compensating the matrix effect that could occur during analysis of variable samples, such as special beers. At final, the calculated mean value of absolute recovery was 102.00% (RSD = 15.55%).

Furthermore, extended uncertainty, LOQ, and LOD (Table 1 – left) of the method were calculated (see 2.5 Method Development). According to the legislative the maximum permitted value of melamine is stated in mg/kg.
units, thus, concentrations LOQ, LOD and limit concentration 2.5 mg/L were recalculated into required units mg/kg (Table 1 – right). As it can be seen from the results, all three concentration levels (LOQ, LOD and limit concentration) in the units mg/L and mg/kg were equivalent with insignificant estimation errors. Hence it can be concluded that the method is sufficient to accomplish the legislative requirements.

### 3.2 Method Application

In order to demonstrate the practical application of the method on real beer samples, 20 samples of commercial lagers and other beer styles were analyzed. The quantification was performed by the method of two-level standard addition using spiked samples of beer to 0.5 and 2.5 mg/L concentration of melamine. The example of sample responses of original and spiked beer is shown in Figure 3, and it could represent all test samples as they were all evaluated to have concentrations of melamine under the limit of quantification (< 0.4 mg/L).

![Figure 3](https://example.com/figure3.png)

**Figure 3** Comparison of peaks of quantification fragment m/z 171 of original and spiked beer sample to 2.5 mg/L

This is the first study that focuses on levels of melamine in beer. Also, the quality of beer is not generally evaluated according to protein content, thus it can be assumed that the probability of purposeful contamination of beer by melamine is very low. On the other hand, it was observed that plastic kitchenware containing melamine can contaminate food after microwave heating (de Lourdes Mendes, 2014), which could lead to a question if contamination from brewing ware or packing material is possible.

### 4 Conclusion

The method for determination of melamine in beer and beer-type samples using GC-MS/MS was developed. The average absolute recovery of the preparation procedure was 102.00% (RSD = 15.55%). Limits of detection and quantification were 0.1 mg/L, and 0.4 mg/L, respectively. The extended uncertainty of the melamine determination is 0.1 mg/L. The quantification method of two-level standard addition was chosen to compensate possible deviations in matrix effect. All twenty test samples had concentration of melamine under the limit of quantification (< 0.4 mg/L).

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