Validation and measurement uncertainty evaluation of the GC-MS method for the direct determination of hexamethylenetetramine from foods

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Abstract A simple GC-MS method has been developed and validated for the direct determination of hexamethylenetetramine (HMT). The separation of HMT was performed using a MXT-1 column. The calibration curve was linear over the concentration range 0.1–25 µg/mL, with a good correlation coefficient ($r=0.9996$). The recoveries of HMT from foods spiked at 1, 5, and 10 µg/g ranged from 91.7% to 115.2%. Intra-day ($n=5$) and inter-day ($n=5$) precision were less than 7%. The limit of detection and the limit of quantification of the method were 0.05 and 0.15 µg/mL, respectively. The uncertainties associated with food matrix and calibration contributed most to the overall expanded uncertainty. The method validation data indicated that quantitative method could be applied to the direct determination of non-hydrolyzed HMT in foods.

Keywords: hexamethylenetetramine, gas chromatography-mass spectrometry, direct determination, method validation

Introduction

Hexamethylenetetramine (HMT), as a preservative, reduces the formation of gas by *Clostridium* genus which causes the cracking of provolone cheese (1). It is manufactured by the reaction of ammonia and formaldehyde in the liquid phase. HMT (E239) can be used as a food additive in provolone cheese at the maximum level of 25 mg/kg, set as formaldehyde, as per European Commission Directive No 95/2/EC (2). In CODEX, HMT is also authorized for use only in provolone cheese at the maximum level of 25 mg/kg (as formaldehyde) (3). However, HMT is used as an unauthorized preservative in the Republic of Korea, USA, and Japan (4–6). Its acceptable daily intake (ADI) has been established as 0.15 mg/kg body weight per day by the Joint FAO/WHO Expert Committee on Food Additives (7).

HMT has been used as a preservative in the food industry and as a pharmaceutical for treating intestinal infections. Its success in those industries is mainly because of its hydrolysis which produces formaldehyde and ammonia under acidic conditions. Formaldehyde is naturally present in various foods, with the concentration ranging from 1 to 20 ppm (8). In some foods, it is impossible to discriminate between naturally occurring formaldehyde and that derived from artificially added HMT. Consequently, there is a need to develop a

highly sensitive method for the direct determination of non-hydrolyzed HMT present in foods.

Chromatographic techniques, including HPLC (9,10) and GC (11,12), have been reported for the direct analysis of non-hydrolyzed HMT. Koga *et al.* (11) reported a direct determination of HMT in river and sewage water by using a gas chromatograph equipped with a flame thermionic detector using an iodine and charge-transfer complex. However, because of insufficient recovery and narrow applicability, this method cannot be applied to the direct determination of HMT in complex food matrices. In addition, Cottin *et al.* (12) reported that the direct determination of HMT in photolyzed and irradiated interstellar cometary ice analogues is possible (12). GC was selected as the technique for this study because of its excellent sensitivity.

The aim of this study was the development of a highly sensitive method for direct determination of non-hydrolyzed HMT in foods. HMT was determined in food samples by GC-MS using a non-polar MXT capillary column. The validation parameters tested included recovery, precision, linearity, limit of detection (LOD), limit of quantification (LOQ), and measurement uncertainty. The proposed method was successfully applied to the direct determination of HMT in spiked samples.

Materials and Methods

Materials Ethanol, methanol, and chloroform were purchased from Merck (Darmstadt, Germany). Ultrapure water was prepared using a Millipore Mili-Q purification system (Bedford, MA, USA). All other materials were of analytical grade.

Food samples were purchased from internet web sites and local markets after checking the origin marks. A total of 40 samples available in domestic markets were categorized into three food classes, namely, 17 glass noodles, 21 cheeses, and 2 tofu snacks. Glass noodles were imported from China; cheeses were the products imported from Europe; tofu snacks were produced in Korea. All samples were stored at 4°C except for glass noodles, which were stored at room temperature.

Preparation of standard solutions An accurately weighed amount (100 mg) of HMT was transferred into a 100-mL volumetric flask, dissolved in 90 mL methanol, and made up to volume with methanol to prepare a stock solution of 1 mg/mL. This stock solution was further diluted with methanol to obtain working standard solutions of 0.1 to 25 mg/mL for linear plots.

Sample treatment Methanol was used for extraction of HMT from foods. Ground samples (5 g) were transferred into a 50-mL volumetric flask, and methanol was added to give a final volume of 50 mL. The contents were mixed with a homogenizer for 10 min at 20,000 rpm. HMT from the mixture was extracted in an ultrasonic bath for 10 min followed by centrifugation at 4,000x *g* for 10 min. The supernatant of 10 mL was transferred into a 50-mL conical tube. The sample solution was filtered through a 0.45-μm membrane syringe filter (Whatman, Maidstone, Kent, UK). The filtrate was directly injected into the GC-MS.

Chromatographic conditions A Shimadzu GC-2010 gas chromatograph, coupled with a GCMS-QP2010Plus mass selective detector and Shimadzu AOC-20i autosampler, was employed for all analyses. Samples were separated on a Restek MXT-1 (30 m x 0.18 mm, 0.6 μm) capillary column (Bellefonte, PA, USA). DB-1 (15 m x 0.25 mm, 0.25 μm) or HP-1 (30 m x 0.25 mm, 0.25 μm) purchased from Agilent technology (Santa Clara, CA, USA) were tested as test columns in this study. The column temperature was initially held at 50°C for 10 min; then, the temperature was raised to 250°C at a rate of 10°C/min and held for 5 min. Ultra-high purity helium was used as the carrier gas at a flow rate of 1 mL/min. The injector temperature was maintained at 300°C, and the injection volume was 1 μL in split mode (1/10). The interface temperature was held at 200°C. Selected ion monitoring (SIM) of ions with *m/z* =42 and 140 was used for the GC-MS analysis. Electron impact ionization energy was 70 eV.

Method validation The validation for HMT analysis in foods was conducted according to the AOAC (13) and the International

Conference on Harmonization (ICH) guidelines (14). Linearity, accuracy and precision were determined according to the AOAC methods, and LOD and LOQ were estimated according to the ICH procedure.

Linearity was established by injecting a series of six standard solutions of HMT with concentrations ranging from 0.05 to 100 μg/mL. Linearity was expressed as the coefficient of correlation (*r*) of all data at the all concentrations. The accuracy was determined by recovery tests using the addition of known amounts of HMT to three food products: glass noodles, provolone cheese, and a tofu snack. All samples were spiked with HMT at 1, 5 and 10 μg/g sample. All recovery samples were prepared at the same time. The precision, expressed as percent relative standard deviation (RSD%), was also determined for HMT by using the same samples. LOD and LOQ were determined according to the method described by the ICH (14) using the formula: LOD= 3.3 σ/*S* and LOQ=10 σ/*S*, where σ is the mean standard deviation and *S* is the slope of the same equation.

Measurement uncertainty of HMT in foods was estimated according to the International Organization for Standardization (ISO) (15) and Eurachem/Citac (16) guidelines based on intra-laboratory validation. The five sources of uncertainty considered for the determination of measurement uncertainty were those arising from balances, volumetric measuring devices, calibration curves, sample preparation, and repeatability. The measurement uncertainty (*U*), which is the expanded uncertainty, was calculated by multiplying the combined standard uncertainty (*u_c*) by a coverage factor, *k*=2, which gives a confidence level of approximately 95%, as follows:

$$U = k u_c$$

HMT stability test The stabilities of HMT in food matrices were investigated. To study storage stability, the three samples (glass noodle, provolone cheese and tofu snack, which were spiked at the level of 20 mg/kg, were stored in 4°C refrigerator for 24 h. The stabilities of HMT were evaluated by measuring the HMT recovered from the samples at 0, 1, 3, 6, 9, 12, and 24 h as described above. The tests were repeated in triplicate at each time point.

Results and Discussion

Optimization of the chromatographic conditions The GC-MS method used provided satisfactory separation of HMT within 20 min. During the optimization of the chromatographic conditions, three capillary columns were evaluated and methanol was used as an extraction solvent of HMT. Using either DB-1 (15 m x 0.25 mm, 0.25 μm) or HP-1 (30 m x 0.25 mm, 0.25 μm) columns with helium as the carrier gas, serious peak asymmetry and tailing were observed. The best chromatogram was obtained using an MXT-1 column (30 m x 0.18 mm, 0.6 μm) and helium as carrier gas at a flow rate of 1 mL/min, as shown in Fig. 1. In the column testing, the gradient program was set up via the control of initial column temperature and raising rate of

Fig. 1. GC-MS total ion chromatogram using three capillary columns with standard solutions containing 5 $\mu\text{g/mL}$ hexamethylenetetramine. (A) DB-1 (15 m $\times$ 0.25 mm, 0.25 μm); (B) HP-1 (30 m $\times$ 0.25 mm, 0.25 μm); (C) MXT-1 (30 m $\times$ 0.18 mm, 0.6 μm). The title of the X-axis is height and that of the Y-axis is retention time.

temperature. A typical standard solution showed a retention time of about 16.6 min. The separation of the HMT peak is necessary to avoid interferences by components existing in foods. Figure 2 shows the chromatograms obtained when the developed method was applied to provolone cheese, glass noodles, and a tofu snack, each spiked with 5 $\mu\text{g/g}$ HMT. The total ion chromatogram indicated the presence of a major peak in the three samples. The HMT peak was identified by using both the retention time and mass spectral data. The ratio of the characteristic ions (m/z 42 and 140) also confirmed the purity of the HMT peak. The more abundant ion in the mass spectrum is that at m/z 42. Typically, the acquired spectra can be contaminated with extraneous mass spectral peaks, such as commonly

arisen from co-eluting compounds, column bleed, and ion-chamber contaminants (17). The m/z 42 ion is not associated with common contaminants such as air (air leak), methanol (extraction solvent), or polysiloxanes (column and septum bleed) (18) and hence, it was chosen as the quantifier ion for HMT detection.

Selection of extraction solvents To select the optimum solvent for HMT recovery, extractions were performed using methanol, ethanol, acetone, and chloroform. Methanol was better than other solvents in extracting HMT from three samples as shown in Fig. 3. Thus, methanol was used as the extraction solvent for method validation and the analysis of commercial foodstuffs. Our laboratory previously

Fig. 2. Chromatograms of three samples spiked at 5 $\mu\text{g/g}$ of hexamethylenetetramine (HMT), respectively (A) glass noodle; (B) provolone cheese; (C) tofu snack; (D) MS spectrum of HMT. The title of the X-axis is height and that of the Y-axis is mass-to-charge ratio (m/z).

reported an HPLC method to quantify HMT in the same three foodstuffs (9) using a sample extraction with methanol. The procedure and conditions for processing from food samples to prepare test solutions in the GC-MS method were the same as the HPLC method. Other researches have also reported the use of methanol as the extraction solvent in the determination of HMT in explosives and cometary ice analogues (12). Where water was used as an extraction solvent, HMT was detected as formaldehyde, the product which was hydrolyzed from HMT in urine as reported by Strom and Jun (19).

Linearity Under the above optimum conditions, a linear relationship with high correlation coefficient ($r=0.9996$, $n=3$) was found between the peak area ratio of HMT and the HMT concentration in the range from 0.1 to 25 $\mu\text{g/mL}$. The mean regression equation obtained from

the six-points calibration curve was: $Y=42967X-8924.1$. This result indicates that the calibration curve could be effectively applied to the quantification of HMT in foods.

Limits of detection and quantification The LOD and LOQ were calculated according to the AOAC guidelines for method validation (13). The calculations were based on the results of five replicate injections and gave a LOD and LOQ of 0.05 and 0.15 $\mu\text{g/g}$, respectively. The LOD and LOQ were about 10 times and 6.7 times lower than the values (0.5 and 1.0 $\mu\text{g/g}$, respectively) observed in the HPLC method for the direct determination of HMT in our laboratory (9).

Accuracy and precision The accuracy of the method was determined by recovery tests conducted in triplicate for different spiking

Fig. 3. Relative peak area of hexamethylenetetramine after extraction using four solvents

concentrations. Glass noodles, provolone cheese, and a tofu snack spiked with different concentrations (1, 5, and 10 $\mu\text{g/g}$) analyzed by the method. The mean percentage recoveries exceeded 90% for all levels, ranging from 91.7 to 115.2% (Table 1). This indicated that the method had good accuracy, within experimental limits. Chromatograms of a standard solution and the spiked samples are shown in Fig. 2, showing that quantification of HMT is possible.

Precision was expressed as percent relative standard deviation (RSD%). Intra-day precision was studied at three HMT concentration levels. Three aliquots were prepared at each level, and injected into the GC-MS system. The RSD% calculated was less than 5.7% for glass noodles, 4.6% for provolone cheese, and 3.2% for tofu crackers, respectively. Inter-day precision was conducted on five different days with the same concentration levels of spiked samples. The RSD% of the determinations was less than 5.8% for glass noodles, 6.6% for provolone cheese, and 6.4% for tofu crackers, respectively.

Measurement uncertainty The number following the symbol $\pm$ is the numerical value of an expanded uncertainty. The expanded uncertainty for the three food matrices studied ranged from 11.7 to 14.5%, compared with each measured value (the number preceding the symbol $\pm$). The uncertainty was based on a level of confidence of approximately 95% using a coverage factor of 2.

The relative uncertainty variance contributions are used to illustrate the relative impact of different uncertainty components. The importance of a source of uncertainty is determined by its quantitative effect on the measurement result. Among the five sources of uncertainty, the uncertainties associated with three food matrices and the calibration curve appears to be the main contributors to the overall uncertainty (Fig. 4). Therefore, analysts should pay most attention to factors affecting recovery tests and calibration.

The expanded uncertainty was satisfactory, based on the criteria stated in the ISO and Eurachem/Citac guidelines (15,16).

Stability of HMT in three matrices The stabilities of HMT in three matrices were examined as shown in Fig. 5, the concentrations of HMT in glass noodles, provolone cheese, and tofu snack slowly decreased to approximately 16.3, 16.3, and 9.8 mg/kg, respectively (data not shown). The decomposition of HMT is carried out in the presence of activated carbon, as well as by acidic conditions and high temperatures (20). Iwakami and Takazono (20) reported that the carbon-rich residue present after HMT is decomposed and acted as a catalyst for the decomposition process. Our results seem to agree with those of these researchers.

Determination of HMT in commercial foodstuffs The procedure was applied to the determination of HMT in 40 food samples (glass noodles, cheeses, and tofu products) imported into Korea. No samples were labeled as containing HMT because it is an unauthorized additive. In all samples, the concentrations of HMT were lower than the LOQ (0.15 $\mu\text{g/g}$).

The aim of this study was to develop a highly sensitive method for

Table 1. Recoveries of hexamethylenetetramine (HMT) from glass noodle, provolone cheese, and tofu snack sample spiked at three different concentrations ($n=3$)

Sample	Spiked level ($\mu\text{g/g}$)	Found HMT ($\mu\text{g/g}$)	Recovery (%)	Precision (RSD%)		Expanded uncertainty ¹⁾ ($\mu\text{g/g}$)
				Intra-day ($n=5$)	Inter-day ($n=5$)	
Glass noodle	1	0.9 $\pm$ 0.1	94.0 $\pm$ 5.4	5.7	5.6	9.6 $\pm$ 1.39
	5	4.6 $\pm$ 0.2	91.7 $\pm$ 3.7	4.0	5.3	
	10	9.6 $\pm$ 0.4	95.8 $\pm$ 3.7	3.9	5.8	
Provolone cheese	1	0.9 $\pm$ 0.01	92.8 $\pm$ 1.1	1.2	6.6	11.5 $\pm$ 1.66
	5	5.4 $\pm$ 0.2	107.8 $\pm$ 4.9	4.6	5.2	
	10	11.5 $\pm$ 0.4	115.2 $\pm$ 3.7	3.2	4.4	
Tofu snack	1	0.9 $\pm$ 0.02	92.8 $\pm$ 1.7	1.9	4.0	9.4 $\pm$ 1.10
	5	5.0 $\pm$ 0.03	99.3 $\pm$ 0.7	0.7	6.4	
	10	9.4 $\pm$ 0.3	93.6 $\pm$ 3.0	3.2	5.5	

¹⁾Basis of approximately 95% confidence

Fig. 4. Uncertainty contributions in hexamethylenetetramine analysis of three samples. (A) glass noodle; (B) provolone cheese; (C) tofu snack

the determination of HMT in foods. The newly developed method is easy to perform and yields satisfactory validation results. Moreover, the method is more sensitive than the LC method previously reported. The method was also successfully used for the direct determination of HMT in the non-hydrolyzed form in foods. Finally, it is useful for the analysis of HMT in non-acidic conditions and allows studies of the stability of HMT in foods and pharmaceutical formulations.

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Disclosure The authors declare no conflict of interest.

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Fig. 5. Stability of hexamethylenetetramine (HMT) in three samples spiked with the standard solution of 20 mg/kg during storage at 4°C (A) glass noodle; (B) provolone cheese; (C) tofu snack

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