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Short communication

Accurate analysis of urea in milk and milk powder by isotope dilution gas chromatography–mass spectrometry

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ABSTRACT

A high order method for measuring urea concentrations in milk and milk powder was developed. The method can be applied to certify the concentration of urea in some new milk and milk powder CRMs. This high accurate method for analysis of milk is valuable given the inherent challenges associated with the complexity of the sample matrix. A measurement procedure based on gas chromatography/isotope dilution mass spectrometry (GC/IDMS) was developed. Samples were pre-treated with acetonitrile to remove proteins and the method was applied to determine urea concentrations in milk and milk powder. Excellent precision was obtained, with within- and between-set coefficients of variation of 0.15–0.46 and 0.18–0.65%, respectively. The measurement uncertainty is evaluated. The method can trace to mass.

1. Introduction

Urea is an important product of the catabolism of purine nucleosides present in organic fluids and it occurs naturally in milk and milk powder [1]. The concentration of urea in milk can reflect the quality of products. Urea is one of the important parameters in dairy product analysis. There are reports that multinational companies, who are so stringent in maintaining quality of products in developed countries, are accused of adulteration of urea not only in milk or milk powder but also in infant feeds. Since urea is a natural constituent of milk, its adulteration is easy. In that respect, an accuracy analysis of urea has societal impact. According to Ontario Dairy Herd Improvement, the normal range for milk urea nitrogen concentration is usually between 6 and 25 mg dL^{-1} [2]. Many methods for analysis of urea in milk have been reported, such as the diacetyl monoxime assay [3,4], the Chemspec 150 instrumental method [5], some enzymatic reactions [6], infrared spectrometric techniques [7], and differential pH assays [8]. Methods can be classified as direct, in which complexation between urea and a suitable reagent is determined colorimetrically, or indirect, in which degradation of urea is determined enzymatically. These methods have a constant bias, in contrast to mass spectrometric methods. Therefore, evaluation of the traceability and in particular the trueness of these results remains a problem. To demonstrate traceability of the results of these methods, control samples for internal and external quality control must be available with urea concentration values assigned by a method of higher metrological order. The only analytical principle that seems to be suitable for establishing such a reference method is isotope dilution mass spectrometry (IDMS). The combination of GC/MS and ID is a reference method that is internationally accepted to yield high specificity and trueness [9]. Moreover, IDMS has been defined by the Comité Consultatif pour la Quantité de Matière as a primary method of measurement [10]. To the best of our knowledge, no GC/MS reference method has been published for milk and milk powder urea with good precision and accuracy. In the present study, a high metrological order method was developed for analysis of urea in milk and milk powder using ID coupled to GC/MS. The experiment results demonstrate that the GC/IDMS method is accurate and precise.

2. Materials and methods

2.1. Materials and instruments

Urea CRMGBW09201 (purity 99.9 ± 0.1%) is certified by National Research Center for Certified Reference Materials (NRCCRM). [^{13}C,$^{15}N_2$]urea (98 at.% ^{13}C, 99 at.% ^{15}N) was obtained from Cambridge Isotope Laboratories. Malondialdehyde bis(dimethylacetal) (MDBMA) and *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) were purchased from Sigma. Hydrochloric acid (250 g L^{-1}), acetonitrile and methanol (HPLC grade) were purchased from Merck. Milk and milk powder were purchased from supermarket and stored at 4 °C before use. Water was prepared using a Milli-Q system (18.2 MΩ cm). Filters with a pore size of 0.22 μm were purchased from Sugelabor S.A. (Madrid, Spain). A Finnigan Polaris

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Q GC–MS (ion trap) system equipped with EI was used for GC/MS analysis.

2.2. Standard solutions

Two independently weighed stock standard solutions containing natural urea at 2 mg g^{-1} in methanol were prepared at 20 °C. These standard solutions are made gravimetrically. The weight was multiplied by a factor of 0.999 according to the purity of the urea reference material. These solutions were stored in a refrigerator at 4 °C in a well-stoppered brown all-glass container. A labeled urea stock standard solution containing [^{13}C,$^{15}N_2$]urea at 2 mg g^{-1} was prepared according to the same procedure.

2.3. Sample preparation

2.3.1. Preparation of milk powder and milk samples

Milk powder samples stored at 4 °C were equilibrated to room temperature prior to analysis. Then 1.0 g of sample was reconstituted by adding 5.0 g of deionized water. Different milk powder samples were reconstituted on separate days. The mass of added water was determined by weight rather than by volume. The milk powder was gently mixed at regular intervals for 30 min until fully reconstituted. Approximately 1.0 g of each milk powder solution was spiked with a known amount of isotopically labeled urea (to give an ~1:1 ratio of analyte to internal standard for each sample). Samples were mixed immediately after addition of the labeled urea. The spiked samples were then allowed to equilibrate for 2 h at room temperature prior to extraction. The concentration of the milk powder samples should be multiplied by the dilution factor. Milk samples were prepared in three different sets on different days. Each set consisted of three vials with three different concentrations for milk samples 1, 2 and 3. The samples were equilibrated at room temperature for 10 min prior to analysis. The same procedure was used for addition of internal standard and equilibration as for the milk powder solutions.

2.3.2. Preparation of calibrators

At the same time that milk and milk powder samples were spiked with labeled internal standard solution, a working standard solution of known urea concentration was prepared and then spiked with the same labeled urea solution used to spike the samples at a natural/labeled urea ratio approaching 1:1. The concentrations of the calibrators were almost the same as that of the natural urea in each set of milk samples. These calibrators were prepared daily and stored in well-stoppered brown all-glass containers at 4 °C prior to analysis.

2.3.3. Sample extraction

Acetonitrile was used to precipitate milk proteins prior to analysis by GC/MS. The volume ratio is 5:1 (acetonitrile/spiked sample) for the precipitation of protein. Once acetonitrile was added and proteins were precipitated by intensive shaking for 8 min. The solution was allowed to stand for 5 min before centrifugation at 8000 rpm for 10 min at 10 °C. A 200-μL aliquot of the supernatant was transferred for subsequent derivatization.

2.3.4. Derivatization

MDBMA solution (0.3 mol L^{-1}, 30 μL) and hydrochloric acid (250 g L^{-1}, 60 μL) were added to each extract sample or calibrator in acetonitrile. The reaction leading to 2-hydroxypyrimidine was complete at room temperature after 1 h. The samples and calibrators were evaporated to dryness under a stream of N_2 at room temperature. The residues were reacted with 30 μL of MSTFA at 60 °C for 1 h. The reaction is shown in Fig. 1. A 1-μL aliquot of the reaction mixture was injected into the GC/MS system. For continuous control of the system stability, calibrators (Fig. 2a) and milk samples (Fig. 2b) were analyzed alternately.

2.4. Analytical recovery

Vials of milk samples were combined and 11 5-mL samples were taken to determine the accuracy of the method. A certain amount of urea was added to nine of the 11 samples at three concentrations (~350, 514 and 1027 mg kg^{-1}). No urea was added to the other two samples. Different amounts of [^{13}C,$^{15}N_2$]urea were added to each sample (natural/labeled ratio of ~1:1 in each sample) and the samples were then processed for extraction and analysis as described above. The same procedure was used to spike milk powder samples at three urea concentrations.

2.5. Equilibration

To test for complete equilibration of labeled urea and natural urea in milk, vials of milk samples were combined and five 5.0-mL samples were taken. A given amount of [^{13}C, $^{15}N_2$] urea was added to each sample after mixing and the samples were equilibrated at room temperature for various times (0.5, 1, 2, 3, and 4 h). The samples were processed as described above.

2.6. Standard cross-checking

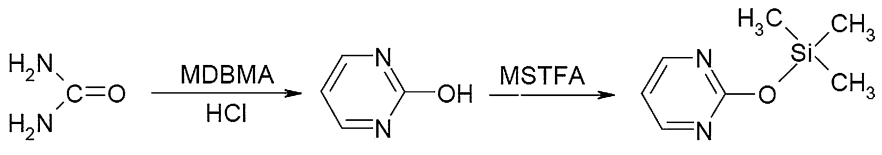
We prepared two independent sets of standards and assayed one by single-point calibration against the other. The weight ratio determined by IDMS measurement was then compared with the weighed weight ratio for that standard.

2.7. Linearity

Two groups of working standard solutions of natural and labeled urea were prepared by diluting the corresponding stock solution with methanol. A set of spiked standard solutions containing designated concentrations of natural urea and the same concentration of labeled urea was then prepared using the two groups.

2.8. Chromatographic separation

A fused-silica capillary column coated with OV-5 (30 m × 0.32 mm i.d., film thickness 0.25 μm; Agilent) was used for chromatographic separation. The carrier gas was helium at a flow rate of 1.0 mL min^{-1} and the split ratio was set to 1:50. The injector temperature was 280 °C. Before sample injection, the column oven



(I) (II) (III)

Fig. 1. Reaction scheme for the derivatization process of urea with MDBMA and MSTFA.

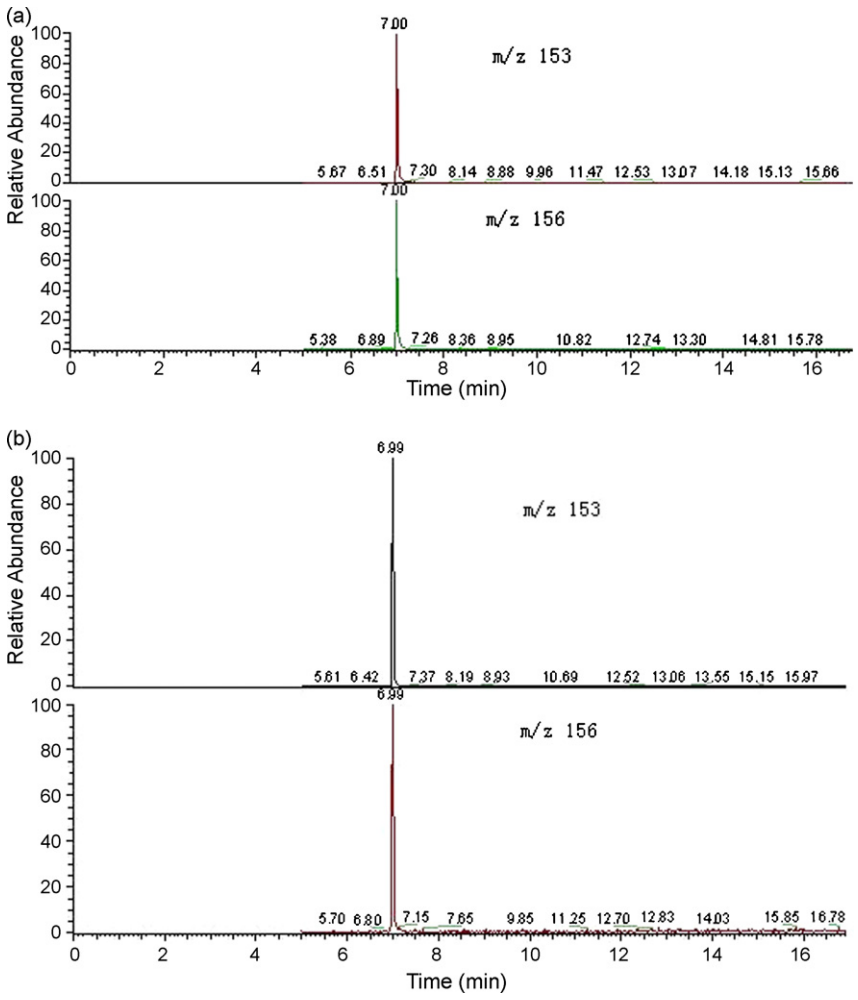


Fig. 2. Selected-ion GC/MS chromatograms for urea and [^{13}C,$^{15}N_2$]urea derivatives in a standard solution and a milk sample with SIM mode.

was heated at 70 °C for 1 min. Then the temperature was increased to 150 °C at 10 °C min^{-1} and to 240 °C at 30 °C min^{-1} and held for 4 min. For MS analysis, the ionization energy was 70 eV and the temperature of the ion source was 250 °C. To calibrate the mass scale of the instrument, the exact positions of the peak maxima for the ions at *m*/*z* 153 and 156 (M^+-15) and *m*/*z* 168 and 171 (M^+) were determined in a separate chromatographic run of a calibrator mixture of labeled and natural urea derivatives before a series of quantitative analyses.

2.9. Calibration methods

To determine the concentration of a given sample, samples of 1.5 mL of milk or milk powder solution were divided into three independent samples and then spiked and extracted. Three repeat injections were performed for each sample extract. The results for IDMS determination of urea were calculated from peak area ratios measured by the ion monitoring technique. Single-point calibration was used to calculate the urea concentration in milk and milk powder samples. Each sample injection was bracketed by injections of a spiked standard of almost equal concentration and isotope ratio to those of the sample. Four calibration injections were used to calculate a sample result.

2.10. The equation for calculation concentration of urea in milk and milk powder

The concentrations of urea in milk and milk powder are calculated using Eq. (1).

$$M = \frac{A_1/A_1'}{A_2/A_2'} \times \frac{M_1'}{M_2'} \times \frac{M_2}{M_S} P_s \quad (1)$$

where

M—the concentration of urea in real sample (μg g^{-1});
A_1—peak area of urea measured by instrument in real sample;
A_1'—peak area of labeled urea measured by instrument in real sample;
A_2—peak area of urea measured by instrument in standard solution;
A_2'—peak area of labeled urea measured by instrument in standard solution;
M_1'—the mass of label urea added into the real sample (g);
M_2'—the mass of label urea added into the standard solution (g);
M_2—the mass of urea in the standard solution (μg);
M_S—the mass of the real sample (g);
P_s—the purity of the urea standard reference material.

3. Results and discussion

3.1. The development of the GC/MS method

3.1.1. Choice of pure material of urea

The use of a certified reference material of high and well-known purity is a necessary prerequisite for establishing a higher order method. The urea reference material from NRCCRM (GBW09201), which was used during this investigation, fulfills this requirement; the certified purity of urea is 99.9 ± 0.1%. Urea of GBW09201 is a higher order certified reference material. This certified reference material of GBW09201 was not only admitted by China as a primary reference material but also approved by JCTLM as a higher order reference material. The purity of urea of GBW09201 was certified using the mass balance approach (summation of impurities) and validated by differential scanning calorimetry method. It can be traced to the mass.

3.1.2. Choice of internal standard

Because an unpredictable portion of the analyte can be lost in the pre-instrumental part of analytical procedure or even during GC, it is necessary to control these losses by using an internal standard. An isotopically labeled urea is the ideal internal standard (IS) because it can be expected to exhibit the same physico-chemical properties as the non-labeled substance during extraction, derivative formation, and GC. So [^{13}C, $^{15}N_2$]-Urea is used as the IS.

3.1.3. Equilibration effects

After the addition of labeled material to the sample in an isotope dilution procedure, equilibration between labeled and unlabeled forms must be attained before the analyte is isolated from the matrix. Incomplete equilibration can lead to different recoveries of the unlabeled and labeled forms, resulting in loss of accuracy. We investigated the time required to reach complete equilibration. Under our conditions, the ion abundance ratios measured for all of these samples were the same after 1 h within experimental error. And the ratio of labeled to natural urea did not change for at least 6 h at room temperature. An equilibration time of 2 h was thus chosen.

3.1.4. Standard cross-checking

The accuracy of results for milk samples depends on the accuracy of the standard mixtures for calibration. We prepared two independent sets of standards and used single-point calibration to measure the concentration of one against the other. The weight ratio determined by IDMS was compared with the weighed weight ratio of that standard. The bias was 0.18 and −0.27% for the two stock standard solutions.

3.1.5. Choice of ions for ID/MS measurements

For quantification, the GC peak area ratios measured at *m*/*z* 153 for the non-labeled derivative *O*-trimethylsilyl-2-hydroxypyrimidine and *m*/*z* 156 for the analogous [^{13}C,$^{15}N_2$]-labeled derivative in calibrators (Fig. 2a) and samples (Fig. 2b) were used. Another independent calculation of the results was based on the ratios of the peak areas for *m*/*z* 168 and 171. The quantification results had bias of 0.07% for the same milk sample when using these two different ion pairs. The ion pair *m*/*z* 153 and 156 was used for the measurement of all samples because of its high relative abundance, and the absence of any detectable interference under our experimental conditions.

3.1.6. Optimization of sample extraction

Ultrafiltration and precipitation using an organic solvent are the best methods for protein precipitation [11,12]. When we used ultrafiltration to remove protein, the repeatability and reproducibility were poor. Moreover, the ultrafiltration tubes are expensive. Thus, we used acetonitrile to precipitate milk proteins prior to derivatization.

Table 1
Recovery of urea added to milk and milk powder.

Added (mg kg^{-1})	Expected (mg kg^{-1})	Measured (mg kg^{-1})	RSD (%)	Recovery (%)
Milk samples				
0		342.52	0.52	
6.84	349.36	345.59	0.46	98.92
171.26	513.78	510.95	0.43	99.45
685.04	1027.56	1039.48	0.58	101.16
Milk powder samples				
0		1830.27	0.35	
18.30	1848.57	1815.48	0.48	98.21
183.03	2013.30	2001.62	0.69	99.42
915.18	2745.45	2741.33	0.52	99.85

The milk powder samples were dissolved in water with weight ratio of 1:1, 2:1, 4:1, 5:1, 6:1, 8:1 (water/milk powder) and precipitated protein by acetonitrile. On the other hand, the milk powder samples were precipitated proteins with acetonitrile directly without water-addition. It seems that the direct protein precipitation with acetonitrile for uneven system cannot get good extract for derivatization. When the weight ratio is 5:1 (water/milk powder), the effect of protein precipitation is the best. We explored the effect of acetonitrile precipitation of protein when the volume ratio of acetonitrile and milk powder solution are 3:1, 5:1, 6:1, 8:1. It is obvious that the solution is cloudy after precipitation and need several hours to clarify, indicating the precipitation of the protein is incomplete. When the volume ratio is 5:1, the upper fluid clarified quickly after precipitation, which means complete protein precipitation. With the increasing of acetonitrile volume ratio, there is no significant change in sedimentation effect and the peak areas for *m*/*z* 153. In order to reduce the amount of acetonitrile, saving the cost of experiments and protecting the environment, the final choice of the volume ratio is 5:1 for the precipitation of protein.

3.1.7. Optimization of derivatization condition

The reaction temperature and time are mainly considered. Fixed other factors remaining unchanged, we carried out the optimized experiment for the two factors. First, set the reaction time as 1 h, investigated the influence for derivatization at room temperature, 45, 60 and 75 °C, respectively. Followed setting the reaction temperature as 60 °C, investigated the influence for derivatization with 15 min, 30 min, 45 min, 60 min, 2 h reaction time. From the experiment results, when the reaction temperature is higher than 60 °C, reaction time more than 45 min, the peak area for *m*/*z* 153 is almost constant. Therefore, the derivatization condition was carried out at 60 °C for 1 h.

Table 2
GC/MS results for urea in milk and milk powder (n = 9).

Sample no.	Average (mg kg^{-1})	RSD[a] (%)	Overall RSD[b] (%)
Milk 1#	342.52	0.46	0.52
Milk 2#	120.85	0.42	0.65
Milk 3#	285.67	0.32	0.49
Milk powder 4#	1830.27	0.29	0.35
Milk powder 5#	2127.64	0.15	0.18
Milk powder 6#	1279.57	0.24	0.32

[a] Relative standard deviation (RSD) of a single measurement within a set.
[b] RSD of the mean for that level.

Table 3
Expanded uncertainties for milk and milk powder urea by GC/MS measurements.

Uncertainty table	Milk 1#	Milk 2#	Milk 3#	Milk powder 4#	Milk powder 5#	Milk powder 6#
Method precision	0.52%	0.65%	0.49%	0.35%	0.18%	0.32%
P_s	0.05%	0.05%	0.05%	0.05%	0.05%	0.05%
Weighings	0.03%	0.03%	0.03%	0.03%	0.03%	0.03%
Combined uncertainty	0.53%	0.66%	0.50%	0.36%	0.19%	0.33%
K factor	2	2	2	2	2	2
Relative expanded uncertainty[a]	1.06%	1.32%	1.00%	0.72%	0.38%	0.66%

[a]Uncertainty of 95% confidence interval.

3.2. The validation of this GC/MS method

3.2.1. Linearity and limit of detection

A regression line for peak area versus mass concentration was generated for each set of calibrators. Excellent linearity was obtained, with correlation coefficients ranging from 0.9987 to 0.9998. A linear response was obtained for urea concentrations from 10 to 1600 mg kg^{-1}. The limit of detection (LOD) evaluated at a signal-to-noise ratio of 3 was 0.11 ng. The limit of quantification (calculated at a signal-to-noise ratio of 10, LOQ) was 0.41 ng. These low LOD and LOQ permit sample dilution to avoid matrix effects.

3.2.2. Recovery of spiked urea

The method recovery was evaluated by assaying milk and milk powder samples spiked with a standard urea solution. The value measured for the non-spiked sample was subtracted from that of the spiked samples. Table 1 lists the percentage recovery of urea from milk and milk powder samples spiked with three different concentrations of standards. The recovery was 98.92–101.16% for milk and 98.21–99.85% for milk powder.

3.2.3. Application of the method: measurement of milk and milk powder materials

The method was used to determine the difference concentration of urea in milk and milk powder samples. Samples were prepared and analyzed as described in Section 2 and the results are given in Table 2. Excellent reproducibility was obtained for all six concentrations: within-set CV ranged from 0.15 to 0.46%, and between-set CV ranged from 0.18 to 0.65%. The data in Tables 1 and 2 demonstrate the good repeatability and reproducibility of the GC/IDMS method.

3.2.4. Uncertainty analysis

The concentration of urea in milk and milk powder measured by GC–IDMS can be calculated by formula (1). The uncertainty of urea includes: the uncertainty of measurement caused by repeatability (A_1, A'_1, A_2, A'_2), the uncertainty of weight (M'_1, M'_2, M_2, M_S) and the uncertainty of purity standard reference material (P_s).

The uncertainty of balance was evaluated as rectangle distribution. The repeatability of Sartorius ME-235S is 0.015 mg when all amounts were adjusted to give masses ≥200 mg, so the weighing uncertainties were kept 0.6×0.015 mg = 0.009 mg. And the relative uncertainty was 0.009/200 = 0.0045%. The repeatability of Mettler Toledo UMX2 is 0.00025 mg when all amounts were adjusted to give masses ≥20 mg, so the weighing uncertainties were kept 0.6×0.00025 mg = 0.00015 mg. And the relative uncertainty was 0.00015/20 = 0.00075%. The uncertainty of urea purity certified reference material was 0.1% and its expanded factor $k = 2$. The relative uncertainty u_1 induced by purity certified reference material can be described as follow,

$$u_1 = \frac{0.10\%}{2 \times 99.9\%} = 0.05\% \quad (2)$$

In summary, the uncertainty associated with the final measured concentration was calculated by combining the relative standard uncertainty for the precision of the method as a whole with the uncertainties associated with weighing and the uncertainty of purity standard reference material (P_s). The total uncertainty of the described method was estimated within a typical 95% confidence interval, as shown in Table 3.

4. Conclusions

A highly accurate GC/MS method was been developed for the determination of urea in milk. The combination of high precision and a lack of significant bias mean that the method is accurate and superior to existing approaches. The method could be used to counter-check the results of conventional methods like diacetyl monoxime assay. In this condition, the GC–MS method can be used to certify the values of these control samples which were used to check the results of conventional methods. Moreover, the method provides a benchmark and could be used to certify the concentration of urea in new standard reference materials of milk and milk powder. Such reference materials are required by field laboratories to test the accuracy of their methods and calibrators and to demonstrate the traceability of measurements.

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