Development of Isotope Dilution-Liquid Chromatography/Tandem Mass Spectrometry as a Candidate Reference Method for the Determination of Folic Acid in Infant Milk Formula

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An isotope dilution-liquid chromatography/tandem mass spectrometric method was developed as a candidate reference method for the accurate determination of folic acid in infant milk formula. Sample was spiked with $^{13}C_5$-folic acid and then extracted with phosphate buffer (pH 6) solution. The extract was further cleaned up by deproteinization followed by a C18 solid-phase extraction cartridge. The extract was analyzed by using LC/ESI/MS/MS with selectively monitoring the collisionally induced dissociation channels of m/z 442 → m/z 295 and m/z 447 → m/z 295, which are the neutral glutamyl loss from the $[M+H]^+$ ions of folic acid and $^{13}C_5$-folic acid, respectively. LC/MS/MS chromatograms showed substantially reduced background from chemical noises compared to LC/MS chromatograms. Repeatability and reproducibility studies showed that the LC/MS/MS method is a reliable and reproducible method which can provide less than 1.5 relative percentage of method precision.

Key Words : Folic acid, Infant formula, ID-LC/MS, Method validation, Reference method

Introduction

Folic acid (or folate, or folacin), a vitamin cofactor, is an essential nutrient for fetus' and infants' growth and development.[1] Fortification programs of staple foods with folic acid are established or under consideration in many countries to help adequate intake of the nutrient especially for infants and pregnant women.[2,3] Many infant milk formula manufacturers also fortify their products with vitamins as well as folic acid to compensate the loss of these vitamins during the heat treatment along the manufacturing processing.[4] Therefore, there is an increased need for reliable monitoring of folic acid in food and infant formula.

Microbiological assay methods have been used for the assay of folate levels in food and biological matrix.[5,6] However, these methods assay total folate levels and do not distinguish derivatives of folic acid which have different biological activities.[7,8] For years, HPLC analysis with electrochemical, UV, and/or fluorescence detection have been developed and applied for differentiation of folates.[7,9-15] However, the HPLC methods are susceptible to interferences from sample matrix and thus require extensive sample clean up procedures, which in adverse make it difficult to correct losses during the sample clean-up processes. Gas chromatography/mass spectrometry (GC/MS) with isotope dilution techniques was developed for the determination of folic acid in biological samples,[16-18] but it is not widely accepted as a choice of methods due to cumbersome derivatization steps required for the GC analysis. Recently, liquid chromatography/mass spectrometry (LC/MS) has been developed for the analysis of folic acid in food and biological samples.[19]

Meanwhile, the Third International Food Data Conference in 1999 reached a consensus that the use of certified reference materials (CRMs) should be encouraged to harmonize the assay results of folates among laboratories.[20] It has been recognized that a definitive reference method should be used for the certification of CRMs to make their certified values acceptable nationally and internationally. LC/MS with isotope dilution techniques (ID-LC/MS) is a promising candidate as a reference method for the accurate certification of folic acid in CRMs without systematic bias as the methods provide adequate correction of recovery of the analyte along the sample clean up processes. Recently, many ID-LC/MS methods have been developed for the determination of folic acid in food stuffs and biological samples.[21-31] Those methods do not have been rigorously evaluated if these methods have adequate metrological quality to be used as reference methods.

We established an ID-LC/MS/MS method for the accurate determination of folic acid in infant milk formula. To test the metrological quality of the method to be used as a reference method, we evaluated its accuracy, repeatability, reproducibility, and checked the presence of possible influence by matrix interferences.

Experimental

Materials. A bottle of infant milk formula powder commercially available nationwide in Korea was purchased at local market. NIST SRM 1846 was purchased from the National Institute of Standards and Technology (Gaithersburg, MD, USA) and used as homogenized infant milk formula powder. Folic acid was purchased from Fluka Chemie AG

(Buchs, Switzerland) and used as a primary reference material without further purification. The purity of the primary reference material was evaluated in our laboratory based on the analytical data in the certificate of analysis provided by the manufacturer. The assigned purity was 90.8 ± 1.0%.[32] $^{13}C_5$-folic acid, which has ^{13}C atoms substituted at the five carbons on the glutamic acid portion, was obtained from Eprova AG (Schaffhausen, Switzerland). HPLC grade organic solvents (Methanol and acetonitrile) were obtained from Burdick and Jackson (Muskegon, MI, USA). Ammonium acetate, potassium phosphate dibasic, 2-mercaptoethanol and formic acid were obtained from Aldrich (Milwaukee, WI, USA). Pure water was prepared by using a membrane-filtering system and further purified by passing through a Millipore Corp Milli-Q RG purification system. All the solvents and buffer solutions used hereafter in this study were degassed by purging helium before usage.

Calibration Standard Solutions. The standard solution used in this study was prepared and verified according to a procedure maintained in our laboratory. The brief description of the procedure is as following. We gravimetrically prepared folic acid standard solutions of a 5 mg/kg level in [acetonitrile 26% + methanol 14% + water 60%] containing 10 mM 2-mercaptoethanol. As the solubility of folic acid in the solvent is poor, weighed portion of 1 mg folic acid was first dissolved in 10 mL of 10 mM ammonium acetate buffer (pH 10) containing 10 mM 2-mercaptoethanol, in which 190 mL of the LC mobile phase, [acetonitrile 26% + methanol 14% + water 60%], was added. Four standard solutions were prepared independently for the cross check. A $^{13}C_5$-folic acid standard solution of a 5 mg/kg level was prepared with the same way used for the folic acid standard solutions. For each of the four folic acid standard solutions, at least two calibration mixtures with 1:1 isotope ratio were prepared by mixing the weighed aliquots of the solution and the $^{13}C_5$-folic acid standard solution. We crosschecked the calibration mixtures by using LC/MS to test the self-consistencies of the standard solutions and the calibration mixtures. For sample analysis, one calibration mixture was selected and used

Sample Preparation. Either commercial infant formula or SRM 1846 was sampled in 1 g unit and taken into a 15 mL conical tube. An appropriate amount of the $^{13}C_5$-folic acid standard solution was spiked to the sample so that the ratio of folic acid to $^{13}C_5$-folic acid was close to 1.0. The exact amount of sample and the $^{13}C_5$-folic acid standard solution taken into the tube was determined by weighing the tube before and after adding each of them into the tube. Sample in the conical tube was dissolved with 10 mL of 0.1 M dibasic potassium phosphate solution (pH 6). The tube was purged with argon gas, caped tightly, and placed at 4 °C for an appropriate period of time, usually overnight for convenience, for the equilibration of spiked $^{13}C_5$-folic acid with folic acid in sample. The tube was then placed in a 100 °C water bath for more than 30 minutes with shaking the tube periodically, and cooled to room temperature. The sample was centrifuged at 3000 *g* for 20 minutes to separate lipid from the aqueous solution. The supernatant lipid layer was then removed from the tube. The sample was added with 70 μL of formic acid to adjust pH to 3.5. The sample was again centrifuged at 3000 *g* for 20 minutes to spin down protein precipitates. The whole aqueous layer was then subject to clean up by a solid-phase extraction (SPE). After testing the performance of a few reversed phase SPE cartridges and anionic exchange cartridges, we decided to perform a single stage clean-up with a C18 solid-phase extraction cartridge. The sample extract was loaded onto a C18 solid-phase extraction cartridge (Supelclean ENVI-18, 500 mg/3 mL from Supelco), which was preconditioned by 6 mL of methanol, 6 mL of water, and 0.03 mM dibasic potassium phosphate solution (pH 3.5). After the sample extract was loaded, the cartridge was washed with 3 mL of 0.03 mM dibasic potassium phosphate solution. The analyte was eluted with 1.6 mL of [acetonitrile 26% + methanol 14% + water 60% + 0.1% formic acid]. The eluent was collected to an HPLC vial after discarding the initial 0.2 mL portion.

LC/MS Analysis. The LC/MS used in this study was a Micromass Quattro Ultima mass spectrometer (Tedington, U. K.) combined with a Waters Alliance LC through its electrospray ionization interface. Sample extracts and the selected calibration mixture (with 1:1 isotope ratio) were loaded in 20 μL units to a Luna C18 column (250 mm length, 4.6 mm i.d., 5 μm particle size) from Phenomenex (Torrance, CA, USA). The mobile phase was a programmed gradient of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile 26% + methanol 14% + water 60% + 0.1% formic acid). The mobile phase started with isocratic elution with 30% solvent B at flow rate of 0.175 mL/min for 9 min. Linear gradient to 100% solvent B at 0.3 mL/min was given from 9 to 14 min and the mobile phase was kept isocratic with 100% solvent B at 0.3 mL/min up to the end of run and changed to the initial mobile phase for the next run. The mass spectrometer was operated in the positive ion mode. The ESI probe voltage was 3.5 kV, and the cone voltage was 45 V. The gas flow rate for nebulization and desolvation were set to 158 and 350 L/hour, respectively. The desolvation temperature was 350 °C and the temperature of the source region was kept at 150 °C. For the LC/MS/MS analysis, the mass spectrometer was operated in a selected reaction monitoring (SRM) mode for monitoring the collisionally induced dissociation (CID) channels of m/z 442 → m/z 295 and m/z 447 → m/z 295, which are the neutral glutamyl loss of the $[M+H]^+$ ions of folic acid and $^{13}C_5$-folic acid, respectively. For the LC/MS/MS analysis the collision cell, second quadrupole of the mass spectrometer, was filled with argon gas at a pressure of 1.0×10^{-3} torr and the collision energy was 20 eV. The same extracts were also analyzed by the LC/MS in SIM mode for comparison with the LC/MS/MS analysis results. For the LC/MS analysis, the mass spectrometer was operated in a selected ion monitoring (SIM) mode for monitoring the $[M+H]^+$ ions of folic acid and $^{13}C_5$-folic acid at m/z 442 and 447, respectively.

Measurement Protocol. The protocols for the ID-LC/MS/MS method in the SRM mode for the determination of

folic acid in infant milk formula were as follows. For each set of sample, a single LC/MS/MS run of calibration mixture was followed by a single LC/MS/MS run of each of sample extracts. The single run cycle was repeated for 4 or 5 times. The same sample extracts were also analyzed by the ID-LC/MS method in the SIM mode in the same run sequence.

Results and Discussion

Sample Cleanup and LC/MS Performance. It is known that optimization of pH of the mobile phase is essential to get adequate retention and separation of folic acid with a C18 reversed phase column as the analyte is a highly polar compound and has both acidic and basic functional groups. The test study carried out in our laboratory showed that the C18 column provide adequate retention and separation of folic acid when the pH of the mobile phase is acidic, as it was described in reference.[19,33] Therefore, we added 0.1% formic acid into the LC mobile phase to keep its pH at 3.5. We also tested both positive and negative ion modes as an operation mode of the LC/MS. A folic acid standard solution of 1 mg/kg was measured by the LC/MS with both positive and negative ion modes under the selected pH condition of the mobile phase. The selected ion chromatogram for $[M+H]^+$ at m/z 442 in positive ion mode showed a 5 fold higher folic acid peak than the corresponding peak on the selected ion chromatogram for its $[M-H]^-$ at m/z 440 in the negative ion mode. Therefore, we decided to run the mass spectrometer in positive ion mode in this study.

A few solid phase extraction methods were tested for the clean up of sample extracts. A C18 solid-phase extraction cartridge (Supelclean ENVI-18, 500 mg/3 mL from Supelco) and a Phenyl cartridge (Supelclean LC-Ph, 100 mg/1 mL from Supelco) were tested. Both cartridges showed a similar level of sample clean up with a good recovery of the analyte. A strong anionic exchange cartridge (Supelclean LC-SAX, 100 mg/1 mL from Supelco) was also tested for further clean up after the reversed phase SPE cleanup. Recovery of the analyte at this stage was not satisfactory. Therefore we decided to perform only a single stage SPE clean-up with the C18 cartridge without further cleanup with the SAX SPE cartridge.

Typical LC/MS SIM chromatograms of folic acid at m/z 442 and $^{13}C_5$-folic acid at m/z 447 in the extracts of a commercial infant formula sample and NIST SRM 1846 infant formula are given in Figure 1. For both infant formula samples, folic acid and $^{13}C_5$-folic acid coeluted at 25 min. In addition to intense folic acid and $^{13}C_5$-folic acid peaks, the chromatograms showed many intense peaks from sample matrix and substantially elevated back ground levels, which cause inaccuracy in the integration of the areas of folic acid and $^{13}C_5$-folic acid peaks. It is noticeable that the commercial infant formula sample and NIST SRM 1846 show different patterns of matrix interferences. These results indicate that the LC/MS method in the SIM mode is prone to be interfered by sample matrix and the levels of interferences vary with sample.

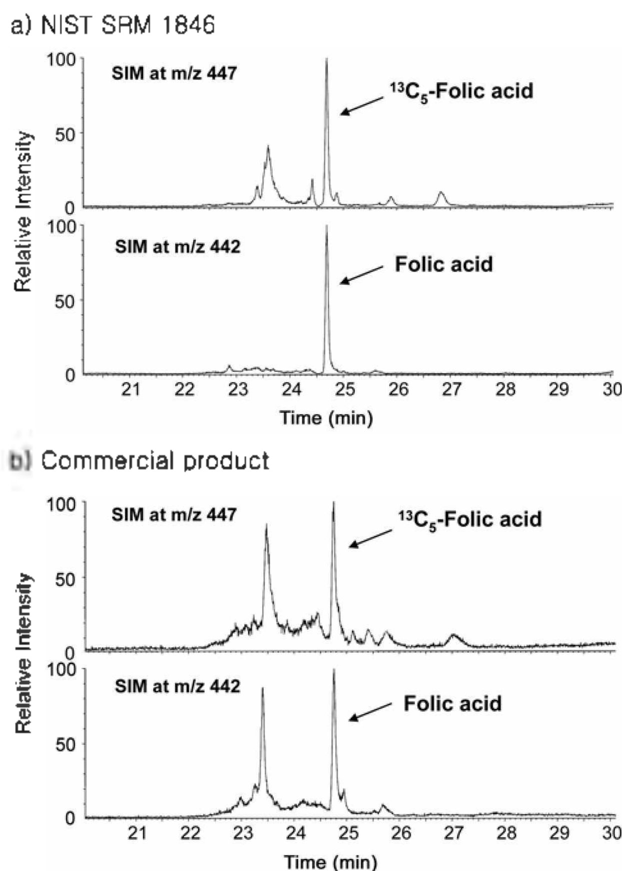

Figure 1. Typical LC/MS SIM chromatograms of folic acid at m/z 442 and $^{13}C_5$-folic acid at m/z 447 from a commercial infant formula sample and NIST SRM 1846.

Figure 2 shows typical LC/MS/MS SRM chromatograms of folic acid and $^{13}C_5$-folic acid in the commercial infant formula sample and NIST SRM 1846 by monitoring the selected CID channels of m/z 442 $\rightarrow$ m/z 295 and at m/z 447 $\rightarrow$ m/z 295, respectively. For both samples, the chromatograms were dominated by the peaks of the target analytes and showed low background noise and no significantly interfering peak from sample matrix. The relative standard deviation of the area ratio of folic acid and $^{13}C_5$-folic acid from repeated LC/MS/MS run of a single sample extract was usually less than 2%.

Comparison of Measurement Results by ID-LC/MS and ID-LC/MS/MS. Table 1 summarizes the measurement results of multiple subsamples of the commercial infant formula and SRM 1846 by the LC/MS and LC/MS/MS methods. The same subsamples were analyzed by the LC/MS and LC/MS/MS methods after sample preparation, and the same calibration standard solution was used for both LC/MS and LC/MS/MS measurements. For both commercial infant formula and SRM 1846, the average of the results of multiple subsamples from the ID-LC/MS method is lower than that from the ID-LC/MS/MS method and the difference between the two methods is larger than the standard deviations among subsamples from the two methods. As indicated on the chromatographic qualities of two methods shown in

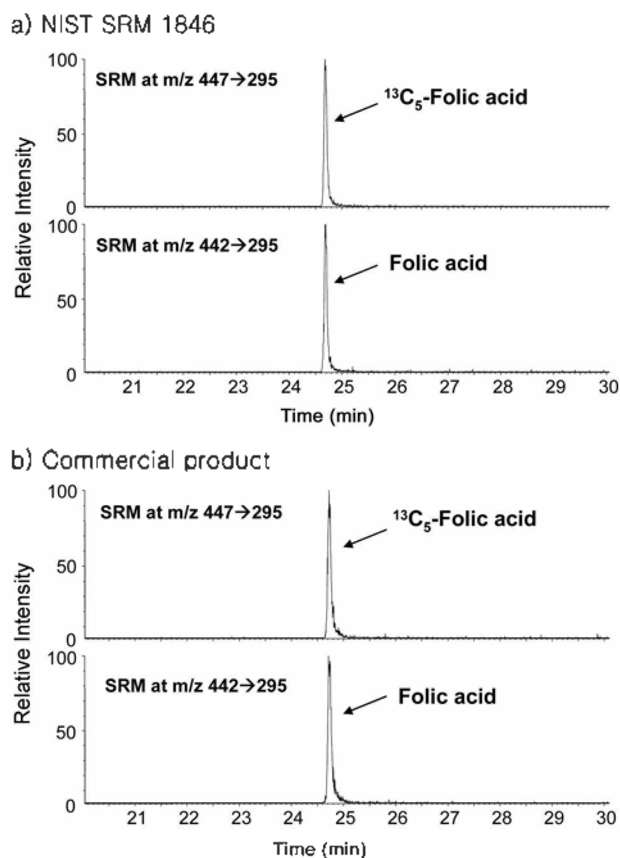

Figure 2. Typical LC/MS/MS SRM chromatograms of folic acid and $^{13}C_5$-folic acid from a commercial infant formula sample and NIST SRM 1846 by selectively monitoring CID channels of m/z 442 → m/z 295 and at m/z 447 → m/z 295, respectively.

Table 1. Comparison of ID-LC/MS and ID-LC/MS/MS results

Sample	Measurement results by ID-LC/MS[a] (mg/kg)	Measurement results by ID-LC/MS/MS[a] (mg/kg)
Infant Formula (A commercial product)	1.134 ± 0.027	1.245 ± 0.016
NIST SRM 1846	1.129 ± 0.007	1.208 ± 0.016

[a]The numbers after "±" are the standard deviation of the measurement results of multiple subsamples. Other uncertainty sources are not considered in comparing the results of ID-LC/MS and ID-LC/MS/MS as the same extracts of subsamples were analyzed by LC/MS and LC/MS/MS with using the same calibration standard solution.

Figures 1 and 2, the discrepancy between the two methods can be explained by the matrix interferences in the LC/MS method due to its low specificity of single stage MS detection compared to the LC/MS/MS method which has additional selectivity by monitoring specific CID channels of the analytes. Therefore, we decided to establish and evaluate the ID-LC/MS/MS method as a candidate reference method.

Method Validation and Analytical Quality Check. Followings are the experimental results we carried out to evaluate if the ID-LC/MS/MS method has an adequate quality as a reference method that can be used in national metrology institutes.

Validation by Well-characterized Reference Materials. To prove that the candidate reference method can link the measurement results traceable to the International System of Units (SI), some degree of validation is always appropriate by using well-characterized standard or published methods. NIST SRM 1846 has a reference value of 1.29 ± 0.28 mg/kg, which was assigned based on the results of microbiological assay methods carried out by NIST's collaborating laboratories and is not assignable as a certified value due to lack of confidence on the measurement method. The assigned value is slightly higher than our value determined by the ID-LC/MS/MS method, 1.21 ± 0.03 mg/kg, but the two results agree within their uncertainties. The higher average value of the microbiological assay results was expected as it measures total natural folates including folic acid.[34,35] Recently, the folic acid level in NIST SRM 1846 was analyzed by Pawlosky *et al.*[21] using an ID-LC/MS method in negative ion mode. Our results agree with the results obtained by Pawlosky' group, 1.13 ± 0.06 mg/kg, within the measurement uncertainties. However, we are very cautious to directly compare these two values as details of uncertainty of Pawlosky group's value are not available in the article. Especially, the primary reference materials, pure folic acid, used for the preparation of the standard solutions in this study and Pawlosky et. al.'s study were not from the same sources, and it was not clearly described in the article how Pawlosky et. al assyed the purity of their primary reference material.

Repeatability and Reproducibility. Due to the lack of well-characterized reference material, careful investigation on repeatability, reproducibility, and sources of uncertainty are required to validate the metrological quality of the method. To test the repeatability of the candidate reference method, multiple subsamples from NIST SRM 1846, a model of homogenized sample batch, went in parallel through the sample preparation processes, and the extracts were analyzed together by the LC/MS/MS method in the SRM mode. To test reproducibility of the method, the same repeatability test

Table 2. Results of the ID-LC/MS/MS measurement of NIST SRM 1846 at two different time periods

	Results by ID-LC/MS/MS (mg/kg)	
Period 1	Subsample 1-1	1.206
	Subsample 1-2	1.221
	Subsample 1-3	1.220
	Subsample 1-4	1.187
	Average	1.208
	Standard Deviation	0.016 (1.33%)
	Exp. Uncertainty (95%)[a]	0.040 (3.27%)
Period 2	Subsample 2-1	1.189
	Subsample 2-2	1.219
	Subsample 2-3	1.202
	Subsample 2-4	1.229
	Average	1.210
	Standard Deviation	0.018 (1.47%)
	Exp. Uncertainty (95%)[a]	0.030 (2.39%)

[a]The expanded uncertainties are with levels of confidence of 95%.

Table 3. Uncertainty Sources in the ID-LC/MS/MS Determination of Folic Acid in Infant Formula

Uncertainty Components	Sources (Evaluation Methods)	Typical value[a] (Relative %)
Folic acid standard solution	Purity of the reference material (from the certificate)	0.5%
	Gravimetric preparation (from cross-check of independent sets of calibration solutions)	0.19%
Calibration mixture	Gravimetric mixing (from cross-check of multiple calibration mixtures from each individual standard solution)	0.4%
Weight of sample taken for analysis	Readability and linearity of the balance used (from the certificate of the balance)	< 0.01%
Weight of $^{13}C_5$-folic acid solution spiked into sample taken for analysis	Readability and linearity of the balance used (from the certificate of the balance)	< 0.01%
Peak area ratio of folic acid and $^{13}C_5$-folic acid from LC/MS measurements of calibration mixtures	Standard deviation of multiple measurements	0.6%
Peak area ratio of folic acid and $^{13}C_5$-folic acid from LC/MS/MS measurements of sample extract	Standard deviation of multiple measurements	1.0%

[a]Typical uncertainty of each source is based on the measurement protocol used in this study.

was carried out after a few days of the first test. The results obtained at two different time period are summarized in Table 2. The relative standard deviation of measurement results among multiple subsamples is around 1.4% within a period, indicating that the ID-LC/MS/MS method has a good repeatability. The measurement results from the two different time periods agree to each other within their uncertainties, indicating that the method has a good reproducibility.

Uncertainty Sources. For a reference method to be fit for its purpose, the uncertainty of the measurement result at a given level of confidence must be evaluated and must be confirmed that the uncertainty is at least many fold smaller than that of the method generally used by field laboratories. Uncertainty sources of the results by the ID-LC/MS/MS method are listed in Table 3. A full discussion of measurement uncertainty is beyond the scope of this article and only a brief description is given here. Most of the information required to evaluate uncertainty were already obtained during the repeatability and reproducibility test. The standard deviation of the measurement results of multiple subsamples within a period is the combination of random uncertainties[36,37] due to the uncertainties in weighing sample taken for analysis, weighing the $^{13}C_5$-folic acid solution spiked to the sample, and the LC/MS/MS measurements of calibration mixtures and sample extracts. The uncertainties in the standard solution and the calibration mixtures give systematic effects on the results and are not included in the repeatability. The relative standard uncertainty of the measurement value for infant formula having typical folic acid level of around 1 mg/kg by this method is expected to be around 1.5%, indicating that the method presents a high metrological quality as a reference method.

Recovery of Folic Acid in Sample Preparation. The overall recoveries of folic acid and $^{13}C_5$-folic acid from infant formula sample are within 40% to 70% range. Further action was not taken to improve the recovery as it is not a critical factor in IDMS methods as $^{13}C_5$-folic acid was spiked to the sample as an internal standard.

Detection Limit and Dynamic Range. As folic acid-free infant formula is not available, the detection limit of the ID-LC/MS/MS method was only estimated based on the signal to noise ratio of the SRM chromatograms as shown in Figure 2. The detection limit of the LC/MS/MS method, with the signal to noise ratio of 3, was estimated to be 0.005 mg/kg. According to the screening of a few commercial samples, the candidate reference method is expected to provide the same levels of analytical quality with similar uncertainties as described above if the folic acid level is higher than 0.5 mg/kg.

Conclusions

An LC/MS/MS-based isotope dilution mass spectrometric method has been evaluated as a candidate reference method for the accurate determination of folic acid in infant formula. Validity of the method was tested by evaluating its performance. The repeatability/reproducibility studies and the uncertainty evaluation results have proven that the candidate method has a metrological quality which is adequate enough to be used as a reference method.

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