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Isotope dilution-liquid chromatography/mass spectrometric method for the determination of riboflavin content in multivitamin tablets and infant formula

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ABSTRACT

Isotope dilution liquid chromatography/mass spectrometry (ID-LC/MS) was developed to measure riboflavin contents in infant formula and multivitamin tablets. $^{13}C_4$, $^{15}N_2$-riboflavin was used as an internal standard. For infant formula, acid hydrolysis was conducted after spiking internal standard to release bound riboflavin. Free riboflavin in the multivitamin tablets was extracted with distilled water for 3 h at 4 °C. The mass spectrometer was operated in selected ion monitoring mode to observe the collisionally induced dissociation channels of m/z 377 → 243 for riboflavin and m/z 383 → 249 for $^{13}C_4$,$^{15}N_2$-riboflavin. The developed method was validated based on tests of repeatability and reproducibility, evaluation of measurement uncertainty, and analysis of available certified reference materials. For infant formula and multivitamin samples, the repeatability was 0.1–0.4% and 0.3–1.2%, and the reproducibility was 1.4 and 1.0%, respectively. The realtive expanded uncertainty of analyzing homogenized sample was ranging 1–4%. Therefore, the developed method showed that it has as a high-order metrological quality. The developed ID-LC/MS method was also tested for the accurate analysis of riboflavin in commercial food products including infant formula, breakfast cereals, milk, and multivitamin tablets.

1. Introduction

Riboflavin is a water-soluble vitamin that is classified as vitamin B2, along with flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Vitamin B2 is found in natural foods in its free form or bounded to protein or sugar (Gregory, 1996). Vitamin B2 deficiency symptoms, such as skin and mucosal disorders and anemia, are rarely observed in individuals who consume well-balanced diet or dietary supplements, because riboflavin is found in various foods such as milk, cheese, leafy vegetables, mushrooms and meats (Gregory, 1996; Sykes et al., 2011; Tang et al., 2006).

Traditionally, riboflavin in foods was analyzed using microbiological and fluorometric methods. These classical approaches have been replaced by liquid chromatography (LC) in combination with various detection techniques (Callinice et al., 2000; Chen et al., 2005). Due to the high fluorescence of riboflavin itself, a fluorescence detector was widely adopted for the LC analysis of riboflavin in food matrices (Gatti and Gioia, 2005; Jakobsen, 2008; Ndaw et al., 2000; Tang et al., 2006).

Prior to the instrumental analysis, samples are treated with proper preparation procedure including extraction, chemical hydrolysis or enzyme treatment depending on the matrices. In the case of infant formula, simple extraction or acid hydrolysis can be accepted as a suitable procedure for sample preparation because the free form of riboflavin is fortified to meet the designed value for the product. In several previous studies, riboflavin contents were reported after simple extraction either with or without acid hydrolysis for milk (Klejdus et al., 2004), infant formula (Goldschmidt and Wolf, 2010) and multivitamins (Chen et al., 2006). In addition, Klejdus et al. (2004) simultaneously determined contents of riboflavin and other vitamins using LC-DAD after simple solubilization for the selected samples, including pharmaceutical products, fortified powdered drinks and food samples.

While analysis techniques have been continuously improved, scientists have pursued more accurate and precise analysis of riboflavin and water-soluble vitamins (WSVs). The isotope-dilution mass spectrometry (IDMS) method based on LC of GC is an accurate method for the quantitative analysis of organic

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compounds in complex matrices because this method overcomes the difficulty in the correction of recovery during the sample preparation and exhibits high accuracy and repeatability (De Leenheer and Thienpont, 1992; De Bièvre, 1993). Our previous papers have reported the development of ID-LC/MS methods dedicated to the analysis of several individual vitamins, such as folic acid (Kim et al., 2010), retinol (Lim et al., 2011), niacin (Shin et al., 2013), and tocopherols (Lee et al., 2013) in infant formula and multivitamin tablets.

The current study was performed to establish an ID-LC/MS method as a higher-order reference method for the accurate determination of the riboflavin content in food samples, focusing on infant formula and multivitamin tablets. The developed method was evaluated through testing repeatability and reproducibility, validation with available certified reference materials, and estimation of overall measurement uncertainties.

2. Materials and methods

2.1. Chemicals and materials

Riboflavin was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany) as a primary reference material, and its purity was determined as (99.8 ± 0.1) % using protocols maintained in this laboratory; LC/UV analysis for structurally related impurities, Karl-Fischer Coulometry for water content, and thermogravimetric analysis for non-volatile impurities (Lee and Kim, 2014). $^{13}C_4$,$^{15}N_2$-riboflavin was purchased from IsoSciences, LLC (King of Prussia, PA, USA). HPLC grade methanol was obtained from Burdick and Jackson (Muskegon, MI, USA). Ammonium formate, ammonium acetate, sodium acetate, acetic acid, formic acid, hydrochloric acid and ammonia were obtained from Sigma-Aldrich. HPLC grade water was prepared using a Milli-Q system (Millipore, Bellerica, MA, USA). Filter cartridges (PURADISC NYL 25 FILTER 13 mm × 0.2 μm and GLASS MICROFIBER, 25 mm × 1.0 μm) were obtained from Whatman (Clifton, NJ, USA). The solid-phase extraction (SPE) cartridges used for sample clean-up were Oasis HLB cartridges (60 mg, 3 mL, Waters, Milford, MA, USA). The certified reference materials, SRM 1849 (infant/adult nutrition formula) and SRM 3280 (Multivitamin/Multielement Tablets), were purchased from the National Institute of Standard and Technology (Gaithersburg, MD, USA). Other testing materials including several multivitamin tablets, infant formulas, breakfast cereals and milks were purchased from local markets.

2.2. Calibration standard solutions

Standard solutions and isotope ratio standards were prepared and verified using the procedure described in our previous publications (Lee et al., 2013; Lim et al., 2011; Shin et al., 2013). Four independent standard solutions (50 mg/kg) were prepared gravimetrically by dissolving 1 mg of the primary reference materials in 20 mL of distilled water. An isotope-labeled riboflavin ($^{13}C_4$,$^{15}N_2$-riboflavin) standard solution was prepared in a similar way. Two isotope ratio standard solutions were prepared from each of the four riboflavin standard solutions by gravimetrically mixing the standard solutions with the $^{13}C_4$,$^{15}N_2$-riboflavin solution to obtain a 1:1 isotope ratio. A total of eight isotope ratio standard solutions were prepared and compared to each other using LC/MS analysis to cross-check the consistency of the prepared standard solutions and isotope ratio standard solutions. One isotope ratio standard solution was selected and used for single point (exact matching) calibration for sample analysis. The preparation of all of the standard solutions was carried out using amber glass bottles under subdued light to minimize the photolysis of riboflavin (Ahmad et al., 2004).

2.3. Sample preparation

Different sample preparation procedures were developed for infant formula, breakfast cereals and milk in comparison to multivitamin products. Infant formula, breakfast cereal and milk were prepared using acid hydrolysis to release riboflavin conjugated to proteins and sugars. The multivitamin products, manufactured by blending free vitamin forms, were analyzed after simple extraction without hydrolysis.

2.3.1. Preparation of infant formula, breakfast cereal and milk samples

Before sample treatment, each type of sample was properly prepared for homogenization by grinding or mixing if necessary. Each sample (1 g) was gravimetrically taken into a 60 mL pre-weighed amber bottle, and an appropriate amount of the $^{13}C_4$,$^{15}N_2$-riboflavin standard solution was spiked into the bottle to generate an isotope ratio close to 1:1. To perform the acid hydrolysis, 20 mL of HCl (0.1 mol/L) was added to the bottle and purged with argon gas. The bottle was placed into a boiling water bath for 30 min with continuous shaking. After cooling the bottle to room temperature, the pH of the sample solution was adjusted to between 4 and 4.5 by adding sodium acetate (2.5 mol/L). After transferring the sample into a conical tube, the sample was centrifuged and the aqueous solution was collected for SPE. An OASIS HLB cartridge was pre-conditioned with 6 mL of methanol and 6 mL of 5 mmol/L ammonium acetate buffer (pH 4–4.5), and all of the collected sample solution was loaded. After washing with 6 mL of 5 mmol/L ammonium acetate buffer, riboflavin was eluted with 3 mL of methanol. The eluent was dried under nitrogen gas at 40 °C and reconstituted with 2 mL of distilled water. The final sample extract was transferred to a 2 mL vial for LC/MS analysis.

2.3.2. Preparation of multivitamin samples

For the analysis of multivitamin products, 0.1 g of ground sample was placed into a 60 mL glass bottle, and 50 mL of distilled water was added to the bottle. The exact amount of sample and extraction solvent were determined by weighing the bottle before and after the addition of each component. The bottle was purged with argon gas. After vortexing for 30 min, the sample solution was kept at 4 °C for 3 h. Then, 1 mL of the sample extract was transferred to a vial and spiked with an appropriate amount of the isotope standard solution to generate an isotope ratio as close to 1:1. The sample was passed through a filter cartridge, and then diluted with water to a level for convenient LC/MS analysis.

2.4. Instrumentation and MS analysis

The LC/MS analysis was achieved using an Agilent 6410 Triple Quadrupole LC/MS system (Santa Clara, CA, USA) connected to an Agilent 1200 Series LC system (Waldbronn, Germany). The chromatographic separation was carried out using a Waters X-Bridge C18 column (4.6 mm i.d., 150 mm length, 3.5 μm particle size) connected to a C18 guard column. The mobile phases were 20 mmol/L ammonium formate in water containing 0.1% formic acid (phase A) and methanol (phase B). The flow rate was 0.3 mL/min. Gradient elution started with 95% A and changed linearly from 95% to 20% A over 5 min. The mobile phase was then kept isocratic with 20% A for 10 min, changed from 20% to 95% A over 1 min, and maintained at 95% A for 14 min (total 30 min). The injection volume was 10 μL. MS analysis was conducted in the positive ion mode of electrospray ionization (ESI). The optimized MS conditions for the detection of riboflavin were as follows. Capillary voltage, 3000 V; nebulizer gas (N_2) pressure, 35 psi; nebulizer gas (N_2) temperature, 350 °C; drying gas flow, 8 L/min; and fragmentor voltage (applied to the extraction skimmer), 150 V. Detection was performed in selected reaction monitoring (SRM) mode, 23 eV of collision energy