Analytical Methods

Investigation of isotope dilution mass spectrometric (ID-MS) method to determine niacin in infant formula, breakfast cereals and multivitamins

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1. Introduction

Niacin is one of the components in vitamin B complex and generally includes both of nicotinic acid (pyridine 3-carboxylic acid) and nicotinamide (pyridine 3-carboxamide). In biological system, niacin involved in various dehydrogenase reactions in forms of nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) (Gregory, 1996). Niacin can be existed as free nicotinic acid, nicotinamide, NAD, or NADP in several foods including poultry, nuts, legumes, milk, cereals, and vegetable. In cereal grain, niacin is also founded as chemically bound form of nicotinic acid to polysaccharides and peptides or glycopeptides. On the determination of niacin in foods, it should be clearly specified that the method measure “free” or “total” niacin. Free niacin has been usually measured by determining nicotinic acid and/or nicotinamide after extraction with acidic aqueous solution (Ball, 2006). For the analysis of total niacin, it is usually required to extract niacin after all forms of niacin convert to free niacin by heating the sample with alkali or acid.

Colorimetric and microbiological methods have been used as an official method to analyse niacin in food matrix (AOAC, 1997a, 1997b). Colorimetric method is based on the König reaction and determines the total niacin with low specificity. Microbiological assay evaluates either total niacin or free niacin depending on the using microorganisms. Free niacin is generally determined by Lactobacillus casei or Leuconost oc mesenteroids subsp. Mesenteroides. Lactobacillus plantarum (ATCC 8014) can determine total niacin because this particular microorganism responds to nicotinic acid, nicotinamide, nicotinuric acid (an inactive metabolite) and NAD (Ball, 2006).

However, chromatographical techniques currently replaced colorimetric and microbiological methods. Several liquid chromatographical methods with various separation and detection techniques have been reported (Lang, Yagar, Eggers, & Hofmann, 2008; Perrone, Donangelo, & Farah, 2008; Rose-Sallin, Blake, Genoud, & Tagliaferri, 2001; Zafra-Gómez, Garballo, Morales, & García-Ayuso, 2006). One of those studies compared the results of total niacin contents measured by a liquid chromatography (LC) with fluorescence detector and by microbiological assay using L. plantarum (Rose-Sallin et al., 2001). The HPLC method showed better reproducibility (0.9–2.7%) compared to the microbiological assay (5.7–6.8%). Also the other method by LC–UV (Zafra-Gómez et al., 2006) showed the relative standard deviation from 0.5% to 3.7% for the simultaneous analysis of nicotinamide and seven other water soluble vitamins in supplemented foods. Mass spectrometry (MS) coupled with LC has been introduced to improve the efficiency of niacin analysis in coffee bean samples, and the repeatability of method for nicotinic acid was maintained as 1.6–4.9% depending on the levels of analytes in the samples (Perrone et al., 2008). An isotope dilution mass spectrometric (IDMS) method based on LC was applied to determine nicotinic acid and nico-
taminamide in foods and biological samples. The ID-LC/MS was shown 1.1% of repeatability for nicotinic acid and 3.1% for nicotinamide in coffee samples (Lang et al., 2008). Adopting isotope dilution tech-
niques eliminates the systemic bias through the accurate correc-
tion of the recovery of the target analyte along the sample clean-
up process (De Bivière, 1993; Dube, Henrion, & Richter, 1997; Jung et al., 2004). Therefore, IDMS method is recognised as one of the potential primary methods in chemistry by CCQM (Comité Consul-
tatif pour la Quantité de Matière) (Quinn, 1997) as it provide higher order metrological quality for the measurement of samples with complex matrices. A few National Metrology Institutes (NMI), National Institute of Standard and Technology (NIST) of US and Na-
tional Institute of Metrology (NIM) China, have established refer-
ence methods by applying the ID-LC/MS method to determine niacin in infant formula containing niacin. (Goldschmidt & Wolf, 2010; Huang et al., 2010). These methods monitored only free nicotinamide in sample without alkaline hydrolysis process, which was required to determine total niacin including bound forms of niacin.

Current study described about the development of an ID-LC/MS method as a candidate reference method for the analysis of niacin in infant formula, breakfast cereal and multivitamin. This method was designed to investigate total niacin in various food types by adopting alkaline hydrolysis step, which can be also applied for the analysis of infant formula or milk powder regardless of existing forms of niacin in the matrix. The uncertainty of the ID-LC/MS was evaluated. Finally, niacin contents in various commercial foods and food supplements were determined to confirm the feasibility of the ID-LC/MS developed.

2. Materials and methods

2.1. Chemicals and materials

Nicotinamide was purchased from Chromadex, Inc. (Santa Ana, CA, USA) as a primary reference standard and its purity was deter-
mined as (99.4 ± 0.3)% by the protocols maintained in this labora-
tory of KRISS (Korea Research Institute of Standards and Science), the National Metrology Institute of Korea. Nicotinamide-(2,4,5,6)-
\(^4\)d was purchased from CDN Isotopes Inc. (Quebec, Canada). Ammonium acetate was obtained from Sigma–Aldrich (Steinheim, Germany). Sodium hydroxide and hydrochloric acid were pur-
chased from Samchun Chemicals (PyungTak, Korea). HPLC grade methanol and formic acid were from Burdick and Jackson (Muske-
gen, MI, USA) and Fluka (St. Gallen, Switzerland), respectively. HPLC grade water was purified in a Milli-Q system (Millipore, Beld-
ford, MA, USA). Filter cartridges (PURDISC NYL 25 FILTER 25 mm × 0.45 μm) were obtained from Whatman (Clifton, Nj, USA), Certified reference materials SRM 1849 (infant/adult nutri-
tion formula) and BCR 431 (Brussels sprouts powder) were pur-
chased from the National Institute of Standard and Technology (Gaithersburg, MD, USA) and Institute for Reference Materials and Measurements (Geel, Belgium), respectively. Other testing materials including several infant formulas, breakfast cereals and multivitamins were purchased from local markets. Two infant for-
mulas were for 3–6 months baby from different producers. Breakfast cereals A and B were products with mainly corn, respectively 62% and 89%, from different producers and breakfast cereal C was products contain 85% of brawn rice. All of three multivitamins were tablet types from different producers.

2.2. Calibration standard solutions

Standard solutions and isotope ratio standard were prepared and verified following the procedure maintained in this laboratory as described in our other articles (Kim, Hwang, So, Son, & Kim, 2010; Lim, Kim, Ahn, & Kim, 2011). Briefly, weighted portion (7 mg) of nicotinamide primary reference material was dissolved into a weighted portion (80 g) of methanol. Four standard solutions were independently prepared. An isotope labelled nicotinamide (nicotinamide-\(^4\)d) standard solution was prepared in the same way. As a calibration standard solution for isotope dilution meth-
od, isotope ratio standard solution was prepared by gravimetrically mixing the nicotinamide standard solutions and the nicotinamide-
\(^4\)d standard solution (1:1 isotope ratio). Two isotope ratio standard solutions were prepared from each nicotinamide standard solu-
tion. After dilution with water (1 mg/kg in this study), the eight isotope ratio standards were analysed by LC/MS in SIM (selected ion monitoring) mode to cross-check the consistencies among them. Repeatability of preparing standard solutions and isotope ra-
tio standards were evaluated by the LC/MS results. One isotope ra-
tio standard was selected and used for the calibration standard on sample analysis.

2.3. Sample preparation

Samples were differently prepared depending on the existing forms of niacin in the samples. Alkaline hydrolysis is necessary to convert into free nicotinic acid from NAD or NADP and bound nicotinic acid in milk or breakfast cereal. Samples like multivita-
mins which contain mainly nicotinamide were analysed after simple extraction without hydrolysis.

2.3.1. Preparation for infant formula and breakfast cereal samples

Breakfast cereal sample has to be homogenised by grinding with a laboratory mill (FRITSCH, Model No. Pulverisette 14, Ger-
many) before sampling. SRM 1849, BCR 431, and infant formula were homogeneous fine powder forms and did not require grinding. Sample (2 g) was taken gravimetrically in 60 mL amber bottle and appropriate amount of nicotinamide-\(^4\)d standard solution was spiked in the bottle to make the isotope ratio to 1.0 as based on the product information. Water (30 mL) was added in the bottle and mixed with sample by vortexing and sonication. A portion (10 mL) of the prepared sample solution was transferred to a con-
cial tube. To perform the alkaline hydrolysis, 5 mL of NaOH (5 mol/ L) was added to the sample solution, then they were heated in a boiling water bath for one hour. After cooling down to room tem-
perature, pH of the sample solution was adjusted between 2.5 and 3.0 by adding hydrochloric acid. For the solid-phase extraction, OA-
SIS HLB cartridge (Waters, Milford, MA, USA) was pre-conditioned with methanol and water, and 2 mL of sample solution was loaded. After washing with 1 mL of 50 mmol/L ammonium acetate buffer, nicotinic acid was eluted with 3 mL of 50 mmol/L ammonium acetate buffer. The isotope ratio standard (10 mL) was subject to the same sample preparation processes including alkaline hydrolysis to be used as a calibrant for sample analysis.

2.3.2. Preparation for multivitamin samples

For the analysis of multivitamin sample, 0.1 g of ground sample was placed into a 60 mL glass bottle and 50 mL of water was added into the bottle. The exact amount of sample and extraction solvent were determined by weighing the bottle before and after addition of each of them. After sonication for 2 h, 2 mL of the sample extract was transferred to a vial and spiked with an appropriate amount of the isotope standard solution to make the isotope ratio as close to 1:1. The sample was passed through a filter cartridge, and then di-
luted with water to a level (1 mg/kg) which is convenient for LC/ MS analysis in comparison with the isotope ratio standard (with-
out further treatment such as alkali hydrolysis).
2.4. Instrumentation and MS analysis

The LC–MS analysis was achieved with Agilent 6410 Triple Quad LC/MS (Santa Clara, CA, USA) connected with Agilent 1200 Series (Waldborn, Germany) containing a quaternary pump, an autosampler, a degasser, a column oven and a diode-array detector.

The chromatographic separation was carried out on the Phenomenex Synergi Fusion RP-C18 (2 mm i.d., 150 mm length, 4.0 μm particle size) connected with a C18 guard column (Lang et al., 2008). Mobile phases were 5 mmol/L ammonium acetate in water (phase A) and 5 mmol/L ammonium acetate in methanol (phase B) both containing 0.1% formic acid. Flow rate was 0.3 mL/min. Gradient elution started with 100% A for 1 min, changed linearly from 100% to 92% A for 4 min, from 92% to 100% A for 4 min, and maintained 100% A for 6 min. The injection volume was 10 μL. MS analysis was conducted in the positive ion mode of electrospray ionisation (ESI). The MS conditions optimised for detecting nicotinic acid and nicotinamide were as following: capillary voltage, 4000 V; nebulizer gas (N2) pressure, 45 psi; drying gas (N2) temperature, 350 °C; drying gas flow, 12 L/min; fragmentor voltage (applied to the extraction skimmer), 120 V. For the verification of calibration standard solution on SIM mode, [M+H]+ ions of nicotinic acid and nicotinamide were monitored at m/z 124 and 123 and corresponding isotope labelled ions were detected at m/z 128 and 127, respectively. On the selected reaction monitoring (SRM) mode, 20 eV of collision energy was applied on the collision cell, and dissociation channels of m/z 124 → 80 and m/z 128 → 84 were chosen for nicotinic acid and the isotope labelled nicotinic acid, respectively, in prepared samples. SRM channels for nicotinamide and nicotinamide-d4 were m/z 123 → 80 and m/z 127 → 84, respectively.

2.5. Calculation and uncertainty evaluation

The mass fractions of niacin (equivalent to nicotinamide) in test samples (mg/kg) were determined by the isotope ratio of nicotinamide/nicotinamide-d4 (for multivitamin samples without alkaline hydrolysis) or of nicotinic acid/nicotinic acid-d4 (for samples with alkaline hydrolysis in the sample preparation processes) in sample, in comparison with that in the isotope ratio standard as following equation (De Bivère, 1993; Dube et al., 1997; Jung et al., 2004; Kim et al., 2010; Lim et al., 2011; Shin, Kim, Lee, & Hwang, 2010). All the measurement data obtained in this study were converted to the value equivalent to nicotinic acid by considering their molecular weights.

\[ C_{\text{sample}} = \frac{M_{\text{sample, spiked}} \times AR_{\text{sample}} \times M_{\text{s-sol, std}} \times CS_{-\text{sol}}}{M_{\text{sample}} \times AR_{\text{std}} \times M_{\text{s-sol, std}}} \]

where, \( C_{\text{sample}} \) = concentration of nicotinamide in the sample; \( C_{\text{s-sol}} \) = concentration of nicotinamide in the standard solution; \( M_{\text{sample}} \) = mass of the sample taken for analysis; \( M_{\text{s-sol, std}} \) = mass of the nicotinamide standard solution spiked into isotope ratio standard solution; \( M_{\text{s-sol, spiked}} \) = mass of the nicotinamide-d4 solution spiked into sample solution; \( M_{\text{s-sol, std}} \) = mass of the nicotinamide-d4 solution spiked into isotope ratio standard solution; \( AR_{\text{sample}} \) = observed area ratio of nicotinamide/nicotinamide-d4, or nicotinic acid/nicotinic acid-d4, for the sample solution from LC/MS measurement; \( AR_{\text{std}} \) = observed area ratio of nicotinamide/nicotinamide-d4, or nicotinic acid/nicotinic acid-d4, for the isotope ratio standard solution from LC/MS measurement.

The uncertainty evaluation in this study was referred to previously published papers (Choi, Hwang, So, & Kim, 2003; Lim et al., 2011; Park, Lee, Lim, & Hwang, So, 2010) and Guide to the Expression of Uncertainty in Measurement (GUM) (BIPM, IEC, IFCC, ISO, IUPAC, IUPAP & OIML, 1995). The uncertainty components in ID-LC/MS measurement were categorised into systematic and random effects according to their influences on the variation of multiple measurements. Consequently, the relative standard uncertainty of the measurement value was combined the categorised uncertainty components by following equation, \( u_c = \sqrt{u^2_{\text{sys}} + u^2_{\text{ran}}} \). The observed results in this study were expressed as mean and expanded uncertainty (\( U \)), and \( U \) were calculated by \( u_c \) multiple with coverage factor (\( k \)), which was determined from the Student’s \( t \)-distribution corresponding to the appropriate degrees of freedom and an 95% confidence for each mean values.

3. Results and discussions

3.1. LC/MS performance

The LC conditions for the separation of nicotinic acid and nicotinamide were adapted from prior study by Lang et al. (2008) and the mobile phase was tested for the effects of compositions and pHs of aqueous phase. Nicotinic acid and nicotinamide were hardly retained on the selected column (Phenomenex Synergi Fusion RP-C18) with methanol, and were properly separated on the column when the mobile phase consists of more than 90% of aqueous solution. The effects by pH of mobile phase were shown in Fig. 1. Mobile phase with higher pH provided better retention of nicotinamide. However, the peak of nicotinamide becomes broader and the peak intensity becomes lower as pH increases. We note that aqueous phase with pH 3.6 provide a proper separation between the two compounds. Therefore, 5 mmol/L ammonium acetate buffer with pH 3.6, which was set by 0.1% formic acid (Fig. 1b), was chosen as an aqueous mobile phase in this study. Gradient elution program, which was run with the selected aqueous phase and methanol containing 5 mmol/L ammonium acetate, carried out to properly separate nicotinic acid and/or nicotinamide from matrix in current study.

The optimisation of the instrumental sensitivity and the collection of structural information on analytes and their isotope labelled compounds were performed with the MS/MS experiments. As shown in Fig. 2a, nicotinic acid ([M+H]+, m/z 124) produced the fragment ions at m/z 80, which were pyridine ring ions after losing carboxylic acid. The similar CID pattern was observed from isotope labelled nicotinic acid (which was hydrolysis product of nicotinamide-d4) with [M+H]+ peak at m/z 128 and [M+H–COOH]+ peak at m/z 84 (Fig. 2b). Nicotinamide ions ([M+H]+ at m/z 123) were dominantly dissociated to m/z 80 as losing the carboxamide from the parent ion (Fig. 2c), however, nicotinamide-d4 ([M+H]+) at m/z 127) showed the fragment ions at m/z 83 as well as 84 (Fig. 2d). The additional peak at m/z 83 was inferred to the result of H–D scrambling in the pyridine ring during the CID process. However, the additional peak (m/z 83) barely affects to the quantification of niacin by ID-LC/MS, because the scrambling was consistently occurred during the CID of nicotinamide-d4 in standard and sample solution.

3.2. Sample preparation

The sample preparation process was selected between chemical hydrolysis and simple extraction methods depending on forms of niacin existing in sample. Infant formula contains free nicotinic acid and nicotinamide as well as low amount of NAD or/and NADP. Breakfast cereal also contains free nicotinic acid and nicotinamide as well as bounded nicotinamide which is originated in cereal grain. Therefore infant formula and breakfast cereal require alkaline hydrolysis, which convert nicotinamide and other forms of niacin into free nicotinic acid to determine total niacin. In the preliminary study, it was confirmed that alkaline hydrolysis with
produced by blending raw materials of vitamins and have niacin conducted. sure that the alkaline hydrolysis procedure had been properly and nicotinic acid were simultaneously monitored in order to as-

for desalting the sample to obtain reproducible data in mass spec-

breakfast cereals. As the sample contains excess amount of salt vert all forms of niacin into nicotinic acid in infant formulas and sodium hydroxide in the boiling water for 1 h was enough to con-

in nicotinamide form according to the information provided by manufacturers. This was confirmed by that the observed results of niacin were \((19.95 \pm 1.05)\) g/kg and \((19.96 \pm 1.06)\) g/kg before and after the alkaline hydrolysis, respectively. Consequently, nico-

taminamide in multivitamin can be analysed after extraction with water and proper dilution without further chemical process. How-

3.3. Method validation and analytical performance check

3.3.1. Validation by reference materials

The validation of the developed ID-LC/MS method was tested by analysing the well-characterised reference materials including SRM 1849 (NIST) and BCR 431 (IRMM). SRM 1849 has a certificate value of \((97.5 \pm 2.3)\) mg/kg as expressed in nicotinamide and this value was assigned by ID-LC/MS without any chemical treatment. Our observed results by the developed ID-LC/MS method were \((97.5 \pm 4.3)\) mg/kg with alkaline hydrolysis and \((96.8 \pm 4.4)\) mg/kg without alkaline hydrolysis (Table 1). The results obtained by this method agree with the certified value within their uncertainties. BCR 431, Brussels sprouts powder, has a certificate value of \((43 \pm 3)\) mg/kg as expressed in niacin, which indicates total niacin because the values were assigned by microbiological assay (L. plantarum) and König reaction according to their certification. As shown in Table 1, free nicotinamide was determined to be \((9.80 \pm 0.48)\) mg/kg without alkaline hydrolysis. After alkaline hydrolysis, however, total niacin was determined to be \((41.2 \pm 2.1)\) mg/kg, which agree with the certified value within uncertainties. This agreement indicates that the applied analytical method with alkaline hydrolysis was properly determined the bounded nicotinamide and other sources of nicotinic acid in BCR 431 sample. Though the certified value was produced by microbi-

ological assay which is not considered to have higher-order metro-

logical quality, the agreement of our results with the certified value partly supports the validity of this method. All the uncertainties above were the expanded uncertainties with 95% of level of confidence.

3.3.2. Repeatability and reproducibility

To test the repeatability of the developed ID-LC/MS method, candidate reference materials of infant formula and multivitamin powder prepared in this laboratory were used as homogenised samples. The reproducibility of the ID-LC/MS method was also con-

firmed by conducting the same repeatability test in different days with one month intervals. In each time period, a new set of multi-

ple standard solutions were prepared and verified by the consist-

ency test among them as described in experimental section. Table 2 shows the observed results for infant formula and multivi-

tamin samples at different time periods. In the case of infant for-

mula, the mean averages of observed results in three different time periods were 80.5, 81.1 and 82.0 mg/kg, and the relative stan-

dard deviations within a period were 0.690%, 1.75% and 0.809%. The mean averages of observed results for multivitamin samples in each time period were 19.9, 20.7 and 20.2 g/kg, and the relative standard deviation within a period was between 1.70% and 3.21%. Observed results from the three different time periods show 0.568% and 1.86% of relative standard deviation for infant formula and multivitamin, respectively. These observed results supported the developed method had good repeatability and reproducibility. Consequently, the developed ID-LC/MS method can be applied to assess total niacin in various types of food matrix, and preparation method can be selected between chemical hydrolysis and simple extraction by the forms of niacin in samples.
3.4. Application of the ID-LC/MS method to various samples

3.4.1. Analysis of homogenised samples

The validated ID-LC/MS method was applied to homogenised samples of infant formula and multivitamin, which were prepared by in-house protocol with the intention of using as candidate reference materials. Commercial products were purchased from different brands containing different levels of niacin. As shown in Table 3, homogenised infant formula and multivitamin samples were determined as (80.5 ± 3.17) mg/kg and (19.9 ± 1.05) g/kg, respectively, which were mean values of multiple sub-samples and expanded uncertainties at 95% of confidence level. These uncertainties contain the uncertainty components from both of analytical method and sample inhomogeneity from measurements of multiple subsamples (n = 10). The relative standard deviations (%) were obtained as 0.829% and 2.25% for infant formula and multivitamin samples, respectively, and these referred sample inhomogeneity of the distribution of niacin in samples. Excluded the

Fig. 2. Product ion spectra of collisionally induced dissociation (CID) of the [M+H]+ ions of nicotinic acid (a), nicotinic acid-d₄ (b), nicotinamide (c) and nicotinamide-d₄ (d). Dissociation mechanism of the dominant channel, [M+H]+ ions to [M+H–COOH]+, was identical between two compounds.
uncertainty by sample homogeneity, the uncertainty of the developed analytical method was expected as 0.8% for both of multivitamins and infant formula. These levels of small uncertainties on measurements support that the method has a high metrological quality as a reference method. Additionally, homogenised multivitamin samples were analysed by two different testing laboratories and reported as 20.5% and 14.8% in kg/kg.

### 3.4.2. Application on the commercial products

Table 4 summarises the observed results and the expanded uncertainties in comparison with values labelled on the commercial products. The observed results for two different brands of infant formula were (70.2 ± 3.96) mg/kg and (69.8 ± 3.28) mg/kg, which were higher than the values labelled by the manufacturers, 60 mg/kg and 65 mg/kg, respectively. The labelled niacin contents of breakfast cereals ranged from 205 mg/kg to 225 mg/kg. The main ingredient for breakfast cereal A and B were corn and breakfast cereal C was brown rice, and nicotinamide was added as an additional source of niacin for all three breakfast cereals. The discrepancy between the declared values and the observed values seems to the results that alkaline hydrolysis released the bounded nicotinamide from corn or brown rice. The developed method is able to determine total niacin including free nicotinic acid and nicotinamide as well as bounded nicotinamide in the sample matrix. In the case of multivitamin, the assessments by the developed ID-LC/MS were similar with the labelled values. All three multivitamins include only nicotinamide as a niacin source.

### 4. Conclusion

An isotope dilution mass spectrometric method based on the LC/MS has been established as a candidate reference method for the accurate determination of niacin in infant formula, breakfast cereal products, and multivitamin. Depending on the existing forms of niacin in the samples, the application of chemical treatment like alkaline hydrolysis helped to release into a free nicotinic acid and to determine the total niacin in samples. The repeatability/reproducibility studies and the uncertainty evaluation results have proven that the developed method has a metrological quality which is adequate to be used as a reference method.
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References


