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Analytical Methods

Metabolomics approach of infant formula for the evaluation of contamination and degradation using hydrophilic interaction liquid chromatography coupled with mass spectrometry

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ABSTRACT

In this study including the field of metabolomics approach for food, the evaluation of untargeted compounds using HILIC–ESI/TOF/MS and multivariate statistical analysis method is proposed for the assessment of classification, contamination and degradation of infant formula. HILIC mode is used to monitor more detected numbers in infant formulas in the ESI-positive scan mode than the reversed phase. The repeatability of the non-targeted contents from 4 kinds of infant formulas based on PCA was less than the relative standard deviation of 15% in all groups. The PCA pattern showed that significant differences in the classification of types and origins, the contamination of melamine and the degradations for one week were evaluated using HILIC–ESI/TOF/MS. In the S-plot from the degradation test, we could identify two markers by comparison to standards as nicotinic acid and nicotinamide. With this strategy, the differences from the untargeted compounds could be utilized for quality and safety assessment of infant formula.

1. Introduction

Infant formula has been recognized as a very important commodity regarding food quality and safety. Additives, nutrients and natural contents are included in infant formulas depending on the various kinds of earmark products worldwide. On a global mass scale, infant formula would be standardized regarding its definitions and analytical methods of existing additives, nutrients and natural contents for food quality and safety. However, in 2008, the highest contamination of melamine was detected in infant and/or follow-up formula foods for children (Tyan, Yang, Jong, Wang, & Shiea, 2009; Xin & Stone, 2008). At that time, unexpected melamine that contains a high percentage of nitrogen to make the protein content of food appear higher than the actual value could not be determined before limiting the extent of damage. It was impossible to predict the artificially enhance protein concentrations using unknown nitrogen-rich compounds in infant formula products (Abernethy & Higgs, 2013; MacMahon, Begley, Diachenko, & Stromgren, 2012). Later on, the nitrogen-rich dicyandiamide was also detected in infant formula (Inoue, Sakamoto, Min, Todoroki, & Toyo'oka, 2014). Thus, we prevent a future recurrence of unexpected accidents of infant formula using untargeted metabolomics strategy.

Based on the evaluation of the unexpected contents for food quality and safety, various ideas have been to use protein oxidation, antioxidant, nutrition and environments (Friel et al., 2013; Heller, Keoleian, & Willett, 2013; Hounsome, Hounsome, Tomos, & Edwards-Jones, 2008; Zhang, Xiao, & Ahn, 2013). Recently, the analytical strategy for non-targeted food ingredients is a major topic in modern Food Analytical Science, as demonstrated by the growing activity in the very new field of *Foodomics* included food metabolomics that is defined as a discipline that studies the food and nutrition domains through the application of *Omics* technologies (Cifuentes, 2009; García-Cañas, Simó, Herrero, Ibáñez, & Cifuentes, 2012; Herrero, Simó, García-Cañas, Ibáñez, & Cifuentes, 2012). A broad vision means not only a broad expertise acquisition, but also the ability and possibility of resolving any unexpected food accidents using metabolomics approach (Cevallos-Cevallos & Reyes-De-Corcuera, 2012). However, due to the unexpected accidents of infant formula, we are still far from an approach that integrates the untargeted metabolomics approach. A chemical

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fingerprint tends to be focused on the application of an interesting method based on the metabolomics approach in human milk (Marincola et al., 2012). The untargeted metabolomics approach to simultaneously measure dynamic changes of many compounds in various food samples has been utilized as high resolution nuclear magnetic resonance (NMR) and mass spectroscopy (MS) coupled with either high or ultrahigh resolution liquid (LC) or gas (GC) chromatographic technique. Compared to many techniques, LC/MS is more sensitive and allows for the measure of a broader array of metabolites in food and/or biological samples (Roux, Lison, Junot, & Heilier, 2011; Toyo'oka, 2008; Rogachev & Aharoni, 2012). LC/MS applications in the metabolomics field have been based on reversed-phase (RP) chromatographic methods, in which only non-polar and medium polarity analytes can be separated and retained on RP columns. On the other hand, hydrophilic interaction chromatography (HILIC) offers a different selectivity, with better retention of polar analytes not easily retained or indeed not retained at all using RP columns (Kawachi et al., 2011). Since infant formula contains about high-percentage constituent of water, many contents are expected to be highly polar and to be better separation of chemical status using HILIC than RP modes. However, there are only a very limited number of studies that have comprehensively performed the coverage and endurance of HILIC method for untargeted, complicated and exhaustive analytes of infant formula. Thus, our LC/MS assay based on HILIC mode can obtain the large number of chromatogram data (m/z, retention time, peak response and detected numbers) from infant formulas for multivariate statistical analysis of untargeted chemicals. Also, due to our approach, this assay are established based on different patterns that showed the visualized integration of data and chemicals that are statistically significant such as kinds, origin, contamination and degradation in infant formula with steady-state homeostasis. Therefore, the chemical profiling and/or fingerprinting of different patterns under steady-state conditions has been demonstrated, and is opening new possibilities for the assessment of infant formulas based on a non-targeted metabolomics approach. In this study, we propose the unique approach of *Foodomics* included metabolomics that the assay of non-targeted low-molecular-weight compounds in infant formulas by HILIC coupled with electrospray time-of-flight MS (HILIC–ESI/TOF/MS) and a multivariate statistical analysis that was applied to the assessment of the unexpected contaminations and degradations.

2. Material and methods

2.1. Reagents and solutions

Melamine was purchased from the Kanto Chemical Co. (Tokyo, Japan). Methanol, acetonitrile, formic acid, ammonium acetate, nicotinic acid and nicotinamide were obtained from the Wako Chemical Co. (Osaka, Japan). For the mobile phase, pure water, acetonitrile and methanol were used from Merk KGaA (Billeria, MA, USA). All other chemicals were of analytical grade. Deionized and distilled water was used throughout the study (Aquarius PWU200 automatic water distillation apparatus, Advantec, Tokyo, Japan). The infant formula samples were obtained from local stores in Japan, China and other countries.

2.2. LC instrument and conditions

The LC system was a Waters Acquity H Class from Waters Co. (Milford, MA, USA). The RP and HILIC separations were performed using an Acquity UPLC BEH C18 column (1.7 μm, 2.1 × 100 mm), TSKgel NH_2 column (3.0 μm, 2.1 × 150 mm) and TSKgel Amide-80 column (2.0 μm, 2.0 × 150 mm) at 40 °C. The injection volume was 5 μL. The mobile phase consisting of solvent A; 10 mM ammonium acetate in water, and solvent B; acetonitrile, was delivered at the flow rate of 0.2 or 0.4 mL/min. Three gradient modes of this mobile phase were used for the simple separation of the reversed phase (Type A), HILIC for NH_2 mode (Type B) and HILIC for Amide-80 mode (Type C) of the non-targeted compounds in the infant formulas. The gradient elution of Type A was as follows: 0.0 min [A/B: 98/2], 3.0 min [A/B: 98/2], 30 min [A/B: 2/98], 35.0 min [A/B: 2/98], 35.5 min [A/B: 98/2] and 50.0 min [A/B: 98/2] delivered at the flow rate of 0.2 mL/min. The gradient elution of Type B was as follows: 0.0 min [A/B: 2/98], 3.0 min [A/B: 2/98], 30 min [A/B: 98/2], 35.0 min [A/B: 98/2], 35.1 min [A/B: 2/98] and 50.0 min [A/B: 2/98] delivered at the flow rate of 0.2 mL/min. The gradient elution of Type C was as follows: 0.0 min [A/B: 2/98], 1.0 min [A/B: 2/98], 11 min [A/B: 90/10], 12.0 min [A/B: 90/10], 12.1 min [A/B: 2/98] and 18.0 min [A/B: 2/98] delivered at the flow rate of 0.4 mL/min.

2.3. MS instrument and conditions

The separated compounds were detected by a Waters LCT Premier XE time-of-flight mass spectrometer (TOF/MS) from Waters Co. (Milford, MA, USA). The electrospray (ESI) (positive ionization mode) conditions were as follows: capillary voltage was 3.0 kV, sample cone was 15 V, source temperature of 120 °C and desolvation temperature of 350 °C. The cone and desolvation gas flows were 50 and 650 L/h, respectively, and were obtained flowing nitrogen. The analytical mode and dynamic range were the V mode and normal, respectively. The aperture 1 voltage was 15 V. For calibration, the reference solution used 4 μg/mL leucine enkephalin (m/z 556.28, 2 ppm) in 0.1% formic acid in water/acetonitrile (5/5, v/v). The scan mode was used from m/z 100 to 1000.

2.4. Sample preparation

The 0.5 g samples were weighed in plastic tubes. Five-mL of water was then added and mixed. The infant formula samples were pretreated using Amicon Ultra-4 (Ultracel-3K, regenerated cellulose 3000 M.W. for volumes <4 mL, Milliore Co., Ltd., Billerica, MA, USA). The 0.5 mL sample solution was eluted through this cartridge by centrifuging at 14,000*g* for 10 min, added of 0.5 mL acetonitrile, filtered through a 0.2 μm filter for LC and measured by LC/MS assay.

2.5. Multivariate statistical analysis

The LC/MS data were analyzed for peak detection and alignment from m/z 100 to 1000, and exported for the principal components analysis (PCA) and orthogonal partial least-squares-discriminant analysis (OPLS-DA) by a MarkerLynx™ XS V4.1 SCN803 (Waters Co., Milford, MA, USA). The method parameters were as follows: mass tolerance = 0.05 Da, apex track peak parameters, peak width at 5% height (seconds) = 15/peak-to-peak baseline noise = 50, apply smoothing = yes, collection parameters, intensity threshold (counts) = 100/mass window = 0.05/retention time window = 0.10, noise elimination level = 6, deisotope data = yes. R2 (cumulative) and Q2 (cumulative) were used to determine the validity of the model. R2 (cum) indicates the variation described by all components in the model, and Q2 is a measure of how accurately the model can predict class membership. For the univariate analysis of the unknown compounds, the decreased and increased m/z values with a correlation were extracted by estimation of the peak shape and complete separation during the extracted ion monitoring.