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Short Communication

NATURAL PRODUCTS

Development of Method for Evaluation of Edible Bird's Nest Content in Ready-to-Eat Beverages

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Abstract

Background: Edible bird's nest (EBN) is one of the most valuable tonic Chinese foods, made from glutinous salivary secretion with highly concentrated mucin glycoprotein. For ease of consumption, manufacturers have marketed different ready-to-eat EBN products, in which the EBN content varies. This is the first study to analyze the EBN content in ready-to-eat beverages.
Objective: To determine the EBN content in ready-to-eat beverages by its active ingredient, *N*-acetylneuraminic acid (sialic acid).
Method: Sialic acid in ready-to-eat beverages and raw EBN was extracted in sodium hydrogen sulfate solution, followed by derivatization using *o*-phenylenediamine dihydrochloride and determination using high-performance liquid chromatography (HPLC). Method precision, recovery of extraction, degradation of sialic acid due to cooking, and measurement uncertainty were evaluated.
Results: The mean concentrations of raw EBN in different origins and colors ranged from 5.77 to 10.92%. Ten different brands of traditional ready-to-eat EBN beverages from the market were analyzed, in which estimated concentrations of EBN were diversified, ranging from 0.014 ± 0.010 to 0.66 ± 0.069% (w/w) (95% confidence level). The concentration of sialic acid was found to range from 11.4 to 527 mg/kg.
Conclusions: Based on the results, sialic acid content can provide a better estimation of the EBN content in traditional ready-to-eat beverages. Neither the selling price nor dried matter could be used as an indicator of the quality of the ready-to-eat EBN beverage among the samples obtained.
Highlights: Sialic acid can be used as an indicator to estimate EBN content, where the sialic acid and EBN content in ready-to-eat beverages from the market were found to vary significantly.

The increase in the use of Chinese tonic foods is a global trend, where falsification of some expensive Chinese tonic foods, such as edible bird's nest (EBN), has become a significant quality issue for the Chinese tonic foods industry. EBN, also called "Yan Wo," is made of condensed salivary secretion containing amorphous mucin glycoprotein, which is obtained from three major species of swiftlets, including *Collocalia esculent* (white belly swifts), *Aerodamus maximus* (black-nest swiftlets), and *Aerodamus fuciphagus* (white-nest swiftlets) (1, 2). Traditionally, in Chinese communities, dried raw EBN would be used in the preparation of a beverage, which is believed to have various functions in health promotion. Studies have shown that EBN may possess therapeutic effects (3), such as inhibiting influenza viral infection (4), anticancer (5), skin lightening (6), and enhancing epidermal growth for fast recovery (7). Some of these therapeutic effects may be caused by the sialyl-sugar chains in EBN (8–10), where one of the major active ingredients in EBN, *N*-acetylneuraminic acid (sialic acid), is responsible for the

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suppression of viruses and regulation of proinflammatory cytokines and chemokines, for influenza, and even for severe acute respiratory syndrome coronavirus (SARS-CoV-2) infections. Although sialic acid can also be found in other animal sources such as beef, the concentration of sialic acid is low (< 1%) when compared with EBN (>5%) (11).

For ease of consumption, manufacturers prepare traditional ready-to-eat EBN beverages by cleaning raw EBN prior to cooking in water with the addition of rock sugar (12). Due to the high value and labor-intensive cleaning process, there are rising concerns about the authenticity of EBN, especially in the preparation of ready-to-eat beverages, as the increase of EBN in ready-to-eat beverage products would significantly increase the cost, which would decrease the price competitiveness of the products. To mislead consumers into believing that beverage products contain more EBN, manufacturers may add hydrocolloids to achieve the desired viscosity and texture of these products (13). Current approaches to determine glycoprotein content (14, 15) and protein profiling (16) can only identify raw EBN qualitatively, which cannot be used in ready-to-eat beverage products mixed with a small amount of EBN and other adulterants. As most studies have focused only on authentication of raw EBN, there is a need to develop a method to authenticate and quantify EBN in ready-to-eat beverage products.

The sialic acid in glycoprotein (17) has been widely used as an indicator to assess the quality of EBN, due to its abundance and uniqueness (18–21). Based on the results from Tung et al. (22), the average concentration of sialic acid found in different dried raw EBNs was about 10.8%. As You et al. (23) also reported a similar concentration (10.47%), the concentration of sialic acid may be used in estimation of the content of EBN in ready-to-eat beverages. In this study, a method from Feng et al. (24) that was originally designed for raw EBN was modified in determining sialic acid content in ready-to-eat EBN beverages. By following the joint EURACHEM/CITAC guide in quantifying measurement uncertainty in analytical measurement (25), we can estimate the percentage of EBN added to the ready-to-eat EBN beverage products.

Experimental

Raw EBN, EBN Products, Chemicals and Reagents

Ten different samples of ready-to-eat EBN beverage products were obtained from the local market, with the details listed in Table 1. Except Samples 1 and 2, which stated the content of EBN added (2.8% and 4.0%, respectively), all other products did not specify the concentration of EBN added. For the volume, except Sample 4, which was packaged in a smaller 45 mL bottle, others were marked with similar volumes (from 70 to 75 mL) or net weight (70 g).

To verify the sialic acid concentration in EBN from the literature, six samples of raw EBN in different colors and origins were obtained (Table 2). All samples were stored at room temperature (~20°C) according to the manufacturers' instructions.

O-Phenylenediamine dihydrochloride, phosphoric acid, sodium hydrogen sulfate, and *n*-butylamine were purchased from Alfa Aesar Co. Inc. (United Kingdom). Sialic acid (CAS number 131-48-6, from *Escherichia coli*, ≥ 98%) solvents of chromatographic grade and others were obtained from Sigma-Aldrich (St. Louis, MO, USA). All solvents were filtered through 0.22 μm PTFE syringe filters (Membrane Solutions, LLC, Shanghai, People's Republic of China) before use. Ultrapure water (resistivity >18.2 MΩ cm^{-1}) from the GenPure system (Thermo Scientific, MA, USA) was used for all sample preparation. All solvents were filtered through 0.22 μm PTFE syringe filters before use.

Extraction of Sialic Acid From Raw EBN and Ready-to-Eat EBN Beverage Products

The sample preparation was modified from Feng et al. (24), in which the ready-to-eat EBN beverage products were homogenized in a stomacher. After homogenization, 100 mg of each of ready-to-eat EBN beverage product was weighed and mixed with 1 mL of 0.5 mol/L sodium hydrogen sulfate solution. The mixture was incubated at 80°C in a water bath for 30 min to facilitate extraction. After cooling, 1 mL of derivatization reagent, which was made by dissolving 2 g of *o*-phenylenediamine dihydrochloride in 100 mL of 0.25 mol/L sodium hydrogen sulfate solution, was added to the mixture. The resulting mixture after derivatization was filtered with a 0.45 μm PTFE syringe filter (Membrane Solutions, LLC, Shanghai, People's Republic of China) prior to high-performance liquid chromatography (HPLC) analysis. All samples were tested in triplicate. A method blank was analyzed per each batch of samples and was prepared by following the extraction procedures above with the use of ultrapure water.

To verify the sialic acid concentration in raw EBN (*see* Table 2), 10 mg of each of raw EBN was weighed and followed the extraction and derivatization above to determine the concentration of sialic acid. All samples were tested in triplicate.

To validate the recovery of sialic acid content by cooking, laboratory-made ready-to-eat beverage samples were made by mixing 10 mg of each of the dry raw EBN (*see* Table 2) with 5 mL of ultrapure water, followed by cooking at autoclave conditions at 15 psi and 121°C for 30 min (26, 27). The resulting laboratory-made ready-to-eat beverage samples were then extracted and tested for the sialic acid content by following the same

Table 1. Ready-to-eat EBN beverage products obtained in this study

Sample	Product	Volume/net weight per unit bottle	Country of origin
1	Blumarine authentic bird's nest	75 mL	Thailand
2	Ninest bird's nest beverage	75 mL	Thailand
3	Bwell bird's nest beverage	75 mL	Thailand
4	Dok Bua Ku Twin Lotus bird's nest beverage	45 mL	Thailand
5	Wing Cheong Tong bird's nest with rock sugar	70 g	Thailand
6	Brand's bird's nest with rock sugar	70 g	Malaysia
7	Indonesian Premium	70 mL	Indonesia
8	Pure Health bird's nest with rock sugar	70 mL	Thailand
9	Fu Kin bird's nest beverage	70 g	Thailand
10	Genuine Swallow bird's nest with natural rock sugar	70 g	Thailand

Table 2. Raw EBN powder obtained in this study

Sample	Color	Country of origin	Sialic acid content in raw EBN (% by weight)	Recovery of sialic acid from laboratory-made ready-to-eat beverages, %
S1	White	Thailand	10.68 ± 2.60	98.7 ± 10.7
S2	Yellow	Singapore	6.45 ± 0.68	97.6 ± 1.1[a]
S3	Golden	Malaysia	5.77 ± 0.78	95.8 ± 10.3
S4	White	Indonesia	10.68 ± 0.72	106.7 ± 9.10
S5	White	Indonesia	10.92 ± 0.96	108.3 ± 5.7
S6	Yellow	Malaysia	6.77 ± 0.66	108.8 ± 11.22

[a] Significant difference from 100% recovery (1-sample *t*-test, $P < 0.05$).

extraction procedures as the market samples, and the recovery by cooking was evaluated by comparing the sialic content with the study of sialic acid in corresponding raw EBNs. All samples were tested in triplicate.

To assess the extraction efficiency, a recovery test ($n = 3$) was conducted by spiking a known concentration of sialic acid to the laboratory-made ready-to-eat beverage samples, followed by the above extraction and derivatization to determine the sialic acid content. Recovery was calculated by deducing the sialic acid found with those in laboratory-made ready-to-eat beverages without any spiking of sialic acid.

Determination of Sialic Acid Content

The sialic acid of all samples was determined by an Agilent 1290 LC system (Agilent Technologies, Santa Clara, CA, USA), which comprises a diode-array detector (DAD) with detection wavelengths at 230 nm. Chromatographic separation was achieved on an Agilent TC-C18(2) column (4.6 × 250 mm × 5 μm) (Agilent Technologies, Santa Clara, CA, USA), with the column temperature at 35°C. The mobile phase was composed of 95:5 1% tetrahydrofuran, 0.5% phosphoric acid, 0.15% *n*-butylamine aqueous buffer, and acetonitrile at a flow rate of 1.0 mL/min with isocratic elution conditions. A series of five calibration standard solutions (1, 5, 10, 20, and 50 mg/L) was prepared by dissolving an appropriate amount of sialic acid in ultrapure water. An equal volume of each of the calibration standard solutions was mixed with the derivatization reagent, and the resulting mixtures after derivatization were filtered with a 0.45 μm PTFE syringe filter prior to calibration of HPLC.

Determination of Mean EBN Content

From both the literature information and our experimental findings below, most of the traditional ready-to-eat EBN beverage products from the market with white or yellow EBN fragment/powders contained a range of 5–11% sialic acid. The mid-point value from the range was thus assigned to be 8%, which was used in estimating the mean EBN content from the mean sialic acid content by the use of Equation 1:

$$mean\ EBN\ (\%\ by\ weight) = \frac{[mean\ sialic\ acid\ content\ (mg/kg)]}{8\% \times 10,000} \quad (1)$$

Determination of Dried Weight Content

The filter papers used were pre-dried in an oven at 105 ± 2°C for 2 h and then cooled in a desiccator prior to weighing. Then, 3 g of each homogenized ready-to-eat EBN beverage sample was weighed and filtered with a pre-dried filter paper (Grade No. 2, Advantec MFS, Inc., California, USA). The residues retained on the filter paper were rinsed with 50 mL water three times. After rinsing, the filter paper with residue was placed in an oven at 105 ± 2°C for 2 h and cooled in a desiccator prior to reweighing. All samples were tested in triplicate. The dried weight of sample *w*, expressed as mass fraction in percent, of the sample on a dry basis is given by Equation 2:

$$w = \frac{m_1 - m_2}{m_0} \times 100\% \quad (2)$$

where, m_0 = mass in grams of the ready-to-eat EBN beverage; m_1 = mass in grams of the dried filter paper with residue; m_2 = mass in grams of the pre-dried filter paper.

Determination of Relative Standard Uncertainty

According to the EURACHEM/CITAC guide (25), we can evaluate the relative standard uncertainty (u) of precision and recovery from experimental observations (type A) by using Equation 3:

$$u = \frac{\left(s/\bar{x}\right)}{\sqrt{n}} \times 100\% \quad (3)$$

where, s = standard deviation; $\bar{x}$ = mean; n = number of trials.

For relative standard uncertainty by means of other than experimental observations (Type B), such as the standard uncertainty due to conversion from sialic acid to ENB content that is based on the literature information on sialic acid in different types of EBN, assuming a rectangular probability density function, it can be estimated by the use of Equation 4:

$$u = \frac{\pm y}{\sqrt{3}} \times 100\% \quad (4)$$

where, u = standard uncertainty (Type B) by means of other than experimental observations, and y = semi-range of probability density function.

After evaluating the standard uncertainty components, the combined uncertainty and expanded uncertainty (with an assumption of coverage factor $k = 2.00$ for 95% confidence level) by using Equation 5 and Equation 6:

$$u_c = \sqrt{(u_{precision})^2 + (u_{recovery})^2 + (u_{sialic\ acid})^2} \quad (5)$$

$$U = k \times u_c \quad (6)$$

where, u_c = combined uncertainty; $u_{precision}$ = standard uncertainty of the precision of sialic acid content; $u_{recovery}$ = standard

uncertainty of the recovery of sialic acid content; $u_{sialic\ acid}$ = standard uncertainty of the variation of sialic acid content in raw EBN; U = expanded uncertainty (with 95% confidence level when $k = 2.00$); k = coverage factor (assuming a Gaussian probability density function of the measurement uncertainty, for a 95% confidence level).

Data and Statistical Analysis

Statistical analysis of the collected data in this study was performed by using GraphPad Prism 8.3.0. The mean and standard deviation of the replicates from each experiment were calculated. To determine any significant difference for recovery of sialic acid content, one sample *t*-test was applied, with *P* value at 0.05.

Results

For the method validation of sialic acid content, the chromatographic peak of sialic acid (with retention time at 27.7 ± 0.05 min) in all samples was resolved to the baseline. Calibration of sialic acid was conducted using linear regression, with the correlation coefficient (R^2) ≥0.99. The limit of detection and limit of quantitation were found to be 0.06 mg/L and 0.2 mg/L, respectively, which were determined by analyzing blank solutions. The limit of quantitation was further verified by the signal obtained from the diluted standard solution against the calibration curve, and it was found to be well below the lowest calibration standard solution (i.e., 1 mg/L) and the concentration of sialic acid in all samples.

Table 3 shows the sialic acid content of the ready-to-eat EBN beverage samples, where the mean concentrations ($n = 3$) varied from 11.4 to 527 mg/kg. Relative standard deviations ranged from 3 to 62%, with an increase in magnitude at lower concentrations.

By the use of Equation 3, the relative standard uncertainty in precision ($u_{precision}$) was found to range from 2.3 to 36%.

For the dried weight content, the mean value ($n = 3$) varied from 0.280 to 1.03%. The relative standard deviations of dried weight were found between 1.5 and 12.8%, representing good homogeneity during sample preparation.

Table 2 shows the sialic acid found in raw EBN samples, with the mean concentrations ($n = 3$) ranging from 5.77 to 10.92% (by weight). Furthermore, the mean recovery of ready-to-eat beverage samples made from these raw EBN samples was found to be between 95.8 and 108.8%. Except sample S2, which was found to be statistically different from 100% recovery ($P = 0.025$), the recovery of other laboratory-made ready-to-eat beverage samples was not significantly different from 100% recovery ($P > 0.05$).

For the recovery study by spiking a known amount of sialic acid to a laboratory-made ready-to-eat EBN beverage (i.e., made of sample S2), good recoveries between 99.3 and 106.7% (with relative standard deviation of 3.94%, $n = 3$) were achieved, and the mean recovery was not significantly different from 100% recovery ($P > 0.05$).

Discussion

This is the first study to investigate the content of EBN in traditional ready-to-eat beverages. Sialic acid was chosen as the indicator to estimate EBN content due to its high abundance, and it was also used as a chemical marker to identify the presence of raw EBN (18, 19) and as an active ingredient for assessing the quality of EBN (20, 21). Although sialic acid and analogues of sialic acid can be synthesized by microorganisms (28–30), the cost to falsify EBN by fortifying synthetic sialic acid in ready-to-eat beverages to is still high when compared with adding authentic EBN. It is thus not very common in the ready-to-eat EBN industry. On the other hand, sialic acid was found in other food sources (11, 31), including crucian egg (0.46%), egg yolk (0.11%), egg white (0.04%), milk (0.02%), cheese (0.02%), and yogurt (0.016%). However, the taste, color, and texture of these ingredients were incompatible with the traditional ready-to-eat EBN beverages made of EBN in water (with the presence of rock sugar or not), and the sialic acid from non-EBN ingredients was 10–100 times less than that in EBN. Therefore, the approach of testing sialic acid in the determination of EBN can be applied to traditional ready-to-eat EBN beverages. In cases where EBN is added to other nontraditional foods and beverages composed of ingredients with high sialic acid content, the method may become invalid and the EBN content may need to be adjusted by deducing proportions from other sources of sialic acid.

Among the 10 samples, Sample 7 was the second most expensive product in the samples, but its sialic acid content found was the second lowest. Samples 4, 6, and 10 contained the top three concentrations of sialic acid, but the selling price was found to be below the average price among the 10 samples. Based on the above information, selling price cannot be used as an indicator of the amount of EBN used and the quality of the ready-to-eat EBN beverage among the samples obtained. By the use of Equation 3 for Type A standard measurement uncertainty, the relative standard uncertainty in precision ($u_{precision}$) of the 10 samples was calculated by dividing the relative standard deviation ($s/\bar{x}$) by the square root of the number of trials (i.e., 3) and was found to range from 2.3 to 36.0% (Table 3). In general, the lower the EBN content, the greater the relative standard uncertainty in precision, which aligns with the findings from Horwitz et al. (32).

For the recovery study, the mean and relative standard deviation of recovery by spiking a known amount of sialic acid to a laboratory-made ready-to-eat EBN beverage were found to be between 99.3 and 106.7% and 3.94%, respectively. By the use of Equation 3 for Type A standard measurement uncertainty, the relative standard uncertainty of recovery ($u_{recovery}$) was around 2.28% (where $n = 3$), calculated by the relative standard deviation of recovery. There was no significant difference when comparing the mean recovery to 100% recovery ($P < 0.05$); thus, no adjustment to the standard uncertainty of recovery was required (25). Among different extraction methods, acid hydrolysis using sodium hydrogen sulfate is the most commonly used extraction solvent for sialic acid in EBN (23, 24, 33–35). Feng et al. (24) reported a recovery between 85.03 and 97.14% for the extraction of sialic acid in raw EBN samples by sodium hydrogen sulfate, which was comparable to the recovery found in this study for spiking of sialic acid in laboratory-made ready-to-eat EBN beverages. Since sialic acid may be degraded during cooking, a study was conducted comparing the sialic acid extracted from raw EBN samples and the corresponding laboratory-made ready-to-eat EBN beverage products (Table 2), in which high mean recoveries were observed (between 95.8 and 108.8%). Cooking of ready-to-eat EBN beverages at temperatures ≥100°C, especially in autoclave conditions, to achieve sterilization is very common for ready-to-eat EBN products (26, 27); autoclave conditions (i.e., 15 psi and 121°C for 30 min) were chosen in this study. From the results of the recoveries, sialic acid content obtained after the cooking process was not significantly different from direct extraction from the raw EBN, which implies negligible degradation of sialic acid by cooking of traditional EBN beverages even at the high temperature and pressure

Table 3. Sialic acid and dried weight content of ready-to-eat EBN beverage products

Sample	Sialic acid concentration, mg/kg	Relative standard uncertainty of precision, %	Dried weight content (% by weight)	Expanded measurement uncertainty of EBN content (95% confident level)	EBN content (% by weight) in sample (with 95% confidence level)
1	15.3 ± 3.6	14	0.280 ± 0.027	± 27.9%	0.019 ± 0.0053
2	124 ± 11.9	5.6	0.995 ± 0.128	± 12.5%	0.15 ± 0.019
3	212 ± 8.6	2.3	0.733 ± 0.078	± 7.4%	0.27 ± 0.019
4	250 ± 10.0	2.3	0.771 ± 0.056	± 7.4%	0.31 ± 0.023
5	11.4 ± 7.1	36	0.839 ± 0.041	± 72.3%	0.014 ± 0.010
6	527 ± 40.0	4.4	0.743 ± 0.037	± 10.5%	0.66 ± 0.069
7	16.9 ± 6.7	22.8	1.038 ± 0.119	± 46.0%	0.021 ± 0.0097
8	53.5 ± 1.6	1.8	0.993 ± 0.026	± 6.7%	0.067 ± 0.0044
9	16.1 ± 7.6	27.3	0.948 ± 0.015	± 55.0%	0.020 ± 0.0011
10	323.4 ± 76.9	13.7	0.802 ± 0.092	± 28.0%	0.40 ± 0.11

conditions of autoclave. Raw EBN is commonly treated by heating (e.g., ≥100°C for 3 h) and hydrogen peroxide cleaning for removal of fungi and/or bleaching (26), but none of these processes have reported any significant deterioration of sialic acid content in raw EBN from literature review. Therefore, the stability of sialic acid as a marker in estimation of EBN content in ready-to-eat beverages is satisfactory.

Although there is no indication from the claims on the labeling whether the contents of EBN added were dry weight, the dried weight contents of Samples 1 and 2 found (0.28 and 0.99%, respectively) in this study were much less than the claimed amount of EBN added (2.8 and 4.0%, respectively). Manufacturers should improve the claims to indicate whether dry or wet weight is referenced in order to let consumers compare between products. From the results obtained, dried weight content may provide some indication as to the falsified claim of EBN added as most EBN contents are insoluble in water even after cooking. Furthermore, Sample 1 contained the lowest value for both the dried weight and sialic acid, reflecting that it may contain the smallest amount of EBN and was the worst quality among the samples. Samples 5, 7, 8, and 9 were found to contain a relatively large amount of dried matter, but the concentration of sialic acid was low, implying that most of the dried weight may be contributed by the addition of non-EBN adulterants (36) such as hydrocolloids, pork skin, and Tremella fungus, to increase the biomass and thus the product's solid texture. Samples 4 and 6 had a similar amount of dried matter, but the sialic acid concentration in Sample 6 was found to be more than double that in Sample 4. Based on the results of the sialic acid and dried weight content, there is no direct relationship between dried matter in the traditional ready-to-eat beverages and the sialic acid/EBN added. However, comparing dried matter and sialic acid content can still provide some indication as to the falsified claim of EBN added and the adulteration of other ingredients.

To estimate the concentration of EBN added in the ready-to-eat beverage samples, a conversion factor is required to be applied to the sialic acid content found. Six raw EBN from different origins and with different colors were tested to determine their sialic acid content. The relative standard deviations were found within 15%, reflecting a good repeatability of the analysis. Low variations from the results observed were also caused by even distribution of sialic acid in the EBN and a stable level of sialic in the saliva of swiftlets (35), which make sialic acid a good marker for estimation of EBN content.

The sialic acid content from the raw EBN obtained was similar to other studies. Kathan and Weeks (37) first reported about 9% sialic acid in collocalia mucoid. Another example, Careena et al. (34), analyzed seven different EBN samples from Malaysia, where the sialic acids ranged from 5.47 to 11.00%; there was no significant decrease in the sialic acid content for EBN collected from a heavily polluted industrial area. Some studies found that EBN with different colors (from swiftlets of different species and with different habitats) resulted in different levels of sialic acid. Yang et al. (21) conducted a survey of sialic acid from EBN with different colors, where imperial (white) EBN had the highest mean concentration (10.86%) and grass (green) EBN contained only 7.61%. Quek et al. (33) also observed a difference between house (white) EBN and cave (colored) EBN, where the mean sialic acid content was 13.6 and 8.3%, respectively. Observations from the above studies were consistent with our results, where white EBN in general contained higher mean sialic acid content (10.68–10.92%). A significantly lower sialic acid was found in EBN with yellow and golden colors (5.77–6.77%). From both the literature information and our experimental findings, since most of the traditional ready-to-eat EBN beverage products from the market used white or yellow EBN powders, sialic acid in a range of 5–11% and the mid-point at 8% with a semi-range of ±3% was deemed to be appropriate to estimate the EBN content in the products. By the use of Equation 4 with the assumption of a Type B measurement uncertainty with rectangular distribution of probability density function (semi-range = ± 3%), the standard uncertainty due to the variation of sialic acid ($u_{sialic\ acid}$) would thus become 1.73%.

After evaluating all the standard uncertainty components (i.e., $u_{precision}$, $u_{recovery}$, and $u_{sialic\ acid}$), the expanded uncertainties of the EBN content of the traditional ready-to-eat products can be evaluated by Equation 5 and Equation 6 and are summarized in Table 3, ranging from ±6.7 to ±72.3% (with a 95% confidence level), with more than 10 times difference between the lowest and highest measurement uncertainty. By comparing with the magnitude of different standard uncertainty components, the major contributor to the expanded uncertainty was found to be the precision (i.e., $u_{precision}$), which may be contributed by factors including sample variations, consistency of operators, stability of equipment, and the effect of the Horwitz function (32). By the

use of expanded uncertainties obtained, the range of EBN content in each of the ready-to-eat beverage samples can be determined by multiplying the mean EBN content with the expanded uncertainty (Table 3). The estimated EBN content from the samples (with a 95% confidence level) ranged from 0.014 ± 0.010% (the lowest, i.e., Sample 5) to 0.66 ± 0.069% (the highest, i.e., Sample 6). From the results obtained, there was more than 47 times difference between the lowest and highest mean EBN content, indicating a large variation of EBN added and/or quality of EBN used in the traditional ready-to-eat beverages in the market.

Conclusions

All in all, the determination of sialic acid content in ready-to-eat beverages can be a way to estimate the EBN content, where the recovery and repeatability of the method in the determination of sialic acid content were found to be acceptable.

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Conflict of Interest

The authors declare no conflicts of interest.

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