PRELIMINARY STUDY ON FREE SIALIC ACID CONTENT OF EDIBLE BIRD NEST FROM JOHOR AND KELANTAN

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ABSTRACT. Edible bird's nest (EBN) is made from the saliva of swiftlet from the *Aerodramus* species. It is one of the most widely consumed traditional health food by the Chinese community due to its claimed medicinal value. EBN contains glycoproteins with abundance of sialic acid (SA). EBN with higher SA content has a potential to command for higher price. The objective of this study was to determine and compare free SA content of EBN collected from Johor and Kelantan. A total of 23 and 30 of raw and unprocessed EBN samples were obtained from Kelantan and Johor, respectively. SA from EBN samples were analysed using LC-MS/MS. Johor showed higher content of free SA with the value of 135.04 ± 29.60 mg/kg compared to Kelantan which has a value of 95.55 ± 25.6 mg/kg. Highest content of free SA was found in EBN from Pontian district, Johor.

Keywords: edible bird's nest, sialic acid, LC-MS/MS

INTRODUCTION

Edible bird's nest (EBN) is made from regurgitated saliva of swiftlet from the *Aerodramus* species. It is one of the premium foods among the Chinese community worldwide and it is one of the most expensive animal products consumed by human. The EBN is also esteemed for its high medicinal value, which includes anti-ageing, growth promoting and immune enhancing properties. Nowadays, the edible bird's nest has been developed into value added products, including mixed congee, can, mask, face cream, etc. (Zhang *et al.*, 2012). Three major categories of bird's nest products (EBN) exported from Malaysia are raw-unclean EBN, raw-clean EBN and its value added products. Projected an export value of EBN by 2020 is RM5.2 billion with increasing annual production of 11.6% (MOA, 2011).

EBN contains glycoproteins with abundant sialic acid-containing sugar chains. Marcone (2005) reported the carbohydrate content of the edible-birdnest

to be 27.26%. According to Kathan and Weeks (1969), the carbohydrate component in edible-birdnest consists of 9% sialic acid, 7.2% galactosamine, 5.3% glucosamine, 16.9% galactose, and 0.7% fructose.

Sialic acids (SA) are a family of nine-carbon acidic monosaccharides that occur naturally at the end of sugar chains attached to the surfaces of cells and soluble proteins. N-acetyl neuraminic acid is one of the most predominant SAs occurring in nature (Shaw *et al*., 2001). Wang and Brand-Miller, 2003 has suggested that the exogenous source of SAs plays a role in brain development and learning ability. In Malaysia, SA could be a potential criteria to be used for determining the price of EBN. Thus, the aim of this study is to determine and compare the free SA content of raw- unprocessed EBN between two states in Malaysia.

MATERIALS & METHOD

Reagents

Reference standard of N-acetylneuraminic acid (sialic acid, SA) was obtained from Sigma-Aldrich Chemie (Steinheim, Germany). For LC/MS/MS analysis formic acid and acetonitrile were purchased from Merck (Darmstadt, Germany). Water was purified by the Milli-Q Biocel (Bedford, MA, USA).

Standard Solutions

Primary stock solution containing 1000 mg/mL of the active substances was prepared in methanol and stored at -20 °C when not used. Working standard solution of SA was freshly prepared by diluting intermediate standard solution with water.

Samples collection and preparations

Raw un-processed white EBN samples were obtained from state of Kelantan and Johor. These nests comprised of man-made bird housed.

The samples were cleaned from dirt and the visible feathers were manually removed using forceps. The nests were finely ground using a grinder. The ground samples were then kept in the container labelled accordingly and kept at room temperature.

Sample Preparation

10 mg each of crushed raw EBN in micro centrifuge tube were dissolved in 1 ml water. The samples were then vortexed thoroughly and sonicated for 5 min. Following centrifugation at 13,000 rpm for 10 min, the clear supernatant was transferred to an autosampler vial and 5 µl of supernatant was injected into the LC/MS/MS system.

Instrumental analysis

For analysis, a 1290 series UHPLC system coupled to a 6490 Triple Quadrupole (QqQ) mass spectrometer (Agilent Technologies, Waldbronn, Germany) was used. The 6490 QqQ system was equipped with an Agilent Jet-Stream ESI interface and was operated by Mass Hunter Workstation B.04.01 software. The analytical column used was a ZORBAX RRHD Eclipse Plus C_{18} (100 × 2.1 mm, 1.8 µm) column from Agilent Technologies. Chromatographic separation was performed at 30 °C with a flow rate of 500 µL min^{-1}. Eluent A was composed of water/formic acid (99.9:0.1, v/v) and eluent B of acetonitrile/formic acid (99.9:0.1, v/v). The total run time of the chromatographic run was 3 min comprising an initial hold time of 0.5 min at 10% B and a linear gradient to 80% B within 2 min. It was then returned to its original proportion of 10% within 0.1 min and held for 0.9 min, which allowed the column for equilibration. Analysis was carried out using the dynamic multiple reaction monitoring mode and fast polarity switching. The general source settings in the negative ionisation modes were as follows: gas temperature, 300 °C; gas flow, 12 $Lmin^{-1}$; nebulizer, 45 psi; sheath gas temperature, 250 °C; sheath gas flow, 11 $Lmin^{-1}$; capillary voltage, 3 000 V and nozzle voltage, 0 V. The fragmentor voltage was 380 V for both mass transitions, and both scanning quadrupoles (Q1 and Q3) were set to unit resolution.

Negatively single charged ion of *m/z* 307.99 was selected as parent ion for SA. For multiple reaction monitoring, the transitions *m/z* 307.99 → 86.9 was selected for quantification of SA and *m/z* 307.99 → 170 was used as qualifiers.

Quantification and Quality control

A five-point matrix calibration was prepared at the concentrations range of 50 mg/kg to 250 mg/kg. A 5 µL aliquot of the obtained solutions was loaded on the HPLC column for the MS/MS analysis. Calibration curve was made by plotting the peak area SA versus the analyte concentration of the calibration standard. A calibration standard, blank and spiked samples were subjected to the same sample preparation procedure. Quality control samples (QC samples) were prepared at three different levels 50 mg/kg, 100 mg/kg and 200 mg/kg.

The limit of detection (LOD) and the limit of quantification (LOQ) were defined as the lowest concentration with a signal-to-noise ratio of 1:3 and 1:10, respectively. The concentrations of unknown samples were then calculated by interpolating their SA peak area on the calibration curve.

Statistical Analysis

Results were statistically analysed using Analysis of Variance (ANOVA) test at 95% confidence interval with Tukey HSD for comparing the mean SA content

between districts (by SPSS® version 16 for Windows).

RESULTS AND DISCUSSION

The detection of SA in EBN was performed using the LC-MS/MS method with an electrospray ion source operating in negative ion mode. Calibration with matrix matched was employed for quantification. All standards and positive controls were spiked with a known SA and were subjected to the same sample extraction procedures.

The correlation coefficient of the linear least-squares fit of the calibration

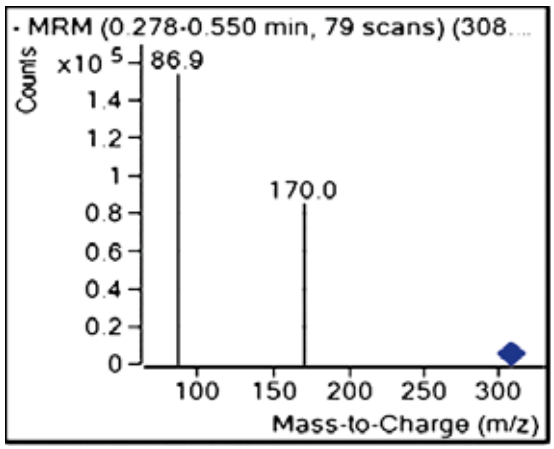

Figure 1. Product ion scan of SA, m/z 307.99, showing the most intense fragment ions at m/z 86.9 and 170

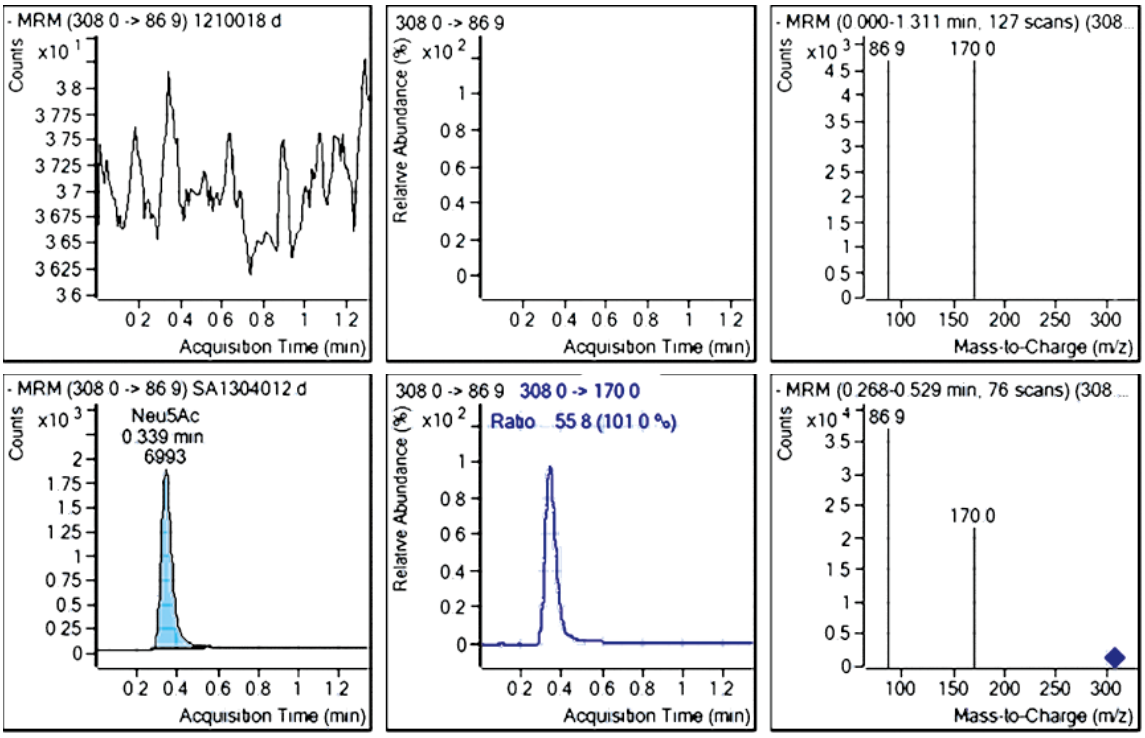

Figure 2. Multiple reaction monitoring (MRM) chromatogram of a blank sample (top row) and EBN sample spiked with 50 mg/kg (bottom row).

Table 1. Mean Sialic Acid Content from Johor and Kelantan

Kelantan (n=23)		Johor (n=30)	
District	**Mean (mg/kg)**	**District**	**Mean (mg/kg)**
Pasir Puteh	68.92[b]	Batu Pahat	107.04[b]
Pasir Mas	73.56[ab]	Kluang	116.25[ab]
Gua Musang	73.65[ab]	Muar	118.98[ab]
Tanah Merah	81.91[ab]	Segamat	122.04[ab]
Gong Manok	89.65[ab]	Kulai	124.00[ab]
Machang	93.86[ab]	Johor Bahru	142.00[ab]
Jeli	94.88[ab]	Tangkak	142.45[ab]
Kota Baru	118.00[ab]	Mersing	154.28[ab]
Bachok	132.00[a]	Kota Tinggi	157.79[ab]
		Pontian	165.57[a]
Average	**95.55A**	**Average**	**135.04B**

[a-b] Mean values with different lowercase superscripts within the same column are significantly different ($p<0.05$)

[A-B] Mean values with different uppercase superscripts within the same row are significantly different ($p<0.05$)

curve was found to be 0.9973. The peak area ratios of the SA transition reactions were found to be within 54.7 ± 0.5. The spiking recoveries were 98.9% ± 4.4. The limit of detection (LOD) was found to be 10 µg/kg with a signal-to-noise ratio greater than 3. This value is sensitive for detection of SA in the samples.

The mass spectrum for SA (Figure 1) exhibits a deprotonated molecular ion at *m/z* 307.99 and major fragment ions at *m/z* 86.9 and at *m/z* 170. The most abundant product ion was *m/z* 86.9, therefore it was chosen for quantification. The respective product ions (*m/z* 86.9 and *m/z* 170) presented in the samples can be used to differentiate between genuine and fake/adulterated EBN in relation to SA. Blank samples did not show interference and the equivalence in analyte was null. Figure 2 shows typical MRM chromatograms of blank and spiked EBN samples.

EBN samples were analysed using the optimised method and the results for SA content in 53 samples from Johor and Kelantan are shown in Table 1. The SA content ranged from 68.92 mg/kg to 132 mg/kg and 107.04 mg/kg to 165.57 mg/kg for samples obtained from 9 districts in Kelantan and 10 districts in Johor, respectively. The mean SA content in EBN from Kelantan (95.55 mg/kg) was significantly lower ($p<0.05$) than that of Johor (135.04 mg/kg). Among different districts in Kelantan, the SA content in EBN from Pasir Puteh was significantly lower ($p<0.05$) than Bachok. The highest SA content in Kelantan was found in samples from Bachok while in Johor, it was samples from Pontian. In fact, the SA content in samples from Pontian was

found to be the highest compared to all other districts in both states. Meanwhile, the mean SA content from other districts in Kelantan and Johor showed no significant difference ($p>0.05$). Colombo *et al.*, 2003 reported that improvement in neurological and intellectual development of swiftlets' infants were associated with SA supplementation. Higher concentration of SA from Pontian compared to other districts in Kelantan and Johor might be due to the condition of surrounding habitats, availability and abundance of food source in this area.

CONCLUSION

In this study, free sialic acid was quantified using rapid and high-throughput method. More comprehensive study needs to be conducted involving more samples from different states in order to confirm the present findings.

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