Determination of Edible Bird's Nest and Its Products by Gas Chromatography

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Abstract

A specific gas chromatographic (GC) detection method for edible bird's nest (EBN) based on identifying the composition of the oligosaccharide chain combined with glycoprotein in EBN is developed. Five monoses (D-mannitose, D-galactose, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, and *N*-acetyl neuraminate) that constitute the oligosaccharide chain are detected using GC and GC–mass spectrometry techniques; their characteristic GC spectrum can reliably be regarded as EBN's fingerprint. The peak-area ratios in GC spectrum of those five monoses are found to be fixed; therefore, the GC technique developed in this work can conveniently be used to determine various raw EBNs and their products both qualitatively and quantitatively, distinguishing between fake and genuine EBN rapidly.

Introduction

Edible bird's nest (EBN) is essentially woven by Swiftlet or Collocalia (*Apodidae*) from gelatinous strands of its saliva mixed with minor feathers. Traditionally, EBN has been considered nourishing and a booster of health for the sick and aging (1). In Swiftlet habitats in southeast Asia and Pacific Islands, collecting their nests on cliffs is a potentially hazardous occupation; therefore, EBN is so expensive that sometimes fake EBN is sold on the market.

There was no previous detection method available to test EBN specifically. Empirical measures such as visual examination, burning tests, and coloring checks have been previously used. Later, EBN content in food products was estimated by way of determining the protein or sialic acid (SA) in it (2,3). Unfortunately, other ingredients in EBN products interfered with the determination. The present goal was to seek a specific and reliable, yet simple and convenient, analytical procedure to detect EBN based on the characterization of the molecular structure of EBN.

It was known that the nest contains 50~60% protein, 20~30% carbohydrate, hexosamine, SA, etc. (1,4). Biochemical judgment was that there must be plenty of glycoprotein in EBN made from Swiftlet saliva, although no previous study on EBN glycoprotein was referenced. Glycoproteins exist universally in organisms, but the structure of their oligosaccharide chains combined with proteins are diversified, offering the possibility to differentiate glycoproteins from each other. Therefore, an EBN detection method based on the identification of EBN oligosaccharide chain composition will be specific and characteristic.

Other edible plant or animal ingredients are often added in large quantities to EBN products sold in the market, seriously interfering with previous methods of EBN detection. Gas chromatography (GC) possesses the capability of separating and determining some derivative monoses simultaneously; it can separate other ingredients or interferences in the sample from EBN's monoses as well if proper pretreatment of the GC sample is adopted. Further investigation of GC spectrum that performed a fingerprint function for EBN showed that the peak-area ratios among those monoses were fixed for EBNs from different places of origin. This made a GC technique a better qualitative and quantitative detection method for EBN than other existing procedures.

Experimental

Reagents

N-Acetyl-D-galactosamine (GalNAc, purity > 98%) and *N*-acetyl-D-glucosamine (GNAc, purity > 99%) were from Fluka (Bellefonte, PA). *N*-Acetylneuraminic acid (NANA, purity > 95%) was from Sigma (Bellefonte, PA). D-Mannose (Man) and D-galatose (Gal) (biochemical reagent) were made in Shanghai. *n*-Nonadecanoic acid was of chromatographic purity. Acetic anhydride, pyridine methanol, sulfuric acid, and barium carbonate were of analytical purity.

Apparatus

The apparatuses consisted of a Varian (Santa Clara, CA) 3400 GC/TSQ-70 mass spectrometer (MS) spectrograph with an electron-impact ion source (70 eV) and a Hewlett-Packard (Palo Alto, CA) HP5890A GC with a flame ionization detector (FID). GC conditions are for a 25-m × 0.32-mm HP-5 (Hewlett-Packard) fused-silica capillary column. The column temperature was programmed as follows: splitless/split injection at 110°C, isothermol for 1 min, a 20°C/min temperature ramp to 210°C, and then a 6°C/min ramp to 270°C, held for 10 min. The injection port was kept at 250°C. The detector was kept at 270°C. The inlet N_2 pressure was 50 kPa.

Sample pretreatment

Raw EBN

Methanolysis. Approximately 7~8 mg of crushed raw EBN was weighed out, and the sample was put into a glass vial with a Teflon sealing screw for derivatization. After 0.5 mL of 1M sulfuric acid–methanol was added, the vial was filled with N_2 and closed. Then the vial was heated in an oven for 90 min at 90°C. Once it cooled down, the reactant in the vial was trasferred into a centrifuge tube, and 0.3 g of barium carbonate was added to neutralize the sulfuric acid in the solution. The tube was placed on an ultrasonic vibrator for 5 min and then centrifuged. The supernatant was transferred into a test tube with a ground-glass cover. The tube was vacuumed at approximately 35°C to evaporate the methanol.

Acetylization. Then, the dried product of methanolysis was in the test tube. Pyridine (1 mL) and acetic anhydride (0.5 mL) were added into the tube. The tube was covered and shaken. Then, the tube was placed into an oven for 30 min at 90°C, taken out of the oven, and allowed to cool. One hour later, the sample was injected for GC.

EBN products

The original contents were taken out from the bottled EBN product (soup) and diluted with an equal amount of water. The diluted sample was homogenized for 100 s at 7000 rpm. An aliquot of ~ 30 mL of the homogenized sample was poured into a preweighed dialysis bag to dialyze the sample for 24 h in a water flow. Then, the contents in the bag were transferred thoroughly into a beaker to dry the sample. The dried, all-solid sample was weighed. The solid sample was ground, and 7~8 mg of the sample powder was weighed out. The sample was processed in the same way as for raw EBN after the internal standard sample *N*-nonadecanoid was added.

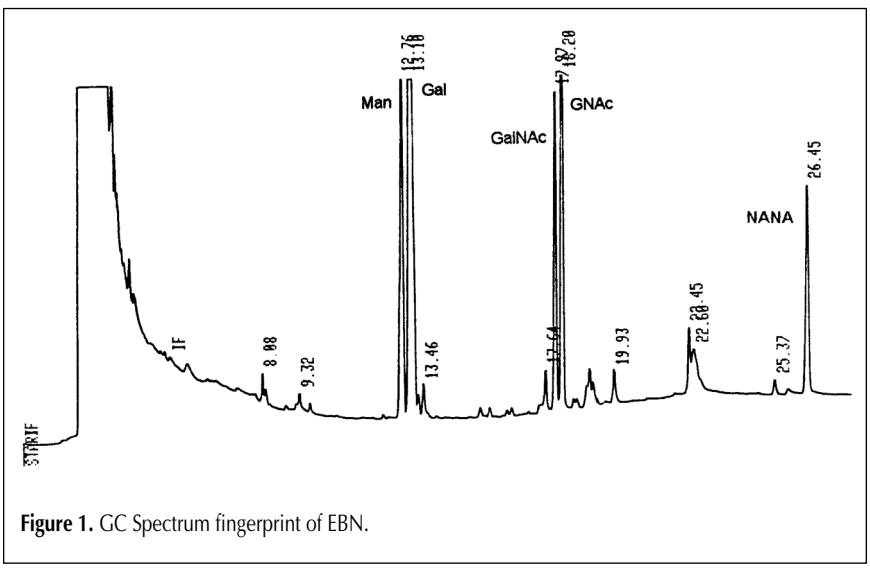

Figure 1. GC Spectrum fingerprint of EBN.

Results and Discussion

Separation and identification of the monose constituents of oligosaccharide chain in EBN glycoprotein

The oligosaccharide chain of glycoprotein in EBN combines with peptides in the form of glucoside. It was necessary that the glucoside be hydrolyzed into monoses and then derivatized into volatile matter for GC detection.

Because the goal of this work was to develop a rapid determination method for EBN, methanolysis was thus chosen to simplify the pretreatment of the sample without separating the protein in advance. Chemicals were tried as received to prepare H_2SO_4–CH_3OH as the reagent for methanolysis of glycoprotein, whereas the usual method is to use HCl–CH_3OH prepared with dried HCl. H_2SO_4–CH_3OH is a suitable and convenient reagent here.

Under the given conditions of methanolysis and acetylizating, the derivatized products from the monoses constituents of the oligosaccharide chain in EBN produced a clear GC spectrum, as shown in Figure 1, after passing through an HP-5 capillary chromatographic column. The GC spectra for different grades of EBN (such as white EBN, black EBN, and blood EBN) collected from various places were all the same with minor differences in the monoses content from each other.

Through GC–MS identification and checks against standard monose samples, it was found that the peaks in EBN GC spectrum (as shown in Figure 1) represented Man, Gal, GalNAc, GNAc, and NANA. All of them were pyranoses in the form of methyl glycoside. Acetylization was complete: one peak in the GC spectrum corresponded one monose only. No multipeak was observed for a monose. No remarkable nonmethylate or divaricate reaction occurred, even when the reagents of sulfuric acid and methanol normally contained some water.

During the course of methanolysis, fatty acid in the sample was also methylesterified. Fortunately, the fatty acid content in EBN was less than 1%; its peak in the spectrum was not notable. Although GC also detected some derivative amino acids from the hydrolysis of proteins, under given pretreatment conditions, only a little protein hydrolyzed, which did not interfere with the separation of monoses. Instead, it performed a supporting role in forming a unique fingerprint for EBN together with

five characteristic peaks of monoses.

Sulfuric acid–methanol dissociation curves for EBN oligosaccharide chain

It was verified that five monoses that constituted the oligosacchride chain in EBN could be divided into three categories: hexose, hexosamine, and sialic acid. Their dissociation rate from the chain could be different if they connected to the chain at different locations. The sulfuric acid–methanol dissociation of the five monoses conducted in this work is shown in Figure 2 in the form of dissociation yield versus dissociation time at different dissociation temperatures.

Two dissociation curves for the two hexoses look similar. The similarity applies to two hexosamines also. The dissociation curve for SA is quite different from the other two groups; in other words, the dissociation yield for SA decreases as the dissociation time is longer and the temperature is higher than 90°C, which means that SA is chemically unstable at high temperatures or in an acidic medium.

There is a property common to the five groups of curves: the higher the temperature, the bigger the dissociation rate will be, and at 90~100°C, the dissociation reaches saturation after 1 h. Therefore, it is appropriate to methanolize EBN for 1~2 h at 90°C so that the methanolysis products of EBN are mainly the aforementioned five monoses with minor methanolysis products from protein or other components in EBN.

One more thing that should be noted is that some pentaacetate pyranoses are produced in the reactant if a small amount of water is involved in the sample or reaction container, because water interferes with the completion of methylation and thus affects the detection quantitatively. Therefore, attention must be paid to the dryness of the sample during the course of its pretreatment.

Comparison among different grades of EBNs collected from various places

GC was conducted on 12 different raw EBNs collected from various places. The results show that their GC spectra were all the same shape and could be regarded as the fingerprint for EBN.

Through the calculation of peak areas in GC spectra representing the contents of five monoses in EBN and the comparison of them with each other, something interesting was revealed. No matter what grade of EBN the samples belonged to or where they were from, the peak-area ratio of GNAc–Man was approximately 0.97 (relative deviation < 10%). The peak-area ratio of Gal–Man remained almost the same for black EBN and blood EBN, except for black and blood EBNs collected from Thailand, whose peak-

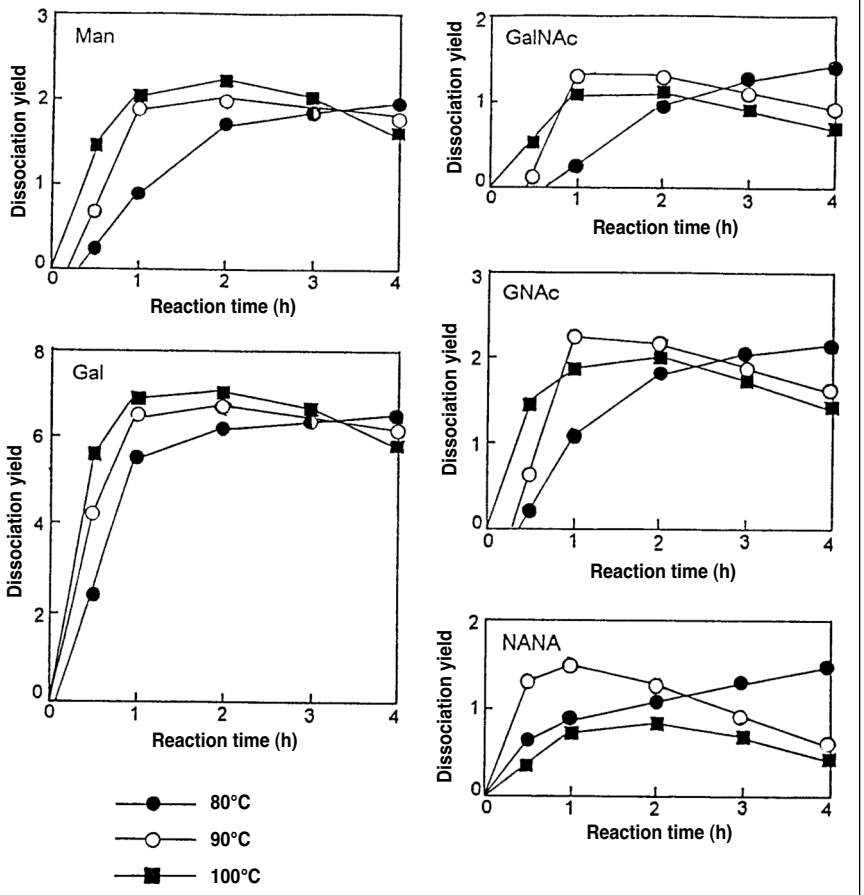

Figure 2. Dissociation curves for methanolysis of EBN.

Table I. Peak-Area Ratios of Monoses in EBN from Different Places of Origin*

Origin	Sample	Group	Monoses		
			Gal	GalNAc	GNAc
Indonesia	house EBN "cup"	1	2.61	0.508	0.974
Malaysia	cavern EBN "cup"	1	2.40	0.523	0.920
Thailand	cavern white EBN "cup"	1	2.51	0.572	0.946
Shanghai	white EBN "cup"	1	2.59	0.524	1.04
Thailand	blood EBN	2	2.88	0.496	0.981
Thailand	EBN pieces	2	2.92	0.542	0.995
Indonesia	black EBN pieces	3	3.61	0.477	0.958
Indonesia	blood EBN pieces 1	3	3.73	0.474	0.967
Indonesia	blood EBN pieces 2	3	3.77	0.556	0.993
Malaysia	black EBN pieces	3	3.21	0.495	0.904
Hong Kong	EBN "ball"	3	3.53	0.543	0.907
Shenzhen	black EBN pieces	3	3.43	0.581	0.946

* Peak area of Man taken as 1.00 (FID).

area ratio of Gal–Man was remarkably lower than those for black and blood EBNs collected from Indonesia and Malaysia. As for white EBNs collected from various places, the peak-area ratios of GalNAc–Man and GNAc–Man were close to those for black and blood EBNs, whereas the peak-area ratio of Gal–Man was ~ 30% lower than that for black and/or blood EBNs (Table I).

The existing data acquired in this work were convincing enough to conclude that the origin of EBN had a minor effect on the composition of oligosaccharide chain in EBN (as far as the twelve samples covered were concerned). The physiological changes of the Swiftlet probably explained the difference in peak-area ratios between white EBN and black or blood EBN.

The composition of oligosaccharide chain combined with glucoprotein in EBN is undoubtedly a specific identity, like a fingerprint for EBN, and can be used to distinguish fake EBN from genuine EBN. The quality grade of genuine EBN can further be determined according to peak-area ratios in GC spectrum for the five monoses in EBN, distinguishing black or blood EBN from the white one.

Distinguishing between genuine and fake EBN

Distinguishing was done straightforwardly for raw EBNs in question or crystal sugar–EBN soups in question by checking their GC spectra against the GC spectrum fingerprint produced by genuine EBN.

It is reported that things often disguised as EBN are plant polysaccharides such as agar, alginate, gum, white fungus and animal glue such as pigskin, egg white, and gelatin. Recently, EBN products sold on the market were mixed soups with additives such as ginseng, hashima, Chinese caterpillar fungus, etc. This makes the identification and detection of EBN even more complicated.

The protein and fat from the aforementioned "disguises" and additives after methanolysis and acetylization as described above did not interfere with the GC spectrum produced from the five monoses in EBN, although the dissociation products from some polysaccharides might introduce some problems. If that is the case, making use of the fixed peak-area ratios among Man, Gal, GalNAc, and GNAc to distinguish the tested samples is recommended. For example, it was determined that monose peak-area ratios (average value taken) for EBNs of different origins were as follows: GNAc–Man = 0.97 ± 0.07, GalNAc–GNAc = 0.52 ± 0.05 (for 12 different raw EBN, Table I), Gal–Man = 3.5 ± 0.3 (for black and/or blood EBN), Gal–Man ≈ 2.9 (for black and/or blood EBN from Thailand), and Gal–Man = 2.5 ± 0.1 (for white EBN).

Attention should be paid to one phenomenon. Hexosamine and SA in EBN decomposed to a certain degree during food processing, so that peak-area ratios of GNAc–Man < 0.9 in the majority of EBN products, and the NANA peak even disappeared from the GC spectrum for some EBN products. Obviously, this was because EBN was in a medium with too high an acidity or was overheated, leading to the decomposition of some hexosamine and SA, lowering the quality of EBN and its effectiveness. The GC technique developed as a detection method of EBN displayed yet one more advantage for the aforementioned problem of EBN's quality.

Figure 3 is a GC spectrum of a rock sugar–EBN soup from Hong Kong. It contained more EBN, and its spectrum was clean. Peak-area ratios of Gal–Man (3.3) and GalNAc–Man (0.47) belonged in typical EBN product; only the lower GNAc–Man (0.75, lower than 0.97) showed that the amino sugar in EBN oligosaccharide chain was damaged slightly. For another rock sugar–EBN made in Singapore (Figure 4), the peak-area ratios of Gal–Man (2.8), GNAc–Man (1.03), and GalNAc–Man (0.55) were considered from EBN of Thailand origin. This GC spectrum looked like some other peaks because a lot of alginate was added to the soup.

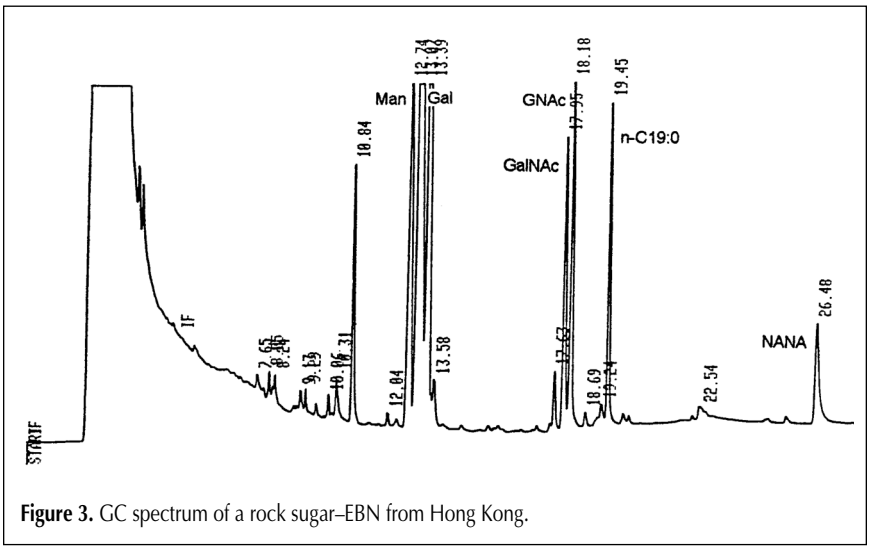

Figure 3. GC spectrum of a rock sugar–EBN from Hong Kong.

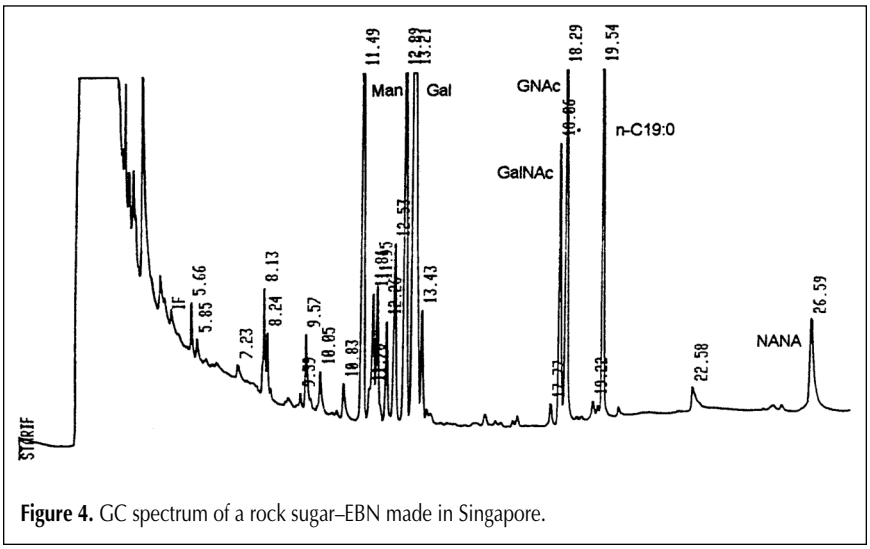

Figure 4. GC spectrum of a rock sugar–EBN made in Singapore.

Stability of derivatives from monoses connected to the oligosaccharide chain in EBN

Samples in this work were preserved in acetylization reagent (pyridine–acetic anhydride solution) after methanolysis and acetylization. The experiment showed no remarkable changes in GC detection quantitatively after the samples were preserved this way for more than 1 week at room temperature, with a coefficient of variation (CV) less than 5% for all the monoses except NANA (Table II), which means that samples preserved in the acetylization reagent were stable. There was no need to transfer samples into other solvents.

One EBN specimen was sampled several times between May and November, pretreated, and GC tested. The results are listed in Table III. The CV for Man, Gal, and GNAc were all within 7%, although NANA had a CV value of 31% and GalNAc (whose content was low in EBN) had a CV value of 9.1%. It is clear that the sample pretreatment procedure adopted in this work acquired stable GC results, making the technique pragmatic for EBN determination with good reproducibility.

Quantitative GC analysis of EBN products

In this work, it was found that the contents of monoses connected to the oligosaccharide chain in EBN were similar for black and blood EBN collected from different places, whereas the contents in white EBN of different origins were slightly lower. That being the case, black and white EBN collected in Indonesia (whose production and marketing are thriving) were used as references for black or blood EBN products and white EBN products, respectively, to conduct quantitative GC analysis.

Among the five monoses combined with glucoprotein in EBN, the content of GalNAc was the lowest, causing a large detection error. NANA was unstable, resulting in poor repetition of its experimental data. All three remaining monoses (Man, GNAc, and Gal) were good enough that their peak areas in GC spectrum could alternatively be used to calculate EBN content in products, depending on what kind of interferences were involved in the sample tested.

The internal standard method was used to minimize experimental errors resulting from sample loss after pretreatment and chromatographic condition uncertainty. The criteria to choose the internal standard sample were that (*a*) it would have a moderate retention time and (*b*) its peak would not be interfered with by miscellaneous peaks. *n*-Nonadecanoic acid was chosen in this work for this purpose.

The FID detector used in this work had a wide response linearity that was fit for quantitative analysis. EBN content as low as 0.01% was detected in 1 mL of sample taken from an EBN soup using the GC technique developed in this work. EBN recovered from crystal sugar–EBN soup processed through the experimental procedure described in this work was 87~108%.

Table II. Preservation Test Results for a Pretreated EBN Sample*

Duration of preservation (days)	GC peak-area ratio							
	Man		GalNAc		GNAc		NANA	
1	0.299	0.293	0.233	0.211	0.345	0.324	0.284	0.218
1	0.302	0.298	0.230	0.228	0.335	0.338	0.287	0.284
2	0.293	0.295	0.231	0.230	0.327	0.341	0.246	0.269
2	0.293	0.303	0.208	0.238	0.315	0.342	0.233	0.316
5	0.298	0.291	0.250	0.223	0.350	0.331	0.238	0.197
5	0.295		0.229		0.334		0.223	
8	0.292	0.292	0.229	0.223	0.335	0.327	0.198	0.188
8	0.293	0.295	0.230	0.234	0.337	0.339	0.190	0.214
Mean	0.295		0.228		0.335		0.237	
CV	1.26%		4.54%		3.04%		16.6%	

* Preservation temperature of the sample was room temperature.

Table III. GC Determination Results of Repeated Samplings from the Same EBN Specimen*

Experiment date	Peak-area ratio†					Pretreatment (temperature, time)	
	Man	Gal	GalNAc	GNAc	NANA	Methanolysis	Acetyzation
960516	1.95	6.85	1.32	2.27	1.51	90°C, 60 min	90°C, 60 min
960516	1.94	6.68	1.31	2.04	1.20	90°C, 120 min	90°C, 60 min
960612	1.93	6.54	1.49	2.20	1.56	90°C, 90 min	25°C, 18 h
960612	2.06	6.58	1.62	2.35	1.69	90°C, 90 min	90°C, 60 min
960613	1.88	6.28	1.43	2.13	1.34	90°C, 90 min	90°C, 60 min
961105	1.97	6.47	1.40	2.09	0.80	90°C, 90 min	60°C, 90 min
961106	1.97	6.60	1.31	2.04	0.69	90°C, 90 min	90°C, 30 min
961106	2.01	6.56	1.67	2.44	0.76	90°C, 90 min	90°C, 30 min
Average	1.96	6.57	1.44	2.19	1.19		
CV	2.6%	2.3%	9.1%	6.3%	31%		

* Every single datum listed in the table is the mean of 3~4 runs.
† EBN specimen with a weight ratio of 58:1 to internal standard sample (*n*-Nonadecanoic acid).

Table IV. Determination EBN Contents (%) in Various EBN Products (Soup) by GC and Other Methods*

EBN Products	GC method				
	Protein method†	Man way	GNAc way	SA SP method‡	Amino acid method
Rock EBN A	1.68	1.50	1.17	1.18	1.33
Rock EBN B	1.49	0.84	0.84	0.73	1.17
Rock EBN C	0.71	0.88	0.73	0.53	0.24
Rock EBN D	0.50	0.27	0.14	0.19	0.21
Rock EBN E	0.52	0.40	0.14	0.036	0.076
Rock EBN F	0.75	0.52	0.28	0.088	0.047
American Ginseng EBN	1.08	0.61	0.33	0.011	0.26
Hashima EBN	0.45	0.37	0.22	0.18	0.11

* Average value of three different black EBN from Indonesian origin was taken as standard for content.
† Kjeldahl–Gunning method for determination.
‡ A spectrophotometry method for measuring sialic acid (3).

Comparison among protein, amino acid, and SA spectrophotometric method

Table IV compares the EBN contents in 8 EBN soup samples determined using different methods. Protein and amino acid methods are applicable only to crystal sugar–EBN soup without additives; unfortunately, the majority of EBN products sold on market contain additives. Spectrophotometric detection of EBN is based on the determination of SA resulting in a lower EBN content than that determined by a protein method and GC detection, because SA decomposed due to high acidity or high temperature during soup processing.

EBN contents detected using GC were calculated based on Man or GNAc peak area. The "Man way" judged the input of raw EBN before soup processing by comparing the results with data from a protein method, whereas the "GNAc way" showed the amount of EBN remaining in its products after soup processing by comparing the results with data from a spectrophotometric method.

Conclusion

Protein and fatty acid present in the sample in large quantities did not interfere with GC detection. Various "disguises" and additives present in the sample on the whole did not affect it either, but they did affect the spectrophotometric detection of the sample. The GC method detected EBN both qualitatively and quantitatively, simultaneously pointing out whether there were disguises and additives in EBN products or not and whether the food processing technology was proper or not. Therefore, the GC technique developed in this work is a promising, specific, and pragmatic determination method for EBNs and their products.

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