Journal of Food and Drug Analysis, Vol. 16, No. 4, 2008, Pages 86-91 藥物食品分析 第十六卷 第四期

Authentic Determination of Bird's Nests by Saccharides Profile

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(Received: March 30, 2007；Accepted: November 24, 2007)

ABSTRACT

A simple high performance ion liquid chromatography with pulsed amperometric detector (HPAE-PAD) was applied to the saccharide profile analysis for the authenticity identification of edible bird's nests. The glycoproteins in authentic edible bird's nests, as reference materials for this study, contain fucose (Fuc) 0.44%, rhamnose (Rha) 0.20%, galactosamine (GalN) 4.19%, glucosamine (GlcN) 5.29%, galactose (Gal) 5.03%, mannose (Man) 0.75% and N-acetylneuraminic acid (NeuAc) 10.8% ± 0.76. The NeuAc content was used as an index to estimate the content of authentic bird's nest contained in dried powder. The dried powder was prepared by using bird's nests obtained from retail stores, or from the gel substance of bottled products of bird's nest steeped in rock sugar solution (BNsrss). The quality of dried bird's nest was also judged by the NeuAc/GalN ratio. The NeuAc/GalN ratios in commercial products claimed as edible dried bird's nest ranged from 2.3 to 3.0, while those in dried powders from gel substance claimed as BNsrss ranged from 0.03 to 3.0.

Key words: bird's nest steeped in rock sugar solution (BNsrss)

INTRODUCTION

The edible bird's nest is built by swiflets of *Collocalia* spp. during the breeding season. The nest is cemented using the solidified saliva. The swiflets are found predominantly in southeastern Asia, and on islands of the Indian and Pacific Ocean(1). Due to the high economic value and limited natural authentic bird's nest, artificial bird's nest products made of various adulterants are quite widespread. Usual adulterants include jelly fungus, agar, sodium alginate, nature plant gum, pork skin, isinglass and egg white. This study was aimed to develop a method for the identification of the authenticity of commercially claimed bird's nest or dried powder of gel substances from products claimed as bird's nest steep steeped in rock sugar solution (BNsrss). High performance ion liquid chromatography with pulsed amperometric detector (HPAE-PAD) was employed to analyze the saccharide profiles of bird's nest and its processed products.

MATERIALS AND METHODS

I. *Reagents*

Fucose (Fuc), rhamnose (Rha), galactose (Gal), N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), glucose (Glc), mannose (Man), N-acetylneuraminic acid and trifluoroacetic acid were purchased from Sigma Chem. Co. (St. Louis, MO, USA). 50% (w/w) sodium hydroxide was obtained from Fisher Scientific Inc.（New Jersey, USA）.

II. *Bird's Nest Samples*

Six crude edible bird's nests mingled with feather were used as reference materials (BN.1-6). Six samples of processed edible bird's nest (CP.1-6) and twenty samples claimed as BNsrss products (S.1-20) were purchased from a grocery store in Taipei.

III. *Hydrolysis*

(I) *Neutral and Amino Sugars*

The of hydrolysis of glycoproteins, described by Fu *et al.*(2), was referred as Scheme 1. After being dried and ground, 50 mg each of bird's nest powder was dissolved in 2 mL 4 N trifluoroacetic acid (TFA) in a hydrolysis tube with a Teflon-lined screw cap. The hydrolysis tube was sealed and incubated at 121°C for 3 hours. After being cooled to room temperature, the hydrolysis tubes were opened, and each reaction mixture was evaporated under reduced pressure to dryness to remove residual

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TFA. The residue was then dissolved with distilled water to 1000 mL.

Gel substances steeped in rock sugar solution were filtered out, and then washed to remove sugars. After being dried and ground, the powders were subjected to the hydrolysis as mentioned above.

(II) *Preparation of Standard Solution of Neutral and Amino Sugars*

100 μg/mL Fuc, Rha, GalNAc, GlcNAc, Gal, Glu, Man were prepared by dissolving in 2 mL 4 N TFA. A series of 10, 5, 1, 0.5 and 0.1 μg/mL was further made, respectively.

(III) *N-acetylneuraminic acid*

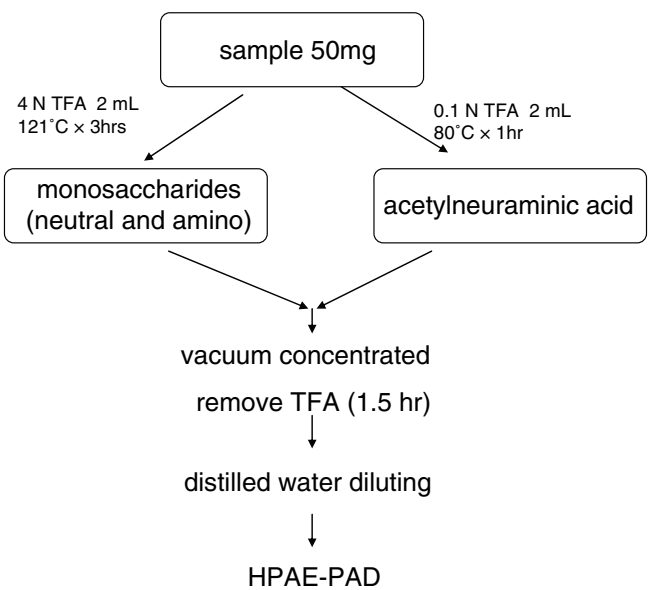
50 mg each of dried bird's nest powder or powder of BNsrss was dissolved in 2 mL 0.1 N TFA and incubated at 80°C for 1 h. The samples were then evaporated to dryness to remove residual TFA under reduced pressure. The residue was then diluted with distilled water to 1000 mL.

(IV) *Preparation of Standard Solution of N-acetylneuraminic acid*

100 μg/mL of N-acetylneuraminic acid was prepared by dissolving in 2 mL 0.1 N TFA and a series of 10, 5, 1, 0.5, 0.1 μg/mL was further made.

IV. *HPAE-PAD Analysis*

Analysis of monosaccharide was carried out by high performance ion liquid chromatography on Dionex DX-600, system equipped with LC20 (chromatography enclosure), ED50 (electrochemical detector, gold electrode), GP50 gradient pump, AS100 autosampler and Dionex peaknet 6.20 chromatography workstation (Sunnyvale, CA). The CarboPac PA1 column was run at isocratic conditions with 16 mM sodium hydroxide as the eluent at a flow rate of 1.0 mL/min. Due to the low concentration of sodium hydroxide used for the separation, the column needs to be regenerated after each run. Otherwise, carbonate will start to contaminate the column irrespective of the care taken to eliminate it from eluents and samples. The column was regenrated by washing it with 200 mM sodium hydroxide for 10 minutes at a flow rate of 1.0 mL/min. The procedure will also remove other strongly bound contaminants such as peptides and amino acids. After washing, the column should be re-equilibrated with 16 mM sodium hydroxide at a flow rate of 1.0 mL/min for ten minutes[3].



Scheme 1.

RESULTS AND DISCUSSION

I. *Determination of the Content of Neutral Sugar and Amino Sugars*

Neutral sugar and amino sugar components of bird's nests have been reported previously, where hexoses were determined by the orcinol method of Winwler[4], and hexosamine were quantitatively determined by the method of Elson and Morgan[5]. Glycoprotein contains about 7.2% galactosamine, 5.3% glucosamine, 16.9% galactose, and 0.7% fucose[6].

In this work, a simple and effective method has been developed by high performance ion liquid chromatography with pulsed amperometric detector (HPAE-PAD) for the determination of saccharide profile of bird's nests contents, which has been proven capable of determining hexoses and hexosamine simultaneously, needless of two separate methods, as mentioned above.

Authenticity of dried edible bird's nests could be identified by the relative composition of Fuc , Rha, GalN, GlcN, Gal, and Man. But most of bird's nest contents in BNsrss ratio is too low, and other materials like rock sugar, jelly fungus, agar and Nature plant gum could interfere the identification. In this paper, authenticity of edible bird's nests was examined by the relative composition of GalN, GlcN and Gal. The migration time correlation coefficient (r^2) for fucose, rhamnose, galactosamine, glucosamine, galactose, glucose, mannose and N-acetylneuraminic acid during HPAE-PED analysis are shown in Table 1. Neutral and amino sugars of dried bird's nests reference materials were analyzed and summarized in Table 2. The chromatogram of HPAE-PAD analysis of neutral and amino sugars derived from dried bird's nest of sample BN.6. is shown in Figure 1. The average ratio of GalN, GlcN and Gal contents in BN.1 to BN.6 was approximately 4.2 : 5.3 : 5.0. GalN content of CP.2 and CP.4 was

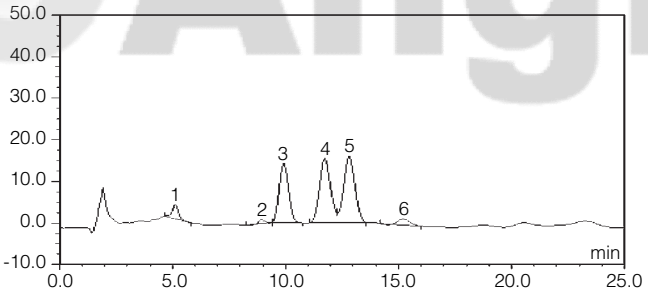


Figure 1. HPAE-PAD analysis of neutral and amino sugars derived from dried bird's nest of sample BN.6. Peaks identification: 1.fucose; 2.rhamnose; 3.galactosamine; 4.glucosamine; 5.galactose and 6.mannose.

Table 1. The migration time and correlation coefficients of 8 sugars standards of the HPAE-PAD method

Peak Name	migration time (min)	correlation coefficients r^2
fucose(Fuc)	5.25	0.9998
rhamnose(Rha)	9.17	0.9991
galactosamine(GalN)	10.25	0.9999
glucosamine(GlcN)	12.08	0.9998
galactose(Gal)	13.25	0.9997
glucose(Glc)	14.42	0.9989
mannose(Man)	15.75	0.9978
N-acetylneuraminic acid(NeuAc)	7.08	0.9999

higher than that of dried bird's nests reference material, whereas GalN content of CP.5 was lower. Gal content of CP.2, CP.3, CP.4 and CP.5 was higher than that of reference dried bird's nests. Reference dried bird's nests did not contain Glc, where as CP.4 contained 2%Glc, indicating that the sample might be non-authentic.

Saccharide profiles of bird's nest adulterants were summarized in Table 3. The jelly fungus contained Fuc (3.5%), Glc (16%) and Man (31%), respectively. Saccharide profiles of gel substance from products claimed as BNsrss were summarized in Table 4. The ratios of GalN, GlcN and Gal contents of S.1, S.2, S.11 and S.14 were similar to those of dried bird's nests, but S2 product contained agar, the Gal content was higher. As to the rest of the samples, some also contained GalN, GlcN and Gal, but their ratio was too low, and the others did not even contain detectable amount of GalN, GlcN or Gal, indicating that bird's nest in the samples might be non-authentic.

II. *Determination of the Content of N-acetylneuraminic acid*

The sialic acid component of bird's nests contained N-acetylneuraminic acid[6-10]. Sialic acid can be quantitatively determined by the thiobarbituric acid method of Warren and glycoprotein contain 9% sialic acid (Kathhan and Weeks, 1969). N-acetylneuraminic acid content and NeuAc/GalN ratio of dried bird's nest reference materials in this experiment were summarized in Table 5. The content of NeuAc ranges between 10.2-12.1%, where the average was 11.4%.

NeuAc/GalN ratio of BN.1-6 ranged from 3.0 to 2.3, NeuAc/GalN ratio of CP.2-5 ranged from 1.7 to 0.04. The

Table 2. Neutral and amino sugars contents in dried bird's nest

Sample Code	Origin	Neutral and Amino Sugars[a] (%)						
		Fuc	Rha	GalN	GlcN	Gal	Glc	Man
BN.1	Vietnam	0.41 ± 0.22	0.33 ± 0.04	4.42 ± 0.06	5.20 ± 0.13	4.72 ± 0.02	ND	0.70 ± 0.01
BN.2	Indonesia(A)	0.37 ± 0.13	0.19 ± 0.08	4.18 ± 0.12	5.54 ± 0.05	4.93 ± 0.08	ND	0.73 ± 0.15
BN.3	Indonesia(B)	0.45 ± 0.03	0.16 ± 0.20	4.03 ± 0.02	5.39 ± 0.08	5.38 ± 0.06	ND	0.99 ± 0.06
BN.4	Indonesia(C)	0.47 ± 0.18	0.22 ± 0.03	4.54 ± 0.04	5.64 ± 0.01	5.43 ± 0.11	ND	0.81 ± 0.00
BN.5	Indonesia(D)	0.36 ± 0.00	0.17 ± 0.05	4.08 ± 0.04	5.22 ± 0.12	4.58 ± 0.06	ND	0.77 ± 0.11
BN.6	Thailand(A)	0.59 ± 0.20	0.13 ± 0.06	3.91 ± 0.11	4.76 ± 0.08	5.11 ± 0.07	ND	0.48 ± 0.06
CP.1	Thailand(B)	0.78 ± 0.14	0.21 ± 0.05	3.79 ± 0.16	5.21 ± 0.04	5.20 ± 0.00	ND	0.66 ± 0.02
CP.2	Unknown1	0.64 ± 0.08	0.56 ± 0.11	6.08 ± 0.06	9.63 ± 0.04	14.03 ± 0.06	ND	0.05 ± 0.01
CP.3	Unknown2	ND[b]	0.28 ± 0.12	3.82 ± 0.02	4.59 ± 0.08	8.51 ± 0.28	ND	0.47 ± 0.06
CP.4	Unknown3	1.33 ± 0.00	0.19 ± 0.06	7.24 ± 0.05	8.80 ± 0.10	10.96 ± 0.17	2.0 ± 0.02	1.17 ± 0.04
CP.5	Unknown4	0.11 ± 0.02	0.02 ± 0.01	2.58 ± 0.14	4.93 ± 0.10	6.91 ± 0.06	ND	0.51 ± 0.10
CP.6	Unknown5	0.54 ± 0.18	0.24 ± 0.12	4.24 ± 0.01	5.72 ± 0.02	4.70 ± 0.13	ND	0.80 ± 0.01

a: mean ± standard deviation, n = 3.
b: ND: not determined.

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Table 3. Comparison of neutral and amino sugars contents of adulterants

Adulterant	Neutral and Amino Sugars[a] (%)						
	Fuc	Rha	GalN	GlcN	Gal	Glc	Man
jelly fungus	3.51 ± 0.01	ND	ND	ND	ND	16.26 ± 0.18	31.18 ± 0.41
agar	0.80 ± 0.06	ND	ND	ND	11.04 ± 0.30	3.51 ± 0.06	0.03 ± 0.00
sodium alignate	0.05 ± 0.00	ND	ND	ND	0.05 ± 0.00	0.02 ± 0.00	0.06 ± 0.02
nature plant gum	0.39 ± 0.12	11.04 ± 0.02	ND	ND	14.28 ± 0.15	ND	ND
pork skin	ND[b]	ND	ND	0.20 ± 0.01	0.22 ± 0.12	0.11 ± 0.06	0.12 ± 0.03
isinglass	ND	ND	0.05 ± 0.01	0.07 ± 0.00	0.11 ± 0.02	0.12 ± 0.03	ND
egg white	ND	ND	0.14 ± 0.05	3.31 ± 0.13	0.23 ± 0.05	ND	1.1 ± 0.16

a: mean ± standard deviation, n=3.
b: ND: not determined.

Table 4. Comparison of neutral and amino sugars contents of gel substance from products claimed as BNsrss

Sample No.	Neutral and Amino Sugars[a]（%）						
	Fuc	Rha	GalN	GlcN	Gal	Glc	Man
S.1	1.10 ± 0.12	0.10 ± 0.02	3.31 ± 0.11	3.12 ± 0.03	4.31 ± 0.05	14.96 ± 0.12	0.37 ± 0.6
S.2	0.48 ± 0.06	0.12 ± 0.01	3.43 ± 0.11	5.62 ± 0.10	8.28 ± 0.02	5.44 ± 0.31	0.49 ± 0.02
S.3	0.78 ± 0.12	0.15 ± 0.02	1.89 ± 0.06	2.94 ± 0.21	4.04 ± 0.02	23.26 ± 0.22	0.20 ± 0.00
S.4	0.81 ± 0.22	0.11 ± 0.03	1.96 ± 0.07	2.83 ± 0.07	3.71 ± 0.05	18.84 ± 0.35	0.24 ± 0.08
S.5	0.62 ± 0.08	0.06 ± 0.00	0.81 ± 0.02	0.78 ± 0.06	0.84 ± 0.00	18.06 ± 0.28	0.07 ± 0.00
S.6	0.48 ± 0.11	1.04 ± 0.03	ND	ND	ND	18.34 ± 0.18	ND
S.7	0.28 ± 0.17	ND[b]	ND	ND	ND	17.06 ± 0.25	0.19 ± 0.05
S.8	0.37 ± 0.04	0.27 ± 0.07	0.01 ± 0.00	ND	ND	20.78 ± 0.40	0.04 ± 0.00
S.9	1.28 ± 0.17	ND	ND	0.12 ± 0.01	ND	28.38 ± 0.26	8.0 ± 0.11
S.10	0.38 ± 0.05	ND	ND	ND	ND	22.08 ± 0.17	0.1 ± 0.05
S.11	1.05 ± 0.10	0.11 ± 0.02	3.3 ± 0.01	4.94 ± 0.06	6.21 ± 0.15	11.19 ± 0.21	0.80 ± 0.02
S.12	0.42 ± 0.00	ND	0.07 ± 0.00	0.07 ± 0.02	0.27 ± 0.05	8.54 ± 0.21	0.53 ± 0.05
S.13	0.65 ± 0.06	0.04 ± 0.00	0.14 ± 0.02	0.12 ± 0.01	0.35 ± 0.01	26.36 ± 0.29	0.04 ± 0.00
S.14	1.84 ± 0.02	0.28 ± 0.06	3.81 ± 0.01	5.45 ± 0.21	6.64 ± 0.16	15.51 ± 0.06	0.96 ± 0.08
S.15	0.75 ± 0.00	0.01 ± 0.00	0.19 ± 0.03	0.24 ± 0.02	0.47 ± 0.04	17.78 ± 0.14	2.42 ± 0.11
S.16	1.63 ± 0.11	0.01 ± 0.00	0.13 ± 0.04	0.22 ± 0.01	0.30 ± 0.06	18.46 ± 0.32	2.14 ± 0.06
S.17	0.81 ± 0.04	0.01 ± 0.00	0.12 ± 0.00	0.22 ± 0.08	0.29 ± 0.01	18.91 ± 0.45	5.36 ± 0.09
S.18	1.16 ± 0.15	0.01 ± 0.00	0.19 ± 0.05	0.23 ± 0.00	0.53 ± 0.05	20.51 ± 0.23	3.95 ± 0.12
S.19	1.64 ± 0.06	0.01 ± 0.01	0.15 ± 0.02	0.21 ± 0.02	0.42 ± 0.05	21.71 ± 0.28	5.02 ± 0.05
S.20	0.77 ± 0.04	0.01 ± 0.00	0.16 ± 0.02	0.20 ± 0.01	0.32 ± 0.08	21.32 ± 0.25	3.16 ± 0.15

a: mean ± standard deviation, n = 3.
b:ND:not determined.

chromatogram of HPAE-PAD analysis of NeuAc derived from dried bird's nest sample BN.1 was shown in Figure 2. CP.1 and CP.6 contained slightly lower sialic acid than dried bird's nest reference materials. CP.2, 3, 4 and 5 contained one half lower sialic acid than reference dried bird's nests. These indicated that CP.2, 3, 4 and 5 might

Table 5. Comparison of N-acetylneuraminic acid from different origin of dried bird's nest

Sample code	Origin	N-acetylneuraminic acid[a]	NeuAc/GalN
BN.1	Vietnam	10.25 ± 0.11	2.32
BN.2	Indonesia(A)	11.80 ± 0.21	2.82
BN.3	Indonesia(B)	12.12 ± 0.08	3.00
BN.4	Indonesia(C)	11.76 ± 0.15	2.59
BN.5	Indonesia(D)	11.70 ± 0.05	2.86
BN.6	Thailand(A)	10.55 ± 0.05	2.70
CP.1	Thailand(B)	9.08 ± 0.09	2.40
CP.2	Unknown1	1.72 ± 0.04	0.28
CP.3	Unknown2	2.77 ± 0.05	0.73
CP.4	Unknown3	0.34 ± 0.02	0.05
CP.5	Unknown4	4.41 ± 0.12	1.70
CP.6	Unknown5	9.19 ± 0.25	2.17

a: mean ± standard deviation, n = 3.

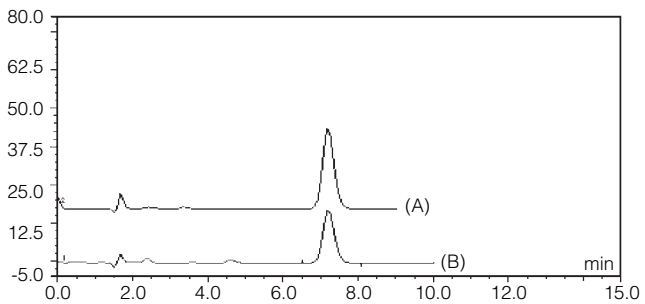

Figure 2. HPAE-PAD analysis of (A)N-acetylneuraminic acid standard (B) N-acetylneuraminic acid derived from dried bird's nest sample BN.1.

be non-authentic. CP. 4 contained only 0.34% sialic acid and the bird's nest might be built by some kind of swallow with materials such as grass, mud, and branch.

Egg white and pork skin contained 0.22% and 0.04% NeuAc respectively. The other adulterants did not contain NeuAc. NeuAc content and NeuAc/GalN ratio of dried powders obtained from gel substance in products claimed as BNsrss were summarized in Table 6. In S.6-9 NeuAc component was not detected. The other samples contained 0.02-5.56% NeuAc. NeuAC/Gal is a quality index for bird's nests, especially for BNsrss, in which not only does the added quantity of bird's nest vary, but also the quality of the added bird's nest raw material varies. Taking samples S12 and S2 in Table 6 for comparison, it can be seen that although the NeuAc content of S12 (i.e. 0.21%) is lower than that of S2 (i.e. 5.56%), the NeuAc/Gal ratio of S12 (i.e. 3.0) is actually significantly higher than that of S2 (i.e. 1.6). Therefore, bird's nest quantity

Table 6. Comparison of N-acetylneuraminic acid contents of BNsrss

Sample Code.	N-acetylneuramic acid[a]	NeuAc/GalN
S.1	3.86 ± 0.12	1.17
S.2	5.56 ± 0.16	1.62
S.3	3.02 ± 0.05	1.60
S.4	3.50 ± 0.02	1.79
S.5	1.45 ± 0.05	1.79
S.6	ND[b]	–
S.7	ND	–
S.8	ND	–
S.9	ND	–
S.10	0.03 ± 0.06	–
S.11	0.16 ± 0.04	0.05
S.12	0.21 ± 0.02	3.00
S.13	0.02 ± 0.00	0.14
S.14	0.11 ± 0.05	0.03
S.15	0.16 ± 0.01	0.84
S.16	0.25 ± 0.05	1.92
S.17	0.21 ± 0.06	1.75
S.18	0.17 ± 0.05	0.89
S.19	0.30 ± 0.05	2.00
S.20	0.45 ± 0.10	2.81

a: mean ± standard deviation, n=3.
b: ND:not determined.

alone is not sufficient to demostrate the quality of BNsrss. Rather, NeuAc/Gal ratio should be used to indicate the real quality, or authenticity, of bird's nest products.

NeuAc content analyzed by HPAE-PAD could be a sensitive indicator for the identification of the authenticity of bird's nest. The content of BNsrss was thus estimated by NeuAc content in our study.

III. *Calculation for Estimated of Percentage of Authentic Bird's Nest in Sample*

Percentage (P1) of N-acetylneuraminic acid in dried bird's nest reference material

= B μg/mL × dilute multiple ÷ 1000 μg/mg ÷ 50 mg (sample weight of dried bird's nest powder) × 100%

Percentage (P2) of N-acetylneuraminic acid in dried powders obtained from gel substance in products claimed as BNsrss

= C μg/mL × dilute multiple ÷ 1000 μg/mg ÷ 50 mg (sample weight of dried gel substance) × 100%

Authentic bird's nest content in each can of product claimed as BNsrss = P2 × A ÷ P1

A: Weight of dried gel substance in each can filter out gel substance from 5 bottles of canned products claimed as BNsrss. After drying gel substance to constant weight, calculate the average weight of the dried gel substance in each can.

B μg/mL: Concentration of N-acetylneuraminic acid (obtained by HPAE-PAD analysis) in sample solution prepared from dried bird's nest reference material.

C μg/mL: Concentration of N-acetylneuraminic acid (obtained by HPAE-PAD analysis) in sample solution prepared from dried gel substance.

CONCLUSIONS

The authenticity of marketed bird's nest can be easily identified through the saccharide profile analysis by HPAE-PAD method. N-acetylneuraminic acid content provides a basis to estimate the content of authentic bird's nest in sample. The content ratio of N-acetylneuraminic acid to GalN can serve as an indicator for identifying the quality of sample claimed as bird's nest.

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