

# Quantitative analysis of sialic acids in Chinese conventional foods by HPLC-FLD

Hongwei Li, Xingdan Fan

Department of Preventive Medicine, School of Public Health, Xiamen University, Xiamen, China; [rocque@xmu.edu.cn](mailto:rocque@xmu.edu.cn)

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## ABSTRACT

**Background:** Sialic acids are a family of nine-carbon sugar compounds with carboxylic acyl derivatives. It exists in bacteria, fish, mammals and other living organisms, participates in and regulates many important life events, such as cell recognition, membrane flow, endocytosis and so on. Sialic acid is usually located in the outermost layer of the sugar part of the cell membrane and the key positions of secreted glycoconjugates (glycolipids, glycoprotein and lipopolysaccharide). Sialic acid (Sia) is an important material foundation for variety of the structure and founction of glycoconjugates. Sia has been known as nearly 50 members, including N-acetylneuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5Gc) and deaminoneuraminic acid (KDN) as its core monomer. The rest of the sialic acids are derived from these three monomers. The contents of Sia in Chinese food products are unknown. **Objective:** To determine the contents of Sia in raw and cooked red meat, seafood, poultry and so on. **Design:** The following food products were purchased from a Chinese supermarket: pork, beef, lamb, salmon, cod, tuna, cow milk, cheese, butter, duck, chicken and chicken eggs. Human milk was collected from Xiamen Maternity and Child Health Care Hospital (Xiamen, China). All tissues were homogenized and hydrolyzed with 0.05 M, 0.1 M and 0.2 M TFA for 150 min at 80°C in dark, respectively. The concentrations of Neu5Ac, Neu5Gc and KDN were determined by using HPLC with fluorescence detector. **Results:** The contents of total Sia ( $\mu\text{g/g}$  tissue or  $\mu\text{g/mL}$  liquid sample) in Chinese raw meat were highest in lamb (269.60), followed by pork (254.88), duck (200.63), chicken

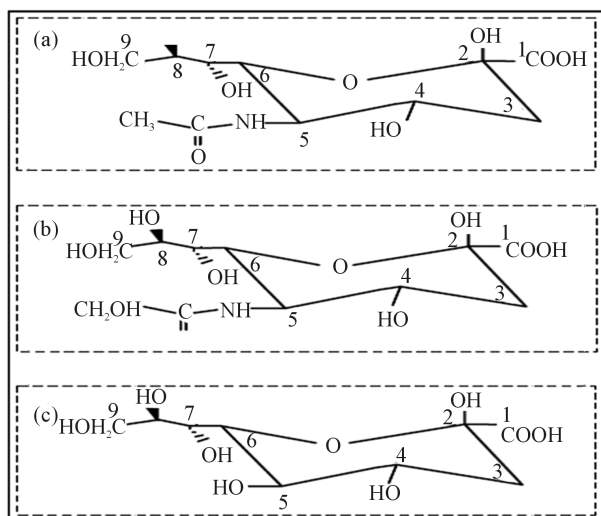
(162.86) and beef (88.03). The percentages of Neu5Gc were 36.08%, 26.48%, 0%, 0% and 28.40%, respectively. Cod contained higher levels of Sia (171.63) than salmon (104.43) and tuna (77.98). Only Neu5Ac was 50 found in detected aquatic product. Egg yolk contained the highest level of Sia (682.04), and a higher level of Sia (390.67) was found in the egg white. Also our result showed that human milk contained extremely high level of Sia (602.55). Neu5Ac was the predominant form of Sia in all the deteced samples. KDN was found in cow milk only among the samples, the content was 1.14  $\mu\text{g/g}$ . **Conclusion:** The highest content of Sia in examined Chinese foods was found in 56 eggs, followed by lamb, pork, duck, cod, chicken, salmon, beef and tuna. Knowledge of the Sia content in conventional foods may help us to better understand possible medical disorders involving the uptake of the “non-human” Neu5Gc from our diet.

## KEYWORDS

Quantitative Analysis; Sialic Acids; HPLC-FLD

## 1. INTRODUCTION

The family of sialic acids comprises approximately 50 members [1], all derivatives of the negatively charged 9-carbon sugar neuraminic acid. The predominant sialic acid family found in humans is from the N-Acetylneuraminic acid (Neu5Ac, **Figure 1(a)**) branch. Hydroxylation of the N-acetyl gives rise to N-glycolylneuraminic acid (Neu5Gc, **Figure 1(b)**), a branch present in animals including the great apes [2], yet absent in healthy humans since the gene responsible for N-acetyl hydroxylation of Neu5Ac to Neu5Gc has been rendered



**Figure 1.** (a) N-acetylneuraminic acid (Neu5Ac), (b) N-glycolylneuraminic acid (Neu5Gc), (c) Ketodeoxynonulosonic acid (KDN).

inactive. The different ratio of Neu5Ac to Neu5Gc in glycans is species-specific, tissue-specific and is specific for different body fluids [3]. An unusual modification is the presence of an additional hydroxyl group instead of the amino group at position 5 of the sugar, leading to 2-keto-3-deoxy-nonulosonic acid (**Figure 1(c)**) (keto-deoxy-nonulosonic acid, KDN) found in fish eggs (caviar) [4] and human ovarian cancer cells and ascites cells obtained [5].

Sia is found in the most vertebrate tissues and also in certain pathogenic bacteria. The mammalian central nervous system is one of the richest sources of Sia; specially brain gray matter, where Sia is mainly bound to gangliosides (65% of total brain Sia) and glycoproteins (37% of total Sia) [6]. Brain sialoglycopeptides have been shown to participate in the establishment of synaptic pathways, calcium transportation, binding of neurotransmitters, cell-to-cell interactions and axon regeneration [7,8].

The need for Sia by the developing brain, both in utero and after birth, cannot be satisfied by anabolic metabolism alone. It is assumed that significant amounts of Sia are provided through the placenta and that human milk is provided to Sia after birth, so an exogenous source of sialic acid plays an important role in rapid brain growth. Neu5Ac is the most common form of Sia in human glycoconjugates, including glycoproteins, glycosaminoglycans, gangliosides and mucins [8], and is expressed ubiquitously throughout the human body. Neu5Gc can be found at low levels in human carcinomas, fetal tissue, and normal tissue types, e.g., endothelium and epithelium of human surgical specimens at autopsy [9]. This finding implies that Neu5Gc originates from exogenous dietary sources [10], generally red meat and milk prod-

ucts [11].

The distribution of Sia is widely expressed on all mammalian tissues as either Neu5Ac, Neu5Gc, or more rarely as KDN. Animal foods, including red meat, fish and poultry, contain high-biological-value protein and important micronutrients that are required to sustain adequate human health throughout life. These animal foods also remain a core food in the diet of Chinese today.

There are no data about the contents of Sia in Chinese conventional foods until now, thus we analyzed them in raw and cooked red meat, seafood and poultry by using an established HPLC-FLD method recently.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals and Reagents

It is essential to use high-quality water of high resistivity (18 M $\Omega$  or better) that contains as little dissolved carbon dioxide as possible. Biological contamination should be absent. Neu5Ac, Neu5Gc and KDN from Sigma (St. Louis, MO, USA) were used as standards for identification and quantitation. 4,5-Methylenedioxy-1,2-phenylenediamine dihydrochloride (DMB) was also from Sigma. Methanol and acetonitrile (HPLC grade) were from SK Chemical (Seoul, Korea). Analytically pure trifluoroacetic acid (TFA), glacial acetic acid, sodium sulfite, sodium hyposulfite, 2-mercaptoethanol were from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 0.45  $\mu$ m microporous membranes were from Shanghai Chu-Ding Analytic Instrument Limited Company (Shanghai, China).

### 2.2. DMB Reagent

8 mM DMB, 1.5 M glacial acetic acid, 0.25 M sodium hyposulfite, 0.25 M sodium sulfite, 0.8 mM 2-mercaptoethanol.

#### Samples and Preparations

Food samples (beef, pork, lamb, chicken, duck, cow milk, cheese, egg, butter, ham, salmon, cod, tuna, corn and so on) were purchased in supermarket (Xiamen, China). Human milk was collected from Xiamen Maternity and Child Health Care Hospital (Xiamen, China).

#### Preparation of Raw Food Samples

1 g of edible part of food was weighed and homogenated with pure water and metered volume at 50 mL, liquid samples were diluted 8 times. 1 mL homogenated or diluent samples were hydrolyzed by 0.05 M TFA or 0.1 M TFA or 0.2 M TFA at 80°C for 1.5 h (according to food's distinct). Once cooled down to room temperature, hydrolyzed samples were filtered through 0.45  $\mu$ m microporous membrane, derived with DMB as followed. Briefly, sample (90  $\mu$ L) was mixed with DMB (10  $\mu$ L) reagent and then kept for 2.5 h at 50°C in dark. Use 10  $\mu$ L of the reaction mixture for HPLC analysis.

### Preparation of Cooked Food Samples

Edible part of food were weighed and recorded. Animal products were richly wrapped up by aluminium-foil paper and baked at 200°C for 20 min; egg and corn were boiled for 30 min. Cooked samples were weighed and recorded for calculating the ratio of raw to cooked. Then followed the methods of raw food samples treatment.

### Preparation of Mixed Standards

Different samples were hydrolyzed by different volume of TFA, mixed standards of Neu5Ac, Neu5Gc and KDN were prepared in parallel to samples. Triplet of mixture of Neu5Ac, Neu5Gc and KDN (1.0 mM each) were subjected to acid hydrolysis by isopyknic 0.2 M TFA, 0.1 M TFA and 0.05 M TFA under the same conditions as described for samples, respectively. Use the resulting mixture (0.5 mM each) for linearity, coefficient of recovery and limit of quantitation studies.

### 2.3. HPLC Separation of DMB Derivatives

HPLC system from Agilent (Santa Clara, USA) was used: an Agilent 1200 HPLC system equipped with a fluorescence detector, an autosampler coupled to a HPLC pump. Separation was carried out on a LiChrosorb RP-18 chromatographic column (250 mm × 4 mm, 5 μm) that was from Merck KGaA (Darmstadt, German). DMB derivatives of Sia were isocratically eluted using 7% (v/v) methanol, 8% (v/v) acetonitrile in high-quality water for 15 min at a flow rate of 0.9 ml/min. All injections were performed at 30°C. The eluant was monitored for fluorescence at 373 nm (excitation wavelength) and 448 nm (emission wavelength).

To ascertain the linearity and minimum detection limits of Neu5Ac, Neu5Gc and KDN, 3 injections (10 μL) of each of the dilutions were made (Table 1). Plot peak area against injection volume to determine linearity.

All samples were double treated under same condition,

**Table 1.** Data for preparing standard curve of sialic acid.

Concentration of standars in injections (μmol/L)	Volume of mixture standars (0.1 mM & 1 mM each) (μ L)	Volume of DMB reagent (μ L)	Volume of water (μ L)
0.5	2	10	88
1.0	4	10	86
1.5	6	10	84
2.0	8	10	82
2.5	10	10	80
5.0	20	10	70
7.5	30	10	60
10.0	40	10	50
12.5	50	10	40

calculate average content of two treatments (CV is less than 10%).

### 2.4. Reproducibility

To determine reproducibility, 90 μl (0.9 picomole each) of the standard mixture prepared for reproducibility studies was analyzed repetitively 10 injections during 10 h after derivatization. Calculate peak area RSDs of 10 injections.

### 2.5. Recovery Rate

Add different concentrations of identical volume mixed standards to samples, according the above method to determine the total quantity of Neu5Ac, Neu5Gc and KDN and calculate recovery rate.

### 2.6. Minimum Detection Limits (MDL)

Defined amount of standards Neu5Ac, Neu5Gc, KDN were separately multiple diluted by water, and 90 μL total dilution was derived at same condition and get different Signal-to-Noise by HPLC. According to the following formula:  $MDL = 3 NW/A$  (W: concentration of object; N: the peak height of noise; A: the peak height of this concentration of Sialic acids), so when Signal-to-Noise (A/N) is 3, this concentration is the limit of quantification.

## 3. RESULTS

### 3.1. Separation of Sia

As can be seen in Figure 2, Neu5Ac, Neu5Gc and KDN are well resolved with this method. The retention times of KDN, Neu5Gc and Neu5Ac are 6.961 min, 7.586 min and 9.669 min, respectively.

Linearity of Sia Analysis with HPAE-FLD Table 2 shows that Neu5Ac, Neu5Gc and KDN have good linearity between 1 μmol/L to 200 μmol/L injections under different the hydrolysis conditions.

### 3.2. Minimum Detection Limits

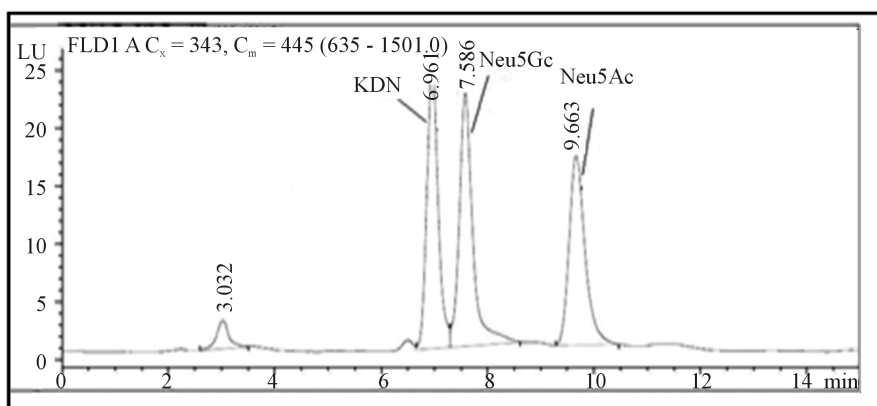
According to the MDL calculation, the MDLs of Neu5Ac, Neu5Gc and KDN are 0.05 μmol/L, 0.06 μmol/L and 0.05 μmol/L, respectively.

### 3.3. Reproducibility

According to above method about determining reproducibility, RSD is 5%, which stand for standard after derivativing is stable in 10 h.

### 3.4. Recovery Rate

According the above method the recovery rate of



**Figure 2.** Chromatogram of Neu5Ac, Neu5Gc and KDN.

**Table 2.** Linear regression equation and correlation coefficient (r<sup>2</sup>) of Neu5Ac, Neu5Gc, and KDN.

Concentrations of TFA to hydrolyze (M)	Neu5Ac	Neu5Gc	KDN
0.05	$Y = 0.0181X + 4.9593$ $r^2 = 0.9927$	$Y = 0.018X + 3.2900$ $r^2 = 0.9922$	$Y = 0.0208X + 3.2501$ $r^2 = 0.9937$
0.1	$Y = 0.0214X + 0.6806$ $r^2 = 0.9940$	$Y = 0.0211X + 0.8134$ $r^2 = 0.9956$	$Y = 0.0241X - 1.8903$ $r^2 = 0.9940$
0.2	$Y = 0.0255X - 4.7169$ $r^2 = 0.9931$	$Y = 0.0258X - 6.2973$ $r^2 = 0.9937$	$Y = 0.021X + 0.3492$ $r^2 = 0.9959$

Neu5Ac, Neu5Gc and KDN are showed in **Table 3**.

### 3.5. Content Analysis of Sia in Samples

Once the method had been fully optimized, we applied it to assay food samples. The results of total Sia in Chinese conventional foods are summarized in **Table 4**. The contents of total Sia ( $\mu\text{g/g}$  wet tissue) in Chinese raw meat was highest in lamb, followed by pork, duck, chicken and beef. The percentages of Neu5Gc were 32.30%, 26.48%, 0%, 0% and 36.08%, respectively. Cod contained higher levels of Sia than salmon and tuna. Only Neu5Ac was found in detected aquatic product. Egg yolk contained the highest level of Sia, and a higher level of Sia was found in the egg white. Also our result showed that human milk contained extremely high level of Sia. Neu5Ac was the predominant form of Sia in all the detected samples. KDN was found in cow milk only among the samples, the content is  $1.14 \mu\text{g/g}$  (did not shown in **Table 4**).

Futhermore, cooked samples were detected. The results of total Sia ( $\mu\text{g/g}$  cooked tissue) are summarized in **Table 5**.

There are little losses of Sia in cooked samples compared to raw. The survival rates are in a range of 59.6% to 96.6% after proofreading by ratio of raw to cooked (**Table 6**). There are less losses of Sia in low water content and boiled samples.

## 4. DISCUSSION

To our knowledge, sialic acids have been determined

**Table 3.** Average recovery rate of Neu5Ac, Neu5Gc, and KDN.

Standard	Average recovery rate
Neu5Ac	95 - 100
Neu5Gc	96 - 99
KDN	95 - 100

only by colorimetric assays [12,13] which are less specific and less sensitive than HPLC owing to the interference of other sugars that undergo Maillard reactions. Sia has no fluorescence in itself, but it can be produced derivatively with DMB, which can be excited by 343 nm exciting light and produce recognized and quantitative strong fluorescent signal without other interferences in HPLC [14]. We made a little bit to modify for component of DMB derived liquid, because we found that 0.25 M sodium thiosulfate and 0.25 M sodium sulfite were used instead of 0.25 M sodium hydrosulfite [15] for DMB reagent, which enhanced the stability of derived Sia.

This quantitative assay relies on the release of sialic acid from food. Total single Sia must also be released by hydrolyzing before the DMB derivatization reaction is possible. We use TFA hydrolysis to accomplish all of this. Sia cannot bear the higher concentration of TFA used to release. To optimize hydrolysis procedure, six different concentrations of TFA to hydrolysis ranging from 0.05 M to 0.5 M for one sample were tested. According to peak area, best condition of hydrolysis for this sample was chosen.

Human milk is a rich source of Sia, and the content of

**Table 4.** Contents of total sialic acids in conventional foods ( $\mu\text{g/g}$  or  $\mu\text{g/ml}$ ).

Raw food sample	Neu5Ac	Neu5Gc	Total	Neu5Gc, % of total
Beef	63.03	25.00	88.03	28.40
Beef fat	178.54	85.17	263.71	32.30
Pork	187.39	67.49	254.88	26.48
Lamb	172.33	97.27	269.60	36.08
Ham	134.76	44.35	179.11	24.76
Chicken	162.86		162.86	
Duck	200.63		200.63	
Egg white	390.67		390.67	
Egg yolk	682.04		682.04	
Salmon	104.43		104.43	
Cod	171.63		171.63	
Tuna	77.98		77.98	
Milk (2% Fat 3% Pr)	93.75	3.51	97.26	3.61
Butter	206.87		206.87	
Cheese	231.10	17.01	248.11	6.86
Human milk	602.55		602.55	

**Table 5.** Contents of total sialic acids in cooked foods ( $\mu\text{g/g}$  cooked tissue).

Cooked food sample	Neu5Ac	Neu5Gc	Total	Neu5Gc, % of total
Beef (cooked)	109.59	45.97	155.56	29.55
Pork (cooked)	226.33	76.97	303.30	25.38
Lamb (cooked)	228.22	111.74	339.96	32.87
Ham (cooked)	147.75	45.27	193.02	23.45
Chicken (cooked)	210.56		210.56	
Duck (cooked)	230.72		230.72	
Egg white (cooked)	357.45		357.45	
Egg yolk (cooked)	757.60		757.60	
Salmon (cooked)	191.73		191.73	
Cod (cooked)	220.77		220.77	
Tuna (cooked)	94.96		94.96	

total Sia in egg is the highest among analysed samples. Majority analysed animal samples are rich with Sia, and the principal constituent is Neu5Ac. Neu5Gc was found in a fewer samples, such as in cow milk. As known by animal experiment, single Sia ganglioside can effectively repair brain damage and remould cranial nervous system.

**Table 6.** Survival rate of Sia in cooked samples (%).

Food Sample	ratio of raw to cooked	Neu5Ac	Neu5Gc
Beef	1:0.41	71.2	75.3
(cooked)	1:0.53	64.0	60.4
Pork	1:0.53	70.1	60.8
(cooked)	1:0.84	92.0	85.7
Lamb	1:0.50	64.6	
(cooked)	1:0.54	64.5	
Ham	1:1.00	91.5	
(cooked)	1:0.87	96.6	
Chicken	1:0.51	93.6	
(cooked)	1:0.46	59.2	
Duck	1:0.69	81.8	

The Sia plays the vital role in the synapse structure and the function formation. The exogenous Sia can promote the brain growth and improve study memory behavior [13]. The examination of content of Sia in food has certain significance for food choice which promotes the brain growth and repairs brain damage.

An accurate and reliable method to quantify Sia content in food would be needed to properly be considered. Several methods to detect and/or quantify Sia have been described. In general, the first step of these protocols is to release the Sia moiety from the parental glycoconjugate. Both enzymatic and chemical reactions conducted under mild acidic conditions are preferred since only mild acid hydrolyses ensure the release of the majority of Sia residues while preserving the integrity of the molecules released. In many instances, there are steps to purify the resulting free Sia prior to quantification or identification of their precise molecular structures. Once purified, Sia can be determined by enzymatic reactions, immunochemical techniques, or by using lectins or viral agglutinins as molecular probes. Quantitative determination has traditionally been performed by colorimetry, and it is the basis of the commonly-used and thiobarbituric acid (TBA) test [16]. Currently, different versions of more laborious and sophisticated analytical technologies, such as high-performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), NMR and GC-MS [17], high-performance anion-exchange chromatography-pulsed amperometric detection [18], and re-versed-phase-ion-pair HPLC [19], have been reported for the analysis of nonderivatized free Sia. Although derivatization constitutes an additional step in analytical methods, in many cases, it increases the specificity as well as the intensity of the instrument signal, while reducing interferences and allowing a more accu-



rate quantification. Derivatization methods that produce adducts in the presence of certain monosaccharides are also used for Sia, although some previous chemical modifications of the Sia structure, such as deacetylation or decarboxylation, are required. The most commonly used conjugation method that is specific for Sia uses the fluorescent compound 1,2-diamino-4,5-methylenedioxy-benzenedihydrochloride (DMB) [15]. Other derivatization methods using periodic acid/TBA [20] and o-phenylenediaminehydrochloride [21] have also been reported. In the present report, we describe the application of the DMB derivatization of Sia from different foods followed by HPLC separation of the resulting adducts. This method has been previously used in different biological sources, but the aim of the work described in this report was to assess its applicability to such complex food matrices.

The distribution of Sia is widely expressed on all mammalian tissues either as Neu5Ac, Neu5Gc or more rarely as KDN. Animal foods, including red meat, fish and poultry, contain high-biological-value protein and important micronutrients that are required to sustain adequate human health throughout life. These animal foods also remain a core food in the diet of China today. We analyzed the contents of Sia in raw and cooked red meat, seafood and poultry by using an established HPLC method. The content of total Sia in Chinese raw meat was the highest in lamb, followed by pork, duck chicken and beef. The percentages of Neu5Gc in each ranged from 0% to 46.08%. Cooked lamb contained the highest percentage of Neu5Gc in the tested foods. Interestingly, both chicken egg yolk and white contained high Sia levels, predominantly as Neu5Ac. Koketsu *et al.* reported the distribution of Sia in the eggs of original Silky fowl and showed that the Sia content [22] in the yolk, albumin and chalaza of a single egg was similar with our data. Our own findings confirmed the previous reports that Neu5Gc was rare in poultry and fish, but common in milk products and enriched in red meats [11].

Importantly, humans lack the ability to synthesize Neu5Gc because of an exon deletion/frameshift mutation in the human CMAH gene 2 - 3 million years ago [11]. This mutation resulted in the complete loss of Neu5Gc expression in all human tissues [11]. However, existing biochemical pathways allow exogenous Neu5Gc to be metabolically incorporated into cultured human cells [23]. Consequently, Neu5Gc can be found at low levels in human carcinomas, fetal tissue, and normal tissue types, e.g., endothelium and epithelium of human surgical specimens at autopsy [9]. This finding implies that Neu5Gc originates from exogenous dietary sources, generally red meat and milk products [11]. As described above, there are some foods containing defined quantity of Neu5Gc. Knowledge of the Neu5Ac and Neu5Gc le-

vels in conventional foods may help us to better understand possible medical disorders involving the uptake of the “nonhuman” Neu5Gc from our diet.

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