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Novel Study on Analysis Method for Measuring Sialic Acid Derived from Edible Bird's Nest in Dementia Drugs and Cognitive Functional Foods

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Abstract: The purpose of this study is to compare, optimize and verify the spectrophotometric and HPLC analysis methods with fluorescence detection to measure sialic acid in dementia drugs and cognitive functional foods containing sialic acid derived from edible bird's nest. A common sample preparation procedure (hydrolysis and purification) for both methods has been proposed. The linearity (from 6 to 150 lg of total sialic acid in the assay for spectrophotometry, and from 12.5 to 250 ng and 1 to 5 ng of Neu5Ac and Neu5Gc, respectively, for HPLC) is adequate. The detection and quantification limits (0.29 and 0.97 mg of total sialic acid/L of reconstituted sample, respectively, for spectrophotometry, and 0.03 and 0.08 mg Neu5Ac/L; 0.003 and 0.009 mg Neu5Gc/L of reconstituted sample, respectively, for HPLC) are low enough for the determination of sialic acid in dementia drugs and cognitive functional foods formulas. The precision of both methods, expressed as relative standard deviation, is less than 6%, and the accuracy evaluated by recovery assays show 104% recovery for spectrophotometry; 95% for Neu5Ac and 109% for Neu5Gc for HPLC. Samples analyzed show no significant differences ($a < 0.05$) attributable to the method used; consequently, both of them could be applied after common sample preparation, the choice of technique depending on the facilities available in the laboratory.
Keywords: Dementia drugs, Cognitive functional foods, Edible bird's nest, Sialic acid, Neu5Ac, Neu5Gc, Spectrophotometry, HPLC-fluorescence.

I. INTRODUCTOION

In previous research, we have manufactured a composition to improve brain function and studied dementia drugs and cognitive functional foods formulas to prepare for the increase in senile dementia patients due to aging [1-3]. For this purpose, we applied the newly designed affinity bead technology (ABT) to extract sialic acid from edible bird's nest, isolate and purify it, and prepare a nutrient delivery system (NDS) [3-6].

Sialic acid is used as a trivial reference to the family of acylated derivatives of a 9-carbon carboxylated monosaccharide [7]. More than 40 compounds have been associated to this family [8]. The main representative forms of sialic acid are N-acetylneuraminic acid (Neu5Ac) and N-glycolyl-neuraminic acid (Neu5Gc).

The measurement of Neu5Gc in dementia drugs and cognitive supplements is very important as it has been reported that sialic acid not only has an effect on intellectual ability in infants and the elderly but also has anti-inflammatory activity that modulates physiological and pathological processes. In addition, sialic acid forms Neu5Ac through a glycosidic bond, and this compound plays an important role in the proper function of synapses and is known to help improve memory [1-6,9] because it does not appear in dementia drugs and cognitive functional foods [1-3,7,8] and can cause disease [1-3,9,10].

Only Kim et al. [1-6] and Martín et al. [11] have developed a HPLC method with fluorescence detection in which Neu5Ac and Neu5Gc could be determined individually, with validation being limited to the determination of Neu5Ac in milk-based foods formulas.

The limited literature data related to the determination of sialic acid in milk-based foods formulas, together with the lack of full validation of the spectrophotometric method in this matrix, and the Neu5Gc determination by HPLC, justify the objective proposed in the present study: to optimize and validate the spectrophotometric and HPLC methods for the determination of total sialic acid, Neu5Ac and Neu5Gc in IF, following a common sample preparation procedure for both methods – thereby helping to simplify the procedure and choose one technique or the other according to the existing laboratory facilities.

Recently, different methods for determining sialic acids in drugs and foods have been reviewed [1-3,

12]. Classically, determination has been carried by spectrometry involving the nonspecific reaction between sialic acids and the reagent resorcinol, and this procedure has been applied to biological samples [13,14], milk-based drugs and foods [18,19]. The technique requires prior purification with an anion exchange resin, in order to eliminate reducing sugars [13]. This method has been used for indirect determination of the total ganglioside content expressed as lipid bound sialic acid in milk-based drugs and foods [15-19]. The method allows determination of the total sialic acid content, and is unable to differentiate among different kinds of sialic acids present in the sample.

II. MATERIALS AND METHODS

2.1 Chemicals

Sodium formate and sodium hydrosulfite were analytical grade and purchased from Fluka (Sigma–Aldrich, St. Louis, MO, USA). Ion exchange Dowex 1 x 8 (quaternary amine as functional group, 200–400 mesh particle size, chloride form), Dowex 2 x 8 (dime-thylethanolamine as functional group, 200–400 mesh particle size, chloride form) and Marathon A2 (dimethylethanolamine as functional group, 570 lm particle size, chloride form), standard reagents of Neu5Ac, Neu5Gc, 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB) and resorcinol, were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile, N-amyl alcohol, copper (II) sulfate pentahydrate, formic acid, glacial acetic acid, hydrochloric acid, methanol, b-mercaptoethanol, sodium hydroxide and sulfuric acid were purchased from Merck (Darmstadt, Germany).

2.2 Samples

Five commercial milk-based ingredients and foods (IF) were used during the study: A1 and B1 were powder starter IF, A2 and B2 were powder follow-up IF, and C was a liquid follow-up IF. All of them were purchased from the same manufacturer.

2.3 Hydrolysis

For hydrolysis of the sample, the conditions described by Kim et al. [1-3] and Svennerholm et al. [14] (1958) were used, with some modifications. Briefly, 0.1 g of powder or lyophilized liquid IF were dissolved in 0.5 mL of distilled water and hydrolyzed with 2.5 mL of H_2SO_4 0.05 M, heated for 60 min at 80 ℃ in heater block (Thermoblock, from Stuart Scientific), cooled to room temperature, and stored at 4 ℃ until analysis.

2.4 Purification

The hydrolyzed sample was centrifuged at 4 ℃ during 10 min at 200 g. The supernatant was passed through 2 mL of Dowex 1 x 8 previously activated following the manufacturer's instructions. This was followed by washing with 20 mL of water and elution with 20 mL of formic acid 0.1 N (Puente et al., 1996). The eluate was collected in 50 mL polypropylene tubes and analyzed by the spectrophotometric method following lyophilisation (CD-4, from Heto Sicc) or directly by the HPLC method.

2.5 Spectrophotometric determination

For the determination of total sialic acid (expressed as Neu5Ac), the method described by Svennerholm (1958) was followed. The lyophilate was reconstituted in 2 mL of deionized water and mixed with 2 mL of resorcinol reagent (10 mL of resorcinol 2% in water (w/v), 80 mL of concentrated HCl containing 0.25 mL of copper sulfate 0.1 M and deionized water for an end volume of 100 mL). After heating the sample for 15 min at 100 ℃ in a heater block with subdued light, it was cooled to room temperature, 5 mL of N-amyl alcohol were added and vigorously mixed, and the sample was left for 15 min in a water ice bath in the dark. Posteriorly, the sample was centrifuged at 4 ℃ during 5 min at 200g. The upper organic phase was transferred to microcuvettes and measured at 580 nm (UV–Vis Lambda 2 pectrophotometer, from Perkin Elmer).

2.6 HPLC determination

Neu5Ac and Neu5Gc were determined by HPLC-fluorescence detection following derivatization with DMB according to the method described by Martín et al. [11]. Briefly, 400 lL of purified sample were ultrafiltered with Microcon Ultracel YM-10 (purchased from Millipore, Milford, MA, USA) at 13000g for 10 min at 4 ℃. Then 50 lL of the filtered sample were mixed with 50 lL of DMB reagent (8 mM DMB, 1.5 M acetic acid, 14 mM sodium hydrosulfite, and 0.8 M b-mercaptoethanol) and heated for 2.5 h in a heater block at 50 ℃ in the dark. After cooling to room temperature, the derivatized sample was ready to be injected.

HPLC analysis was carried out on a Waters chromatography system composed of a Waters 600 quaternary pump, Waters 474 fluorescence detector, Waters 717 plus autosampler, Waters 600 degassing system and a Jones chromatography 464 oven. The mobile phase used was water : methanol : acetonitrile 85 : 7 : 8 (v/v/v), with a flow of 0.9 mL/min (mobile phase was filtered using a Millipore system with 0.20 lm Millex-GN

membrane filters). The detector parameters were: λexc = 373 nm, λem = 448 nm, gain: 1, attenuation: 64, response: 5 s. The column used was a Hidrosorb RP-18 (250 x 4.6 mm, 5 µm) with a Hidrosorb RP-18 (5 µm) guard column, both purchased from Merck (Dalmstaad, Germany). The column was kept at a constant temperature of 33 °C. Under these conditions, Neu5Gc and Neu5Ac elute at 9–10 and 11–12 min, respectively, as seen in Fig. 1, where a chromatogram of Neu5Ac and Neu5Gc standards (150 and 5 ng, respectively) and of a sample (IF A1) are shown.

Data were collected and analyzed using the Millenium[32] Chromatography Manager Simple System software package.

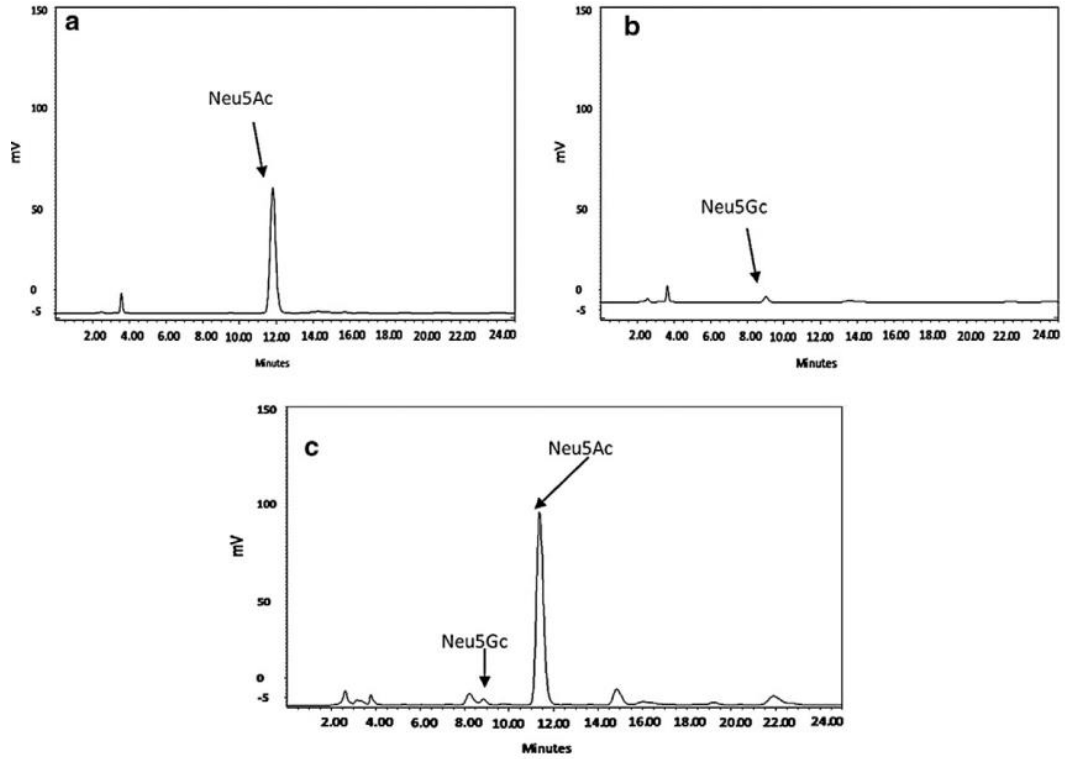

Fig 1: Chromatogram of the Neu5Ac–DMB standard 150 ng (a), Neu5Gc–DMB standard 5 ng (b), and IF A1 sample (c).

2.7 Mass spectroscopy analysis

The corresponding chromatographic peaks of derivatized sample IF A1 and Neu5Ac standard (100 ng) were collected (Waters Fraction Collector II) from the HPLC system and delivered directly by a syringe pump 60061 (Cole-Parmer) into an ESI source (Bruker Esquire 3000 plus, from Bruker) at a flow rate of 240 mL/h. The conditions used for the analysis were: positive ion mode; capillary -4300 V; capillary exit offset, 120 V; plate offset, -500 V; skimmer, 40 V; dry gas, N2, 5 L/min; dry temperature, 300 °C; nebuliser, 10 psi; and scan range 50–1000 *m/z*.

2.8 Statistical analysis

A paired t-test was used to evaluate the differences between both methods applied to one same sample, and to evaluate matrix interferences – significance being fixed at a = 0.05 for both analyses.

All analyses were done with the Statgraphics Plus 5.0 statistical package (Statistical Graphics Corp., Rockville, MD, USA).

III. RESULTS AND DISCUSSION

3.1 Preliminary studies with standard solutions

In order to adapt and validate the spectrophotometric [1-3,13] and HPLC methods [1-3,11] for sialic acid determination in IF in our laboratory, a previous study with standard solutions of sialic acid was carried out.

3.1.1 Linearity

To test the linearity of the spectrophotometric and HPLC methods, five calibration curves were assayed

on the same day (intra-day) or on different days (inter-day) in the range of 6–150 lg of Neu5Ac for the spectrophotometric assay, and 12.5–250 ng of Neu5Ac and 1.0–5.0 ng of Neu5Gc for the HPLC method. Both methods presented good linearity in the studied range, with an RSD of under 2% for spectrophotometry and under 8% and 6% for Neu5Ac and Neu5Gc, respectively, with the HPLC (see Table 1).

On contrasting the information in the literature, no data were found on linearity in the spectrophotometric method neither the Neu5Gc determination by HPLC, while a similar linear range for Neu5Ac (25–250 ng, with RSD = 5%) was reported for HPLC [1-3,11].

Method	Analyte	Linearity (RSD%)		Calibration curve	
		Intra-day (n = 5)	Inter-day (n = 5)	Intra-day (n = 5)	Inter-day (n =5)
Spectrophotometry	Total sialic acid	1.0	1.6	y = 0.0048x - 0.0067 (R 2 = 0.9996)	y = 0.0051x - 0.0073 (R 2 = 0.9997)
HPLC	Neu5Ac	4.6	7.9	y = 15870x -40419 (R 2 = 0.9999	y = 16262x + 1291 (R 2 = 0.9999)
	Neu5Gc	3.7	6.1	y = 10507x - 2354 (R 2 = 0.9999)	y = 10596x - 3744 (R 2 = 0.9999)

Table 1: Sialic acid: linearity of the spectrophotometric and HPLC methods.

3.1.2 Precision

Inter- and intra-day precision were estimated. The standard amount used for the spectrophotometric method was 98.9 lg of Neu5Ac, versus 98 ng of Neu5Ac and 2 ng of Neu5Gc for HPLC. With both methods, the inter- and intra-day precision values were lower than 10% and 3.2%, respectively (see Table 2). In order to get a correct precision for the Neu5Gc determination no more than 12 h should pass between the derivatization and the analysis.

Method	Analyte	Intra-day[a] (n = 5)	Inter-day[a] (n = 3)	LOD[b] (ng assay – mg/L[c])	LOQ[b] (ng assay – mg/L[c])
Spectrophotometry	Total sialic acid	1.1	1.6	560–0.29	1860–0.97
HPLC	Neu5Ac	3.2	9.8	4.22–0.03	12.79–0.08
	Neu5Gc	2.9	4.3	0.46–0.003	1.40–0.009

Table 2: Precision and limits of detection and quantification of the spectrophotometric and HPLC.

[a] Precision parameters are expressed as RSD (%).

[b] LOD (limit of detection), LOQ (limit of quantification).

[c] mg sialic acid per liter of infant formula reconstituted to 13% (w/v).

3.1.3 Limits of detection and quantification (LOD and LOQ)

The LOD (signal-to-noise ratio 3) and LOQ (signal-to-noise-ratio 10) were evaluated using the response of six blanks.

Results expressed in ng assay and mg/L of sample are shown in Table 2. Both methods allow the analysis of sialic acid, Neu5Ac and Neu5Gc, respectively, in IF, comparing LOD and LOQ with the results shown in Table 3.

With the HPLC method, the LOD and LOQ found in this study were lower (approximately one-half – see Table 2) than the values reported by Kim et al. [1-3] and Martín et al. [11] for Neu5Ac (LOD = 9.9 and LOQ = 29.9 ng). In the case of the spectrophotometric method and Neu5Gc determination by HPLC no data have been found in the literature.

3.2 Samples

3.2.1 Selection of resin

The resin described initially in the literature [14] and used after by all authors who have determined sialic acid in the past is presently out of catalogue. Accordingly, a first requirement was the selection of a new resin, as well as optimization of the sample size for the new resin, and the conduction of a validation study.

Two different kinds of resins, with characteristics similar to Dowex 2 x 8, advised by the manufacturer, were evaluated by spectrophotometric method to determine total sialic acid and by HPLC method for determining Neu5Ac, because in the literature only could be found data about this one. For the assays, the amount of each resin used was 2 mL per column, as described in previous studies (Svennerholm, 1958). The

first resin (Marathon A2) has the same functional exchange groups but a larger particle size than Dowex 2 x 8, while the second resin (Dowex 1 x 8) has the same particle size, but less exchange power than Dowex 2 x 8. A sample, the starter IF A1, was purified using both resins and determined by both methods (results are shown in Table 4).

Sample	**Spectrophotometry**	HPLC		
	Total sialic acid	Neu5Ac	Neu5Gc	Total sialic acid (Neu5Ac + Neu5Gc)
A1	140.8 ± 4.7[a]	147.6 ± 4.5	5.2 ± 0.1	152.8 ± 4.5
A2	143.3 ± 3.8	145.9 ± 4.9	4.7 ± 0.1	150.6 ± 4.9
B1	163.6 ± 8.0	157.4 ± 4.3	4.5 ± 0.3	161.9 ± 4.3
B2	154.4 ± 3.6	157.4 ± 3.5	5.5 ± 0.1	162.9 ± 3.5
C	200.2 ± 2.1	199.7 ± 7.5	3.8 ± 0.2	203.5 ± 7.5
5	5.88	5.25	2.78	2.50

Table 3: Contents of sialic acid in IF analyzed by the HPLC and spectrophotometric methods.

[a] Results are expressed as mean ± standard deviation (n = 3) in mg of sialic acid per liter of infant formula reconstituted to 13% (w/v).

The sialic acid contents in the IF A1 obtained using the resin Marathon A2 are lower than the contents reported in the literature and ranging from 100 to 250 mg/L for milk-based IF [18,19]. In addition, the variability obtained is very high compared with the concentration of sialic acid found for this sample. It was shown that sialic acid was not retained in the resin because eluting with double the volume (40 mL of formic acid 0.1 M) yielded no changes in the contents. In view of these observations, the use of Marathon A2 was discarded.

On the other hand, the results obtained with the Dowex 1 x 8 resin had an RSD <5% (see Table 4). The sialic acid concentrations found with each method in IF were not in concordance (52.3 mg total sialic acid/L with the spectrophotometric method versus 145.7 mg Neu5Ac/L with HPLC). In view of this problem, the same sample was assayed with Dowex 2 x 8, kindly provided by Lee and Nam from Kyung Hee University Laboratories, and determined by HPLC. The result was 147.8 mg Neu5Ac/L, which is closer to the value afforded by Dowex 1 x 8 than with Marathon A2. Due to the different amounts obtained with both methods, and since the precision was adequate, a series of assays were performed: mass analysis in the HPLC method to discard the co-elution of interferences with Neu5Ac in the same chromatographic peak, and a matrix interference assay in the spectrophotometric method to discard that the low concentration obtained with this method is not due to lowering of the signal associated to the matrix.

Resin	Spectrophotometry		Calibration curve	
	Total sialic acid (mg/L)[a]	RSD (%)	Neu5Ac (mg/L)[a]	RSD (%)
Marathon A2	48.0 ± 7.3	15.1	40.4 ± 5.6	13.9
Dowex 1 x 8	52.3 ± 2.5	4.8	145.7 ± 3.2	2.2

Table 4: Selection of anionic exchange resin for purification.

[a] Results are expressed as mean ± standard deviation (n = 3) in mg of sialic acid per liter of infant formula reconstituted to 13% (w/v).

3.2.2 Mass analysis

The mass spectra obtained in the derivatized sample IF A1 and a Neu5Ac standard (100 ng) are shown in Fig. 2. In both cases the same profile is observed. It therefore can be concluded that no different substances are present in the sample and standard. The Neu5Ac-DMB complex has an m/z = 535.

3.2.3 Mass analysis

The matrix interference study was carried out by the standard addition method applied to the hydrolyzed and purified IF A1. Two sets of aqueous standards of Neu5Ac in the range 6–150 lg were prepared, and the matrix of one of them was added in a proportion of 50%. Sialic acid of both sets was analyzed by the spectrophotometric method. A t-test was applied to compare the slopes of the regression equations corresponding to the added matrix with those of aqueous standards. No significant differences were found between the confidence interval of the slopes ($p < 0.05$). Thus, sialic acid determination in IF by the spectrophotometry method was free from matrix interferences.

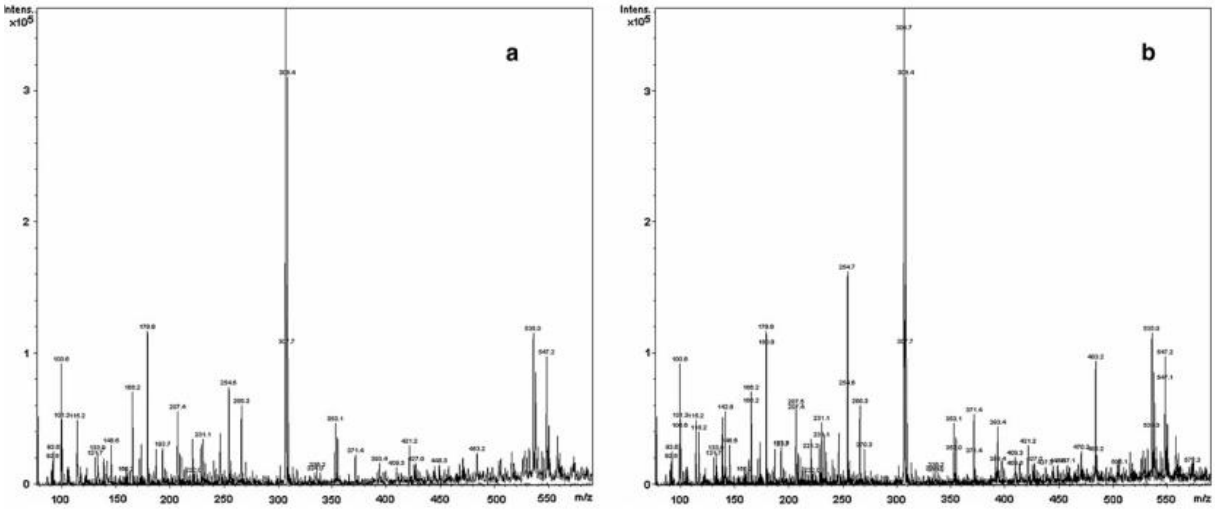

Fig 2: Mass spectrum of the Neu5Ac–DMB standard 100 ng (a) and the IF A1 sample (b).

3.2.4 Sample size

On the basis of the results obtained by the mass and matrix interferences assays, and considering that sample size was very different in the original studies (0.25 g in spectroscopy – [1-6,19]; 0.068 g in HPLC – [1-6, 11], we decided to optimize sample size in order to establish a common procedure for the hydrolysis and purification steps in both methods. A centrifugation step (200g, 10 min, 4 x C) was added after hydrolysis. This step was not described before, and although turbidity was noted, it could be critical in order to clarify the sample and thus homogenize flow through the resin during the purification step.

Different sample amounts (0.10, 0.25 and 0.50 g) and reconstitution volumes (0.5, 5.0, 10.0 and 25.0 mL) were hydrolyzed and purified in Dowex 1 x 8 resin, before analysis by spectrophotometry and HPLC. We selected 0.1 g reconstituted in 0.5 mL of distilled water, considering the fact that with this amount the resin bed was not saturated, and similar results for both methods were obtained (140.8 ± 4.7 mg total sialic acid/L and 154.3 ± 4.5 mg Neu5Ac + - Neu5Gc/L with spectrophotometry and HPLC, respectively). This selection implies a reduction of sample weight (40%) with respect to the original spectrophotometric method [1-6, 14] and an increase (32%) in the amount used in the HPLC method [1-6, 11].

3.2.5 Validation

3.2.5.1 Precision

For both methods, the intra-day precision was lower than 4%, and the inter-day precision was lower than 6% (see Table 5). On comparing with the literature, the spectrophotometric method [1-6, 18] reported RSD = 1% in biological samples, including milk formula, while Kim et al. [1-6] and Martín et al. [11] for the HPLC method reported an RSD = 4.35% for Neu5Ac in dementia drugs, cognitive functional food formula. Both of these values coincide with our own results.

As in the case of standard solutions of Neu5Gc, in order to get a correct precision, the analysis should be done within the first 12 h after the derivatization.

3.2.5.1 Accuracy

For evaluating accuracy with the spectrophotometric method, a recovery assay was made spiking the IF A1 sample with 23 lg of Neu5Ac standard, and obtaining a recovery of about 104% (see Table 5). Kim et al. [1-6] and Svennerholm et al. [1-6, 14] obtained recoveries in the order of 89–95% in model systems of standards of Neu5Ac.

To test the accuracy of the HPLC method, an assay was made using a commercial sialic acid-free soy-based IF. 120 ng of Neu5Ac and 4.7 ng of Neu5Gc were added to the sample and were analyzed, and the percentage recovery was 94% and 109.1% for Neu5Ac and Neu5Gc, respectively. Kim et al. [1-6] and Martín et al. [11] also obtained recoveries between 97% and 98% in the case of Neu5Ac, using the same kind of spiked matrix, for Neu5Gc no data was available (see Table 5).

3.2.6 Application to samples

Sialic acid content was measured in samples A–C by the spectrophotometric method (total sialic acid) and HPLC method (Neu5Ac and Neu5Gc), applying the common sample preparation. Results ranged from 140.8 to 200.2 mg total sialic acid/L applying the spectrophotometric method and from 145.9 to 199.7 mg Neu5Ac/L and from 3.8 to 5.5 mg Neu5Gc/L with the HPLC method (all results considering 13%, w/v) (see Table 3). Using the spectrophotometric method, Kim et al. [1-6] and Sánchez-Díaz et al. [19] reported contents of total sialic acid between 233 and 266 mg/L, while Neeser et al. [18] reported contents ranging from 92 to 284 mg/L. With the HPLC method, [11] found contents ranging from 108 to 166 mg Neu5Ac/L. Our results are of the same order, and the minor differences found could be attributed to variability of the raw materials used for manufacturing the IF. No previous data for Neu5Gc is known. No significant differences were found between both determinations, comparing total sialic acid with Neu5Ac + Neu5Gc by spectroscopy and HPLC, respectively, on applying a paired t-test ($\alpha = 0.05$).

Method	Analyte		Intra-day (n = 5)	Inter-day (n = 3)	Accuracy (% recovery) (n =4)
Spectrophotometry	Total sialic acid	RSD (%)	3.3	4.9	104.3 ± 3.3
		Content (mg/L) [a]	104.3 ± 3.3	142.1 ± 6.9	
HPLC	Neu5Ac	RSD (%)	3.1	5.7	94.1 ± 4.9
		Content (mg/L) [a]	147.6 ± 4.5	145.4 ± 8.3	
	Neu5Gc	RSD (%)	3.9	4.3	108.5 ± 3.9
		Content (mg/L) [a]	4.1 ± 0.2	4.2 ± 0.1	

Table 5: Analytical parameters of the spectrophotometric and HPLC methods in infant formulas (IF A1).
[a] Results are expressed as mean ± standard deviation in mg of sialic acid per liter of infant formula reconstituted to 13% (w/v).

IV. CONCLUSION

A common sample hydrolysis and purification procedure with a new resin (Dowex 1 x 8) is proposed for the determination of Neu5Ac by spectrophotometric and HPLC assays. The HPLC method is more sensitive and faster than the spectrophotometric method, since the lyophilization step before determination is not required, and the chromatographic run time is less than 30 min. Furthermore, the Neu5Gc form can be determined in dementia drugs and cognitive functional foods containing sialic acid derived from edible bird's nest, applying this method. This new procedure allows preparation of the sample and choosing the determination method according to the existing laboratory facilities.

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ABBREVIATIONS

ABT: Affinity bead technology; NDS: Nutrient delivery system; Neu5Ac: N-acetylneuraminic acid; Neu5Gc: N-glycolyl-neuraminic acid; HPLC: High Performance Liquid Chromatography; DMB: 1,2-diamino-4,5-methylenedioxybenzene; IF: Ingredients and foods; RSD: Relative standard deviation; LOQ: limit of quantification; LOD: limit of detection.

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