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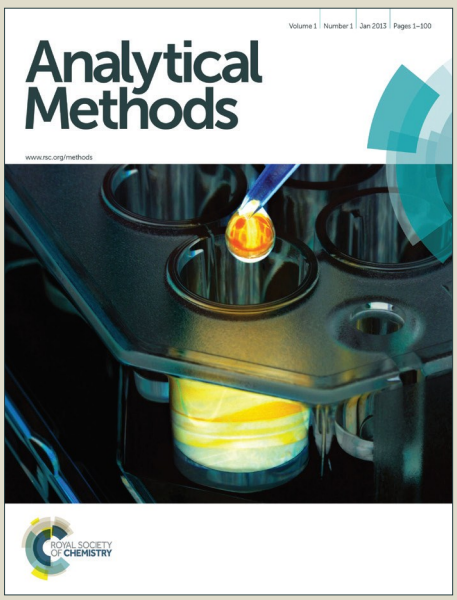

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A novel, sensitive and convenient method for determination of sialic acids in human serum utilizing the ultrasonic-assisted closed in-syringe hydrolysis and derivatization prior to high performance liquid chromatography

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Abstract: A novel, sensitive and convenient method, utilizing the ultrasonic-assisted closed in-syringe hydrolysis and derivatization (UCSHD) prior to high performance liquid chromatography (HPLC) coupled with fluorescence detection (FLD) and online mass spectrometry (MS) identification, has been developed for determination of sialic acids. The pivotal parameters affecting the release of sialic acids from serum and the derivatization were investigated with response surface methodology (RSM). Under the optimized conditions, the two sialic acids were released maximum and labeled successfully in a relative short time of 72 min (traditional time > 3 h) for the reason of the combination of hydrolysis steps with derivatization in a closed system with assistance of ultrasonic. Excellent linearity (R^2 > 0.9991) in the calibration range of 0.5–16 μmol/mL and quite low detection limits (LODs) (0.30 pmol for Neu5Ac and 0.21 pmol for Neu5Gc) were achieved. When the established UCSHD-HPLC-FLD-MS method was applied for the analysis of sialic acids in various human sera, low relative error (RE: -3.4% to 2.5%), high recoveries (90-96%) and intra- and inter-day precisions (RSD, 0.9-2.2% for Neu5Ac and 1.4-2.8% for Neu5Gc) were also obtained, demonstrating the obvious advantages for the accurate, sensitive and convenient determination of sialic acids in bio-samples.

Keyword: N-acetylneuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5Gc), 2-[2-(7H-dibenzo[a,g]carbazol-7-yl)-ethoxy]-ethyl carbonylhydrazine (DBCEEC), Ultrasonic-assisted closed in-syringe hydrolysis and derivatization (UCSHD), High performance liquid chromatography-fluorescence detection-tandem mass spectra(HPLC-FLD-MS/MS), human serum

1. Introduction

Sialic acids, acetylated derivatives of neuroaminic acid, are widely distributed in mammals' tissues.[1] They are typically found attached to the non-reducing terminus of glycan chains. N-acetylneuraminic acid (Neu5Ac) and its hydroxylated form, N-glycolylneuraminic acid (Neu5Gc), where a glycolyl group is bound to the amino

group at C5, are the main representative and most abundant forms of sialic acid.[2] By far Neu5Ac is the most widespread form of sialic acids and almost the only found in humans. Neu5Gc is not expressed in normal human body due to the evolutionary loss of the gene encoding the enzyme that converts Neu5Ac into Neu5Gc (CMP-Neu5Ac hydroxylase).[3] However, studies have demonstrated the metabolic incorporation of Neu5Gc into glycoproteins in individuals affected by certain types of cancers such as colon and breast cancers.[3-8]

The serum is the most important human biofluids containing sialic acids and especially valuable in clinical diagnosis of several disease.[9-14] Some studies have reported that serum sialic acid was over-expressed in patients with inflammatory diseases and cancer, which could be a useful marker for cancer screening.[15, 16] A recent study proposed by Gruszewska et al.[17] described the marker capability of serum sialic acids for diagnosis and evaluation of tumor location in patients with primary pancreatic cancer. So the measurement of serum sialic acid could be valuable in earlier diagnosis of malignant disease[18] or monitoring the tumour bulk in response to treatment.[17]

Many quantitative analytical methods have been reported for serum sialic acid. Historically, the earliest methods for serum sialic acid tended to be colorimetric,[19] but some suffered from sensitivity or specificity problems and consequently are rarely used routinely. In recent decades, commonly employed methods for the analysis of serum sialic acids included capillary gas chromatography-mass spectrometry(GC-MS),[20, 21] liquid chromatography (LC),[22-25] μ-liquid chromatography-laser induced fluorescence (μ-LC-LIF)[26] or liquid chromatography–tandem mass spectrometry (LC-MS)[27] after derivatization[28, 29] with a chromophore or fluorophore for sensitive detection. Among the derivatization reagents for the determination of Neu5Ac and Neu5Gc in human serum, 1,2-diamino-4,6-dimethoxybenzene (DDB) and 1,2-diamino-4,5-methylenedioxybenzene (DMB) have been frequently used by LC.[22, 24, 26, 27] But the two labeling reagents have been reported with several limitations, such as time-consuming, the fussy operation, instability, unknown by-products and serious interferences. In this study, an excellent probe 2-[2-(7H-dibenzo[a,g] carbazol-7-yl)-ethoxy] ethyl carbonylhydrazine (DBCEEC), which had been reported for aldehydes derivatization,[30] was employed to label sialic acids for trace determination with high satisfactoriness. In contrast with DDB/DMB, the

reagent of DBCEEC was used to label sialic acids directly for the first time, which could simplify operation processes, provide faster derivatization without photopathic operation, improve the stability of the product owing to its larger conjugated substructures, and produce more intense ion current signals for MS.

Critical step in sialic acid analysis is their liberation from human serum, which entails isolation of sialic acids from the parental glycoconjugate. The blood composition is more complex, multi-step manual operations such as solid-phase extraction (SPE)[31] are usually required to remove impurities from the sample. However, multi-step operations are tedious, time-consuming, and more seriously, tend to cause the loss of analytes and high reagent-consumption, which will pose a potential threat to experimenters and environment. In a recent study,[26] a simple protocol based on ultrasound as auxiliary energy has been proposed to shorten hydrolysis and derivatization time and steps. Therefore, combining the ultrasonic-assisted trace hydrolysis with the in-syringe derivatization[32, 33] in closed system as a novel pretreatment technique for HPLC will make it possible to establish the desired method. Except for the assistance of ultrasonic energy, the method of ultrasonic-assisted closed in-syringe hydrolysis and derivatization (UCSHD) has one major differentiating characteristic: the hydrolysis and derivatization were performed in closed syringe system. UCSED technique allows for a simple, convenient operation in relatively short time, and has several additional advantages than conventional tube method: first, a certain amount of solution was drawn accurately by the syringe without the aid of other auxiliary equipment; second, volatilization and loss could be prevented, which was necessary for accurate quantification and it would prevent a potential threat to experimenters and environment; third, it was convenient for filtration. After finishing the reaction, the resulting mixture was cooled to room temperature and filtered through a syringe filter (0.22 μm) without the aid of additional syringe, which can practically avoid additional operations for filtration.

In this study, a method of UCSHD prior to high performance liquid chromatography (HPLC) coupled with fluorescence detection (FLD) and tandem mass spectra (MS/MS) technique has been developed and applied to the quantification of Neu5Ac and Neu5Gc. By derivatization, DBCEEC, an excellent fluorogenic reagent for α-keto acids, was introduced into the molecules of Neu5AC and Neu5Gc to enhance the HPLC sensitivity.

Meanwhile, the introduction of strong hydrophobic DBCEEC moiety into the hydrophilic sialic acid molecules also greatly increased the retention of the analytes on a reversed phase column. Therefore, the two sialic acids with similar properties could be separated. In order to obtain the optimum UCSHD condition, Box-Behnken design (BBD) from response surface methodology (RSM) was used to optimize the main parameters affecting the derivatization and hydrolysis yield. Under the optimal conditions, the proposed method has been successfully applied to the analysis of sialic acids in various sera with cancers (lung, liver, breast, esophageal, gastric, colorectal, intestinal cancer) and healthy control group, which was proven to be simple, efficient, sensitive and accurate for sialic acids analysis in biological samples.

2. Experimental section

2.1. Instruments and conditions

The HPLC analysis was performed using an Agilent 1100 series HPLC system, equipped with an on-line-degasser, a binary pump, an autosampler and a thermostated column compartment. A fluorescence detector (model G1321B, Agilent, USA) was adjusted at wavelengths of 300 and 400 nm for excitation and emission. Chromatographic separation was achieved on a ZORBAX SB-C18 column (4.6 × 150 mm, 5 μm, Agilent, USA). Solvent A was 5% acetonitrile in water and B was acetonitrile. The flow rate was constant at 1 mL/min and the column temperature was kept at 30 °C. The gradient condition of mobile phase was as follows: 40-50% B from 0 to 5 min; 50-100% B from 5 to 6 min and then hold for 4 min. The column was equilibrated with the initial mobile phase for 5 min before the next injection. The injection volume was 10 μL. The liquid analytes were filtered through a 0.22 μm Nylon membrane filter (Alltech, Deerfiled, IL, USA).

The mass spectrometer 1100 Series LC-MSD Trap-SL (Agilent, USA) equipped with an Agilent Jet Stream, which was controlled by Esquire-LC NT software, version 4.1. MS/MS measurements were conducted using an electrospray ionization source (ESI) instrument operated in the positive ion mode. Ion source conditions were: spray pressure 241.3 kPa; dry gas temperature 350 °C; dry gas flow rate 5 L/min; capillary voltage 3.5 kV. Full scan MS was operated in positive mode over a mass range of *m/z* 100-900 with the number of parents 2, fragmentation amplitude of 1.00 V and SmartFrag on (30-200%).

The mobile phase was filtered through a 0.22 mm nylon membrane filter (Alltech, Deerfiled, IL, USA) and the injection volume was 10 μL. The polymer filter (0.22mm) was bought from Jiangyan Kangtai medical equipment company. TGL-16M refrigerated centrifuge (Xiangzhi Co., Changsha, China) was used for sample preparation. UCSHD was carried out using a temperature- and time-adjustable of ultrasonic cleaner (KQ-100B, Kunshan Ultrasonic Instrument Co., Kunshan, China).

2.2. Chemicals and Reagents

Neu5Ac and Neu5Gc standards were purchased from Sigma Co (St. Louis, MO, USA). DBCEEC was synthesized in author's laboratory as described in our previous study.[34] Acetonitrile was of HPLC grade commercially available (Sigma-Aldrich, USA). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). Glacial acetic acid was purchased from Yuwang Company, China. All other reagents including glacial acetic acid were also of analytical grade unless otherwise stated. Normal control blood samples were obtained from the Qufu Blood Center (Shandong province, China) for serum analyses. The representative samples are drawn from persons screened by a physician and found to be in good health and, therefore, suitable as blood donors. For patients with cancer, all patient serums were drawn at the time of hospital admission from the People's Hospital in Qufu. To prepare sera for testing, blood samples were permitted to clot at room temperature for 20 min and then at 4 °C for 20 min, after which they were centrifuged at 2000 rpm in an International refrigerated centrifuge for 5 min.[35] All sera were stored at -20 °C in a freezer until the time of analysis.

All experiments were performed in compliance with Blood Management System Laws of the People's Republic of China, the experimental procedure for the present study has been approved by the ethical committee of Qufu Normal University, China, and the informed consent provided by patients was obtained for any experimentation with human subjects.

2.3. Preparation of standard solutions and labeling reagent

Individual stock standard solutions at a concentration of 10^{-2} mol/L for Neu5Ac and Neu5Gc were prepared by dissolving appropriate amounts of sialic acid standards in 10 mL of pure water, respectively. The mixed standards at the concentration of 5×10^{-4} mol/L were prepared by diluting the corresponding stock solution with pure water. The

derivatizing reagent solution (5×10^{-2} mol/L) was prepared by dissolving 206.5 mg of DBCEEC in 10 mL of acetonitrile and the corresponding low concentration solutions were diluted by acetonitrile. When not in use, all standards were stored at 4 °C in a refrigerator.

2.4. Samples pretreatment procedure

To improve efficiency of pretreatment, an UCSHD technique was developed. Accurately measured 10 μL of serum sample was added into a syringe barrel (2 mL), and then 100 μL of 2 mol/L acetic acid solution was drawn. The syringe was sealed with screw-cap and sonicated at 75 °C for 35 min. Meanwhile, a mixture of 21 μL of glacial acetic acid and 140 μL of DBCEEC solution(5×10^{-3} mol/L) was prepared, and the obtained mixture was drawn into the syringe (the volume ratio of acetic acid to the final solution in syringe was 12%). The syringe was immediately re-sealed and put into an ultrasonic water bath (70 °C) for 37 min. The resulting mixture was cooled to room temperature and filtered through a syringe filter (0.22 μm) for the direct HPLC analysis.

Standard sample was obtained by mixing 33 μL glacial acetic acid and 140 μL DBCEEC (5×10^{-3} mol/L) with 100 μL of standard solutions (5×10^{-4} mol/L) and pretreated identically. It is noteworthy that the volume ratio of acetic acid to the final solution in syringe was 12%, which was identical with real sample pretreatment procedure. The scheme of derivatization reaction is shown in Fig. 1.

2.5. Optimization of UCSHD

Optimization of derivatization condition. Single-variable experiments were carried out to evaluate the factors on the yield of derivatization, and some factors such as molar ratio (derivatization reagent/analytes), concentration of catalysts, temperature and time would interact with each other, thus they were further optimized by a multivariate method. A Box-Behnken Design (BBD) with four variables, the molar ratio of DBCEEC to the total sialic acids (X_1), derivatization temperature (X_2), derivatization time (X_3), the volume ratio of catalyst to the final solution (X_4), was applied to optimize derivatization conditions, which were statistically analyzed by the software Design Expert (Version 8.0.6, Stat-Ease Inc., Minneapolis, MN, USA). BBD for the combinations of four variables (X_1 (2-10), X_2 (50-100°C), X_3 (10-60 time) and X_4 (1%-20%)) are listed in Table S1. According to the principle of RSM, all the 29 randomized experiments, including the

repeated combination, in Table S1 were repeated for three times.

Optimization of hydrolysis condition. Acid hydrolysis, a relatively inexpensive and quite effective method, was used to release sialic acids from serum in this study. After single-variable experiments, considering the interaction of factors with each other, BBD with three variables, X_1, hydrolysis temperature; X_2, hydrolysis time; and X_3, acid concentration in the final solution, was applied to optimize hydrolysis conditions. Three variables, X_1 (50-100 °C), X_2 (10-60 min) and X_3 (1-3 mol/L), are listed in Table S2. All the 17 randomized experiments (including the repeated combination) in Table S2 were repeated for three times.

2.6. Method validation

The developed analysis method was validated by evaluation of the linearity, repeatability, sensitivity, accuracy and precision. The mixtures of the two sialic acid standards at different concentrations, in the range of 0.5-16.0 μmol/mL for each of the sialic acids, were analyzed to study the linearity under the optimal hydrolysis and derivatization conditions. The repeatability was investigated by spiking a known amount of standard solution (three concentration levels) in real samples (n = 6) and was reflected by relative standard deviations (RSDs) of peak area and retention time. Analytical sensitivity were reflected by limit of detection (LOD) and limit of quantification (LOQ) tested at the signal-to-noise ratio (S/N) of 3:1 and 10:1, respectively. The recovery of the method was evaluated by spiking a known amount of standard (three different levels) into real samples, after addition, each sample was hydrolyzed and labeled by the method described above and analyzed by HPLC. The recovery was determined according to the formula of (measured value - original value)/added value×100%. Relative error (RE) and relative standard deviations (RSD) were calculated to evaluate the accuracy and precision, respectively (n = 6).

3. Results and discussion

3.1. Optimization of UCSHD

Optimisation of derivatization parameters. Table S1 described 29 randomized experimental runs and results. The analysis of variance (ANOVA) was used to assess the significance of each factor and interaction terms. Results of the analysis showed that F value was significant at the level of $p < 0.0001$ and the lack of fit was no significant,

indicating that the second-order polynomial model was sufficiently accurate for predicting the relevant responses. The coefficient of determination (R^2) was the proportion of variability in the data explained or accounted for by the model.[36] Validity of the model was determined by comparing the experimental and predicted values. The R^2 was 0.9421 and the adjusted R^2 was 0.9041, which revealed that the experimental data were in good agreement with the predicted values of peak area. Coefficient of variation (C.V.) of less than 5.15% indicated that the model was reproducible. The 3D surface plots (Fig. 2) were drawn on the basis of the model equation to illustrate the interaction among the independent variables and to determine the optimum conditions for derivatization.

In conclusion, on the basis of RSM and experimental evidence, the optimum conditions for the derivatization reaction were defined as: reaction temperature: 70 °C, reaction duration: 37 min, added amount of DBCEEC: 7-fold molar excess to total molar sialic acids; the volume ratio of acetic acid in the final solution: 12%.

Optimization of hydrolysis condition.

For efficient pretreatment, a thorough optimization with 17 runs of experiments for interactive variables was listed in Table S2, and the 3D surface plots were plotted in Fig. 2. The ANOVA results showed the model was significant with p-value < 0.01 and F-value for the lack of fit was insignificant (P > 0.05), which all proved the model can be used accurately. For the model fitted, the R^2 was 0.9809 for Neu5Gc and 0.9858 for Neu5Ac. Therefore both the two multivariate models proved to be competent for predicting the optimal combination of variables. As a result, two variable combinations with comparable experimental responses (16 for Neu5Gc and 32 for Neu5Ac) were obtained. In view of the better validation of BBD model, the variable combination (hydrolysis time: 35 min, acid concentration: 2 mol/L and hydrolysis temperature: 75°C) was recommended.

The overall results of the optimization illustrated the enhancement effect of ultrasound, which reduced the hydrolysis time from 180 min required in conventional protocol to only 35 min and reduced the derivatization time to 37 min with ultrasonic assistance. To test the validity of response surface analysis method, the hydrolysis and derivatization were carried out under the optimal condition. The experimental values were found to be in agreement with the predicted ones, indicating that the experimental

design model may better reflect the derivatization parameters.

3.2. HPLC-FLD-MS method

With the thorough optimization, a simple UCSHD pretreatment for serum samples has been developed. Containing several hydroxyl groups, sialic acids are quite hydrophilic and thereby they usually elute at early retention times in HPLC analysis. As shown in Fig. 5A, Neu5Ac and Neu5Gc were detected by HPLC-FLD system at 2.43 min and 2.82 min respectively, and the excess labeling reagents eluted after the elution of sialic acids and had no influence on the detection. To guarantee the chromatogram peaks of analytes were not overlapped with impurity, the chromatogram peaks of analytes were confirmed by both retention time and online mass spectrometry identification. For obtain abundant MS and MS/MS data, two MS ion modes (negative and positive) of ESI were used to investigate the sialic acids derivatives. Although both ion modes have high response, ESI^+ mode was chosen since more fragments were obtained by ESI^+ mode. For example, DBCEEC-Neu5Ac comprises peaks of *m/z* 687.7, 495.2, 413.6, 397.4 and 280.4 by ESI^+ mode, whereas, only *m/z* 687.7 $[M+H-H_2O]^+$ was detected by ESI^- mode. The resulting MS and MS/MS spectra of representative DBCEEC-Neu5Ac are shown in Fig. 3. As can be seen from Fig. 3a, DBCEEC labeled Neu5Ac derivatives showed excellent ionization efficiency and produced intense molecular ion peak at *m/z* $[M+H]^+$ of 705.1. The MS/MS spectra(Fig. 3b) of DBCEEC-Neu5Ac showed that there were abundant fragment ions of *m/z* 687.7, 495.2, 413.6, 397.4 and 280.4, the characteristic fragment ions and cleavage modes for labeled Neu5Ac are shown in Fig. 3c. The ions of *m/z* 687.7 represented $[M+H-H_2O]^+$ by losing a molecule of H_2O from the protonated molecular; another fragment ion of *m/z* 495.2 corresponded to the C_4-C_5 bond breakage of the sugar chain by losing a molecule of H_2O; and the ion at *m/z* 397.4, which resulted from the cleavages between the C-N bond of the N-linked side chain and the simultaneous loss of acetyl group. The corresponding cleavage mode and MS/MS analysis for Neu5Gc was shown in Fig. 4. As expected, the DBCEEC-Neu5Gc derivative produced an intense molecular ion peak ($[M + H]^+$) at *m/z* 720.1. The MS/MS spectra of molecular ion ($[M + H]^+$) produced intense and stable fragment ions at *m/z* 702.9, 685.1, 483.7, 397.4, 413, 306.6 and 280 (Fig. 4b).With MS/MS, the ions at *m/z* 397.4 and *m/z* 280 were specific fragment ions for the identification of sialic acid derivatives. In short, with this

UCSHD-HPLC-FLD-MS method, the two sialic acids in serum samples can be detected in a more accurate and rapid way.

3.3 Method validation

Calibration curves were obtained according to Experimental Section. As shown in Table 1, excellent linearity for Neu5Ac and Neu5Gc was achieved in the concentration range from 0.5 to 16 μmol/mL with the correlation coefficient of $R^2 \geq 0.9991$. Sensitivity of this method was determined by LODs and LOQs. As expected, very low LODs (0.3 pmol for Neu5Ac and 0.21 pmol for Neu5Gc) and LOQs (0.90 pmol for Neu5Ac and 0.63 pmol for Neu5Gc) are achieved, which was superior to that of reported HPLC methods with NQAD[37] or UV.[38] Moreover, the LODs of this HPLC method (< 0.30 pmol) was on the same level or a bit higher than those of traditional GC-MS methods,[38] which requires sophisticated instrumentation and a rather tedious sample clean-up procedure. According to the results obtained from the reproducibility test, the RSDs for the retention time and peak area were less than 0.02% and 1.5%, respectively.

Intra- and inter-day variations for Neu5Ac and Neu5Gc are listed in Table 2, where it could be seen that the intra-day and inter-day accuracy ranged from -2.8% to 2.5% and from -3.4% to 1.4%, respectively. The inter-day precision values shown by RSD vary from 0.9% to 2.2%, and the intra-day precision values vary from 1.4% to 2.8%. As shown in Table 3, the recovery was measured by adding known amounts of Neu5Ac and Neu5Gc at three different concentration levels to human serum samples, the results showed that the present method provides good recoveries of 93.0 ± 3.0% for Neu5Ac, 92.5 ± 2.5% for Neu5Gc.

3.4 Analysis of real samples

In order to verify the practical applicability, the method was applied to the determination of Neu5Ac and Neu5Gc in the serum of normal and cancer patients. The typical chromatograms of standard solution and representative normal and breast cancer serums are illustrated in Fig. 5 (A, B and C), and the total analytical results are listed in Table 3. As can be seen from Table 3, Neu5Ac was found in all examined samples, but the concentrations in the samples of normal and cancers serum were significantly different (from 1.55 to 3.34 nmol/mL). The concentration of Neu5Ac in serum of cancer patients (>1.88 μmol/mL) was much higher than that of in the healthy group

(1.55μmol/mL), which is in agreement with the previous findings.[18, 24, 26] Concerning Neu5Gc, it has been only detected in certain types of samples such as human cancer cells[3] and serum from patients with endometrial cancer;[39] therefore, our results are consistent with these findings since Neu5Gc was not detected in any of human serum analyzed.

4. Conclusions

A simple, sensitive and novel method using UCSHD with a labeling reagent DBCEEC was established for determination of sialic acids based on HPLC-FLD-MS/MS. Owing to the combination of hydrolysis with derivatization steps in a closed system with assistance of ultrasonic, the UCSHD technique was proved to be a more convenient sample pretreatment method for determination of sialic acids than ever reported. What's more, good linearity ($R^2 > 0.9991$), quite low LODs (0.30 pmol for Neu5Ac and 0.21 pmol for Neu5Gc) and satisfactory recovery (93.0 ± 3.0% for Neu5Ac and 92.5 ± 2.5% for Neu5Gc) were achieved, which indicated that it is efficient, sensitive, accurate and reliable for sialic acids analysis in biological samples. The proposed UCSHD-HPLC-FLD-MS/MS method was successfully applied to the simultaneous determination of Neu5Ac and Neu5Gc in sera of normal and cancer patients, the experimental data demonstrated that Neu5Ac in serum of cancer patients is remarkable elevated compared with that in normal serum, which suggest that Neu5Ac can be a valuable marker for early diagnosis and prognosis analysis of patient with cancer. To the best of our knowledge, this is the most convenient and sensitive method for analysis of sialic acid in serum, so it exhibits powerful potential for accurate detection of sialic acid from other biological samples.

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Fig. 1 Scheme of ultrasonic-assisted closed in-syringe hydrolysis and derivatization (UCSHD) technique (1: ultrasonic-assisted hydrolysis and 2: in-syringe ultrasonic-assisted derivatization) and the derivatization process between the two analytes (Neu5Ac and Neu5Gc) and fluorescence reagent 2-[2-(7H-dibenzo[a,g] carbazol-7-yl)-ethoxy] ethyl carbonylhydrazine (DBCEEC).

Fig. 2 3D surface for the derivatization (a(1-6)) and liberation (b(1-3)) of sialic acid using the BBD obtained by plotting: a(1-6) showing the effects of the molar ratio of DBCEEC to the total sialic acids, volume ratio of catalyst to the final solution, derivatization temperature and time on the derivatization yield; b(1-3) showing the effects of acid concentration, hydrolysis temperature and time on the liberation of Neu5Gc

Fig. 3 MS spectra of representative Neu5Ac and the cleavage mode of protonated molecular ion (a: MS, b: MS/MS)

Fig. 4 MS spectra of representative Neu5Gc and the cleavage mode of protonated molecular ion (a: MS, b: MS/MS)

Fig. 5 The chromatograms of Neu5Ac and Neu5Gc from standard solution and typical chromatograms of Neu5Ac and Neu5Gc in sera of normal and patients with breast cancer. (A: the standard solution; B: the normal serum; C: the serum of breast cancer)

Table1

Linear regression equation, correlation coefficients, LODs, LOQs, reproducibility of retention time and peak area

Component	Regression equation[a]	R	LOD[b] (ng/mL)	LOQ[c] (ng/mL)	Reproducibility (RSD, %, n =6)	
					Retention time	peak area
Neu5Ac	y = 2.4459x – 0.8557	0.9995	1.08	3.59	0.02	1.3
Neu5Gc	y = 2.4054x – 1.0903	0.9991	0.97	3.35	0.01	1.5

[a] y = peak area; x = theoretical concentration of sialic acids (μmol/L).

[b] Signal/noise ratio = 3:1.

[c] Signal/noise ratio = 10:1.

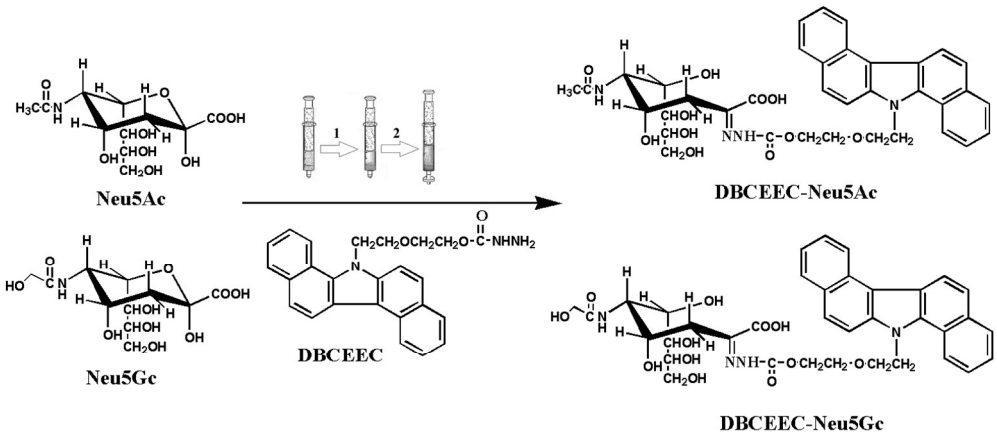
Table 2
Intra- and inter-day accuracy and precision of Neu5Ac and Neu5Gc at three concentration levels (n = 6)

Serum sample	Spiked (μmol/mL)	Inter-day			Intra-day		
		Mean ± SD	Accuracy (RE%)	Precision (RSD%)	Mean ± SD	Accuracy (RE%)	Precision (RSD%)
Neu5Ac	1	0.98 ± 0.02	-2.0	1.5	0.99 ± 0.03	-1.0	2.0
	5	4.94 ± 0.11	-1.2	1.4	4.93 ± 0.14	-1.4	1.6
	10	9.72 ± 0.10	-2.8	0.9	9.66 ± 0.19	-3.4	1.5
Neu5Gc	1	1.01 ± 0.04	1.0	2.2	0.99 ± 0.02	-1.0	2.8
	5	4.93 ± 0.09	-1.4	1.3	5.07 ± 0.12	1.4	1.8
	10	10.25 ± 0.13	2.5	1.2	9.79 ± 0.19	-2.1	1.4

Table 3
Determination of Neu5Ac and Neu5Gc in real samples under the optimized experimental conditions

No.	Samples	Components	Content (μmol/mL)	Added (μmol/mL)	Found amount (μmol/mL)	Recovery[b] (%)
1	Normal serum	Neu5Ac	1.55	1	2.51	93
				5	6.20	
				10	10.55	
		Neu5Gc	ND[a]	1	0.90	
				5	4.80	92
				10	9.00	
2	Serum of patient with intestinal cancer	Neu5Ac	2.67	1	3.55	
				5	7.22	90
				10	11.77	
		Neu5Gc	ND	1	0.95	
				5	4.50	93
				10	9.40	
3	Serum of patient with lung cancer	Neu5Ac	3.34	1	4.25	
				5	7.89	91
				10	12.44	
		Neu5Gc	ND	1	0.95	
				5	4.70	94
				10	9.30	
4	Serum of patient with liver cancer	Neu5Ac	2.89	1	3.84	
				5	7.29	92
				10	12.19	
		Neu5Gc	ND	1	0.92	
				5	4.60	92
				10	9.20	
5	Serum of patient with breast cancer	Neu5Ac	2.65	1	3.54	
				5	7.15	90
				10	11.75	
		Neu5Gc	ND	1	0.99	
				5	4.60	95
				10	9.40	
6	Serum of patient with esophageal	Neu5Ac	1.88	1	2.79	
				5	6.43	91
				10	10.98	
		Neu5Gc	ND	1	0.94	
				5	0.45	92
				10	9.2	
7	Serum of patient with gastric cancer	Neu5Ac	2.24	1	3.23	
				5	6.84	96
				10	11.94	
		Neu5Gc	ND	1	0.90	
				5	4.25	90
				10	9.5	
8	Serum of patient with ovarian cancer	Neu5Ac	2.12	1	3.02	
				5	6.67	91
				10	11.32	
		Neu5Gc	ND	1	0.90	
				5	4.55	91
				10	9.20	

[a] Not detected; [b] Data are expressed as mean recovery(%)



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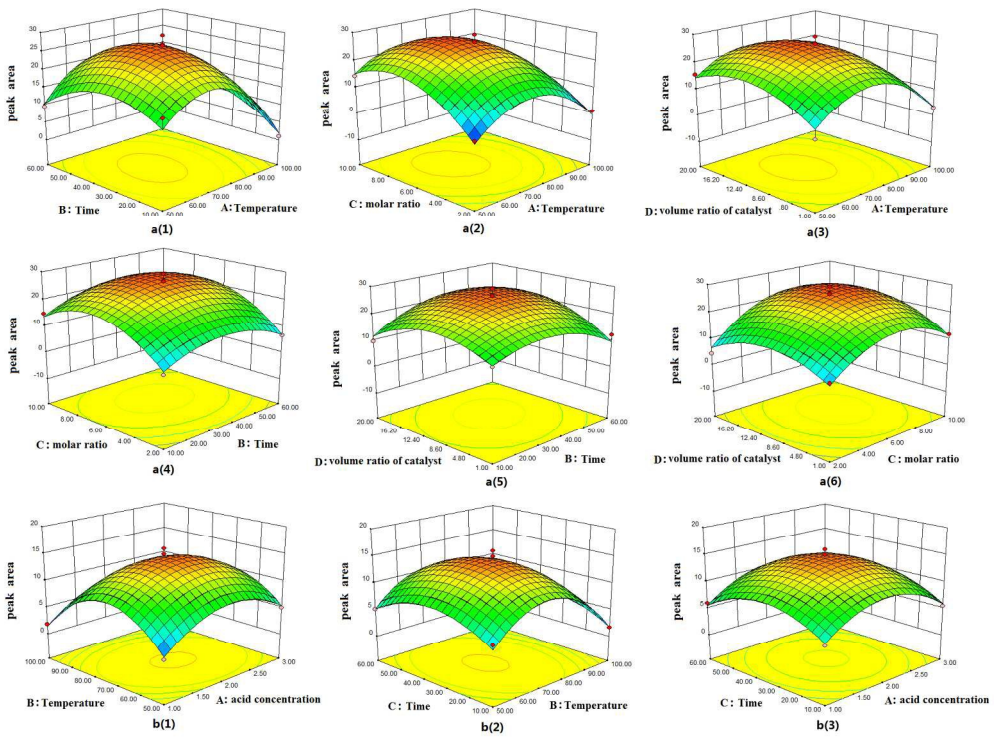

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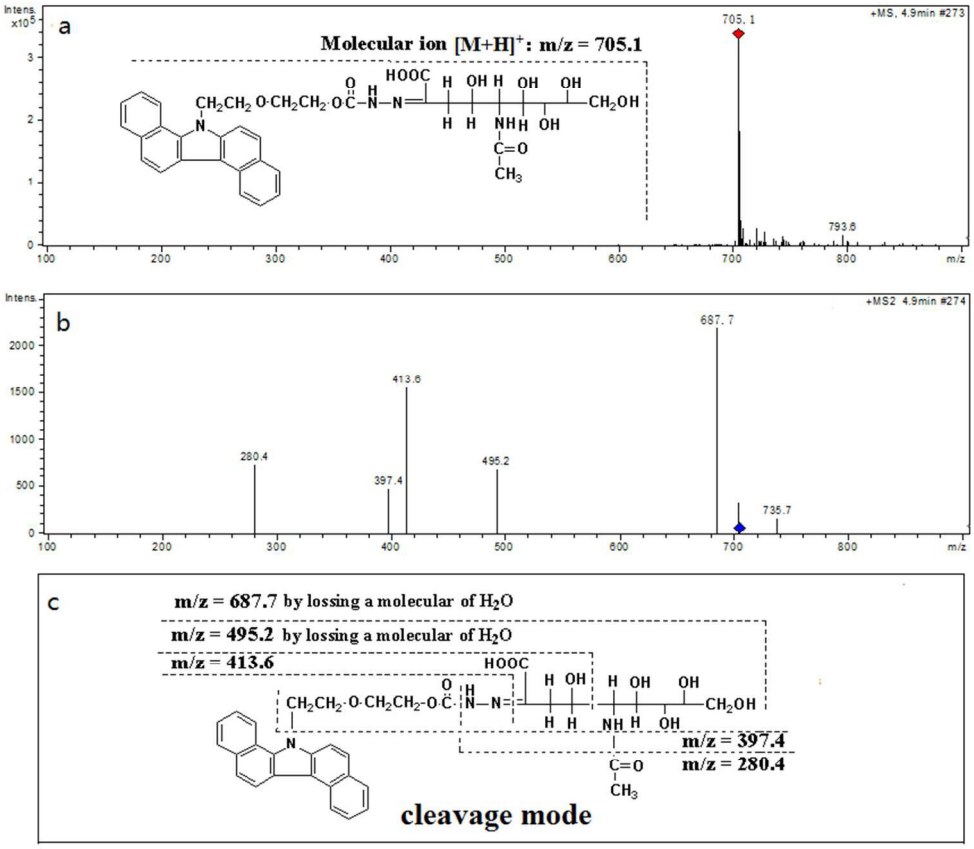

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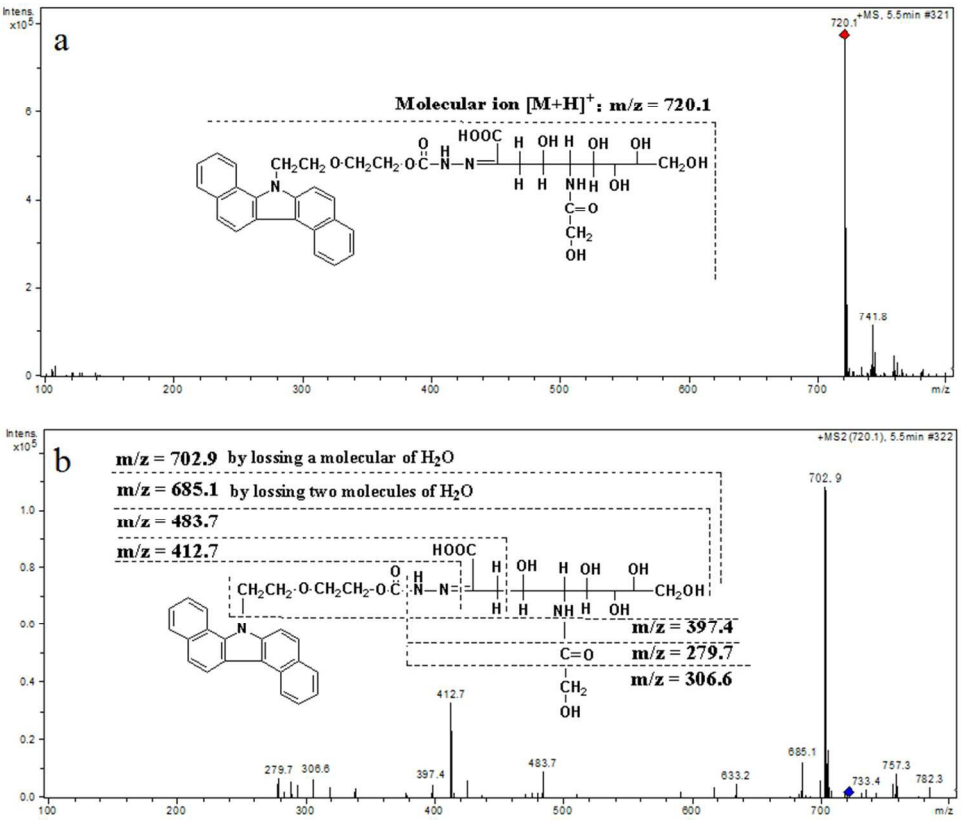

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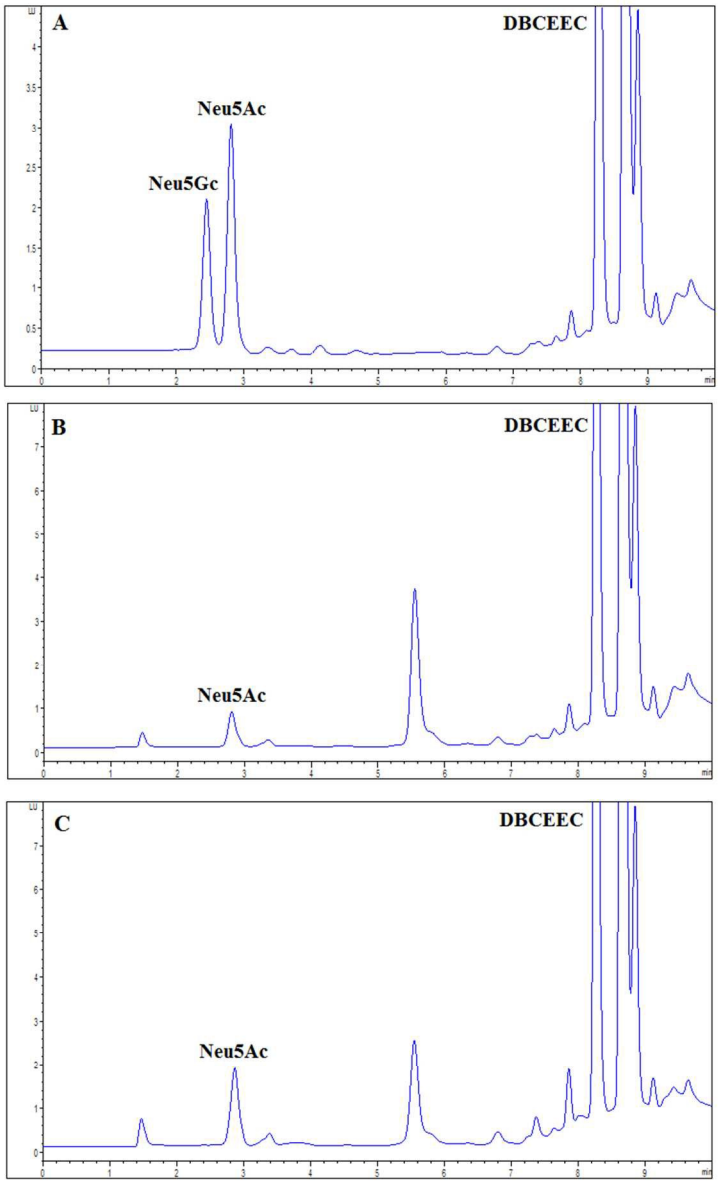

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