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ARTICLE

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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Validation and Quantification of genomic 5-carboxylcytosine (5caC) in mice brain tissues by liquid chromatography-tandem mass spectrometry

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Abstract 5-carboxylcytosine (5caC) is one of the most important oxidant product of 5-methylcytosine, an epigenetic biomarker generated from cytosine (Cyt). Although several methods have been carried out to detect 5caC, they still can not accurately quantify trace amount of 5caC. To conquer the challenge, we developed and validated a simple, robust method for the quantification of 5caC level in mammals' tissue by LC-MS/MS. Tissue DNA was isolated by commercial kit, hydrolyzed using 88% formic acid at 140 °C, separated using a bridged ethylene hybrid HILIC column, and analyzed by tandem MS. The linearity was evaluated in the concentration range of 40 to 4000 ng/mL for Cyt and 1to 100 ng/mL for 5caC, and both the correlation coefficients were higher than 0.99. Limits of detection were 0.05 ng/mL for Cyt and 0.1 ng/mL for 5caC, and limits of quantification were 0.1 ng/mL for Cyt and 1 ng/mL for 5caC, respectively. All the relative standard deviation (RSD) of intra-day precisions was lower than 6%. The recovery of the method was ranged from 93.42% to 96.54% with RSD low than 0.6%. Using this method, we illustrated 5caC was distributed in the tissues of mice brain, and the content of 5caC in cerebrum was higher than that in cerebellum and brainstem. Our studies indicated that the LC-MS/MS method was adequate to analyze 5caC level in biological samples.

Keywords: DNA demethylation; 5-carboxylcytosine; LC-MS/MS; epigenetics; Hydrophilic interaction liquid chromatography

1. Introduction

DNA cytosine (Cyt) modification is a dynamic process in mammalian[1]. The conversion from Cyt to 5-methylcytosine (5mC) conducted by DNA methyltransferase (DNMTs) has been well studied, and the roles of 5mC, including inactivation of the X chromosome[2], embryogenesis, carcinogenesis[3] and epigenetic regulation of gene expression[4, 5], were also demonstrated. The discovery of ten-eleven translocation (TET) protein family improved our understanding on the demethylation pathway. TET family members oxidized 5mC to 5-hydromethylcytosine (5hmC)[6, 7], and further into 5-formylcytosine (5fC) and 5-carboxylcytsine (5caC) in an enzymatic activity-dependent manner[8-10]. Compared to clarified 5mC, little is known about how 5caC functionally take part in DNA demethylation pathway. Nevertheless, the oxidation products 5fC and 5caC were found that can be recognized and cleaved by thymine-DNA glycosylase (TDG), which led to restore unmethylated cytosine via the DNA base excision repair (BER) pathway[8, 11, 12]. And 5caC was suggested as an important intermediate in the demethylation process.

Quantification of 5caC in the genomic was the premise of exploring its biological function. Several methods, including thin-layer chromatography, high performance liquid chromatography mass spectrometry[8, 13], chemical derivatization coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) [14, 15] etc, have been successfully found 5caC existed in genome from several biological samples However, 5caC exhibited a trace amount which was much lower than other intermediates in DNA demethylation pathway[16]. Accurate quantification for 5caC in mammal genome is still a challenge. One preliminary study reported the presence of 5caC in follicular cells of axolotl ovary by immunochemistry and immunofluorescence assay [17], but it didn't verified by absolute quantification method such as mass spectrometry yet.

Here, we demonstrated a simple and robust approach for 5caC quantification in which DNA sample was hydrolyzed by formic acid and analyzed by hydrophilic interaction liquid

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chromatography-tandem mass spectrometry (HILIC-MS/MS). With this method, we successfully qualified and quantified the 5caC in mammalian tissues and illustrated the 5caC distribution in different brain tissue. It demonstrated that the assay with a high sensitivity and accuracy can be used for the 5caC detection in various biological samples.

2. Experimental

2.1 Chemicals and reagents

Cyt ($C_4H_5N_3O$, M=111.1 g/mol), 5mC, 5hmC and ammonium formate (chromatographic grade, purity >99.995%) were purchased from Sigma (St. Louis, Missouri, USA). 5caC ($C_5H_5N_3O_3$, M=155.1g/mol), which purity was higher than 98% (the validation materials was not presented in this study), was synthesized by our laboratory. The isotope cytosine Cyt^{13}C^{15}N$_2$ was purchased from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). Methanol, acetonitrile (chromatographic grade), and formic acid were purchased from Merck (Darmstadt, Germany). All other reagents were of analytical grade. Purified water was obtained in our laboratory using a Milli-Q water system (Millipore, Massachusetts, USA).

2.2 Stock and working standard solutions

Stock solutions (40 μg/mL) of target analytes Cyt and 5caC were accurately prepared in methanol. In order to evaluate the linearity of the method, stock standard solutions of two analytes were further diluted with matrix to obtain a series of mixed working solutions that contained Cyt at a range of concentrations (40, 80, 200, 400, 800, 2000, 4000 ng/mL) and 5caC at a range of concentration (1, 2, 5, 10, 20, 50, 100 ng/mL). Internal standard Cyt^{13}C^{15}N$_2$ was dissolved in acetonitrile-water (83:17, v/v) to a final concentration of 50 ng/mL. All the stock and working solutions were kept in amber bottles at -20 ^{o}C.

2.3 DNA extraction and hydrolyzation

The animal experiments in this study were approved by the Committee on the Ethics of Animal Experiments of Guangdong Medical University and followed the guidelines of The Rules for Management and Implementation of Medical Experimental Animals issued by National Health and Family Plan Commission, P.R. China. All efforts have been made to minimize animal suffering. Two-month-old mice were purchased from Animal Centre of Guangdong Medical University and six of them were randomly selected to acquire cerebrum, cerebellum and brainstem tissues. Genomic DNA from mice brain was extracted by Genomic DNA Isolation Kit (Tiangen, Beijing, China) according to the manufacturer's protocols, and precipitated by 1/10 volume of sodium acetate (pH 5.4; 3 mol/L) and 2.5-fold volume of ethanol. About 30 μg DNA sample was hydrolyzed according to previously reported methods[18, 19]. Briefly, the residual was mixed with 0.2 mL isotope cytosine in a 2 mL clear glass vial and dried under nitrogen. Then, 0.2 mL of 88% formic acid was added and hydrolyzed at 140 ^{o}C for 90 min. The sample was cooled to room temperature and dried under nitrogen. The residue was dissolved in 0.2 mL acetonitrile-water (83:17, v/v) and centrifuged at 15 000 g for 5 min, and the final supernatant was directly injected into LC-MS/MS for analysis.

As the 5caC existed in both DNA and RNA [16, 20], RNA contamination will cause the error of detection, so two steps were employed to eliminate the influence. First, RNA was eliminated by adding RNase during DNA extraction. Secondly, the uracil content was investigated to exclude RNA contamination according to previous studies[21, 22].

2.4 Side reaction experiment

To investigate whether 5caC come from side reaction of DNA chemical hydrolysis, individual standard solutions of cytosine, 5mC, and 5hmC, which concentration was 10 μg/mL, 0.5 μg/mL and 1ng/mL respectively, were prepared in mobile phase. Next, 0.3 mL of them were mixed after adding 0.3 mL 88% (v/v) formic acid, and then hydrolyzed at 140 ^{o}C. The hydrolytic solution was dried under nitrogen and the residue was dissolved in mobile phase. Finally, the solution was analyzed in multiple reaction monitor mode by LC-MS/MS.

2.5 LC-MS/MS analysis

Samples were analyzed using LC (The LC system consisted of an Agilent 1200 series binary pump, an autosampler, and a thermostatted column compartment) attached to a mass spectrometer (Agilent 6430A triple quadrupole mass spectrometer equipped with an electrospray ionization source).

Target compounds were separated by a bridged ethylene hybrid (BEH) HILIC (50 × 2.1 mm id, 1.7 μm) column (Waters Chromatography Ireland Ltd., Dublin, Ireland) equipped with an inline filter. The flow rate was 0.3 mL/min and the column temperature was set at 20 ^{o}C. Injection volume was 5 μL. Gradient elution was performed with (A) 7 mM ammonium formate and 0.1% ammonia in Milli-Q water and (B) 0.1% ammonia in acetonitrile. The following gradient was used: 83% B for 0.5 min to 50% B over 5.50 min, held for 0.9 min; increased to 83% B at 7.0 min, and held for 2 min before column re-equilibration at 83% B for 1.7 min.

Mass spectrometric detection was achieved with an ESI source using nitrogen as the nebulizer gas. To achieve maximal sensitivity, the acquisition parameters of mass spectrometer were optimized by injecting 1 μg/mL standard solution of 5caC with a flow rate of 0.3 mL/min. Full scan mass spectra was recorded in the range of *m/z* 50-300 with the ESI source using both positive and negative modes. Due to higher response, positive ESI mode was selected for subsequent detections. Quantification and qualification were accomplished in multiple-reaction monitoring (MRM) mode, and the MS was operated at unit mass resolution for both precursor and product ions. The Quantification of both Cyt and 5caC was conducted by internal standard method with an isotope Cyt^{13}C^{15}N$_2$. The parameters of QQQ Source settings were as follows: nebulizer gas pressure, 35 psi; curtain gas flow, 10 L/min; capillary voltage, 4000 V; and source temperature, 350 ^{o}C.

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2.6 Methodological validation

The prepared mix working solutions of two analytes Cyt and 5caC were determined in sextic replicates by means of LC-MS/MS method. Calibration curves were established using ratios of the observed analytes peak area to internal standard versus molar concentration of analytes. Three folds of signal to noise ratio (SNR) were confirmed as limit of detection (LOD), and ten times of SNR were defined as limit of quantification (LOQ).

In precision and accuracy experiments, mix working solutions of Cyt and 5caC at three concentration levels (corresponding to the concentration level of point 2, 4 and 6 of each calibration curve) were analysed in sextic replicates using LC-MS/MS, respectively.

The reproducibility experiment was performed on three DNA samples in parallel. They were treated using the hydrolysis procedures mentioned above and then detected in triplicates by LC-MS/MS.

The stability of the method was determined by inter-day and intra-day assays. Mix working solutions of Cyt and 5caC at known concentration levels (in line with the concentration level of point 2, 4 and 6 of each calibration curve) were prepared in accordance with the above hydrolyzation protocols. LC-MS/MS detection of Cyt and 5caC performed in the 1, 3, 5, 7, 9 h of one validated day was used to assess inter-day difference, and that performed in 8 consecutive validated days was used to evaluate intra-day difference. LC-MS/MS was conducted in triplicate for each sample.

For the recovery test of the method, three amount levels including 6, 10 and 20 ng of standard 5caC was added into an identical DNA sample, respectively. Then, the mixtures were treated using the treatment process of DNA samples and LC-MS/MS analysis was carried out in six times for each composite sample.

All the methodological data in this study was represented as relative standard deviations (RSD).

2.7 Data analysis

The percentage of carboxylation was calculated using the following formula:

$$\text{Carboxylation\%} = Q_{5caC}/Q_{Cyt}\times 100\%;$$

Q_{5caC} and Q_{Cyt} represented the molar quantity of 5caC and Cyt determined in DNA sample, respectively.

3. Results and Discussion

3.1 Mass Spectrometric Characterization of 5caC

Figure 1a displayed a full scan mass spectrum of 5caC, which is noted that the ion m/z 156.0 corresponded to the protonated molecular ion $[M+H]^+$. Figure 1b showed that the product ions *m/z* 138.1, 94.9 and 68.0 were generated from the precursor ion *m/z* 156.0. Therefore, the product ion at *m/z* 156.0/138.1 was chosen for quantification of 5caC in the MS/MS section, and the product ion at *m/z* 156.0/94.9 and 156.0/68.0 was used as qualitative ions. The fragmentor of 5caC was optimized and a value of 120 V was selected to give the highest signal in further experiments. The parameters of Cyt and $Cyt^{13}C^{15}N_2$ listed in Table 1 were referred to our previous articles [21, 22].

3.2 Optimization of the mobile phase

We previously reported the quantification of 5hmC in colorectal cancer tissue using HILIC-MS/MS [23]. However, none 5caC signal was acquired from aforementioned method. In order to satisfy the detection of 5caC in genomic DNA, we attempted to optimize the composition of mobile phase. When the ammonia was added in mobile, the peak of 5caC was peaked magically. So, five concentration levels of ammonia (0.01%, 0.05%, 0.1%, 0.5% and 1.0%, v/v) was added into both default aqueous phase (7 mM ammonium formate in Milli-Q water) and organic phase (acetonitrile), and the best sensitivity and peak shape of 5caC were gotten when adding 0.1% ammonia (shown in Figure 2). Thus， 0.1% ammonia was integrated into the mobile phase to enhance the sensitivity of the method. As shown in Figure 3, two analytes Cyt and 5caC can be completely separated by the combination of optimized mobile phase and established gradient elution program. A DNA sample was also tested to validate the separation, sensitivity and peak shape of 5caC.

Theoretically, the existence of acids would be beneficial to the electrical spray ionization of containing nitrogenous compounds and further enhances the sensitivity. However, we did not detect 5caC signal when formic acid was added. Instead, the peak of 5caC exhibited when the ammonia was added in mobile. It indicated that the enhancement of 5caC sensitivity was not realized via affecting on ionization efficiency of 5caC, but the alteration of chromatographic behaviours. Added a certain amount of ammonia was able to effectively decrease adsorption between 5caC and packing materials of HILIC column, which was beneficial to 5caC separation. Nevertheless, the excess ammonia would predominantly restrain the ionization of 5caC and finally reduce the detection sensitivity of 5caC. Therefore, the optimal concentration of ammonia was selected as 0.1% to experimentally offer the best detection sensitivity for 5caC.

3.3 Calibration curves, limitation of detection and quantification

Calibration curves of 5caC and Cyt were obtained from MRM mode with optimized conditions described above. As shown in Figure 4, the assay was found to have good linearity in the tested range for 5caC and Cyt, and both the correlation coefficients were higher than 0.99, indicating that these calibration curves were adequate for quantitative purposes. According to the calibration curves, the LOD of Cyt and 5caC were calculated and the values were 1.6 and 3.2 fmol, and the LOQ was confirmed as 3.2 and 16.0 fmol for Cyt and 5caC respectively.

In previously published methods for 5caC quantification[14-16], the LODs of 5caC were ranged from 10 to 103 fmol. Compared with these methods, our method exhibited better sensitivity. Additionally, Tang et al[15] reported a chemical derivatization coupled with LC-MS/MS method with a LOD of 0.23 fmol;

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however, the sample preparation in this method was more complicated and time-cost as it needs enzymatic digestion and chemical derivatization.

3.4 Precision, accuracy, and stability

The RSD of precisions ranged from 1.10% to 3.09% and from 1.60% to 4.81% were obtained for Cyt and 5caC, respectively. Accuracy was ranged from 85% to 105%. The RSD of inter-day precision was ranged from 1.92% to 3.30% for Cyt and from 1.87% to 3.42% for 5caC, and those of intra-day precision was varied from 2.59% to 5.01% for Cyt and from 3.77% to 4.98% for 5caC, which suggested a good stability of this method.

The existence of RNA residues would interfere the accurate quantification of genomic 5caC. Herein, we detected the amount of uracil using our established LC-MS/MS method with a LOD of uracil at 8.9 fmol[21], and ensured DNA samples were avoided RNA contamination.

3.5 Recovery

The recovery of the method was varied from 93.42% to 96.54% and the RSD values were ranged from 0.24% to 0.59%. The favourable recoveries recorded in Table 2 demonstrated that our method was suitable to analyse the 5caC from biological tissues.

3.6 Oxidative products of nucleobases

The results of side reaction experiment was showed in Figure 5, there were not any 5caC generated from the chemical hydrolysis of Cyt, 5mC and 5hmC, which demonstrated that 5caC was only obtained from the DNA demethylation.

3.7 Application to real samples

By means of established HILIC-MS/MS method, we successfully identified and quantified 5caC in cerebrum, cerebellum and brainstem of mice. The MRM chromatograms of a cerebrum samples were showed in Figure 6. In order to improve reliability, two qualitative ions (*m/z* 156->94.9, 156->68.0) was applied in real samples.

Characteristic results from the analysis of all the tissue samples are shown in Figure 7. Data are the means ± standard deviations from triplicate analyses and the carboxylation level of cerebrum, cerebellum and brainstem was $(8.93\pm0.21)\times10^{-6}$, $(6.24\pm0.13)\times10^{-6}$, $(7.15\pm0.22)\times10^{-6}$, respectively. The results suggested that method is s suitable for determination 5caC level in DNA samples.

4. Conclusions

In summary, a novel HILIC-MS/MS method for the quantification of 5caC contents in mice brain has been developed and fully validated. The method has superior speed, specificity, and sensitivity. In comparison with previously reported methods for 5caC detection, chemical hydrolyzation of DNA used in this study highly shortened the time consumption of entire experiment protocols (more than 24h was saved) and avoided the incomplete action in enzymatic hydrolyzation, which was probably crucial point to improve the sensitivity in our HILIC-MS/MS assay. In addition, HILIC separation gives a number of advantages when mass spectrometric methods are used as detection devices, and HILIC elution with high portion organic phase enhances overall sensitivity by increasing desolvation and reducing surface tension.

In spite of utilization of an apparatus (Agilent 6430A) with relatively low sensitivity, we were still able to accurately detect and quantify 5caC, indicating the superiority of our method. Considering the sensitivity, accuracy and simplicity, this HILIC-MS/MS technology is suitable for measuring the degree of carboxylation cytosine in various DNA samples. Using the established method, we provided direct evidence for the presence of 5caC in DNA isolated from brain tissue. Moreover, the distribution of 5caC in cerebrum, cerebellum and brainstem of brain suggested an epigenetic regulative role of 5caC in mammal neurons.

Acknowledgements

This work was supported by grants from the National Nature Science Foundation of China (NSFC 21375029), Discipline Construction in Higher Education of Guangdong, P. R. China (2013CXZDA018).

Conflict of Interest

The authors have declared no conflict of interest.

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Figure Captions

Figure 1 Full scan (a) and product ion scan (b) of 5caC.

Figure 2 The effect of ammonia concentration on the peak area of 5caC. The different ammonia concentrations were adjusted to form an eluent consisting of acetonitrile and water.

Figure 3 Chromatograms of 5caC, Cyt and Cyt$^{13}C^{15}N_2$ in standard solutions. Total ion chromatogram of 5caC and Cyt (a) displays a better separation, the chromatograms of the quantifier transition of 5caC (b), Cyt (c) and Cyt$^{13}C^{15}N_2$ (d). The retention times were 0.67, 1.10 and 1.10 min, respectively.

Figure 4 Liner ranges, regression equations, correlation coefficients R^2 for Cyt and 5caC. (a)5caC; (b)Cyt.

Figure 5 The MRM of Cyt, 5mC and 5hmC after DNA chemical hydrolysis. (a) 5caC standard solution (1 ng/mL); (b) Cyt standard solution (10 μg/mL); (c) 5mC standard solution (0.5 μg/mL); (d) 5hmC standard solution (1 ng/mL).

Figure 6 Chromatograms of qualitative and quantitative ions for 5caC from a cerebrum sample in MRM. a. quantitative ion (*m/z* 156->138.1) of 5caC in standard solution; b. quantitative ion (*m/z* 156->138.1) for 5caC in tissue samples; c. qualitative ion (*m/z* 156->94.9) for 5caC in tissue samples; d. supplemental qualitative ion (*m/z* 156->68.0) for 5caC in tissue samples.

Figure 7 The percentages of 5caC in the DNA of cerebrum, cerebellum and brainstem. 5caC contents were expressed as the percentages of 5caC in the total pool of cytosine.

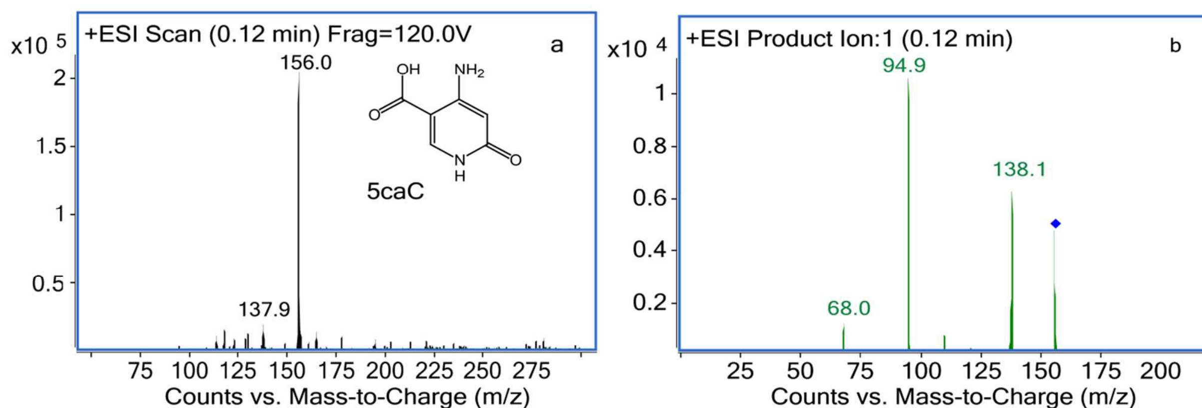

Figure 1

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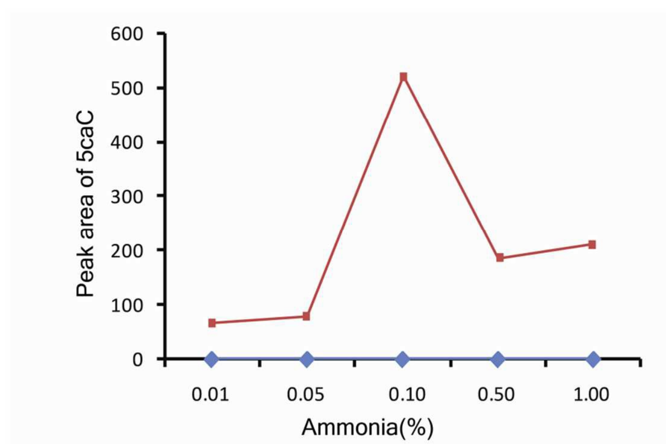
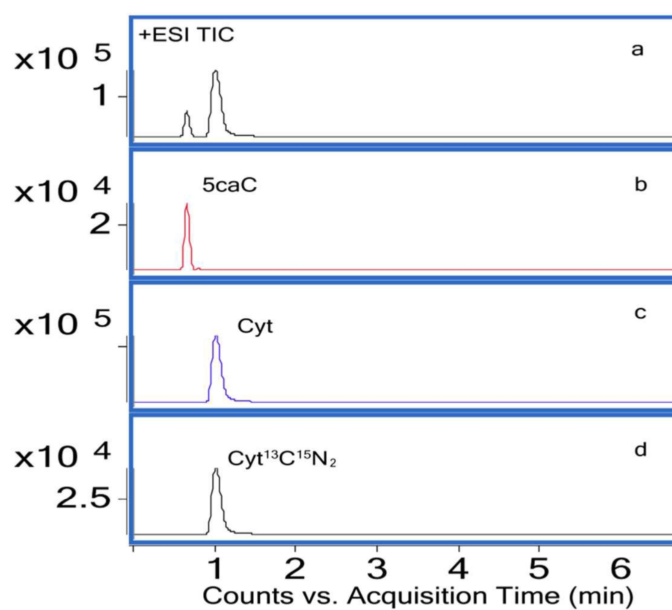


Figure 2



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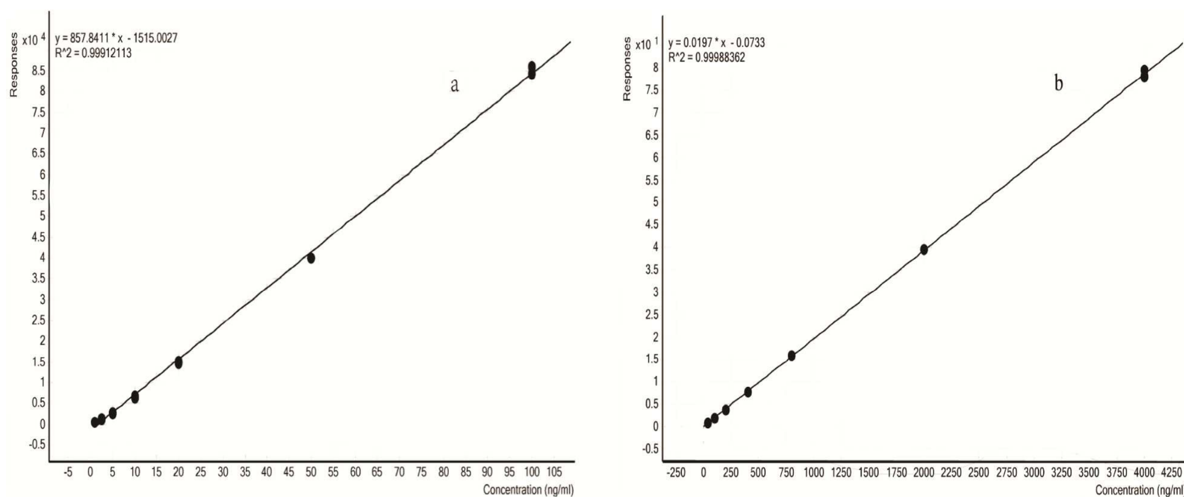

Figure 4

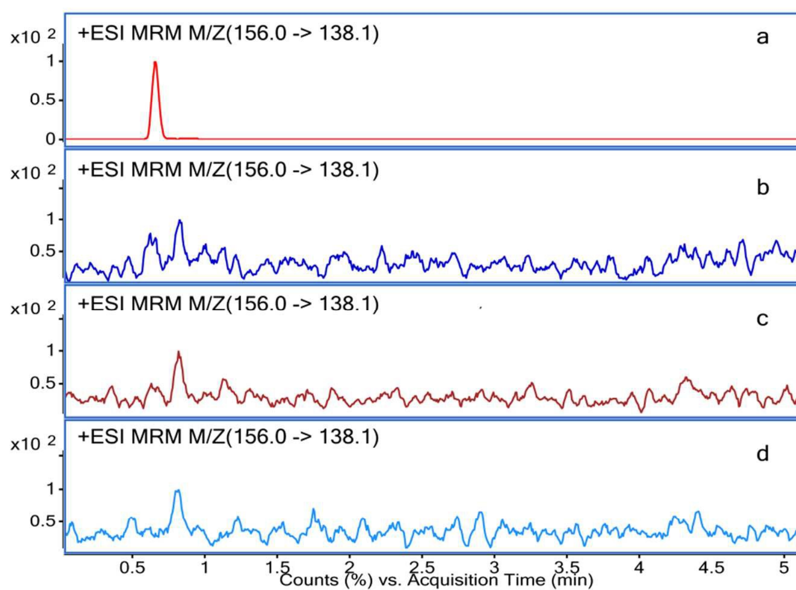

Figure 5

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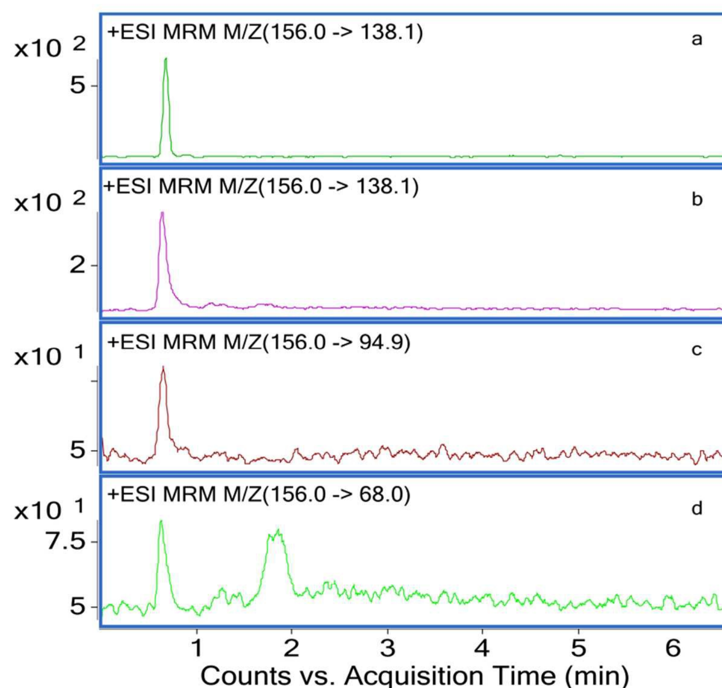

Figure 6

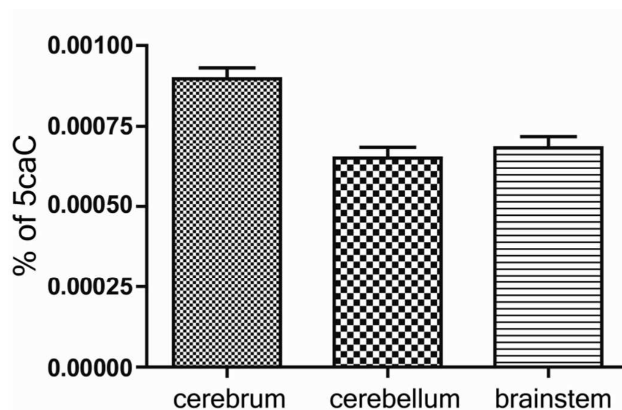

Figure 7

Table 1 Mass spectrum parameters of Cyt, Cyt$^{13}C^{15}N_2$ and 5caC

Analytes	precursor ion (*m/z*)	Fragmentor (V)	quantifier product ions (*m/z*)	quantifier collision energy (V)	qualifier product ions (*m/z*)	qualifier collision energy (V)
Cyt	112.1	114	95.0	19	69.1	18
Cyt$^{13}C^{15}N_2$	115.1	114	97.1	19	70.1	22
5caC	156.0	120	138.1	10	94.9	20

Table 2 Recoveries of 5caC (n=6)

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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

Sampled (ng)	Added(ng)	Found±SD (ng)	Recovery (%)	RSD (%)
12.5	6	17.62±0.81	95.04	0.46
12.5	12	23.69±1.40	96.54	0.59
12.5	20	30.40±0.73	93.42	0.24

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