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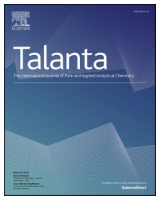

Quantitative analysis of RNA by HPLC and evaluation of RT-dPCR for coronavirus RNA quantification

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ABSTRACT

Nucleic acid detection and quantification have been known to be important at various fields, from genetically modified organisms and gene expression to virus detection. For DNA molecules, digital PCR has been developed as an absolute quantification method which is not dependent on external calibrators. While when it comes to RNA molecules, reverse transcription (RT) step must be taken before PCR amplification to obtain cDNA. With different kinds of reverse transcriptase (RTase) and RT reaction conditions being used in laboratory assays, the efficiency of RT process differs a lot which led variety in quantification results of RNA molecules. In this study, we developed HPLC method combined with enzymatic digestion of RNA to nucleotides for quantification of RNA without RT process. This method was metrologically traceable to four nuceloside monophosphate (NMP) Certification Reference Materials of National Institute of Metrology, China (NIMC) for insurance of accuracy. The established method was used to evaluate the reverse transcription digital polymerase chain reaction (RT-dPCR) of three target genes of Middle East Respiratory Syndrome Coronavirus (MERS-CoV) RNA, including open reading frame 1ab (*ORF1ab*), nucleocapsid protein (*N*) and envelope protein (*E*) gene. Three available RT kits had been evaluated and disparities were observed for the RT efficiency varied from 9% to 182%. It is thus demonstrated that HPLC combined with enzymatic digestion could be a useful method to quantify RNA molecules and evaluate RT efficiency. It is suggested that RT process should be optimized and identified in RNA quantification assays.

1. Introduction

Nucleic acid detection and quantification play a crucial role at various fields, such as genetically modified organisms [1], gene expression and regulation [2–5] and virus detection [6–10]. Ribonucleic acid (RNA) is the genetic material of RNA virus, which may cause many human diseases, including the most recently emerged severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) which has spread rapidly all over the world [11,12]. The pathogen was identified as a novel coronavirus, which belongs to the family *Coronaviridae* [12,13]. There has been two major epidemics caused by members of the coronaviruses in history, including severe acute respiratory syndrome coronavirus (SARS-CoV) occurred since 2003 [14,15], and middle east respiratory syndrome coronavirus (MERS-CoV) occurred since 2012 [16]. For RNA measurement, reverse transcription real-time quantitative polymerase chain reaction (RT-qPCR) has been considered as the primary method and found wide applications. Reverse transcription digital polymerase chain reaction (RT-dPCR), due to its sensitivity and accuracy, has also been developed and used in RNA quantification [8,17]. RT-dPCR allows absolute quantification of cDNA without calibrant by partitioning the PCR solution into small reactions and applying Poisson statistics to the proportion of the negative partitions [18,19]. However, all PCR-based detection methods of RNA are dependent on reverse transcription (RT) of RNA to cDNA, which is essential prior to PCR but may induce biases to the results [20]. This fact has been noticed but often neglected, for example, in the expression levels of mRNA and its clinical significance where variability up to100-fold were observed [20–23]. The variability depends on the choice of reverse transcriptase (RTase) as well as the reaction conditions like temperature and priming strategy (oligo (dT), random hexamer, or gene specific primer) [21–25].

Thus, in this study, we focus on developing accurate RNA quantification method not depending on reverse transcription process. Most regularly used method like ultra-violet (UV) and fluorescence spectroscopy can be simple but lack of specificity and traceability to quantify

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RNA, due to the interference of other impurities such as organics, proteins or DNA [26,27]. Snake venom phosphodiesterase I (SVP) has been used in quantification of 20mer oligonucleotide by digesting DNA to deoxynucleotide monophosphates (dNMPs) followed by isotope dilution mass spectrometry (IDMS) [28,29], and ultrasonic treatment was needed prior to SVP digestion for quantification of large dsDNA [30]. RNA can be hydrolyzed to nucleotide monophosphates (NMPs) by SVP whereas RNA quantification method of this kind has not been reported to date as we know.

The purpose of this study is to investigate the ability of digestion of RNA by SVP followed by high performance liquid chromatography (HPLC) to quantify RNA molecules using four NMP Certification Reference Materials obtained from National Institute of Metrology, China (NIMC) as calibrators for insurance of accuracy. The established method was applied to quantify an *in vitro* transcribed MERS-RNA comprised of three major target genes termed open reading frame 1ab (*ORF1ab*), nucleocapsid protein (*N*) and envelope protein (*E*) gene. Meanwhile, the *in vitro* transcribed MERS-RNA was measured by RT-dPCR using different RT kits, so that the RT efficiency was evaluated.

2. Experimental section

2.1. RNA samples

An *in vitro* transcribed RNA molecule consisting of three genes of MERS (partial *ORF1ab,* full length *E* and *N*) was used in the whole study. Gene synthesis and construct containing MERS-*1ab-E-N* were ordered from Sangon Biotech Co., Ltd (Shanghai, China). Plasmid containing synthetic genes was linearized with BamHI at the 3'-end and purified as the linear template DNA. *In vitro* transcription reaction was performed using MEGAscript™ T7 Transcription Kit (Thermo Fisher Scientific, USA) according to the manufacturer's suggested protocol, and 1 μL of TURBO DNase were added after transcription to remove the template DNA. Transcribed RNA was purified with MEGAclear™ Kit (Thermo Fisher Scientific, USA). Purity of the RNA transcript was measured using the RNA 6000 Pico kit and analyzed on a 2100 Bioanalyzer Instrument using their 2100 Expert Software (Agilent Technologies, USA). RNA stock solution was diluted to 1.2 ng/μL with RNA storage solution (Thermo Fisher Scientific, USA), aliquoted to 100 μL/tube, stored at −80 °C, and used as RNA template throughout this study.

2.2. Standards and reagents

Four Certified Reference Materials of adenosine 5'-monophosphate (AMP, GBW(E)100054), guanosine 5'-monophosphate disodium salt (GMP, GBW(E)100068), cytidine 5'-monophosphate (CMP, GBW(E) 100067) and uridine 5'-monophosphate disodium salt (UMP, GBW(E) 100069) at purities of 98.9%, 98.8%, 99.3% and 99.4% were developed and stored by NIMC. Uncertainties of 0.7%, 0.6%, 0.6% and 0.4% were assigned to the corresponding values for AMP, GMP, CMP and UMP. The NMP standards were dried at 80 °C for 4h before use. Snake venom phosphodiesterase I (SVP) was purchased from Sigma-Aldrich (phosphodiesterase I from *Crotalus adamanteus* venom, 0.46 units/vial).

2.3. Digestion of RNA samples

A total volume of 100 μL mixture containing 90 μL of RNA, 9 μL of SVP buffer (100Mm Tris-HCl pH 8.8, 10mM ammonium acetate, and 100 mM Magnesium acetate), and 1 μL of SVP (0.00023 U/μL) were incubated at 37 °C for 15 min. No enzyme control (NEC) and no template control (NTC) were prepared by adding water in place of enzyme or template RNA.

2.4. HPLC analysis

The HPLC system of Agilent 1200 series was used in this work. The separation of the four nucleotides was achieved using an SB-AQ C_{18}, 2.7 μm particles, 4.6 mm × 150 mm analytical column (Agilent) maintained at 25 °C. The mobile phase was 0.1% formic acid in a flow rate of 1 mL/min. The four nucleotides were eluted within 5 min after injecting 20 μL of samples, with UV detector wavelength of 260 nm and 280 nm.

Stock standard solution of about 1 mg/g for each NMP was prepared by weighing each solid standard and dissolved in water (18 MΩ). Stock solutions of four NMPs were two-fold serially diluted and mixed to obtain calibrators which were separated and analyzed by HPLC. Calibration curve of HPLC was generated by plot mass concentration and peak area of five-point NMP calibrators. Accurate mass concentrations of NMP calibrators were used with consideration about purity of NMPs and weight of the solvent to calculate the concentration of NMPs released from RNA samples. Four digestion mixtures of RNA samples were prepared to be analyzed by HPLC. Each sample was injected and analyzed in triplicate for both RNA samples and calibrators.

2.5. Reverse transcription digital PCR (RT-dPCR)

The primer and probe sequences for detecting *ORF1ab* and *E* gene target of the MERS-CoV published by world health organization (WHO) were used for this study [31]. For detecting *N* gene target, primer and probe published by Center for disease control and prevention (CDC) was used [32].

One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad, USA) was used to optimize the assays. The reaction mixture in a final 20 μL volume included 5 μL of supermix, 2 μL of reverse transcriptase, 1 μL of 300 mM DTT, 1μL of mixture of forward and reverse primers and probe, 6 μL of RNase-free water and 5 μL of template. The final concentrations of primers and probes were 900 nM and 100 nM for ORF1ab, 500nM and 200nM for E gene and N gene respectively. Each reaction mix was converted to droplets using the QX200 Droplet generator (Bio-Rad, USA). After generation, the emulsion of droplets was transferred to a 96-well plate, heat sealed and amplified in a GeneAmp System 9700 thermal cycler (Applied Biosystems, USA). The thermal cycling conditions were as follows: 45 °C for 60 min (reverse transcription); 95 °C for 10 min; and 40 cycles of 95 °C for 30 s, and 58 °C for 30 s; 98 °C for 10 min. The cycled plate was then transferred to the QX200 reader (Bio-Rad, USA) and analyzed using QuantaSoft Version 1.7.4.0917 (Bio-Rad, USA).

For two-steps RT-dPCR assays, ThermoFisher SuperScript III First-Strand synthesis supermix for qRT-PCR and SuperScript IV First-Strand synthesis system had been used to generate cDNA following the protocols of the kits. cDNA was then applied to dPCR with a 20 μL reaction including 10 μL of supermix for probes (Bio-Rad, USA), 1μL of mixture of forward and reverse primers and probe, 4 μL of RNase-free water and 5 μL of template. The primers and probes concentration and the droplet generation procedure were the same as above. The thermal cycling conditions were the same as above except that the RT process was omitted. The primers and probes and the reaction conditions were identified to be suitable when cDNA was used in dPCR assays.

3. Results and discussion

3.1. Separation of NMPs by HPLC

SB-AQ is a reversed-phase packing which had been used for separation of dNMP [30] when quantifying DNA molecules with mobile phase of ammonium acetate buffered to pH 3.5. Initially NMPs digested from RNA were applied to the column with the same condition, but the four NMPs failed to separate entirely from each other. Optimization of ammonium acetate buffer with different pH including 3.5, 4.5, 5.5 and 8.5 didn't improve the result. When the mobile phase was changed to 0.1% formic acid, under isocratic elution condition, complete separation of the four NMPs was ultimately achieved. The retention time of the latter two NMPs was observed to increase so that they could be

separated better when the column temperature reduced to 25 °C. The four NMPs was eluted under the optimized condition within 5 min with retention time of 2.07 min, 2.52 min. 3.27 min and 3.67 min respectively for CMP, UMP, AMP and GMP (Fig. 1A). Data at 260nm was chosen to be analyzed for AMP, UMP and GMP, and data at 280 nm was chosen for CMP, because the signal intensity of CMP was remarkably higher at 280 nm than at 260 nm.

3.2. Digestion of RNA samples

The total sequence length of the *in vitro* transcribed RNA, including the transcription initiation site of T7 RNA polymerase, was 4610 nucleotides according to the synthetic report of manufacturer. Purification of transcribed RNA was applied to remove nucleotides, proteins and salts. Purity and homogeneity of the transcribed RNA were checked and confirmed through chip assay where only one band exited. When it came to DNA, long fragment nucleic acid could not be completely digested by using only enzymes [30]. However, as single-stranded RNA is sensitive to RNase and degrades more easily, it was deduced that the *in vitro* transcribed RNA could be digested completely by using SVP without any pretreatment. In this study, digestion optimization was carried out with the peak area of each NMP analyzed at time point of 5 min, 15 min, 30 min, 60 min and 120 min. Over the 2 h time course, no significant change was observed in the amount of hydrolysis products from 15 min to 1 h, yet slight reduction appeared at 2 h (Fig S1). Furthermore, in the chromatography result of HPLC, the profile of digestion products released from RNA sample was comparable with the that of NMP calibrators (Fig. 1B). Therefore, RNA was considered to be completely digested with hydrolysis time of 15 min to 1 h at 37 °C. Further hydrolysis of nucleotides may occur after 2 h.

3.3. Quantitative analysis result of RNA by HPLC

In view of the dependence of the accuracy of our analysis method on calibrators, they were gravimetrically prepared and metrologically traceable to CRMs of NIMC. Standard curve was established to calculate the unknown concentration of NMP digested from RNA sample. The four standard curves corresponding to CMP, UMP, AMP and GMP exhibited excellent linearity with R^2 of 0.9999, 0.9999, 1.0000, 1.0000 (Fig S2). Nucleotides digested from the transcribed MERS-CoV RNA sample thus could be detected and analyzed by the standard curves. With the exact sequence of the RNA sample being known, concentration of the intact RNA was calculated based on the detected mass concentration of each NMP. The calculated concentrations of the RNA sample derived from CMP, UMP, AMP and GMP are 1.05 ± 0.05 ng/μL, 1.06 ± 0.05 ng/μL, 1.06 ± 0.06 ng/μL, and 1.09 ± 0.06 ng/μL respectively (Table 1). These results show that the RNA concentration determined by each of the NMP agrees well with each other and the mean value is 1.07 ± 0.06 ng/μL. Expanded uncertainty ($k = 2$) which combined standard deviation and uncertainty of dilution factors and reference materials were considered. Because the RNA concentration is too low to be measured accurately using absorbance at 260nm, it was calculated to be 1.20 ± 0.06 ng/μL through dilution factor and the stock solution concentration of 25.8 ng/μL. The predicted value from HPLC analysis was slightly lower than from UV absorption (Fig. 2), which was may due to that the HPLC method would not be interfered with other impurities such as proteins or DNA.

HPLC analysis combined with digestion of RNA described here is a useful method to quantify RNA which has some favorable features. Firstly, this is a quantification method that does not require reverse transcription step. The total amount of RNA could be measured, and when the exact length and sequence of the RNA is known, the copy number concentration can be calculated. Furthermore, because of the different profiles of NMPs and dNMPs in the HPLC chromatography, it is able to differentiate DNA from RNA, which is not workable when using UV absorption. Another important aspect is the accuracy of the method could be assured by tracing to Certified Reference Materials of NMPs, in which case, it is applicable to any homogeneous RNA sequence. The sensitivity of the HPLC method was assessed to be 0.02 ng/μL with UV detectors. Other methods of detection, such as mass spectrometry, may improve the sensitivity and accuracy of analysis with use of internal standards. Finally, the performance of the method doesn't cost a lot, because phosphodiesterase I is the only special reagent used that is not expensive, and the two main experiments include digestion and separation of nucleotides could be completed within 15 min and 5 min, respectively. Thus the method should be easy to be carried out.

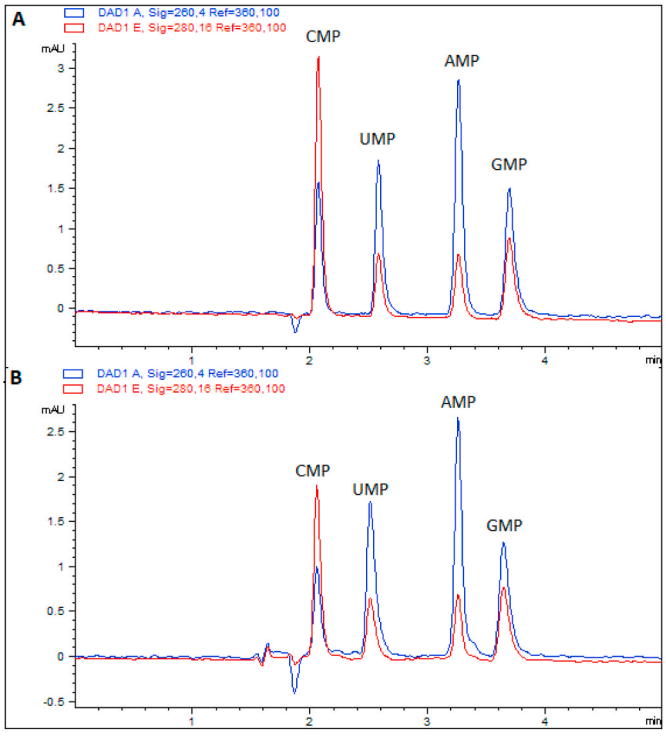

Fig. 1. HPLC profile of AMP, UMP, CMP and GMP. (A) NMP reference materials. (B) NMP released from RNA samples after enzymatic digestion.

3.4. Optimization of RT-dPCR assays of MERS-RNA

Partial *ORF1ab,* full length *E* and *N* gene of MERS were constructed onto the same plasmid to evaluate the amplify and reverse transcription efficiency of the RT-dPCR assays. Primers and probes sequence used in this study origin from former publications [31,32]. Optimization for dPCR has been carried out including primers and probes concentration and annealing temperatures on Bio-rad QX200 platform using one-step RT-dPCR kit. Validation experiments were performed to assess the performance characteristics of the RT-dPCR including repeatability, linearity, specificity and sensitivity of the assay (supplemental Fig S3 and Table S1.)

The *in vitro* transcribed RNA was 10-fold diluted into RNA storage buffer (Thermofisher) and measured. Good linearity was observed

Table 1
Quantitative Results for the intact *in vitro* transcribed MERS-RNA from HPLC analyses. Expanded uncertainty (U, $k = 2$) combined standard deviation and uncertainty of dilution and reference materials.

	CMP	UMP	AMP	GMP
RNA Mass fractions (ng/μL)	1.05	1.06	1.06	1.09
SD (ng/μL)	0.01	0.01	0.02	0.02
% CV	1.4	1.0	1.6	1.7
U ($k = 2$) (ng/μL)	0.05	0.05	0.06	0.06

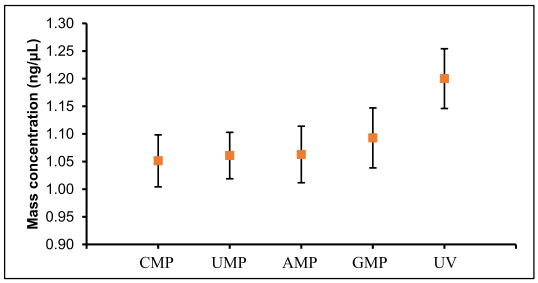

Fig. 2. Comparison of the RNA mass concentration calculated from four NMPs and UV absorption. Error bar represents expanded uncertainty ($k = 2$).

across the dilution range from approximately 10^5 to 10^0 copies/reaction for the three target genes with $R^2 \geq 0.9999$. The LoQ of the RT-dPCR assay was assess to be 64 copies/reaction for *ORF1ab*, 167 copies/reaction for *E* and 156 copies/reaction for *N* gene, with a criterion of CV value lowering than 25%.

The specificity of the assays for three genes has been tested in the previous report [31,32]. In this study, we further validated that the assays had no positive results when testing the SARS-CoV-2 RNA reference material which was prepared by National Institute of Metrology, China.

3.5. Evaluation of RT-dPCR assays of MERS-RNA

The transcribed MERS-RNA solution prepared in this study was quantified by RT-dPCR using the established assays. Additional to one-step RT-dPCR kit of Bio-rad, two cDNA synthesis kits of Thermofisher mentioned above were used to generate cDNA, and supermix for probes of Bio-Rad was subsequently used to quantify the cDNA. For one-step RT-dPCR, three tubes of RNA were tested with triplicate assays for each tube; for two-steps RT-dPCR, three tubes of RNA were tested with each tube being reverse transcribed respectively and triplicate assays of dPCR were performed for each cDNA. The quantification results were showed at Fig. 3.

Indeed, what the RT-dPCR determined was the copy number concentration of the reverse-transcribed cDNA molecules. The HPLC method established here could determine the accurate mass concentration of RNA molecules, and the copy number concentration of the RNA molecules could be calculated based on RNA mass concentration from HPLC quantification result, applying the following equation:

$$C_{RNA} = \frac{m_{RNA} \times N_A}{M_{RNA}}$$

C_{RNA} RNA copy number concentration (cp/μL) m_{RNA} RNA mass concentration (ng/μL) N_A Avogadro constant (cp/mol) M_{RNA} molar mass of the transcribed MERS-RNA (ng/mol)

Calculation of the molar mass of the transcribed MERS-RNA was based on the sequence of the RNA and the molar mass of each nucleotide monophosphate. Thus, the efficiency of the reverse transcriptional conversion of RNA template into cDNA was determined and expressed as the ratio of RNA copy number concentration of RT-dPCR and HPLC (Table 2).

From Fig. 3 and Table 2, we noticed dramatical disparities in the quantification results and RT efficiency among different methods. The efficiency of One-step RT-dPCR kit and Superscript III kit was range from 9% to 71%, and that of Superscript IV was more than 100%. The reverse transcriptase in both the two ThermoFisher kits are MMLV RT and different reaction conditions have been optimized and developed by manufacture for them. For Superscript III kit, the reverse primers existed in the mix of RT reaction including both oligo$(dT)_{20}$ and random hexamers and other components. Its suggested reaction temperature was 50 °C, and higher temperatures up to 60 °C was suggested for difficult templates. But we didn't get higher yields of cDNA at 60 °C than at 50 °C. For Superscript IV kit, a mix of template RNA, dNTP and the reverse primers was heated at 65 °C for 5min followed by incubation on ice for at least 1min to get the annealed RNA prior to RT reaction. We suspected non-specific amplification occurred during this reaction leading to excessive quantification result.

For one-step RT-dPCR, the reverse transcriptase was integrated within one tube with PCR reactions. Its efficiency was higher than Superscript III kit for both *E* and *N* gene, but lower for *ORF1ab* gene. Because the three genes of MERS-RNA were constructed to one single strand, their copy number concentrations were theoretically expected to be the same. Nevertheless, different quantification results and RT efficiency were observed for the three genes, no matter which kit was used. This may due to the variability of G-C content or secondary structures of the RNA template.

As indicated above, absolute quantification of RNA via RT-dPCR relies heavily upon the efficiency of the reverse transcription process. For accurate quantification of RNA molecules, work flow of optimization should be to designed to identify the most appropriate RT type and reaction conditions for target genes.

4. Conclusions

A quantification method of RNA molecules with no need of reverse transcription by using enzymatic digestion followed by HPLC was successfully applied for the quantification of *in vitro* transcribed MERS-RNA. This method was demonstrated to be accurate for the metrological traceability to certification reference materials. RT-dPCR method of MERS-RNA was established and evaluated through the HPLC result and dramatical disparities of RT efficiency were observed, thus RT type and reaction conditions were recommended to be optimized and identified.

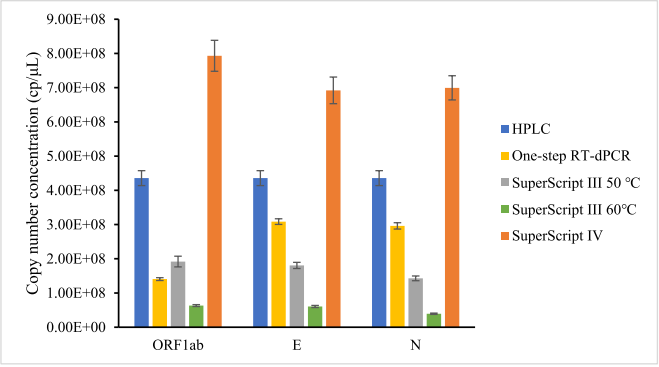

Fig. 3. Comparison of the copy number concentration result of the three genes of MERS-RNA.

Table 2
Conditions and efficiency of reverse transcript reaction.

RT kit	Primer type	Temperature (°C)		RT Efficiency[a]		
		Suggested	Used	ORF-1ab	E	N
One-step	Gene-specific primers	42–50	45	32%	71%	68%
SuperScript III	Random hexamers and oligo $(dT)_{20}$	50–60	50	44%	41%	33%
			60	14%	14%	9%
SuperScript IV	Random hexamers	55	55	182%	159%	160%

[a] RT Efficiency = $\frac{\text{copy number concentration of cDNA measured from RT – dPCR}}{\text{copy number concentration of RNA calculated from HPLC}}$.

Credit author statement

Chunyan Niu wrote the manuscript and analyzed the data. Chunyan Niu, Lianhua Dong, and Yunhua Gao designed the study. Chunyan Niu, Yongzhuo Zhang, and Xia Wang did the HPLC and RT-dPCR measurements. Lianhua Dong, Yunhua Gao and Jing Wang supplied critical suggestions to the manuscript. And all authors have reviewed the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2021.122227.

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