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ARTICLE

Direct and quantitative detection of dicyandiamide (DCD) in milk using surface enhanced Raman spectroscopy

Xiang Lin,[a] Wu-Li-Ji Hasi,*[a] Xiu-Tao Lou,[a] Si-qin-gao-wa Han,[b] Dian-Yang Lin,*[a] Zhi-Wei Lu*[a]

Dicyandiamide (DCD) is a contaminant found in New Zealand's milk which needs to be examined for security. In this study, the feasibility of using SERS technique to detect DCD in milk was evaluated. Assignments of the DCD vibrational spectra have been conducted by DFT calculations. The influences of different aggregating agents and pH have been investigated in terms of their efficiency for detecting DCD. Two detection modes were established for detecting DCD in aqueous solution. Among the three ions, SO_4^{2-} provides the best performance in both acidic and alkaline conditions and the limit of detection in alkaline condition (with an enhancement factor of 2.88×10^5) is lower than in acidic condition. For real milk sample, based on a particular order of agent addition, DCD can be quantitatively detected without any sample pretreatment through an internal standard method. The linear coefficient is 0.99747 at a concentration range from 1×10^{-4} to 1×10^{-3} g/mL. By exploiting the multi-component detection ability and high sensitivity of SERS, our scheme has advantages such as simplicity, less time and good accuracy over other methods, which shows great promise for on-site screening DCD in milk products.

Introduction

Dicyandiamide (DCD), similar to melamine, is a nitrogen-rich compound and putative contamination to enhance the apparent protein content of milk.[1] It can be used to reduce the negative effects of greenhouse gas emissions and nitrate leaching into waterways.[2] And it is always used by dairy farmers to promote the growth of grass. Unluckily, in January 2013, it was reported that New Zealand's milk and milk products were found to contain dicyandiamide (DCD) which can be accumulated into milk through the food chain. The New Zealand government ordered a ban on the sale of milk products containing DCD. Subsequently, DCD was added to the list of materials that need to be detected for security by the US Food and Drug Administration (FDA). To date, there is no agreed standard for acceptable levels of DCD in food products, while high doses of DCD are considered toxic to humans. Therefore, it would be important to at least detect the DCD contamination in milk and infant formula.

In this context, many methods such as ion exclusion chromatography,[3] UV spectroscopy coupled with chemometrics,[4] hydrophilic interaction liquid chromatography with tandem mass spectrometry (HILIC–MS/MS)[5] and liquid chromatography–tandem quadrupole mass spectrometry (UFLC–MS/MS)[6] have been developed for detecting DCD in milk since 2013. However, these methods always require large-scale instruments, complicated process and extensive sample preparation, therefore not well suited for rapid on-site assessment and identification of DCD in numerous milk and milk products. Thus, there is a need for a rapid, simple and portable method that can effectively screen milk products for DCD adulteration without using hazardous solvents and complex pretreatment processes.

Surface enhanced Raman spectroscopy (SERS), as an emerging analysis technology,[7, 8] has been proven to effectively detect various food contaminants such as melamine,[9–11] thiram[12] and crystal violet[13] in many kinds of matrix. Although various kinds of SERS substrates have been developed so far,[14–17] metal colloids[18, 19] are still the most commonly used SERS substrates for trace detection of target analyte due to its easy preparation method and ultra-high sensitivity (down to single molecule level).[20] However, the Raman enhanced effect of metal colloid is easily affected by many conditions such as pH,[21] aggregating agents,[22] concentration of metal nanoparticles[23] and analyte species. Therefore, when detecting a new chemical substance, many parameters need to be optimized.

For quantitative detection, a high sensitivity is meaningless without reproducibility. Changes in experimental parameters, such as fluctuations in laser power, focus position onto the sample and different Raman enhanced effect of substrates, make SERS detection hard to achieve good signal reproducibility. To reduce these interferences, SERS was combined with internal standard method to obtain quantitative SERS measurements.[24–26] By using an analyte band (internal standard) which lies in a region free from bands of interest, spectra can be corrected to compensate changes in experimental parameters. This allows SERS to be a quantitative analytical technique.[27]

In this paper, we proposed a SERS method to detect DCD in milk which could eliminate complicated pretreatment procedure and greatly reduce the testing time. Density functional theory (DFT) calculation has been applied to assign all vibrational bands of DCD. In addition, the influences of three aggregating agents with different binding ability to Ag NPs, including Cl^-, NO_3^- and SO_4^{2-}, and PH on the detection of DCD have been evaluated. Two modes, acidic and alkaline condition, were proposed to detect DCD in aqueous solution. After further optimization of the detecting condition, combined with an internal standard method, this method enables quantitatively detection of DCD in the range of 1×10^{-4} to 1×10^{-3} g/mL without any sample pretreatment.

Experimental section

2.1 Reagents

Silver nitrate ($AgNO_3$), sodium citrate and dicyandiamide (DCD) were obtained from Sinopharm Chemical Reagent Co., Ltd., hydrochloric acid (HCl), sulfuric acid (H_2SO_4) and nitric acid (HNO_3) were obtained from Beijing Yili Fine Chemicals Co., Ltd., Sodium chloride (NaCl), sodium sulphate (Na_2SO_4) and sodium nitrate ($NaNO_3$) were purchased from Xilong Chemical Co., Ltd., Deionized water was used for all procedures. Milk was purchased from a local grocery store.

2.2 Sample preparation

DCD standard stock solution (1×10^3 ppm) was prepared at first. A series of concentrations of standard DCD solutions were prepared by diluting the stock solution with water. The solutions of milk spiked with 0.1, 0.25, 0.5 and 1 mg/mL DCD were prepared by adding 1 mL of each standard DCD solution into 9 mL of milk. 1 mL of deionized water was added into 9 mL of milk as a blank control.

2.3 Synthesis of Ag NPs

Silver colloid was prepared according to the method of Lee and Meise.[28] Briefly, 45 mg of silver nitrate was added to 250 mL of deionized water, which was then brought to a boil in a flask under vigorous stirring. Sodium citrate (5 mL, 1%) was added, and the solution was kept boiling for 1 hour. Finally, the solution turned greenish brown, which indicated the formation of silver colloid, it was then cooled naturally. The morphology of silver colloid was observed by a Hitachi SU8010 field emission scanning electron microscope (SEM) at an accelerating voltage of 15 kV. The photograph, SEM image and UV-vis spectrum of the colloidal AgNPs are shown in Fig. 2A. After synthesis silver colloid were centrifuged and redispersed in deionized water to obtain a concentrated silver colloid ($10\times$).

2.4 SERS measurement

360 μL of DCD solution was added to 40 μL of $10\times$ silver colloid, then 50 μL of the aggregating agents was added into the mixture, finally, 10 μL of mixing solution was taken to subject SERS detection.

Raman spectra were recorded by a portable compact laser Raman Spectrometer BWS415-785H (B&W Tek, Inc.) equipped with a 785 nm Laser. On one hand, the surface plasma resonance (SPR) absorption peak of silver colloid would shift to red once silver nanoparticles aggregate. On the other hand, fluorescence is a common problem in Raman spectroscopy, whereas longer laser excitation wavelengths do not have enough energy to excite molecular fluorescence. Therefore 785 nm laser can be used to eliminate or reduce the fluorescence emission which is helpful to SERS detection. The spectrometer provides Raman spectrum over the range of 68 to 3200 cm^{-1} with a spectral resolution of batter than 3 cm^{-1}. The SERS detections were conducted with a 5 s exposure time and laser power of 150 mW unless otherwise stated. All the experiments were performed in triplicate unless otherwise stated. Boxcar averaging was used to smooth the raw spectrum, and a baseline correction routine was performed to obtain the final spectrum with the background subtracted.

2.5 Computational details

Gaussian 09 program was used for all calculations. The geometry optimization and vibrational spectrum calculation for DCD in gas phase were performed with the B3LYP hybrid exchange–correlation function and the standard 6-31G(d) basis set. Gaussview 05 program has been considered to obtain visual animation and complete the assignment of normal modes.

Results and discussion

3.1. Molecular geometry and Normal Raman of DCD

To the best knowledge of the authors, no vibrational mode assignments for DCD have been made to date. Therefore, DFT calculation was applied to assign all vibrational bands of DCD. DCD molecule was subjected to geometry optimization and vibrational spectrum calculation in the ground state by employing Becke 3-parameter-Lee-Yang-Parr (B3LYP) hybrid functional[29, 30] with the basis set 6-31G(d). In B3LYP, the exchange term is described by the exchange-functional of Becke, the non-local correlation is described by the LYP expression, and VWN functional III for local correlation. It has become a standard method used to study organic chemistry in the gas phase.[31] Melamine, which has similar chemical groups and elemental composition with DCD, has been calculated by DFT using B3LYP functional and show a good result.[32, 33] Furthermore, to improve the chemical accuracy of DFT method,

a constant coefficient (scale factor) has been introduced to calibrate the vibrational frequency. According to the scale factor database for vibrational frequencies,[34] a scale factor of 0.991 was used.
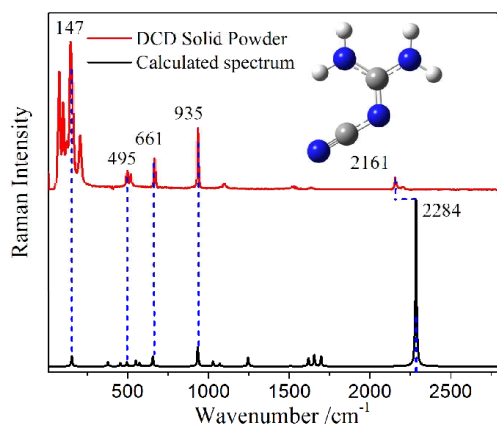
Fig. 1 Experimental and density functional theory calculated Raman spectra of dicyandiamide (DCD). Insert: B3LYP6-31G(d) optimized molecular structure of the neutral species of DCD.

The optimized structure of DCD, the observed Raman spectra of solid DCD along with the DFT calculated spectrum of DCD molecule were shown in Fig. 1. As can be seen, the most intensity Raman bands of the predicted spectrum agree well with the experimental result except some slight discrepancies, which may be due to that the theoretical calculation is attributed to an isolated molecule in the gas phase whereas the experimental detection is attributed to DCD molecules in the solid phase.[32]

The experimental and calculated wavenumbers and assignments of the Raman bands are presented in Table 1. The most intense bands present in the DCD Raman spectrum are also present in its calculated spectrum with minute changes in the position of the bands. The most intensity Raman bands of DCD solid powder were located at 147 cm^{-1}, 495 cm^{-1}, 661 cm^{-1}, 935 cm^{-1} and 2161 cm^{-1}.[35, 36] The strongest band at 147 cm^{-1} is due to the bending mode of C-N=C. The weak band at 495 cm^{-1} is attributed to the N–C rocking coupled with the N-C≡N bending mode. The medium band at 661 cm^{-1} is assigned to the N-C≡N bending mode coupled with the C-N rocking mode. The strong band at 935 cm^{-1} is due to the C–N stretching mode coupled with the C-N=C symmetric stretching and bending mode. The weak band at 2161 cm^{-1}, which had red-shift about 123 cm^{-1} (to 2284 cm^{-1}) in the predicted spectrum, is due to the -C≡N asymmetric stretching vibration.[37, 38]

**Table 1** Experimental and Calculated Vibrational Frequencies (cm^{-1}) and Assignments for dicyandiamide.

NRS	Calculated wavenumbers	assignment
76		
100		
123		
147	153	C-N=C bending
	159	H-N-C=N wagging + C≡N rocking
206		
	377	N-C-N twisting
	453	N–C rocking
497	495	N–C rocking + (N-C≡N) bending
520		
	550	NH$_2$ twisting + (N-C≡N) bending
	572	NH$_2$ twisting
667	655	(N-C≡N) bending + C–N rocking
	712	CN$_3$ out-of-plane bending
935	934	C–N stretching + (C-N=C) symmetric stretching and bending
	1028	
		NH$_2$ rocking
1097	1068	
	1244	NH$_2$ rocking + C-N=C asymmetric stretching
1525	1507	N-C-N asymmetric stretching
1543	1617	N=C stretching + NH$_2$ scissoring
1650	1654	N-C-N asymmetric stretching + NH$_2$ scissoring
	1697	N=C stretch + N-C-N symmetric stretching and bending + NH$_2$ scissoring
2159&2205	2284	(N-C≡N) asymmetric stretching

3.2. Effect of aggregating agents on SERS spectra of DCD

Currently, many kinds of anions have been used as aggregating agents to improve the SERS enhancement of silver colloid. The most critical difference between these agents is their affinity to Ag NPs. In this paper, three salts with different affinity to Ag NPs including NaCl, NaNO$_3$ and Na$_2$SO$_4$ were used as aggregating agents for SERS detection. The affinity order among the three kinds of anions is: Cl$^-$ > NO$_3^-$ > SO4^{2-}.[39] In order to evaluate the discrimination between SERS spectra of DCD solution and the blank control group, both DCD solution and water were submitted to SERS detection using the three agents, as shown in Fig. 2B, Fig. 2C and Fig. 2D. Previous investigation has shown that the concentration of aggregating agent is a key factor for SERS detection,[22] therefore the concentrations of the three salts were optimized for the detection of DCD. The concentration is 2M for NaNO$_3$ and Na$_2$SO$_4$ and 0.1M for NaCl. The two spectra using NO$_3^-$ and SO4^{2-} as aggregating agents are identical. Bonds at 611, 922, 952, 1024, and 1396 cm^{-1} show up in both SERS spectra of DCD solution and control group. They are originated from citrates adsorbed on Ag NPs.[40, 41] Due to the binding ability of SO4^{2-} and NO$_3^-$ to Ag NPs is weaker than citrate which serve as the stabilizer of silver colloid, SO4^{2-} and NO$_3^-$ could lead to aggregation without replacement of citrate on Ag NPs.[41, 42] And the Raman signal of citrate will be enhanced by SERS hot spots located at the junction of nanoparticles. Besides these bonds, Raman peaks of DCD, which locate at 663, 925 and 2161 cm^{-1}, are also exist on the spectra. This shows that the affinities of DCD and citrate to Ag NPs are comparable. Among Raman bands of citrate, the band at 922 cm^{-1} will cause interference for detecting DCD, as it locates too close to the DCD Raman band

at 925 cm^{-1}. Whereas, when Cl$^-$ was used, the bands of citrate disappear and only a band at 1032 cm^{-1} appears with no interference for discrimination of DCD.
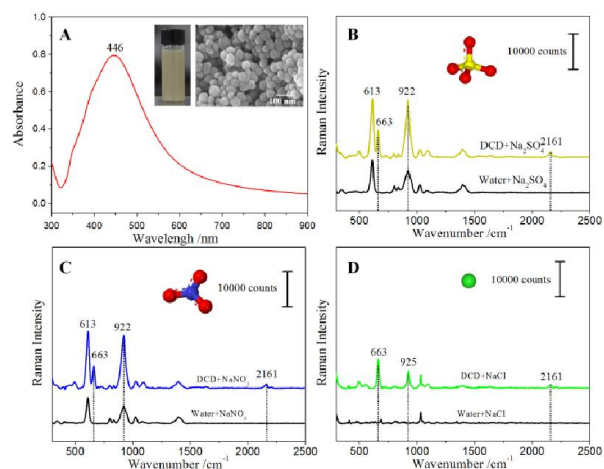
Fig. 2 (A) UV–visible adsorption spectra, photograph and SEM image of silver colloidal Ag NPs; (B-D) SERS spectra of DCD using different aggregating agents. Insert: 3D balls of sulphate, nitrate and chloride ion.

It's worth noting that the concentration of Cl$^-$ (0.1M) is much lower than SO$_4^{2-}$ and NO$_3^-$ (2M). When the concentration of Cl$^-$ is 2M, the bonds of DCD disappear, as shown in Figure S2. That's because the affinity of Cl$^-$ to Ag NPs is stronger than both citrate and DCD. Suitable concentration of Cl$^-$ could replace citrate and adsorb on the surface of Ag NPs, meanwhile provide sites for DCD to access Ag NPs. However, when the concentration of Cl$^-$ is high, Ag nanoparticles would be covered fully by Cl$^-$ and this strongly bonded surface layer would prevent adsorption and detection of DCD molecules. Therefore, the affinity of DCD to Ag NPs is roughly between that of Cl$^-$ and citrate.

The intensity of Raman band at 663 cm^{-1} was used to compare the enhancement effect of the three salts for detecting DCD. As shown in Fig. 1, Cl$^-$ yields the best result without any interference, meaning that both the bands at 663 cm^{-1}, 935 cm^{-1} and 2161 cm^{-1} could be used to identify DCD. In conclusion, among the three ions, Cl$^-$ is the best choice for detecting DCD in neutral condition.

3.3. Effect of pH on SERS spectra of DCD

Many SERS studies reveal that pH can cause band shift and intensity variation by induced geometric orientations of the analyte on the metal surface.[21, 43, 44] In order to further optimize the parameters for detecting DCD, the influence of pH to SERS performance was investigated. H$_2$SO$_4$, HNO$_3$, HCl and NaOH were used to adjust the pH without introducing other anions. The concentrations of the agents used were adjusted to optimize the SERS detection of DCD.

3.3.1. Under acidic condition. To compare the difference between the three agents, their corresponding acids, HCl, HNO$_3$ and H$_2$SO$_4$, were used to activate silver colloid. The concentration is 2M for HNO$_3$ and H$_2$SO$_4$ and 0.002M for HCl. As shown in Fig. 3, in acidic condition, Raman bands of DCD

at 663 cm^{-1}, 926 cm^{-1} and 2161 cm^{-1} were consistent with that in neutral condition. Moreover, SERS spectra of water with acids show no Raman bands of citrate. Among the three acids, H$_2$SO$_4$ provided the highest signal intensity at 663 cm^{-1} and 926 cm^{-1}. Importantly, the band intensity using H$_2$SO$_4$ is much stronger than that using NaCl in neutral condition.
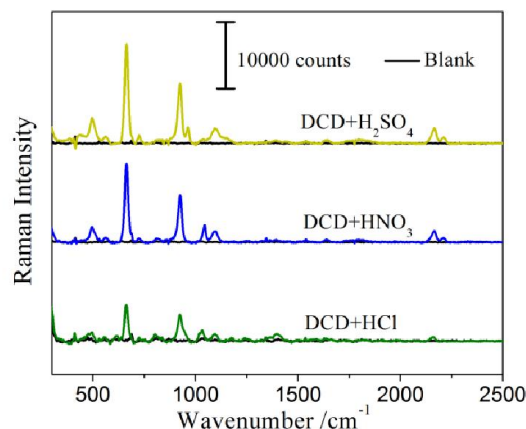
Fig. 3 SERS spectra of DCD using different acids, black lines are the corresponding control groups.

3.3.2. Under alkaline condition. Fig. 4A shows the normal Raman spectrum of DCD solid powder and SERS spectra of DCD solution in acidic and alkali condition. In acidic condition, the Raman band at 667 cm^{-1} was slightly shifted to 663 cm^{-1} and the Raman band at 935 cm^{-1} was shifted to 926 cm^{-1}. In alkali condition, Raman band at 667 cm^{-1} was shifted to 700 cm^{-1} and the band at 935 cm^{-1} was also shifted to 926 cm^{-1}. In addition, the shoulder band at 2205 cm^{-1} of band at 2159 cm^{-1} was shift to 2100 cm^{-1}. This is because different pH conditions produce different interactions at the interface between the nanoparticles and DCD molecule, thereby giving different SERS spectra.
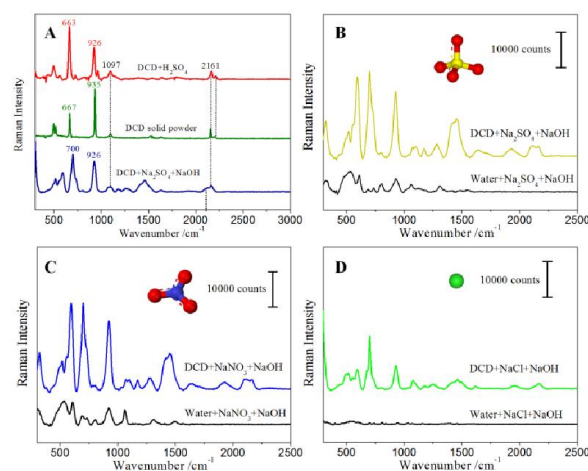
Fig. 4 (A) the influence of pH to Raman band position; (B-D) SERS spectra of DCD using different aggregating agents with NaOH.

DCD solution and water were submitted to SERS detection using three agents with NaOH, as shown in Fig. 4B, Fig. 4C and Fig. 4D. The concentration is 0.1M for all agents. It's worth noting that SERS spectra of control groups using the three salts also have a small Raman band at 933 cm^{-1} which may cause interference for detecting DCD. Even so, there still has a significant difference of band intensity values between DCD solutions with the control groups. In this case, the Raman intensity of bands on SERS spectra using SO_4^{2-} and NO_3^- are about the same height, while Cl^- provides a lower height. Generalizations can be made about the detection of DCD using different aggregating agents and under different pH conditions. Both acidic and alkaline conditions could be used to detect DCD in aqueous solution, and SO_4^{2-} is the best choice for both conditions.

### 3.4. Comparison of the two detection mode
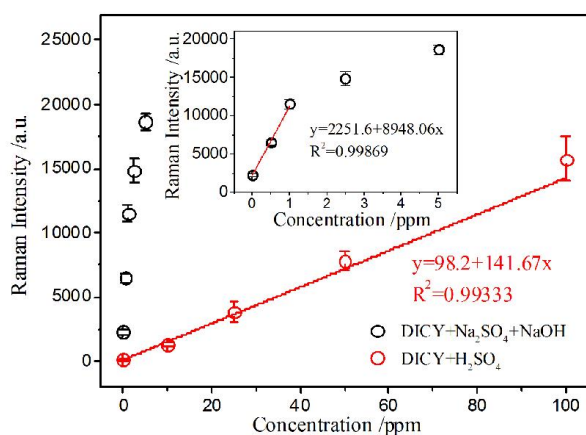
Fig. 5 Calibration curves of DCD in two conditions. Insert: The magnified plot of the Raman intensity versus the concentration of DCD under alkaline condition.

The typical Raman bands of 663 and 926 cm^{-1} on SERS spectra under acidic and alkaline conditions were used to identify DCD. Fig. 5 provides calibration curves of DCD using the two detection modes. The result reveals that even acidic mode has a wider linear range, alkaline mode has a lower detection limit than acidic mode. The Raman intensity of 926 cm^{-1} under alkaline condition was linear over the range 0 to 1 ppm and had a correlation coefficient R^2=0.9987. Therefore, Na_2SO_4 coupled with NaOH were the best combination for the detection of DCD in aqueous solution. On that basis, an enhancement factor (EF) of 2.88×10^5 was achieved, see supplementary material.

3.5. Detection of DCD in milk

According to our previous study,[45] adding aggregating agents into silver colloid before milk sample introduction could effectively enhance the Raman signal of milk. For practical sample detection, after optimizing the detection condition, 100μL of aggregating agent, 2M NaCl/2M NaOH (1:1, v/v), was firstly added in to 50μL of 10× silver colloid, then 200μL of milk sample was introduced into the mixture solution.

Finally, 10μL of mixing solution was used to subject SERS detection. Raman bands at 614 cm^{-1}, 652 cm^{-1}, 701 cm^{-1}, 865 cm^{-1}, 929 cm^{-1}, 1424 cm^{-1} and 1459 cm^{-1} will arise on the SERS spectrum of milk (Fig. 6A). These Raman fingerprint bands, which come from milk components, have constant relative intensity to each other and could be used as internal standards for detecting containments in milk. Using this internal standard method, the variation caused by environments is negligible.
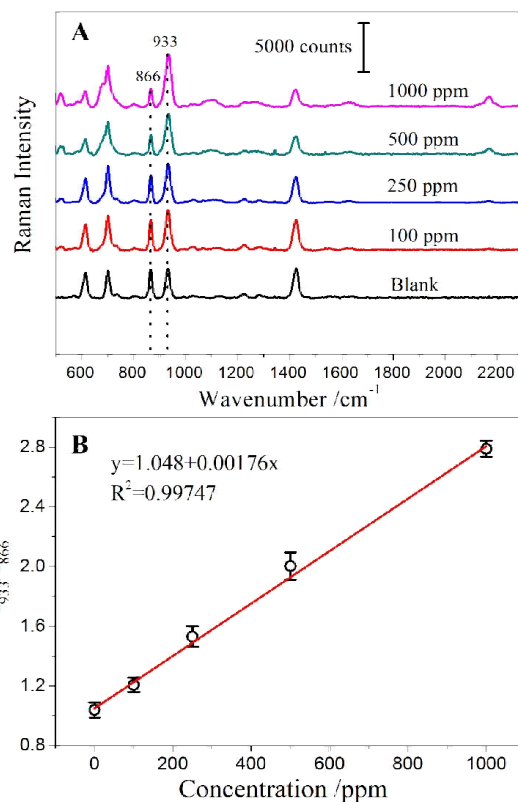
Fig. 6 (A) SERS spectra of DCD spiked milk sample with concentrations range from 0.1 to 1 mg/mL ppm; (B) Calibration curve for DCD spiked milk sample.

Raman band at 866 cm^{-1} was selected as the internal standard to quantitatively detect DCD in milk. Fig. 6A shows SERS spectra of milk samples spiked with DCD at different concentrations. And the calibration curve for DCD in milk was shown in Fig. 6B. It was found that the normalized Raman signal intensity (933/866 intensity ratio) of milk sample increases linearly with the increasing concentrations of the DCD. The sensitivity of this approach was determined to be 0.1 mg/mL with a relative standard deviation (RSD) of 5.43% (n=5). From the correlation plot of SERS intensity against the concentration of DCD, the linearity range is determined to be between 1×10^{-4} and 1×10^{-3} g/mL with a correlation coefficient of 0.99747. The result reveals that quantitative detection of DCD is possible down to a level of 1×10^{-4} g/mL without any sample pretreatment.

To evaluate the accuracy of proposed method for detecting DCD in milk, recovery experiments were carried out by spiking two milk samples with standard DCD solution of 100, 500 and

1000 ppm. The results of analyzing are shown in Table 1. As seen from Table 1, the recoveries of melamine are in the range of 97.9~112.0 %, with RSDs between 1.4% and 9.9 %.

Table 2 Analytical results for the two milk samples (*n*=3)

Sample	Spiked (ppm)	Found (ppm)	RSD (%)	Recovery (%)
S$_1$	100	97.94	2.2	97.94
	500	490.62	3.7	98.12
	1000	996.11	1.5	99.61
S$_2$	100	108.11	1.4	108.1
	500	494.70	9.9	98.94
	1000	1119.7	8.1	112.0

3.6. Comparison with other methods

The performance of our method was further evaluated by comparing it with those of the reported methods[3-6] with respect to limit of detection (LOD), linear range and time for sample pretreatment, as shown in Table 3. Although LODs of these methods are smaller than that of SERS method. The detection instrumentations of these methods are expensive and bulky. The sample pretreatment procedures are complicated and time consuming, while always using organic solvents and laboratory equipment such as centrifuge, ultrasonic cleaning machines and various extraction devices. Therefore, the most advantage of SERS method compare with other approach is its simplicity.

Table 3 Comparison of methods for detection of DCD in milk products.

method	Linear range (mg L^{-1})	LOD (mg L^{-1})	Time for sample pretreatment (min)	reference
SERS	100-1000	100	0	This work
Ion exclusion chromatography UV	0.05-300	0.0125	36	3
spectroscopy & chemometrics	-	6.91	18	4
HILIC–MS/MS	-	0.0791	36	5
UFLC–MS/MS	0.0001-0.02	2×10^{-5}	19	6

Conclusions

In this contribution, a SERS method has been developed to fast screen DCD in milk using silver colloid as SERS substrates. Assignments of the vibrational spectra have been conducted by DFT calculations. A good correlation was found between the calculated and experimental spectra. The influences of different aggregating agents and pH have been tested in terms of their efficiency to detect DCD. Among the three ions, SO_4^{2-} yields the best performance in both acidic and alkaline conditions. Therefore, two detection modes were established for detecting DCD in aqueous solution and the limit of detection in alkaline condition (0.5 ppm with an enhancement factor of 2.88×10^5) is much lower than in acidic condition (10 ppm). Furthermore, using a particular aggregating agent addition order, DCD in milk can be quantitatively detected without any sample pretreatment through an internal standard method and the LOQ

can reach 1×10^{-4} g/mL. In summary, our results indicate that, based on the multi-component detection ability and high sensitivity, SERS technique offers great practical potentials for rapid screening DCD in milk, especially for consumer applications.

Notes and references

[a] National Key Laboratory of Science and Technology on Tunable Laser, Harbin Institute of Technology Harbin 150001, China: hasiwuliji@126.com, dianyanglin@hit.edu.cn, zw_lu@sohu.com.

[b] Affiliated Hospital of Inner Mongolia University for the Nationalities, Inner Mongolia Tongliao 028007, China

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