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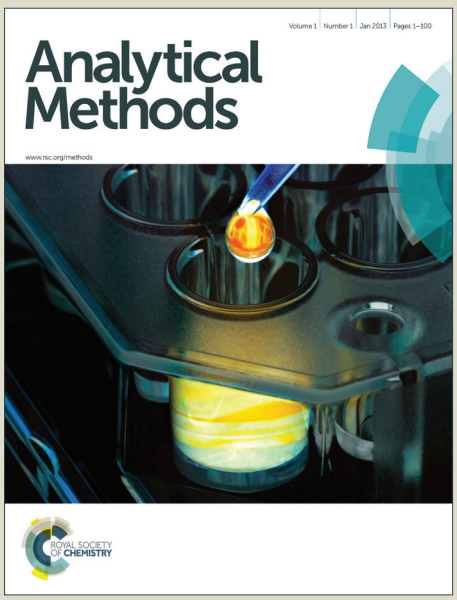

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Single point calibration for semi-quantitative screening based on internal reference in thin layer chromatography-SERS: the case of Rhodamine B in chili oil

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Thin layer chromatography (TLC) has been used in combination with surface enhanced Raman spectroscopy (SERS) for onsite screening of various analytes. In this work, we propose a novel concept for future field semi-quantitative SERS screening applications, where calibration curves were pre-built in the lab but subjected to onsite single point known standard amending. Rhodamine B (RhB) in chili oil, a case of food scandal reported in China, was chosen as our model sample. A standard calibration curve of RhB was built using melamine as internal standard and used throughout the assay. Before the analysis of samples, one single known RhB standard mixed with melamine was tested and used to calibrate the previous built standard calibration curve. RhB in chili oil was separated through TLC method. Then, it was extracted and mixed with melamine. The signal of mixture was recorded and compared with the single point calibrated calibration curve, instead of building a new curve each time. A limit of quantification (LOQ) of RhB from chili oil by SERS, 1×10^{-7} M, was first time realized, and the recovery range was from 66.1% to 110%, despite the fact that the cheapest and non-uniform SERS substrate was used. We expect such a protocol could be used for future fast onsite food quality assurance inspection.

Introduction

Rhodamine B (RhB) is a xanthene dye that commonly applied in textile, mining industry, steel sector, analytical chemistry and biological studies.[1-4] Due to its intensive color, reports show that RhB has been illegally added to food products (such as chili oil, preserved sausages and red pepper paste) to enhance the color appearance in recent years.[5-7] However, RhB is not a permissible substance in food at any level because of its potential genotoxicity and carcinogenic property.[8, 9]

Several methods have been reported for the detection of RhB. High performance liquid chromatography coupled with mass spectroscopy (HPLC-MS) is one of the popular methods currently used for the detection and quantification of RhB.[10, 11] Meanwhile, enzyme-linked immunosorbent assays (ELISAs) are also used for detection the dye.[2, 12] Although the above methods have advantages of high sensitivity and high recovery rate, they are not cost-effective tools for onsite screening of RhB. For example, many factors could affect the detection results by ELISA method, such as time, temperature, pH and so on. Therefore, it is very important to develop more convenient, cost-effective and sensitive methods for onsite screening of RhB in foodstuff.

Discovered in 1970s', SERS has become one of the few spectroscopic analytical methods that can give both qualitative and quantitative information of minute amount of analyte.[13-15] Furthermore, Raman instrument can be easily miniaturized for field applications with little or no sacrifice on its performance.[16] Thus, there are tremendous interests in taking advantage of the technique for applications in, for example, biomedicine,[17-19] homeland security,[16] environmental monitoring,[20-22] and food quality assurance.[23-29] Illegal additives in food have been paid special attention, such as melamine,[25, 30, 31] Sudan Red dyes,[32-34] and so on.[35, 36] However, using SERS technique for both onsite identification and (semi-)quantification of illegal additives in adulterated food is still challenging. Difficulties currently facing include: the lack of cost-effective and highly reproducible substrate, difficulties in building calibration curves and cumbersome pre-separation process for complicated sample matrices, to name a few.[21, 36-38]

In our previous work, we showed that pesticide methidathion in tea leaves can be separated and quantified by the using of TLC-SERS method.[36] The recovery rates determined from spiked tea samples were found to be around 10% of variation, which is comparable with other groups' work.[21] However, other than complicated sample metrices, TLC-SERS also suffers from the tedious calibration curve

building for quantification, which makes it difficuilt to be used in onsite fast (semi-)quantitative screening. In addition, to the best of our knowledge, unlike Rhodamine 6G, the SERS analysis of RhB in foodstuff has not been reported.

Here in this work, we show that by introducing internal standard,[39-42] a common practice to minimize the signal fluctuations caused by the SERS substrate and improve the accuracy of quantitative measurements, semi-quantification of analytes can be realized through a pre-built calibration curve in TLC-SERS. RhB in chili pepper oil was selected as our model system. Melamine was mixed with RhB as internal reference, and the ratio of the SERS intensity of RhB at 1356 cm^{-1} and that of melamine at 681 cm^{-1} was plotted against the concentration of RhB. Unlike previous works reported, the calibration curve is pre-built and used throughout the work. A known RhB standard sample was used to amend the prebuilt calibration curve, and the concentration of RhB in the spiked chili oil sample was calculated using the single point amended calibration curve. The results were then compared with standard HPLC protocol. We show that, by carefully choosing internal standard along the analysis, semi-quantitative determination of RhB in chili oil can be realized, even with the most common and non-uniform citrate reduced Ag NPs and complicated sample metrices. Thus, the proposed method could be used in future onsite screening of interested analytes.

Experimental

Chemicals and reagents

The following reagents were used without further purification: Rhodamine B (RhB, dye content: 95%, Aldrich), Melamine (content: 99%, Sigma). Methanol, ethanol, acetic acid and acetic ether were all analytical grade which were purchased from Kelong, China. High-purity water (Nanopure, Thermo) was used throughout the experiment. Chili oil was bought from a local supermarket.

Instruments

All SERS spectra were obtained with a Raman microscope system (B&W TEK Raman, BWS445 innoRam). A 785 nm laser source and 20× microscope objective (N. A. =0.25) were used throughout the test. Unless otherwise specified, 10 % of the laser power (30 mW) and 3s of the integration time was used througout the work.

For HPLC measurement, Aglient 1100 series HPLC system with a fluorescence detector was used. The column was a TC-C18 (4.6×250 mm, 5 μm, Aglient). The injection volume was 20 μL and the oven temperature was 25℃. The excitation and emission wavelength were 550 nm and 580 nm, respectively. The mobile phase was a mixture solution containing 80% (v) methanol and 20% (v) water. All analysis was used at a flow-rate of 0.8 mL/min.

Sample preparation

A RhB standard stock solution of 1 mM was prepared by dissolving RhB powder in ethanol. Standard solutions with different concentrations (3.00×10^{-7} M~1.00×10^{-4} M) were prepared by stepwise diluting with ethanol.

Chili oil samples spiked with various concentrations of RhB were prepared by adding appropriate quantity of RhB standard stock solution, and the final RhB concentrations were 1.00×10^{-5}, 5.00×10^{-7} and 1.00×10^{-7} M, respectively.

5.00×10^{-5} M melamine aqueous solution was prepared as an internal reference by dissolving it in water.

Sample pre-treatment

For SERS analysis, 0.5 g of spiked chili oil was mixed with 0.6 mL methanol and centrifuged for 5 min at 7000 r/min. Then, 20 μL of the supernatant of each sample was dropped on TLC plate (silica gel 60 F_{254}, Merck, Germany) and developed using 2:1 v/v% ethyl acetate/ethanol mixture solvent. Different from our previours work,[36] after development and drying on the TLC plate, 5 mm×5 mm of silica gel powder where the seperated RhB spot covered was scratched off carefully and collected into a centrifuge tube. 40 μL of 30% acetic acid aqueous solution was used to dissolve RhB from silica gel powder. Then, supernatent was collected after 15 min centrifugation at 130,000 r/min. Note that directly adding Ag NPs onto TLC plate[36] would not produce detectable SERS signal even at 10^{-5}M of R6G (See Electronic Supplementary Information, Fig. s1).

For HPLC analysis, chili oil was extracted by MeOH with the ratio of 1:5. Extracts were further purified by centrifugation at 4000 r/min for 5 min. The supernatant was then transferred into another centrifuge tube and dried by nitrogen flow. Later, 1 mL of methanol was added to dissolve the remaining RhB. Then, the solution was filtered through a 0.45 μm nylon syringe filter. RhB standard stock solution (1.00×10^{-5} M) was prepared with methanol. A series of concentrations of standard RhB solutions were also prepared by diluting the stock solution with methanol.

SERS measurement

Synthesis of Ag NPs: 1 mM of Ag NPs was prepared according to our previous work.[36, 43, 44]

Sample solution preparation for SERS measurement: To obtain the RhB calibration curve, 20 μL RhB standard solution, 0.0035 g silica gel powder and 40 μL 30% acetic acid aqueous solution were mixed. After centrifuging for 15 min at 130,000 r/min, 12 μL of the supernatant was mixed with 4 μL of melamine/HCl ($V_{(melamine)}/V_{(0.1\ M\ HCl)}$=10/1) solution.

SERS measurement: 5 μL of Ag NPs (1mM) was deposited onto aluminum foil which was rinsed with ethanol and nano-pure water before use and dried under the flow of N_2. After that, 3 μL of the sample solution was added onto Ag NPs spot and dried at 40 □. 5 μL of Ag NPs was supplemented at the same spot. Three parallel samples were tested for each concentration, and 20 SERS spectra were recorded for each sample.

Results and discussion

SERS calibration curve

In quantitative SERS, the practice of introducing of an appropriate internal reference is widely adopted to compensate any variation caused by the instrument as well as SERS substrate.[45] In this work, melamine was selected as the internal reference since it has a very simple Raman spectrum whose bands don't interfere with those of RhB. SERS spectra of pure melamine and the mixture of RhB/melamine are shown in Fig. 1. It is clear that the peak at 681 cm^{-1} is the characteristic band of melamine, and the rest are all from RhB. The highest and independent band at 1356 cm^{-1} of RhB was selected for future calculation.

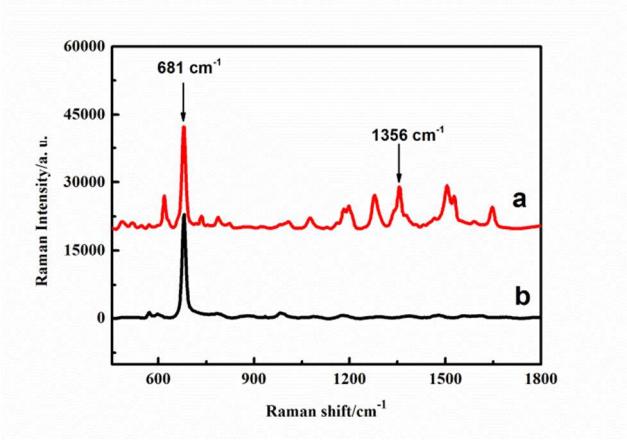

Fig.1 Representative SERS spectra at 785 nm exciting wavelength. (a) the mixture of melamine (5.00×10^{-5} M) and RhB (5.00×10^{-6} M). (b) melamine (5.00×10^{-5} M).

Fig. 2 shows that the presence of internal reference melamine can greatly improve the reproducibility of SERS measurements. In Fig. 2a, the *raw* data at 1356 cm^{-1} from RhB obtained from different locations of one sample spot are shown. It is extremely inhomogeneous (relative standard deviation, RSD, of 98.1%), which is not acceptable but is common in fractal like Ag NPs aggregates.[46] However, by introducing internal reference (melamine) and normalizing the SERS signal of RhB with that at 681 cm^{-1} from melamine, the RSD% (Fig. 2b) was greatly improved to 24%, comparable with our previous work on self-assembled substrate.[43] Thus, it is clear that the introducing of internal reference is necessary.

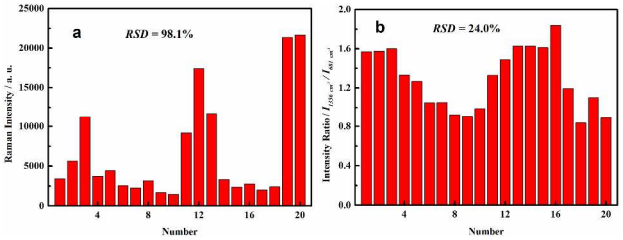

Fig.2 Variation of SERS signal at 1356 cm^{-1} (a) and normalized with internal reference melamine (b). Concentration: RhB, 1.00×10^{-5} M; melamine, 5.00×10^{-5} M.

The representative SERS spectra of RhB/melamine mixture with different concentrations of RhB ranging from 3.00×10^{-7} to 1.00×10^{-4} M are shown in Fig. 3a, the concentration of melamine in all the samples is 5.00×10^{-5} M. The ratio of $I_{1356\ cm^{-1}}$ and $I_{681\ cm^{-1}}$ (Raman intensity ratio at the two positions) was plotted against the concentration of RhB. A good linear response in the range of 3.00×10^{-7}~1.00×10^{-4} M of RhB is obtained (Fig. 3b), where y in the equation is the ratio of the two peaks ($I_{1356\ cm^{-1}}$/ $I_{681\ cm^{-1}}$) and C is the concentration of RhB. The square of regression coefficient (R^2) is 0.9881, slightly better than similar work using Ag-Cl band as internal reference, suggesting the success of utilizing melamine as internal reference.[46] This calibration curve will be used for future quantitative detection of RhB throughout the whole work (after adjusting).

Note in this work the building of calibration curve is extremely labor intensive and time consuming. Partially because the TLC separated RhB has to be collected to produce detectable SERS (supporting material). The whole process took about 3~5 hours.

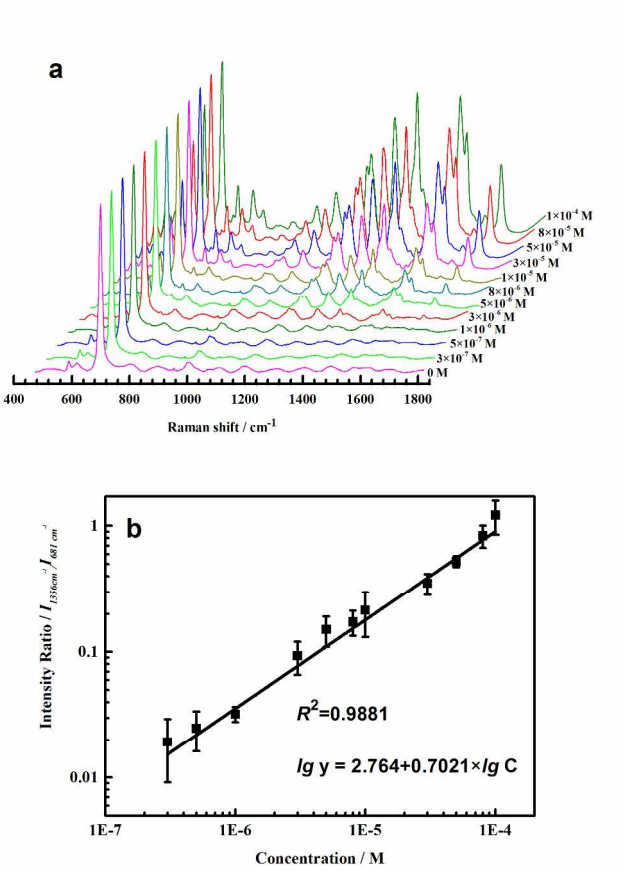

Fig.3 (a) SERS spectra of varying concentrations of RhB and melamine (5.00×10^{-5} M), the spectra were scaled for comparison. (b) Concentration dependence of SERS intensity ratio at 1356 cm^{-1} and 681 cm^{-1} versus different concentrations of RhB, both in logarithmic scale.

Single-point calibration and application to semi-quantification of RhB in spiked chili oil samples

Ideally, the calibration curve shall work for any future onsite SERS analysis of RhB in chili oil. However, day to day variation of instrument is common. Most importantly, during the TLC separation and sample pretreatment, loss of analyte (RhB in our case) is inevitable. These factors can not be thoroughly compenstated by using internal reference. Thus, we further introduce the single point amending for the calibration curve to account the instrument variation and any possible loss during sample pretreatment. The experiment was performed as following:

After spotting 20 μL of the extracted sample onto the TLC plate (Fig. 4 inset, b), 20 μL of RhB standard solution was spotted onto the same TLC plate side by side (Fig. 4 inset, a), followed by developing using 2:1 v/v% ethyl acetate/ethanol mixture. The purpose of spot "a" (RhB standard) here was also meant to ascertain the position of "b" spot (spiked chili oil sample), in case that the concentration of RhB in chili oil sample was too low to be seen under UV irradiation. After that, the silica gel covered by RhB at both spots were scraped off

and collected into a centrifuge tube, respectively. It was dissolved in 30% acetic acid aqueous solution and centrifuged. The supernatant mixed with melamine/HCl solution and tested on Raman spectrometer following the procedure described above. The standard (spot a in Fig. 4) was used to amend the calibration curve, i.e. the interception of the equation in Fig. 3b (assuming there is no change on the slope). It was then used for semi-quantitative analysis of RhB from spiked chili oil on the same TLC plate.

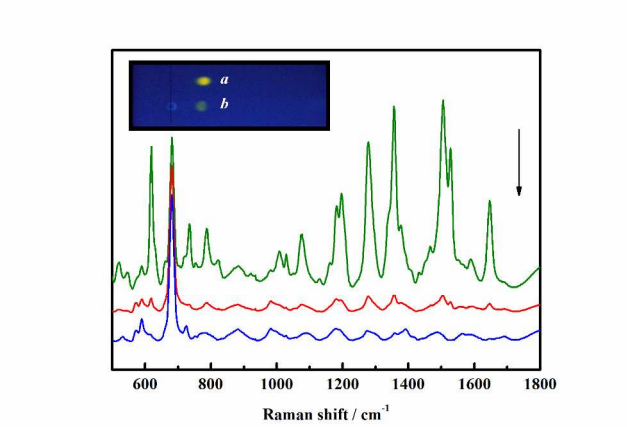

Fig.4 SERS spectra of different concentrations of RhB from chili oil samples, the spectra were scaled for comparison. Top to bottom: 1.00×10^{-5} M, 5.00×10^{-7} M and 1.00×10^{-7} M. Inset, a typical photo of RhB separated spots in TLC plate under UV illumination. a, RhB spot from RhB standard solution; b, spiked chili oil sample.

SERS spectra of RhB spiked chili oil samples at different concentrations were recorded (Fig. 4), the determined concentrations of RhB from spiked chili oil samples and the recovery rates are shown in Table 1, where with and without single point standaprd amending, and HPLC method are compared. It is found that from single point calibrated SERS method, the recovery rates of these samples are between 66.1~110%, and the RSDs are from 9.60% to 10.4%. While the recovery rates from the situation where there is no amending with the calibration curve, are extremely far from the actural spiked amount (1000~1670%).

HPLC analysis

To evaluate the viability for the detection and semi-quantification of RhB by single point calibrated SERS method, HPLC was applied to analyze the concentrations and calculate the recovery rates of RhB in chili oil. Spiked chili oil samples that analyzed by SERS above were all measured by HPLC method again. The content and recovery rates of RhB in the chili oil sample determined by HPLC were also listed in Table 1. It is clear for all the 3 samples tested, the recovery rates were among 111~151%, and the RSD were from 2.05~9.62%. It is then believed that the extraction protocol used in single point calibrated SERS method is valid, and the semi-quantitative as well as qualitative analysis of RhB in chili oil is satisfactory (lowest recovery rate of 66.1%). Most importantly, the results in Table 1 indicate that single point amending of calibration curve based on internal reference could be used for future onsite qualitative and semi-quantitative screening of potential illegal food additives (since there is no need to build calibration curve on-site).

Table 1. RhB content and recovery rate in chili oil analyzed by single point calibrated SERS, SERS* and HPLC method.

RhB spiked (M)	Single point calibrated SERS method			SERS method*			HPLC method		
	RhB found (M)	Recovery rate (%)	RSD (%)	RhB found (M)	Recovery rate (%)	RSD (%)	RhB found (M)	Recovery rate (%)	RSD (%)
	9.25×10^{-6}	92.5		1.40×10^{-4}	1400		1.33×10^{-5}	133	
1.00×10^{-5}	1.10×10^{-5}	110	9.60	1.67×10^{-4}	1670	150	1.29×10^{-5}	129	9.62
	9.48×10^{-6}	94.8		1.43×10^{-4}	1430		1.11×10^{-5}	111	
	3.30×10^{-7}	66.1		5.00×10^{-6}	1000		7.30×10^{-7}	146	
5.00×10^{-7}	3.98×10^{-7}	79.6	10.2	6.02×10^{-6}	1204	109	7.25×10^{-7}	145	2.05
	3.42×10^{-7}	68.3		5.17×10^{-6}	1034		7.53×10^{-7}	151	
	1.04×10^{-7}	104		1.58×10^{-6}	1580		1.37×10^{-7}	137	
1.00×10^{-7}	8.67×10^{-7}	86.7	10.4	1.31×10^{-6}	1310	162	1.41×10^{-7}	141	5.01
	1.05×10^{-7}	105		1.60×10^{-6}	1600		1.28×10^{-7}	128	

* *SERS method here means introducing internal reference only, with no single point calibration. The data is calculated by prebuilt calibration curve: lgy=2.764+0.7021×lgC, where y is the SERS ratio and C is the concentration of R6G.*

Conclusions

As a follow-up and improvement of our previous work where TLC-SERS method was used for the quantification of methidathion in tea leaves, we show that by adding an internal reference, calibration curve for SERS analysis can be pre-built in lab and used in field for semi-quantification with single-point-standard amending. The results from single point calibrated SERS method are comparable with HPLC. Meanwhile, it is demonstrated the first time that RhB, an illegal additive found in chili oil, could be detected by the using of TLC-SERS with LOD of 1.00×10^{-7} M. The using of internal reference and amending of prebuilt calibration curve with known standard eliminate the need for building calibration curve onsite, which not only shortens the analysis time, but also is capable of providing quantitative information. Thus, this strategy could be utilized for future fast onsite qualitative and semi-quantitative analysis of illegal food additives.

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Notes and references

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Electronic Supplementary Information (ESI): SERS spectra of RhB and blank silver colloids solution on TLC plate. See DOI: 10.1039/b000000x/

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