

# Determination of chloramphenicol and crystal violet with surface enhanced Raman spectroscopy

Keqiang Lai · Yuanyuan Zhang · Rui Du ·  
Fuli Zhai · Barbara A. Rasco · Yiqun Huang

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**Abstract** Residual chloramphenicol, crystal violet and other illegal drugs in fish pose potential health risks and adverse impact to the aquatic environment, and are important concerns of consumers and regulatory agencies. Surface enhanced Raman spectroscopy (SERS) with two different types of SERS-active substrates were used to collect the spectra of chloramphenicol and crystal violet over a concentration range of 10 ng/mL to 10 µg/mL. Partial least squares regression and multiple linear regression models were developed for quantitative prediction of these drugs from their spectral data ( $n = 32$ ). The limit of detection for chloramphenicol and crystal violet was 50 and 20 ng/mL, respectively, and  $R^2$  values of chemometric models were from 0.82 to 0.87, indicating potential of applying SERS for determination of trace amounts of prohibited substances in food.

**Keywords** Raman · Surfaced enhanced Raman spectroscopy · Chloramphenicol · Crystal violet · Fish drug

## Introduction

The world aquaculture production of fisheries has expanded rapidly, with an annual growth rate at about 7% since 1996–2007 [1, 2]. Because of limited natural resources, the

growth of fishery production relies heavily on intensified aquaculture production. This leads to an increase of waste materials and organic compounds in fish ponds, and therefore an increase risk of fish diseases [3]. The use of banned or unapproved antibiotics and other drugs, such as chloramphenicol and crystal violet (Fig. 1), in aquaculture operation is an important concerns of consumers and regulatory agencies.

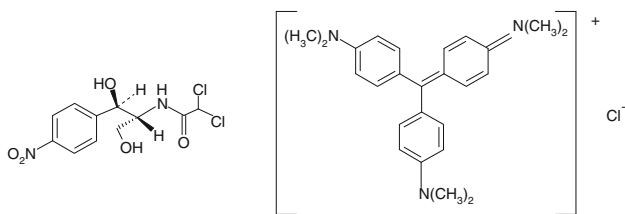
Chloramphenicol is a broad-spectrum antibiotic that can inhibit various fish diseases at different stages [4]. Crystal violet, a chemical dye, is an effective anti-fungal agent [5]. The residues of chloramphenicol and crystal violet in food pose potential health risks, such as aplastic from exposure to chloramphenicol, and cancer caused by long term exposure to crystal violet [3, 5]. Therefore, the use of chloramphenicol and crystal violet in aquaculture is banned by many countries, including but not limited to, the USA, European Union, Japan, and China [4, 5]. Yet, due to their low cost and high effectiveness against certain fish diseases, both of these drugs are still used illegally in aquaculture on a relatively large scale [6]. Residues of illegal drugs in fish products have become a trade barrier for products from developing countries. To ensure safety of imported fish, testing and inspection of fish products at ports of entry is the most commonly used measure in developed countries.

The commonly used official methods for prohibited fish drugs analyses are HPLC and LC–MS, which are time-consuming and require highly skilled operators [6, 7]. Development of simple and rapid methods for screening and quantitative analysis for prohibited drugs in fish products is much needed to ensure safer aquaculture products.

Surface enhanced Raman spectroscopy (SERS) technology has the advantages of spontaneous Raman spectroscopy which include rapidity, non-destructivity and high

K. Lai · Y. Zhang · R. Du · F. Zhai · Y. Huang (✉)  
College of Food Science and Technology, Shanghai Ocean University, Shanghai 201306, China  
e-mail: yqhuang@shou.edu.cn; yiqunh@hotmail.com

B. A. Rasco  
School of Food Science, Washington State University,  
Pullman, WA 99164-6376, USA



**Fig. 1** Chemical structures of chloramphenicol (*left*) and crystal violet (*right*)

molecular specificity, but overcomes the problem of low sensitivity with conventional Raman [8]. In SERS, Raman scattering signals of an analyte are greatly enhanced through adsorption of the analyte onto a roughened metal (typically Au, Ag, and Cu) surface [8, 9]. Many studies have indicated the great potential of applying SERS as an analytical tool in various areas; yet SERS is still in its early development stage in food science and aquaculture [10–14]. The main factors impeding the successful application of SERS by the food science researchers are the high cost and lack of resource to make effective surface enhanced substrates. However, more affordable user-friendly Raman instruments and commercial substrates have recently become available, making it possible to consider this technique in food analysis.

The objective of this study was to determine chloramphenicol and crystal violet with SERS. Standard solutions of fish drugs instead of a specific food product were used to gain a better understanding of the limit of detection of the tested chemicals as well as the possibility for quantitative analysis of these chemicals with current SERS technology. This study could be used as the basis for further studies on determining antibiotics and other chemical hazards in various food systems.

## Materials and methods

### Preparation of sample solutions

Chloramphenicol (S-7653, HPLC reagent) and crystal violet (C6158, ACS reagent) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol (Sigma, HPLC reagent) was used as solvent for chloramphenicol and crystal violet to prepare solutions (10, 20, 50, 100, 200, 300, 500,  $10^3$ ,  $5 \times 10^3$  and  $10^4$  ng/mL) ranging from 10 ng/mL to 10  $\mu$ g/mL.

### Raman spectral acquisitions

Surface enhanced Raman spectra ( $400\text{--}3500\text{ cm}^{-1}$ ) were recorded with a Nicolet DXR microscopy Raman spectrometer (Thermo Fisher Scientific Inc., Waltham, MA,

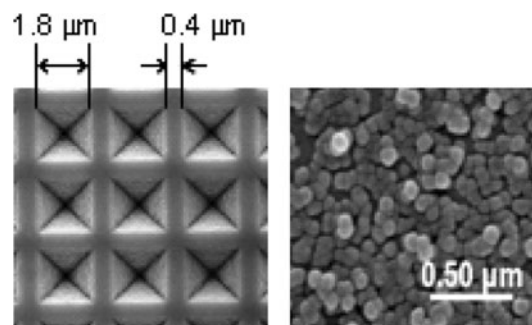
USA) and SERS-active Klarite™ (D3 technologies Ltd., Hampshire, UK) or Q-SERS substrates (Nanovia Inc., Columbia, MO, USA). A 780 nm laser source and  $10\times$  microscope objective were used for all spectral acquisitions. The laser power reaching the sample was about 14 mW. The exposure time was 10 s per scan, and each spectrum was the average of five scans.

The Klarite and Q-SERS were the only two solid surface based substrates that we could find in the global market at the time of this study. There are some commercial colloid-based SERS substrates available, but colloid-based substrates normally did not produce consistent signals for food sample extract with organic solvent. Klarite substrates contain an active area (4 mm  $\times$  4 mm) with gold coated textured silicon surface. The active surface of Klarite (Fig. 2) has regular arrays of inverted square pyramid subunits which are about 1  $\mu$ m deep and 1.8  $\mu$ m long, while the distance between each subunit is about 0.4  $\mu$ m [15]. The active surface of Q-SERS is much more irregular than that of Klarite, containing gold particles of about 40–60 nm in diameter distributed on the surface of a glass slide as shown in Fig. 2.

To acquire surface enhanced Raman spectra, 1.0  $\mu$ L of chloramphenicol or crystal violet solution was deposited onto the active area of Klarite or Q-SERS substrate. After the solvent being evaporated, nine Raman spectra of chloramphenicol or crystal violet were recorded at different points of the substrate, and four of the spectra were selected for data analyses. In addition, spontaneous Raman spectra of solid chloramphenicol and crystal violet were recorded.

### Data analysis

Surface enhanced Raman spectral data were analyzed with DeLight 3.2 software package (DSquared Development Inc., La Grande, OR, USA). Prior to multivariate data analysis, spectra pre-transformations including binning, smoothing and second derivative transformation were conducted to remove background interference, and increase the signal to noise ratio [16]. Partial least square regression



**Fig. 2** Active surface of Klarite™ (*left*) and Q-SERS (*right*)

(PLS) and multiple linear regression (MLR) were selected to interpret spectral data [16, 17]. Leave-one-out cross validation was used to evaluate the model predictability. The  $R^2$  of predicted analyte concentrations against their actual value was used to evaluate the performance of models [16].

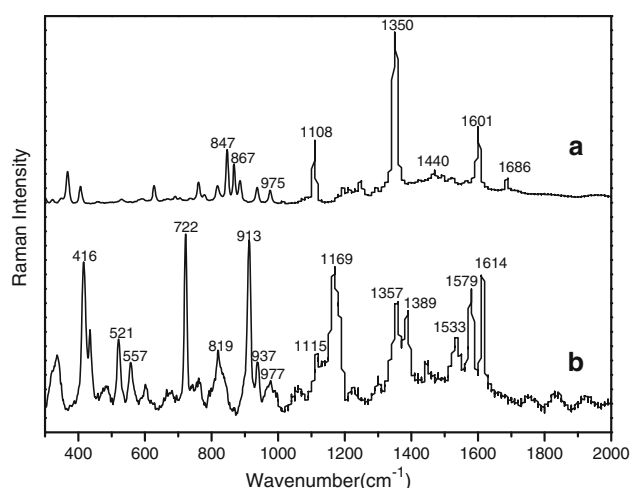
## Results and discussion

### Spectral features of chloramphenicol

Raman spectrum of chloramphenicol was shown in Fig. 3. The three most prominent peaks at 1108, 1350, 1601  $\text{cm}^{-1}$  were attributed to the N–H in-plane bending, N–O<sub>2</sub> symmetric stretching, and ring stretching vibration of chloramphenicol molecule, respectively [18]. In addition, the C=O stretching, C–H out-of-plane bending, NO<sub>2</sub> scissoring, and ring breathing were observed at 1686, 975, 867 and 847  $\text{cm}^{-1}$ , respectively (Table 1) [18].

The Q-SERS substrate did not work well for chloramphenicol analysis. Even when the chloramphenicol concentration was up to 10  $\mu\text{g/mL}$  (about 10 ppm), there were no obvious Raman scattering signals of chloramphenicol observed with Q-SERS substrates. However, with Klarite substrate, the signals of Raman scattering were greatly enhanced (Fig. 4). The characteristic peaks at around 857, 998, 1440 and 1601  $\text{cm}^{-1}$  could be clearly identified for chloramphenicol at as low as 50 ng/mL level, although the two characteristic peaks at around 1108 and 1350  $\text{cm}^{-1}$  were very weak at this low chloramphenicol level.

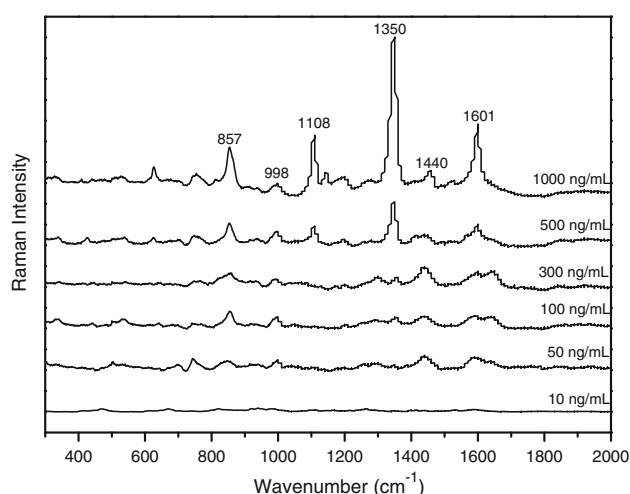
The prominent peaks (such as 1108 and 1350  $\text{cm}^{-1}$ ) in the spontaneous Raman spectrum of chloramphenicol may not be the prominent peaks in the SERS spectrum. In addition, the main peaks appeared in the spontaneous



**Fig. 3** Raman spectrum of **a** chloramphenicol and **b** crystal violet

**Table 1** Assignments of vibrational bands in Raman and SERS spectra of chloramphenicol [18]

Raman ( $\text{cm}^{-1}$ )	Klarite ( $\text{cm}^{-1}$ )	Assignment
847	–	Ring breath
867	857	NO <sub>2</sub> scissoring
975	–	C–H out-of-plane bending
1011	998	Ring in-plane bending
1108	1108	N–H in-plane bending
1350	1350	N–O <sub>2</sub> symmetric stretching
1440	1440	CH <sub>2</sub> scissoring
1601	1601	Ring stretching
1686	–	C=O stretching



**Fig. 4** Representative SERS spectra (acquired with Klarite substrate) of chloramphenicol

Raman spectrum could be downshifted, upshifted, broadened, or even disappeared in the SERS spectrum (Fig. 4) [11, 18]. The peaks at 857 and 998  $\text{cm}^{-1}$  in the SERS spectrum may be due to the downshift of 867 and 1011  $\text{cm}^{-1}$  with respect to Raman spectrum, though it is also possible that they were due to the upshift of 847 and 975  $\text{cm}^{-1}$ . Chloramphenicol molecules may be adsorbed onto the Klarite substrate at different orientations, and the types of adsorption sites on the substrate also varied, which contributed to significant broadening of Raman signals [11]. The molecular orientations when attached to the substrate and the types of adsorption sites also affect the wavenumbers of the Raman signals. The wavenumbers of vibrational modes are normally found downshifted when the bonds of pertinent functional groups were weakened, but upshifted when the bonds were strengthened [18].

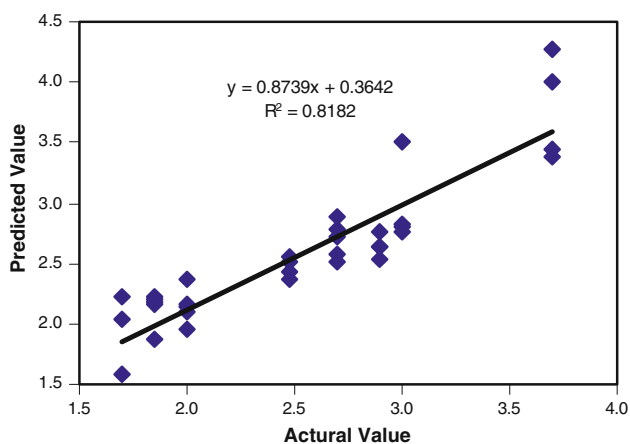
The intensity of some prominent peaks, such as those at around 857, 998, 1108, 1350, 1440 and 1601  $\text{cm}^{-1}$ ,

increased with an increase of chloramphenicol concentration. This makes it possible to determine chloramphenicol content with SERS and chemometric methods.

### Quantitative analysis of chloramphenicol

When chemometric method was used to correlate the actual log value of chloramphenicol with the SERS spectral data acquired through Klarite, PLS model with three latent variables yielded the best result. The  $R^2$  of log value of chloramphenicol versus their values predicted by the model was about 0.82, indicating the potential of applying SERS with chemometric method for determination of chloramphenicol (Fig. 5). The model predictability was adversely affected by a relatively small sample size ( $n = 32$ ) applied over a wide range of chloramphenicol level (10 ng/mL to 10  $\mu$ g/mL) in an attempt to limit the number of analyses due to the cost of substrates.

The quantitative analysis of chloramphenicol was also adversely affected by the heterogeneous nature of Klarite substrate as well as the complicated mechanism of SERS enhancement. The Klarite substrate consists of regular arrays of inverted square pyramids (Fig. 2). The binding sites of chloramphenicol molecules onto the substrate varied because of the heterogeneous nature of the substrate. This resulted in big difference in SERS enhancement effects and inconsistent results with the molecules trapped in the “hot-spot” giving the most intensive scattering effect [9]. In addition, the molecular orientations of chloramphenicol adsorbed onto the substrate influence the SERS enhancement. Some chloramphenicol molecules may be flatly adsorbed on the surface between pyramid subunits, while others may be only partly adsorbed through benzene ring moiety on the surface inside the pyramid unit. The



**Fig. 5** Actual log values of chloramphenicol concentrations versus their PLS model predicted values. The unit of chloramphenicol concentration was ng/mL

disparity in adsorption not only affects the intensity of SERS spectral signal, but also causes fluctuations in band positions, which is a typical problem for applying SERS technology in quantitative analysis [11].

### Spectral features of crystal violet

Raman spectrum of solid crystal violet is shown in Fig. 3. The strong bands at 416 and 722  $\text{cm}^{-1}$  were caused by out-of-plane bending mode of C–C<sub>center</sub>–C and C–N–C symmetric stretching vibration of dimethylamino group, respectively (Table 2) [19–21]. The medium bands at 521 and 557  $\text{cm}^{-1}$  were attributed to bending mode of C–N–C and out-of-plane aromatic C–C deformation [21]. The C–H out-of-plane bending modes were observed at 913 and 819  $\text{cm}^{-1}$ , while the C–H in-plane bending mode was observed at 1169  $\text{cm}^{-1}$  [19, 20]. The bands at 1357, 1389 might be caused by overlap of stretching vibration of C–C<sub>center</sub>–C and stretching vibration of nitrogen and phenyl ring [19]. The prominent bands at 1579 and 1614  $\text{cm}^{-1}$  were due to in-plane aromatic C–C stretching vibration [20].

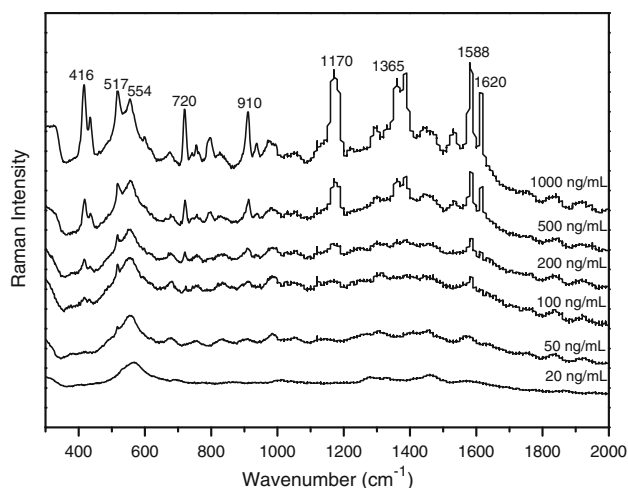
Unlike the analysis results for chloramphenicol, the Q-SERS substrate works well for crystal violet analysis. With Klarite substrate, crystal violet could only be detected at or above 1  $\mu$ g/mL (about 1 ppm); while with Q-SERS substrate, crystal violet could be detected at 20 ng/mL (about 20 ppb) (Fig. 6). With the Q-SERS substrate, the characteristic bands of crystal violet at around 416, 722, 913, 1170, 1580 and 1620  $\text{cm}^{-1}$  exhibited an increase in intensity of Raman scattering signals as the concentration of crystal violet increased (Fig. 6).

Table 2 shows the assignments of some major bands in Raman and SERS spectra of crystal violet. The wavenumbers of the characteristic bands were quite similar between the Raman spectrum and SERS spectra; except for 1357, 1579 and 1614  $\text{cm}^{-1}$  with some noticeable shifts, other bands exhibited no major shifting. This indicated that a rather weak interaction occurs between crystal violet molecules and Q-SERS or Klarite, which was consistent to other studies for large dye molecule like crystal violet [21].

As a tri-*p*-dimethylaminophenyl carbonium ion, crystal violet molecule has three phenyl rings systematically arranged around a central carbonium atom at 30° in a propeller-like shape [21]. The active surface of Klarite substrate has inverted square pyramid subunits, which may cause difficulty for the large propeller-like-shape molecules to be effectively attached to the substrate surface. Since the angle of the analyte to the surface of substrate has great impact on the intensity of Raman scattering, if the crystal violet molecules cannot be effectively adsorbed to the right sites of the substrate surface at desired angles, there would be no or weak enhancement of Raman

**Table 2** Assignments of vibrational bands in Raman and SERS spectra of crystal violet [19–21]

Raman ( $\text{cm}^{-1}$ )	Klarite ( $\text{cm}^{-1}$ )	Q-SERS ( $\text{cm}^{-1}$ )	Assignment
416	418	416	Out-of-plane bending mode of C–C <sub>center</sub> –C
521	521	517	Bending mode of C–N–C
557	558	554	Out-of-plane aromatic C–C deformation
722	723	720	C–N–C symmetric stretching vibration
913	913	910	C–H out-of-plane bending modes
1169	1171	1170	C–H in-plane bending mode
1357	1366	1365	Stretching vibration of nitrogen and phenyl ring
1389	1386	1390	Stretching vibration of nitrogen and phenyl ring
1579	1584	1587	In-plane aromatic C–C
1614	1617	1620	In-plane aromatic C–C

**Fig. 6** Representative SERS spectra (acquired with Q-SERS substrate) of *crystal violet*

scattering. On the other hand, the active surface of Q-SERS consists of nano scale particles, which may pose no or limited adverse effect on the adsorption of crystal violet molecules to the substrate surface, and thus provide higher sensitivity for Q-SERS compared to Klarite for crystal violet detection.

#### Quantitative analysis of crystal violet

For Q-SERS spectral data, both PLS and MLR models showed potential for quantitative analysis for crystal violet. The  $R^2$  of log values of crystal violet concentrations versus their values predicted by PLS or MLR models were about 0.84–0.87. At some wavenumbers, such as 720, 1170 and 1530  $\text{cm}^{-1}$ , the Raman scattering intensity had high linear relationship ( $R^2$  is about 0.81) with crystal violet concentration. Similar to the quantitative analysis for chloramphenicol, a relatively small sample size ( $n = 32$ ) as well as the heterogeneity of substrate surface were the main factors affecting the accuracy for quantitative analysis of crystal violet. Q-SERS active surface contains unevenly distributed

nano-particles varying in sizes, which affects the reproducibility of SERS spectra.

#### Conclusions

The exact mechanism for SERS enhancement effect is still disputable, but electromagnetic and chemical enhancement mechanisms are two generally accepted theories [9, 22, 23]. The electromagnetic enhancement is attributed to the excitation of localized surface plasmons that create an intensive electromagnetic field [23]. The chemical enhancement is due to the increase of molecular polarizability from interactions between analyte and metal surface, and photo-driven charge-transfer between adsorbed molecules and metal surface [9, 22]. For chloramphenicol, crystal violet and two substrates used in this study, chemical enhancement played a less important role than physical enhancement as indicated by limited band shifts in their SERS spectra compared to their Raman counterparts; however, the involvement of chemical enhancement is also significant as shown in the change of relative intensity of the spectral bands in the SERS spectrum of chloramphenicol or crystal violet compared to its Raman spectrum [22].

Surface enhanced Raman scattering is a rather complicated process with its enhancement effect influenced by many factors including, but not limited to, excitation frequency, electronic structure of the metal, surface roughness, and molecular structure of analyte. Different analytes may require different substrates to achieve significant SERS enhancement as shown in this study. Using SERS as quantitative method is still at early development stage with many questions unresolved. However, the potential of applying SERS for quantitative analyses is realized in more and more research fields. In this study, we were able to detect fish drugs at about 20–50 ppb level with commercial substrates, indicating potential for this method for determination of trace amounts of prohibited substances in food. The study results could be used as basis for further studies

on determination of antibiotics and other chemical hazards in various food systems.

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