Rapid determination of histamine concentration in fish (*Miichthys Miiuy*) by surface-enhanced Raman spectroscopy and density functional theory

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Abstract: Histamine is a type of biogenic amine, which plays a major role in the health problems associated with seafood consumption. Legislative limits of histamine level in seafood have been set in many countries. This study focuses on investigating the feasibility and potentiality of determining histamine concentration in fish (*Miichthys miiuy*) by surface-enhanced Raman spectroscopy (SERS) combined with density functional theory (DFT). Both a gold colloid and a silver colloid were used to determine the enhancement effect for SERS detection of histamine standard solution, and the gold colloid exhibited more effective as compared to the silver colloid. The protocol on extraction of histamine with 12% trichloroacetic acid and adjustment of pH with NaOH solution (5 mol/L) could significantly shorten sample preparation (20 min) and provide clear SERS spectra of histamine. The peaks of histamine molecules were classified using the DFT and five spectra (953 cm^{-1}, 992 cm^{-1}, 1106 cm^{-1}, 1262 cm^{-1} and 1317 cm^{-1}) were selected as the characteristic bands of histamine discrimination. Moreover, the intensity of the peak at 1262 cm^{-1} had a good linear relationship with histamine concentration at 5-400 mg/kg with R^2=0.9755. It is concluded that the SERS-DFT approach will be a potential method for rapidly and reliably detecting histamine at levels from 5 mg/kg to 400 mg/kg in fresh fish.

Keywords: surface-enhanced Raman spectroscopy (SERS), density functional theory (DFT), gold nanoparticles, histamine, rapid determination, fish (*Miichthys miiuy*), food safety

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1 Introduction

Biogenic amine (BA) is a general name for non-volatile aliphatic, alicyclic or heterocyclic nitrogen-containing organic compounds. BA has some biological activities, which is widely found in organisms and a variety of food[1]. The proper amount of BA in the body has vital physiological activities, such as regulating the secretion of neurotransmitters, controlling blood pressure, and participating in immune response[2-4]. However, when its intake is too high or there is too much accumulation in the body, it may be toxic to human health. Among BA, histamine is the most toxic one. Studies have shown that it can lead to mild poisoning when the intake of histamine content reaches 8-40 mg, and the histamine concentration of more than 100 mg may cause vomiting, dizziness, diarrhea, allergies and other serious poisoning reactions, and even lead to shock or death[3]. The National Food Safety Standards of China has directed that the average of histamine content in fresh fish is set at 400 mg/kg for high histamine-contained fish (such as mackerel, tuna, et al.) and 200 mg/kg for other fish for food safety[5]; while the European Union (EU) limit is more strict and set at

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100 mg/kg[6]. In addition, the Food and Drug Administration (FDA) sets histamine content of more than 50 mg/kg as a standard to assess the deterioration of fish[7]. Therefore, a reliable and rapid detection method of histamine in fish is of great significance to ensure the quality of fish and the health of consumers.

Traditional methods for the detection of histamine in fish are mainly by high-performance liquid chromatography (HPLC), fluorescence quantification, ion exchange chromatography, capillary electrophoresis and enzyme-linked immunosorbent assay[8-11]. Although the sensitivities of these methods are high, the cumbersome pre-test, time-consuming detection, inconvenient instrument, expensive reagents and other shortcomings limit their applications, e.g. the pre-processing time for the histamine determination in fish takes up to 5 h according to the Chinese national standard method[5]. Surface-enhanced Raman spectroscopy (SERS) is a highly sensitive fingerprint. The "surface-enhanced" describes the characteristic that the Raman signal of a compound molecule can be enhanced in geometric multiple when it adsorbs on some nanoscale rough metals surface (such as gold, silver and copper) or in the sol[12-14]. Thus, the SERS technology can achieve rapid detection of trace samples and single molecules. At the same time, SERS has the advantages of simple pretreatment method, convenient equipment and fast detection speed, and is widely used in the rapid screening of pesticide residues in agricultural products, the detection of trace substances in food, and the detection of harmful substances in the environment[15-17]. Gao et al.[18] successfully established a method for rapid detection of histamine content in canned tuna by molecularly imprinted polymers combined with the SERS technique, which used gold nanoparticles as a substrate. The detection range was 3-90 ppm. Xie et al.[19] established a method for rapid screening of histamine in fish by SERS combined with a thin-layer chromatography method. The accuracy and sensitivity of the method were comparable to those used by the EU. Zhang et al.[20] used the SERS technique to analyze the content of calcium dipicolinate (CaDPA), a biomarker of *Bacillus anthracis*. The results showed that data collection could rapidly analyze CaDPA corresponding to the amount of 10^4 spores with only 5 s spent based on silver nanoparticle, which showed a good prospect for SERS in environmental hazard detection. Nevertheless, few studies have been comprehensively conducted on detecting the histamine content in fresh fish using SERS. In this study, a simple sample preparation protocol and SERS combined with DFT were used to test the applicability of the approach for rapid determination of histamine in the muscle of fresh fish.

2 Materials and methods

2.1 Materials

Histamine (purity>97.0%) was obtained from Sigma-Aldrich (St Louis, MO, USA); trichloroacetic acid and sodium hydroxide were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China); silver nitrate, chloroauric acid, sodium citrate and sodium chloride were obtained from Jingchun Industrial Co., Ltd (Shanghai, China). All the reagents are analytical pure.

Fresh fishes (*Miichthys miiuy*) were provided by Fenghua Xingyang Aquatic Food Co., Ltd., which were collected from Ningbo Fenghua seafood fishing base and shipped to the laboratory (Hangzhou, China) at –20°C within 12 h. The experimental water is double deionized water (ultra-pure water).

2.2 Instrumentation

RamTracer-200-HS portable Raman spectrometer equipped with a 785 nm excitation wavelength of a diode frequency stabilizer (OptoTrace Technologies, USA); a ZNCL intelligent thermostat magnetic stirrer (Zhengzhou Yalong, China); a Vortex-Genie 2/2T vortex mixer (Shanghai Lingling, China); An FEI Tecnai G2 F20 S-TWIN transmission electron microscope (FEI, USA); a BSA124S-CW electronic balance with the accuracy of 0.1 mg (Sartotius, Germany); a JW-1024 low-speed centrifuge with the maximum speed of 4500 r/min (Anhui Jiawen, China).

2.3 Sample preparation

The back muscles of *Miichthys miiuy* were chopped and minced by a meat grinder. Seventeen samples were accurately weighed (5 g each) and spiked with 0.1 mL of different concentrations of histamine standard solution (with 12% trichloroacetic acid) to obtain the final

concentrations of 5 mg/kg, 25 mg/kg, 50 mg/kg, 75 mg/kg, 100 mg/kg, 125 mg/kg, 150 mg/kg, 175 mg/kg, 200 mg/kg, 225 mg/kg, 250 mg/kg, 275 mg/kg, 300 mg/kg, 325 mg/kg, 350 mg/kg, 375 mg/kg, 400 mg/kg. Meanwhile, the fresh muscle spiked without histamine was set as a blank sample, which was confirmed to be histamine free according to the method described in Chinese national standard method (GB/T 5009. 208-2008)[21].

The histamine standard working solution at a concentration of 10.0 mg/L was prepared with 12% trichloroacetic acid.

2.4 Sample treatment

The spiked samples (5 g) were mixed with trichloroacetic acid (12%) at a 1:10 solid-liquid ratio[22]. They were continuously mixed during ultrasonic extraction which was at 60 W for 5 min at 50°C. The samples were spun in a centrifuge at 3600 r/min for 5 min. The supernatant was filtered through two layers of quantitative filter paper to obtain the extract and then filled with trichloroacetic acid (12%) to 50 mL. Ten milliliter of the extract was taken and put in a 25 mL glass tube with plug, 2.5 mL of 5 mol/L NaOH solution was added and vortex mixing, so as to adjust pH over 12. And 100 μL of the mixture was taken for the SERS machine detection. Total time of sample preparation was approximately 20 min.

After the same pretreatment (adjust pH), two portions (10 mL each) of 10.0 mg/L histamine standard working solution were used to determine nanoparticles (gold or silver) for histamine SERS.

2.5 Preparation of silver and gold nanoparticles

The trisodium citrate heating reduction method was slightly modified according to the literature for the preparation of gold nanoparticle[23]. The process was as follows. A chloroauric acid solution (50 mg/L) was poured in a flask, which was heated to boiling at 120°C on a constant temperature magnetic stirrer. Then 4 mL of trisodium citrate solution (5 mg/mL) was added. The mixture was stirred at the speed of 100 r/min to prepare gold sol into the color of the wine red. After the solution was cooled, the above gold gel solution was poured into a centrifuge tube. The supernatant (2 mL) was removed after centrifugation. And then 1 mL ultra-pure water was added to the centrifuge tube and mixed with ultrasonic oscillation. After repeating purification, the gold colloid was stored in dark.

The preparation of silver nanoparticle was established on the basis of the Lee-Meisel trisodium citrate heating reduction method[24]. A silver nitrate solution (180 mg/L) was poured into the flask, which was heated quickly to boiling at the high temperature on a constant temperature magnetic stirrer. A 1% trisodium citrate solution (60 mg of trisodium citrate dissolved in 6 mL of ultra-pure water) was gradually added dropwise over 2 min while stirring at 200 r/min. Then it was stored in dark after the solution turned to gray-green in 25 min.

2.7 Raman spectroscopy acquisition

The acetonitrile was utilized for instrument calibration with an excitation wavelength of 785 nm before Raman spectra were collected. Herein, the power, the scanning range, the optical resolution and the integration time were set as 200 mW, 200-3300 cm^{-1}, 2 cm^{-1}, and 10 s, respectively. The average spectral value was collected three times. The histamine powder was planished with a glass slide on the quartz plate, which was collected with a matching microscope platform.

SERS acquisition: 500 μL of the nano-enhancer, 100 μL of samples and 100 μL of sodium chloride (1%) were added in turn into a quartz vial, which was used for SERS spectral acquisition after mixed.

2.8 Data analysis and processing

All computations and data analyses were performed with the aid of MATAB R2014a, Gaussian.v09, OMNIC v8.2, Origin v8.0, and SPSS V17.0.

3 Results and discussion

3.1 Raman spectroscopy analysis of histamine

The theoretical Raman spectroscopy of histamine (molecular formula $C_5H_9N_3$) was calculated by using the B3LYP hybrid function of DFT. Meanwhile, B3LYP was the Becke3 parameter density functional model. The molecular structure is shown in Figure 1, which is mainly composed of imidazole ring, C-N, C=N, C=C, C-C, C-H, N-H and other groups. The Raman spectra peaks of histamine can be resolved according to the

characteristic frequency of functional groups.

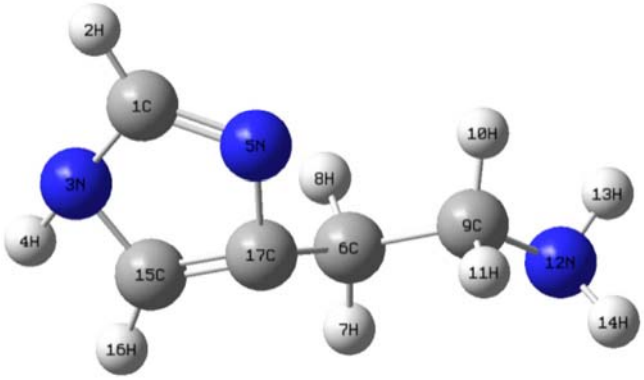


Figure 1 Schematic drawing of the structure of histamine

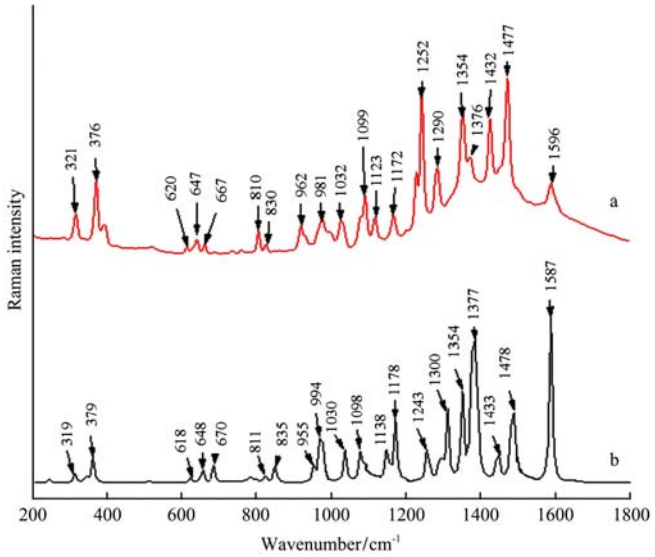


Figure 2 Raman spectra of histamine solid (a) and the theoretical calculation (b)

Raman peaks of histamine solid (321 cm^{-1}, 376 cm^{-1}, 647 cm^{-1}, 810 cm^{-1}, 1032 cm^{-1}, 1099 cm^{-1}, 1376 cm^{-1}, 1432 cm^{-1} and 1477 cm^{-1}) (Figure 2a) were basically in line with those (319 cm^{-1}, 379 cm^{-1}, 648 cm^{-1}, 811 cm^{-1}, 1030 cm^{-1}, 1098 cm^{-1}, 1377 cm^{-1}, 1433 cm^{-1} and 1478 cm^{-1}) calculated by the DFT (Figure 2b). Table 1 presents the peaks of histamine reviewed by the references[19,25]. Among them, 321 cm^{-1} and 376 cm^{-1} were attributed to the outward bending vibration of the skeleton in the histamine molecules, and the peaks at 647 cm^{-1} and 810 cm^{-1} were the outer ring vibration of the histamine molecules. The peaks at 1032 cm^{-1} and 1099 cm^{-1} were due to the C-H surface deformation vibration and the C-N stretching vibration of the histamine molecules, respectively. Moreover, the peaks at 1376 cm^{-1} and 1477 cm^{-1} were the ring stretching vibration, and the peak at 1432 cm^{-1} was the ring vibration of the histamine molecules and the in-plane bending vibration of the N-H group. These peaks could serve as the Raman characteristic peaks of histamine.

Table 1 Proposed assignment of Raman bands of histamine

Calculation[a] /cm^{-1}	Solid[b] /cm^{-1}	SERS[b] /cm^{-1}	Assignment
319	321(m)	313(w)	δ Skeletal
379	373(s)	363(m)	δ Skeletal
618	620(w)	-	γ Ring
647	648(w)	647(w)	γ Ring
670	667(w)	667(w)	γ Ring
811	811(m)	811(w)	δ(C-H)
835	830(w)	838(w)	δ Ring
955	962(m)	953(w)	δ Ring, γ(N-H)
994	981(m)	992(w)	δ(C-H)
1030	1032(m)	1030(m)	δ(C-H)
1098	1099(s)	1106(w)	υ(C-N)
1138	1123(m)	1129(w)	Y(N-C-N), δ(N-H)
1178	1172(m)	1186(w)	δ(N-H)
1243	1252(vs)	1262(s)	δ(C-H), Ring breathing
1252	1252(vs)	1262(s)	Ring breathing
1300	1290(s)	1317(s)	υ Ring
1344	1354(vs)	-	δ Ring
1377	1376(w)	1377(w)	υ Ring
1433	1432(s)	1425(w)	δ(N-H), υ Ring
1478	1477(vs)	-	υ Ring
1587	1596(m)	1593(vs)	υ Ring, υ (C=C)

Note: [a] Calculated wavenumbers at B3LYP/6-31G(DFT). [b] vs = very strong; s = strong; m = medium; w = weak. υ = stretching; δ = in-plane bending; γ = out of plane bending.

3.2 Comparison of the enhancement effects of silver or gold nanoparticle on histamine

The SERS spectra of the histamine standard solution (10 mg/mL) with silver and gold nanoparticle are shown in Figure 3 (a and b), where the Raman peaks at 953 cm^{-1}, 992 cm^{-1}, 1030 cm^{-1}, 1106 cm^{-1}, 1186 cm^{-1}, 1262 cm^{-1}, 1317 cm^{-1}, 1425 cm^{-1} and 1593 cm^{-1} were strong, and the wavenumbers of these characteristic peaks are shown in Table 1. However, there were only five weak peaks (432 cm^{-1}, 749 cm^{-1}, 844 cm^{-1}, 939 cm^{-1} and 1339 cm^{-1}) in the Raman spectra of histamine untreated with surface enhancer (Figures 3 c and 3d).

Structures of silver and gold nanoparticles were characterized by transmission electron microscopy (TEM). Results showed that the diameter of silver nanoparticles was about 60 nm and that of gold nanoparticles was 40 nm. The sizes of the two nanoparticles were uniform. The enhancement effect of gold nanoparticles on histamine molecules was higher significantly at 953 cm^{-1}, 992 cm^{-1}, 1262 cm^{-1}, 1106 cm^{-1},

1262 cm^{-1}, 1317 cm^{-1}, 1425 cm^{-1} and 1593 cm^{-1} as compared with silver colloid. Follow-up study would use gold nanoparticles.

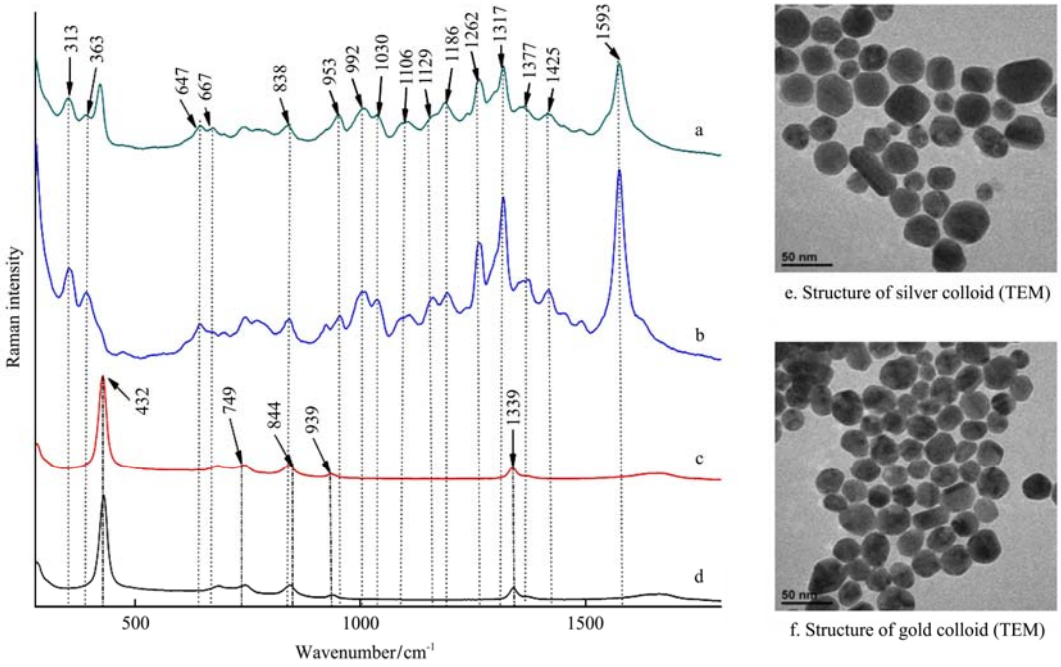


Figure 3 Raman spectra of histamine treated with silver colloid (a) and its structure characterized by TEM (e) or gold colloid (b) and its structure characterized by TEM (f) or histamine untreated with surface enhancer (c) and blank (12% trichloroacetic acid) (d)

3.3 Qualitative and quantitative analyses of SERS on histamine concentration in *Miichthys Miiuy*

SERS spectra of all samples were processed via smoothing and baseline correction in order to remove the effects of noise and baseline drift. As the extract was not degreased, the SERS results showed a large number of impure peak signals. However, except for the blank SERS spectra signal of *Miichthys Miiuy* in Figure 4 (q), five histamine Raman characteristic peaks (953 cm^{-1}, 992 cm^{-1}, 1106 cm^{-1}, 1262 cm^{-1} and 1317 cm^{-1}) could be identified clearly, and the intensities of peaks gradually decreased as the concentration of histamine decreased. Additionally, when the concentration of histamine in spiked fish was 5 mg/kg, the signal could still be effectively identified. Therefore, the five characteristic peaks were selected as the basis for qualitative and quantitative determination of histamine in *Miichthys Miiuy* at the concentration of 5-400 mg/kg.

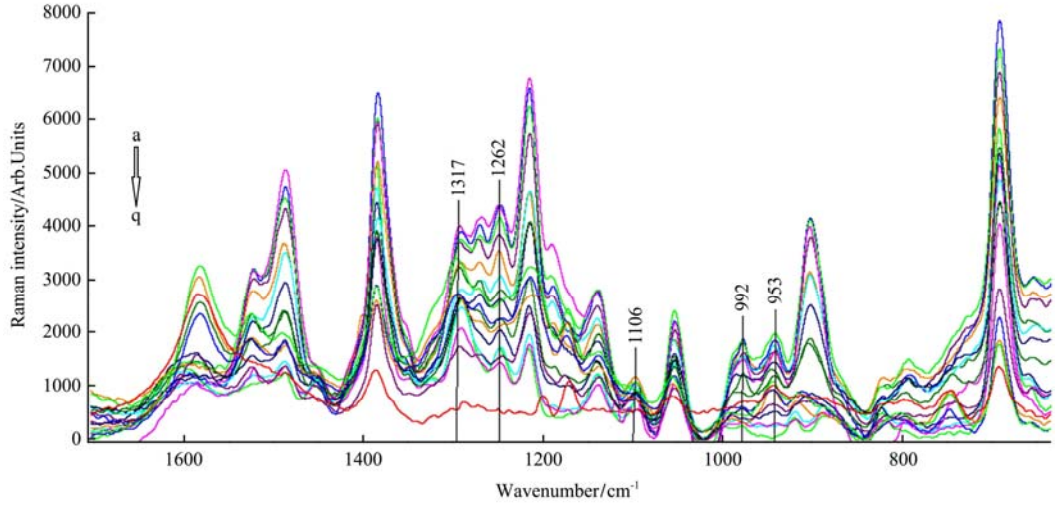
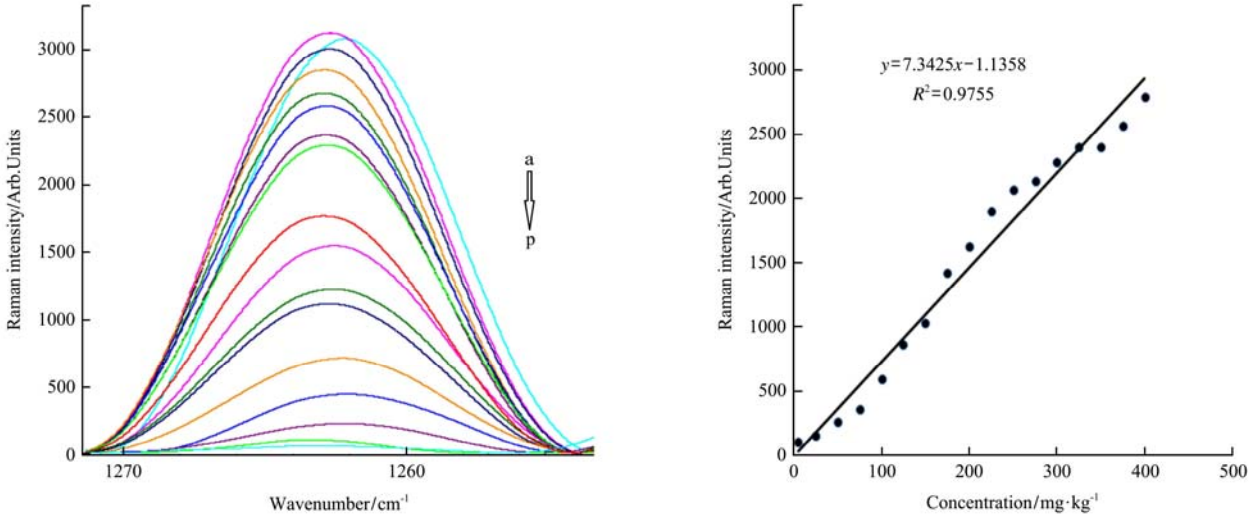


Figure 4 SERS spectra of different concentrations of histamine in *Miichthys miiuy*
(a-q: 400 mg/kg, 375 mg/kg, 350 mg/kg, 325 mg/kg, 300 mg/kg, 275 mg/kg, 250 mg/kg, 225 mg/kg, 200 mg/kg, 175 mg/kg, 150 mg/kg, 125 mg/kg, 100 mg/kg, 75 mg/kg, 50 mg/kg, 25 mg/kg, 5 mg/kg, 0 mg/kg)

Because the intensity was high at 1262 cm^{-1} and there were no peaks piled and impure peaks in the vicinity, the intensity of this peak was chosen to establish a quantitative model for histamine content determination in fresh fish. Figures 5a and 5b showed a linear standard curve between the SERS characteristic peak intensity (1262 cm^{-1}) and the concentration of histamine (5-400 mg/kg). The linear equation was y=7.3425x–1.1358, and the correlation coefficient (R^2) was 0.9755. These results demonstrated that the sample preparation protocol and SERS combined with DFT in this study could qualitatively and quantitatively analyze the histamine in fresh fish at the levels covering legislative limit in EU countries, US and China. The whole process was simple, which did not include extracting by n-hexane, degreasing, derivatization and other steps as compared to the Chinese national standard method (approximately 5 h). Moreover, the time required for pre-processing was only one-fifteenth of that of the traditional method.



a b

Figure 5 SERS spectra (1262 cm^{-1}) of *Miichthys miiuy* spiked with different concentrations (a-p: 400 mg/kg, 375 mg/kg, 350 mg/kg, 325 mg/kg, 300 mg/kg, 275 mg/kg, 250 mg/kg, 225 mg/kg, 200 mg/kg, 175 mg/kg, 150 mg/kg, 125 mg/kg, 100 mg/kg, 75 mg/kg, 50 mg/kg, 25 mg/kg and 5 mg/kg) of histamine (a) and its linear equation (b)

4 Conclusions

In this study, a simple sample pretreated protocol was developed to significantly shorten the sample preparation time as compared to the Chinese national standard method. Gold nanoparticles had a better effect on histamine molecules for the SERS spectra collection than silver nanoparticles. Five Raman characteristic peaks (953 cm^{-1}, 992 cm^{-1}, 1106 cm^{-1}, 1262 cm^{-1} and 1317 cm^{-1}) obtained by SERS-DFT can be used as a qualitative and quantitative basis for the determination of histamine in fresh fish. The intensity of the characteristic peak of spectra at 1262 cm^{-1} was linearly correlated with the histamine at the concentration of 5-400 mg/kg with R^2=0.9755, implying that the SERS-DFT approach could rapidly and reliably detect histamine at levels from 5 mg/kg to 400 mg/kg in fresh fish.

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