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DIFFERENTIATION OF BACTERIAL STRAINS BY MEANS OF SURFACE ENHANCED FT-RAMAN SPECTROSCOPY

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The silver nanoparticle colloid was used to obtain surface enhanced Raman spectra of *Listeria monocytogenes, Salmonela enterica,* and *Esherichia coli* bacteria. The SERS spectra were captured using for excitation the near-infrared (1064 nm) laser radiation with reduced intensity, which ensured the prevention of the fluorescence background as well as photo- and thermal decomposition of the samples. It was found that the optimal size of silver nanoparticles for the enhancement of the Raman signal in the near-infrared spectral region is ca. 50 nm. The spectral data obtained in this study indicate that relative intensities of SERS spectral bands of bacteria can be used for spectral differentiation of bacteria. In case of *Listeria, Salmonela*, and *Esherichia* cells, the intensity ratio of spectral bands of adenine and cysteine can be used as a spectral marker for differentiation of the bacteria.

Keywords: SERS, silver colloid, bacteria identification and differentiation

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1. Introduction

Recent reports from the World Health Organization have concluded that the incidence of foodborne diseases is a growing public health problem in both developed and developing countries [1]. Contaminated food consumed in the United States causes an estimated 48 million illnesses, 128,000 hospitalisations, and 3,000 deaths annually [2].

Spectroscopic techniques that detect molecular vibrations have been used for analysis and identification of microorganisms for past years. Infrared spectroscopy and Raman spectroscopy provide complementary technologies for rapid and precise detection of microorganisms and are emerging methods in food analysis.

Meanwhile, the IR spectroscopy has some drawbacks concerning sample preparation and spatial resolution, which limit *in situ* and non-invasive applications in the micro regime. Raman scattering is superior to IR spectroscopy with respect to these issues, especially for aqueous solutions. For many molecular systems it is possible to enhance the Raman signal. The surface enhanced Raman scattering (SERS) spectra can be obtained either using a colloid with silver nanoparticles or on a nanoscale corrugated noble metal (silver, gold, platinum) surface. Even single microorganisms can be identified by applying SERS microspectroscopy [3, 4, 5]. The wavelength of laser radiation used for the excitation of the Raman signal is an important issue. Generally, the intensity of the Raman signal strongly depends on the frequency of radiation ($\sim\nu^4$), but high frequency of laser radiation in many cases is causing a strong fluorescence background appearing in the spectrum. For a particular sample, the optimal laser frequency has to be chosen. Theoretically, the Raman signal can be increased using elevated laser power, but practically, due to the thermal heating of the sample, such approach has limited use. The signal-to-noise ratio of Raman spectra obtained with near-infrared (e. g. low frequency) excitation can be substantially increased using sensitive detectors. The choice of the detector in a modern Raman spectrometer equipped with the Nd:YAG laser operating in the NIR spectral region is a liquid

nitrogen cooled germanium diode. The noise equivalent power (NEP) of such a detector is less than 10^{-5} W $Hz^{-1/2}$, which is 100 times lower than in case of a conventional room temperature DTGS detector used in routine FT-Raman spectrometers (*Bruker Optic*s, product note M25-10/08).

The first attempt to apply SERS spectroscopy with NIR excitation for discrimination of bacterial pathogens is presented in [6]. The studies were performed with a conventional room temperature DTGS detector. In order to have a reasonable signal-to-noise ratio the SERS spectra were exited using high (1 W) Nd:YAG laser power. Such power cannot insure prevention of decomposition of the sample. SERS experiments with low power NIR excitation are needed in order to make proper assignment of SERS spectral bands of bacterial pathogens.

The aim of this study is focused on the possibility to obtain and identify SERS spectra of different surface-adhered food pathogens: *L. monocytogenes, S. enterica,* and *E. coli,* by using for excitation low power near-infrared radiation.

2. Materials and methods

2.1. Bacterial cultures

Three food pathogens were used for experiments: *Listeria monocytogenes* $ATC_{L3}C$ 7644 (3rd passage of ATCC7644-test organism), *Salmonella enterica* serovar Typhimurium strain DS88 [SL5676 SmR (pLM32)], resistant to tetracycline, and *Escherichia coli* O157:H7. All bacteria were maintained at 37 °C for 24 hours onto Luria Bertani Agar (LBA; *Liofilchem*, Roseto degli Abruzzi, Italy). Later *Listeria* culture was grown overnight (~16 h) at 37 °C in 20 ml of Tryptone Soya medium supplemented with 0·6% Yeast Extract (TSYE) (*Liofilchem*), whereas *Salmonella* and *Escherichia* cultures were grown in 20 ml of Luria-Bertani medium (LB; *Liofilchem*), with agitation of 120 rev min^{-1}. Afterwards bacterial cultures were 20 times diluted by the fresh TSYE or LB medium (OD_{540} = 0 164) and grown at 37 °C in a shaker to the mid-log phase (~1.16×10^9 colony forming units (CFU ml^{-1}), OD_{540} = 0.9 for *Listeria*; ~5×10^8 CFU ml^{-1}, OD_{540} = 1.3 for *Salmonella,* ~1×10^8 CFU ml^{-1}, OD_{540} = 0.9 for *Escherichia*). Cells were then harvested by centrifugation (10 min, 3420×*g*), washed twice with sterile water and finally resuspended in 0.1 ml of sterile water to give the final concentration of $5 \cdot 10^9 – 1.5 \cdot 10^{10}$ CFU ml^{-1} of bacterial cells. This suspension was used for further experiments.

2.2. Electron scanning microscopy of bacteria

The morphology of *Listeria* and *Salmonella* was examined by scanning electron microscopy (SEM). Bacterial cultures were grown as described above and diluted to ~$1 \cdot 10^{-7}$ CFU ml^{-1} 0.1 mol l^{-1} phosphate-buffered saline (PBS, pH 7 2). 20 μl of bacterial suspension were withdrawn, mounted onto aluminium stubs, air-dried and sputter-coated with a 15-nm gold layer using Q150T ES (*Quorum Technologies*, England). The scanning was performed with the Apollo 300 scanning electron microscope at an accelerating voltage of 20 kV.

2.3. Silver colloid preparation

Silver colloids for SERS were prepared via reduction of silver nitrate ($AgNO_3$) with trisodium citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$) as described by a modified method of Lee and Meisel [7]. Briefly, distilled water (50 mL) was heated in a 100 ml flask with vigorous stirring on a Corning stirrer/hot plate (Model PC-620, *Fisher Scientific Ltd*., Ottawa, Ontario, Canada). At 45 °C, 9 mg of $AgNO_3$ (*Sigma*, St. Louis, MO) was added. This solution was heated until boiling (100 °C). Then, a 1 ml aliquot of 1% (w/v) trisodium citrate (*Sigma*) was added into the solution and boiling was maintained for appropriate time. The flask was covered with aluminium foil to minimise evaporation loss of water. The obtained silver colloids were stored at 4 °C until use. Variation in the duration of heating during the reduction process was employed to obtain four different silver colloids with 15, 30, 60, and 90 min of boiling.

2.4. Raman system

The SERS spectra were obtained using the FT–Raman spectrometer MULTIRAM from *Bruker*. In order to avoid fluorescence background the Raman experiments were performed in the near-infrared spectral region, using the Nd:YAG laser. The laser operates at 1064 nm with variable power from 1 to 1000 mW which can be changed with 1 mW step. The ultrahigh sensitivity liquid nitrogen cooled Ge detector was used for capturing the Raman signal.

An Au flat mirror was used as a plate for placing the samples. The plate was attached to a motorised x, y, z stage. The sampling area was monitored by a video camera. The SERS spectra were recorded in back-scattering geometry. In order to increase the signal-to-noise ratio of the spectra 1000 scans were co-added for each spectrum. The laser power was set at 150 mW. Such power reasoned only small heating of the samples. The heating was monitored by intensity of a broad thermal peak, which appears in the spectral region of 2300–3400 cm^{-1} (see Fig. 3).

3. Results

3.1. Identification of bacterial morphology by electron scanning microscope

First of all we analysed the morphology of *Listeria* and *Salmonella* by an electron scanning microscope. The SEM images of the above-mentioned bacteria are presented in Fig.1. The data indicate that intact *Listeria* differs from *Salmonella* by size, surface structure and form. *Listeria* cells are ca. 0.6 μm long and 0.2 μm thick while *Salmonella* are 1 μm long and 0.3 μm thick.

3.2. Identification of size of silver nanoparticles

In order to have a sufficient enhancement factor of the SERS spectra, the silver colloid particles have to be much smaller than the wavelength of laser radiation used for the excitation of SERS spectra. In our experiments the laser wavelength was much larger than the size of colloid particles (100–200 nm), which ensured a sufficient enhancement of the Raman signal. The colloid nanoparticle size was monitored by an atomic force microscope (AFM). An AFM image of the colloid which was prepared using 60 min boiling time is presented in Fig. 2. A thorough examination of the image reveals that the average diameter of nanoparticles of this colloid is 35–40 nm, while thickness is only 3 nm.

3.3. SERS spectra of bacteria

For SERS studies, a 6-μl aliquot of bacterial suspension was mixed with a 6-μl aliquot of silver colloids that had been preaggregated with NaCl in a 2:1 ratio (v/v) of colloids to NaCl (0.05 M). The sample was prepared on a conventional gold platted mirror and allowed to dry for about 2 h. The sampling area of ~100 μm^2 was defined by the diameter of the focused Nd:YAG laser beam used for the excitation of the SERS spectra. Having in average $1 \cdot 10^8$–$5 \cdot 10^8$

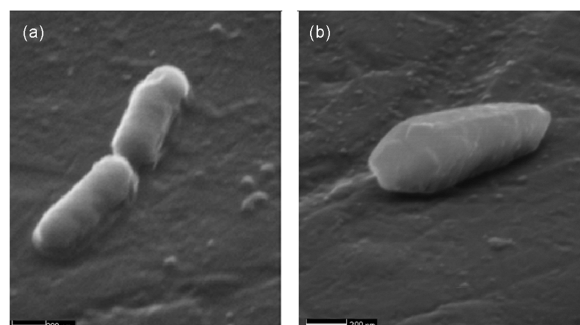

Fig. 1. SEM images of (a) *L. monocytogenes* ATC$_{L3}$C 7644 and (b) *S. enterica* DS88 cells.

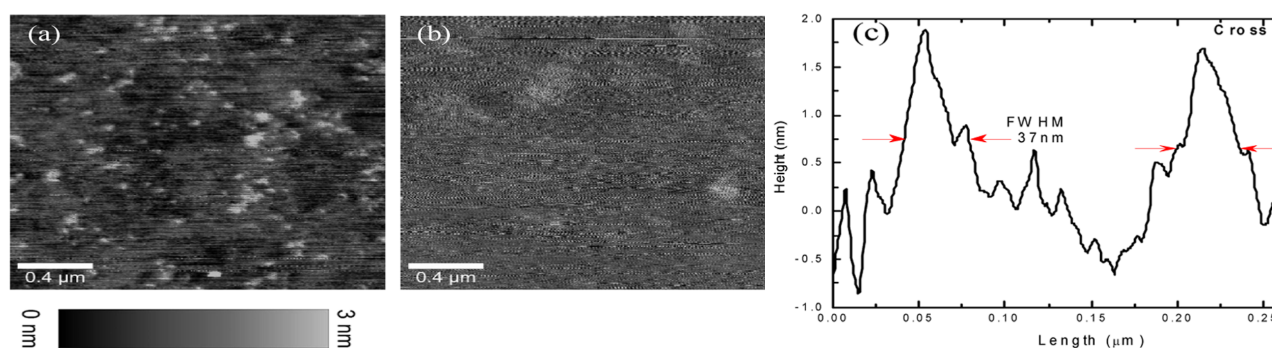

Fig. 2. (a) AFM image of silver colloid nanoparticles. (b) AFM image of glass substrate used for placing a drop of the colloid. (c) Diagram for measuring diameter and thickness of the nanoparticles. The bar at the bottom of the figure shows correlation between thickness of the nanoparticle and intensity of its grey colour in the image.

bacterial cells in cm^2, the SERS spectra resulted from ~100–500 bacterial cells within the field of laser illumination.

A survey of the SERS spectrum of *Listeria monocytogenes* mixed with the silver colloid (heating time 60 min) is presented in Fig. 3(a). The spectrum consists of many spectral bands. A broad spectral feature in the region of 2400–3400 cm^{-1} is related to the thermal radiation of the sample. The intensity of this band correlates with Nd:YAG laser power. Small downward looking spectral bands in the region of 2000–2400 cm^{-1} are related with the absorption of a SERS signal by atmospheric water vapour persisting in the spectrometer. The bands in the region of 400–2000 cm^{-1} can be attributed either to *Listeria* cells or colloid. The Raman spectrum of a pure colloid is presented in Fig. 3(b). The colloid bands can be easily subtracted from the SERS spectrum of bacterial cells. It is notable that the concentration of bacterial cells used in this experiment ($1 \cdot 10^8$–$5 \cdot 10^8$ cells in cm^2) does not produce any (or produces an extremely weak) conventional Raman spectrum (Fig. 3(c)).

In order to optimise the SERS experimental conditions, colloids with various boiling times were used in the experiments (Fig. 4). The enhancement of the Raman signal factor was increasing with a longer heating time of the colloid. Such increase of enhancement was observable up to 60 min heating time. Further increase of the heating time had no effect on the Raman signal enhancement (see Fig. 4(c) and (d)). Consequently, the silver nanoparticle colloid produced using 60 min heating time was chosen for further SERS experiments.

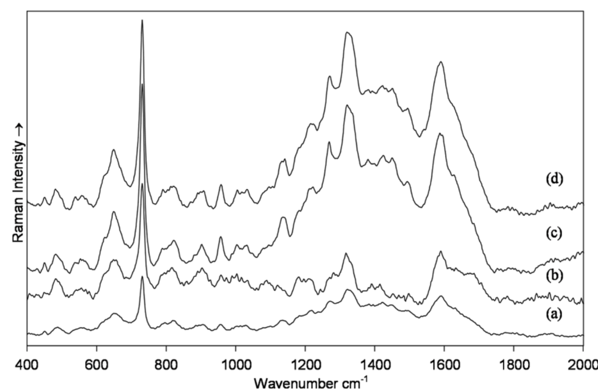

Fig. 4. Surface enhanced Raman scattering with NIR excitation (1064 nm) spectra of *L. monocytogenes* $ATC_{L3}C$ 7644 in a spectral region of 400–2000 cm^{-1}. The silver colloids were prepared with various heating times: (a) 15 min, (b) 30 min, (c) 60 min, and (d) 90 min.

SERS spectra for *Listeria*, *Salmonella*, and *Escherichia* are presented in Fig. 5. Differences in the position of the spectral bands for all the bacteria are insignificant and only intensities can be used for differentiation of the bacteria. Indeed, chemical constituents of bacteria are the same and only concentrations of the constituents can differ. Exact assignment of spectral bands of such chemically complicated system is not possible, but tentative

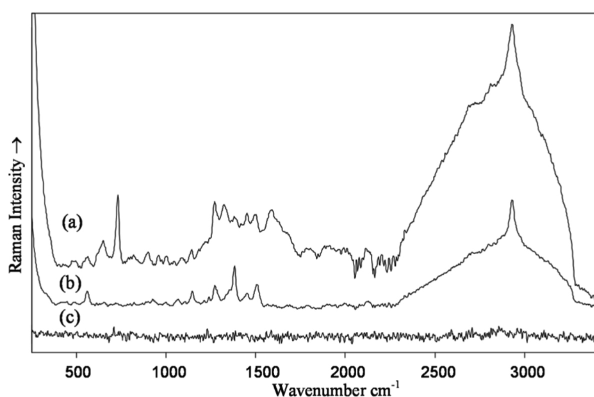

Fig. 3. (a) SERS spectrum of *L. monocytogenes* $ATC_{L3}C$ 7644. (b) Raman spectrum of the silver colloid. (c) Conventional Raman spectrum of *L. monocytogenes* $ATC_{L3}C$ 7644.

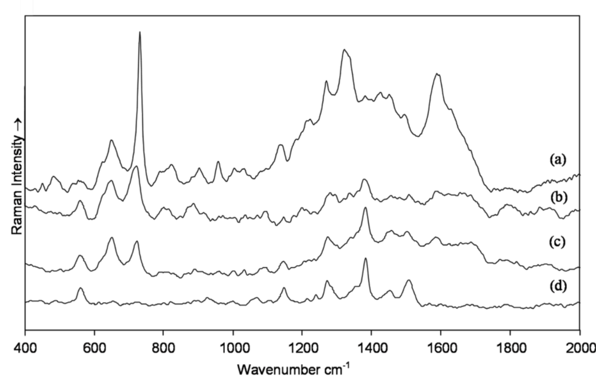

Fig. 5. SERS with NIR excitation (1064 nm) spectra of (a) *L. monocytogenes* $ATC_{L3}C$ 7644, (b) *S. enterica* DS88, and (c) *E. coli* O157:H7 in a spectral region of 400–2000 cm^{-1}. (d) Raman spectrum of a silver colloid (heating time 60 min) used for enhancement of Raman spectra of the bacterial pathogens.

interpretation can be performed. Results of such assignment are presented in Table.

The bands can be attributed to the main chemical species of the bacteria – guanine, adenine, phenylaniline, cysteine, tryptophan and other amino acids, which are known to have large enhancement factors in SERS spectra. Due to the complexity of bacteria as a molecular sample for vibrational spectroscopy, precise assignment of the spectral bands is not possible. The spectral bands do not represent pure molecular normal vibrations and result by overlapping the bands arising from various vibrations of molecules constituting bacterial cells. There are many ambiguities in literature concerning assignment of vibrational spectral bands of bacteria. For instance, the spectral band at 646 cm^{-1} of bacterial pathogens is assigned to tyrosine skeletal vibration in [8], while in [6] it is assigned to the COO^- band of amino acids or C–S stretch mode of cysteine. It is well established that in case of tyrosine water solution the most intense Raman spectral bands are located at 826, 846, 1210, and 1615 cm^{-1} [9]. There are no bands with intensities higher than intensity of the band at 646 cm^{-1} in our SERS spectra of the bacterial pathogens in the 820–850 cm^{-1} region; therefore, this band cannot be attributed to tyrosine. Other candidates for assignment of this band are COO^- band of amino acids and C–S stretch of cysteine. Both these chemical groups can be responsible for binding amino acids to metal nanoparticles, and high enhancement factors are expected for the SERS bands corresponding to various vibrations of these groups. In case of *Listeria, Salmonella,* and *Escherichia* the band at 646 cm^{-1} is asymmetric, e. g. consists of at least two bands; therefore, we consider this band a result of overlapping of COO^- band in amino acids and C–S stretch in cysteine.

Despite the similarities between SERS spectra of the bacteria, some spectral features can be used for discrimination of these bacteria. The ring stretching spectral band of guanine and adenine at 1588 cm^{-1} is rather strong for *Listeria*, while it has medium intensity for *Salmonella* and is weak for *Escherichia.*

From all the spectral differences it seems that the intensity ratio of the spectral bands of adenine (722–731 cm^{-1}) and cysteine/amino acids (646 cm^{-1}) is best suited for the spectral differentiation of the bacteria. This ratio is 3.1 for *Listeria*, 1.5 for *Salmonela*, and 0.9 for *Esherichia*.

4. Discussion

Vibrational spectroscopy, unlike other techniques used in microbiology, is a relatively simple method for studying structural changes occurring within a microbial cell following environmental stress and applications of food processing treatments. Vibrational spectroscopy provides a wide range of biochemical properties about bacteria in a single spectrum, most importantly characteristics of the cell membrane. These techniques are especially useful for studying properties of bacterial biofilms on contact surfaces, the presence and viability of

Table. Tentative assignment of the surface enhanced Raman spectral bands of *L. monocytogenes* $ATC_{L3}C$ 7644, *S. enterica* DS88, and *E. coli* O157:H7 in the region of 400–2000 cm^{-1} [8, 35].

L. monocytogenes $ATC_{L3}C$ 7644 (cm^{-1})	*S. enterica* DS88 (cm^{-1})	*E. coli* O157:H7 (cm^{-1})	Tentative assignment
485	–	–	–
623	620	620	phenylalanine (skeletal)
646	646	646	COO^- bend of amino acids, C–S stretch mode of cysteine
731	720	722	glycosidic ring, adenine
895	898	–	COC stretching / tryptophan
955	960	–	N–C stretching
1003	1003	1003	phenylalanine
1090	1090	1088	C–C skeletal and COC stretching from glycosidic link
1323	1331	1336	CH_2 deformation
1453	1453	1453	CH_2 deformation
1588	1588	1588	guanine, adenine (ring stretching)

bacterial vegetative cells and spores, the type and degree of bacterial injury, and assessment of antibiotic susceptibility [10].

Compared to the traditional morphological and biochemical tests, recently developed genetic methods, namely analysis of 16S ribosomal deoxyribonucleic acid (DNA) or 16S ribosomal ribonucleic acid (RNA), have been designated the "gold standard" for bacterial identification. However, these genetic methods have drawbacks, specifically, that they are time consuming and require expensive reagents and expendables [10]. Both infrared and Raman spectroscopies are forms of vibrational spectroscopy and can provide "whole organism fingerprinting" through an examination of spectral features corresponding to a wide range of important functional groups that together can provide important information about the biochemical constituents of each bacterial cell and also identify and discriminate bacteria at a species level or strain level [10].

Infrared and Raman spectroscopies have been extensively applied in various research areas: for detection of food toxicants and chemical adulteration [11-13], bioprocessing and fermentation monitoring [14-17], enzyme activity [18], microorganism identification and segregation [19-26], microbial cell injury identification [27, 28], virus identification [29], and prion structure elucidation [30].

Raman spectroscopy provides some major advantages over infrared spectroscopy for investigations of biological samples [31] since interference of water spectral features is less problematic. In addition, more spectral features are detectable in a Raman spectrum than in an infrared one over the same wave number (Raman shift) range. The Raman bands tend to be narrower than those in the mid-IR range due to the fact that molecular electrical polarisability is less sensitive to intermolecular interactions than the dipole moments. There is a wide range of potential analytical wavelengths and laser resources (UV, near-infrared or visible) for Raman what allows substantially increase sensitivity of Raman spectroscopy by using resonance effect (the so-called resonance Raman scattering) [32]. In our experiments we used a 1064 nm excitation laser to reduce the interference from fluorescence and photodecomposition. The reduced power (150 mW) of excitation laser ensured prevention of thermal decomposition in the sample. In order to find conditions for the highest enhancement of the Raman signal, silver colloids of various heating times were used. Primarily we optimised the preparation protocol of a silver colloid and chose the optimal 60 min heating time as further prolongation did not affect the resulted spectra (Fig. 4). The average size of the silver colloid nanoparticles was about 35–40 nm. Other studies [33, 34] demonstrated a similar diameter of silver nanoparticles produced by the same method.

The SERS spectral bands of analyte using silver nanoparticles were recorded with a reasonable signal-to noise-ratio while they were too weak to be observable in conventional Raman spectra obtained without the colloid, indicating significant enhancement factors (Fig. 3). The ability of the surface enhanced Raman spectroscopy in the NIR spectral region to identify bacteria was evaluated by the analysis of three foodborne pathogens: *Listeria*, *Escherichia,* and *Salmonella*. Slightly different Raman spectra were obtained for different types of bacteria. Bacteria with similar structures exhibited more similar Raman spectra (Gram-negative *Escherichia* and *Salmonella*), whereas Gram-positive *Listeria* exhibited a stronger spectrum compared to that of the Gram-negative bacteria (Fig. 5). It is important to highlight the presence of a strong band at 731 cm^{-1} in *Listeria*, in contrast to a relatively weak band at 722 cm^{-1} in *Escherichia* and 720 cm^{-1} in *Salmonella*. These results are very similar to those obtained by Luo and Lin [35]. They observed an intensive band at 732 cm^{-1} for *Listeria* and weaker bands at 723 and 720 cm^{-1} for *Escherichia* and *Salmonella*, respectively, using a 784.8 nm semiconductor laser and silver colloid for bacterial spectra acquisition. The authors suggest that the band around the region of 720–735 cm^{-1} is because of the glycosidic ring mode of *N*-acetyl-D-glucosamine (NAG). Taking into account that there is a higher content of peptidoglycan (rich in NAG) in Gram-positive than in Gram-negative bacteria it is not surprising that *Listeria* exhibited a stronger peak in this region.

Comparison of bacterial SERS measurements presented in this study with other studies reported in literature is difficult due to a large variety of results obtained at different experimental conditions (various SERS substrates, preparation protocols for colloids and bacteria, different species and strains of

bacteria, excitation wavelengths, etc.). For instance, Raman spectra of bacteria prepared in distilled water (Fig. 5) differ from the previously published spectra of the same bacteria prepared in the growth media [6]. Despite that, the results obtained in this study are in good agreement with that measured by Luo and Lin [35].

The data obtained clearly indicate that relative intensities of SERS spectral bands of bacteria can be used for spectral differentiation of bacteria. In case of *Listeria, Salmonela,* and *Escherichia* cells, the intensity ratio of the spectral bands of adenine and cysteine can be used as a spectral marker for differentiation of the bacteria. The SERS spectra excited with the 1064 nm Nd:YAG laser allow to differentiate bacterial species, suggesting a potential application of this method for identification and differentiation of foodborne bacteria on food matrixes or food-related surfaces in the future.

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PAVIRŠIUMI SUSTIPRINTOS FT RAMANO SPEKTROSKOPIJOS TAIKYMAS BAKTERIJŲ PADERMIŲ NUSTATYMUI

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Santrauka

Listeria monocytogenes, Salmonela enterica ir *Esherichia coli* bakterijų Ramano spektrai buvo užregistruoti naudojant sidabro nanodalelių koloidinį tirpalą. Bakterijų paviršiumi sustiprintiems Ramano sklaidos (SERS) spektrams žadinti pirmą kartą buvo panaudotas 1064 nm bangos ilgio lazeris. Nustatyta, kad naudojant 50 nm skersmens sidabro nanodalelių koloidą stebimas didžiausias bakterijų Ramano sklaidos signalo stiprinimas. Galima daryti išvadą, kad bakterijų diferencijavimui SERS spektriniu metodu yra naudotini ne spektrinių juostų dažniai, o jų santykiniai intensyvumai. *Listeria monocytogenes, Salmonela enterica* ir *Esherichia coli* ląstelių diferencijavimui tinkamiausias yra SERS spektrinis žymuo – adenino ir cisteino spektrinių juostų 600–750 cm^{-1} srityje intensyvumų santykis.