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Combination of laser-induced breakdown spectroscopy and Raman spectroscopy for multivariate classification of bacteria☆

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ABSTRACT

In this work, we investigate the impact of data provided by complementary laser-based spectroscopic methods on multivariate classification accuracy. Discrimination and classification of five *Staphylococcus* bacterial strains and one strain of *Escherichia coli* is presented. The technique that we used for measurements is a combination of Raman spectroscopy and Laser-Induced Breakdown Spectroscopy (LIBS). Obtained spectroscopic data were then processed using Multivariate Data Analysis algorithms. Principal Components Analysis (PCA) was selected as the most suitable technique for visualization of bacterial strains data. To classify the bacterial strains, we used Neural Networks, namely a supervised version of Kohonen's self-organizing maps (SOM). We were processing results in three different ways - separately from LIBS measurements, from Raman measurements, and we also merged data from both mentioned methods. The three types of results were then compared.

By applying the PCA to Raman spectroscopy data, we observed that two bacterial strains were fully distinguished from the rest of the data set. In the case of LIBS data, three bacterial strains were fully discriminated. Using a combination of data from both methods, we achieved the complete discrimination of all bacterial strains. All the data were classified with a high success rate using SOM algorithm. The most accurate classification was obtained using a combination of data from both techniques. The classification accuracy varied, depending on specific samples and techniques. As for LIBS, the classification accuracy ranged from 45% to 100%, as for Raman Spectroscopy from 50% to 100% and in case of merged data, all samples were classified correctly.

Based on the results of the experiments presented in this work, we can assume that the combination of Raman spectroscopy and LIBS significantly enhances discrimination and classification accuracy of bacterial species and strains. The reason is the complementarity in obtained chemical information while using these two methods.

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1. Introduction

A conventional identification of bacteria requires some knowledge of their morphological, biochemical, physiological and genetic characteristics. A bacteria identification procedure consists of bacteria cultivation, separation, its study under an optical microscope, etc. The whole procedure is time-consuming, inefficient and requires an expertise of a trained microbiologist. Therefore there is an effort to develop a faster and automatic method for bacteria identification.

One of the promising methods for biological samples identification is Laser-Induced Breakdown Spectroscopy (LIBS) [1–6]. The potential of LIBS for bacteria study and identification is well demonstrated in several publications. Here will be mentioned only several manuscripts, interesting from the point of view of goals of this work. S. Morel et al. [7] performed discrimination of bacteria species and strains based on the cumulative spectral line intensities ratio. It was presented that this approach works well, however, in the case of vast datasets it is still time-consuming and inefficient. M. Baudelet et al. [8] studied the influence of laser pulse duration on analytical performance of LIBS for bacteria identification. In the manuscript it is shown that in a direct comparison of nanosecond and femtosecond laser pulse the femtosecond has less interference with emissions from the ambient air.

Improved figures of merit can be achieved by employing multivariate data analysis (MVDA, in spectroscopy often called chemometrics).

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It has been shown that MVDA algorithms is a powerful tool for biological samples identification. Manzoor et al. [9] employed neural networks (NN) to discriminate and identify different strains of fungi *Candida* measured by LIBS. They successfully distinguished 21 strains of *Candida* based mainly on emission from organic compounds of yeast cells (such as signal from H, N, O, CN, C_2 and other molecular bands). In another work, Manzoor et al. [10] adopted a similar approach for bacteria strains identification and discrimination. It shows that even if the elemental composition is approximately the same, the NN can discriminate the differences. Similar work was performed by Marcos-Martinez et al. [11], who successfully identified and distinguished different bacterial strains using LIBS and NN. Likewise, the group of S.J. Rehse presented a possibility of bacteria strains discrimination by using chemometrics (namely discriminant function analysis - DFA). J. Diedrich and S.J. Rehse employed DFA to discriminate the *E coli* strains [12]. In the later works, S.J. Rehse et al. [13] studied the influence of bacteria dilution and sample mixing on the discrimination precision. Their study presents that in a mixture of two different bacteria, the identification was possible up to the 80:20 mixing ratio. Moreover, in a mixture of two bacteria in any mixing ratio, the spectrum was always identified as a strain of one of the two bacteria which were mixed.

In the present work, our goal is to enhance the classification accuracy of various bacteria by a combination of LIBS and Raman spectroscopy. These two methods can be used beneficially not only because of the complementarity of yielded chemical information (elemental and molecular composition) but also because of the similar instrumentation (a laser and a spectrometer are main parts of both methods' instrumentation). The combination of LIBS and Raman spectroscopy has recently been used for various applications. A comprehensive study of the historical development of the combination of LIBS and Raman analysis up to the recent applications was published in 2013 by Lin et al. [14]. M. Sovago et al. [15] used a combination of LIBS and Raman for off-line monitoring of nanoparticles in dispersion. While Raman spectroscopy provides information about nanoparticle size, LIBS gives information about the elemental composition of the particles. Pořízka et al. [16] utilized combination of LIBS and Raman spectroscopy for detection of fluorine in CaF2 crystals. In this work, they investigated the possibility of fluorine detection via atomic line and also molecular bands emission. Noteworthy is a work of M. Hoehse et al. [17] dealing with classification of pigments and inks for forensic purposes using chemometrics on merged LIBS and Raman spectra. Their study showed that the fused data of both methods can improve the classification accuracy of several chemometric methods, namely Principal Compounds Analysis (PCA), Soft Independent Modeling of Class Analogy (SIMCA), Partial Least Squares Discriminant Analysis (PLS-DA) and Support Vector Machine (SVM).

For the purpose of our experiment, five bacterial strains of *Staphylococcus aureus* and one strain of *Escherichia coli* were selected. The measurements were performed using Raman spectroscopy first and shortly afterward using LIBS. The data obtained were filtered, scaled and subsequently, chemometric algorithms, specifically PCA and NN, were applied to spectroscopic data from each technique separately as well as to merged datasets.

2. Samples

Microbial samples used in the study included following six bacterial strains (abbreviations in brackets are used in graphs legends):

1. *Escherichia coli* CCM 3954 (E coli)
2. *Staphylococcus aureus* CCM 4223 (S aur)
3. *Staphylococcus aureus* CCM 4750 - methicillin resistant (MRSA)
4. *Staphylococcus aureus* CCM 3953 - methicillin sensitive (MSSA)
5. *Staphylococcus sciuri* (S sci)
6. *Staphylococcus pseudointermedius* (S pse)

The set of analyzed strains (1–4) was obtained from the Czech Collection of Microorganisms (Brno, Czech Republic). Strains 5 and 6 were obtained from the Culture Collection of the Department of Microbiology, St. Anne's Faculty Hospital (Brno, Czech Republic). Identification of strains was verified by mass spectroscopy MALDI-TOF-MS and biochemical methods. All of the strains were stored at −70 °C.

Before the experiment, the strains were thawed and cultivated on Mueller-Hinton agar plates (MH, Oxoid, Basingstoke, UK) for 24 h at 37 °C.

3. Experimental

3.1. LIBS

LIBS measurements were performed on commercially available device Sci-Trace (AtomTrace, CZ) composed of instrumental compartment and an interaction chamber [18]. As the laser source was employed Nd:YAG laser CFR-400 (Quantel, FR; 20 Hz, 532 nm, 10 ns). The laser energy was set by a motorized attenuator (Eksma Optics, LT). The laser beam was focused on the sample surface with glass triplet (Sill optics, DE) with 32 mm focal length. Plasma radiation was collected using two UV grade lenses with total focal length 75 mm and led via optical cable (400 μm, Thorlabs, US) on the entrance of an echelle spectrometer (Emu-65, Catalyna Scientific, US). The radiation was recorded on the EMCCD camera Falcon blue (Raptor photonics, IE).

The measurements were performed directly on the agar plate at atmospheric pressure and room temperature. The laser energy was selected with respect to the obtained LIBS signal on 50 mJ per pulse. For each sample, a region with high bacteria concentration was chosen. Within this region, a matrix of 25 × 10 points (one laser pulse per spot) with step size 0.65 mm was measured. This measurement resulted in 250 unique LIBS spectra (one spectrum per spot). We opted for this approach for the sake of simplicity. On the other hand, for each sample a number of measurements came from pure agar and this spectra had to be filtered afterwards. The spot size diameter ranged from 0.2 to 0.6 mm depending on the height of the sample on the respective position. The thickness of bacterial colonies varied from approximately a tenth of millimeter to units of millimeters and the laser beam was focused slightly above the agar plate surface. The sample (*Staphylococcus aureus* CCM 4223) after LIBS measurement is depicted in the Fig. 1. The selected gate delay was 1.2 μs and gate width 50 μs.

3.2. Raman spectroscopy

The Raman spectroscopy measurements were performed on commercially available device *inVia* Reflex (Renishaw, UK). We used a laser on wavelength 785 nm. The laser radiation was focused on

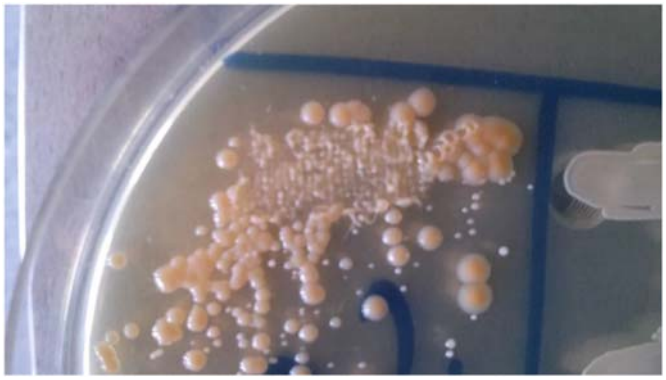

Fig. 1. *Staphylococcus aureus* CCM 4223 (S aur) - after measurement.

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