Discrimination of microbiological samples using femtosecond laser-induced breakdown spectroscopy

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Using femtosecond laser-induced breakdown spectroscopy, the authors have analyzed five different species of bacterium. Line emissions from six trace mineral elements, Na, Mg, P, K, Ca, and Fe, have been clearly detected. Their intensities correspond to relative concentrations of these elements contained in the analyzed samples. The authors demonstrate that the concentration profile of trace elements allows unambiguous discrimination of different bacteria. Quantitative differentiation has been made by representing bacteria in a six-dimension hyperspace with each of its axis representing a detected trace element. In such hyperspace, representative points of different species of bacterium are gathered in different and distinct volumes. © *2006 American Institute of Physics*.
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Laser-induced breakdown spectroscopy (LIBS) is an elemental analytical technique which can provide sensitive, multielemental, and real time chemical composition analysis.[1] Recently LIBS has been used to analyze microbiological samples such as bacteria, molds, or pollens.[2–10] Data processing methods usually associated to such analysis are statistical methods such as linear correlation or principal component analysis,[2–5] which provide discrimination of different biological matters. On the other hand, the advantages related to the use of femtosecond pulses in LIBS (femto-LIBS) have been demonstrated.[11–13] In particular, a highly sensitive detection of trace mineral elements is provided by femto-LIBS in bacterial sample analysis.[9,10] In this letter we report femto-LIBS analysis of five different species of bacterium, *Acinetobacter baylyi, Bacillus subtilis, Erwinia chrysanthemi, Escherichia coli*, and *Shewanella oneidensis*. We demonstrate that profiles of relative concentrations of trace mineral elements extracted from LIBS spectra provide full discrimination of the analyzed bacteria. We present a data processing method that quantifies the differences between trace element profiles of different bacteria by representing them in a hyperspace where each axis corresponds to a detected trace mineral element.

Detailed description of our experimental setup can be found elsewhere.[9,10] Briefly femtosecond pulses (120 fs, 3.8 mJ at 810 nm) were used to generate plasma. Five nonpathogen bacteria able to grow in a same nutrition medium have been prepared according to the following procedure: growth of bacteria overnight up to the stationary phase in liquid Luria broth to obtain 800 mℓ culture for each bacterium; the culture was centrifuged and the powder washed with distilled water. The cells (estimated to a number of 10^{12}) were resuspended in 4 mℓ of distilled water and lyophilized. The dried matter was transformed into a pastille with 20 mm in diameter and 1–2 mm in thickness. Samples were fixed on a rotation stage in order to have a fresh surface for each laser shot. Emission from plasma was coupled to an Echelle spectrometer equipped with an intensified charge-coupled device camera (Andor Technology, Mechelle and iStar). For each bacterial sample 50 spectra were taken. Each individual spectrum was accumulated over 1000 laser shots. For each laser shot, a detection window was open with a delay of 100 ns and a width of 5 μs. Laser energy controls made during the spectral measurements showed a fluctuation of less than ±5%. Spectra were recorded continuously in order to keep the experimental conditions as identical as possible for all samples.

Raw spectra were analyzed in order to identify trace mineral elements that present line emissions strong enough to be considered. Six elements have been included in our consideration: Na, Mg, P, K, Ca, and Fe. From each individual spectrum, a line intensity was extracted for each of these six elements, which is the sum of all lines belonging to this element. For the data processing we use I_{ij}^{k} to represent a specific raw total line intensity, where k represents different samples ($k=1,\ldots,L$; $L=5$), j refers to an individual spectrum ($j=1,\ldots,N$; $N=50$), and i refers to a specific element ($i=1,\ldots,M$; $M=6$). For given experimental conditions, we can consider the line intensity of a trace element as a protocol-dependent relative concentration of the element. Line intensities associated to the ensemble of trace elements for a given bacterium species provide a profile of relative concentrations of trace elements (or simply trace element profile).

In order to present a normalized trace element profile, two different procedures have been applied. In the first one, the mean value and the standard deviation of line intensities

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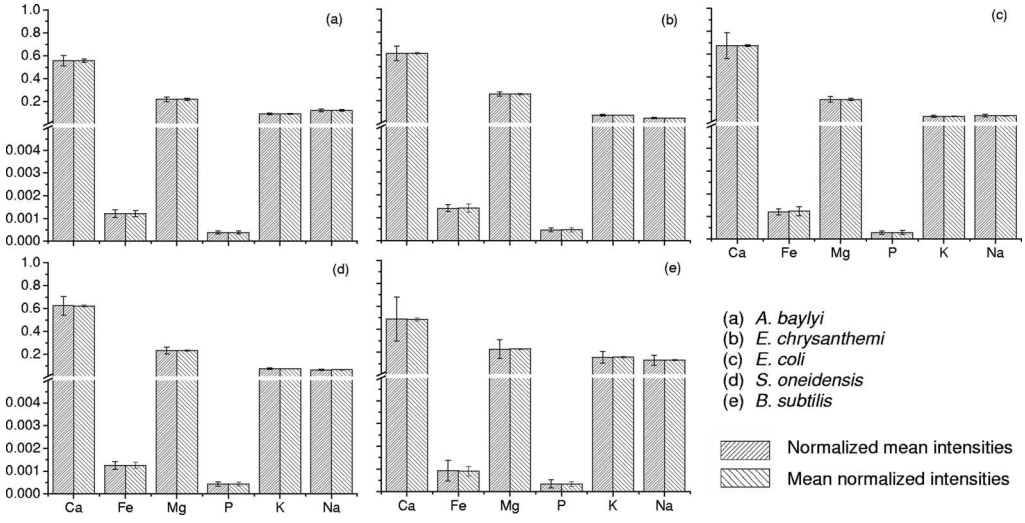

FIG. 1. Profiles of relative concentrations of trace elements for the five analyzed bacteria. Both $\bar{x}_i^k \pm \sigma_i^k$ (normalized mean intensities) and $\bar{y}_i^k \pm s_i^k$ (mean normalized intensities) are presented. The error bars in the figure are standard deviations of the corresponding data sets.

of an element are calculated for 50 individual spectra of a given bacterium:

$$\bar{I}_i^k = \frac{1}{N}\sum_{j=1}^{N} I_{ij}^k,$$

$$\tilde{\sigma}_i^k = \sqrt{\frac{1}{N-1}\sum_{j=1}^{N} (I_{ij}^k - \bar{I}_i^k)^2}.$$

Normalization is then made for the six trace elements:

$$\bar{x}_i^k = \bar{I}_i^k \Big/ \sum_{i=1}^{M} \bar{I}_i^k,$$

$$\sigma_i^k = \tilde{\sigma}_i^k \Big/ \sum_{i=1}^{M} \bar{I}_{ij}^k.$$

In the second procedure, line intensities are first normalized for each individual spectrum:

$$y_{ij}^k = I_{ij}^k \Big/ \sum_{i=1}^{M} I_{ij}^k.$$

Mean values of normalized line intensities and associated standard deviations are then calculated:

$$\bar{y}_i^k = \frac{1}{N}\sum_{j=1}^{N} y_{ij}^k,$$

$$s_i^k = \sqrt{\frac{1}{N-1}\sum_{j=1}^{N} (y_{ij}^k - \bar{y}_i^k)^2}.$$

In Fig. 1, normalized trace element profiles are shown with either $\bar{x}_i^k \pm \sigma_i^k$ (normalized mean intensities) or $\bar{y}_i^k \pm s_i^k$ (mean normalized intensities) for the five bacteria. Comparing first the two different data sets from the two different normalization procedures, we see that in general, s_i^k are significantly smaller than σ_i^k, while $\bar{y}_i^k$ represent similar profiles as $\bar{x}_i^k$. This means that the normalization within each individual spectrum reduces significantly experimental fluctuations. We can also remark from Fig. 1 that each bacterial sample has a characteristic trace element profile.

To quantify the difference between trace element profiles of different bacteria, we represent them in a hyperspace with a dimension equal to the number of considered trace elements. Such hyperspace representation of bacteria appears significant because of the close relationship between trace elements and biological processes in a living matter. For instance, a six-dimension hyperspace is used to represent the five analyzed bacteria. Each individual spectrum of a given bacterium generates a point in the hyperspace with its six coordinates corresponding to the normalized line intensities. Experimental fluctuation leads to dispersion of representative points of a given sample. Discrimination of two different bacteria is made if the corresponding representative points of the bacteria occupy two distinguished volumes in the hyperspace. Identification of an unknown bacterium can be performed by comparing its hyperspace coordinates to a database providing positions of reference bacteria in the hyperspace. We call this method trace element hyperspace classification (TEHC). It is important to emphasize that this data processing method associated to the measurement of trace element profiles with LIBS might be generalized to other biological samples.

In order to perform quantitative analysis, a vector is associated to each representative point of bacterium in the hyperspace. Each bacterium sample thus corresponds to an ensemble of 50 vectors. Difference between two species of bacterium can then be quantified by calculating the angles between two corresponding ensembles of vectors. The calculation is carried out by evaluating scalar products in two ways: (i) between vectors belonging to the same bacterium (self scalar products),

$$\theta_{j'\neq j}^k = \arccos\left[\frac{\sum_{i=1,j'\neq j}^{M} y_{ij}^k y_{ij'}^k}{\sqrt{\sum_{i=1}^{M} (y_{ij}^k)^2}\sqrt{\sum_{i=1}^{M} (y_{ij'}^k)^2}}\right],$$

and (ii) between vectors belonging to the two different bacteria (cross scalar products),

TABLE I. Mean values of angular distances in degrees between representative points of the same bacterium (diagonal elements) and between representative points of couples of different bacteria (off-diagonal elements).

	A. baylyi	*E. coli*	*E. chrysantemi*	*S. oneidensis*	*B. subtilis*
A. baylyi	1.56±1.22				
E. coli	9.18±1.13	1.03±0.66			
E. chrysantemi	7.90±0.65	6.23±1.06	0.93±0.63		
S. oneidensis	6.66±0.89	4.18±0.91	2.71±0.84	1.03±0.53	
B. subtilis	8.32±1.49	16.39±1.36	13.58±1.20	13.27±1.34	1.82±1.05

$$\theta_{jj'}^{k'\neq k} = \arccos\left[\frac{\sum_{i=1}^{M} y_{ij}^{k} y_{ij'}^{k'}}{\sqrt{\sum_{i=1}^{M} (y_{ij}^{k})^2}\sqrt{\sum_{i=1}^{M} (y_{ij'}^{k'})^2}}\right].$$

From these two sets of values, we calculate $\bar{\theta}^k$, mean angular distance between different representative points of a given bacterium, and $\bar{\theta}^{k'\neq k}$, mean angular distance between representative points of two different bacteria. Table I shows the results, where the diagonal elements correspond to $\bar{\theta}^k$ and the off-diagonal elements to $\bar{\theta}^{k'\neq k}$. The uncertainties presented in this table are standard deviations of corresponding data sets. We see that the mean angular distances between different bacteria are all significantly larger than those between different vectors of a same bacterium. The five studied bacteria are thus distinguished in the trace element hyperspace.

Another interesting way to explore the hyperspace is the use of projections to lower dimension spaces. Specific pairs of elements or specific group of elements can be selected to provide a representation of analysis results in a lower dimension space. The choice of the representative elements can be guided by biological arguments. This is actually the advantage of the TEHC method, which not only provides discrimination of biological matters but also intends to provide an analysis of biological properties of the studied sample. Figure 2 shows a three-dimension projection with respect to the group of three elements, Ca, K, and Na. We remark first that representative points corresponding to different bacteria occupy distinguished volumes. This means that in such three-dimension representation the five analyzed bacteria are already discriminated. Notice that all the four Gram$^-$ bacteria (*A. baylyi, E. chrysanthemi, E. coli*, and *S. oneidensis*) have a significantly higher concentration of Ca than the Gram$^+$ bacterium (*B. subtilis*). This result fits well with the property of Gram$^-$ bacteria, in which divalent cations maintain the cohesion of proteins in their outer membrane.[14] Finally, the addition of the third element, Na, allows full discriminations among the Gram$^-$ bacteria (especially between *E. chrysanthemi* and *S. oneidensis*). This illustrates the general principle of the TEHC method: the ability of discrimination should increase when the dimension of the hyperspace increases.

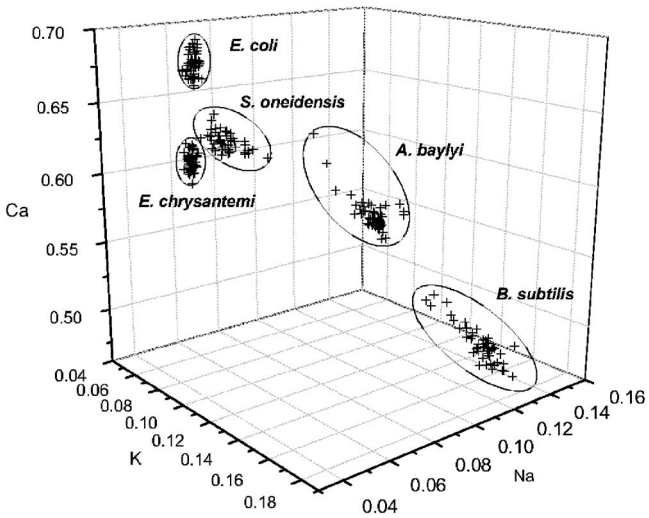

FIG. 2. Three-dimension projection of representative points of the five bacteria with respect to the group of three elements, Ca, K, and Na.

In conclusion, we have demonstrated that a microbiological sample such as bacteria can be characterized by its profile of relative concentrations of trace mineral elements. Such relative concentrations can be precisely determined by LIBS, especially by femto-LIBS which provides a sensitive detection of a large number of trace elements contained in a biological medium. The data processing method presented in this work based on the classification of biological samples in a trace element hyperspace enables LIBS to provide biologically significant analysis of living matters.

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