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Elemental analysis of fingernail of alcoholic and doping subjects by laser-induced breakdown spectroscopy

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Abstract Laser-induced breakdown spectroscopy (LIBS) is applied to investigate the effect of alcoholism and doping on elemental composition of fingernails of subjects. Measurements are made on 36 fingernail clippings including 8 doping, 8 alcoholic and 20 normal subjects. Classification of normal, alcoholic and doping subjects based on 46 atomic and ionic emission lines belonging to 13 elements of fingernail is examined using discriminant function analysis (DFA) method. The most affecting elements in classification of groups are discussed. In order to improve the repeatability of LIBS measurements, an auto-focus system has been designed and used in experiments. Results are promising and show that by improving the repeatability of experiments through improving the setup, some evidence of the impact of the alcohol and doping on elemental composition of fingernails is observed.

1 Introduction

Laser-induced breakdown spectroscopy (LIBS) is an analytical technique for the determination of elemental composition of samples. In this technique, a focused laser beam ablates a small area and creates hot plasma on sample surface. The plasma contains the elemental constituents of the sample material. As the plasma cools down, atoms in plasma emit their characteristic spectral lines [1, 2]. The elemental composition of sample can be determined by spectral analysis of plasma emission. LIBS has been applied extensively for analysis of different materials down to trace concentrations [3, 4]. Analysis of bacteria [5–10], teeth [11, 12], hairs [13], bones [14] and fingernails [15–17] are some examples of LIBS application in biological samples. Other methods used for elemental analysis of biological matrix are neutron activation analysis (NAA) [18], atomic absorption spectrophotometry (AAS) [19], inductively coupled plasma mass spectrometry (ICP-MS) [20], X-ray fluorescence (XRF) [21] and proton particle-induced X-ray emission (proton PIXE) [22]. LIBS offers many advantages compared to the aforementioned methods, including no sample preparation, extremely fast measurement time, broad elemental coverage, versatile sampling protocols and thin-sample analysis without the concern of substrate interference. These advantages made LIBS suitable for biological sample analysis [23–26].

The use of banned performance-enhancing drugs in human sport is commonly referred to as doping [27]. Under established doping control protocols, the participant will be asked to provide a urine sample [28]. Athletes, seeking to avoid testing positive for doping, use various methods to cheat on the drug tests. For detection of doping substances, apart from urine and blood, specialists are discussing other endogenous excretions or parts (e.g., saliva, sweat, hair, nails) as being suitable [29]. Among human tissues, nail tissue can be a useful sample for clinical investigations since metabolic events occurred during the time of its formation can influence the components of nail [30–32]. Thus, in spite of urine and blood, nail would be a suitable sample for doping test as it cannot be influenced by temporary actions. Owing to this, nail analyses were used for detection and quantification

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of illicit drugs [33]. Kim et al. [34] developed a method for the simultaneous qualification and quantification of amphetamine, methamphetamine, MDA, MDMA, ketamine and norketamine in nail clippings. The results indicated that nail clippings are potentially useful specimens for the detection of past illicit drug use. Lemos et al. [35] demonstrated the analysis of nail clippings for the drugs of abuse.

Nail and hair samples are also used for alcohol testing [36]. It is mentioned that because cosmetic treatments destroy the presence of some drugs and biomarkers in hair, nail samples are the preferred specimen over hair samples [37].

In the present study, laser-induced breakdown spectroscopy is used for analysis of fingernail belonging to doping and alcoholic subjects. Elemental analyses of fingernails of opium addicts, osteoporotic and hyper- and hypothyroidism subjects by means of LIBS have been investigated by our group, previously [16, 17, 38]. In previous works, in some cases, we could find some correlation between disease and fingernail elements. In those experiments, we could not be sure that the differences between the fingernail element intensities are due to the disease and are not due to the experimental fluctuations. So, in the present study, an auto-focus system is applied to prepare the same situation for all fingernails and improve the repeatability of the LIBS experiments. A statistical multivariate method named discriminant function analysis (DFA) is used to discriminate and classify samples into different groups, and also a comparison between some line intensities between the groups has been made.

2 Experiment

The experimental setup used to analyze fingernails includes two main sections of LIBS and auto-focusing (shown in Fig. 1). At the LIBS section, a Q-switched Nd:YAG laser pulse at wavelength of 1,064 nm, pulse energy of 65 mJ/pulse, repetition rate of 1 Hz and pulse duration of 6 ns is focused to create a plasma on the sample surface. Plasma emission is guided by an optical fiber to an Echelle spectrograph with resolving power of 1,700. The spectrograph is equipped by an intensified charge-coupled device (ICCD) camera for time-resolved detection. A part of laser beam is sent to a photodiode by means of a beam splitter, and then a signal is send to a delay generator. ICCD is triggered by the delay generator, 1 μs after the plasma initiation. Each spectrum is recorded with a gate width of 20 μs.

A sample like nail is an uneven sample with a slight convexity, and it is required to find the focal point on every fingernail separately. In order to maintain the distance between lens and all sample surfaces in similar situation, an auto-focusing system is used. The auto-focusing system includes a CCD with a resolution of 500 TV line, a doublet lens with 10 cm focal length and a mirror. A diode laser beam is directed from one side of a lens and reaches the sample (see Fig. 1). The scattered light from the sample surface becomes parallel by the other side of the lens and imaged on a CCD. If the sample is located farther from or closer to the focal point, the reflected beam on the CCD is directed to the left or right, respectively. A home-written program controls a motorized stage. By processing the CCD image, sample distance from the focal point is determined and a command be sent to the motorized stage to return the sample to the focal point. To keep CCD and the lens at fixed distance from each other and to prevent additional environmental lighting, an aluminum holder was designed. The doublet lens is common between two sections.

In order to assure the accuracy of the auto-focusing system, an experiment was designed. First, a reference

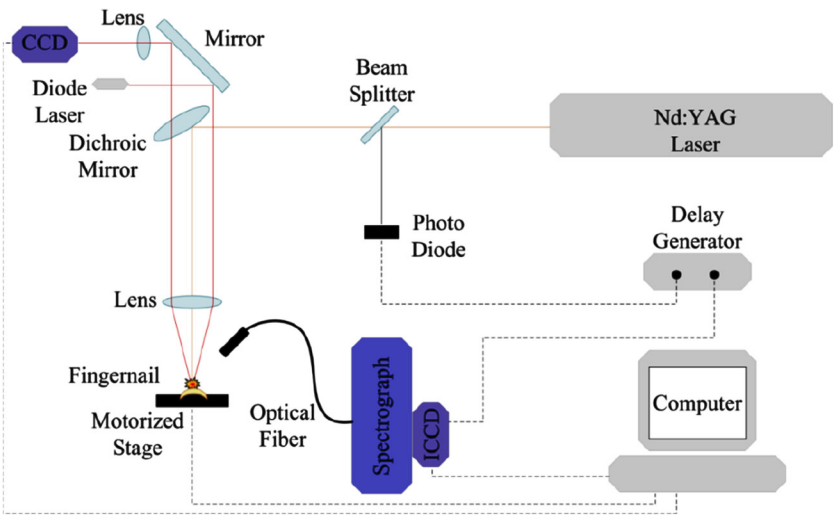

Fig. 1 Schematic diagram of the experimental setup of the LIBS auto-focus measurements

Table 1 Results of auto-focus system testing

Reference position (mm)	Position 1 (mm)	Position 2 (mm)	Position 3 (mm)
12.0397	12.0562	12.0526	12.0601

image is taken by CCD from nail in one position. Then, the nail was manually moved by a step motor to another position. The program controls the step motor to return the sample to the reference position. This procedure was repeated for three times, and the result is shown in the Table 1. The absolute error of our auto-focus system is about 20 microns which is less than the depth of focus of our optical system (approximately 668 microns). So this system is very good for our LIBS experiment.

All nail samples were fixed on a specialized sample holder in which the situation of all nail with respect to laser pulse is the same. During experiment the case-control status of samples was unidentified. Five points are selected on each nail sample and 10 laser shots hit on each point. So, the LIBS spectrum of each nail sample is obtained by accumulation of 50 laser shots.

3 Subjects

The subjects of this study were randomly chosen from the population of Rasht, in the North of Iran. The purpose of the study was explained to everyone and then, informed consent for participation was obtained. All subjects filled out questionnaires containing individual information such as personal information, medical history, drug consumptions and special diets. A total of 36 subjects participated in this study and all of them were males. These 36 subjects were categorized into four groups including 8 alcoholic and 9 related control subjects, and 8 doping and 11 related control subjects. Our alcoholic cases had median age of 47.5 with standard deviation of 8.9 years old. They consumed at least once a day for at least 4–5 months before. Our controls had median age of 47 with standard deviation of 10.25 years old. Our doping cases had median age of 25 with standard deviation of 5.25 years old. They used steroid drugs at least for 4 months before. Our controls had median age of 24 with standard deviation of 4.5 years old.

The free edge of the fingernails was taken by a stainless steel nail clippers and kept separately in plastic envelopes at room temperature until analysis. In order to eliminate any surface contamination, specimens were washed by soaking in acetone, alcohol and distilled water for 2 min for each one. All of them were dried at room temperature.

4 Statistical method

Discriminant function analysis (DFA), a multivariate statistical method, has been used for classification of subjects into case and control groups. DFA is a multivariate analysis of variance which forms linear combinations using variables to identify group memberships. In our study, for example, the spectra acquired from fingernails of cases should constitute one group, and fingernail spectra of controls would constitute another group. In DFA, for discrimination among N groups, $N-1$ discriminant function scores for each case are calculated. Then, the unknown case is assigned a group membership based on those $N-1$ scores. Since here we are discriminating among two groups, only one score is calculated. So, all variables from each spectrum belonging to each case are used by DFA to calculate a discriminant function score and, then, to predict the group membership of that particular case. All the variable vectors from all groups were analyzed simultaneously by a commercial DFA program (SPSS Inc., SPSS v17.0) to construct the canonical discriminant functions. DFA is processed in three basic steps: construction of discriminant functions, test of significance, and classification. A useful quantity in DFA is Wilks' lambda. Wilks' lambda is a statistical test used in multivariate analysis of variance (MANOVA) to test whether there are differences between the means of identified groups of subjects on a combination of dependent variables. The smaller the lambda for a variable, the more that variable contributes to the discriminant function. Lambda varies from 0 to 1, with 0 meaning group means differ (thus the more the variable differentiates the groups), and 1 meaning all group means are the same. Further detailed information on the statistical procedures can be found in our previous works [16, 17].

5 Results and discussion

A typical LIBS spectrum obtained from fingernail is given in Fig. 2. A total of 46 emission lines belonging to 13 elements including calcium, magnesium, silicon, sodium, potassium, titanium, strontium, iron, aluminum, carbon, nitrogen, hydrogen and oxygen have been identified in fingernail in our previous works [16, 17]. These emission lines are the basis of our spectroscopic analysis. The intensities of all 46 observed lines in each spectrum are summed which is called the total spectral power, and then the intensity of each spectral line is divided by this total spectral power to obtain the fractional spectral power. These fractional spectral powers are used as variables in the statistical method. DFA statistical method performs two multivariate analyses with these normalized lines and categorizes 36 subjects into two groups including 8 alcoholic

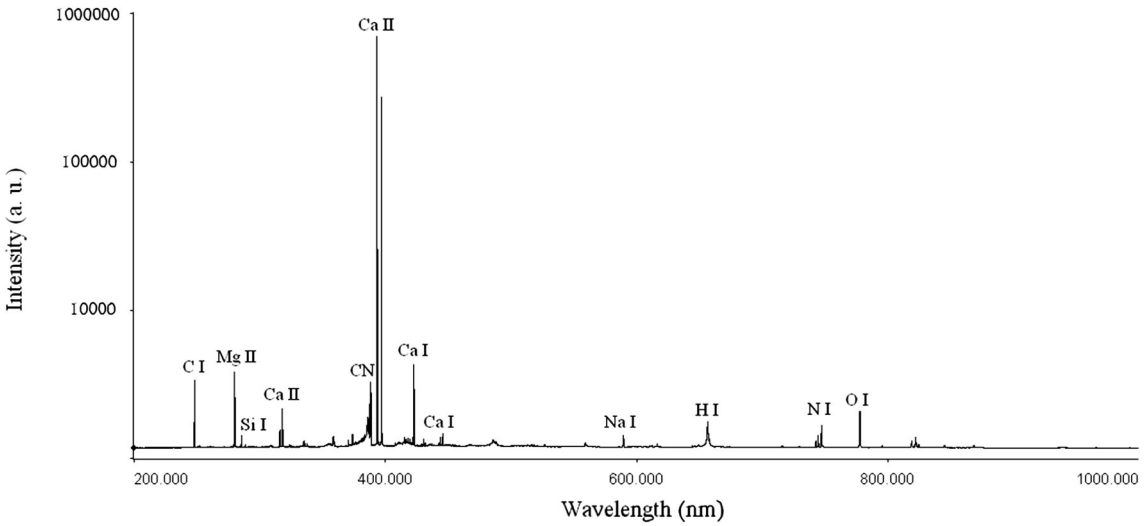

Fig. 2 A typical fingernail LIBS spectrum

and 9 related control subjects, and 8 doping and 11 related control subjects. So, the results have two main parts belonging to alcoholic and doping subjects.

In the part of alcoholic subjects, a plot of first discriminant function scores of every sample belonging to classification of two groups is shown in Fig. 3 and the results of this classification are shown in Table 2. All line intensities used in this analysis are shown in Table 3. Figure 3 shows the only one canonical discriminant function which is calculated for classification and accounts for 100 % of the variance. As it is shown in the graph, there is a good discrimination between two groups. In this analysis, 100 % of

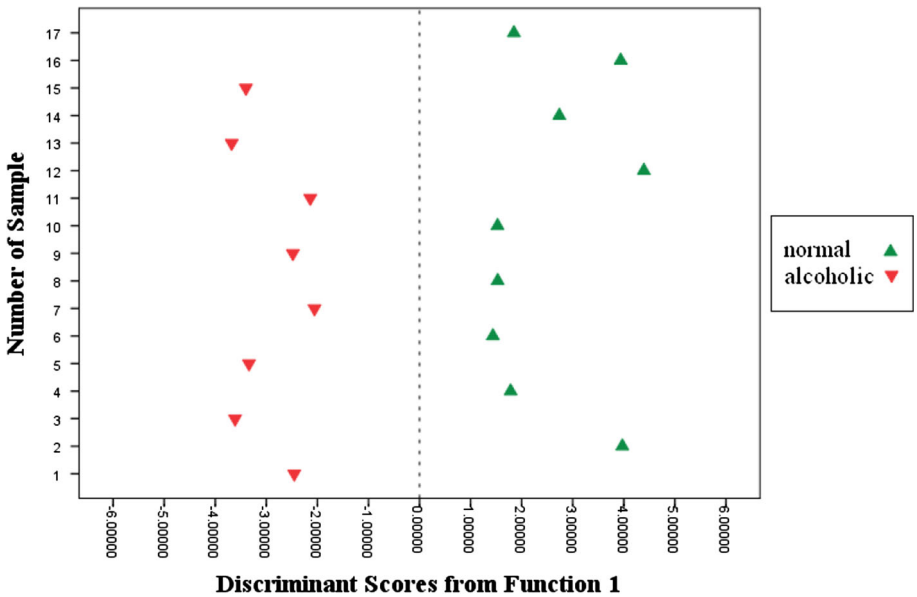

Fig. 3 Discriminant function analysis plot showing the first discriminant function scores of LIBS spectra obtained from fingernails of two different health statuses (alcoholic and normal subjects)

Table 2 Results of classification between alcoholic and normal subjects

		Category	Predicted group membership		Total	Correctly classified
			Alcoholic	Normal		
Cross-validated	Count	Alcoholic	7	1	8	76.5 %
		Normal	3	6	9	
	%	Alcoholic	87.5	12.5	100.0	
	%	Normal	33.3	66.7	100.0	

Table 3 Neutral and ionic lines and related Wilks' lambdas used in the classification of alcoholic and normal subjects

Line identification	Wilks' lambda
C I (247.85)	0.972
Mg II (279.55)	0.967
Mg II (280.27)	0.965
Mg I (285.21)	0.965
Si I (288.15)	1.000
Ca II (393.36)	0.982
Ca II (396.84)	0.990
Ca II (315.88)	0.767
Ca II (317.93)	0.795
Ca II (370.46)	0.997
Ca II (373.69)	0.995
Ca II (854.20)	0.996
Ca II (866.18)	0.912
Ca I (422.67)	0.997
Ca I (612.22)	0.990
Ca I (616.22)	0.861
Ca I (646.25)	0.844
Ca I (643.90)	0.801
Ca I (442.54)	0.799
Ca I (610.27)	0.969
Ca I (558.87)	0.966
Ca I (649.37)	0.988
Ca I (445.47)	0.984
Ca I (443.49)	0.932
Al I (394.40)	0.991
Al I (396.15)	0.994
Al I (309.27)	1.000
H I (656.28+656.27)	0.988
Na I (588.99+589.59)	0.979
N I (742.36)	0.999
N I (744.22)	0.988
N I (746.83)	0.986
N I (868.02+868.34)	0.904
O I (777.19+777.41 +777.53)	0.976
K I (766.48)	1.000
K I (769.89)	0.797
Ti II (334.94)	0.996
Ti II (336.12)	0.997
Ti II (337.27)	0.988
Ti II (338.37)	0.997
Ti II (323.45)	0.945
Ti I (399.86)	0.979
Ti I (365.38)	0.966
Fe I (248.32)	1.000
Fe I (260)	0.995
Fe I (302.107)	0.998
Sr II (407.77)	0.769

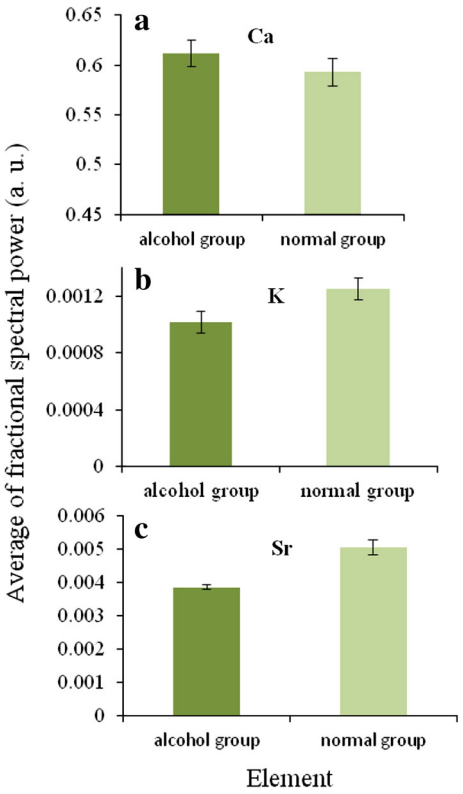

Fig. 4 Graphical representation of comparison between average of (**a**) sum of all Ca (**b**) sum of all K and (**c**) Sr *line* intensities in human nail of alcoholic and normal subjects

original grouped cases are correctly classified which shows that there is a suitable discrimination between two groups based on fingernail elements (not shown in the table). However, the validation results show that 76.5 % of cross-validated grouped cases are correctly classified. In cross-validation analysis, one sample is assumed to be unknown and separated from other samples, and then, a discriminant function is derived from all other samples and the membership of unknown sample is determined by the function. So, the result of cross-validation analysis is important. The true accuracy of this test can only be obtained by the cross-validated results. This satisfactory preliminary result shows that using LIBS spectra of fingernail accompanied with DFA can be acceptable in classification of alcoholic and normal subjects.

In Table 3, the quantity called Wilks' lambda of every variable in the classification can be observed. This quantity can be as an indicator of the effect of every variable in classification. So, the smaller the lambda for a line, the more that line contributes to the classification. As the experiment has been performed in the air, the elements which are common between air and nail including O, C, H and N cannot be appropriate indicators of status of our cases. We choose 5 lines which have the smallest Wilks'

lambda in this classification—except N line—and performed a comparison between line intensities of different elements in alcoholic and healthy subjects. There are three Ca lines including Ca II (315.88), Ca II (317.93), Ca I (422.67) and K I (769.89) and Sr II (407.77). In order to show whether there are remarkable differences between fractional spectral powers of these elements in two groups, the graph of comparison of the sum of every element lines is shown in Fig. 4. As it can be seen in Fig. 4a, there is difference in sum of all Ca lines between two groups but it is not bigger than the standard error of each group. Anyway, it can be seen that Ca is higher in fingernail of alcoholic subjects. Alcohol intake is one of the multiple risk factors for developing osteoporosis [39, 40]. Alcohol interferes with pancreas performance and its absorption of calcium and vitamin D. Alcohol also affects the liver, which is important for activating vitamin D which is also important for calcium absorption [41, 42]. So, maybe in alcoholic subjects, the excretion of Ca via fingernail is more than that of normal subjects. However, our analysis cannot approve this hypothesis. So, Ca in fingernail cannot be used as an indicator of alcohol status. As it can be seen in the graph (Fig. 4b), the difference in the fractional spectral power of K between two groups is statistically meaningful and lower in alcoholic subjects. Alcoholism leads to low potassium level (i.e., hypokalemia) [43]. Normally the kidneys are a major route of potassium ion excretion and serve as an important site of potassium regulation. Alcohol consumption historically has been found to reduce the amount of potassium excreted by the kidneys [44]. So, our results in this part are in good agreement with these explanations. Also, it is shown in the Fig. 4c that the difference in the fractional spectral power of Sr between two groups is more obvious and it is lower in alcoholic subjects. So, Sr in fingernail may be an indicator of alcohol status. However, there is no discussion in the literatures about Sr in alcoholic subjects and we cannot explain the difference between two groups.

In the part of doping subjects, a plot of first discriminant function scores belonging to classification of two groups is shown in Fig. 5 and results of this classification are shown in Table 4. All line intensities used in this analysis are shown in Table 5. In Fig. 5, the only one canonical discriminant function which is calculated for classification is demonstrated. As it is shown in the graph, there is a good discrimination between two groups. Also, in this analysis, 100 % of original grouped cases are correctly classified which shows that there is a suitable discrimination between two groups based on fingernail elements (not shown in the table). However, the validation results show that 73.7 % of

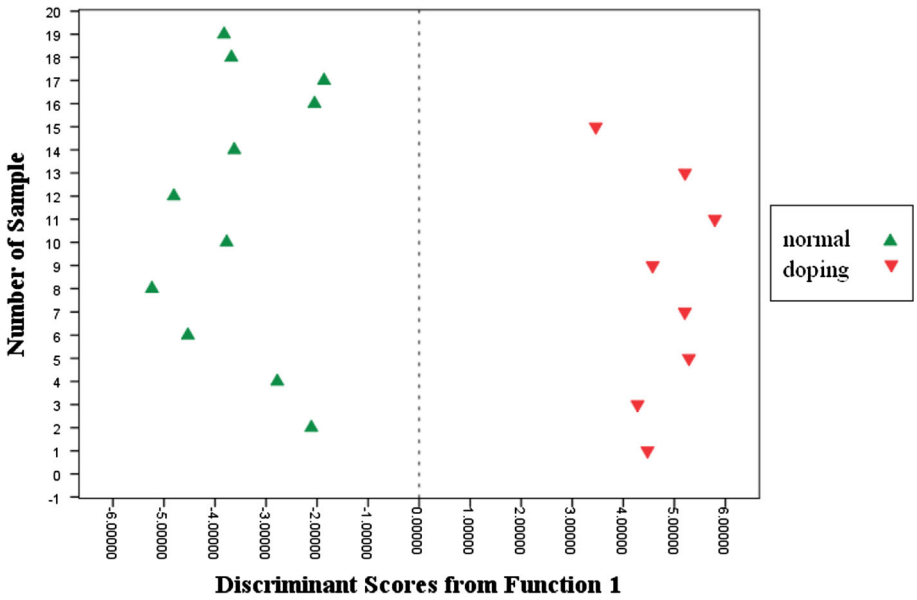

Fig. 5 Discriminant function analysis plot showing the first discriminant function scores of LIBS spectra obtained from fingernails of two different health statuses (doping and normal subjects)

Table 4 Results of classification between doping and normal subjects

		Category	Predicted group membership		Total	Correctly classified
			Doping	Normal		
Cross-validated	Count	Doping	6	2	8	73.7 %
		Normal	3	8	11	
	%	Doping	75.0	25.0	100.0	
	%	Normal	27.3	72.7	100.0	

Table 5 Neutral and ionic lines and related Wilks' lambdas used in the classification of doping and normal subjects

Line identification	Wilks' lambda
C I (247.85)	0.916
Mg II (279.55)	0.802
Mg II (280.27)	0.832
Mg I (285.21)	0.911
Si I (288.15)	0.999
Ca II (393.36)	0.991
Ca II (396.84)	0.999
Ca II (315.88)	0.891
Ca II (317.93)	0.897
Ca II (370.46)	0.913
Ca II (373.69)	0.920
Ca II (854.20)	0.999
Ca II (866.18)	0.927
Ca I (422.67)	0.992
Ca I (612.22)	0.983
Ca I (616.22)	0.825
Ca I (646.25)	0.871
Ca I (643.90)	0.976
Ca I (442.54)	0.977
Ca I (610.27)	0.965
Ca I (558.87)	0.978
Ca I (649.37)	0.818
Ca I (445.47)	0.978
Ca I (443.49)	0.997
Al I (394.40)	0.990
Al I (396.15)	0.996
Al I (309.27)	0.957
H I (656.28 + 656.27)	0.931
Na I (588.99 + 589.59)	0.927
N I (742.36)	0.846
N I (744.22)	0.827
N I (746.83)	0.796
N I (868.02 + 868.34)	0.727
O I (777.19 + 777.41 + 777.53)	0.810
K I (766.48)	0.656
K I (769.89)	0.977
Ti II (334.94)	0.956
Ti II (336.12)	0.964
Ti II (337.27)	0.961
Ti II (338.37)	0.968
Ti II (323.45)	0.735
Ti I (399.86)	0.992
Ti I (365.38)	0.800
Fe I (248.32)	0.985
Fe I (260)	0.987
Fe I (302.107)	0.998
Sr II (407.77)	0.991

cross-validated grouped cases are correctly classified which are normally lower than the original classification result. The result of this classification is also satisfactory.

From Table 5, it can be seen that 2 lines belonged to K I (766.48) and Ti II (323.45) have the smallest Wilks' lambda in this classification. So, the graph of comparison of the elements of these lines is shown in Fig. 6. These lines and also K I (769.89) are small in nail spectrum as they are shown in Figs 7 and 8, but they are trustworthy. As it can be seen in the Fig. 6a, b, the difference in the fractional spectral power of both K and Ti between two groups is bigger than the standard error of every group. This difference is more obvious in the fractional spectral power of K which is more in normal subjects. However, about Ti it is vice versa and it is more in doping subjects. There is not any information about special elemental metabolism in doping subjects. Hence, we cannot find any explanation for these differences.

6 Conclusion

Laser-induced breakdown spectroscopy has been applied to investigate the effect of alcoholism and doping on elemental composition of fingernails. An auto-focus system has been designed to improve the repeatability of the

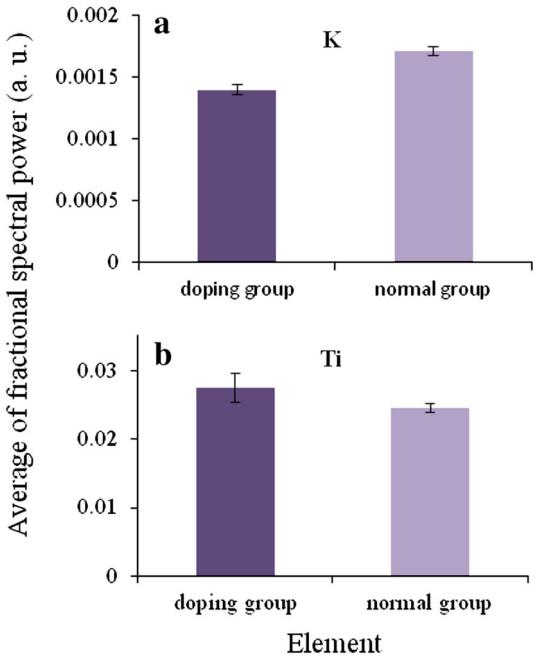

Fig. 6 Graphical representation of comparison between average of sum of all (**a**) K and (**b**) Ti *line* intensities in human nail of doping and normal subjects

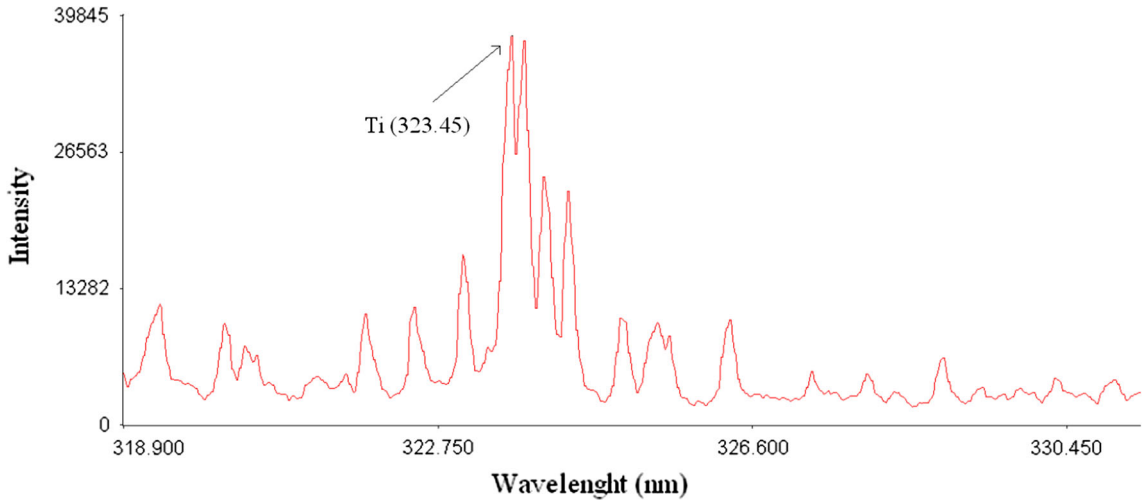

Fig. 7 The regions of the fingernail spectrum containing Ti II (323.45) *line*

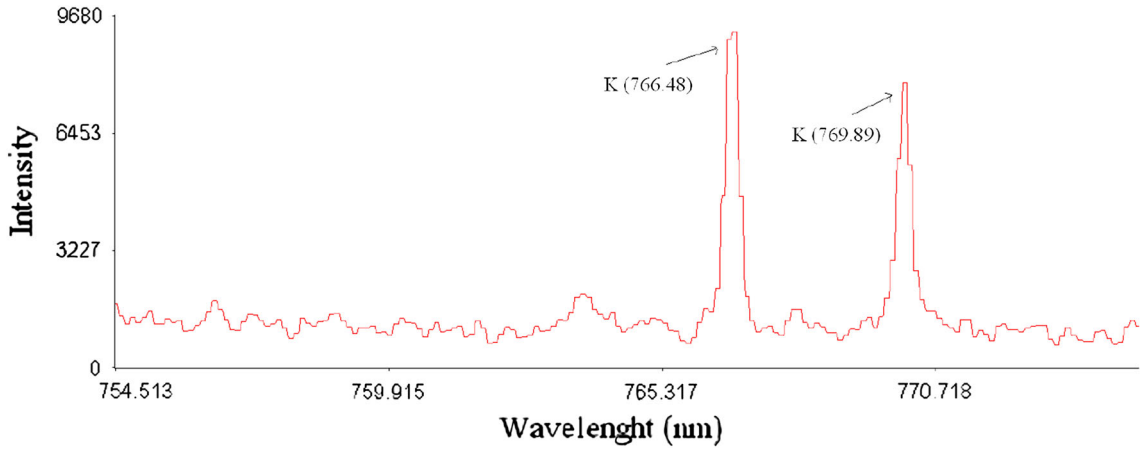

Fig. 8 The regions of the fingernail spectrum containing K I (766.48) and K I (769.89) *lines*

experimental LIBS results. A total of 36 fingernail clippings belonging to two sets of 8 doping and 8 alcoholic subjects and 20 normal subjects have been analyzed. The ability to classify subjects based on 46 atomic and ionic emission lines belonging to 13 elements of fingernail has been examined by a statistical multivariate method called DFA. DFA has been used for classification of subjects into two groups, and the results of classification are so good. Also, a comparison of some important elements has been made, and some useful information about metabolic differences between subjects of two groups can be deduced from their fingernails.

Overall, the results of this analysis show that by reducing the variation in LIBS spectra based on experimental conditions, some evidence of association between unusual feeding habits and elemental composition of fingernails can be obtained. However, this is a preliminary work with the limited number of samples. In order to reach a reasonable conclusion, the results of this study should be proved by further work and larger sample size.

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