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# Characterization of Milk from Mastitis-Infected Cows Using Laser-Induced Breakdown Spectrometry as a Molecular Analytical Technique

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**Abstract** The present work focuses on the possibility of using the evolution of the spectral molecular bands of cyanide (CN) and carbon ($C_2$) in the laser-induced breakdown spectra of cow's milk for mastitis characterization. It has been found that the intensities of these molecular bands are directly proportional to the mastitis symptoms and consequently to the somatic cells count (SCC). The results obtained using laser-induced breakdown spectroscopy (LIBS) technique in the present measurements proved that it is a direct, straightforward, and easy method for discrimination between mastitis milk and normal milk. A linear relation between the CN and $C_2$ spectral line intensities and the corresponding average of the SCC has been obtained. Such linear relationship can be easily exploited in the identification and characterization of mastitic milk samples.

**Keywords** Mastitis · Bovine milk · LIBS · CN and $C_2$

## Introduction

Mastitis is a serious and common disease in the dairy industry which causes heavy economic problems for milk producers and adversely affects welfare of infected animals. It is mainly due to inflammation of the milk secreting tissues of the udder, caused by microbial infections in one or more quarters. The warm, moist, and rich nutrient environment inside the udder provides adequate conditions for fast bacterial multiplication. Pathogens that cause mastitis induce pronounced changes in milk composition besides steady increase in the somatic cell count (SCC). Concerning milk composition, proteins may suffer from dramatic changes. Low-quality whey proteins increase on the account of decreasing of casein, which is the most important protein in milk ( Melanie and Murray 1999). This affects negatively the quality and flavor for various dairy products, namely butter and cheese (Auldist and Hubble 1998; Lotte et al. 2004).

Milk protein may break down as a result of having proteolytic enzymes in cow's milk together with mastitis. Plasmin increases proteolytic activity due to incidence of mastitis to twice as high, this can lead SCC to cause serious damage to casein in the udder before milking (Politis et al. 1989). Degradation of milk protein in the presence of mastitis may continue during milk storage and manufacturing of dairy products (Mungatana et al. 2011). Elevated levels of sodium and chlorine concentrations with declination of potassium and increased conductivity show up clearly in milk due to mastitis (Auldist and Hubble 1998). It is well known that calcium is linked with casein in milk; hence, hindering of casein synthesis leads to lowering of calcium in milk as a result (Roig et al. 1999; Ogola et al. 2007).

It is of great importance to have a reliable diagnostic technique for mastitis. Nowadays, mastitis is diagnosed via clinical symptoms such as changes in the udder's natural form and/or in milk's normal appearance. On the other hand, subclinical mastitis is diagnosed by chemical tests in the laboratory. Estimation of the milk somatic cell count (SCC) via the California Mastitis Test (CMT) is very common. The use of fluoro-opto-electronic cell counting technique is also used for estimating SCC in some cases. Mastitis detection has been also performed via measurement of electrical conductivity for milk though with varying results (Hillerton and Semmens 1999; Andrews 2000; Shosani et al. 2000). Other

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techniques have adopted measurements of serum proteins and enzymes as markers of inflammatory reaction in the mammary glands (Pyörälä 2003). Anyway, all the above-mentioned methods have drawbacks that hinder its routine use in animal production farms. New techniques for mastitis diagnostic are needed that satisfies the requirements of the very fast development in quantitative and qualitative milk production technology.

In fact, great attention has been given to the use of laser spectrochemical analytical techniques in diagnosis of mastitis in dairy cows. Abdel-Salam et al. 2015 demonstrated the potential of laser-induced breakdown spectroscopy (LIBS) and laser-induced fluorescence (LIF) techniques for the diagnosis of mastitis in milk. They found that the spectral line intensities of calcium and sodium in LIBS spectra are relevant to the SCC in the milk samples under investigation. The fluorescence intensities of mastitis milk and healthy milk have been also correlated to the SCC in such samples.

LIBS is a spectrochemical elemental analysis technique in which a short laser pulse is focused onto the samples resulting in the ablation of minute amount, typically a few micrograms. The ablated material is heated up by the laser pulse and produces the so-called plasma plume which is a collection of ions and swirling electrons at elevated temperature (6000 to 60,000 K). As the plasma plume cools down, it gets rid of the previously absorbed energy in the form of emitted light. Spectroscopic analysis of such emitted light provides us with a typical spectrum including the characteristic spectral lines of the elements existing in the target material. Quantitatively, there is proportionality between the spectral lines intensity for a certain element and the concentration of such element in the target taking into consideration self-absorption and the matrix effects. LIBS has many advantages; it is very fast, needs very little or no sample preparation, is quasi-nondestructive, can be used in hostile environments remotely using fiber optics, can be used in situ with the modern developed portable LIBS systems, and is relatively cost-effective compared with other conventional elemental analysis techniques. LIBS can be also used successfully for the detection of some molecular species, such as cyanide (CN), carbon ($C_2$), OH, etc. This extends the analytical potential of LIBS to the molecular analysis as well.

The objective of this work is to study the feasibility of making use of the molecular emission bands of CN and $C_2$ in the LIBS spectrum of milk samples, for the early detection of mastitis. This of course depends on the direct relation between the somatic cell count (SCC) and the molecular lines of both CN and $C_2$ in the laser-induced plasma. There is a correlation between such molecular lines and the SCC in the milk samples that will be constructed and exploited for characterization of mastitis. These investigations will provide a fast, reliable, and cost-effective technique (LIBS) that can be used in animal production farms for the early diagnosis of mastitis.

## Materials and Methods

### Milk Samples Collection

150 pure milk samples have been collected from healthy Holstein cows at nearly same age and weight from a standard animal production farm at Salhia in the middle of the Egyptian delta. The samples were kept into sterile plastic tube, where part is taken from each sample for somatic cell counting and the other part was cooled down to −20 °C and stored carefully at such low temperature. Droplets of milk (about 0.5 mL/droplet) that have been thawed at room temperature from the frozen samples were taken onto ashless filter paper to be used in LIBS measurements. Before analyzing it via LIBS, the milk droplets were left to be absorbed and expand on the filter paper in clean air for nearly 15 min.

### Mastitis Identification

In order to diagnose cows as suffering from mastitis, it is not possible to depend on the visible observable signs, which are mostly undetectable. In choosing such infected animals, the milk somatic cells count (SCC) (Sundekilde et al. 2013) and some udder symptoms should be considered as being the most decisive factors (Kramer et al. 2009; Miekley et al. 2012). In the present work, a conventional somatic cell counting system (Somacount 150 Bentley, USA) has been used. The fluorescent dye added to the milk sample in this conventional counting system leads to dispersion of the globules and staining of the DNA in the somatic cells. The stained somatic cells are then separated after injection into a laminar stream of carrier fluid. In exposing the cells to laser light, they fluoresce and the light pulses are converted to countable electrical pulses using a photomultiplier tube. The counted pulses are then classified and translated to SCC via special software. Samples with SCC of less than 300,000 cells/mL are classified as mastitis-free while those with SCC higher than 300,000 cells/mL are classified as mastitic. It should be taken into consideration that disease identification in this way depends on the animal's health regulations in each geographical region (Pinto et al. 2013). The average of every 15 samples having almost the same SCC has been considered.

### LIBS Arrangement

During the last three decades, laser-induced breakdown spectroscopy (LIBS) has been adopted as a reliable spectrochemical analytical technique in many applications including biological ones. LIBS mainly is an elemental analysis technique; however, and as mentioned before, it has been used in the molecular analysis of organic materials via detection of some molecular bands in the LIBS spectra such as CN and $C_2$. Numerous books and review papers have described in details the scientific and technical basics of LIBS (Miziolek et al.

2006; Singh and Thakur 2007; Singh et al. 2009; Kaiser et al. 2012). A typical single-pulse LIBS setup (described in details elsewhere (Abdel-Salam et al. 2012) has been used in the present work. The laser used was a Q-switched Nd:YAG (Brio, Quantel, France) delivering 50 mJ/pulse at its fundamental wavelength ($\lambda = 1064$ nm) with 5-ns pulse duration and 1-Hz repetition rate. A plano-convex quartz lens of focal length 10 cm has been used to focus the laser pulses onto the milk sample on the ashless filter paper. A micrometric X-Y translational stage has been used to move the target in front of the incident laser beam in order to have fresh target spot for each laser pulse. The emission of the laser-induced plasma is collected via a 600-mm diameter-fused silica optical fiber and fed to the entrance slit of an echelle spectrometer (Mechelle 7500, Multichannel, Sweden). The echelle spectrometer has a focal length of 17 cm with f-number of 5.2. It provides a constant spectral resolution of 7500 corresponding to 4-pixel FWHM, over a wavelength range of 200–1000 nm, displayable in a single spectrum. The spectrometer was coupled to an ICCD camera (DiCAM-PRO, PCO-Computer optics, Germany) for detection of the dispersed light. The ICCD was triggered optically, at a typical delay time of 2000 ns and gate width of 2000 ns. Wavelength calibration of the system is performed every 1 h (it takes about 5 min). Spectra were collected from five fresh spots on the milk sample on the ashless filter paper. Each LIBS spectrum is the average of 25 spectra taken as five spectra at five different positions. The spectra were normalized to the average background after subtracting the spectrum of the blank filter paper. The obtained spectra have been analyzed using the LIBS++ software (Corsi et al. 2001). It is worth to mention that the time duration of a single sample measurement via LIBS is almost 15 min or less, requiring no sample treatment. Conventional techniques for SCC require 2 to 3 h for single-sample measurement besides the time required for sample preparation and transportation to the laboratory.

## Results and Discussion

From the analytical point of view, LIBS is essentially an elemental analysis technique as mentioned above. Although of that, it has been demonstrated by many researchers that it is feasible to use LIBS in detection and follow up of some molecules, e.g., CN, $C_2$, and OH, through its molecular emission bands in the LIBS spectra (Elnasharty et al. 2011; Kasem et al. 2011; Abdel-Salam et al. 2012).

The interpretation of the presence of CN and $C_2$ bands in the LIBS spectra can be understood through two main mechanisms. Recombination is the first mechanism in which $C_2$ recombine with nitrogen from the air or from the target material to form CN, while formation of $C_2$ goes through fragmentation of molecules containing carbon–carbon double bonds (Baudelet et al. 2007; Lucena et al. 2011). CN and $C_2$ are produced in the second mechanism through the direct ablation of both molecular species from the target material, namely the milk sample in the present case (Kasem et al. 2011). This, of course, facilitates the evaluation of total proteins including somatic cells and IgG (Dohoo and Meek 1982; Cinar et al. 2015) through detection of CN and $C_2$ bands in the LIBS spectra of milk samples. It is well known that the somatic cells as other biological cells and all proteins

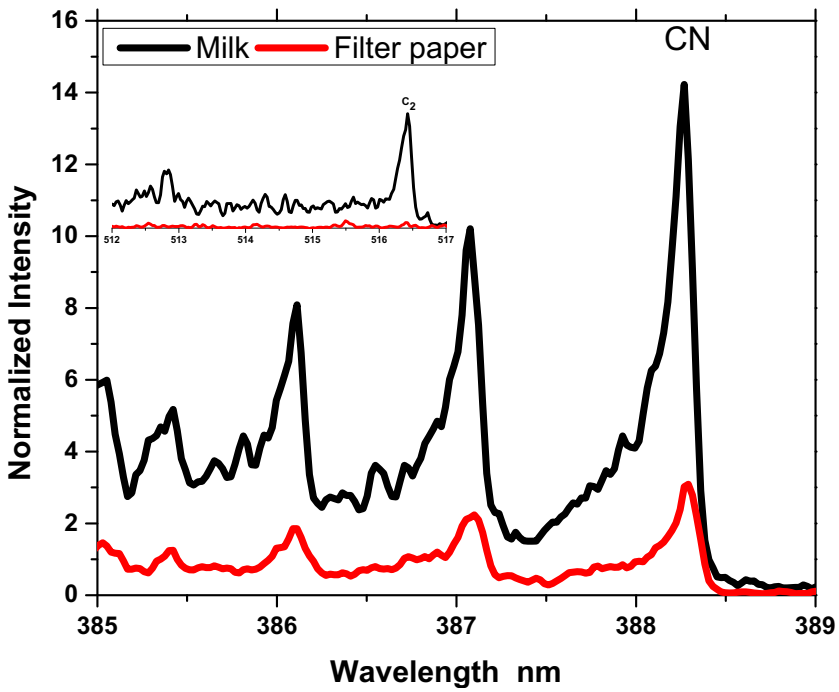


**Fig. 1** Typical LIBS Spectra of the violet CN emission bands ($B^2\Sigma^+ \rightarrow X^2\sum$) and the $C_2$ Swan band ($A^2\Pi \rightarrow X^2\sum^+$) in the visible region (*inset*) for milk samples compared with the blank filter paper spectrum

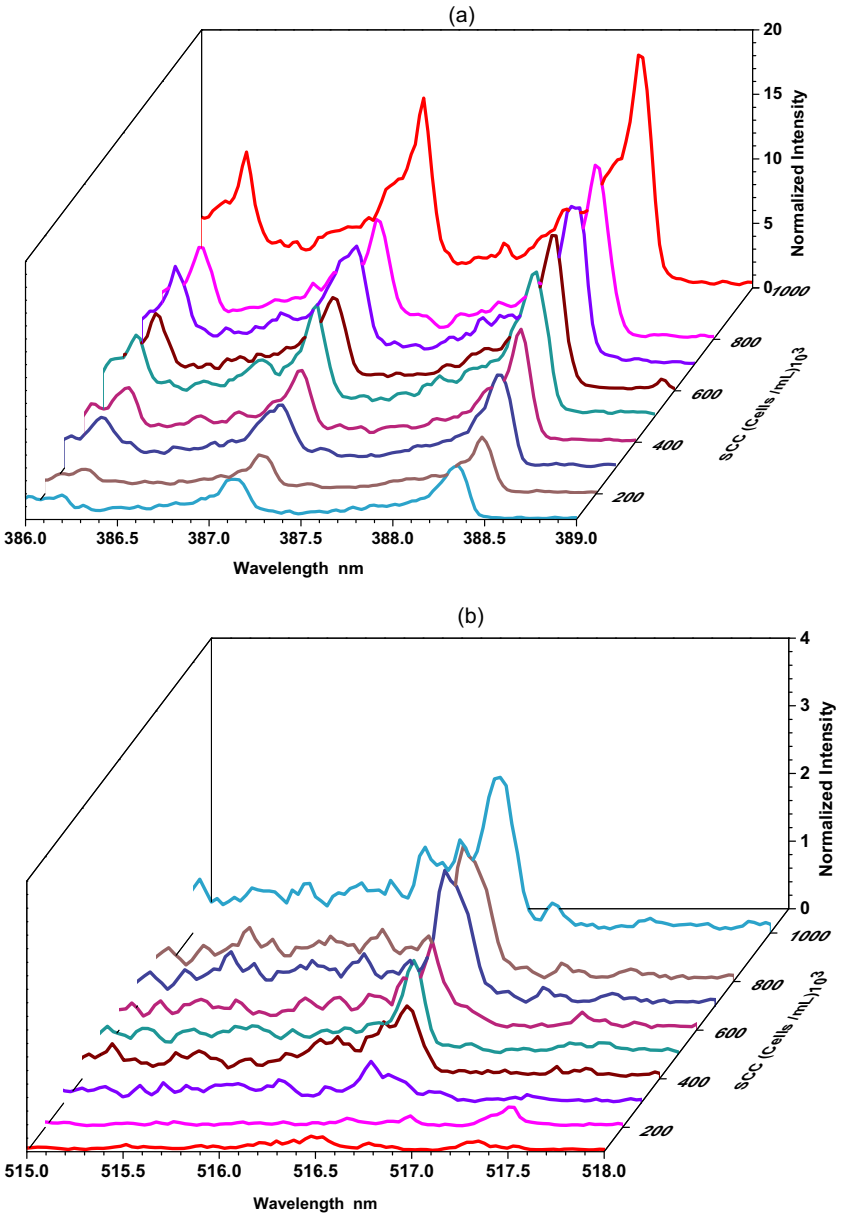


**Fig. 2** Typical LIBS spectra of CN (**a**) and $C_2$ (**b**) versus SCC (cells/mL) × $10^3$

constituents include amines ($NH_2$) and carboxylic groups (COOH) and their degradation under focused laser effect leads to the formation of CN and $C_2$ species as described above. Figure 1 shows the violet CN emission bands ($B^2\Sigma^+ \rightarrow X^2\sum$) and the $C_2$ Swan band ($A^2\Pi \rightarrow X^2\sum^+$) in the visible region for milk samples and the blank filter paper. The pronounced difference in the intensity of the spectral bands demonstrates that the origin of the CN and $C_2$ bands is from the milk samples. The time evolution curve of the CN bands (not shown) has an exponentially decaying trend which ensures that they are not originating from recombination reaction in air.

Figure 2 depicts the increase of the intensities of the CN and $C_2$ molecular lines in the LIBS spectra with the increase of the somatic cell count (SCC cells/mL ×$10^3$) in the analyzed samples of milk. It is clear that the intensities of CN and $C_2$ spectral lines were lower in case of normal milk with low SCC (>300 × $10^3$ cells/mL) than in mastitis milk with higher SCC (<300 × $10^3$ cells/mL) as shown in the figure. According to DePeters and Fergusson (1992), the major proteins in milk are synthesized in the mammary glands. The most important milk protein, casein, suffers from reduction in its concentration in case of mastitis due to the damage of the mammary glands and destruction of blood-born proteases (Holdaway 1990). Therefore, the contribution of the casein in the increase of the CN and $C_2$ intensity can be neglected because of its reduced quantities with the increase of SCC and other proteins.

Results presented in Fig. 3 show a comparison between the net CN intensities at different wavelengths in the UV region at 386.2, 387.1, and 388.1 nm for normal milk with low SCC

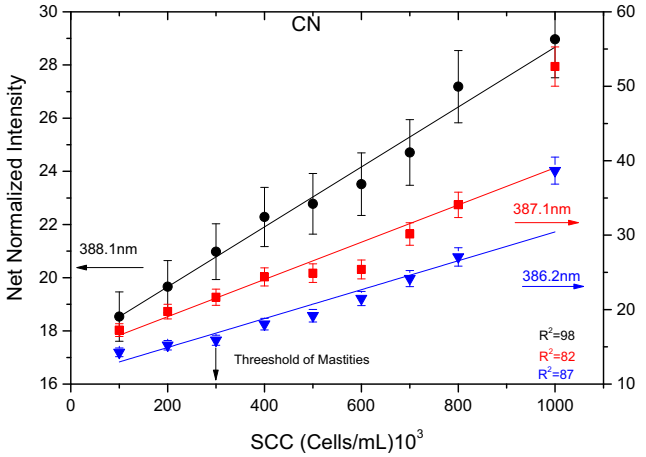


**Fig. 3** Trends for integrated net intensity values for CN emission lines at different SCC (cells/mL) × $10^3$. The threshold of mastitis is indicated on the *x*-axis. The *error bars* are the standard deviation of the experimental data

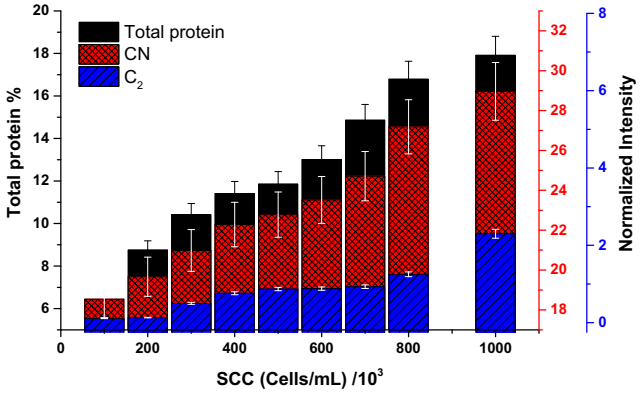


**Fig. 5** Trends of summation of the normalized integrated line intensities for CN and $C_2$ bands and the percentage of the total protein for different samples. The *error bars* represent the standard deviation of the experimental data of each group

(>300 × $10^3$ cells/mL) and for mastitis with higher SCC (>300 × $10^3$ cells/mL). Figure 4 describes similarly the behavior of $C_2$ emission band net intensity at 516.65 nm versus the SCC. This agrees with previous studies by (Politis et al. 1989). The presence of proteolytic enzymes in cow's milk with mastitis can lead to the breakdown of the milk protein which can lead to the decrease of CN and $C_2$ emission band intensities. On the contrary, a pronounced increase in the intensity of such molecular bands has been obtained due to the high SCC and the high immunoglobulin too as depicted in Figs. 3 and 4. In addition, according to (Roux et al. 2003) the quality of milk is lowered due to the fact that higher SCC results in higher enzymatic activity of proteinases, namely plasmin, causing reduction in mammary glands function and an increase in barrier permeability in infected milk. Decrease of protein, lactose, fat, casein micelle size, and increase of immunoglobulin, sodium, and chloride are expected following effects (Bruckmaier et al. 2004; Moslehishad et al. 2007).

Amines and carboxylic groups are increasing in the mastitic milk samples due to the increase of somatic cells and IgG. To confirm this fact in the investigated samples adopting the obtained LIBS spectra, the trend of total protein concentration with SCC has been studied. The intensity of three CN violet bands at 387.1, 386.2, and 388.1 nm as well as the intensity $C_2$ emission band at 516.65 nm (function of protein content) has been plotted versus the SCC in the investigated samples in the bar graph in Fig. 5. The percentage of the total protein,

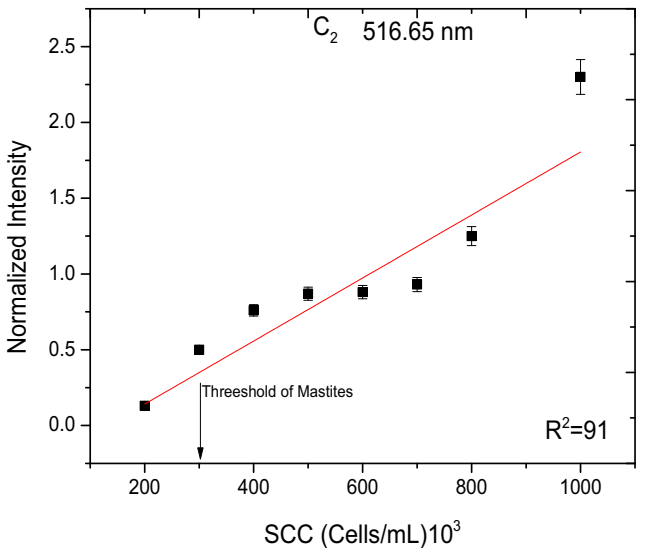


**Fig. 4** Trends for integrated net intensity values for $C_2$ emission lines at different SCC (cells/mL) × $10^3$. The threshold of mastitis is indicated on the *x*-axis. The *error bars* are the standard deviation of the experimental data

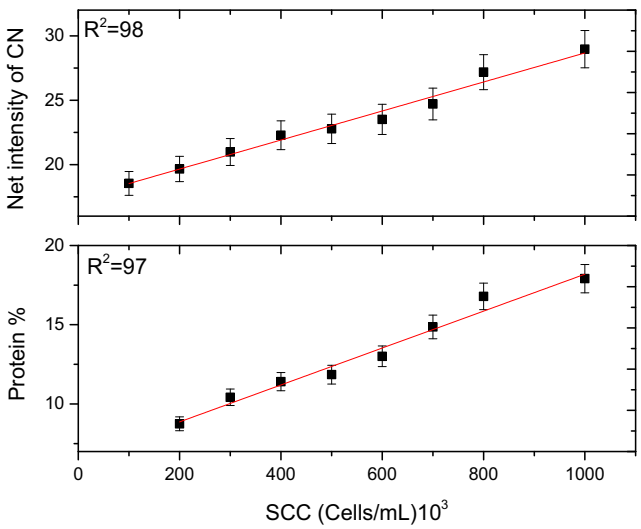


**Fig. 6** Net intensity of the CN bands (*top*) and protein % (*bottom*) versus the somatic cell count. The *error bars* represent the standard deviation of the experimental data

measured with conventional laboratory procedure using an automated infrared technique (Bentley 150 infrared milk analyzer (Chaska, MN, USA) is also depicted in the same figure. The results shown demonstrate clearly that the higher the total protein content is (somatic cells, IgG, rest of casein, and other proteins), the higher the CN and $C_2$ spectral lines intensities in the milk samples are. This result shows that proteins have the same qualitative trend in all samples.

To validate the obtained spectrochemical results, Fig. 6a, b depicts the linearity of both CN net intensity and the protein (%) with the SCC. Both linear relations have almost the same slope (0.0112 and 0.0117, respectively), confirming the validity of using LIBS technique as an easy and straightforward method in the early detection of mastitis.

## Conclusion

In the present study, it has been shown that in adopting LIBS, it is possible to use the changes in the spectral intensity of CN and $C_2$ bands as characteristic for diagnosing mastitis of milk cows. This demonstrates of course the spectrochemical analytical potential of LIBS technique for the qualitative evaluation of healthy and infected milk. Compared to the classical techniques used in similar studies, LIBS is fast, safe, simple, and can be used in situ. As depicted by the obtained results, the SCC in the milk has been evaluated using the spectral lines of molecular bands of CN and $C_2$ in LIBS spectra. This study shows clearly that LIBS is not only useful as an elemental analysis technique in the field of dairy science and technology, as has been demonstrated early, but also it can be used successfully as a molecular analysis technique in one of the most important applications in the same field, namely mastitis characterization in milk.

**Compliance with Ethical Standards**

**Conflict of Interest** Z. Abdel-Salam declares that she has no conflict of interest. S. Abdelghany declares that he has no conflict of interest. M.A. Harith declares that he has no conflict of interest.

**Ethical Approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Informed Consent** Not applicable.

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