Talanta 99 (2012) 140–145

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Laser spectrochemical characterization of semen

Z. Abdel-Salam, M.A. Harith *

National Institute of Laser Enhanced Science (NILES), Cairo University, Egypt

ARTICLE INFO

Article history:
Received 6 March 2012
Received in revised form
17 May 2012
Accepted 18 May 2012
Available online 24 May 2012

Keywords:
LIBS
LIF
Buffalo semen
Seminal plasma
Artificial insemination (AI)

ABSTRACT

The overall objective of this paper is to use a fast, more sensitive and less costly spectrochemical analysis laser techniques for estimation of seasonal variation of elements present in seminal plasma as well as for semen sperm count. For these two tasks we used Laser Induced-Breakdown Spectroscopy (LIBS) as an elemental analysis technique and Laser Induced Fluorescence (LIF) as a molecular analysis technique for sperm count estimation. The samples investigated via both techniques were buffalo semen from the artificial insemination center at the faculty of agriculture. The obtained LIBS data helped to assess indirectly the semen quality, sperm motility and spermatozoa count, relevant to the studied elements in different seasons. In addition it has been demonstrated that LIF can be adopted directly in centers of artificial insemination as a simple and fast method for the essential step of semen counting instead of the lengthy and inaccurate conventional techniques.

1. Introduction

Elemental and molecular analysis by means of laser-spectroscopic techniques such as LIBS and LIF has now become one of the most popular methods for direct sample analysis with least or no sample preparation. In addition both LIBS and LIF can perform *in situ* and real-time measurements using the nowadays available compact and portable systems. Accurate and precise determination still remains an important challenge for both techniques. In fact LIBS and LIF have been exploited in many biological and medical applications [1–5]. To the best of our knowledge both techniques have not been used in semen investigations. However Smuk et al. studied random laser action in bovine semen without any analytical investigations [6]. To increase the animal production wealth, artificial insemination (AI) represents a very important and promising issue. It is expected that improvement of the sperm quality will have a direct impact on the artificial insemination of animals both qualitatively and quantitatively.

In the present work LIBS and LIF were used for the characterization of the elemental and molecular composition of buffalo semen. Seminal plasma contains several trace elements that play an important role in the normal function of sperm. Some metals are essential for life, others have unknown biological functions, either favorable or toxic, and some others have the potential to cause disease [7]. On the other hand semen analysis, especially sperm counting, is an important test performed in artificial insemination centers.

Most works dealing with trace elements investigation in semen adopt conventional techniques such as Atomic absorption spectroscopy (AAS), and Inductively Coupled Plasma mass-spectrometry (ICP-MS). LIBS is as reliable as ICP in the analysis of most complex liquids. However, LIBS suffers from relatively poor sensitivity and relatively high detection limits compared to ICP. This may represent a problem when the elements concentrations are below the ppm range which is not the case in semen. Adamson and Rehse [8] used LIBS for the detection of Al in surrogates of human tissue up to parts per million levels. The authors used standard reference samples of known Al concentrations to build a calibration curve and consequently determine the limit of detection (LOD) which was less than 1ppm. LIBS limits of detection of Na and Mg in water have been found to be between 1 and 2.5 ppm [9]. In general LIBS has an inferior LOD with respect to other spectroscopic techniques such as ICP-MS, LA-ICP–MS, AES and AAS. However to follow up minor elements concentration variations, in semen for example, using LIBS, qualitative analysis would be satisfactory. On the other hand LIBS furnishes the possibility of *in situ* and real-time measurements contrary to the other spectroscopic techniques [1].

The unique advantage of LIBS in allowing the study of a broad variety of samples without sample preparation is attractive for the analysis of biological samples. Therefore, the LIBS technique is being utilized promisingly for the analysis of biomaterials [1]. With LIBS it is possible to perform also localized elemental analysis of the samples with a relatively high spatial resolution especially in biological samples where sample homogeneity represents a real difficulty [4]. Moreover, as mentioned before LIBS can be used to perform *in situ* measurements which facilitate its use in the field [10]. This, of course makes LIBS one of the most appropriate analytical techniques in biological applications. Recent approaches have been followed for qualitative and quantitative compositional analysis of calcified tissues, namely bones

* Corresponding author. Tel./fax: +202 3567 5335.
E-mail address: mharithm@niles.edu.eg (M.A. Harith).

0039-9140/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2012.05.031

and teeth, using (LIBS) [11]. LIBS has been also used as a simple method to identify and characterize some types of human malignancies [3]. Zheng et al. [12] have recently investigated the feasibility of using LIBS to characterize animal tissues. Tameze et al. [13] performed empirical analysis of LIBS images for ovarian cancer detection. Using LIBS, plasma images of the blood samples were generated and analyzed. LIBS has been used for the *in situ* quantitative estimation of the elemental constituents in different parts of kidney stones obtained during surgery [14].

Buffalo seminal plasma contains several trace elements that play an important role in the normal function of sperm. Trace elements are essential for the function of various enzymes and other proteins. Zinc and magnesium concentrations in seminal plasma correlate with spermatozoa count. Strong correlations were found between calcium, magnesium, and zinc in seminal plasma [15]. According to Vallee and Falchuk [16], zinc is involved in a number of functions of importance to sperm physiology. Although of the iron importance in proteins, it has been found that excess of iron is strongly related to male infertility. Detailed investigations of trace elements of seminal plasma in humans have been reported, but there is a little information available about trace elements contents in the buffalo seminal plasma. The conventional techniques for sperm counts are time consuming and include many sources of errors since it depends mainly on the accuracy of the person performing the counting procedure. Due to the unavailability of external proficiency testing programs for semen analysis, it has recently been shown that there is wide variation in sperm counts between conventional techniques. Conventional technique such as haemocytometer, Cell-VU, flow cytometry and Makler chamber require semen dilution, and microscopic counting as described in details by different authors [17,18].

The main motivation of the present work is to make use of two laser spectroscopic techniques, namely LIBS and LIF, in the field of artificial insemination in animals by semen characterization. LIBS is used to follow up the variation of elements contents of seminal plasma in high and low seasons and LIF technique is exploited as a simple, and time saving technique in the estimation of sperm count in semen.

2. Materials and methods

2.1. Semen samples

120 semen samples used in the present study were collected from six healthy and sexually mature buffalo bulls (*Bubalus bubalis*) three to five years old. The animals were nearly at the same body conditions, with an average body weight of 592 kg at the Artificial Insemination Laboratory, Animal Production Department, Faculty of Agriculture, Cairo University.

Semen was collected from the bulls early in the morning with the help of sterile artificial vagina, which was prepared to have internal temperature of 41–44 °C. The collecting tube was covered by means of a rubber sheath to eliminate light and heat effects. Ejaculates were transferred immediately to the laboratory and were kept in water bath at 37 °C until the respective examinations.

2.2. Seminal plasma samples for LIBS analysis

10 samples were taken from each sire in high season (winter) with short season between January and March, and another 10 samples in low season (summer) with short season between June and August. Samples were collected every nine days in most cases. Seminal plasma has been obtained by centrifuging the semen at 5000 rpm for 10 min [Variable Speed Micro Centrifuge 1020D.E, Centurion Scientific Ltd, UK]; the supernatants were transferred into 1.5 ml tubes, re–centrifuged to eliminate the remaining cells [17]. For LIBS analysis each seminal plasma sample was added drop wise onto a substrate of high quality ash less filter paper (50 mm diameter). Samples were left to spread on the filter paper and dry partially for 15 min where they were still wet before analysis. Filter paper was then mounted on an X–Y micrometer translation stage to move the sample during the measurements to ensure that a fresh surface location is available for each laser shot. To optimize the signal-to-noise ratio and guarantee results reproducibility LIBS spectra were collected from five fresh spots where five consecutive shots were recorded for each spot. The average of the 25 spectra for each sample was analyzed. The LIBS spectra obtained from the samples have been normalized on the strong carbon line at 247.8 nm to compensate for any experimental fluctuations. The spectral lines are identified and labeled in all spectra of different samples.

2.3. Semen samples for LIF analysis

Semen samples from the 60 high season collected samples have been prepared at different degrees of dilution to have samples of different sperm count. Semen was diluted with 0.7% NaCl. A drop of diluted semen (10 μl) was placed onto a haemocytometer covered with a cover slip and left for 10 min. Total number of spermatozoa is recounted as spermatozoa concentration × sperm volume. Determination of spermatozoa concentration was measured by counting cells following the method described by Alavi et al. [17]. A haemocytometer was used to determine spermatozoa concentration at the Artificial Insemination Laboratory. Semen samples (sperm counted) were taken into quartz cuvettes, for further LIF analysis.

Adenosine tri-phosphate (ATP) in semen is detected by reaction with Luciferin/Luciferase mixture in buffered solution. The light emitted in the process is detected and displayed in relative luminescence unites (RLU) by the HY-LiTE luminometer (Merck KGaA, Germany).

2.4. LIBS arrangement

A typical LIBS experimental setup has been used in the present work. The used laser source is a Q-switched Nd:YAG laser (BRIO, Quantel, France), operating at the fundamental wavelength ($\lambda = 1064$ nm), with a pulse duration of 5 ns. The laser pulse energy was set to 50 mJ and the repetition rate to 1 Hz. The laser beam was tightly focused onto the sample surface using a 10 cm focal length planoconvex quartz lens. The collected plasma emission is then fed *via* the optical fiber to the entrance slit of an echelle spectrometer (Mechelle 7500, Multichannel, Sweden). Gateable ICCD camera, DiCAM-Pro (PCO, computer optics-Germany), coupled to the spectrometer, was used for the detection of the dispersed light. Spectra display, processing and analysis were performed using the commercial 2D- and 3D- Gram/32, software programs (National Instruments, USA). In addition to the atomic data base used by the mentioned software, further spectral lines identification was accomplished using LIBS++ software [19]

2.5. LIF arrangement

The experimental set up of the equipment used throughout the present work for LIF measurements is shown in Fig. 1. The excitation light source was a continuous wave (CW) DPSS laser [Changchun new industries optoelectronics tech Co, Ltd. (CN)] with an average laser output power of 40 mW at a wavelength of 405 nm. The laser beam is focused into one end of an optical fiber while the other end of the fiber delivers the beam onto one side of

the quartz cuvette containing the semen sample. The emitted fluorescence is collected perpendicularly via another fiber used to deliver the fluorescent light to the spectrometer (USB2000 FLG, Ocean Optics, USA). Acquisition and analysis of the spectra obtained from the spectroscopic system is accomplished using the commercial SpectraSuit software (Ocean Optics, USA). All the spectra were further processed using computer software (Origin, Origin Lab. Corp., USA, Version 8).

3. Results and discussion

3.1. LIBS

To follow up the relative concentration of zinc, iron, magnesium and calcium, in the seminal plasma samples under investigation, the intensities of their corresponding spectral emission lines in the obtained LIBS spectra have been studied.

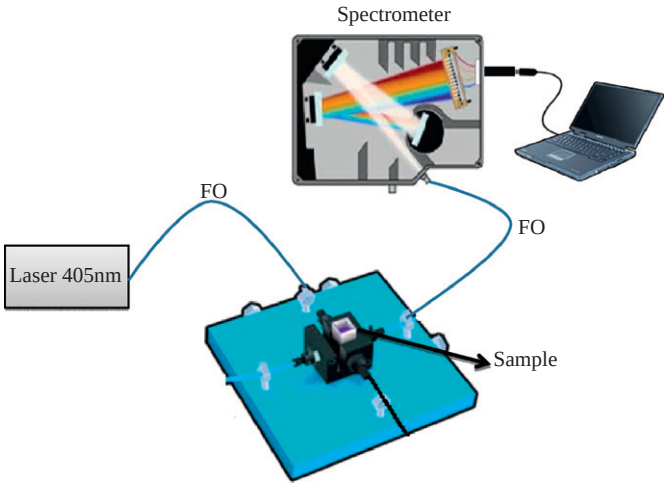

Fig. 1. Setup for the laser induced fluorescence experiment. FO: Fiber Optics.

Fig. 2 shows a typical LIBS spectrum of buffalo seminal plasma. The spectrum depicts the spectral lines of the elements of interest in the present study. Certain well resolved spectral lines for each element that have reasonable signal to noise ratio have been systematically followed up. The chosen lines were Mg at 285.22 nm, Ca at 428.30 nm, Fe at 430.83 nm and Zn at 330.29 nm. These spectral lines were well resolved, free of interference with other spectral lines and suffer very little from self-absorption.

Fig. 3a–d shows that the spectral lines of the four studied elements have higher intensity values in high season than in low season. This is a consequence of an increased seminal plasma concentration of the corresponding elements in high season. Seminal plasma contains variety of biochemical components, some of which are relatively specific for the regulation of sperm function [20]. In previously published works the presence of abnormal levels of Ca, Mg and of trace elements, in particular Zn may affect spermatogenesis with regard to production, maturation, motility, and fertilizing capacity of the spermatozoa [21]. The obtained higher values of Ca, Mg, Fe and Zn in seminal plasma in winter season than in the summer season indicate that the impact of such elements may be used in future studies to evaluate semen parameters including motility, and morphology, since such elements are believed to be important for spermatogenesis [15]. The relatively high zinc values in buffalo seminal plasma in the present study were comparable with those reported elsewhere in the literature [22]. This indicates that high semen zinc concentrations are related to the increase of sperm motility in this case. Zinc has also a fundamental role in the antimicrobial activity of the seminal plasma [23]. Calcium is an essential element, which is a crucial regulator of many physiological processes in every living cell including spermatozoa. Calcium ion is the trigger of the acrosome reaction in mammalian spermatozoa, and there is substantial evidence that the calcium ion is differentially involved in sperm motility depending on the

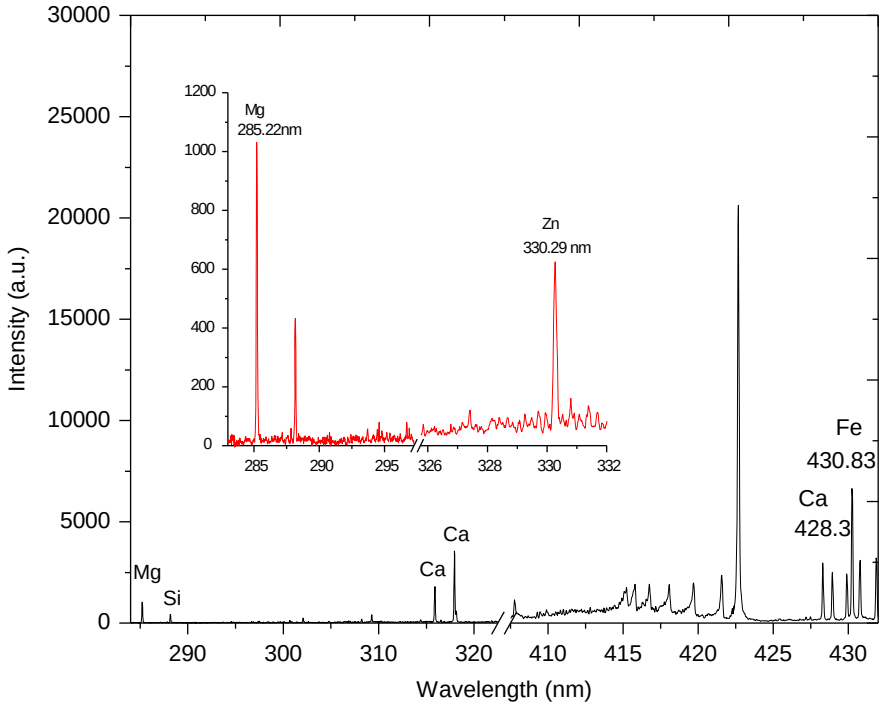

Fig. 2. Typical LIBS spectrum of buffalo seminal plasma. The inset shows a zoomed segment of Mg and Zn spectral lines.

stage of sperm maturation. It has been demonstrated that the prostate, seminal vesicles, and epididymis are also very rich in calcium that is why several studies have investigated the association between calcium and male subfertility [24,25]. Biologically magnesium is an important cation found in nearly all enzymatic systems. It modifies specific enzyme substrates and plays a fundamental role as a cofactor in more than 300 enzymatic reactions involving energy metabolism and nucleic acid synthesis. Magnesium may play a role in spermatogenesis, in particular in sperm motility. Furthermore, Mg is regarded as a marker of the secretions of the seminal vesicles and acts as an intracellular calcium antagonist [26]. Iron is an important component in proteins such as hemoglobin, ferritin, lactoferrin and catalase. On the other hand, iron is involved in the production of reactive oxygen species (ROS) via the Fenton and the Haber–Weiss reaction processes which produce the hydroxyl radical and hydrogen peroxide, respectively. An excess of ROS has been suggested to be the main cause of male infertility [26].

Fig. 4 shows that the spectral lines intensities corresponding to the mean values of Mg, Zn and Ca content of seminal plasma were significantly high in high season than in low season, but in case of Fe the increase of the mean value in high season with respect to the low season was non-significant. It is also noticeable that the Zn intensity value is the lowest significant ($p < 0.05$) compared with the other three elements. This may be due to the essential biological role that zinc plays in different sperms parameters that could not be affected strongly by seasonal variations. In case of Iron though the winter intensity value is high but the difference is statistically not significant.

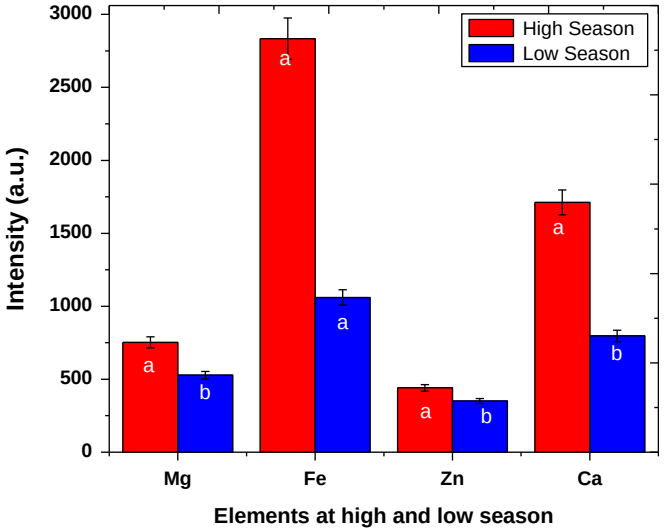

Fig. 4. LIBS intensity of different elements in seminal plasma of buffalo in different seasons. The error bars represent the typical standard deviation.

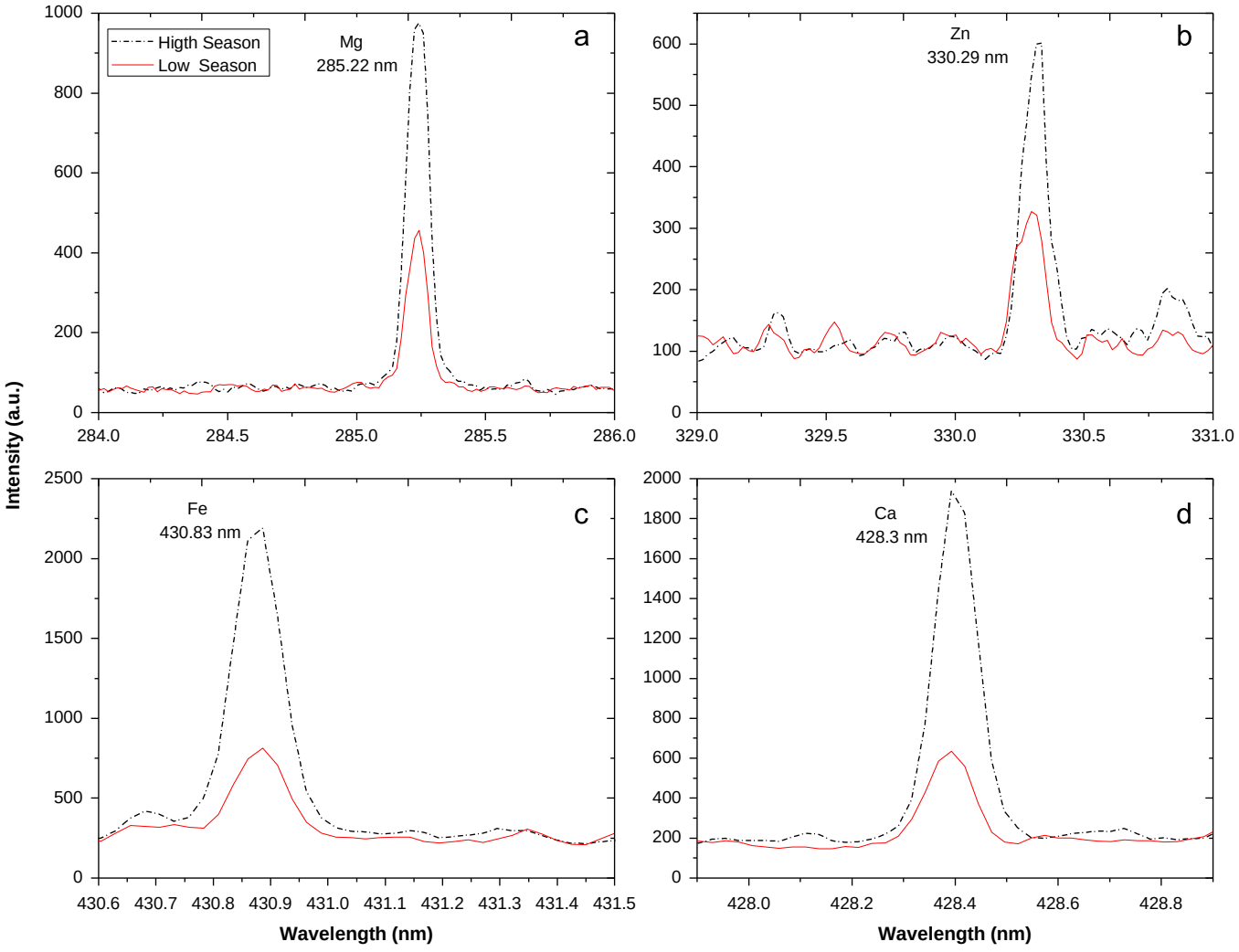

Fig. 3. Mg, Zn, Fe and Ca spectral emission lines in the LIBS spectra in high and low seasons.

In this study it has been found that seasonal variations are strongly affecting trace elements values in buffalo seminal plasma. It is well known that the reproductive efficiency in rams is influenced by season in temperate zones of the northern hemisphere, affecting, among other features, quantitative and qualitative sperm production and the fertilizing ability of cryopreserved spermatozoa [27]. Smith et al. [28] described the seasonal changes in ram semen volume and sperm concentration as well as the seasonal influence on the total protein concentration in ram seminal plasma, with significant differences between the breeding and non-breeding seasons, in both the southern [28] and northern hemispheres [29].

The obtained results show that LIBS technique is advantageous for biological samples since it is a rapid technique, is not time consuming, and does not require sample preparation. In addition, with the present availability of portable LIBS systems, it is possible to perform *in situ* sperm elemental analysis, i.e., directly in the farm without transportation of the samples to the lab.

3.2. LIF

Semen samples with different sperm counts have been used to study their fluorescence upon exposure to laser light. Fig. 5 shows typical LIF spectra of samples of different sperm count. The fluorescence emission intensity has been found to correlate with the sperm count of the individual samples, high sperm count (500×10^6) has higher fluorescence intensity value than the low sperm count (12×10^6) as shown in Fig. 5.

Concerning the sperm itself, it is well known that it consists of a head, a mid-piece and a tail. ATP is one of the basic components in a sperm cell and is used not only as an energy source but also for protein phosphorylation in cell signaling and as a cofactor regulating protein function [30]. The fact that the ATP can fluoresce upon exposure to a suitable radiation wavelength [31] promoted the idea of using laser induced fluorescence to detect, and consequently indirectly count the sperms in the semen. In mammalian sperm, ATP production supports multiple cellular activities and biochemical events required for successful fertilization to occur, [30]. The fluorescence band obtained in the present measurements extends from 450 to 650 nm peaking at 492 nm upon excited with 405 nm laser light for all samples as shown in Fig. 5. It is well known that the adenosine-phosphate molecules fluoresce in the range 325–500 nm [31]. In the present measurement we have a well resolved fluorescence peak at 492 nm free of interference of any other emission peaks and its intensity is clearly correlated to the sperm count. It has been reported that when excited with 260 nm light ATP has greater fluorescence in the 310–500 nm range than adenosin mono- and di-phosphate (AMP and ADP) [31,32]. However, irradiation of ATP with UV light may result in conformational changes that alter its chemical behavior. Our results demonstrated clearly that the intensity of the observed fluorescence increases gradually to reach its maximum value for highest sperm count which is proportional to the ATP concentration [31]. Fig. 6 depicts the proportionality relation between the ATP luminescence in semen (measured by a luminometer) and the sperm count. This shows that it is feasible to depend on the ATP fluorescence intensity in obtaining the sperm count after building the relevant calibration curve.

LIF method, as any other spectrochemical analytical technique, requires calibration to obtain optimum results for quantitative analysis. For this reason, active calibration is typically employed with the calibration curve developed using a set of known authenticated samples having a composition as close as possible to the unknown samples. In the present case the samples used for constructing the calibration curves are semen samples with a known sperm count. Such known semen samples have been originally counted using haemocytometer method. Plot of the

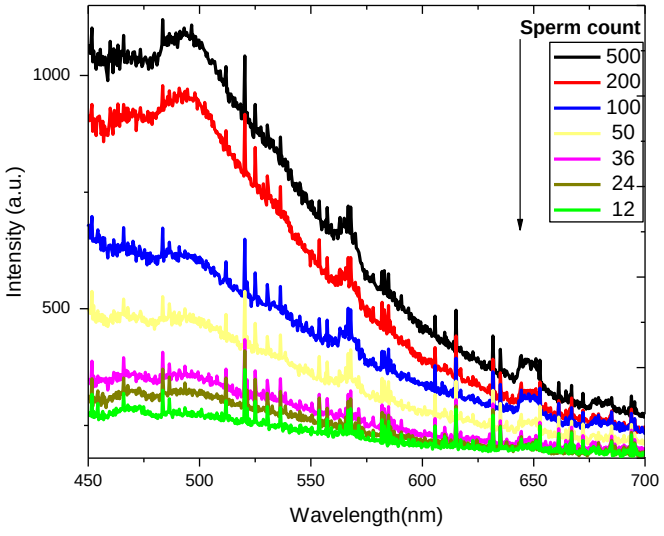

Fig. 5. LIF Spectra of semen samples of different sperm count in millions.

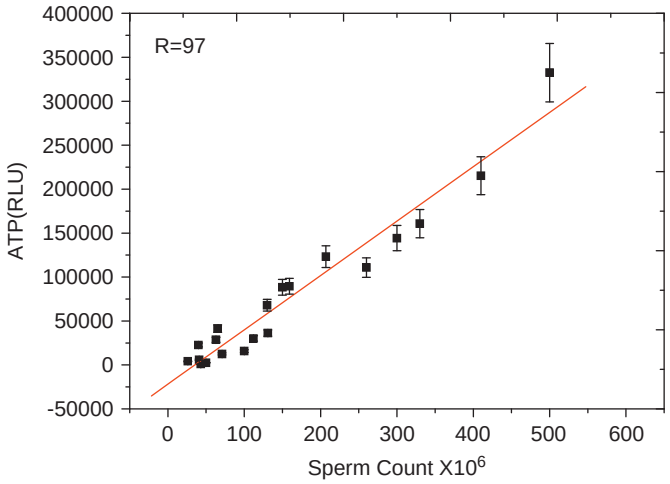

Fig. 6. ATP luminescence in semen versus the sperm count.

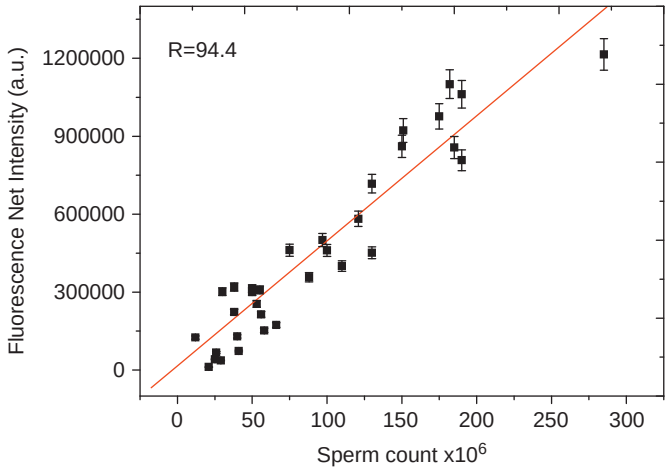

Fig. 7. Calibration curve for net fluorescence Intensity values versus the sperm count.

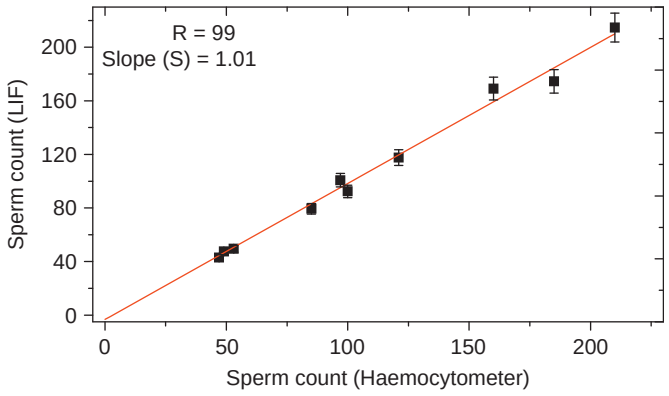

Fig. 8. Comparison between the haemocytometer sperm count and the corresponding values obtained from the LIF net intensity values.

fluorescence net intensity (area under the peak) versus the number of sperms are shown in Fig. 7. The linearity was not that good in case of using the peak intensity instead of the net intensity. The obtained relation can be considered as calibration curve for the count of this kind of buffalo semen. The results obtained depict a linear relationship between the fluorescence intensity response and the sperm count over the entire investigated range of sperm count from 10×10^6 up to 500×10^6 (50 folds). The slope of the calibration curve at a certain concentration is termed the sensitivity and it gives the change in the signal (fluorescence intensity) for a given incremental change in the sperm count. The regression factor (R-value) is given on the calibration curve in Fig. 7.

A comparison between the sperm count obtained conventionally and that obtained from the fluorescence calibration curves is given in Fig. 8. The slope S and the regression factor R of the obtained straight line, 1.01 and 99, respectively, show clearly that the data obtained adopting the net intensity values of the fluorescence band is in better agreement with the conventionally obtained data than that obtained using the peak intensity where S was 2.51 and R was 95. This means that, it is advisable to use the net intensity values and not the peak values in constructing such calibration curves. The obtained results are applicable on same semen species for sperm count in artificial insemination centers, i.e., *in situ*.

4. Conclusion

In conclusion, LIBS, and LIF as spectrochemical analytical techniques have been used in characterization of semen samples. LIBS provided information about the elemental seasonal variation in the seminal plasma. The obtained results demonstrated that buffalo seminal plasma contents of Ca, Mg, Zn and Fe are higher in winter (high season) than in summer (low season). Such elements have direct relation to the sperm parameters and consequently LIBS can be used to asses these parameters indirectly. Laser induced Fluorescence that is normally used for detection of selective species and studying structure of molecules has been exploited in the present work to estimate sperm count in semen. The results indicated that sperm count can be correlated to the intensity of the fluorescence emission and can provide the basis for instrumentation to rapidly determine sperm counts without the need for using conventional microscopic and/or imaging techniques. Future work is planned to study semen of other species adopting both spectrochemical analytical techniques. In addition design of compact and portable LIBS and LIF systems will be beneficial in performing all measurements in artificial insemination centers. Compared with other previously published works in the same filed, this work presents a successful exploitation of two spectroscopic techniques, namely LIBS and LIF, as noninvasive, nondestructive, and fast semen analysis method.

Acknowledgements

Samples used in this work have been obtained from the laboratories of Artificial Insemination, Faculty of Agriculture, Cairo University. We are grateful to Dr. S.H.M. Dessouki for providing the samples and Dr. S.A.M. Abdel-Salam for helping in the statistical analysis.

References

[1] V. Singh, A. Rai., Lasers Med. Sci. 26 (2011) 673–683.
[2] S.J. Rehse, H. Salimnia, A.W Miziolek, J. Med. Eng. Technol. 36 (2012) 77–89.
[3] A. El-Hussein, A.K. Kassem, H. Ismail, M.A. Harith, Talanta 82 (2010) 495–501.
[4] M.A. Kasem, R.E. Russo, M.A. Harith, J. Anal. At. Spectrom. 26 (2011) 1733–1739.
[5] A. El-hussien, H.Ismail, A.K. Kassem, M.A. Harith, The 7th International Conference on Laser Applications ICLA 2009, American Institute of Physics Proceedings 1172. Cairo, Egypt, 17–21 May 2009.
[6] A. Smuk, E. Lazaro, L. Olson, N.M. Lawandy, Opt. Commun. 284 (2011) 1257–1258.
[7] J. Zemanova, N. Lukac, P. Massanyi, J. Trandzik, M. Burocziova, P. Nad, M. Capcarova, M. Stawarz, R. Toman., B. Korenekova, D. Jakabova, J. Vet. Med. A 54 (2007) 281–286.
[8] M.D. Adamson, S.J. Rehse, Appl. Opt. 46 (2007) 5844–5852.
[9] B. Charfi, M.A Harith, Spectrochim. Acta, Part B 57 (2002) 1141–1153.
[10] F.G. Fortes, T. Ctvrtnícková, M.P. Mateo, L.M. Cabalín, G. Nicolas, J.J. Laserna, Anal. Chim. Acta 638 (2010) 52–57.
[11] Z.A. Abdel-Salam, Z. Nanjing, D. Anglos, M.A. Harith, Appl. Phys. B 84 (2009) 141–147.
[12] N.H. Zheng, F.Y.Yueh, S. Burgess, J.P. Singh, Technical Digest. (Optical Society of America, (2006) paper TuE7.
[13] C. Tameze, R. Vincelette, N. Melikechi, V. Zeljkovic, E. Izquierdo, 8th International Workshop on Image Analysis for Multimedia Interactive Services (2008). IEEE, Piscataway, NJ, 300.
[14] V.K Singh, A.K Rai, P.K Rai, P.K Jindal, Lasers Med. Sci. 24 (2009) 749–759.
[15] W.Y. Wonga, G. Flikc, M.W. Groenena, D.W. Swinkels, C.M.G. Thomase, H.J. Jenny, J.H.J. Copius-Peereboom, H.M.W. Merkus, R.P.M. Steegers–Theunissena, Reprod. Toxicol. 15 (2001) 131–136.
[16] B.L. Vallee, K.H. Falchuk., Physiol. Rev. 73 (1993) 79–118.
[17] S.M. Alavi, P. Kozak, A. Hatef, J. Hamackova, O. Linhart, Theriogenology 74 (2010) 317–325.
[18] J. Chun Lu, C. Fang, Hui Ru Xu., H.Yu Feng, Nian-Qing Lu, Labmedicine (2007) 38.
[19] M. Corsi, G. Cristoforetti, V. Palleschi, A. Salvetti, E. Tognoni, Eur. Phys.J. D 13 (2001) 373–377.
[20] J. Strzezek, F. Saiz-Cidoncha, P. Wysocki, A. Tyszkiewicz, M. Jastrzebski, J. Anim. Sci. 20 (2002) 255.
[21] K.P Skandhan, Rev. Fr. Gynecol. Obstet. 87 (1992) 594–598.
[22] S.M. Alavi, R.S Asri, M.H. Kh.Ansari, A. Khaki, Pakistan J. Biol. Sci. 12 (2009) 139–143.
[23] A. Carreras, C. Mendosa, Andrologia 22 (1990) 279–283.
[24] C.Y. Hong, B.N. Chiang, P. Turner, Lancet 2 (1984) 1449–1451.
[25] S.D. Prien, C.D. Lox, R.H. Messer, F.D. DeLeon, Fertil. Steril. 45 (1990) 171.
[26] F.R. Abou-Shakra, N.I Ward, D.M. Everard, Fertil. Steril. 52 (1989) 307–310.
[27] R. Pérez, A. López, A. Castrillejo, A. Bielli, D. Laborde, T. Gastel, Acta Vet. Scand. 38 (1997) 109–117.
[28] J.F. Smith, J. Parr, G.R.M. Murray, R.M. Mcdonald, R.S.F Lee, Anim. Reprod. Sci. 59 (1999) 223–225.
[29] R. Pérez-Pé, B. Barrios, T. Muinõ-Blanco, J.A. Cebrián-Pérez, J. Chromatogr. B (2001) 113–132.
[30] K. Miki, Soc. Reprod. Fertil. Suppl. 65 (2007) 309–325.
[31] A. Amat, R. Joseapa, W. Ronald, K. Ilko, T. Josep., A. Juanita, J. Photochem. Photobiol., B 81 (2005) 26–32.
[32] H. Moll, G. Geipel, G. Bernhard, Inorg. Chim. Acta 358 (2005) 2275–2282.