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Short communication

Assessment of sheep colostrum *via* laser induced fluorescence and chemometrics

Z. Abdel-Salam[a], S.A.M. Abdel-Salam[b], I.I. Abdel-Mageed[b], M.A. Harith[a,*]

[a] National Institute of Laser Enhanced Science (NILES), Cairo University, Egypt
[b] Faculty of Agriculture, Department of Animal Production, Cairo University, Egypt

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ABSTRACT

In the present work sheep colostrum and milk at different milking times postpartum were qualitatively evaluated using laser-induced fluorescence (LIF). First and second milking samples showed higher fluorescence intensity compared with the subsequent milking samples. Such increase in the fluorescence intensity in first and second milking samples can be interpreted in view of the presence of high levels of immunoglobulins (IgG) and lactoferrin in the colostrum. The LIF results have been confirmed by the quantitative evaluation of the IgG in the same samples adopting the enzyme-linked immunosorbent assay (ELISA) as validation technique and a very good agreement has been obtained. Principal component analysis (PCA) of the obtained data led to the conclusion that the fluorescence band from 475 nm to 560 nm is strongly connected to changes during milking time from colostrum to milk. The fluorescence band is linked to changes in the concentration of proteins (IgG, lactoferrin) from colostrum to milk. According to the presented results, LIF spectroscopy can be used as a reliable, accurate, and fast method for real time and *in situ* evaluation of sheep colostrum. LIF coupled with the chemometric analysis of the data can be utilized in designing feeding strategies for newly born lambs.

1. Introduction

In mammals, colostrum is secreted from the mammary glands directly in the very early days postpartum. Especially high levels of immunoglobulins in the first milking have been reported by many researchers (Perez et al., 1990; Levieux and Ollier, 1999; Mainer et al., 2000). A sharp drop in the IgG concentration in the colostrum takes place in few days after parturition (Mainer et al., 2000; Levieux et al., 2002). It is well known that in ruminants, the placenta prevents the passage of antibodies from the mother to the fetus during pregnancy. Therefore, providing the newly born lambs with colostrum is essential in the early 12 h after birth till they gain the needed active immunity. In fact, ruminant embryo is capable to produce protecting antibodies to bacteria and viruses which penetrated through the placenta, at the third term of pregnancy. In other words, the immune system of the newborn is quite well developed, but being protected by the placenta, it is not immunologically challenged. Passive IgG absorption in lambs, takes place in the first 24 h after birth, hence after 12 h the absorption is negligible and has no practical value. After this short period, transmission of immunoglobulin through intestinal epithelium in the digestive system of the newly born animal is not possible (Dominguez et al., 2001). IgG levels can be taken as indicator of the milk status compared to the colostrum (Levieux and Ollier, 1999; Mata et al., 2001; Conesa et al., 2005; Raynal-Ljutovac et al., 2005).

Measurement of IgG levels can be performed by various techniques, namely radial immunodiffusion (Fleenor and Stott, 1980), nephelometry (Collin et al., 2002) or enzyme-linked immunosorbent assay (ELISA) (Kummer et al., 1992). Many researches have been performed on the colostrum of cows and goats where high IgG levels were measured in the first milking (30–200 mg/mL in cows and 48 mg/mL in goats) (Brian et al., 2016; Levieux et al., 2002). However, no thorough investigations have been performed on ewe's milk.

It should be taken into consideration that the above mentioned conventional methods for determination of IgG are expensive and time consuming. In addition, such techniques are not suitable for real time and *in situ* analysis. This shows that, it is of great importance to develop another method to evaluate IgG in colostrum avoiding the above mentioned drawbacks of the conventional methods.

Laser Induced Fluorescence (LIF) is a well-known highly sensitive spectrochemical analytical technique widely used in molecular analysis. This analytical technique is characterized by being completely nondestructive, noninvasive, needs no or minimal sample preparation, fast and cost effective. This makes LIF most suitable for the analysis of biological samples, including milk and colostrum.

* Corresponding author.
E-mail address: mharithm@niles.edu.eg (M.A. Harith).

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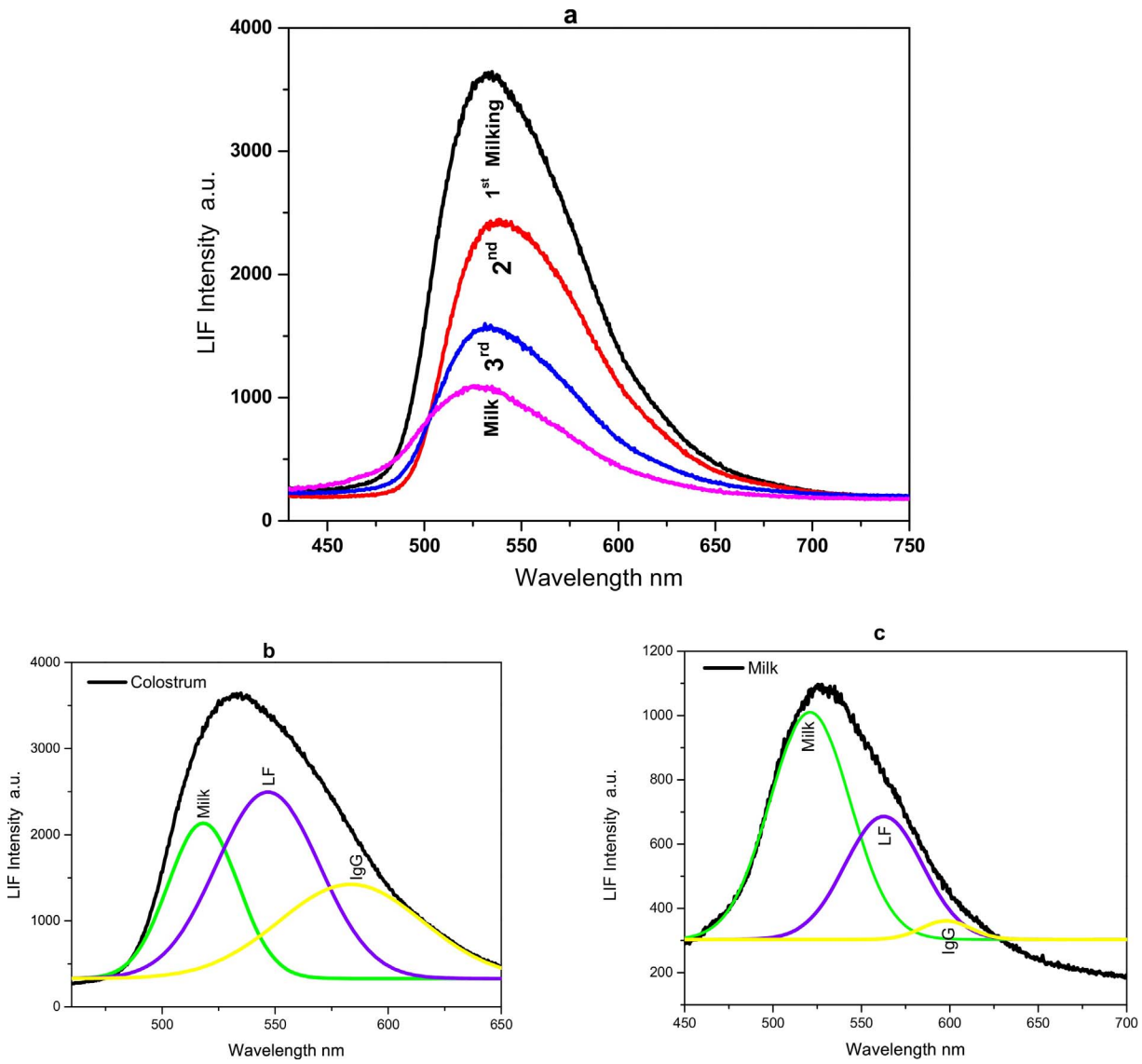

Fig. 1. Typical LIF spectra of the first three milking compared with that of mature milk sample (a). Deconvolution of the fluorescence peak shows the contribution of the IgG and Lactoferrin fluorescence with respect to the normal milk contribution in colostrum (b) and in milk (c).

In fact, LIF applications in biology are not new, and it has been reported previously in the literature by many researchers (Mu et al., 2016; Yang et al., 2017). Simplicity of running the compact equipment of LIF setup makes it suitable to be used for real time and *in situ* measurements in biological applications. This can be performed for example, in animal production centers and dairy farms. The technique has been used in diagnosis of animals' diseases (Abdel-Salam et al., 2015), sperm count (Abdel-Salam and Harith, 2012), evaluation of colostrum in milk (Abdel-Salam et al., 2014), follow up of spoilage of white meat (Abdel-Salam et al., 2017) and numerous other applications.

The aim of the present work was to explore the potential of LIF as an accurate and fast technique to evaluate proteins in sheep colostrum (especially immunoglobulin and lactoferrin) at different milking times. This is very important for setting feeding programs for newly born lambs and for the successful transfer of passive immunity from the ewe to the lamb.

2. Material and methods

2.1. Collection of sheep colostrum

Samples were obtained from Barki ewes in the sheep farm located in agricultural research and experimental station, Faculty of Agriculture, Cairo University. Colostrum milk samples were collected from each of 30 ewes, from the first three milking postpartum. The three milking times were 12 h apart, *i.e.* samples have been collected 0, 12 and 24 h after lambing. The colostrum samples were immediately frozen and stored at −20 °C. A fourth sample representing the ewes' milk has been obtained from milking one week after lambing from each ewe.

2.2. Determination of total IgG and total protein

IgGs in sheep's colostrum and milk were analyzed using enzyme-linked immunosorbent assay (ELISA- Biotek ELX808, USA) with Sheep IgG ELISA Kit, E-35G (ICL Inc. OR, USA) that allows determining the concentration of specific IgG in sheep's milk. Quantitative determination of total IgG in colostrum and milk was performed using the ELISA kit according to the manufactures' instructions. IgG concentration of each sample was determined from the calibration curve of the absorbance values obtained for the standards.

Total protein content in all samples under investigation was determined by routine laboratory procedures using an automated infrared milk analyser (MilkoScan FT1, FOSS, Demark) at the central laboratory of Faculty of Agriculture, Cairo University.

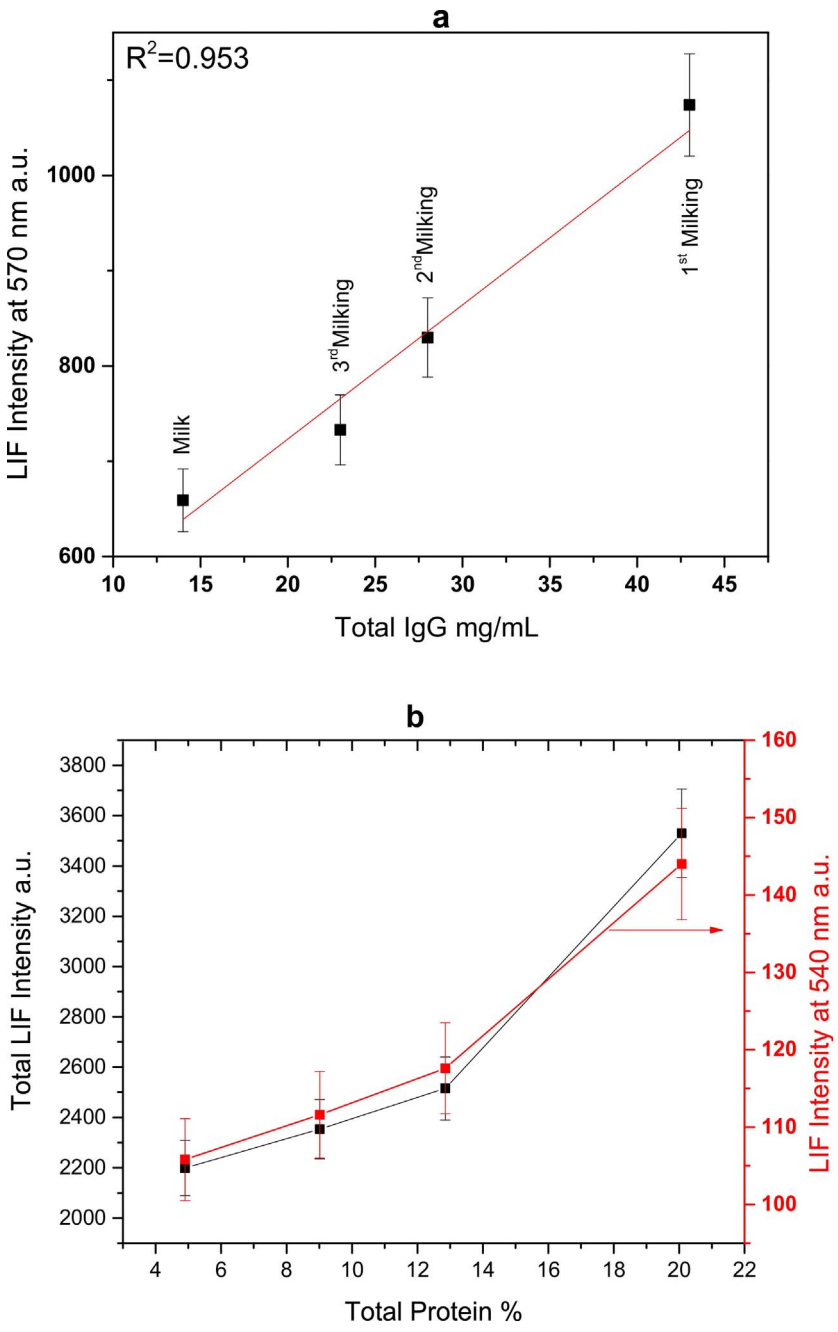

Fig. 2. The relation between the total IgG mg/mL (measured) versus the LIF peak intensity of IgG at 570 nm in sheep colostrum and milk samples (a). Plot of the total protein content (%) versus the sum of LIF intensity (area under the whole fluorescence band) and the fluorescence intensity of the lactoferrin peak at 540 nm (b).

2.3. Confocal laser scanning microscopy

A loop full of each sample was applied to the surface of a wipe-clean glass slide then covered with a slide cover. To visualize the fluorescence emission of the cells, a Confocal Laser Scanning Microscope (CLSM) (LSM 710, Carl Zeiss, Jena, Germany) has been utilized with software version ZEN 2009 for image analysis. The samples were excited *via* diode laser at $\lambda_{ex.} = 405$ nm. A 418–718 nm band-pass filter was used for scanning the emissions.

2.4. LIF arrangement

In LIF, laser light excites the atoms or the molecules which send out radiation when they undergo de-excitation. Emission spectrum of the fluorescence can be observed at several wavelengths as a result of the population of many different excited states. Because both the wavelength that excites the molecule and the one that is observed could be chosen, this makes an excellent choice to study atoms and smaller molecules. The excited species will, after some time usually in the order of few nanoseconds to microseconds, de-excite and emit light at a characteristic wavelength longer than the excitation wavelength. Such de-excitation emitted light can be measured for the identification of the species under investigation and for other different tasks.

The experimental setup of the LIF equipment used throughout the present work is described in details elsewhere (Abdel-Salam et al., 2014) The used laser system was a CW-DPSS laser [Changchun New Industries Optoelectronics Tech Co., Ltd. (China)] of 40 mW power at $\lambda = 405$ nm. The focused laser beam is directed *via* an optical fibre to one side of the quartz cuvette containing 1 mL colostrum or milk sample. The fluorescent light is collected perpendicularly *via* another optical fibre coupled to the spectrometer (USB2000 FLG, Ocean Optics, FL, USA). Acquisition and further processing and analysis of the obtained spectra are accomplished using the commercial SpectraSuit software (Ocean Optics, FL, USA). Final processing of the data for

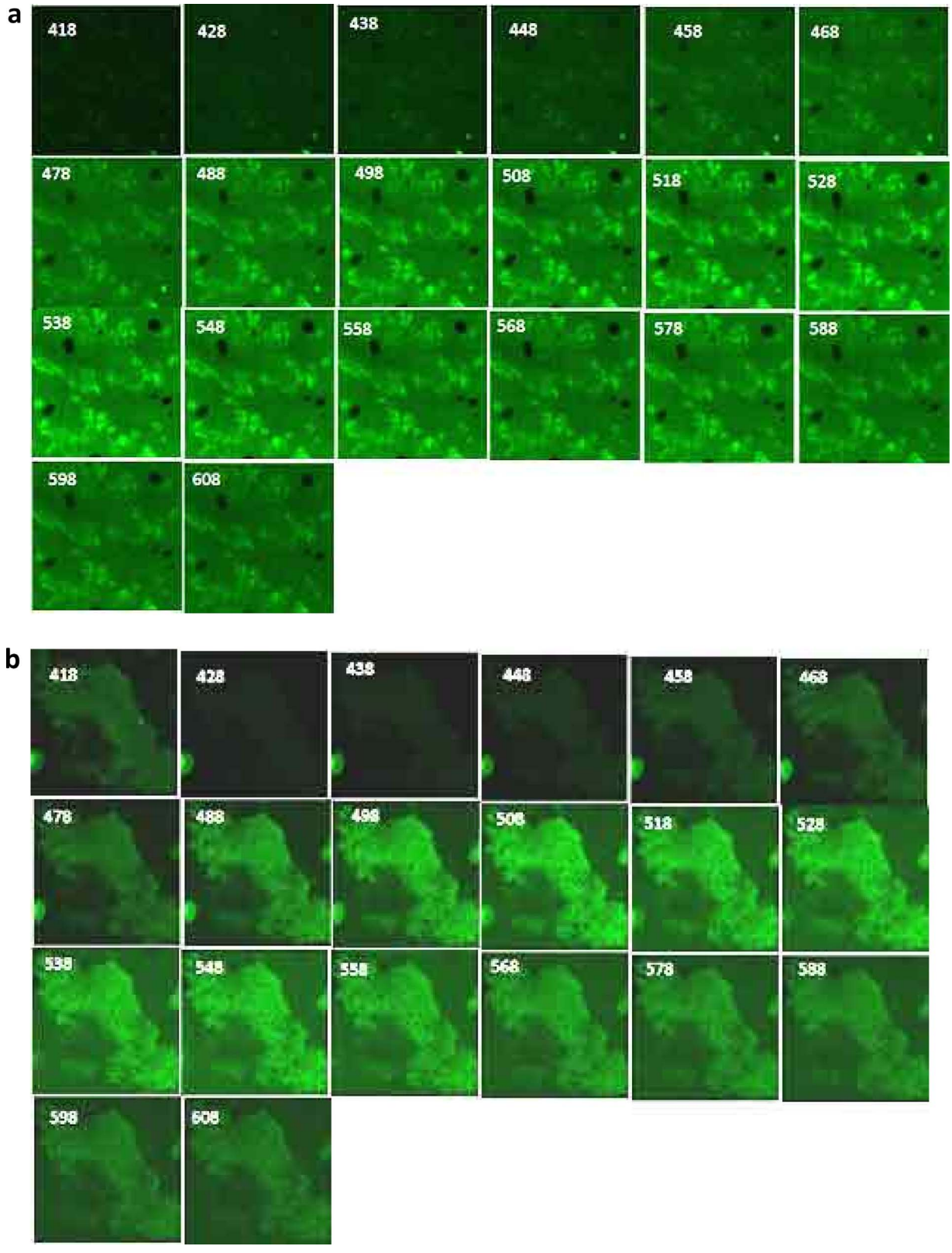

Fig. 3. Spectral images acquired in a sequential band width of 10 nm, representing sheep colostrum fluorescence at first milking (a) & mature milk fluorescence in day 7 postpartum (b).

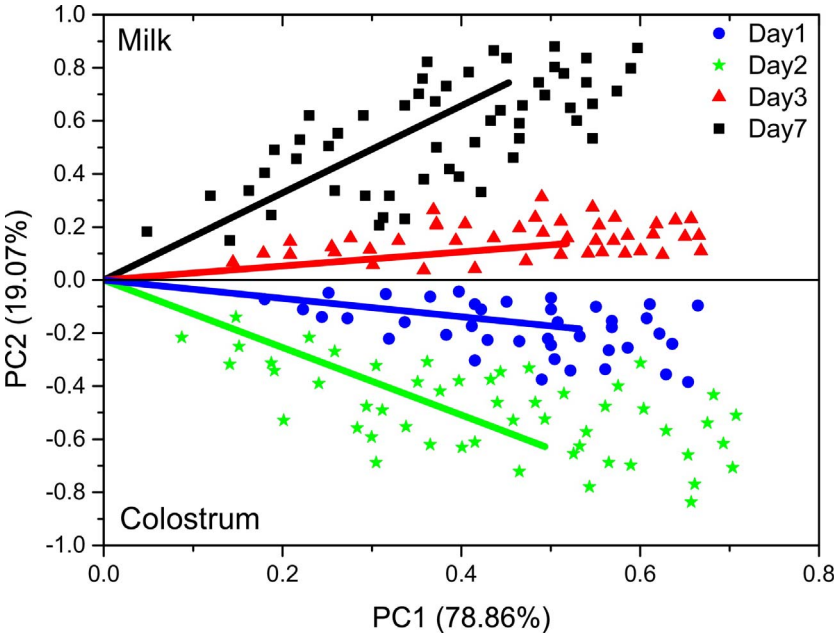

Fig. 4. PCA based comparison of spectral data obtained from colostrum (1st milking, 2nd milking and 3rd milking) and milk (day 7 postpartum) of sheep colostrum and milk.

presentation is performed with the Origin 8.5 software (Origin Lab. Corp., MA, USA).

2.5. Statistical analysis

Principal Component Analysis (PCA) is an efficient statistical multivariate analysis method. In PCA the dimensionality of the spectra is reduced to extract the most crucial spectral feature variables through correlating the input data. The resulted new variables, normally called principal components (PC), are calculated as linear combinations of the original variables.

In the present work the measured data are analyzed statistically *via* PCA using the commercial software (Origin Lab 9). The principal component analysis (PCA) is employed to examine the variations in the LIF spectral data from colostrum to milk in the fluorescence band from 475 nm to 560 nm during milking times.

3. Results and discussion

Fig. 1a shows typical LIF spectra of sheep colostrum and milk samples at different milking times. A pronounced decrease in the fluorescence intensity of the samples is depicted with the increase of the milking number. Samples excited *via* laser light of wavelength 405 nm have fluorescence emission extending from 475 nm up to 650 nm and peaking at 530 nm. The increase in the fluorescence intensity with first and second milking can be interpreted in view of the presence of high levels of immunoglobulins (IgG) and lactoferrin (LF) in the colostrum samples. Previous measurements on bovine colostrum (first milking postpartum) (Abdel-Salam et al., 2014) demonstrated that IgG has a fluorescence peak at 570 nm. Carmona et al. (2014) reported that lactoferrin fluorescence spectrum is peaking at 540 nm. This means that the total fluorescence peak covers three overlapping peaks at 530 nm (milk), 540 nm (lactoferrin) and 570 nm (IgG). The deconvolution of the fluorescence peak (Fig. 1b and c), results in the separation of these three emission peaks. Fig. 1b depicts the three deconvoluted fluorescence peaks of colostrum (1st milking), while the three peaks in case of milk (7th day postpartum) are shown in Fig. 1c. The peaks depicted in Fig. 1c demonstrate the strong decrease in the intensity of the peaks corresponding to lactoferrin and IgG in case of mature milk. This is of course reasonable and also expected in view of the change in composition from colostrum to milk where the first loses lots of its components of lactoferrin and IgG.

In order to confirm the above obtained results, the same samples subjected to the LIF measurements have been used to evaluate their quantitative contents of IgG *via* the well-known classical ELISA technique. The linearity between the IgG concentration and the corresponding laser induced fluorescence peak at 570 nm (R^2 = 0.953) validates the above obtained results (see Fig. 2a). This plot shows the clear proportionality between the IgG fluorescence intensity and the IgG concentration.

El-Hommosi et al. (1998) and Abd-El-Gawad et al. (1996) reported that the colostrum is rich in proteins, (immunoglobulins, lactoferrin). Fig. 2b shows the proportionality between the fluorescence peak intensity of lactoferrin at 540 nm and the sum of the fluorescence band (area under the peak) of the colostrum and milk samples versus the total protein % content. This plot demonstrates that the sum of the fluorescence intensity and total protein have the same trend with respect to the total protein content. It should be mentioned that colostrum contains high levels of lactoferrin which has inhibition effects on bacteria (Baker and Baker, 2009). Lactoferrin is in fact strongly relevant to the immune system as a result of its anti-pathogens effects (Carmona et al., 2014).

Interesting results have been obtained from the examination of samples of first milking after lambing and mature milk samples (obtained seven days after lambing) *via* the CLSM fluorescence, as shown in Fig. 3 a and b respectively. As has been already mentioned, the fluorescence emission band covers the range from (475 nm) up to (650 nm). Fig. 3a depicts that the highest fluorescence intensity shows up in the wavelength region 478–578 nm covering the three distinct peaks of milk (530 nm), lactoferrin (540 nm) and IgG (570 nm) appearing in in Fig. 1b. In case of mature milk samples, IgG and lactoferrin exhibit much lower fluorescence emission intensity due to the drop in their concentrations in milk (Fig. 1c). Obviously, this can be compared with CLMS photos presented in Fig. 3b for milk samples. In general, the confocal scanning laser microscope fluorescence measurements confirmed clearly the results obtained *via* the laser induced fluorescence on the same samples.

The multivariate statistical method of PCA has been applied to obtain more detailed information about the LIF spectral changes. A plot of the LIF data for the first two principal components (PCA) is displayed in Fig. 4. PCA was then performed using wavelength range from 475 to 650 nm, to compare the spectra obtained from sheep colostrum and

milk samples at different milking times (1st, 2nd and 3rd milking) and the milk obtained on day 7 postpartum. The observation diagram shown in Fig. 4 explains the total variance as defined by discriminant factors PC1 and PC2. As shown in the figure, there is a good discrimination with 78.86% rate between the first and second milking samples as indicators of colostrum and the milk.

Hereby, the discrimination is not only due to the above mentioned increased fluorescence intensity. In fact, conformational changes in the protein content (IgG and Lactoferrin) and the sequential losses that take place contribute also to the PCA classification. Therefore, the specific information on the molecular level provided by LIF spectroscopy is necessary to allow for a discrimination of sheep colostrum and milk samples.

4. Conclusion

In the present work the fluorescence band in colostrum and milk LIF spectra is linked to changes in the concentration of proteins (IgG, lactoferrin) from colostrum to milk. The increase in the fluorescence intensity in first and second milking samples has been interpreted in view of the presence of high levels of immunoglobulins (IgG) and lactoferrin, and generally total proteins content in colostrum. Mature milk loses lots of its IgG and lactoferrin contents, and hence the fluorescence intensity of such components in the relevant LIF spectra suffers from a pronounced decrease. The principal component analysis (PCA) has been used for the statistical analysis of the obtained data and led to the conclusion that the fluorescence band from 475 nm to 560 nm is strongly related to changes during milking time from colostrum to milk.

The obtained results demonstrate the potential of LIF as an easy, simple and fast analytical technique for field measurements contrary to the conventional analytical techniques used not only for the analysis of sheep colostrum and milk but also all types of ruminants' colostrum and milk.

Conflict of interest

All authors declare that they have no conflict of interest.

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