

## Accepted Manuscript

Qualitative and quantitative analysis of milk for the detection of adulteration by Laser Induced Breakdown Spectroscopy (LIBS)

S. Moncayo, S. Manzoor, J.D. Rosales, J. Anzano, J.O. Caceres

PII: S0308-8146(17)30564-2  
DOI: <http://dx.doi.org/10.1016/j.foodchem.2017.04.017>  
Reference: FOCH 20887

To appear in: *Food Chemistry*

Received Date: 31 October 2016  
Revised Date: 7 February 2017  
Accepted Date: 2 April 2017

Please cite this article as: Moncayo, S., Manzoor, S., Rosales, J.D., Anzano, J., Caceres, J.O., Qualitative and quantitative analysis of milk for the detection of adulteration by Laser Induced Breakdown Spectroscopy (LIBS), *Food Chemistry* (2017), doi: <http://dx.doi.org/10.1016/j.foodchem.2017.04.017>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



## Qualitative and quantitative analysis of milk for the detection of adulteration by Laser Induced Breakdown Spectroscopy (LIBS)

S. Moncayo<sup>1</sup>, S. Manzoor<sup>1</sup>, J. D. Rosales<sup>1</sup>, J. Anzano<sup>2</sup> and J.O. Caceres<sup>1\*</sup>

*1 Laser Chemistry lab, Department of Analytical Chemistry, Faculty of Chemical Sciences, Complutense University, 28040 Madrid, Spain*

*2 Laser Laboratory and Environment, Department of Analytical Chemistry, Faculty of Sciences, University of Zaragoza, Pedro Cerbuna 12, 50009, Zaragoza, Spain*

### ABSTRACT

The present work focuses on the development of a fast and cost effective method based on Laser Induced Breakdown Spectroscopy (LIBS) to the quality control, traceability and detection of adulteration in milk. Two adulteration cases have been studied; a qualitative analysis for the discrimination between different milk blends and quantification of melamine in adulterated toddler milk powder. Principal Component Analysis (PCA) and Neural Networks (NN) have been used to analyze LIBS spectra obtaining a correct classification rate of 98% with a 100% of robustness. For the quantification of melamine, two methodologies have been developed; univariate analysis using CN emission band and multivariate calibration NN model obtaining correlation coefficient ( $R^2$ ) values of 0.982 and 0.999 respectively. The results of the use of LIBS technique coupled with chemometric analysis are discussed in terms of its potential use in the food industry to perform the quality control of this dairy product.

Keywords: Laser Induced Breakdown Spectroscopy; Milk Adulteration; Melamine; Neural Networks

\*Corresponding author. Tel.: +34 91 3944322; fax: +34 91 3944329

E-mail address: jcaceres@ucm.es (J.O. Caceres).

## 1. Introduction

Milk due to its nutritional importance is among the most consumed food, containing the main ingredients to sustain life, especially in early stages such as childhood, where it represents the principal intake. In 2016, the world milk production reached 816 million tons per year where the European states have taken the lead among the major milk exporters with 19.2 million tons (Griffin, 2016). Consequently, there is a great economic importance of milk products and therefore, milk is one of the main targets of adulteration. The adulteration consists of the addition of any substance to the natural milk, which changes its composition and may occur in many different forms. The most common, in order of importance, is adding water to raw milk, mixing milk of different animal species, fat replacement and modification of the protein content by introducing an adulterant as melamine ( $C_3H_6N_6$ ). The addition of water has only economic and nutritional effects, and does not produce any health hazard. However, the mixture of milk from different animal origin can produce adverse allergic reaction and the addition of melamine to increase the protein content causes serious health problems such as kidney disorder and even death of the consumer (Pei, Tandon, Alldrick, Giorgi, Huang, & Yang, 2011). Thus, milk quality control benefits both consumer and dairy industry from the nutritional, economic and legal point of view. According to European laws, the origin of milk must be stated and labeled for its consumption or production of other dairy products (EU-Comm, 2001). The main quality control procedure of milk and its derivatives include organoleptic analysis, such as appearance, taste and smell, compositional analysis (fats, solids and protein content), physical and chemical assays together with the analysis of adulterant and fraud detection. The principal analytical methods to carry out the control of adulteration are based on chromatographic techniques such as reversed phase high performance liquid chromatography (HPLC) (Tay, Fang, Chia, & Li, 2013), gas chromatography coupled to mass

spectrometry (GC-MS), (Rebecchi, Vélez, Vaira, & Perotti, 2016), mid-infrared microspectroscopy (MIR- microspectroscopy) (Santos, Pereira-Filho, & Rodriguez-Saona, 2013), nuclear magnetic resonance (NMR), matrix assisted laser desorption/ionization time of flight (MALDI-TOF) (Garcia, Sanvido, Saraiva, Zacca, Cosso, & Eberlin, 2012) and Raman spectroscopy (Nieuwoudt, Holroyd, McGoverin, Simpson, & Williams, 2016). These techniques produce accurate results, however, the large amount of sample needed for analysis, the use of complex and voluminous equipment, time delays in providing the results and expensive consumables required, increase the economic and temporal cost of the analysis. Moreover, the milk adulteration with melamine is not easily identified in routine analysis because the non-protein nitrogen cannot be detected by usual procedures for protein determination (Cremers & Radziemski, 2013; Gottfried, Frank C, & Miziolek, 2009; Grégoire, Motto-Ros, Ma, Lei, Wang, Pelascini, et al., 2012). This leads to the need of developing methods that are simple, sensitive and reliable to perform such analyses with the possibility to be employed for an online analysis. LIBS technique has been a subject of research for the past few decades due to the advantages, it offers becoming a powerful analytical tool in different fields of application. LIBS analyses a sample by direct measurement of the emission of plasma generated by ablation of the sample, providing an immediate spectral fingerprint that is representative of its elemental composition (Cremers & Radziemski, 2013). Although there is a need in the food industry to improve quality and process controls, the application of LIBS technique in food control analysis has not been sufficiently explored and only few studies can be found in literature (Bilge, Boyacı, Eseller, Tamer, & Çakır, 2015; Bilge, Sezer, Eseller, Berberoglu, Topcu, & Boyaci, 2016; Bilge, Velioglu, Sezer, Eseller, & Boyaci, 2016; Mbesse Kongbonga, Ghalila, Onana, & Ben Lakhdar, 2014). The combination of LIBS with chemometric methods offers the possibility to be used in a fast, automatic and on-line manner as has already been demonstrated with

successful results for sample classification and quantification (Caceres, Moncayo, Rosales, de Villena, Alvira, & Bilmes, 2013; Curteanu & Cartwright, 2011; Huang, Kangas, & Rasco, 2007; Moncayo, Manzoor, Navarro-Villoslada, & Caceres, 2015; Moncayo, Rosales, Izquierdo-Hornillos, Anzano, & Caceres, 2016; Torrecilla, Cámara, Fernández-Ruiz, Piera, & Caceres, 2008). Some authors have shown that the molecular LIBS signal is dependent on the molecular nature of the sample (Anzano, Casanova, Bermúdez, & Lasheras, 2006; Gregoire, Boudinet, Pelascini, Surma, Detalle, & Holl, 2011). Although there is a loss of molecular information in plasma, the intensities of C, H, N, and O lines and the C/H, C/O, and C/N intensity ratios have provided excellent results for the identification of many samples of organic nature (Lasheras, Bello-Gálvez, Rodríguez-Celis, & Anzano, 2011; Lucena, Doña, Tobaría, & Laserna, 2011; Marcos-Martinez, Ayala, Izquierdo-Hornillos, de Villena, & Caceres, 2011). A wide study on the formation of CN fragments present in the plasma and its correlation with the carbon content of the sample is shown by Baudelet et al. (Baudelet, Boueri, Yu, Mao, Piscitelli, Mao, et al., 2007). In the case of the milk analysis by LIBS, G. Bilge et al. (Bilge, Sezer, Eseller, Berberoglu, Topcu, & Boyaci, 2016) demonstrate that LIBS in combination with multivariate methods can be successfully used to discriminate between skimmed milk powders and whey powders, and to quantitatively determine the adulteration ratio of skimmed milk powder with sweet/acid whey powder.

In this work, Laser Induced Breakdown Spectroscopy (LIBS) technique has been evaluated for the study of two different cases of adulteration practices in milk. As in the case of animal milk, the adulteration is done by mixing liquid animal milk of different origin and in powdered toddler's milk by the addition of melamine, two different methodologies have been developed based on LIBS and NN combination to deal with such cases. Therefore, the aim of this study was improving the recognition capacity of extremely similar samples that have negligible physical and spectral differences between them. Firstly, a methodology combining

LIBS and NN has been developed to detect the adulteration by analyzing the mixtures of milk from different animal species (cow, goat and sheep) in different proportions. Secondly, the quantification of melamine in toddler milk powder samples was performed, particularly observing the CN band emission correlating it to adulteration ratios. Conventional univariate calibration curve and a multivariate NN quantification procedure have been compared.

## 2. Materials and Methods

### 2.1 Milk samples

For the qualitative analysis, two type of samples were analyzed. Firstly, ten commercial pure semi-skimmed samples; four different commercial brands of cow milk, four of goat milk and two of sheep milk all purchased at local markets from Spain. The information about the studied samples including sample ID and respective commercial brand is shown in Table 1. Secondly, twelve milk samples were blended with fixed-volume percentages of different milk used as adulterants. These samples were prepared by mixing the respective pure milk with the percentages ranging from 25 to 75 % v/v of adulterating milks as detailed in Table 2 and agitating continuously using a magnetic stirrer for 15 minutes. For the quantitative analysis, commercial toddler powder milk was used and melamine was obtained from Sigma-Aldrich Company with 99% purity.

<< Table 1 >>

<< Table 2 >>

### 2.2 Sample preparation

For the qualitative analysis, all samples were left to freeze for 24 hours and lyophilized for the next 24 hours, to remove their water content, obtaining powdered sample. For LIBS measurements, 250 mg of milk powder was compressed into compact pellet using a hydraulic press at 10 Ton/cm<sup>2</sup>. The thickness and diameter of the pellets were about 2 mm and 12 mm, respectively. The pellets were directly analyzed without further preparation. In the second adulteration case, quantification of melamine, a blank-value and five different mixtures of 1, 2, 3, 4 and 6 % (w/w) concentrations were prepared. To validate the model three test samples (2, 4 and 5 %) were also produced independently. To prepare each sample, 1 g of commercial toddler milk powder was homogenized in an agate mortar with the corresponding amount of melamine to ensure the uniform distribution and 250 mg of the mixture was taken and compressed to form a pellet as mentioned previously. Three replicates for all samples were prepared to provide statistic and improve the correctness, reproducibility and accuracy of the measurement.

### 2.3 LIBS Set-up

LIBS technique, experimental configuration and conditions used in this study have been previously described (Caceres, Moncayo, Rosales, de Villena, Alvira, & Bilmes, 2013). Herein, the parameters more relevant to the set-up have been given. LIBS measurements were obtained using a Q-switched Nd:YAG laser (Quantel, Brio model) operating at 1064 nm, with a pulse duration of 4 ns full width at half maximum (FWHM), 4 mm beam diameter and 0.6 mrad divergence. Samples were placed over an X–Y–Z manual micro-metric positionator with 7.5 cm of travel at every coordinate. The laser beam was focused on the surface of the pellet with a 100 mm focal-distance lens, producing a spot of 170  $\mu$ m in diameter. The plasma radiation was collected using an optical fiber with 200  $\mu$ m aperture and 2 m long, coupled to a fused silica collimator with a focal distance of 8.7 mm and analyzed with spectrometer

AvaSpec from Avantes, covering the spectral interval between 190 and 450 nm (with spectral resolution of 0.1 nm). The laser pulse energy was fixed to 100 mJ and repetition rate was 1 Hz. LIBS spectra were acquired with a delay of 2.5  $\mu$ s after the laser pulse. The integration time was set to 20 ms (lowest possible).

#### 2.4 LIBS analysis

All samples were measured at room conditions. In the qualitative analysis, for each sample, 100 LIBS spectra were obtained where each spectrum was an average of 5 laser shots at the same position. To expose a fresh portion of the sample surface, the sample was moved by 0.3 mm along a vertical straight line avoiding the areas altered by the previous shots using a micrometer stage. The total acquisition time was less than 10 min/sample taking into account the spectrometer integration time and the laser pulse repetition. In order to reduce spectral variations, all spectra were normalized by using the intensity value of the Ca II emission line at 393.37 nm. Once the normalization was done, all the spectra for each sample were compiled in a data set composed by 3648 rows and 100 columns, intensity value and number of spectra respectively. Only in the case of the C1, G1 and S1 samples, two data sets of 100 spectra were obtained: the first one (training library) was used to calibrate the NN model, and the second data set (replicate library) was used to validate the model (Moncayo, Rosales, Izquierdo-Hornillos, Anzano, & Caceres, 2016). For the quantification analysis of melamine, three pellets for each concentration were prepared, 10 spectra for each pellet were recorded, where each spectrum was the average of five laser shots at a single position. Therefore, for each concentration a total 150 spectra were considered, which is comparable with the qualitative analysis. To build the calibration model six samples at different concentrations were contaminated *ex profeso*. Only on milk toddler brand was used to build and validate the model, however our system is adaptive and more brands can be introduced in further studies.



### 2.5. Wavelength range selection

In order to improve the performance of NN classification model, a reduction in the number of variables was carefully done by selecting only several wavelength ranges encompass useful peaks observed in the spectra. This has previously demonstrated that the variable selection has advantages in providing an increment in the robustness of the models and a higher discrimination capability (Moncayo, Manzoor, Navarro-Villoslada, & Caceres, 2015; Moncayo, Rosales, Izquierdo-Hornillos, Anzano, & Caceres, 2016). Herein, nine wavelength ranges containing the emission lines from C (247.4- 248.4 nm), Mg (278.5 - 281.3 nm), Ca (314.7 - 320.1, 368.6 - 376.1, 389.49 - 401.2, 442.6 - 446.5 nm), Sr (405.5 - 409.0, 420.5 - 423.4 nm) and CN (382.1 - 390.0 nm) were selected, reducing the variables from 3648 to 635.

### 2.6 Data analysis

In this work two chemometric methods were applied, Principal Component Analysis (PCA) and Neural Networks (NN). PCA was employed only as an exploratory data analysis to display the structure of the multivariate data. PCA provides useful plots by representing the first principal components (PCs), linear combination of original variables that retains the maximum amount of spectral variability. Therefore, PCA allows an easy visualization of the distribution of samples, the observation of anomalous spectra (outlier) and the reduction of variables. Although PCA by itself is not a classification method, a previous PCA analysis is always useful to give a general overview of the sampling space improving the analysis. Herein, using PCA provides a fast and simple way to visual and detect such outliers. The introduction of outlier spectra into the classification models go in detrimental of the classification ability of the model and therefore filtering the outliers by PCA is an effective

way to improve analysis. On the other hand, NN analysis has been applied in two independent ways. First for the classification of milk mixtures in a qualitative way and second in a quantitative approach for the quantification of melamine. In both cases, a multilayer perceptron, feedforward, supervised neural network was used, consisting of several neurons (information processing units) arranged in three layers (input, hidden and output) where each receives information from all of the neurons in the previous layer. The connections are controlled by weights able to modulate the output from the neuron before inputting its numerical content into a neuron in the next layer. The process that optimizes the weights, i.e., the training process was based on a backpropagation (BP) algorithm (Bishop, 1996). The main difference between both qualitative and quantitative approach is given by the type of target used (expected output of the NN). The target in the qualitative application is labelled with categorical values (dummy variable) from one to the total number of classes. In the melamine quantification, the target has a chemical meaning corresponding to a concentration and therefore the output value is a continuous number, not restricted to defined values but any infinite number in between. Moreover, in the case of qualitative analysis we used a scaled conjugate gradient backpropagation training function (SCG), whereas in the quantitative analysis we used a Bayesian regularization (BR) training function (MacKay, 1992; Møller, 1993). A detailed description of the calculation process is provided in the literature (Moncayo, Manzoor, Ugidos, Navarro-Villoslada, & Caceres, 2014; Moncayo, Rosales, Izquierdo-Hornillos, Anzano, & Caceres, 2016). In this case, C1, G1, and S1 were selected to train the neural network model. As a part of the training process of the neural network, the dataset was randomly divided into two subsets: 80% in training and 20% in the self-validation of the estimated model. During the training process, C1, G1 and S1 were assigned as 1, 2 and 3 respectively in the NN output layer. PCA and NN models were calculated using home-made software developed in Matlab (Mathworks, 2014b). The “spectral correlation” parameter

(equation 1), defined as the percentage of test spectra correctly classified, was assessed as model accuracy (Moncayo, Rosales, Izquierdo-Hornillos, Anzano, & Caceres, 2016).

$$SC = \frac{100}{N} \sum_{i=1}^N \delta_i \quad (\text{Eq. 1})$$

where  $\delta_i$  is either 1 when a spectrum is classified correctly or 0 otherwise and N is the total number of spectra.

To consider a milk sample correctly classified, the prediction of the model must match with the actual class by an arbitrary threshold of SC higher than 80% and less than 20% to the other classes, otherwise considered incorrectly classified. A milk sample was assigned as unclassified when the SC was less than 80% for all the three type of milks.

A SC higher than 80% is consider as a quality control of our classification system. As far as we know, there is no methodology to estimate the threshold and therefore we consider 80% as suitable value for this kind of classification system. This threshold has already been used in our previous works (Moncayo, Manzoor, Navarro-Villoslada, & Caceres, 2015; Moncayo, Rosales, Izquierdo-Hornillos, Anzano, & Caceres, 2016).

### 3. Results and Discussion

As stated earlier, two separate adulteration tests were performed, one for qualitative milk mixtures detection and the other for quantitative analysis of melamine in adulterated toddler milk powder.

#### 3.1 Qualitative Analysis: Detection of milk mixtures

Ten pure milk samples (four from cow and goat and two from sheep), and twelve mixtures between them as indicated by Table 1 & 2, were analyzed. The spectra were recorded within a range of 190 to 450 nm and a representative spectrum of each milk species is shown in Fig. 1a.

The observed emission lines are identified using the spectroscopic information tabulated in NIST Atomic Spectral Database (US Department of Commerce, 2015). Emission lines from C, Ca, Mg, Sr, and molecular species such as CN and Swan carbon system, among others, are mainly present in the LIBS spectra. A visual comparison between the spectra reveals very similar composition, however, clear differences can be seen in the intensity of Sr emission line. In case of goat milk, an increase in the emission intensity can be observed, which can be attributed mainly to the food consumed and the metabolism of the animals.

Fig. 1b shows the training spectra set (one hundred spectra for each type of milk) plotted according to the values of the PCA scores. It has been represented that the scores of the second PC against the first PC, accumulate 86.02% of the total variance. Each milk type (class) is shown with different color and shape for a better visualization. The confidence ellipses of each class were calculated statistically using SAISIR source code (Cordella & Bertrand, 2014), with  $\alpha = 0.05$ . Although the origin of the milk is different, the discrimination based on PCA is not clear; the LIBS spectra look similar and it is hard to distinguish by linear methods due to overlapping. For this purpose NN classification model was developed, where Fig. 1c shows the output results of the self-validation step of the training process for each type of milk. Although the spectra considered to calibrate the NN are the same as used in PCA model, it is clear to see that NN model, due to its ability to model complex non-linear input target relationships, is able to discriminate between types of milk outperforming PCA.

<< Figure 1 a, b & c >>

It has been demonstrated in several studies that NN outperforms other chemometric methods (Inakollu, Philip, Rai, Yueh, & Singh, 2009; Moncayo, Manzoor, Navarro-Villoslada, & Caceres, 2015; Shaffer, Rose-Pehrsson, & McGill, 1999). However, overfitting is very

common issue for NN and in order to ensure that the NN model is able to generalize, milk samples not included in the training process, were tested. The generalization ability demonstrates the performance of the model to classify new objects taking into account the possible overfitting. In our case, the generalization ability was evaluated testing pure milk samples (C1 - S2). Table 3 shows the classification results obtained. All samples were correctly classified ensuring a non-overfitted model. It is interesting to note that in spite of using only one milk brand for each animal origin for calibrating the NN model, different brands are correctly assigned to their class membership, showing the capacity of the NN model to generalize and obtain a high rate of correct classification (100 %) for all the pure milk samples. Once the NN model was validated, the adulteration test was performed by testing binary blended samples (M1 - M9) and ternary blends of milk samples (M10 - M12). The results of detection of adulteration are also shown in Table 3. Binary adulterated milk samples M1 to M9 were not classified to any class of pure milk used in training and therefore were classified as “unknown” by the NN model, indicating the presence of adulteration. Similar results were obtained in case of ternary mixtures (M10 - M12), where all samples were also correctly classified as unknown. These results were very encouraging, as the adulterated samples were not assigned any class membership. Moreover, irrespective of the type (binary/ternary) and the proportions of mixing, NN model could clearly detect the adulterated milk. It is important to note that none spectra used in the training has been used for any validation purposes. In general, the high spectral correlation demonstrates the high accuracy of the proposed methodology to discriminate and detect blended milk adulteration.

A good quality control system must be able to detect any kind of milk mixture adulteration with a high accuracy but also it has to be able to correctly classify non adulterated sample even when those samples do not belong to the training data set. In here, we demonstrate that the developed NN model accomplished both premises. First, the 100% of correct

classification of pure milk samples reveals the high generalization ability of the model and the lack of overfitting. Secondly, the model is able to detect all the blended samples as adulterated, by not classifying to any pure sample that shows the robustness and capacity of NN to detect this type of adulteration.

<< Table 3 >>

### **3.2 Quantitative Analysis: Adulteration with melamine**

A commercial toddler milk sample was adulterated with different amounts of melamine at concentrations between 1% and 6% to plot the calibration curve. The validation sets comprised of samples at concentrations of 2, 4 and 5%.

For the quantification of melamine in the milk samples, CN band emissions were analyzed. Fig. 2a shows the CN spectral emission range of milk adulterated with melamine at different adulteration ratios where variation in the intensity can be seen. Considering the nature and composition of melamine ( $C_3H_6N_6$ ), an increase in the CN molecule signal with increasing concentrations of melamine was observed.

For the quantification purpose, two calibration procedures were employed. Firstly, a conventional calibration curve was calculated by integrating the CN emission band in the spectral range between 382 – 389 nm. Fig. 2a shows the correlation between the integrated area and the nominal adulterated ratio. Each point represents the value averaged of 30 LIBS spectra (10 for each of the three replicates at the same adulteration percentage) and the error bars show the relative standard deviation (RSD) between the three measurements. Reference values have been represented in black squares whereas the test value are in blue circles. The regression coefficient value found was 0.982.

A multivariate prediction curve using NN (Fig. 2b) was also studied. For this purpose, the same wavelength ranges as in the qualitative analysis has been used as input to the NN model. In this case, an improvement in the correlation coefficient was found, obtaining a value of 0.999 that shows perfect agreement between the actual and NN predicted concentration.

<< Figure 2a & b >>

To compare both methods (univariate and multivariate), the mean prediction error (MPE) was calculated following equation 2: (Cámara, Torrecilla, Caceres, Sánchez Mata, & Fernández-Ruiz, 2010)

$$MPE = \frac{1}{N} \sum_{i=1}^N \frac{|r_i - y_i|}{r_i} \times 100 \quad (\text{Eq. 2})$$

Where N is the total number of spectra,  $r_i$  the prediction output and  $y_i$  the actual value. A MPE of 24% and 5% was obtained for the univariate and multivariate methods, respectively. Table 4 shows an overview of the quantification parameters, it is clear that the multivariate NN approach provided better results for the quantification of adulterated milk samples.

<<Table 4>>

Taken together, these results seem to indicate that NN is more accurate than the conventional univariate method. This can be attributed to the fact that NN is able to model complex non-linear structure of the data. Although NN may be more difficult to implement than others standard chemometric methods because of its diversity of functions and architectures, the results obtained in this study demonstrate that LIBS combined with NN offers significant advantages in the quantitative analysis as well as for the classification purpose.

#### 4. Conclusions

Laser Induced Breakdown Spectroscopy (LIBS) technique has been evaluated to be applied for a real world application for a fast and robust control of adulteration of milk. Two different types of adulterations have been studied, including the binary/ternary blending of milk of different animal species and addition of melamine to toddler milk powder. The LIBS/NN combination allowed to distinguish clearly the blended milk from the pure milk as well as differentiating the pure milk samples with a 100% of correct classification in all cases. On the other hand, the quantification of melamine in toddler powder milk sample showed that the multivariate analysis by NN quantitative model produced better results than conventional calibration. Although the methodology requires further studies to increase the reliability and accuracy, the developed LIBS/NN methodology provided a sensitive and robust analysis to detect and quantify melamine in powder milk sample. It has been demonstrated that the emission band of the CN correlates to the molecular nature and concentration of melamine. The results demonstrate that LIBS/NN combination, supported by its speed of analysis, reduced cost, and ease of use has the potential to serve as a useful screening tool in the quality control of milk, both quantitatively and qualitatively.

### **Acknowledgements**

The authors gratefully acknowledge financial support from the Complutense University of Madrid.

### **Conflict of interest**

The authors declare no conflict of interest.



## References

- Anzano, J., Casanova, M.-E., Bermúdez, M.-S., & Lasheras, R.-J. (2006). Rapid characterization of plastics using laser-induced plasma spectroscopy (LIPS). *Polymer Testing*, 25(5), 623-627.
- Baudelet, M., Boueri, M., Yu, J., Mao, S. S., Piscitelli, V., Mao, X., & Russo, R. E. (2007). Time-resolved ultraviolet laser-induced breakdown spectroscopy for organic material analysis. *Spectrochimica Acta Part B: Atomic Spectroscopy*, 62(12), 1329-1334.
- Bilge, G., Boyacı, İ. H., Eseller, K. E., Tamer, U., & Çakır, S. (2015). Analysis of bakery products by laser-induced breakdown spectroscopy. *Food Chemistry*, 181, 186-190.
- Bilge, G., Sezer, B., Eseller, K. E., Berberoglu, H., Topcu, A., & Boyaci, I. H. (2016). Determination of whey adulteration in milk powder by using laser induced breakdown spectroscopy. *Food Chemistry*, 212, 183-188.
- Bilge, G., Velioglu, H. M., Sezer, B., Eseller, K. E., & Boyaci, I. H. (2016). Identification of meat species by using laser-induced breakdown spectroscopy. *Meat Science*, 119, 118-122.
- Bishop, C. M. (1996). *Neural Networks for Pattern Recognition*: Oxford University Press, USA.
- Caceres, J. O., Moncayo, S., Rosales, J. D., de Villena, F. J., Alvira, F. C., & Bilmes, G. M. (2013). Application of laser-induced breakdown spectroscopy (LIBS) and neural networks to olive oils analysis. *Appl Spectrosc*, 67(9), 1064-1072.
- Cámara, M., Torrecilla, J. S., Caceres, J. O., Sánchez Mata, M. C., & Fernández-Ruiz, V. (2010). Neural Network Analysis of Spectroscopic Data of Lycopene and  $\beta$ -Carotene Content in Food Samples Compared to HPLC-UV-Vis. *Journal of Agricultural and Food Chemistry*, 58(1), 72-75.
- Cordella, C. B. Y., & Bertrand, D. (2014). SAISIR: A new general chemometric toolbox. *TrAC Trends in Analytical Chemistry*, 54, 75-82.
- Cremers, D. A., & Radziemski, L. J. (2013). *Handbook of laser-induced breakdown spectroscopy* (2nd Ed ed.). Oxford - UK: Wiley-Blackwell.
- Curteanu, S., & Cartwright, H. (2011). Neural networks applied in chemistry. I. Determination of the optimal topology of multilayer perceptron neural networks. *Journal of Chemometrics*, 25(10), 527-549.

- EU-Comm. (2001). Methods for the analysis and quality evaluation of milk and milk products. *Official Journal of the European Communities*(213/2001), L37/31–L37/99.
- Garcia, J. S., Sanvido, G. B., Saraiva, S. A., Zacca, J. J., Cosso, R. G., & Eberlin, M. N. (2012). Bovine milk powder adulteration with vegetable oils or fats revealed by MALDI-QTOF MS. *Food Chemistry*, 131(2), 722-726.
- Gottfried, J. L., Frank C, D. L., & Miziolek, A. W. (2009). Discrimination of explosive residues on organic and inorganic substrates using laser-induced breakdown spectroscopy. *Journal of Analytical Atomic Spectrometry*, 24(3), 288-296.
- Gregoire, S., Boudinet, M., Pelascini, F., Surma, F., Detalle, V., & Holl, Y. (2011). Laser-induced breakdown spectroscopy for polymer identification. *Anal Bioanal Chem*, 400(10), 3331-3340.
- Grégoire, S., Motto-Ros, V., Ma, Q. L., Lei, W. Q., Wang, X. C., Pelascini, F., Surma, F., Detalle, V., & Yu, J. (2012). Correlation between native bonds in a polymeric material and molecular emissions from the laser-induced plasma observed with space and time resolved imaging. *Spectrochimica Acta Part B: Atomic Spectroscopy*, 74–75, 31-37.
- Griffin, M. (2016). Food Outlook - Milk and milk products. In, vol. 2016).
- Huang, Y., Kangas, L. J., & Rasco, B. A. (2007). Applications of Artificial Neural Networks (ANNs) in Food Science. *Critical Reviews in Food Science and Nutrition*, 47(2), 113.
- Inakollu, P., Philip, T., Rai, A. K., Yueh, F.-Y., & Singh, J. P. (2009). A comparative study of laser induced breakdown spectroscopy analysis for element concentrations in aluminum alloy using artificial neural networks and calibration methods. *Spectrochimica Acta Part B: Atomic Spectroscopy*, 64(1), 99-104.
- Lasheras, R. J., Bello-Gálvez, C., Rodríguez-Celis, E. M., & Anzano, J. (2011). Discrimination of organic solid materials by LIBS using methods of correlation and normalized coordinates. *Journal of Hazardous Materials*, 192(2), 704-713.
- Lucena, P., Doña, A., Tobaría, L. M., & Laserna, J. J. (2011). New challenges and insights in the detection and spectral identification of organic explosives by laser induced breakdown spectroscopy. *Spectrochimica Acta Part B: Atomic Spectroscopy*, 66(1), 12-20.
- MacKay, D. J. C. (1992). Bayesian Interpolation. *Neural Computation*, 4(3), 415-447.
- Marcos-Martinez, D., Ayala, J. A., Izquierdo-Hornillos, R. C., de Villena, F. J. M., & Caceres, J. O. (2011). Identification and discrimination of bacterial strains by laser induced breakdown spectroscopy and neural networks. *Talanta*, 84(3), 730-737.
- Mbesse Kongbonga, Y. G., Ghalila, H., Onana, M. B., & Ben Lakhdar, Z. (2014). Classification of vegetable oils based on their concentration of saturated fatty acids using laser induced breakdown spectroscopy (LIBS). *Food Chemistry*, 147, 327-331.

- Møller, M. F. (1993). A scaled conjugate gradient algorithm for fast supervised learning. *Neural Networks*, 6(4), 525-533.
- Moncayo, S., Manzoor, S., Navarro-Villoslada, F., & Caceres, J. O. (2015). Evaluation of supervised chemometric methods for sample classification by Laser Induced Breakdown Spectroscopy. *Chemometrics and Intelligent Laboratory Systems*, 146, 354-364.
- Moncayo, S., Manzoor, S., Ugidos, T., Navarro-Villoslada, F., & Caceres, J. O. (2014). Discrimination of human bodies from bones and teeth remains by Laser Induced Breakdown Spectroscopy and Neural Networks. *Spectrochimica Acta Part B: Atomic Spectroscopy*, 101, 21-25.
- Moncayo, S., Rosales, J. D., Izquierdo-Hornillos, R., Anzano, J., & Caceres, J. O. (2016). Classification of red wine based on its protected designation of origin (PDO) using Laser-induced Breakdown Spectroscopy (LIBS). *Talanta*, 158, 185-191.
- Nieuwoudt, M. K., Holroyd, S. E., McGoverin, C. M., Simpson, M. C., & Williams, D. E. (2016). Raman spectroscopy as an effective screening method for detecting adulteration of milk with small nitrogen-rich molecules and sucrose. *Journal of Dairy Science*, 99(4), 2520-2536.
- Pei, X., Tandon, A., Alldrick, A., Giorgi, L., Huang, W., & Yang, R. (2011). The China melamine milk scandal and its implications for food safety regulation. *Food Policy*, 36(3), 412-420.
- Rebecchi, S. R., Vélez, M. A., Vaira, S., & Perotti, M. C. (2016). Adulteration of Argentinean milk fats with animal fats: Detection by fatty acids analysis and multivariate regression techniques. *Food Chemistry*, 192, 1025-1032.
- Santos, P. M., Pereira-Filho, E. R., & Rodriguez-Saona, L. E. (2013). Rapid detection and quantification of milk adulteration using infrared microspectroscopy and chemometrics analysis. *Food Chemistry*, 138(1), 19-24.
- Shaffer, R. E., Rose-Pehrsson, S. L., & McGill, R. A. (1999). A comparison study of chemical sensor array pattern recognition algorithms. *Analytica Chimica Acta*, 384(3), 305-317.
- Tay, M., Fang, G., Chia, P. L., & Li, S. F. Y. (2013). Rapid screening for detection and differentiation of detergent powder adulteration in infant milk formula by LC-MS. *Forensic Science International*, 232(1-3), 32-39.
- Torrecilla, J. S., Cámara, M., Fernández-Ruiz, V., Piera, G., & Caceres, J. O. (2008). Solving the Spectroscopy Interference Effects of  $\beta$ -Carotene and Lycopene by Neural Networks. *Journal of Agricultural and Food Chemistry*, 56(15), 6261-6266.
- US Department of Commerce, N. (2015). NIST Atomic Spectra Database. In).

**Figure Captions**

**Fig. 1.** (a) Typical spectrum of (i) cow, (ii) sheep and (iii) goat. (b) PCA analysis of Cow, Sheep and Goat milk (c) NN outputs of the training samples

**Fig. 2.** (a) Spectral magnification on CN molecular band at different adulteration ratios and Conventional Calibration curve for melamine quantification by integration of the CN band (b) multivariate NN calibration model of adulterated milk powder

ACCEPTED MANUSCRIPT

**Table 1.** Pure milk samples used in the study

<b>Animal Origin</b>	<b>Sample ID</b>	<b>Commercial Brand</b>
<b>Cow</b>	C1	Hacendado
	C2	Lauki
	C3	Pascual
	C4	Asturiana
<b>Goat</b>	G1	Hacendado
	G2	Lauki
	G3	Puleva
	G4	Covap
<b>Sheep</b>	S1	Gaza
	S2	Lauki

ACCEPTED MANUSCRIPT

**Table 2.** Samples composition of the adulterated milk in binary and ternary

Sample ID	Composition (% v/v)		
	C1	G1	S1
M1	25	75	-
M2	50	50	-
M3	75	25	-
M4	25	-	75
M5	50	-	50
M6	75	-	25
M7	-	25	75
M8	-	50	50
M9	-	75	25
M10	50	25	25
M11	25	50	25
M12	25	25	50

ACCEPTED MANUSCRIPT

**Table 3.** Identification and robustness tests results for NN method

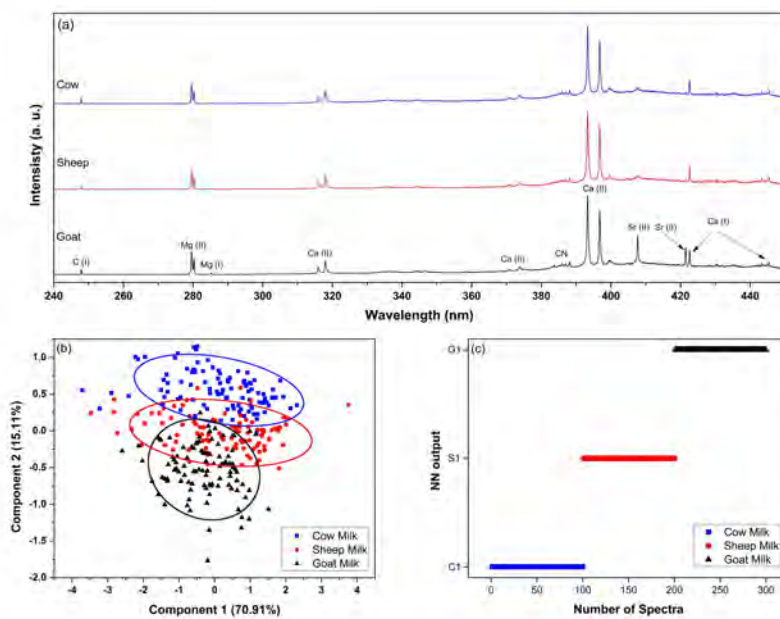
	Sample	Predicted Group Membership (Spectral Correlation, %)				Correctly Classified
		Cow 1	Goat 1	Sheep 1	Unknown	
Pure milk	C1	100	0	100	0	✓
	C2	85	0	0	15	✓
	C3	90	0	0	10	✓
	C4	94	2	2	2	✓
	G1	0	100	0	0	✓
	G2	0	100	0	0	✓
	G3	0	100	0	0	✓
	G4	0	96	2	2	✓
	S1	0	1	100	1	✓
	S2	0	2	96	2	✓
Binary mixtures	M1	0	0	0	100	✓
	M2	0	0	0	100	✓
	M3	0	0	0	100	✓
	M4	0	0	0	100	✓
	M5	0	0	0	100	✓
	M6	0	2	0	98	✓
	M7	42	16	0	42	✓
	M8	6	12	42	40	✓
	M9	40	12	2	48	✓
Ternary mixtures	M10	30	4	0	66	✓
	M11	0	60	0	40	✓
	M12	44	4	4	46	✓

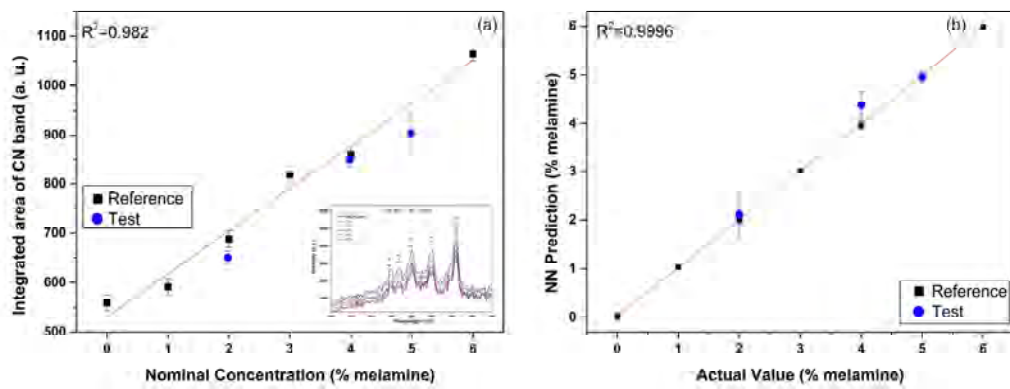
**Table 4.** Statistical Results of the quantification test for melamine adulteration by LIBS

	Univariate method	NN model
Correlation coefficient, $R^2$	0.982	0.999
Mean prediction error, MPE (%)	24	5
Standard deviation (%)	2.2	0.3

ACCEPTED MANUSCRIPT







ACCEPTED MANUSCRIPT

### Highlights

1. LIBS based methodology was developed to detect adulteration in animal milk.
2. Pure and blended milk from different animal species were analyzed by LIBS-NN.
3. Quantification of melamine by univariate calibration and multivariate NN model was done.
4. CN band emission showed linear variation with melamine added.
5. NN outperformed both in classification and quantitative analysis.

ACCEPTED MANUSCRIPT