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Feasibility of laser-induced breakdown spectroscopy (LIBS) as an at-line validation tool for calcium determination in infant formula

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ABSTRACT

In this study, a 150 mJ laser-induced breakdown spectroscopy (LIBS) system was assessed to determine calcium content in infant formula (IF) samples. LIBS is a promising emission spectroscopic technique for elemental analysis, which offers advantages over conventional methods such as real-time analyses, little to no sample preparation and ease of use. The aim of this work was to evaluate the feasibility of LIBS as an at-line tool for IF manufacturing. To this end, IF mixtures with varying content of calcium were prepared over a range (approx. 1.5–7 mg/g of calcium) selected to be in conformity with the guidelines provided by the Codex Alimentarius Commission. Atomic Absorption Spectroscopy (AAS) was used as the reference method for mineral content determination in IF. Partial least squares regression (PLSR) was applied to the LIBS spectral data to develop a calibration model for calcium content quantification. A validation approach was then carried out to investigate the robustness of the model, which showed a good fit with values of R^2_{cv} of 0.90 for cross-validation and a R^2_p 0.85 for external validation. Low values for root mean square errors of cross-validation (RMSECV) and prediction (RMSEP) of 0.62 mg/g and 0.68 mg/g were obtained. Furthermore, this study also illustrated the possibility of LIBS to provide mineral prediction maps as a useful tool for testing sample homogeneity.

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1. Introduction

Calcium is an essential nutrient and one of the most important minerals for human health. It is mostly found in the form of salts in the human body, the functions of which are primarily related to maintenance of bones and growth (Lima et al., 2016). Furthermore, in its ionized form, calcium plays a key role in many vital processes such as nerve conduction, hormone secretion and blood coagulation (FAO/WHO, 2004; Guo, 2014).

Milk and dairy products provide a high amount of calcium in the human diet (Vavrusova & Skibsted, 2014), particularly with regard to infants. Infant Formula (IF) is a milk-like product intended to supplement or act as a substitute for breast milk (Sola-Larrañaga & Navarro-Blasco, 2006). Thus, IF may be the infant's only source of calcium. IF is primarily derived from cow's milk (Murgia et al., 2016). However, the composition of this milk differs significantly

from breast milk and it must be modified to be suitable for infant consumption (Cama-Moncunill et al., 2016). In this regard, after overall mineral content adjustment, calcium needs to be increased to meet the optimal levels for the infant's requirements (Blanchard, Zhu, & Schuck, 2013). Calcium carbonate, among other commercial calcium salts, is a common additive used to enhance the calcium content of various foods (Smith, Gordon, & Holroyd, 2013), including IF (Guo & Ahmad, 2014). According to the Codex Alimentarius Commission, IF should not contain less than 12 mg of calcium per 100 kJ. Although a maximum level for calcium is not provided, it is recommended not to exceed (guided upper level) 35 mg per 100 kJ (Codex, 2007). These guidelines provide a range of calcium over which any novel technology for IF quality assessment should be reliable.

Laser-Induced Breakdown Spectroscopy (LIBS) is a promising emission spectroscopic technique for elemental analysis. It is described as a real-time method with little to no sample preparation (Anabitarte, Cobo, & Lopez-Higuera, 2012; Mehder, Habibullah, Gondal, & Baig, 2016) which also holds the possibility of spatial distribution analysis (Pathak et al., 2012). In LIBS, a laser is focused

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onto the sample surface to excite or ionize its elements. Typically a few micrograms of the surface material is ablated in this process (Andersen, Frydenvang, Henckel, & Rinnan, 2016), generating a high temperature plasma which contains all the excited species. Light emission occurring from the plasma is subsequently analysed by spectrometers to infer the chemical composition of the sample (Anabitarte et al., 2012).

LIBS has been exploited for elemental analysis in a wide variety of fields (El Haddad, Canioni, & Bousquet, 2014; Jantzi et al., 2015). Nonetheless, within food research it is still in its early days (Andersen et al., 2016). LIBS has been used previously for the analysis of milk and IF powders in other studies (Abdel-Salam, Al Sharnoubi, & Harith, 2013; Lei et al., 2011). Abdel-Salam et al. (2013) used LIBS for the qualitative assessment of spectral lines of nutritionally important minerals in breast milk and infant formulas, as well as the evaluation of proteins using the molecular bands of CN and C₂. In another publication, Lei et al. (2011) conducted a quantitative study in milk powders and IF. The authors employed LIBS for the determination of calcium content and other minerals by two different approaches: using calibration curves, which were obtained by mixing a reference sample with cellulose, and utilizing a calibration-free procedure which aided in overcoming matrix effects.

In the present work, a 150 mJ LIBS system was assessed to predict the calcium content in IF samples in combination with chemometric techniques. A different approach as reported in the literature is proposed here aiming to demonstrate the feasibility of LIBS to become an at-line validation tool for the IF manufacturing industry. To this end, the studied range of calcium was selected to be in conformity with the Codex Alimentarius. Moreover, the calcium contents of the samples were adjusted by adding lactose or calcium carbonate to IF. Both of these compounds occur naturally in IF and are permitted to vary within a certain range. The ability of LIBS as a method for exploring sample homogeneity is also illustrated via chemical prediction maps.

2. Material and methods

2.1. Sample preparation

Commercial powdered IF was acquired from the Irish market. Lactose (α -lactose monohydrate $\geq 99\%$) and calcium carbonate ($\text{CaCO}_3 \geq 99\%$) were purchased from Sigma Aldrich (St. Louis, MO, USA). Five samples with varying content of calcium (approx.: 1.1, 2.3, 3.7, 4.9, 6.1 mg/g) were obtained by mixing IF with lactose or calcium carbonate in order to reduce or increase respectively the calcium content. Proportional weights of lactose or calcium carbonate were added to IF to make a final weight of 225 g. Calculations were made on the basis of calcium contents in IF provided by the manufacturer. Calcium contents for lactose and calcium carbonate were approximated to 0% and 40% (w/w) respectively. Blending of the samples was carried out using a laboratory V-mixer (FTLMV-1L&, Filtra Vibracion S.L., Spain). Sample preparation was repeated three times providing three independent sample sets (batches). Additionally, a batch contained 2 extra samples with a calcium content of approximately 3.0 mg/g and 5.5 mg/g for validation purposes, hence giving a total number of 17 samples. For LIBS analysis, pellets were prepared by pressing approx. 400 mg from each IF sample at 10,000 kg with a single die manual hydraulic press (Specac Ltd, UK) for 3 min. Three replicate pellets were prepared per sample, thus, a total of 51 pellets were made (17 samples x 3 replicates).

2.2. Atomic absorption spectroscopy (AAS)

Calcium contents of the aforementioned IF mixtures along with pure lactose and pure calcium carbonate samples were determined by AAS (Varian 55B AA, Agilent Technologies, United States). The samples were dried overnight in an oven and the loss of weight was recorded. Organic matter was then decomposed by dry ashing in a muffle furnace according to the AOAC (Official Methods of Analysis of AOAC International) standard method for mineral determination in IF (method 985.35). Calibration curves were established by using aqueous standards, which were prepared from a calcium stock solution (Calcium standard for AAS – 1000 g/L, Sigma-Aldrich). All AAS analyses were carried out in triplicates. Results were calculated as milligrams of calcium per gram of dried sample. Each of the three independent batches was assessed separately on a different day.

2.3. LIBS instrumentation

LIBS spectra were recorded using a LIBSCAN-150 system (Applied Photonics, UK) equipped with a 150 mJ passively Q-switched Nd:YAG laser operating at 1064 nm with a pulse duration of 5 ns. A schematic setup of the LIBS system is shown in Fig. 1. Light was collected by an array of 6 lenses: 1 for the collection of deep UV, 2 for UV-VIS and 3 for VIS-NIR. The spectral range covered was 181–904 nm. Plasma emission was analysed at a 1.27 μs delay time to avoid the strong background continuum which characterizes the early stages of the plasma lifetime (Rakovský, Čermák, Musset, & Veis, 2014), with an integration time of 1.1 ms. In addition, the system was fitted with a CCD camera which enables the size and shape of the craters generated by laser ablation to be observed.

Individual pellets were placed into a sample chamber equipped with a motorized three-axis translation stage (Applied Photonics, UK) which allowed the acquisition of LIBS data at multiple locations. For each pellet, single laser shots were recorded at 100 different spatial positions, following a 10×10 grid pattern of approximately 0.7 mm distance between each measurement (Fig. 2). Such measurements were conducted operating the laser in a continuous mode with a repetition rate of 1 Hz, giving an approximate measurement time of 1 min and 40 s per pellet (100 laser shots). The three independent batches were also analysed separately on different days in order to evaluate the reproducibility of the method.

Fig. 1. Schematic LIBS system setup.

Fig. 2. Image of an IF and lactose pellet (sample 1 batch 1) showing the craters formed on its surface by the 100 laser pulses in a 10×10 grid pattern.

2.4. Data analysis

Data analysis was performed with R (R Core Team, 2014). The LIBS data was first averaged to obtain a single spectrum per analysed pellet, that is, the average of the 100 laser shots. Combinations of pre-processing techniques were applied to the spectra as part of the model building process. The Standard Normal Variate (SNV) transformation was then selected based on model performance.

The package “pls” (Mevik, Wehrens, & Liland, 2015) was used for conducting multivariate analysis with Partial Least Squares (PLS) regression. PLS was employed to relate the LIBS spectral data to the variation of calcium content. The pre-processed data was divided into a training set ($N = 30$), which was used to obtain the calibration model, and a validation set ($N = 21$) by which model robustness was assessed as external validation. Furthermore, the model was built using the cross-validation leave-one-out method as internal validation.

The model performance was evaluated by the coefficients of determination and the values of root mean square error for calibration (R^2_c , RMSEC) and cross-validation (R^2_{cv} , RMSECV) as well as for prediction (R^2_p , RMSEP) when the validation data set was used. The PLSR commands used for the calculations of these figures of merit are included in the “pls” package for R. The bias between

predicted and reference values was also investigated as further evaluation of the calibration model.

To provide comparison with PLS regression, univariate analysis was conducted. Simple linear regression models were developed by correlating the intensities of several Ca emission lines to the calcium contents determined by AAS. Similarly as for PLS, the training data set ($N = 30$) was used to build the calibration curves, while the validation set ($N = 21$) was employed to assess the predictive ability of the univariate approach. Figures of merit such as the relative standard deviation (RSD), the limit of detection (LOD) and the RMSEP were estimated for the best calibration curve.

2.5. Spatial distribution of calcium

In order to generate chemical maps showing calcium distribution within the samples, the PLS calibration model previously built was used to predict the calcium content of each measurement carried out on the sample's surface. As previously mentioned, measurements were performed at 100 locations per pellet in a 10×10 grid pattern. Therefore, the chemical prediction maps can be considered as images made of 10×10 pixels, in which each pixel displays the calcium content predicted for a specific location or crater formed on the sample's surface. The craters were estimated to measure an average distance of 0.5 mm in diameter. Pixels in the chemical maps, however, display the spectral information collected of measurements spatially separated by 0.7 mm, as such distance was kept to avoid overlapping of the craters.

Chemical maps corresponding to the two validation samples containing approx. 3.0 and 5.5 mg/g of calcium were analysed in this study. In addition, 200 mg of IF with a calcium content of approx. 1.1 mg/g and 200 mg of IF with a calcium content of approx. 6.1 mg/g were placed side by side in the die at the hydraulic press and a single pellet was obtained. This image was included to allow a better understanding of homogeneity.

3. Results and discussion

3.1. AAS

AAS was conducted as a reference method for calcium determination in IF. Calcium concentrations in the IF mixtures of the three batches are presented in Table 1. The calibration curves established by aqueous standards exhibited an excellent linearity with high coefficients of determination ($R^2 \geq 0.998$). The results obtained for the low calcium content samples (lactose blends) showed consistency over the analysed replicates with standard deviations in the range of 0.01–0.08 mg/g and little variation was observed between different batches. Similar results were found for the pure IF samples ($SD \leq 0.09$) with slightly higher variation

Table 1

Calcium contents in milligrams per gram of dried sample in the IF mixtures and the pure lactose and CaCO_3 samples determined by AAS.

No. sample (additive)	Batch 1	Batch 2	Batch 3, validation
	Ca content (mg/g) ^a	Ca content (mg/g) ^a	Ca content (mg/g) ^a
1 (lactose)	1.49 ± 0.04	1.52 ± 0.03	1.40 ± 0.05
2 (lactose)	3.06 ± 0.01	3.06 ± 0.02	3.05 ± 0.08
3 (pure IF)	4.86 ± 0.09	4.74 ± 0.09	4.23 ± 0.03
4 (CaCO_3)	5.17 ± 0.18	5.66 ± 0.12	5.14 ± 0.03
5 (CaCO_3)	7.01 ± 0.31	7.41 ± 0.08	6.56 ± 0.16
6 (lactose)	—	—	3.51 ± 0.09
7 (CaCO_3)	—	—	6.54 ± 0.07
8 (pure lactose)	0.07 ± 0.01	—	—
9 (pure CaCO_3)	433.93 ± 8.78	—	—

^a Mean ± standard deviation ($N = 3$).

occurring between batches. The high calcium content samples (calcium carbonate blends) presented standard deviations over replicates in the range of 0.03–0.31 mg/g and a noticeable variation between the batches, especially with regard to the highest level.

Calcium contents corresponding to pure lactose and calcium carbonate samples were also determined by AAS. As expected, lactose showed a considerably low concentration of calcium: 0.07 mg/g, whereas a high calcium content of 433.93 mg/g was found for calcium carbonate.

3.2. LIBS spectral features

The averaged LIBS spectra obtained for three different samples are presented in Fig. 3. For clarity, only the samples corresponding to the lowest calcium concentration of the second batch (IF and lactose blend at 1.52 mg/g), the highest (IF and calcium carbonate blend at 7.41 mg/g) and pure IF (4.74 mg/g) were included. The graph revealed the main emission spectral lines occurring in the LIBS spectra of the IF mixtures. Such spectral lines were associated with the most probable elements emitting at the observed wavelengths (Table 2), using the spectral lines provided by the NIST database as a reference (Kramida, Ralchenko, Reader, & NIST ASD team, 2016). Good agreement was found with identified spectral lines reported in other studies (Bilge, Sezer, et al., 2016; Bilge, Velioglu, Sezer, Eseller, & Boyaci, 2016), as well as for the molecular emission of the C₂ and CN bands (Abdel-Salam et al., 2013).

Some differences between the samples can be observed in Fig. 3. For instance, the IF and calcium carbonate mixture exhibited higher intensity at the calcium emission line at 616.28 nm, whereas lower intensity was found for the lactose mixture. The same result was found for several calcium emission lines. For other minerals such as sodium, a decrease in intensity was found in the calcium carbonate mixture at 589.05 nm (Na I).

For the emission of molecular species identified in the spectra, no appreciable difference between the different samples was observed for the CN bands occurring in the range of 385.03–388.31 nm. However, the emission of C₂ bands at 512.87–516.47 nm for lactose mixtures exhibited lower intensities when compared to pure IF or the calcium carbonate blend.

Fig. 3. Averaged LIBS spectra of samples: (a) IF and calcium carbonate blend (sample 5 batch 2) at 7.41 mg Ca/g, (b) pure IF (sample 3 batch 2) at 4.74 mg Ca/g and (c) IF and lactose blend (sample 1 batch 2) at 1.52 mg Ca/g. Spectra are vertically offset for clarity.

Table 2

Observed spectral lines occurring in the LIBS spectra and their possible associated elements. These associations were made using the NIST database as a reference (Kramida et al., 2016).

Observed wavelength (nm)	Elements/molecular emission
247.87	C I
279.56	Mg II
385.03–388.31	CN
393.34	Ca II
396.86	Ca II
422.70	Ca I
445.50	Ca I
512.87–516.47	C ₂
558.90	Ca I
589.05	Na I
612.25	Ca I
616.28	Ca I
643.93	Ca I
656.35	H I
746.91	N I
766.46	K I
769.93	K I
777.43	O I

3.3. Calibration model

The calibration model was built by applying PLS regression to the training data set. The concentrations determined by AAS were used as the reference values for PLS, thus correlating the assessed calcium contents to the pre-processed LIBS spectral data.

The first 3 main PLS factors of the model explained approximately 85% of the total variance. The PLS loading plot for the first factor of the calcium model is shown in Fig. 4, which explained 60% of the total variance. Several calcium (Ca I) emission lines were found to contribute to the loading values of the first factor. The most prominent lines occur approximately at 422.65 nm, 558.90 nm, 612.25 nm and 616.80 nm. This result, along with the presence of singly ionized calcium lines (Ca II), demonstrated that most of the variation occurring in the samples was related to the calcium content. However, other spectral lines with a noticeable contribution were that of the sodium (Na I) line at 589.05 nm and potassium (K I) at 766.45 nm. Both of these emission lines showed negative loading values indicating that for an increasing content of sodium and potassium, a decreasing content of calcium was expected in the predicted values. The presence of sodium and potassium lines in the loading values was an expected outcome as

Fig. 4. Loading plot showing the contribution of each wavelength to the first PLS factor.

when varying the composition of IF by adding lactose or calcium carbonate, the levels of these minerals are also modified.

The performance of the calibration model obtained for calcium prediction is summarized in Table 3, which contains the coefficients of determination and the root mean square errors for calibration (R^2_c , RMSEC), cross-validation (R^2_{cv} , RMSECV) and prediction by an independent data set (R^2_p , RMSEP). The model showed good linearity, with calibration coefficients of determination (R^2_c) of 0.95 and 0.96, for 3 and 4 PLS factors respectively. When interrogated by cross-validation, the model was found to fit reasonably well with an R^2_{cv} value of 0.90 for 3 PLS factors. Although the coefficient of determination R^2_c was higher using 4 components, the value for cross-validation R^2_{cv} together with the minimum value found for RMSECV, indicated that the model would perform better for estimating calcium content when 3 PLS factors were used.

3.4. Validation of the calibration model

Validation was performed in order to evaluate the robustness of the calibration model. In this regard, the 3 PLS factors model was used to predict the calcium contents of the validation data set.

The predicted calcium values for the IF samples are shown in Fig. 5. The RMSEP value obtained was similar to the RMSECV value: 0.68 mg/g and 0.62 mg/g respectively. This result indicated that the model was not overfitted. It can be observed in Fig. 5 that the predictions established for the highest levels of calcium (calcium carbonate blends) were more accurate than the predictions for the lowest levels (lactose blends). This may be due to the fact that a considerably lower amount of calcium carbonate was required to bring concentrations to the desired levels, compared to the amounts of lactose needed to decrease the calcium content in the mixtures. Thus, the addition of lactose could possibly lead to a major change in the matrix embedding the calcium. The matrix is known to have notable effects on the emission intensities of the LIBS measurements (Ferreira et al., 2010; Lei et al., 2011; Tognoni, Cristoforetti, Legnaioli, & Palleschi, 2010).

Fig. 5 also displays the values of the coefficients of determination of calibration and external validation (prediction). As previously stated, the model showed good linearity with an R^2_c of 0.95. Regarding the predictions, the R^2_p value was 0.85. This result indicated reasonable model's performance for an independent batch which has not been used for developing the calibrations. The bias value, found between the reference values and the predictions, was 0.56 mg/g indicating that in terms of predictive accuracy the method still needs further enhancement to be fully developed as a validation tool for industry. It should be noted, however, that the lowest calcium content sample, which contains a large amount of lactose, had a negative contribution to both the R^2_p and the bias, whereas the predictions for the other levels were considerably more accurate.

3.5. Comparison to univariate analysis

For completeness, univariate analysis was conducted to

Table 3

PLS regression model performance evaluated by cross-validation (leave-one-out) and external validation (using the validation data set).

No. PLS factors	Calibration (N = 30)				Validation (N = 21)		
	R^2_c	RMSEC ^a	R^2_{cv}	RMSECV ^a	R^2_p	RMSEP ^a	Bias ^a
2	0.93	0.53	0.89	0.66	0.46	1.28	1.17
3	0.95	0.44	0.90	0.62	0.85	0.68	0.56
4	0.96	0.38	0.89	0.64	0.82	0.75	0.66

^a Expressed in milligrams per gram of calcium.

Fig. 5. PLSR calibration curve with reference values and calcium predicted contents for the validation data set.

provide calibration models for each of the calcium emission lines reported in Table 2. Overall, a low calibration performance was observed for all the assessed spectral lines. The model using the Ca I emission line at 616.28 nm yielded the highest R^2 with a value of 0.73 and a RSD of 13.66%. The calibration curve of this model is shown in Fig. 6(a). As in PLS, the model was employed to predict the calcium content of the validation sample set. Predicted versus measured values are displayed in Fig. 6(b). The values of bias and RMSEP were then investigated in order to compare the univariate prediction ability with that of the PLS regression. The bias value was found to be 0.80 mg/g of calcium and the RMSEP value was 1.09 mg/g. Both values were considerably higher than those obtained with PLS (bias: 0.56 mg/g and RMSEP: 0.68 mg/g) substantiating that better accuracy and precision can be obtained with PLS regression. These results indicated that PLS may help overcome matrix effect as reported elsewhere (El Haddad et al., 2014). Additionally, the LOD serves as an indicator of the detectable concentration limits and was computed as 3 times the standard deviation of the regression divided by the slope of the calibration curve. The LOD value for the calibration curve using the 616.28 nm Ca line was 3.69 mg/g.

Fig. 6. (a) Univariate analysis conducted using the calcium emission line at 616.28 nm. (b) Calcium predicted contents versus reference values for the univariate calibration and validation data set.

Fig. 7. (a–b) Chemical predicted maps obtained for the two validation samples (batch 3: samples 6 and 7) at 6.54 mg Ca/g and 3.51 mg Ca/g. (c) Shows the calcium distribution of a single pellet consisting of two different concentrations of calcium arranged side by side (low: 1.52 mg/g, high: 7.41 mg/g), included for illustration purposes only.

3.6. Chemical maps of calcium

In order to study calcium distribution within the samples, the PLS model was used to predict the calcium content of each spatial position, therefore, allowing the visualization of the sample's homogeneity.

Chemical predicted maps obtained for the two additional validation samples at 3.51 mg/g and 6.54 mg/g are displayed in Fig. 7(a–b). It can be observed that a few pixels in the chemical maps are higher or lower than the neighbouring pixels, however, none of the predicted values were found to be extreme. Although some variability was found, the evaluation of the results suggested that overall the pellets were homogeneous with no clumps or aggregates occurring. In order to show a case of non-homogeneity, the figure includes a chemical map (c) of a single pellet consisting of two different concentrations of calcium arranged side by side. In this chemical map, a high variation can be observed between the two concentrations of the pellet. This variation contrasted with the homogeneity observed for the two IF validation pellets with calcium contents of 3.51 mg/g and 6.54 mg/g.

4. Conclusions

In the present work, a 150 mJ LIBS system in combination with chemometric techniques was successfully employed to predict the calcium content of IF samples over a relevant range (approx. 1.4–7.0 mg/g) for IF manufacturing, as it was selected to be in agreement with the guidelines provided by the Codex Alimentarius. The most remarkable advantages of the LIBS system compared to conventional methods for calcium content determination are, its speed, ease of operation and minimal sample preparation.

PLS regression was applied to the LIBS spectral data to build a calibration model for calcium content prediction. The data consisted of three independent samples sets in order to evaluate the reproducibility of the method. When examined by cross-validation and external validation, the model exhibited high values of R^2_{cv} and R^2_p corroborating a good fit over the selected range. The predictive accuracy was considered acceptable. Although these results need further enhancement in terms of predictive accuracy, the study presented here illustrates the potential of LIBS to be applied as an at-line validation method for the quantification of calcium in the IF manufacturing industry.

Furthermore, the chemical mapping conducted in this experiment allows a better understanding of the calcium distribution within the samples. Moreover it demonstrates the possibility of LIBS to extract not only chemical, but also spatial information from the sample surface, even within the selected relevant range for IF manufacturing.

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