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Effect of DL-Methionine Concentration, Moisture Content and Bulk Density of Animal Feed on the Light-Induced Fluorescence as a Process Analytical Tool

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ABSTRACT

Mix uniformity is a critical quality control point in food manufacturing. Process analytical technology (PAT) provides new technological opportunities for fulfilling and perhaps replacing conventional sampling methods by proposing spectroscopic analyzers for measuring blend homogeneity. Many spectroscopic analyzers have been used in powder blending processes. Light-induced fluorescence (LIF) is the most rapid and consistent underutilized PAT. An experiment was conducted to evaluate the effects of DL-methionine concentration, moisture content (MC) and bulk density (BD) on LIF responses. Fluorescent responses to powder mixtures comprising 0.05–0.50% w/w fluorescent active pharmaceutical ingredient (API) were reported. Approximate density ranges of 6.5–20% w/w for MCs and 0.55–0.65 g/ml for animal food were also evaluated. Results indicated that DL-methionine concentration and MC were statistically significant factors affecting the LIF response, but the effect of BD was not statistically significant. DL-methionine concentration from 0.05% to 0.50% caused a linear increase of LIF signals with $y = 41.04x + 715.8$, $R^2 = 0.990$ fitted to the data. Increasing MC from 6.5% to 20% w/w caused decreasing LIF although $y = -45.50x + 1037$ could not explain LIF variation versus MC because of low coefficient of determination ($R^2 = 0.851$).

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INTRODUCTION

Today, process analytical technology (PAT) is increasingly being applied to better understand and control and monitor pharmaceutical units operation. Robust, reliable, accurate and fast, techniques that do not require sample preparation are essential for the in-line monitoring of blending. The Food and Drug Administration (FDA) has defined PAT as a system for controlling, analyzing and designing manufacturing by timely measuring the performance attributes and critical quality of in-process and raw materials and processes to ensure the quality of final product (U.S. Food and Drug Administration, 2014). Chanda et al. (2014) used an active pharmaceutical ingredients (API) process work-flow from the raw-material identification to the finished API to provide representative examples such as how and why the pharmaceutical industry used PAT tools in API development. Nutrient uniformity is crucial for proper nutrition when animals such as nursery pigs and baby chickens are on low daily intake of food (Ensminger et al., 1990; Clark et al., 2007). According to Beumer (1991), mix uniformity is a critical quality control point in food manufacturing. New technologies have the opportunity to fulfill and potentially replace conventional sampling methods by more consistent and rapid techniques of blend homogeneity measurement. Bakeev (2010) updated the application and implementation of spectroscopic process analyzers. Many spectroscopic analyzers have been utilized in powder blending process.

Near-infrared (NIR) spectroscopy has been widely accepted as a PAT tool for quantifying and identifying API and formulation excipients in the noninvasive monitoring of powder blends (Wargo and Drennen, 1996). NIR uses a complex reflectance spectrum specific to the analyzed substance and records the results of analyzing extensive data to reduce the spectra to a representative pattern for the mixture. This method can provide a foundation for the convergence and monitoring of the expected aggregate spectra and establish blend homogeneity. Given that the reflectance NIR is not usually a strong signal, except for water, the sensitivity of this method is limited for highly potent drug formulations in which drug contents are below 1% w/w in the mixture (Lai et al., 2001).

Raman spectroscopy has also been found to be a useful technique as a PAT tool in API and dosage form manufacturing and development, and for identifying pseudo-polymorphic, polymorphic and amorphous API forms (Jestel, 2010). Raman spectroscopy has also been applied to monitoring API crystallization, gel manufacturing processes, emulsion and wet granulation (Islam et al., 2004; Strachan et al., 2007).

Other PAT analyzers include process nuclear magnetic resonance spectroscopy (Edwards & Giammatteo, 2010), microwave resonance sensors (Corredor et al., 2011), acoustic emission (Tok et al., 2008), laser-induced breakdown spectroscopy (Madamba et al., 2007), acoustic resonance spectroscopy (Medendorp et al., 2006), far-infrared spectroscopy (Zeitler et al., 2007) and ultraviolet (UV) visible spectroscopy (Liauw et al., 2010).

LIF is an underutilized PAT. In 2000, eight out of the top ten worldwide pharmaceutical products were reported to have fluorescent properties. Over sixty percent of the top 200 selling medicines were also found to have structural-associated fluorescent properties (Lai & Cooney, 2004). GlaxoSmithKline (GSK) found 50% out of more than 27 new medical compounds tested to have fluorescent properties (Ishan et al., 2015).

Numerous advantages of fluorescence over absorption spectroscopy include NIR and UV. The Stokes shift characterizes the fluorescence excitation and emission spectra and causes differences in the peak wavelength of emission and excitation. The fluorescent intensity equation used for low fluorophore concentrations offers another advantage:

$$I_f = P_o \gamma \phi \varepsilon b C = P_o k C \qquad (1)$$

in which k represents the proportionality constant, C the fluorophore concentration, b the optical path length, ϕ the molar absorption at the fluorophore excitation wavelength, ϕ the fluorophore quantum efficiency,the P_0 fluorescent collection efficiency, P_0 the incident irradiance or power per unit area a$_n$d I_f the fluorescent intensity (Dickens, 2010; Brittain, 2006; Harris, 2010). According to Equation 1, a limited increase in the incident excitation irradiance proportionally increases the fluorescence intensity, which provides an advantage for LIF process analysis and higher detection flexibility. In absorption spectroscopy techniques, including UV and NIR, increasing the incident irradiance does not affect absorbance given that absorbance is a function of the $(P_0)/(I)$ ratio, in which I denotes the intensity of light emerging from the sample and absorbance equals log $(P_0)/(I)$. Fluorescence is influenced by the matrix and environmental conditions, including temperature, pH, moisture, the presence of metal ions and viscosity. pH can influence the charge and resonance of fluorophore and change the fluorescence intensity. An increase in temperature increases the kinetic energy of molecules and the potential for collision deactivation through intermolecular energy transfer and thereby usually decreases the fluorescence intensity.

PAT studies have rarely been conducted on LIF so far, and the few key ones are presented as follows: LIF responses were used for the real-time monitoring of mini-scale dry powder blending. Lai et al. (2001) reported that an increase in powder bulk densities proportionally increased LIF signals at a single bulk powder concentration and an approximate density range of 0.45–0.80 g/cm^3. Lai and Cooney (2004) developed a portable system using LIF as an analytical tool for on-line monitoring of various manufacturing process applications. Their LIF system was verified in several laboratory scale process applications specifically in noninvasive real-time observations of blend kinetics in tumbler blenders. They showed the actual blend characteristic behavior of powder mixtures such as homogeneity end point and blend stability and consistent blend homogeneity end point. A correlation between LIF signal and drug powder concentration was established with limits of detection below 0.02% w/w for the API, Triamterene. Dickens et al. (2011) described a

compact and portable LED array-based fluorescence sensor for rapid real-time monitoring. For the sensor configured for reflectance detection, the response sensitivity ranged from 100 to 60,000 and an estimated tryptophan detection limit was ~0.001% (w/w) in lactose. They discussed excitation parameter effects on the emission signal behavior, fundamental response functions, and standard analytical merits (sensitivity and detection limits) to highlight and benchmark the unique capabilities of their new sensor technology.

Brittain (2006) and Harris (2010) provided a nonlinear series expansion that described the measured fluorescent intensity as a function fluorophore concentration:

$$I_f = kP_o[C - C^2/2 + C^3/3! - \ldots C^n/n!] \quad (2)$$

where I_f is the measured fluorescent intensity, k is the proportionality constant, P_0 is the incident irradiance, and C is the concentration of the fluorophore. At low fluorophore concentrations, the higher-order terms can be dropped leaving the linear form of the equation (Eq. 1).

Karumanchi et al. (2011) reported curvilinear relationships between increasing fluorescent powder concentrations and LIF signals.

The present study was conducted to evaluate the impact of API concentration, moisture content (MC) and bulk density (BD) of animal food powder on LIF responses. Obtaining knowledge about these effects is crucial for the development of LIF as a PAT.

MATERIALS AND METHODS

Materials

The chicken feed powder was prepared in Kian chicken farm of Qom province in Iran with mean particle size of 700 μm. The inactive powder made from meal-based corn-soybean diet was fist formulated for layer chickens (Corn 54.57%, Soybean Meal 27.73%, Calcium Carbonate 9.95%, Bran 2.61%, Wheat Flour 3.43%, Soybean Oil 1.40% and Salt 0.31%). Then, inactive diet was mixed using a vertical mixer (a 5-ton capacity) for the optimum blending time (20min). The inactive sample had no API so that no confounding effects on fluorescent signals. The feed grade MetAINO® (DL-methionine 99%; a necessary amino-acid in chicken diet manufactured by Evonik Antwerpen N.V. of Belgium having mean particle size 600 μm and Density 0.7 g/mL) was the API used in all the experiments.

LIF Sensor

According to Figure 1, LIF sensors use an LED light as the excitation source of light. LED beams were directed into a fiber-optic cable linked to a photo-sensor module including the detector photomultiplier tube and the lens. A dichroic mirror in the photo-sensor module reflected laser beams at ninety degrees to the sample. This lowered the scattered light reaching the detector and enhanced the signal to noise ratio. The fluorescent signal emitted

from the sample was collimated with the lens and passed through the dichroic mirror and an emission filter to the detector. The signals were converted to voltage and then recorded by a multi-meter interfaced through a hyper-terminal and RS-232 to a computer.

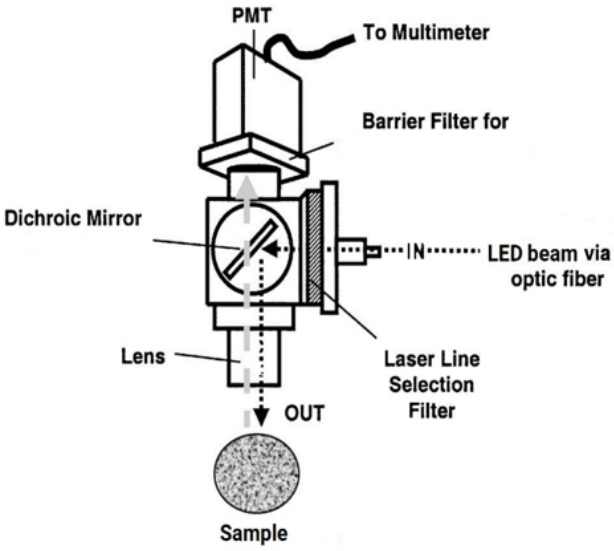

Figure 1. LIF Sensor; LED beam via optic fiber is directed to dichroic mirror. It enhances the beam and then reflects it 90° to the sample. Fluorescent API within sample is excited and emits fluorescent signal. Then, emitted light-induced fluorescence is collimated, filtered and converted to voltage and finally recorded to a computer.

Source: Lai et al. (2001)

An Avalight-LED® compact (Avantes Co., Netherlands) purchased for fluorescence applications produces pulsed or continuous spectral outputs at different wavelengths and with a spectral range of 530/590/780 (nm, an FWHM of 30 nm and an optical power of 25µW associated with a 600-µm-fiber in the light source. Illumination in the probe was twelve 200-µm UV/VIS fibers, wavelength range was 200-800 (nm) UV/VIS and detection was through a 600-µm fiber. The AvaSpec-ULS3648 high-resolution spectrometer wavelength range was 200-1000 nm, its resolution 0.05-20nm, stray light 0.04-0.1%, signal to noise ratio 350:1 and sensitivity 160000 counts/µW per ms of integration time.

Calibration of the LIF Sensor

AvaSpec spectrometer has a standard wavelength calibration as well as coefficients for calculating wavelength based on the pixel number. These data were installed on the AvaSpec's EEPROM. Given the absence of moving elements inside the spectrometers, it did not require wavelength recalibration under normal conditions. Spectrometers could be recalibrated by the use of the auto-calibration software routine in AvaSoft-Full when a wavelength shift was measured in terms of the original wavelength calibration.

Preparing the Concentrations of Test Blends

The inactive blends of powder were prepared using the procedure discussed in materials. These blends were independently used in the experiments. The mass of 0.05–0.50% w/w API samples were obtained in two steps. First, inert powder diet was used to dilute a 10.00 %-w/w fluorescent API achieving various equivalent concentrations of API. The inactive powder with a known weight was charged into a commercial mini-blender (Pars Khazar Co., Iran), and a pre-weighted amount (10.00% w/w) of diluted DL-methionine was layered on the top of the inactive powder to provide a total weight of 10 g of the mixture (Table 1). In order to provide a uniform material blending action for all the samples (0.05%, 0.10%, 0.15%, 0.20%, 0.25%, 0.30%, 0.40%, and 0.50% w/w), the mini-blender was rotated at 1000 rpm for five seconds 3 times.

Preparing the Test Blends for the MC Experiments

Moisturizing of samples was carried out in spectroscopy laboratory at the same time with moisture analyzing and LIF spectroscopy. First, samples were left in oven for 4 hours at 108°C. Four 5g samples with 6.57, 10.37, 12.39 and 21.52 % w/w MC were prepared through handy weighting and titration in laboratory conditions (35°C, 33% RH). Then, 5g of each moist sample was placed in moisture analyzer (MX-50 model by A&D Co., JAPAN), and simultaneously, another 5g was placed under LIF Aventus probe. Samples cooling within desiccator (from 108°C to 35°C) and transferring into moisture analyzer and LIF setup was done quickly to minimize any change in their moisture content. Analyzer determined powder moisture content after about 5 minutes but LIF signals was obtained very fast (about 1 minute).

Table 1

API and inactive diet for 0.05–0.50% w/w concentrations

% API (%w/w)	inactive diet (mg)	10% API (mg)	API Consentration (w/w)
0.05	9950	50	0.0005
0.10	9900	100	0.001
0.15	9850	150	0.0015
0.20	9800	200	0.002
0.25	9750	250	0.0025
0.30	9700	300	0.003
0.40	9600	400	0.004
0.50	9500	500	0.005

API concentrations were composed in two steps. First, inactive powder was mixed with 10% of API. Then, various weighted amounts of two materials were mixed that sum of two columns (10% API + inactive diet) of all samples will be 10000 g.

Preparing the Test Blends for BD Studies

Uniformly mixing the test API throughout the batch was important for determining the effect of material density on LIF responses. In order to break up the agglomerates potentially formed during the storage while avoiding the change in the nature of the material, an amount of the powder adequate for completing the test was passed through a sieve with 1.0 mm apertures. Fifty (50) g of the test sample (m) weighing with a 0.01 g accuracy was gradually introduced into a 100-ml (readable to 1 ml) dry graduated cylinder without compacting. The powder was then leveled carefully while avoiding compacting, and the unsettled apparent volume (V_0) was measured as the nearest graduated unit. BD was calculated in g/ml using the formula m/V_0. These measurements were performed 3 times for each sample.

LIF Tests

The sensor uses Avalight-LED as the excitation light source (Figure 2). DL-methionine generated a specific emission peak at 492 nm when was excited at 405 nm. The fluorescent counts were tested in powder mixtures including 0.05%–0.50% w/w of API. A BD range of approximately 0.55–0.65 g/mL and an MC of approximately 6.5%–20% w/w were also evaluated.

RESULTS AND DISCUSSION

The Impact of API Concentrations on LIF Responses

LED light excitation with wavelength 405 nm created a fluorescent emission wavelength with a peak in 492 nm. The difference in the wavelength of the excitation and emission peak maxima (87nm) is named as *Stokes shift*. This shift allows for a low detection limit to increase sensitivity while decreasing the background noise.

Figure 2. The LIF Test; Avalight-LED® compact produces pulsed or continuous spectral outputs, when a sample is excited at 405 nm, generating a specific emission peak at 492 nm.

Figure 3 shows the impact of API concentrations on LIF responses. LIF peak was in 492 nm for all concentrations with 10% w/w moisture content and 0.65 g/mL bulk density. The lowest line peak (blue) was created with least API (0.05 %w/w) and it increased in the more concentrations in the first and second replication (r_1, r_2). The third test of LIF test (r_3) showed inconsistent peaks for 0.3 and 4.0 % w/w

of API. This seems to be an experimental error. Therefore, it is derived that increasing API concentration increased LIF counts.

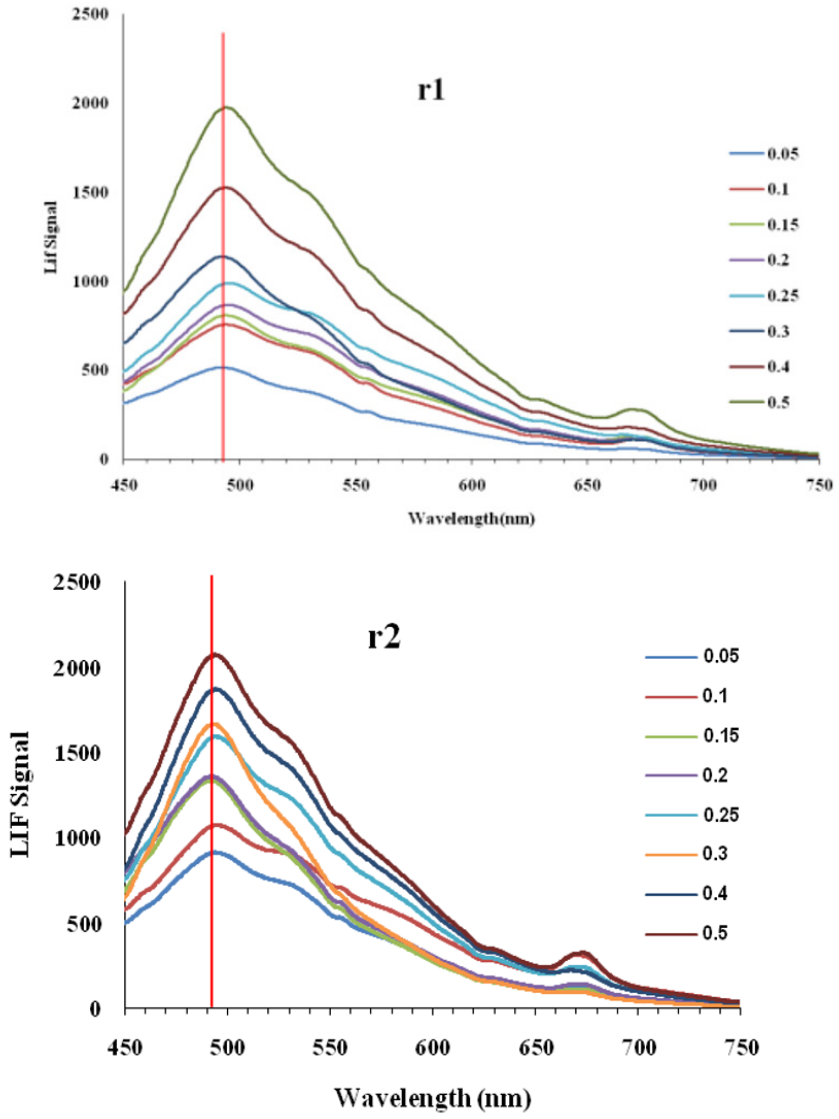

Figure 3. LIF peak was in 492 nm for all concentrations with 10% w/w moisture content and 0.65 g/mL bulk density in all three replications (r_1, $r_{2,}$ r_3).

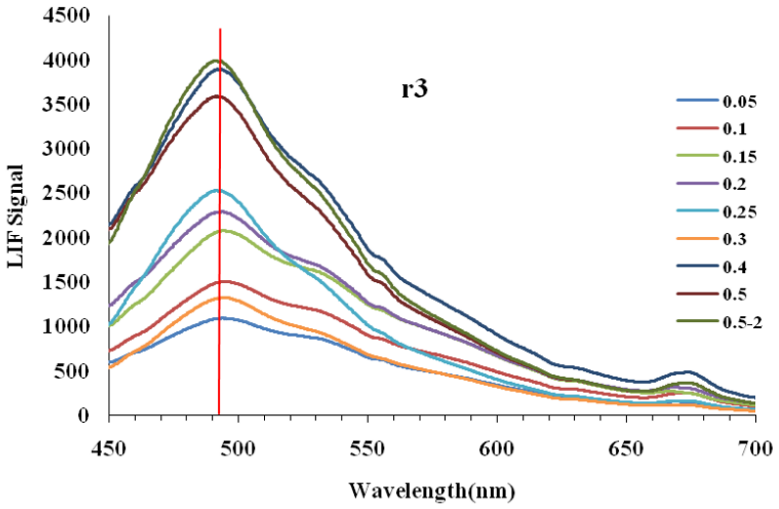

Figure 3. (Continued)

DL-methionine concentration from 0.05%–0.50% caused a linear increase of LIF signals (Figure 4). The model $y = 41.04x + 715.8$ was fitted to the data with $R^2 = 0.990$ where y was LIF counts and x was DL-methionine concentration weight to weight percentage. Methionine was the fluorophore (x) and it was straightly correlated with LIF counts (y). Whatever methionine concentration was increased consequently fluorescence intensity would be increased. Analysis of variance (ANOVA) was performed for LIF counts with eight API concentration and three replications plotted in Table 2.

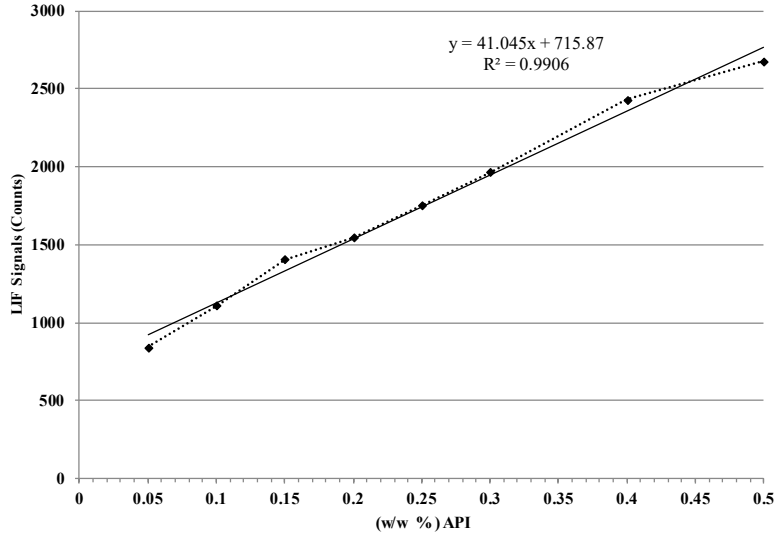

Figure 4. Linear variations in LIF with increasing API; increasing API caused a linear increase of fluorescence.

Table 2

ANOVA for LIF counts by API concentrations with replication

ANOVA	Some of Squares	D_f	Mean Squares	F_c	Significant
R	9089933	2	4544966	31.969	0.00**
LIF	8275918	7	1182274	8.316	0.00**
Error	1990374	14	142169		
Total	19356226	23			

** P<0.01 means the groups aren't statistically equivalent with a 99% confidence. In other words, at least difference of one pair of replications is significant. Also, the difference of fluorescence produced by API in various concentrations is significant.

Results show that at least one pair of group means was not statistically equivalent with 99% confidence. This was the same for between groups (Treatment) and within groups (Replication). Treatment group differences were supported by the Duncan's significant difference test (P<0.01). It compared all the mean pairs of the concentration groups. The Duncan test made up a more conservative alternative to ANOVA. All means were not statistically equivalent with 99% confidence as shown in Table 3. LIF signals in 0.15% and 0.20% w/w API were in one group. It means these were statistically equivalent.

Effect of BD on LIF Response

When corn-soybean meal-based diet was milled (in the range of 0.561- 0.649 g/mL), the volume of the materials was increased. Therefore, BD was decreased with having constant

Table 3

Duncan Compare means of LIF signals in API concentrations

SD	Mean	DL-methionine (w/w)
294	840^{e}	0.05%
374	1112^{de}	0.10%
636	1406^{cde}	0.15%
670	1546^{cde}	0.20%
712	1751^{bcd}	0.25%
1010	1964^{bc}	0.30%
1279	2430^{ab}	0.40%
1137	2679^{a}	0.50%

The group that has "a" index with 95% of confidence is different with other group (without "a" index). Therefore, mean LIF emitted by group 0.40% and 0.50% API have significant difference with group 0.25%, 0.20%, 0.15%, 0.10% and 0.05% API and so on and so forth.

mass. Decreasing density means API decreasing. Fluorescent experiments on bulk densities 0.561, 0.609 and 0.649 g/mL showed that LIF counts of 0.561 and 0.649 g/mL were more than 0.609 g/mL (Figure 5).

In all three replication LIF signals of powder with BD of 0.609 g/mL were least as shown in Figure 5. Therefore, in range of 0.561-0.649 g/mL BD had no increasing or decreasing trend. BD was analyzed through a three-replication randomized complete block design. Results showed that statistically there was no significant deference between BD groups. Whereas Lai et al. (2001) observed a proportional increase ($y = 9.29x + 16.86$, R^2=0.975) of LIF signal with increasing BD from 0.45-0.82 g/cc. However, Ishan et al. (2015) studying API in Lactose Monohydrate observed different results. Linear variation was observed in material densities of 0.6-1.4 g/cm^3 at 0.25%, 0.50%, 1.00%, 2.00% and 2.50% w/w concentrations of API which contradicted the present findings shown in Figure 5.

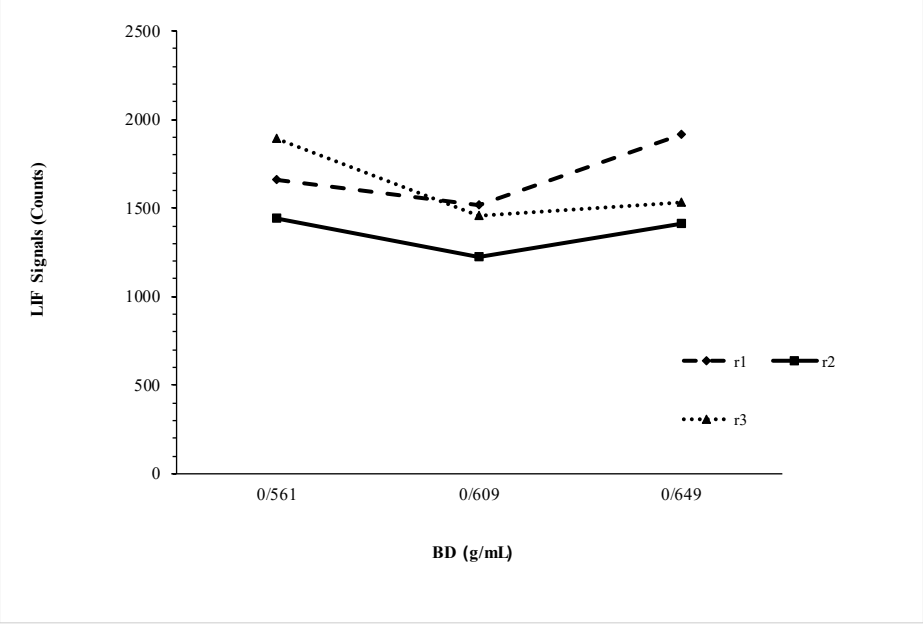

Figure 5. LIF signals in three bulk densities; the experiments was performed in three replications (r_1, r_2, r_3) that LIF falling of BD 0.609 g/mL was the same for all.

Effects of MCs on LIF Responses

Results showed that increasing MC caused decreasing of LIF signals. In all three replications MC from 6.57% w/w increasing to 20.83% w/w decreased LIF signals as shown in Figure 6. When moisture content was increased, really water content as a polar solvent was increased. Water using electrostatic interactions such as hydrogen bonding and influencing on electron shells was caused various deactivation phenomena such as

non-radiative deactivation. It caused reduction of fluorophore quantum efficiency (ϕ) mentioned in Equation 1.

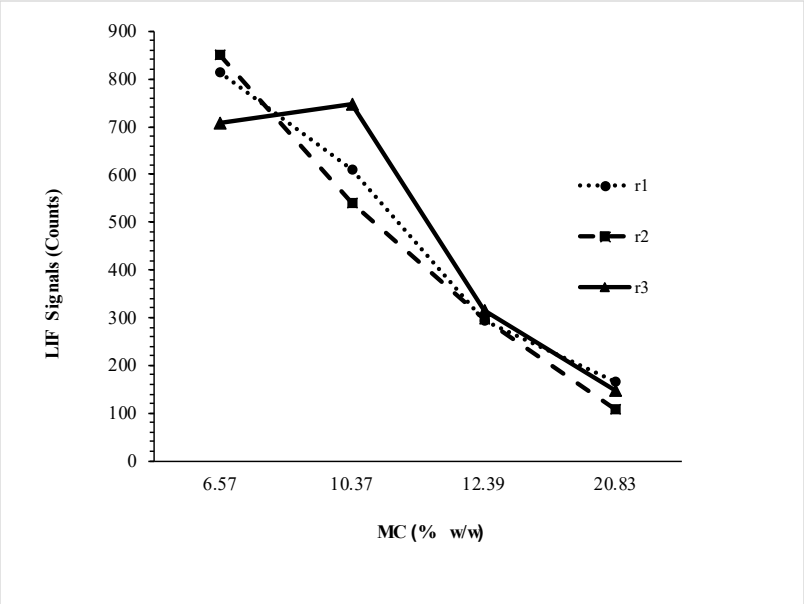

Figure 6. LIF signals in four MCs; in three replications (r_1, r_2, r_3) LIF decreases with MC increasing. The rising LIF of MC 10.37 in third replication (r_3) might be an outlier data.

There was an error signal in third replication that LIF in 10.37% w/w MC was more than 6.57% w/w. One-way ANOVA analyze was done for acceptance or rejection hypothesis of LIF signals equality on the various MCs (Table 4).

Table 4

ANOVA for LIF counts by MC

ANOVA	Some of Squares	D_f	Mean Squares	F_c	Significant
LIF	797414	3	265805	0.980	0.000**
Error	35452	8	4432		
Total	832866	11			

** If PValue<0.01 this means the groups are not statistically equivalent with 99% confidence

MC means were not statistically equivalent with 99% confidence as shown in Table 5.

The Duncan test supported results with significant difference ($p<0.01$) comparing all group mean pairs. It was not any pair means statistically equivalent with 99% confidence

as shown in Table 5. Shah and Stagner (2015) used a Karl Fischer water titrator to make water measurements of granulations stored at 4.16, 4.51, 4.88, 5.89 and 6.53 % w/w MC. They found that increasing Lactose monohydrate MC caused a non-linear decrease in LIF responses. As shown in Figure 7, even though infrared moisture analyzer and handy titration used in this work but total trend of LIF signal variation with increasing MC was similar to their observation. Results of infrared moisture analyzer and handy titration used in this work were similar to Shah and Stagner (2015) who used a Karl Fischer water titrator to make water measurements. Total trend of LIF signal variation with increasing MC was the same.

Table 5

Duncan's multiple range test of LIF signals in MCs

SD	Mean	MC (w/w)
75	791[d]	6.57%
105	633[c]	10.37%
13	302[b]	12.39%
30	142[a]	20.83

All groups with individual "a,b,c,d" indexes are different from the others.

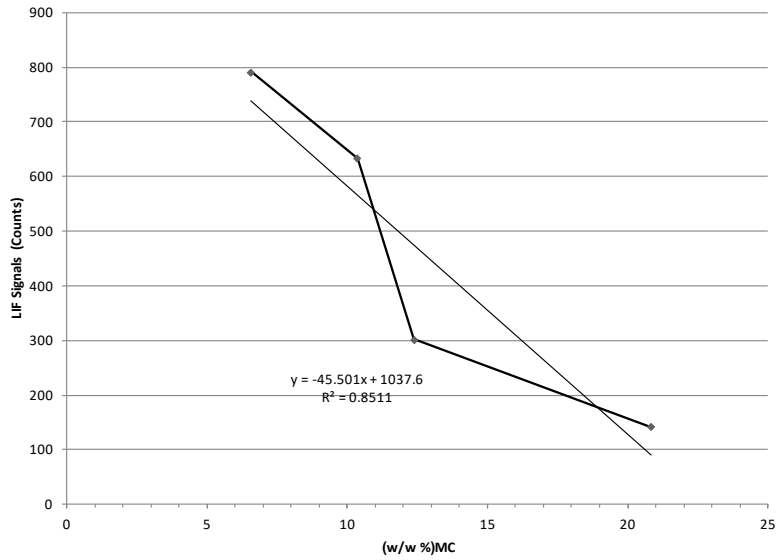

Figure 7. LIF variations with increasing MC; 85% of the moisture contents variations from 6.57% w/w increasing to 20.83% w/w can be explained by the linear model.

CONCLUSION

Nutrient uniformity is crucial for proper nutrition when animals are on low daily intake of food. An LED light excitation with wavelength 405 nm on corn-soybean meal-based diet created a fluorescent emission wavelength with a peak in 492 nm. LIF response increasingly affected by increasing API concentration from 0.05% to 0.50%. DL-methionine concentration caused a linear increase of LIF that fitted to $y = 41.04x + 715.8$ with $R^2 = 0.990$. This means that 99% of the experimental variation can be explained by the linear model and the relationship between LIF and API is statistically significant. Analyze of variance (ANOVA) showed that group means are not statistically equivalent with 99% confidence and also supported by the Duncan test. LIF counts of 0.561 and 0.649 g/mL were more than 0.609 g/mL BD. There was no increasing or decreasing trends in a range of 0.561-0.649 g/ml BD. ANOVA tests indicated that statistically there wasn't significant deference between BD groups. MCs in all three replications from 6.57% w/w increasing to 20.83% w/w decreased LIF Signals. One-way ANOVA analyze was done for acceptance or rejection hypothesis of LIF signals equality on the various MCs. The equality hypothesis (H_0) was rejected and MC means were not statistically equivalent with 99% confidence.

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