

SHORTER COMMUNICATION

APPLICATION OF FLUORESCENT MARKERS FOR HOMOGENEITY
ASSESSMENT OF GRAIN MIXTURES BASED ON MAIZE CONTENTDominika B. Matuszek^{*1}, Krystian Wojtkiewicz²¹Opole University of Technology, Department of Biosystems Engineering, ul. Prószkowska 76, 45-758 Opole, Poland²Wrocław University of Science and Technology, Department of Information Systems, Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland

The paper presents test results for the assessment of the tracer content in a three-component (green peas, sorghum, maize) feed mixture that is based on the fluorescent method. The homogeneity of mixtures was determined on the basis of the maize content (as the key component), which was treated with fluorescent substance: tinopal, rhodamine B, uranine and eosin. The key components were wet-treated with fluorescent substances with different concentrations. Feed components were mixed in a vertical funnel-flow mixer. 10 samples were collected from each mixed batch. Samples were placed in a chamber equipped with UV light and, then, an image recorded as BMP file was generated. The image was analysed by means of the software programme Patan. On the basis of the analyses conducted, data on the maize content marked with a fluorescent marker were obtained. Additionally, the content of the key component was determined in a conventional manner – using an analytical scale. Results indicate the possibility of using this method for homogeneity assessment of the three-component grain mixture. From these tests, fluorescent substances that can be applied in the case of maize as a key component, together with their minimum concentrations, were identified: tinopal 0.3%, rhodamine B 0.001%.

Keywords: grain mixture, fluorescent, homogeneity, image analysis

1. INTRODUCTION

The quality of feed mixtures is determined by means of a number of factors. An adequate feed homogeneity guarantees proper nutrition of livestock intended for food production. The adequate homogeneity can be mentioned as the most relevant among numerous feed quality determinants (Çiftçi et al., 2003; Djuragic et al., 2007; Królczyk 2016).

The mixing process as well as the assessment of its efficiency are becoming more and more important. Nowadays, feeds contain a lot of components often added in small doses (e.g. flavours, vitamins, minerals); therefore a proper level of the homogeneity of such a mixture ensures that the animal feed with it will make a full use of its components.

The ideal, yet theoretical, composition of the mixture characterised by an identical amount of all components in each collected sample of the feed is not possible to be obtained in practise (Rocha et al., 2015). During the mixing of granular and bulk components, particular particles tend to occupy specific places in the bed. The location of particles depends on various factors such as: density, size of particles, interactions between them and design parameters of a mixer (Hogg, 2009). In most cases, the aim under industrial conditions is to obtain a homogeneous mixture which is characterised by the lowest possible

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CV coefficient (not higher than 10%) while maintaining its minimum differentiation between mixing cycles (Rocha et al., 2015).

Reference methods for the assessment of the homogeneity of feedingstuffs are usually based on the determination of various key components, including, *inter alia*, those that are naturally present in the feed, such as: chlorides, carbonates, zinc, copper, manganese, cobalt, methionine or lysine. The second group of methods based on key components added to the feeding stuffs includes iron filings (Microtracer) (Eisenberg, 2008) or amoxicillin, chlortetracycline, doxycycline, lincomycin, tiamulin and tylosin - in the case of medicated feeds (Przeniosło-Siwczyńska et al., 2010). In Poland, the reference method for assessing homogeneity is the determination of the content of chlorides and calcium (Walczyński and Korol, 2007). This article describes the use of high sensitivity photometers to record the light reflected by the coloured particles of the granular mixture. This method was the subject of research of also other authors (Alonso and Alguacil, 1999; Poux et al., 1995; Weinekötter and Reh, 1994). The method of computer image analysis of granular mixtures with different color was described and applied by Boss, Tukiendorf and Matuszek (Boss et al., 2002; Matuszek and Tukiendorf, 2007). This method uses the RGB model to estimate the content of a key component. Among many innovative methods, there are also cases where homogeneity assessment requires manual separation using a set of sieves (Królczyk and Tukiendorf, 2006).

The authors have examined methods for the quality assessment of multicomponent mixtures that can be used for the evaluation of the feed homogeneity. Among the variety of tools, particular attention was paid to the methods using the fluorescence phenomenon and image analysis. These instruments are used in the following industrial sectors: food, agricultural, pharmaceutical and chemical industries (Berthiaux et al., 2006, Coppeta et al., 1995; Dauman et al., 2008; Karumanchi et al., 2011; Lai et al., 2001; Matuszek, 2015; Realpe et al., 2003; Zelko et al., 2012).

The conceptual work (Matuszek et al., 2013) includes attempts to pre-verify the suitability of the proposed method developed by the authors. This article presents the results of the subsequent analyses.

The paper presents the results of homogeneous tests carried out in relation to the three-component grain mixture with the use of fluorescent markers. The suitability of maize as the key component was evaluated and the types and concentrations of the fluorescent solution for this method were determined.

Due to the authors experience the selected method was tested using available equipment. Results are a continuation of the research aiming at developing a fluorescence method applicable for assessing the homogeneity of granular mixtures focusing, above all, on the simplicity, accuracy and reproducibility of results. In order to determine the accuracy of the proposed method, the homogeneity of each of the collected samples was assessed using two methods:

1. by specifying the percentage share of the tracer coated with a fluorescent substance by means of computer image analysis,
2. by specifying the percentage share of the tracer coated with a fluorescent substance by means of the analytical balance.

2. MATERIALS AND METHODS

The solution composed of four fluorescent substances, such as tinopal, fluorescein, eosin and rhodamine B, was used in the research (Table 1). One of the mixtures' components – maize was applied as a tracer. Maize was wet-treated with solutions of fluorescent substances presented in Table 1. After soaking, the grain was naturally dried at the expense of the ambient heat in the laboratory room. After obtaining the appropriate moisture, the tagged grains were stored in appropriately labeled containers at room temperature under the same conditions as the rest of the ingredients.

Table 1. Fluorescence substance used in the tests

Fluorescence tracers	Excitation, nm	Emission, nm	CAS Registry Number	Molecular weight	Formula	Solution, %
Tinopal	350	430	27344-41-8	562.6	$C_{28}H_{20}Na_2O_6S_2$	0.3 0.03
Fluorescein (Uranine)	494	520	518-47-8	376.3	$C_{20}H_{10}Na_2O_5$	0.3 0.03 0.01
Eosin	525	545	15086-94-9	647.9	$C_{20}H_8Br_4O_5$	0.3
Rhodamine B	553	627	88-81-9	479.02	$C_{28}H_{31}ClN_2O_3$	0.3 0.03 0.01 0.007 0.001

Using maize as the key component results from observations and tests conducted and collected at the first stage of the research. The same research excluded the possibility of utilising sorghum or peas for this purpose (Matuszek et al., 2013). The feed mixture contained a three-component structure (green peas, sorghum, maize where the last one was as the key component) with the characteristics presented in Table 2.

Table 2. Characteristics of mixed components

Component	Bulk density, $kg \cdot m^{-3}$	Mean particle size, mm	Share of component, %
green peas	771	6.95	60
sorghum	697	3.94	30
maize	745	8.20	10

These components are often used for the production of feed mixtures. Since multi-component systems are mixed in the industrial practice, tests on multi-component mixtures carried out under laboratory conditions are highly recommended.

The mixing was carried out using a funnel-flow mixer. However, the mixer used is merely a tool for conducting the mixing process which is not the subject of this paper. More details concerning the parameters of the test stand and the mixing process using the pouring method can be found elsewhere (Matuszek et al., 2008). The total mass of the mixed material was 1000g. The mixer tank was filled with 600g of green peas, 300g of green sorghum and 100g of coloured maize. The mixing process was started basis on the flow from one tank to another. Each time, 10 dumps were made. At the end of the mixing process, 10 samples ($N = 10$) of feed from each tank level were taken (thanks to a special demountable construction of the mixer). The weight of each sample was 40 g. Taken samples before further analysis were placed in sun-proof packs.

For each sample, the share of the tracer was assessed by two methods:

2.1. Method with the use of computer analysis of image

Each collected sample was placed on a clean glass – the Petri plate - of diameter 120 mm and then subsequently immersed in UV-chamber. The chamber was made of the material impermeable to light

rays, equipped with UV light (two 15 W fluorescent lamps without housing). A camera was fixed in the upper part of the chamber to record images in BMP format. A sample of the mixture was placed inside a sealed chamber made of a material not impermeable to external rays. A proper design of the test stand is very important to maintain the reproducibility of measurements (Fig. 1). The sample was illuminated with UV rays and then a picture was taken. Photos of each sample – three shots at the resolution of 1600x1200 pixels were taken. Images obtained in this manner were subjected to computer analysis, using original PATAN® software programme. On the basis of a value scale, the RGB-256 components of the test areas were defined and divided into classes: 1st class – tracer, 2nd class – green peas, and 3rd class - sorghum. Then, the area designated for analysis, i.e. circular area, was determined. Thanks to the analysis of the designated area, information on the percentage share of each class (Fig. 2) in the entire area was obtained. The data on the content of tracer – 1st class (average of three measurements) were used for further analysis. It should also be noted that the size obtained as a result of computations performed using the computer-aided image analysis is a dimensionless parameter, and more precisely, it describes the sum of the area of individual elements belonging to the „tracer” class in relation to the total surface. Therefore, an extremely important aspect is that specific measurements were carried out under the same metrological conditions.

These conditions include, for instance, the distance between the camera and the test sample, lighting conditions, and sample dimensions. The reproducibility of measurements was achieved by means of the specialized test stand ensuring control over the relevant image acquisition parameters. A verification test consisting in the determination of the weight of the tracer in relation to the mass of the entire sample was the reference for the computer-aided image analysis. In order to satisfy the requirements of the research work, it was assumed that this research method is unequivocal.

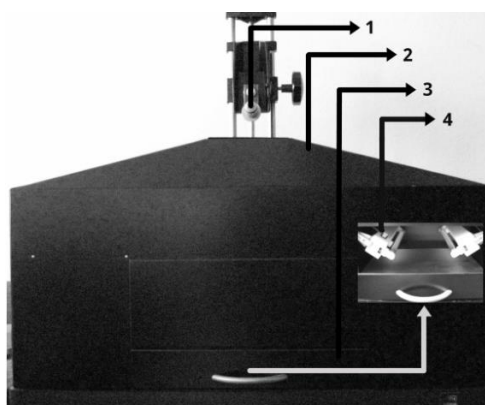


Fig. 1. Stand to take pictures, 1. Camera tripod, 2. Chamber casing, 3. Tray for material samples, UV lighting

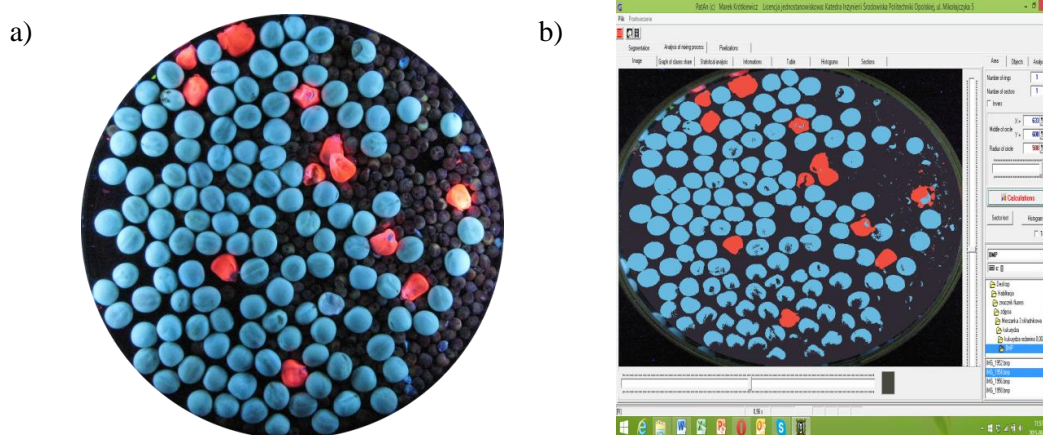


Fig. 2. Image of feed mixture sample (Rhodamine B solution 0.001%) a) image under UV light recorded as .gmp file, b) image after analysis in the Patan® programme.

2.2. Method with analytical scale (control research)

This method based on the precise assessment of the tracer mass in each sample with electronic scales with an accuracy of 0.01 g. Then, in order to compare results obtained from a computer-aided image analysis and the gravimetric method, the so-called percentage content of the tracer (maize) was determined. This parameter was evaluated based on the juxtaposition of the mass (electronic scales) and the share of the tracer (computer-aided image analysis) for various amounts of grains - from 5 to 100. Table 3 obtained in such a way is a useful template for the recalculation of the mass of the tracer to express it as a percentage share of this component (Table 3).

Each series of tests was carried out under identical laboratory conditions as described above.

3. RESULTS AND DISCUSSION

Statistical calculations were performed with Statistica version 12.0 software (StatSoft 2015).

For calculations of t-Student statistics, the following assumptions were made:

- Each of the two populations (population should be understood as the method for the assessment of the share of the tracer) is normally distributed (this assumption was verified using the Shapiro-Wilk test).
- Both populations have equal variances (this assumption was verified by the Fisher test).

The null hypothesis is:

$$H_0: \mu_1 = \mu_2 \quad (1)$$

The means of the populations of interest are equal at the value of $r = 2$.

The alternative hypothesis is:

$$H_1: \mu_1 \neq \mu_2 \quad (2)$$

The means of the populations of interest are different at the value of $r = 2$.

Verification of assumptions (points 1 and 2) was based on the Shapiro-Wilk test (test for normality) and the Fisher's test (F-test of equality of variances). The analysis of hypotheses was performed on the basis of t-Student test for independent samples, by assuming the value of significance level equal to $\alpha = 0.05$ (Aczel 2012).

The results of research and statistical calculations are presented in Table 3.

As a result of the analysis of obtained average and standard deviation values, it can be observed that there are significant differences in the share of the tracer on the basis of results obtained with the computer-aided image analysis method and the gravimetric method. It can be noticed that the concentration of the solution is crucial for the suitability of the applied method. It can be presented using the example of Rhodamine B solution, which, at the concentration of 0.3%, is not suitable to be used in the proposed method, while at the concentration of 0.007%, allows to obtain the best results.

Based on the probability analysis with p-value (at $\alpha = 0.05$) presented in Table 3, there is no reason to reject the null hypothesis on the equality of average shares for the main component in the following solutions: 0.3% Tinopal, 0.3% Fluorescein, 0.01%, 0.001% and 0.007% Rhodamine B. It can be assumed that the results are homogeneous and the mixtures analysed by means of these two methods have similar compositions.

Table 3. Results of the assessment on the share of tracer in feed mixture

Fluorescent solution in %	Computer-aided image analysis ^a , %	Gravimetric method ^a , %	<i>t</i> value ^b
Tinopal			
0.3	2.98±0.61	3.05±0.64	0.23*
0.03	2.11±0.84	3.79±0.76	-4.23**
Fluorescein			
0.3	4.92±1.06	4.03±0.98	1.47*
0.03	9.02±1.79	4.20±1.27	6.28**
Rhodamine B			
0.3	10.01±2.52	4.03±0.85	6.44**
0.03	5.06±0.76	3.69±0.40	4.52**
0.01	5.48±1.15	5.70±1.11	-0.39*
0.001	6.18±1.4	6.32±1.54	0.18*
0.007	6.31±0.77	6.43±0.82	-0.31*
Eosin			
0.3	8.21±1.65	4.27±0.82	6.14**

^a Mean of three analyses and then samples ± standard deviation.

^b *t* value with the compare of two methods of mixture homogeneity assesment ($n = 2$, $N = 60$)

* Not significance difference beetwen populations at $p < 0.05$

** Significance difference beetwen populations at $p < 0.05$

For other solutions of fluorescent substances (0.03% Tinopal, 0.03% Fluorescein, 0.3% and 0.03% Rhodamine B, 0.3% Eosin) there is no reason to approve the null hypothesis. Therefore, it is concluded that the results obtained by means of these two methods are statistically different.

In case of Rhodamine B, satisfactory results were obtained for three different solutions: 0.01%, 0.007% and 0.001%, respectively. However, it seems that the 0.001% solution is the best (due to the results and economic aspects).

From the experiments and conducted computations, the following solutions of fluorescent substances can be used to assess the homogeneity of the maize-based feed: 0.3% Tinopal, 0.001% Rhodamine B.

The share of tracer in the grain mixture obtained by the fluorescence method can be subsequently applied for standard computations of homogeneity parameters (for example the CV value).

The tests were carried out as they should be in laboratory conditions. The specificity of fluorescent markers requires certain conditions during analysis. An example is the need to protect tags and samples of mixtures from external influences, excluding liquid additives for mixing and wear protective clothing. Fluorescent markers should be considered as chemical reagents that require appropriate conditions (information in safety data sheets). Therefore the possibility of applying the method in industrial scale is considerably reduced and certainly requires additional analysis. Additionally the authors are planning to do more tests with different multi-component grain mixtures of different dimensions of grains and grains after grinding. At this stage of research this method may be used to evaluate the homogeneity of selected granular feeds or granular mixtures before grinding.

4. CONCLUSIONS

It has been shown that there are no statistically significant differences in the share of the key component obtained by means of the two methods for the following fluorescent substances: 0.3% Tinopal, 0.3% Fluorescein, 0.01%, 0.001% and 0.007% Rhodamine. However, statistically significant differences in the share of the tracer were found for substances such as: 0.03% Tinopal, 0.03% Fluorescein, 0.3% and 0.03% Rhodamine B, 0.3% Eosin. The proposed solution can be applied for the determination of the share of the tracer for three-component grain mixtures. Two liquids, namely: Tinopal 0.3% and Rhodamine B 0.001%, are suggested in the methodology based on the analysis of the maize (as a tracer) content.

SYMBOLS

CV	coefficient of variation
H_0	null hypothesis
H_1	alternative hypothesis
p	probability value
α	significance level
μ	mean
r	number of population

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