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STEREOLOGICAL ANALYSIS OF PEA PROTEIN IN MODEL SAMPLES

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ABSTRACT

With the growing popularity of various plant proteins used as raw materials for meat production, interest of manufacturers to extend the range of such raw materials is increasing as well. Manufacturers are trying to minimize the cost of manufacturing their products with simultaneous preserving the nutritional value of their products to the maximum extent possible. Such cheaper raw materials, which are also nutritionally rich, include pea protein. Another advantage for manufacturers is the fact that legislation does not order them to indicate pea protein presence in case of its addition, as it does for other allergenic ingredients, although this legume contains storage proteins which can cause a variety of allergic reactions, just like other legumes. Currently no method used for its qualitative determination has been described in literature, let alone its quantitative determination. Our work describes a possible method that can be applied for its quantification. It is a stereological method applied to microscopic sections stained by immunohistochemical staining based on the avidin-biotin complex using monoclonal legumin (1H9) as the primary antibody. The stereological method is based on geometry, it applies knowledge of geometry to analyze a sample of diverse origin, size and internal structure. Despite potential shortcomings in staining microscopic preparations, stereology allows us to perform quantification based on knowledge of morphology of the observed structures. This work describes a procedure of a known pea protein addition quantification in model meat products by means of Ellipse software. Pea protein quantification was performed in two ways. In the first case ten microimages of all sections prepared were examined, while in the second case one scan of the entire section was analyzed. Based on the results, Spearman's correlation coefficient was calculated, which confirmed our assumption of correlation between the protein added into the product and the measured area in microimages. In both ways Spearman's correlation coefficient was rSp = 1000. We obtained regression equations in MS Excel, which can be used for calculation of pea protein addition based on measured area of this protein in microscopic section.

Keywords: Vegetable proteins; microscopy; immunohistochemistry; allergens; meat products

INTRODUCTION

In the meat industry, raw materials in the form of vegetable proteins, which are used as a meat substitute, are very common **(Modi et al., 2004)**. During meat production is frequently used various vegetable and animal proteins. The most commonly employed plant - origin proteins are wheat and soy proteins. Meat product also contain from animal - origin proteins as plasma, collagen or milk protein **(Petrášová, 2015)**. Some of these vegetable proteins are classified as allergens in the legislation **(Regulation (EU) No. 1169/2011)**. Besides other reasons, this motivates the producers' efforts to replace them with other vegetable proteins that are not ranked among the allergens that must be indicated in harmony with the aforementioned legislation. Pea protein belongs among the most common ones **(Baticz, 2001)**. Like other legumes, however, pea proteins also include storage proteins which can cause allergic reactions. The literature identifies analysis of polyphenols characteristic of certain legumes and HPLC method which can detect up to 0.1% addition of soy protein in a meat product, as potential detection methods. Detection of lupine can be performed similarly, nevertheless, reliable detection of pea has not been achieved yet **(Mession et al., 2012; Mellenthin and Galensa, 1999)**. Other possible methods that can be used to detect vegetable protein are microscopic methods.

Microscopic methods belong among the oldest analytical methods and can be applied to demonstrate food components. These methods are simple, able to differentiate and identify individual basic components in the foodstuffs. The most commonly used methods in practice are histochemical methods, but now there is a wide range of options for processing and preparation of samples and also investigative techniques from classic to those that apply the most innovative technical equipment. Imaging techniques belong among the most suitable techniques to examine the structure of food **(Kaláb et al., 1995)**. As argued by **Tremlová et al. (2013)**, addition of vegetable protein can be detected using microscopic methods if they are present in the product in a sufficient size for light microscopy.

Javůrková et al. (2015) mentioned the use of modern microscopy methods for a qualitative as well as quantitative examination of the products. These methods provide information about location all components of the sample examined. One of the methods is image analysis.

Image analysis is often using as qualitative methods for meat products. The image obtained by microscopic methods can undergo quantitative analysis while preserving all the advantages of microscopy. In such a case, the input is image data and the output is a description of the image. Quantitative microscopic examination may be indicative or accurate **(Pospiech, 2008)**. Quantitative image analysis allows us to describe and specify all information obtained by microscopic (as well as macroscopic) scanning. It allows a detailed comparison of samples, accurate processing of information obtained and different ways of expressing the results achieved. The procedure for image analysis consists of creating photographs and their subsequent analysis using a program. To scan microscopic slides, a set composed of a light microscope and a digital camera or camcorder can be utilized. The very analysis involves creating a template (colour and brightness are usually selected from among image parameters) to identify the selected components and subsequently to measure their surface area and the entire section area. This results in numerical data obtained from the image, thereby permitting a detailed comparison of different samples, accurate processing of information obtained and different ways of expressing the results. Recorded data can be evaluated using different statistical methods. Another great advantage is the ability to compare objects scanned currently with objects stored previously. Integration of image analysis into the manufacturing process allows on-line measuring which is very useful, even necessary, in the inspection process in food production. The main advantage is the possibility to obtain a result without direct contact with the sample. This completely minimizes the risk of e.g. cross-contamination **(Javůrková, 2014)**. Image analysis based on computer technology is developing rapidly and allows to obtain objective results, because it uses a large number of images in statistical processing. This means that one of the biggest pitfalls of microscopy can be avoided, namely selecting and publishing only the best images as sample „representatives“ for demonstration of results and publishing **(Tremlová et al., 2013)**. Therefore, the literature considers the results obtained by image analysis in the examination of meat and meat products objective, accurate and comparable with data produced by chemical methods.

Development of image analysis in the field of microscopy largely coincides with the development of stereology. Stereology is based on geometry, it applies its knowledge to analyze samples of diverse origin, size and internal structure. It deals with statistical derivation of geometric properties of the examined structures and object from test probes applied to oriented sample sections **(Glaser and Glaser, 2000)**. Stereology is used by **Flintová and Meech (1978)** in their work. They used a method based on counting the points in a grid in quantifying textured soy protein, where estimated surface area of the object being measured was based on counting the area belonging to one point and the number of grid intersections with the object being measured. The advantage of this measurement includes its ease and affordability and the possibility to examine the image not only based on colour contrast, but also on the basis of morphological criteria. The disadvantage of stereology is manual processing that is time consuming and not always more accurate than automatic examination. Image analysis used as quantification method requires optimum contrast between the monitored component and other components in the product **(Aguilera and Stanley, 1990)**, while stereology does not have this requirement **(Lukášková Řezáčová, 2011)**.

An integral part of quantitative studies is statistical evaluation of results. Correctness of the analysis may be affected by so-called deflections of the measuring system itself, processing (various thickness of sections, uneven stainability, creation of artifacts, change of protocols etc.), examiner (whether in manual measuring or error rate in mathematical processing of results) or improper calibration of the digital recording collection equipment. In current practice, variability of sample processing can be reduced by standardizing and automating the examination workflow **(Tonar, 2008)**.

Currently, there is no commercially available method for demonstrating the addition of pea protein, let alone its quantification in a meat product. Therefore, the aim of our work was to create a method and protocols for its quantitative determination in meat products.

MATERIAL AND METHODOLOGY

Model meat products (MMP) containing pea protein additions in concentrations of 0.1, 1.0, 2.0, 3.0, 4.0 and 5.0% were produced for the examination. The products were made of ground chicken breast meat with the addition of pea protein. These model products were cooked at 70 °C for 10 minutes. Four blocks (A, B, C, and D) of 1 mL were collected from each product and frozen. These blocks were then sliced into sections 10 µm thick using cryostat HM 550 (Germany, Microm).

Subsequently, these cryosections were stained with immunohistochemical (IHC) staining method of ABC complex. The primary antibody used was monoclonal legumin (1H9). With respect to previous testing of immunohistochemical staining and consideration of costs, antibody concentration of 1:1000 was selected.

Quantification of the immunohistochemical examination results was performed in two different ways. First, quantification was performed in digital images of MMP, which had been taken in Eclipse E200 microscope (Nikon, JPN) using EOS 1100D camera (Canon, JPN) and processed by DSLR REMOTE Ver. 2.2.2.1 (UK) at a magnification of 100x (Ellipse 1). The entire sections were scanned in this way and a random selection of 10 images from all blocks of the sample was performed. As reported by **Řezáčová Lukášková (2011)**, who used stereology to quantify the addition of wheat protein in her work, in order to achieve the coefficient of error (CE) <0.2, at least 8 images of the sample with added proteins should be investigated.

Also, these samples were scanned using Eclipse Ci-L microscope (Nikon, JPN), DFK 23U274 camera (Imaging Source, GER) and motorized stage of Prosca III (Prior, USA) in NIS Elements Basic Research 04.13.04 software (Laboratory Imaging, Czech Republic) at magnification of 40x (Ellipse 1). Thanks to the motorized stage and NIS software, the entire sections could be scanned and

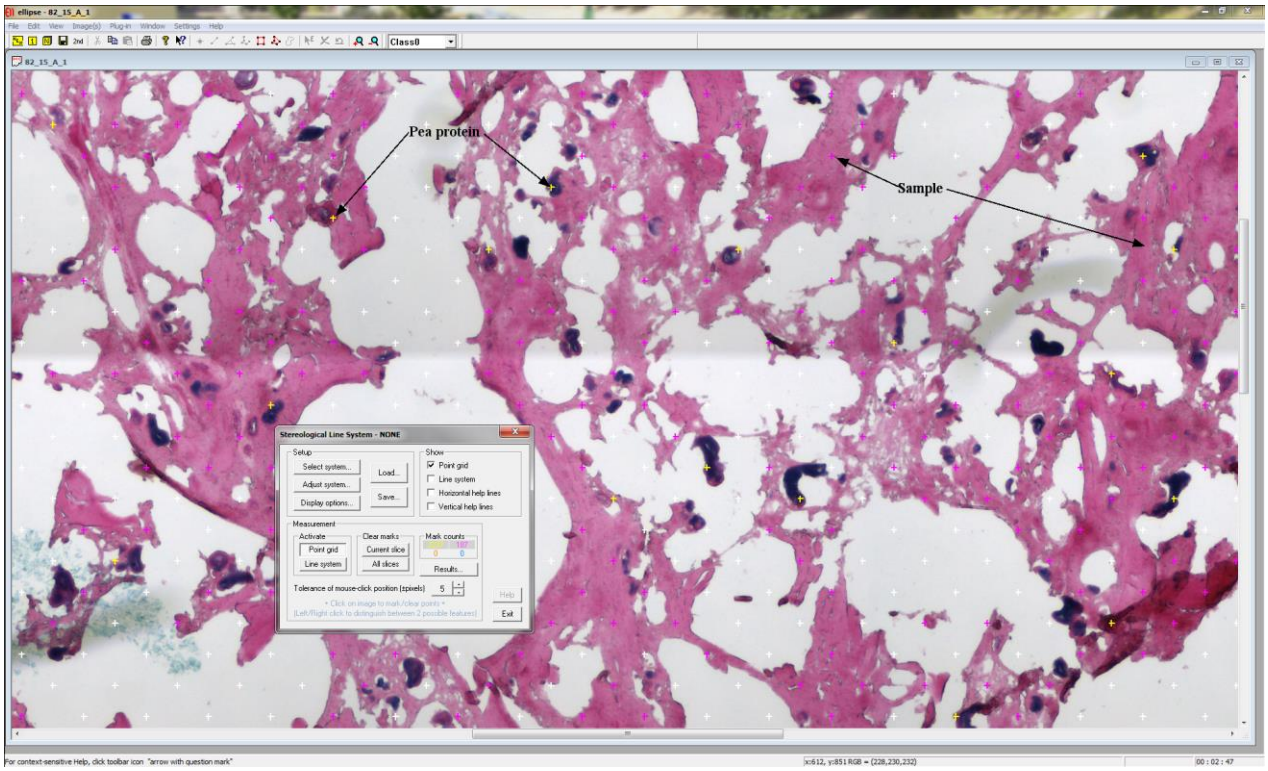

Figure 1 Example of pea protein quantification in the Ellipse software, sample no. 82_15 with 4% pea protein addition, IHC staining method, 40x magnification.

subsequently merged into a single image by the program and thus the Ellipse 1 software was able to examine 1 image (the entire section) from each sample.

Subsequently, the actual quantification of the pea protein addition was performed using Stereological Line System program by Ellipse version 2.0.7.1. (ViDiTo, Slovakia) (Figure 1) with adjusting the size of the grid point for the quantification of individual images and for the entire sections to 20745.5 μm^2 (a total of 157 points in the image) and 20764.8 μm^2 (a total of 7616 points in the image), respectively.

Results obtained by the stereological method of microimages of model meat product sections were contrasted to the contained values in the prepared concentrations of protein additions by means of the Spearman's correlation coefficient rSp (a nonparametric method that uses the order of values of the monitored variables in the calculation, and which can be used to describe any relation (linear and nonlinear). Relation of variables may have a generally upward or downward character **(Bedáňová and Večerek, 2007)**. The coefficients were calculated in the UNISTAT ver. 6.0 software. Moreover, a regression analysis (studying what relationship exists between the variables – linear, quadratic, logarithmic, etc. – and how a dependent variable Y changes depending on changing its predictor (independent variable) X. It is thus a one-sided dependence, unlike the correlation analysis studying bilateral reciprocal relation between two random variables was performed in MS Excel **(Bedáňová and Večerek, 2007)**.

RESULTS AND DISCUSSION

The measured areas of pea protein added for each concentration for both methods of scanning are listed in Table 1 and Figure 2. Table 1 and Figure 2 compare addition of proteins in the weight percentage and section areas in area percentages measured. The results indicate that with increasing addition of the proteins increases the measured area in section by lowest concentration (0.1 percentage).

Figure 3 illustrates a comparison of the examined areas of microscopic slides of model meat product samples when the examined area was 2.9x to 4.7x greater in the event of Ellipse 2 than the examined area Ellipse 1.

Using the first method of capturing images (Ellipse 1) by means of the Ellipse SW, a total of 60 images (10 images of a sample for each concentration) were quantified. Protein surface areas of 1.71%, 3.40%, 6.18%, 8.47%, 9.42%, and 11.26% were detected for the meat product samples with pea protein additions of 0.1%, 1.0%, 2.0%, 3.0%, 4.0%, and 5.0%, respectively. Based on the calculated Spearman's correlation coefficient, statistical dependence (rSp = 1000) was demonstrated for each concentration of pea proteins addition in model meat products.

In the latter method of capturing images (Ellipse 2), where sections were scanned whole, six images (one image of the entire section for each concentration) were examined. Protein surface areas of 0.66%, 2.82%, 4.46%, 6.09%, 7.71%, and 9.52% were measured for pea protein additions of 0.1%, 1.0%, 2.0%, 3.0%, 4.0%, and 5.0%, respectively. Statistical relation was also confirmed by the calculated Spearman's correlation coefficient (rSp = 1000), which confirm high dependence between pea protein addition and measured area of this protein in microscopic sections.

Table 1: Measured areas of pea protein for each MMP concentration.

Prepared MMP concentrations	Measured area of proteins [%] by Ellipse SW	
	Ellipse 1	Ellipse 2
0.1	1.71	0.66
1.0	3.40	2.82
2.0	6.18	4.46
3.0	8.47	6.09
4.0	9.42	7.71
5.0	11.26	9.52

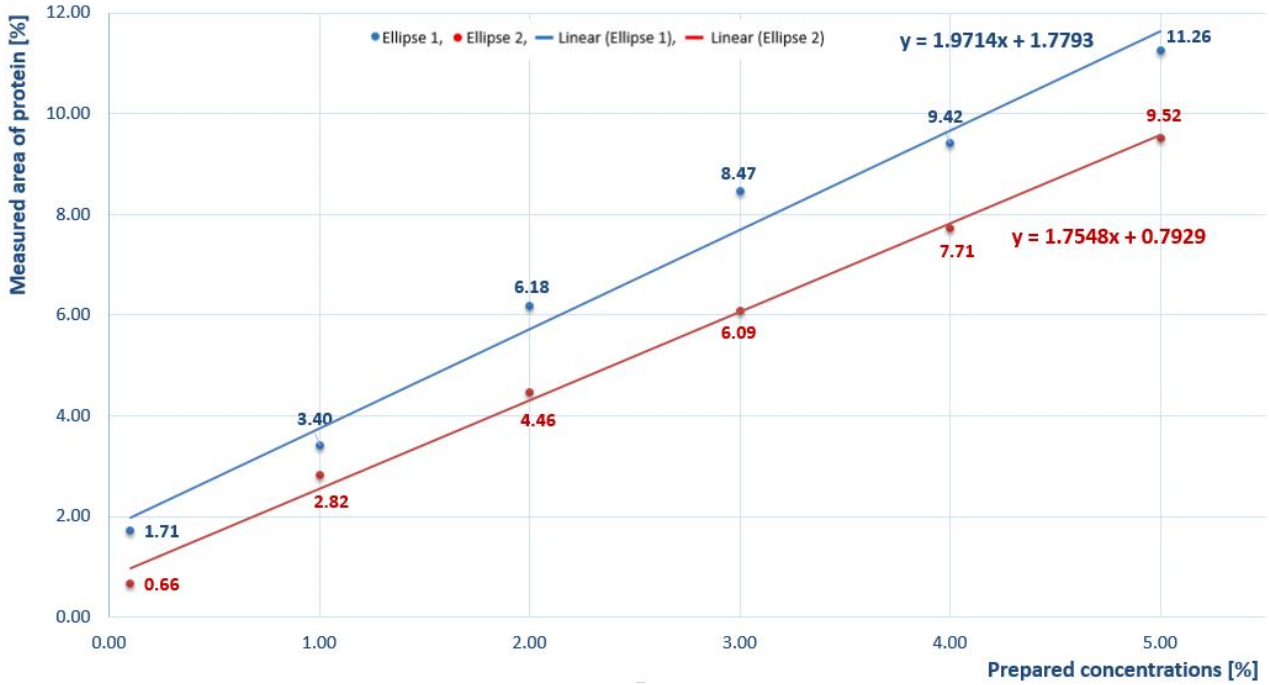

Figure 2 Dependence of protein area measured by Ellipse on the prepared concentrations.

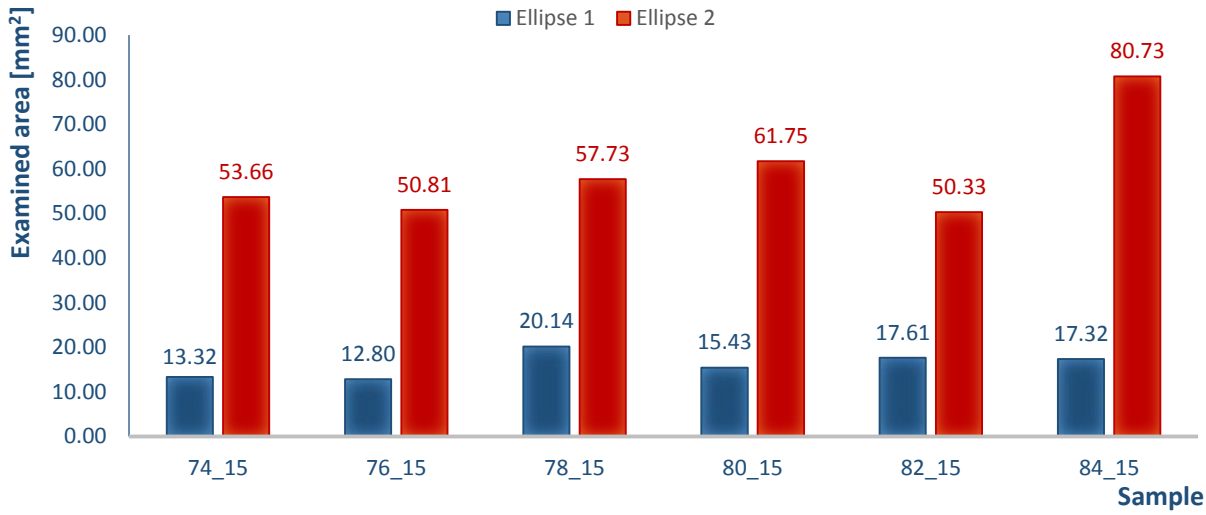

Figure 3 Comparison of areas of the examined sections.

Note: Sample number 74_15 contains 0.1% of pea protein, sample number 76_15 contains 1.0% of pea protein, sample number 78_15 contains 2.0% of pea protein, sample number 80_15 contains 3.0% of pea protein, sample number 82_15 contains 4.0% of pea protein and sample number 84_15 contains 5.0% of pea protein.

In addition to evaluating both procedures of capturing images and their results, regression analysis of the results obtained, which evaluates the dependence of quantitative statistical features, was also conducted. Obtained regression equations are shown in Figure 2. Regression equations can be used for calculation of pea protein addition based on measured area of this protein in microscopic section.

CONCLUSION

Based on the results obtained and calculated Spearman's correlation coefficients, hypothesis regarding the suitability of stereology for the quantitative determination of pea protein additions in model meat products was confirmed. As reported by **Aguilera and Stanley (1990)**, stereological quantification is more time consuming than image analysis. However, in view of incompletely 100% results of immunohistochemical staining, where the image analysis software would fail to mark the protein automatically leading to an incorrect result, this method appears to be appropriate. Also, reduction of stereological points in the grid and thus shortening the time for the quantification itself is worth considering. In case of using scans of entire section, one section would be enough for the quantification, which would also shortened the examination.

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