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DETERMINATION OF HMW – GS IN WHEAT USING SDS – PAGE AND LAB-ON-CHIP METHODS

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ABSTRACT

SDS-PAGE is widely used to determine the amounts of the different gluten protein types. However, this method is time-consuming, especially at early stages of wheat breeding, when large number of samples needs to be analyzed. On the other hand, LoC (Lab-on-Chip) technique has the potential for a fast, reliable, and automatable analysis of proteins. Benefits and limitations of Lab-on-Chip method over SDS-PAGE method in gluten proteins evaluation were explored in order to determine in which way LoC method should be improved in order to make its results more compliant with the results of SDS-PAGE. Chip electrophoresis provides a very good reproducibility of HMW-GS patterns. Moreover this approach is much faster than the conventional SDS-PAGE methods requiring several hours for an analysis. Another advantage over traditional gel electrophoresis is lower sample and reagent volume requirements, as well as specialized protein standards for accurate reproducibility and quantification. In the present study, we identified novel complex allele located at the locus *Glu-1B*.

Keywords: wheat; HMW-GS; SDS-PAGE; LoC

INTRODUCTION

Wheat (*Triticum*) is one of the key crops for nutrition, being the second most grown crop in 2016 with a global production of 749.3 million tons **(FAO, 2017)**. Mainly hexaploid bread wheat or common wheat (*Triticum aestivum* L.) is used as forage and for bread making pointing out the economic importance of these varieties as well as durum wheat (*Triticum durum* L.), a tetraploid species, especially used for pasta **(Cauvain and Young, 2003)**. It is generally known, that wheat gluten has a major effect on the end-use quality of baking industry products, since it is responsible for the visco-elastic properties of the dough. This protein macromolecule is composed of two components, gliadins and glutenins. Gliadins are responsible for the extensibility of dough, whereas glutenins for the dough elasticity **(Payne et al., 1984)**. Glutenins are classed as high molecular weight (HMW) encoded at *Glu*-1 loci and low molecular weight (LMW) encoded at *Glu*-3 loci. HMW glutenin subunits are further subdivided into high M_r x – type with 80 – 88 kDa and low M_r y – type with 67 – 73 kDa subunits **(Payne, Holt and Law, 1981)**.

Correlations and genetic studies of HMW-GS **(Payne et al., 1987)** established subunits with both positive (5 +10) and negative (2 +12) effects on bread making quality. Other allelic variant pairs showed similar results **(Payne et al., 1987)**. In general, a null at *Glu-1A* locus, subunit 6 +8 encoded at *Glu-1B* and 2 +12 at *Glu-1D* are negatively related with the quality parameters **(Weegels, Hamer and Schofield, 1995)**. The highest polymorphism of HMW – GS is regularly detected on 1B chromosome **(Li et al., 2010; Gregová et al., 2011; Hernández et al., 2012; Kuťka Hlozáková, Gregorová and Gálová, 2015)**. A scoring system for HMW-GS has been developed **(Payne et al., 1987)** as the sum of the contributions of each of the three HMW-GS loci. The breeder particularly needs this information to predict the dough-strength potential of the large numbers of lines involved at the earliest stages of breeding, thus to ensure that the poor-quality lines are not unnecessarily propagated. In officially accepted food testing procedures **(Wrigley, 1992)**, SDS – PAGE is used to assess this presence and quantity.

However, the traditional SDS-PAGE method for analysing glutenin subunit composition has several disadvantages. For instance, SDS-PAGE is time-consuming and includes a number of necessary manual steps, such as staining, destaining, imaging, analyzing **(Hsieh and Chen, 2007)**. Quantification can also be difficult **(Hou and Ng, 1995)** and one of the used chemical is acrylamide, a potential neurotoxin. The new, promising, fast electrophoretic technique for protein examinations is a microfluidic or Lab-on-Chip (LoC) method, which allows the integration of electrophoretic separation, staining, destaining, and fluorescence detection into a single process which can be combined with data analysis. This new technique is comparable to time consuming

SDS-PAGE stained with standard Coomassie in sensitivity, sizing accuracy and reproducibility **(Kuschel et al., 2002)**. However, the sizing accuracy of SDS-PAGE and chip – based analysis depend on the protein characteristics and may therefore vary for individual proteins. Some proteins may not migrate according to their molecular weight. In general, the sizing reproducibility of the LoC method is excellent, commonly achieving a sizing reproducibility of 5% or better **(Kuschel, 2000)**.

Scientific hypothesis

This aim of this work was to explore the possibilities of application of novel LoC method for analysis of glutenin subunits in comparison to SDS-PAGE, especially for HMW-GS, isolated from European common wheat varieties, where novel HMW-GS were found.

MATERIAL AND METHODOLOGY

Plant material

The European wheat genotypes used in this study were obtained from the collection of wheat genetic resources maintained in the Gene Bank of the Slovak Republic (National Agricultural and Food centre, Plant Production Research Center, Piešťany).

Protein extraction and SDS-PAGE

Seed storage proteins were isolated from the endosperm of intact, dry and mature single seeds. There were analysed one hundred individual grains from each genotype. Seed homogenization was carried out by grinding. Glutenins were extracted by standard referee method ISTA and were performed by discontinuous PAGE based on ISTA methodology **(Wrigley, 1992)** using the electrophoretic unit Protean II (BioRad). Protein fractions were stained by Coomassie Brilliant Blue R – 250. The separate gluten subunits were identified by the nomenclature of **Payne and Lawrence (1983)**.

Protein separation on chip electrophoresis

The commercial Agilent Protein 230 analysis kit and the 2100 Expert software were used for the glutenin fractionation. The Agilent automated electrophoresis system applies a combination of microfluidic separation technology and sensitive fluorescent detection of proteins. It automatically performs all steps of gel-based electrophoresis (sample separation, staining, destaining, imaging, band detection, data analysis). The analysis of each chip takes 30 min for a set of ten samples. The 2100 Expert software displays the separation results in both the electrophoreogram (peak) and simulated gel views.

RESULTS AND DISCUSSION

Protein content and composition are key parameters related to various aspects of end-use quality of bread wheat *(T. aestivum* L*).* The classification of these proteins into solubility groups is used in the nomenclature for a long time. The UPOV method **(Wrigley, 1992)** for wheat variety differentiation is based on the comparison of Coomassie-stained polyacrylamide gels after one-step grain extraction of weight-matched single grains. For protein band assignment a system according to **Payne and Lawrence (1983)** is accepted where molecular weights of the subunits were determined by SDS-PAGE and the largest HMW-GS, in terms of molecular weights, got the lowest number (Figure 1a, Figure 1b). Typically, only HMW-GS with a molecular weight larger than 100 kDa are considered for variety assignment.

Protein sizing with the chip-based protein analysis system was performed by running the protein sizing standard on each chip from a designated well. Following the analysis of this sizing standard, the software generated a standard curve of the measured migration time versus the known molecular weight of each standard protein which was used to determine the size of each of the proteins detected within the sample **(Kuschel et al., 2002)**. Internal standards, the lower and upper marker, were included in each sample. The protein 230 assay exhibited clear protein patterns in the desired molecular weight range between 14 and 230 kDa which was moreover similar to the SDS-PAGE pattern. Distinct molecular weights could be assigned to interesting protein signals (Figure 2). Therefore, the protein 230 assay was chosen for all further experiments. It is obvious that color intensity of protein bands is caused by concentration of individual HMW-GS.

In this study, the HMW-GS composition is known for all samples and Table 1 lists the names of the wheat varieties together with all HMW-GS. Nevertheless, it has to be mentioned that the determined molecular weights were, in all cases, higher than those derived from SDS-PAGE. Six protein bands corresponding to HMW-GS were in a molecular weight range from 100 to 220 kDa which is in accordance with findings **Marchetti-Deschmann et al. (2011)**; **Balázs et al. (2011)** and **Chňapek et al. (2015)**. This fact was already observed for the first generation of CGE-on-a-chip protein assays (protein 200+) **(Uthayakumaran, Batey and Wrigley, 2005; Uthayakumaran et al., 2006)** where the authors could assign certain HMW-GS to distinct peaks based on the analysis of wheat mutants lacking certain alleles. The determination of higher molecular weights for the extracted proteins can only be explained by a lower electrophoretic mobility of the HMW-GS in the given capillary-based gel system caused maybe by interaction of the proteins with the capillary walls or hindered mobility of protein aggregates in miniaturized systems. Additionally, some of the HMW-GS are migrating in a different order in comparison to SDS-PAGE.

Figure 1a a Figure 1b show the inverted migration order of HMW-GS numbers 5 and 1. This finding corroborates the assumption that the electrophoretic mobility of HMW-GS is highly affected by the chosen capillary-based separation system. One possible explanation can be the fact that glutenins are glycine- and glutamine-rich proteins (>50% of amino acids composition) with average isoelectric points of 5 to 6. It is a known fact that acidic proteins tend to bind SDS poorly **(Eley et al., 1979)** which subsequently affects electrophoretic mobility adversely. The phenomenon of changed migration order in electrophoresis was also already observed for non-miniaturized CGE systems **(Weegels, Hamer and Schofield, 1995; Sutton and Bietz, 1997; Bean and Lookhart, 1999)**.

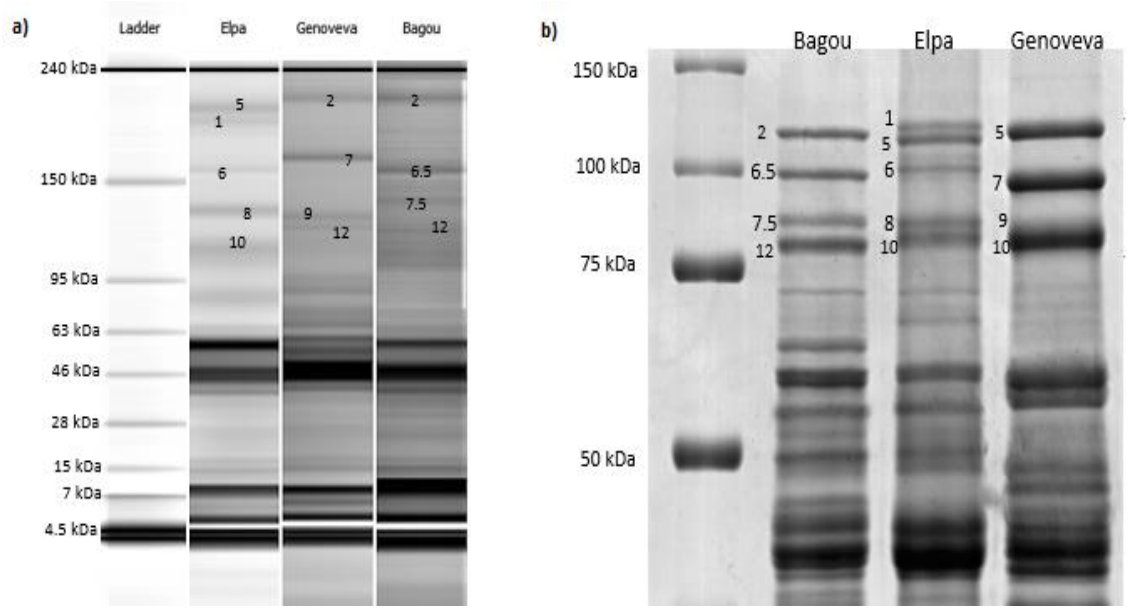

Figure 1 Comparison of the protein patterns of chosen varieties from chip electrophoresis (a) and SDS-PAGE (b).

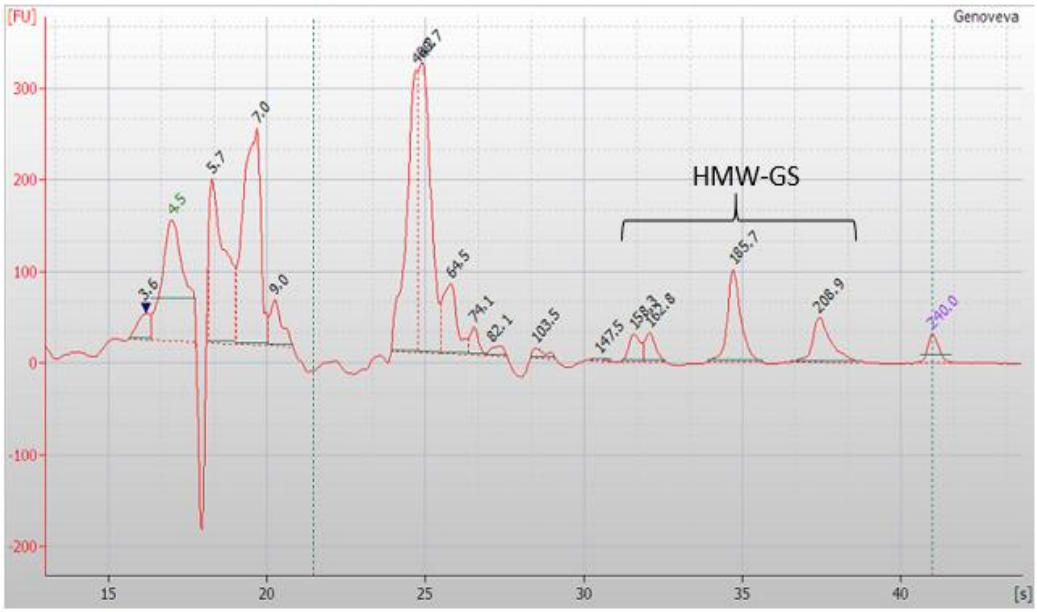

Figure 2 Electrophoreogram of chosen wheat variety Genoveva with HMW-GS identification.

Table 1 Determined molecular weights for proteins extracted by single-grain extraction by means of SDS-PAGE and Lab-on-chip analysis.

HMW – GS	SDS – PAGE (kDa)	LOC (kDa)
1	113	206 – 212
2	108	214 – 216
2*	108	205
5	105	216 – 224
6	100	180
6.5	99	159
7	98	169 – 188
17	90	171
18	89	138
7.5	88	146
8	86	148 – 153
9	83	130
10	82	126 – 134
12	80	129 – 131

Several authors used LoC method for identification and quantification HMW-GS in different wheat varieties **(Uthayakumaran, Batey and Wrigley, 2005; Uthayakumaran et al., 2006; Marchetti-Deschmann et al. 2011)**, whereas **Balázs et al. (2011)** confirmed that influence of environmental conditions on quantity of wheat protein subunits could be monitored using this method. **Chanvrier, Uthayakumaran and Lillford (2007)** followed the polymerization of protein of wheat gluten under processing such as extrusion, while **Maforimbo et al. (2008)** studied the interaction of glutenin subunits and soy proteins by LoC method. Furthermore, molecular weight and concentration of different compounds which are involved in biochemical processes such as nicotinamide adenine dinucleotide phosphate NAD(P)+ isolated from pea, soybean, and wheat proteins **(Nagaoka, 2003)** and Kunitz trypsin inhibitor in soybean varieties **(Torbica et al., 2010)** can be determined by the LoC method.

CONCLUSION

The aim of this study was to evaluate a Lab-on-chip system for fast and reliable wheat variety control. During the study it turned out that the on-chip reproducibility of LoC system in terms of molecular weight determination and protein quantification is very good. Nevertheless, the fast analysis of one-step one grain extracts showed protein pattern which can directly be compared to SDS-PAGE performed according to UPOV method, the internationally accepted method for wheat control. This suggests that this method could be used as a high-throughput alternative for the time- and labour- consuming SDS-PAGE.

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