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APPLICATION NOTE

AUTOMATION OF GLUTEN QUANTIFICATION WITH THE CROCODILE MINIWORKSTATION

Abstract

Celiac disease is a chronic disorder that can only be treated by adherence to a strict gluten-free diet. The reliable quantification of gluten in foodstuff is important not only to identify and label foods that are safe for celiac patients, but also to develop new ingredients and foods that are free of gluten. In this application note, the Crocodile miniWorkstation is used to automate the quantification of gluten in different genotypes of einkorn, a promising alternative to bread wheat with low celiac immunogenicity, using two popular commercially available ELISA assays. Automation was successful and results confirmed a lower content of celiac toxic amino acid sequences in the selected einkorn genotypes.

Introduction

Celiac disease is a chronic, immune-mediated intestinal disorder, which can cause a range of

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symptoms, including diarrhoea, abdominal pain, and bloating, but also slowed growth, dermatitis, fatigue, neuronal disorders, and more; in addition, non-treated celiac disease can lead to the development of other disorders and increase the risk of cancer [1, 2, 3, 4]. The prevalence of celiac disease is typically between 0.5% and 1%, but it can go above 2% of the population, depending on the country [4], and is hence a serious health problem.

Celiac disease is caused by a reaction to gluten, a mixture of prolamin and glutenin proteins presented in wheat, rye, barley, and oats [5]. Currently, the only treatment for celiac disease is lifelong adherence to a strict gluten-free diet [4], but the use of wheat flour and gluten in foodstuffs is extremely common because of their heat stability and useful effects on e.g., texture, moisture retention and flavour. Thus, it is very important for celiac patients to be able to reliably identify food and beverage free of gluten. To this end, regulations in most countries state a limit value of 20 mg of gluten/kg of food (20 ppm) for "gluten-free" foods.

ELISA is the assay most frequently used by food manufacturers and control authorities to assess the gluten content in food products. Different kits are endorsed by a variety of organizations, including the Codex Alimentarius Commission and the Association of Analytical Communities

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(AOAC) [6]. Two of the most frequently used ELISAs are the R5 ELISA RIDASCREEN® from R-Biopharm, and the AgraQuant® Gluten G12™ test kit from Romer Labs. The first assay is based on the R5 monoclonal antibody to the potentially celiac toxic epitope QQPFP and some closely related sequences that are present in all wheat gliadins, rye secalins, and barley hordeins [6] and it is accepted as AOAC Official Method of Analysis (OMA) (2012.01), certified at AOAC-RI (120601) and Codex Alimentarius Method (Type I). The second one uses the G12 antibody, that binds to the celiac toxic amino acid sequence QPQLPY and related sequences in rye and barley [7] and is approved as AACC International Method 38-52.01 and AOAC-OMA 2014.03.

Determination of the gluten content in foods and foodstuff is important not only to appropriately label food, but also to find and develop foodstuff and ingredients with reduced gluten content. In this application note, the Crocodile mini-Workstation was used in combination with the R5 ELISA RIDASCREEN® and the AgraQuant® Gluten G12™ to assess the gluten content of specific genotypes of einkorn (*Triticum monococcum* L. ssp. *monococcum*), as part of a project focusing on the immunoanalytic investigation of this cereal [8].

SINGLE PLATE ELISA WALKAWAY AUTOMATION

The **Crocodile 5-in-one ELISA miniWorkstation** is a compact liquid handling system integrating dispenser, shaker, incubator, washer, and reader into a single system, using the bench space of an ELISA reader only.

The use of the Crocodile increases reproducibility, reduces human error and reduces assay time by eliminating the need to move plates between dispenser, shaker, incubator, washer, and reader.

- All-in-One ELISA automation
- Ultra-compact footprint saving precious bench space
- User-friendly open system software for maximum assay flexibility
- Plug & Play setup
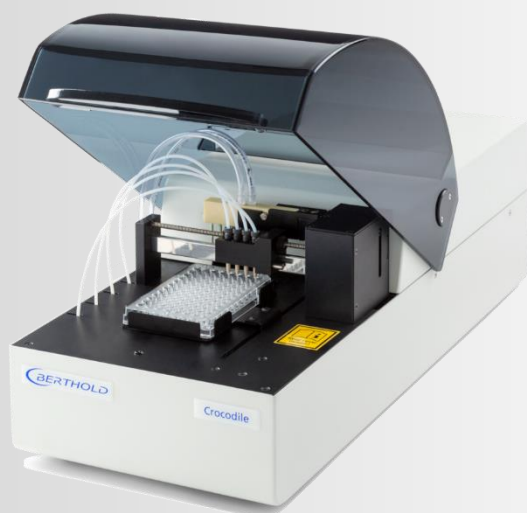
Materials

- Generic laboratory materials for sample extraction: blender, balance, centrifuge, water bath (50° C), shaker, graduated cylinders and tubes of various sizes.
- Flour from einkorn of genotypes MVGB40, MVGB748, MVGB770, MVGB786, MVGB787 and MVBG1177. Bread wheat flour was used as control.
- R5 RIDASCREEN® Gliadin ELISA (R7001) from R-Biopharm.

- AgraQuant™ Gluten G12 ELISA (COKAL0200) from Romer Labs.
- Crocodile 5-in-one miniWorkstation from Berthold Technologies.
- Crocodile Control Software from Berthold Technologies for instrument control and acquisition of data.
- MyAssays Online (www.myassays.com) and RIDASOFT® Win.NET Food & Feed for data analysis.

Methods

From the 208 einkorn genotypes 6 were selected for detailed analysis because they produced the lowest immune reactivity in indirect serological ELISA, used serum from celiac patients [8].

All reagents were prepared following the instructions of the manufacturer of the respective kits. Prolamin extraction and dilution of einkorn samples was performed in four replicates according to the kit manufacturer's instructions. Einkorn and wheat extracts were diluted in order to bring the concentrations within the range of the standard curve.

The ELISA assays were performed using the Crocodile 5-in-one miniWorkstation using the settings detailed in Table 1 (R5 ELISA) and 2 (G12 ELISA).

To calculate gliadin concentrations, the cubic spline algorithm was used for the standard curve construction. Results were corrected by the dilution factor used for each sample. Gluten content was calculated by multiplying the gliadin content by a factor of 2.

Results

According to the results of the ELISA, there were no statistically significant differences in gluten content between einkorn genotypes, but the gluten content of einkorn was much lower than that of bread wheat: at least 10000 times lower according to the R5 ELISA (Fig. 1) and at least 1000 times lower according to the G12 ELISA (Fig. 2). However, SE- and RP-HPLC results showed comparable gluten values of all einkorn genotypes to bread wheat, and thus the very low results obtained by ELISA probably indicate not the low gluten content of the studied einkorn genotypes,

but a different gluten protein composition ([8], data not shown here). It must be kept in mind that both ELISA use antibodies that bind specific celiac toxic amino acid sequences, and that the different composition of the gluten proteins of einkorn could be one of the factors explaining its low immunogenicity.

Results of the ELISA fulfilled the validation criteria of both kits (data not shown), indicating the parameters programmed in the Crocodile Control Software were suitable.
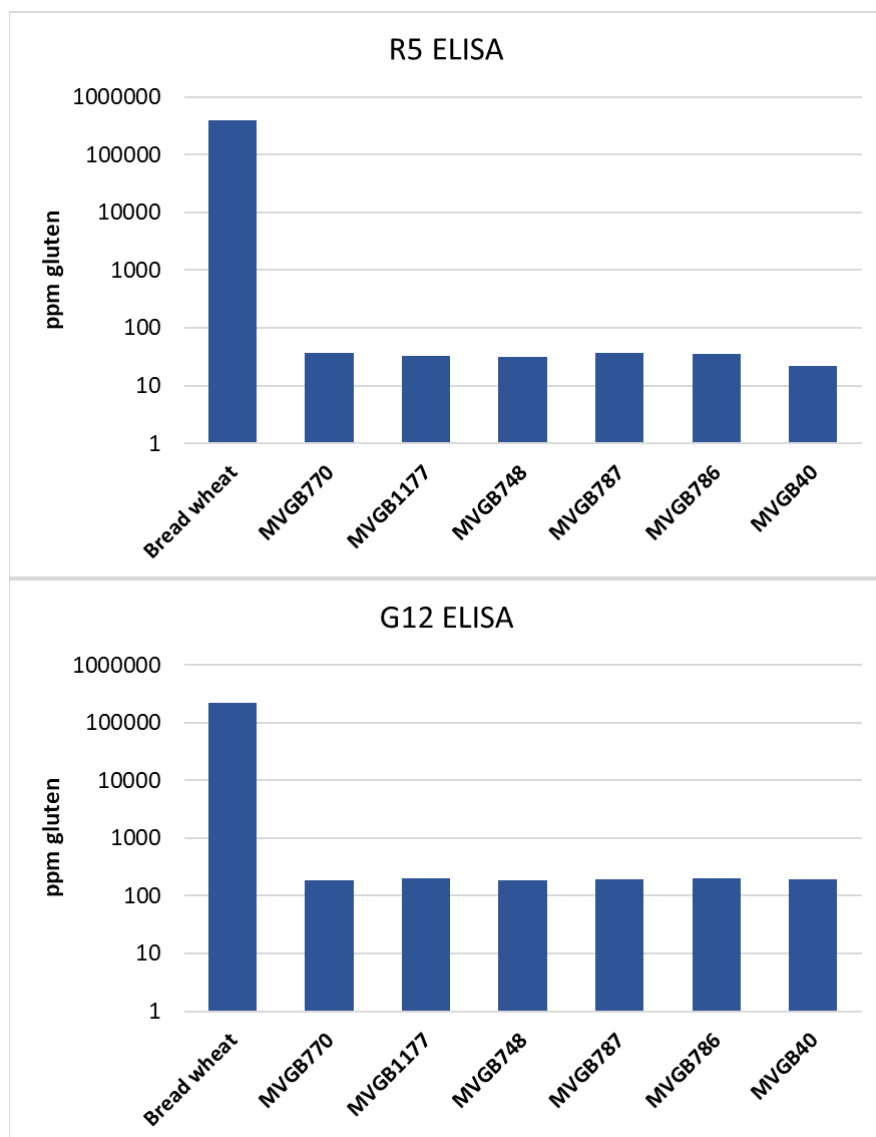

Figure 1. Gluten content measured with the R5 Ridascreen Gliadin ELISA of the bread wheat control and the 6 einkorn genotypes tested.

Figure 2. Gluten content measured with the AgraQuant™ Gluten G12 ELISA of the bread wheat control and the 6 einkorn genotypes tested.

Table 1. Summary of steps programmed in the Crocodile Control Software for the R5 ELISA.

#	Step name	Description and parameters
1	Sample Incubation	**Incubation** Incubator ON, Temperature: 24° C, Duration: 00:30:00
2	Aspiration	**Washing*** Method: Aspirate Only, Wash Solution Inlet: 1, Cycles: 1, Volume: 100 µL, Delay: 1 s, Wait: 100 ms, Dispenser Depth: 1300 (Plate Offset: 0), Aspiration Depth: 2960 (Plate Offset: -29), Sweep: 4 mm @ 2 mm/s
3	Wash Solution priming	**Washing** Method: Prime Washer, Wash Solution Inlet: 1, Cycles: 6, Volume: 1000 µL
4	Washing	**Washing*** Method: Soak Wash, Wash Solution Inlet: 1, Cycles: 3, Volume: 250 µL, Delay: 1 s, Wait: 100 ms, Dispenser Depth: 1300 (Plate Offset: 0), Aspiration Depth: 2969 (Plate Offset: -29), Sweep: 4 mm @ 2 mm/s
5	Aspiration	**Washing*** Method: Aspirate only, Wash Solution Inlet: 1, Cycles: 2, Volume: 100 µL, Delay: 1 s, Wait: 100 ms, Dispenser Depth: 1300 (Plate Offset: 0), Aspiration Depth: 2970 (Plate Offset: -29), Sweep: 4 mm @ 2 mm/s
6	Conjugate priming	**Dispensing** Volume: 850 µL, Inlet: 1, Method: Priming
7	Conjugate addition	**Dispensing** Volume: 100 µL, Inlet: 1, Method: Standard
8	Conjugate incubation	**Incubation** Incubator ON, Temperature: 24° C, Duration: 00:30:00
9	Washing	**Washing*** Method: Soak Wash, Wash Solution Inlet: 1, Cycles: 3, Volume: 250 µL, Delay: 1 s, Wait: 100 ms, Dispenser Depth: 1196 (Plate Offset: -53), Aspiration Depth: 2935* (Plate Offset: 52)
10	Aspiration	**Washing*** Method: Aspirate only, Wash Solution Inlet: 1, Cycles: 2, Volume: 100 µL, Delay: 1 s, Wait: 100 ms, Dispenser Depth: 1300 (Plate Offset: 0), Aspiration Depth: 2935 (Plate Offset: 52), Sweep: 4 mm @ 2 mm/s
11	Substrate priming	**Dispensing** Volume: 850 µL, Inlet: 2, Method: Priming
12	Substrate addition	**Dispensing** Volume: 50 µL, Inlet: 2, Method: Standard
13	Chromogen priming	**Dispensing** Volume: 850 µL, Inlet: 3, Method: Priming
14	Chromogen addition	**Dispensing** Volume: 50 µL, Inlet: 3, Method: Standard
15	Mixing	**Shaking** Duration: 00:01:00, Amplitude: 1 mm, Speed: 5 Hz, Shake in Incubator: No
16	Substrate incubation	**Incubation** Incubator ON, Temperature: 24° C, Duration: 00:30:00
17	Turning incubator Off	**Incubation** Incubator Off
18	Stop solution priming	**Dispensing** Volume: 850 µL, Inlet: 4, Method: Priming
19	Stop solution addition	**Dispensing** Volume: 100 µL, Inlet: 4, Method: Standard
20	Mixing	**Shaking** Duration: 00:01:00, Amplitude: 1 mm, Speed:5 Hz, Shake in Incubator: No
21	Measurement	**Reading** Single Measurement, Filter 1: 450 nm
		Wash settings (mainly depth and offset) have to be optimized for each individual Crocodile unit

Table 2. Summary of steps programmed in the Crocodile Control Software for the G12 ELISA.

#	Step name	Description and parameters
1	Sample Incubation	**Incubation** Incubator ON, Temperature: 24° C, Duration: 00:20:00
2	Aspiration	**Washing*** Method: Aspirate Only, Wash Solution Inlet: 1, Cycles: 1, Volume: 100 µL, Delay: 1 s, Wait: 100 ms, Dispenser Depth: 1300 (Plate Offset: 0), Aspiration Depth: 2953 (Plate Offset: -29), Sweep: 4 mm @ 2 mm/s
3	Wash Solution priming	**Washing** Method: Prime Washer, Wash Solution Inlet: 1, Cycles: 6, Volume: 1000 µL
4	Washing	**Washing*** Method: Soak Wash, Wash Solution Inlet: 1, Cycles: 5, Volume: 300 µL, Delay: 1 s, Wait: 100 ms, Dispenser Depth: 1300 (Plate Offset: 0), Aspiration Depth: 2969 (Plate Offset: -29), Sweep: 4 mm @ 2 mm/s
5	Aspiration	**Washing*** Method: Aspirate only, Wash Solution Inlet: 1, Cycles: 2, Volume: 100 µL, Delay: 1 s, Wait: 100 ms, Dispenser Depth: 1300 (Plate Offset: 0), Aspiration Depth: 2970 (Plate Offset: -29), Sweep: 4 mm @ 2 mm/s
6	Conjugate priming	**Dispensing** Volume: 850 µL, Inlet: 1, Method: Priming
7	Conjugate addition	**Dispensing** Volume: 100 µL, Inlet: 1, Method: Standard
8	Conjugate incubation	**Incubation** Incubator ON, Temperature: 24° C, Duration: 00:20:00
9	Aspiration	**Washing*** Method: Aspirate only, Wash Solution Inlet: 1, Cycles: 1, Volume: 300 µL, Delay: 1 s, Wait: 100 ms, Dispenser Depth: 1196 (Plate Offset: -53), Aspiration Depth: 2935 (Plate Offset: 52), Sweep: 4 mm @ 2 mm/s
10	Washing	**Washing*** Method: Soak Wash, Wash Solution Inlet: 1, Cycles: 5, Volume: 300 µL, Delay: 1 s, Wait: 100 ms, Dispenser Depth: 1196 (Plate Offset: -53), Aspiration Depth: 2935 (Plate Offset: 52)
11	Aspiration	**Washing*** Method: Aspirate only, Wash Solution Inlet: 1, Cycles: 1, Volume: 100 µL, Delay: 1 s, Wait: 100 ms, Dispenser Depth: 1300 (Plate Offset: 0), Aspiration Depth: 2935 (Plate Offset: 52), Sweep: 4 mm @ 2 mm/s
12	Substrate priming	**Dispensing** Volume: 850 µL, Inlet: 2, Method: Priming
13	Substrate addition	**Dispensing** Volume: 100 µL, Inlet: 2, Method: Standard
14	Substrate incubation	**Incubation** Incubator ON, Temperature: 24° C, Duration: 00:20:00
15	Turning incubator Off	**Incubation** Incubator Off
16	Stop solution priming	**Dispensing** Volume: 850 µL, Inlet: 4, Method: Priming
17	Stop solution addition	**Dispensing** Volume: 100 µL, Inlet: 4, Method: Standard
18	Mixing	**Shaking** Duration: 00:10:00, Amplitude: 1 mm, Speed: 5 Hz, Shake in Incubator: No
19	Measurement	**Reading** Single Wavelength, Filter 1: 450 nm
		Wash settings (mainly depth and offset) have to be optimized for each individual Crocodile unit

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Summary

In this application note, the Crocodile miniWorkstation was used to automate the quantification of gluten in the flour from bread wheat and einkorn using the R5 ELISA RIDASCREEN® from R-Biopharm, and the AgraQuant® Gluten G12™ test kit from Romer Labs. The amount of celiac toxic amino acid sequences in all einkorn genotypes tested was found to be much lower than in bread wheat, consistent with its lower immunogenicity.

Both the R5 and the G12 ELISA are endorsed by several organizations for the determination of gluten in foodstuff. For food testing laboratories that have to perform gluten and other ELISA assays, automation is highly desirable, as it increases the reproducibility of tests, reduces human error, and frees up human resources for other important tasks. As shown in this application note, the Crocodile provides an easy and cost-effective way of automating gluten quantification.

Acknowlededgments

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