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# Validation of a Competitive Elisa Method on Supplemental Enzyme Matrices

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## Abstract

**Introduction**: Supplemental enzymes are becoming increasingly used in the food industry. Consequently, they also need to be analyzed for gluten due to labeling reasons for food manufacturers to provide food allergen detection. Gluten is analyzed by using a Sandwich ELISA using the R5 antibody. However, sandwich ELISAs are not suitable for the analysis of fragmented gluten since detection is based on the size of the fragments. As a result, competitive R5 ELISAs have been implemented for use. When a competitive ELISA is used to analyze enzymes without gluten, the results for gluten contamination are very high and its cause is unknown. It has been suggested that the enzymes destroy antibodies of the test format and therefore false positive results are obtained. This study aimed to investigate if the competitive ELISA can be used for the gluten analysis in supplemental enzymes by the adaption of the extraction method.

**Methods:** Enzyme solutions were spiked with known concentrations of gluten then tested for gluten content using sandwich ELISA kits and competitive ELISA kits per manufacturer's instructions. Additional enzyme samples were inactivated by raising the extraction temperature to 100°C to inactivate the enzymes and also tested using both sandwich and competitive ELISA kits.

**Results:** Enzymes were spiked with gluten and analyzed with the two different ELISAs showed false negative results with the sandwich ELISA and false positive results with the competitive ELISA. Preincubation experiments showed that the enzymes destroyed the antibody used in the competitive ELISA. On the other hand, extracts extracted at 100°C did not show that effect.

**Conclusion:** In conclusion, competitive ELISA kits may be used to test fermentation products such as enzymes when the adapted extraction method is used. Spiking experiments clearly showed a good recovery of gluten in the competitive ELISA with the modified extraction, showing that the boiling step does not affect existing gluten content in the samples. This method can be used for supplemental enzymes for the analysis of gluten content in such products.

**Keywords:** Competitive ELISA; Allergen; Gluten; Enzymes; Proteases

## Introduction

The efficiency of qualitative and quantitative methods for allergen testing is important for people who have allergic reactions to certain foods such as gluten [1,2]. Unfortunately, food laboratories have encountered barriers to developing efficient methods [3]. Bottlenecks include the lack of adequate standards, limits of detection and matrix interferences [4]. The accurate and reliable detection of allergens ensures the safety and quality of products and processes, verifies food properties for labelling to avoid product recalls and ultimately safeguards brand and consumer [1,2,5]. Sandwich enzyme linked immunoassays (ELISAs) have been designed to adequately detect many of the leading allergens in standard food applications. Although many methods are available, widely recognized and laboratory validated, certain food preparations still prove difficult to test [6]. According to the Codex Alimentarius Commission, International food standards, processed foods that are naturally "gluten-free" are based on an allowable limit of no more than 20 mg/kg (ppm) in total (Codex Alimentarius). However, there are still limitations in regard to fermented foods which may contain small fragments of these allergens that can simply not be detected by a sandwich ELISA (FDA). Sandwich ELISAs need at least two binding sites for antibodies to bind to, this requires the allergen to be at least 30-40Kda in size [7]. While this is adequate for some fragments of allergens, it does not include all allergen fragments, commonly found in fermented foods rendering the method unsuitable for such matrices [8]. The competitive ELISA only needs one binding site, able to detect peptide fragments as small as 10-15Kda [9]. Therefore, competitive ELISAs, which only need one antigen binding site, have been designed to adequately test this food category [10]. This study will further validate a protocol for using competitive ELISAs on fermented products with protease activity.

## Materials and Methods

### Enzymes and treatment

The enzymes to be tested included, bacterial protease, fungal amylase, papain 66000, papain 48000, lipase Y, lipase O, fungal lactase, acid fungal protease, alpha amylase, cellulase AN, protease S and bromelain 3000 all enzymes were in powdered form. To inactivate the enzymes, 1 g were weighed in a glass vial and 10 ml of 60% ethanol were added. The samples were vortexed until a homogenous mixture was obtained. Subsequently, the solutions were incubated for 10 min at 100°C in a boiling water bath. Samples were then cooled in an ice bath for 5 mins and centrifuged for 10 mins at 2500 g. Furthermore, the samples were filtered through a fluted filter to obtain a clear supernatant. The supernatants were then diluted 1:50 (1+49) in the provided sample buffer of the used commercial gluten detection kits.

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### Gluten ELISA test kits

Two commercial ELISA test kits for the detection of gluten were used. The sandwich ELISA RIDASCREEN® FAST Gliadin used for the quantitative analysis of prolamins from wheat (gliadin), rye (secalin) and barley (hordein) in as gluten-free declared food. Secondly, the competitive ELISA RIDASCREEN® Gliadin competitive was used for fermented and hydrolyzed food samples such as syrup, beer, sour dough and food treated with supplemental enzymes. Comparatively, the competitive enzyme immunoassay quantitates peptide fragments of prolamins from wheat (gliadins), rye (secalin) and barley (hordein). Additionally, the used R5 monoclonal antibody recognizes among others, the potentially toxic sequence QQPFP, which occurs repeatedly in the prolamin molecules.

### Preparation of the samples

For the analysis with the sandwich ELISA, enzymes were either extracted with the Cocktail according to the test kit insert or by the addition of 10 ml 60% ethanol to 1 g sample, followed by 10 min shaking at room temperature and 10 min centrifugation at 2500 rpm or with 10 ml 60% ethanol at 100°C following the same procedure. For the analysis with the competitive ELISA, samples were extracted with 60% ethanol at room temperature or at 100°C. These two preparations served as the control tests to show the degradation of the ELISA antibodies by the enzymes. In a third extraction the sample (enzyme) was incubated for 10 min with 10 ml 60% ethanol at room temperature, following 90 min incubation with a protease inhibitor mix.

### Preincubation with coated plates or ready to use conjugate

Different diluted sample extracts (as described above) were incubated either 10 min on a blank plate (used from the test-kit) or 10 min 1:2 (1+1) with the component conjugate delivered with the test kit, prior to testing.

### Spiking with PWG gliadin

Four enzymes (fungal amylase, bacterial protease, acid fungal protease and protease) were spiked with different known concentrations (5, 10 and 20 mg/kg) of PWG Gliadin to serve as a control, prior to extraction. The PWG Gliadin was dissolved in 60% ethanol. For analysis with the sandwich ELISA or competitive ELISA, enzymes were extracted with 60% ethanol at room temperature or with 60% ethanol at 100°C as described above.

### Spiking with hydrolyzed gluten

To verify the recovery of fragmented gluten in the competitive ELISA, enzymes were spiked with peptic-tryptic hydrolyzates of prolamins from different cereals produced by Gessendorfer et al. [11] at a concentration of 10 mg/kg gluten. For analysis with the competitive ELISA, enzymes were either extracted with 60% ethanol at room temperature or with 60% ethanol at 100°C as described above.

### Competitive and sandwich ELISA tests

All treated and untreated samples were tested using the competitive and sandwich ELISA kits provided following Gessendorfer [11], Koehler P and Wieser H.

### Statistics

For statistical analysis and graph design the software GraphPad Prism version 5.03 for Windows was used. Normal distributed data were analyzed using Shapiro-Wilk-Test, thereafter the data were tested for significance by using One-Way ANOVA with Tukey-HSD post-hoc test. Significances were indicated as $P<0.05$ ($^{*}$) significant and $P<0.001$ ($^{***}$) highly significant, while results not significant were not indicated. All results are presented as mean with standard deviation (SD). All experiments were repeated three times [12-15].

## Results and Discussion

All enzymes were tested for intact/non-fragmented gluten content by a sandwich ELISA. The enzymes were tested negative for intact gluten (results not shown). That was true for two methods of extraction, first extraction with Cocktail (patented) as recommended in the test kit insert and extraction with 60% ethanol for 10 min at room temperature. Only the sample bromelain 3000 seems to contain a low level of 4.67 mg/kg of processed intact gluten, shown by an enhanced value after extraction with Cocktail (patented), extraction with the ethanol showed a negative result. That result indicates, that the sample seems to contain processed gluten. Only extraction with the Cocktail is able to extract processed intact gluten from samples for the detection with the ELISA. However, the value is well below the accepted 20 mg/kg gluten by Codex Alimentarius. In conclusion, sandwich ELISA is showing negative results for all enzymes.

But enzymes may degrade gluten in fragments, since they are used to fragment different proteins in food. The sandwich ELISA may not detect the fragments, since they are too small. Therefore, a competitive ELISA RIDASCREEN® Gliadin competitive was used for the analysis of the enzymes as shown in Table 1. Fragments cannot be analyzed with a sandwich ELISA, since that format needs at least two antibody binding sites for detection. These small peptides often have only one antibody binding site. So, results for the analysis with the sandwich ELISA may be false negative. Therefore, enzymes also need to be analyzed using a competitive ELISA, which is able to detect these fragments.

Lipase O, an enzyme showing no gluten contamination in the sandwich ELISA (results not shown), showed a slight contamination above 20 mg/kg gluten. Furthermore, enhancing the extraction temperature to 100°C seems to have no effect on the amount of contamination. Extraction with the protease inhibitor cocktail seems to have also no effect on the contamination and on the enzymatic activity itself, shown for all other enzymes. Results in Table 1 show, that samples extracted at room temperature and incubated with the protease inhibitor cocktail have a very high amount of gluten for bacterial protease and papain 66000. These high results may indicate a high contamination level with gluten fragments or could also be false positive results due to active enzymes, destroying the antibody components of the ELISA kit. However, both enzymes can be inactivated by cooking within the extraction.

The results show, that first, the amount of gluten is not influenced by the higher extraction temperature and second, enzymes can be inactivated by heating to 100°C within extraction.

Furthermore, a positive control was obtain using a sample of fungal amylase and spiking the solution with three different concentrations to check for recovery in sandwich or competitive ELISA, as shown in Figures 1 and 2, respectively. Recovery was around 100% for all different spiking levels. The biochemical method ELISA has a well-accepted limit of variation of +/-30% (R-Biopharm 2016), so over and underestimation around the 100% are in that limit. One spike level was significant different for the extractions; however, the value was well between 70 and 130% recovery. Therefore, a matrix effect or effect of the enzyme itself could be excluded for fungal amylase. Further spiking experiments could show that effect also for other enzymes like bacterial protease or fungal protease (results not shown).

| Sample | Extraction | mg/kg Gluten |
|---|---|---|
| Bacterial Protease | 1 | >270 |
| Bacterial Protease | 2 | 4 |
| Bacterial Protease | 3 | >270 |
| Fungal Amylase | 1 | 1 |
| Fungal Amylase | 2 | 2 |
| Papain 66000 | 1 | >270 |
| Papain 66000 | 2 | 4 |
| Papain 66000 | 3 | >270 |
| Papain 48000 | 1 | 5 |
| Papain 48000 | 2 | 2 |
| Lipase Y | 1 | 3 |
| Lipase Y | 2 | 1 |
| Lipase O | 1 | 27 |
| Lipase O | 2 | 27 |
| Lipase O | 3 | 29 |
| Fungal Lactase | 1 | 3 |
| Fungal Lactase | 2 | 2 |
| Acid Fungal Protease | 1 | 2 |
| Acid Fungal Protease | 2 | 10 |
| Alpha Amylase | 1 | 4 |
| Alpha Amylase | 2 | 3 |
| Cellulase AN | 1 | 3 |
| Cellulase AN | 2 | 3 |
| Bromelain 3000 | 1 | 2 |
| Bromelain 3000 | 2 | 3 |
| Protease S | 1 | 5 |
| Protease S | 2 | 1 |

**Table 1:** Gluten content measured with the competitive ELISA RIDASCREEN® Gliadin competitive after extraction with different extraction buffers. 1) 10 min incubation with 60% ethanol at room temperature, 2) 10 min incubation with ethanol at 100˚C, 3) incubation with a protease inhibitor cocktail.

To verify if the cooking step influences the recovery of gluten in the competitive ELISA, additional positive controls were obtained, enzymes were spiked with known concentrations of gluten. In Figure 2 recovery of the spikes is shown for fungal amylase. There was no significant extraction effect on the recovery for that specific enzyme. That experiment shows, that the cooking step does not have an influence on the gluten or its detection with both ELISA formats, as also shown in Table 1 for lipase O a naturally contaminated enzyme.

To verify the effect of enzymes on kit components, plate or conjugate (delivered with the commercial ELISA kit) were preincubated with extracted but unheated enzymes. The plate was preincubated to show the effect of untreated enzymes on the coating material (gluten coated on the plate). Furthermore, the conjugate was also preincubated to show the effect of the untreated enzyme on the conjugate antibody. Results are shown in Figure 3. The enzymatic effect on the plate could be significantly reduced by 100˚C extraction of the enzyme before preincubation. This was observed for both bacterial protease and papain 66000. Since lipase O has no effect on the plate or the conjugate (lipase O is a fatty acid cutting enzyme) the signal was the same for all different treatments (no significant difference).

The bacterial protease was able to destroy the antibody in the conjugate and on the plate, since a low signal was observed resulting in a high concentration. On the other hand, papain showed a high signal after preincubation with the conjugate, resulting in a low concentration. Papain 66000 and lipase O have no effect on the conjugate, whereas bacterial protease showed an effect. A protease is able to cut the structure of an antibody. Since antibodies can be found in the conjugate and on the plate, the protease will destroy both. Hence, the signal will be false positive.

Hence, high signals in bacterial protease and papain 66000 in Table 1 can be explained by false positive results due to enzymatic activity on the kit components.

To show that the sandwich ELISA is not suitable for the analysis of supplemental enzymes, further spiking experiments in proteases were performed. Extracts were analyzed with either the competitive ELISA, results shown in Figure 4 or the sandwich ELISA results shown in Figure 5. Cocktail (patented) extracts are not suitable for the analysis with the competitive ELISA as recommended by the manufacturer.

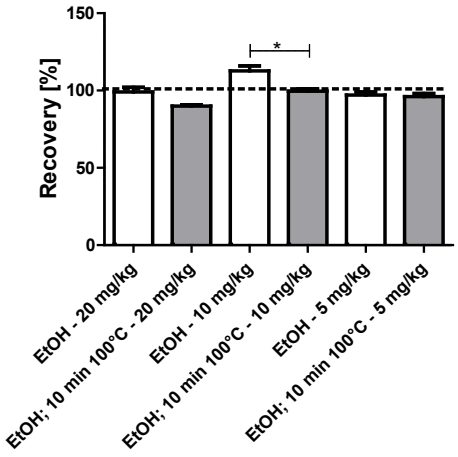


**Figure 1:** Recovery of gluten spikes 20; 10 and 5 mg/kg gluten in fungal amylase prior extraction with ethanol either at room temperature or at 100˚C, analyzed with the sandwich ELISA. Concentration was determined by means of ELISA with SD, samples were extracted n=3, *P<0.05, ANOVA with Tukey-HSD.

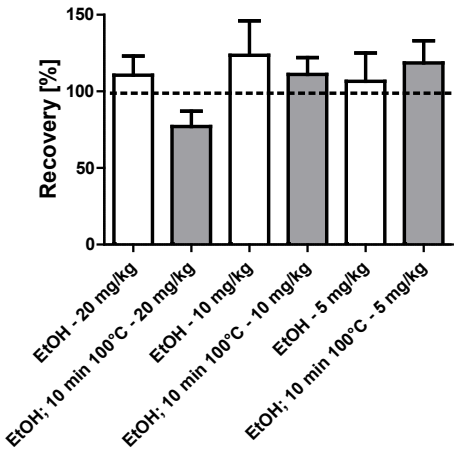


**Figure 2:** Recovery of gluten spikes 20; 10 and 5 mg/kg gluten in fungal amylase prior extraction with ethanol either heated or unheated, analyzed with the competitive ELISA. Concentration was determined by means of ELISA with SD, samples were extracted n=3, ns, ANOVA with Tukey-HSD.

Two of the proteases showed a very high recovery after extraction with ethanol without cooking step. Showing the enzymatic activity on the coating of the ELISA-plate as explained before. So, in an ethanol extract as recommended in the test kit insert, the enzymatic activity could not be reduced, consequence of which is the high gluten result. However, if the samples were cooked for ten minutes in ethanol, the spike could be found very well. These results show that extraction according to the cooking protocol inactivates the enzymes for a better and more exact analysis.

For verification, spiked samples were also analyzed with the sandwich ELISA (Figure 5). There, it was clearly shown that spikes could not be recovered after extraction if compared to spiking controls (all within the range of 70-130%). The main reason is that enzymes degrade the spike to small peptides before extraction. An analysis with the sandwich ELISA is not possible anymore, since that ELISA needs large fragments with at least two binding sites for analysis due to the building of a sandwich by two antibodies. Competitive formats only need one binding site, therefore also fragments can be recognized.

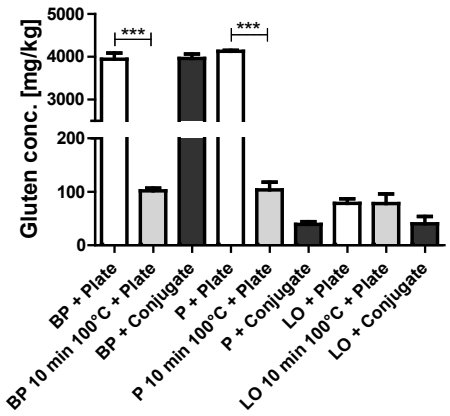


**Figure 3:** Preincubation of untreated or treated (10 min 100°C) enzymes with the component plate or conjugate from the kit RIDASCREEN® Gliadin competitive. BP= bacterial protease; P=papain 66000 and LO=lipase O. Concentration was determined by means of ELISA with SD, samples were extracted n=3, ***P<0.001, ANOVA with Tukey-HSD.

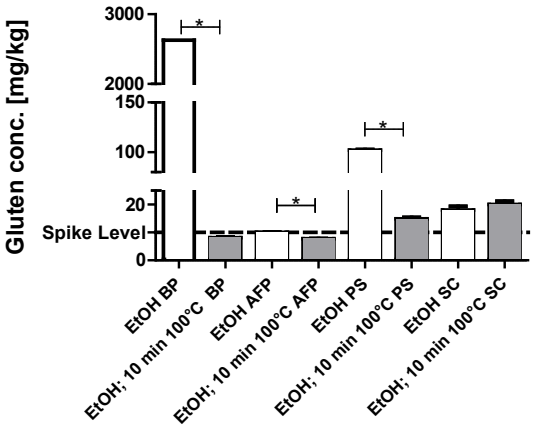


**Figure 4:** Spiking experiments with 3 different proteases, spike was performed with hydrolyzed gluten. Samples were spiked with 10 mg/kg hydrolyzed gluten and extracted with Ethanol or Ethanol cooked for 10 min. Samples were then analyzed with the RIDASCREEN® Gliadin competitive. BP=bacterial protease; AFP=acid fungal protease; PS=protease S; SC=spike control, Concentration was determined by means of ELISA with SD, samples were extracted n=3, *P<0.05, ANOVA with Tukey-HSD.

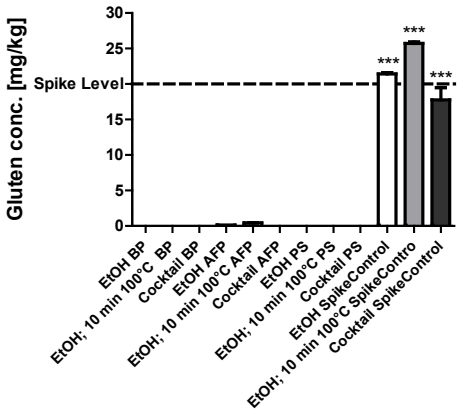


**Figure 5:** Spiking experiments with 3 different proteases, spike was performed with PWG gliadin. Samples were spiked with 20 mg/kg PWG gliadin and extracted with ethanol, ethanol cooked for 10 min or Cocktail (patented). Samples were then analyzed with the sandwich ELISA RIDASCREEN® FAST Gliadin. BP=bacterial protease; AFP=acid fungal protease; PS=protease S; SC=spike control, Concentration was determined by means of ELISA with SD, samples were extracted n=3, ***P<0.001, ANOVA with Tukey-HSD.

## Conclusion

The competitive ELISA kit is able to quantify gluten fragments on fermented products with protease activity. Results confirmed that it is not possible to use the sandwich ELISA for the detection of gluten in supplemental enzymes due to their enzymatic activity. As gluten is digested by the enzymes into small fragments, they become too small to be analyzed with a sandwich ELISA leading to false negative values. Although the competitive ELISA is more sensitive to smaller fragments, an adapted extraction for the samples must also be performed. The extraction includes a cooking step of the sample to inactivate the enzymes. This is necessary, especially for proteases which have an influence on the components of the assay. Plates of a competitive assay are coated with proteins, which can be degraded by the enzymes in the extract if not inactivated as shown in this experiment. Enzymes also have an influence on the conjugate, since in the competitive assay includes a step with preincubation of conjugate and sample. The enzyme can degrade the antibody in the conjugate, leading to false signals in the ELISA. Therefore, Competitive ELISA kits can be used to quantify specific allergens in fermented matrices, but the matrix must be validated each time.

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