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Detection and quantification of gluten as allergen in whisky samples by validated immunoassay method

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Abstract
Gluten is an insoluble, elastic thin film like protein typically present in grains such as wheat, barley and oats. The gliadin and glutenin fractions collectively form gluten which is known to causes celiac disease in genetically predisposed individuals. Incidentally, whisky, the World's most popular alcoholic drink, prepared from fermentation and distillation of aforesaid grains, bears a threat to contain gluten due to improper distillation process and pose a potential threat to individuals having Celiac disease. The present study is aimed to estimate the gluten content in the different brands of whisky samples by means of "sandwich" enzyme immunoassay (Eurofins Immunolab Gliadin kit). The gluten contents in 10 different brands of whisky differed considerably from >0.1 mg/L to 12.7 mg/L, although all the samples were found to be well within the stipulated FDA requirement of 20 mg/L of gluten. The said immunoassay method was validated for linearity, range, precision, accuracy as recovery, limit of detection and limit of quantitation.

Keywords: Gluten, immunoassay, whisky, validation

Introduction
Gluten is a prolamin found primarily in wheat, rye and barley that has been associated with Celiac disease (CD), a severe digestive disease which damages the small intestine resulting in the deprivation of nutrients from food [1]. It has been estimated that approximately 60,000 annually suffer from CD in United States [2]. Gluten is made up of two peptides, namely, gliadin and glutenin [3]. Among them, gliadin appears to be the primary cause for CD. During digestion, glutenin is enzymatically converted into di and tri peptides rather easily due to its molecular mass which gives it a large surface area for the enzymes to bind, leading to easy digestion [4]. On the contrary, densely packed gliadin is having lower surface area, thus digested poorly and leads to production of oligopeptides, resulting in inflammatory response due to stimulation of helper T-cells [5]. This subsequently results in the autoimmune response in the human body as the antibodies attacks gliadin [6]. This autoimmune response is called CD and the only therapy available is the lifelong avoidance of gluten [7, 8].
Whisky is known to be one of the most popular alcoholic drinks made from a variety of grains such as wheat, corn and barley. The general process of making whiskey involves distillation of fermented grain mash. Whisky is often believed to be gluten-free as it is being distilled from gluten-containing grains, as gluten doesn't evaporate, thus should not come into distillate [9, 10]. However, this belief may be far from true as there are several instances of individuals showed negative reactions arising from gluten to consuming whiskey. While distillation separates most of the gluten, there's a risk that it doesn't remove all, due to improper performance of distillation process [11]. Additionally, cross-contamination of gluten-containing ingredients with the whiskey is always a risk. Also, possible mixing of gluten-containing ingredients to whiskey after distillation, for flavor or caramel coloring is also a potential risk for CD patients. Therefore, to predict whether whisky is gluten free or not, the only way is to estimate the gluten content. The present study is aimed to validate the immunoassay method for routine analysis of gluten in whisky samples.

Materials & Methods
Test Kit Information

a. Kit name: Gliadin/Gluten ELISA kit.
b. Cat. No: GLU-E02/E04.

Reagents
1. Microtiter plate consisting of 12 strips with 8 breakable wells each, coated with anti-gliadin.
2. Gliadin standards (0, 2, 6, 20, 60 mg/L Gliadin): 1 x 5 vials.
3. Conjugate (anti-gliadin-peroxidase): 15 ml, one bottle.
4. Enzyme substrate (TMB solution): 15 ml, one bottle.
5. Stop solution (0.5 M sulphuric acid): 15 ml, one bottle.
6. Sample dilution solution (Tris): 60 ml, one bottle.
7. Washing solution (PBS+Tween 20): 60 ml, one bottle.

Additional instrumentation and reagents
1. 5-1000 µl micropipettes
2. ELISA reader (450 nm)
3. Centrifuge
4. Distilled water

Sample Preparation: 0.1 ml of each samples were diluted to 4.9 ml by sample dilution buffer. The samples were centrifuged for 10 minutes at 2500 rpm. The particle free supernatant was subjected to further analysis.

Preparation of Matrix Spiked with Gliadin or Gluten: Matrix samples were aliquoted into 10 ml amounts into individual 50 mL polypropylene tubes before they were directly spiked by the addition of 100 µL of the respective spiking solution. Once spiked, they were blind coded and held for 24 h at 4°C before analysis.

Procedure: The working wheat protein standards (1, 2, 4, 6, 8, 10 and 20 mg/L) and working sample solution (100 µL of each) were added into individual wells in the antibody-coated microplate module. These were prepared in triplicate. All samples were incubated in the wells for 20 minutes at 25°C. After incubation, the solutions in the wells were removed, and the wells were washed six times with 300 µL washing solution per well. After washing, 100 µL enzyme-conjugated antibody was added to each well and incubated for 20 min at 25°C. After incubation, the solutions in the wells were removed, and the wells were washed six times by adding 300 µL washing solution per well. After washing, 100 µL enzyme substrate was added to each well and incubated for 20 min at 25°C. To avoid light exposure, the microplate was covered during the enzyme reaction. After incubation, the enzyme reaction was stopped by the addition of 100 µL stop solution to each well, and the absorbance was measured at 450 nm using a microplate reader.

Method Validation
Linearity: The calibration curve was linear over the concentration range of 1-20 mg/L for gliadin/gluten.

Precision: Five replicated injections at seven different concentration of gluten (1.0, 2.0, 4.0, 6.0, 8.0, 10.0 and 20.0 mg/L) were made and analyzed to examine the precision of the method.

Accuracy: Accuracy of the method was determined by recovery experiments. The recovery of the method was determined at three levels by adding a known quantity of gluten standard to the pre analyzed samples and the mixtures were analyzed according to the proposed method.

Sensitivity: The sensitivity of measurement of gluten by the use of proposed method was estimated in terms of Limit of Detection (LOD) and Limit of Quantitation (LOQ). 5 replicates at lowest level was carried out and from the standard deviation (SD) Limit of Detection was calculated as 3 x SD and Limit of quantitation was calculated as 10 x SD. The limit of detection is the smallest concentration of the analyte that gives a measurable response. The limit of quantitation is the smallest concentration of the analyte which gives a response that can be accurately quantified.

Results
The developed method was then validated and successfully applied for quantitation of gluten from the samples. Regression analysis data is shown in Table 1.
The calibration curve of gluten was linear in the range of 1.0 to 20.0 mg/L. The standard curve is depicted in Figure 1.
Precision, expressed in terms of % RSD, analyzing the substances at seven different concentrations, five times replicated, summarized in Table 2.
To ensure the accuracy of the method, recovery studies were performed by standard addition method at three different levels, to the pre-analyzed samples and the subsequent solutions were re-analyzed. At each level, three determinations were performed and the results obtained are shown in Table 3.
The sensitivity of measurement of gluten by the use of proposed method was estimated in terms of Limit of Detection (LOD) and Limit of Quantitation (LOQ). The LOD and LOQ were given in Table 4.
The method was applied to the ten brands of whisky samples. The gluten content found in the samples are given in Table 5, Figure 2.

Table 1: Statistical validation data of Gluten

Parameters	Results
Linearity range	1-20 mg/L
Slope	0.025
SD of slope	0.001
Intercept	0.067
SD of intercept	0.004
Regression Equation	y = 0.025x+0.067
Correlation Coefficient	0.995

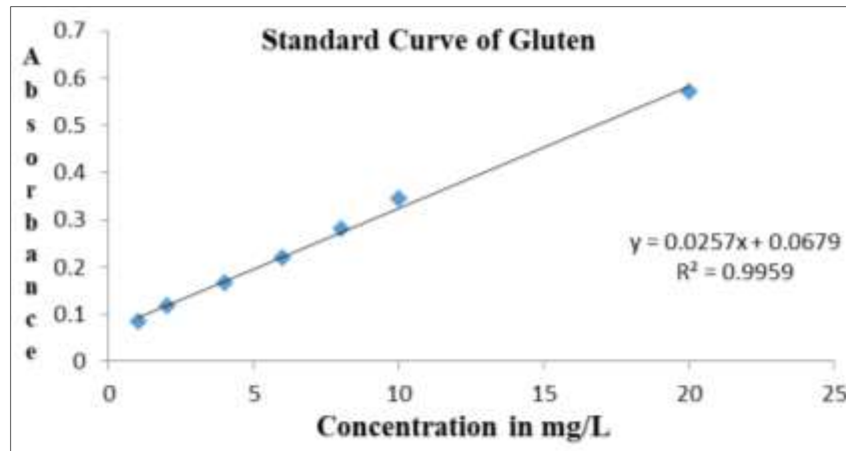

Fig 1: Calibration Curve of Gluten

Table 2: Precision data for gluten standard

Concentration in mg/L	Absorbance (n=5)± SD
1.0	0.084 ± 0.008
2.0	0.119 ± 0.009
4.0	0.166 ± 0.005
6.0	0.220 ± 0.010
8.0	0.282 ± 0.007
10.0	0.344 ± 0.006
20.0	0.571 ± 0.014

Table 3: Recovery data for determination of gluten in whisky

Amount of gluten present in sample (mg/L)	Amount Added (mg/L)	% Recovery ± RSD
3.5	1.0	99.86 ± 4.96
	5.0	98.47 ± 6.90
	10.0	99.77 ± 3.86

Table 4: Results of sensitivity data for gluten

Parameters	Results
LOD (mg/L)	0.03
LOQ (mg/L)	0.1

Table 5: Estimation of gluten content in different brands of Whisky

Sample Name	Gluten content in mg/L
Whisky Brand 1	3.5
Whisky Brand 2	10.4
Whisky Brand 3	<0.1
Whisky Brand 4	4.8
Whisky Brand 5	<0.1
Whisky Brand 6	3.8
Whisky Brand 7	5.9
Whisky Brand 8	12.7
Whisky Brand 9	4.1
Whisky Brand 10	<0.1

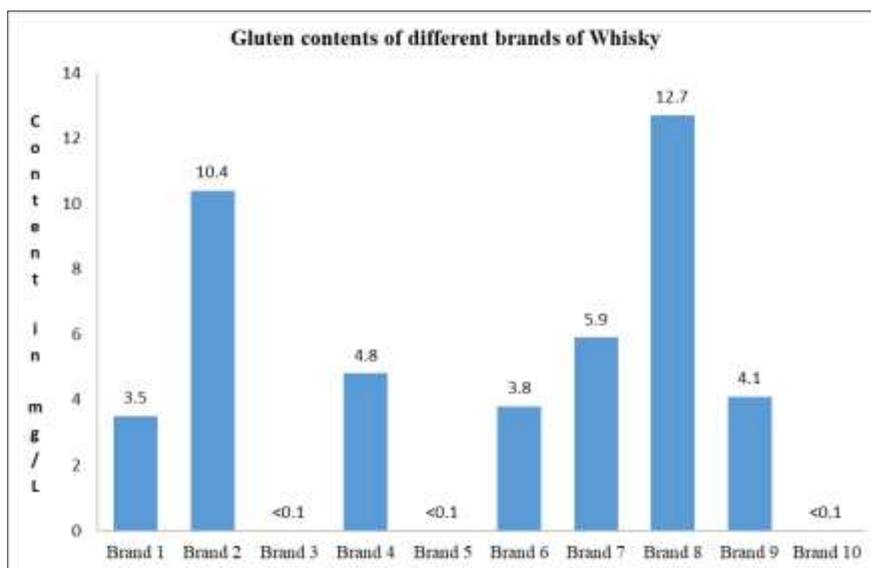

Fig 2: Gluten contents of different brands of Whisky

Conclusion

In this proposed method the linearity was observed in the concentration range of 1.0 – 20.0 mg/L for gluten with co-efficient of correlation, R^2 = 0.995 at 450 nm. The result of the analysis by the method was found to be highly reproducible and reliable. The matrix and other ingredients present in the product did not interfere with determination of gluten. So, the developed immunoassay method is simple, precise and accurate and can be used for routine analysis of gluten in whisky samples.

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