Ind. J. Pharm. Edu. Res., 2026; 60(4):1-8.
https://www.ijper.org

Original Article

Hydrolysis of Gluten in Glucose Biscuits Using Protease Enzymes and Validation of Gluten Content Determination Using ELISA

Amol Vishwanath Tonde, Santosh Subhash Chhajed*

Department of Pharmaceutical Chemistry, MET's Institute of Pharmacy, Bhujbal Knowledge City (Affiliated to Savitribai Phule Pune University, Pune), Adgaon, Nashik, Maharashtra, INDIA.

ABSTRACT

Background: Gluten present in foods such as wheat, barley, and rye, is a protein that triggers allergies in some individuals. Gluten is listed in top 9 allergens in USA and top 14 allergens in Europe. As per USFDA regulation 21 CFR § 101.91, foods that say they are "gluten-free" must not have more than 20 µg/g (20 ppm) of gluten. **Aim:** It is crucial to verify the suitability of the ELISA approach for quantifying the digested gluten, as enzymes have the ability to degrade gluten. **Materials and Methods:** The Enzyme-Linked Immunosorbent Assay (ELISA), a highly specific, sensitive and widely used method for detecting gluten in food additives and products. In the ELISA immunological assay, antibodies adhere to the target structure and quantitatively determine the amount of gluten in sample. **Results:** The ELISA method accurately identified gluten in the enzyme matrix without any interference from the enzyme matrix. The validation of method confirms the suitability and consistency of the method. **Conclusion:** The ELISA method can be used to identify and quantify gluten, in both unhydrolysed form and after partial hydrolysis, without interference of the enzyme matrix. The results demonstrate application of the ELISA in evaluation of commercial products.

Keywords: Development, Validation, ELISA-Based Method, Enzymatic Hydrolysis and Gluten.

Correspondence:
Dr. Santosh S. Chhajed
Department of Pharmaceutical Chemistry, METs Institute of Pharmacy, Bhujbal Knowledge City (Affiliated to Savitribai Phule Pune University, Pune), Adgaon, Nashik, Maharashtra, INDIA.
Email: chhajedss@gmail.com

Received: x-x-x;
Revised: x-x-x;
Accepted: x-x-x.

INTRODUCTION

The texture and elasticity of a variety of food products are greatly influenced by gluten, a composite of storage proteins present in wheat, barley, and rye.[1-3] But for people who have wheat allergy, gluten sensitivity, or celiac disease, its presence in food poses major health risks.[4-7] The elastic network of gliadin and glutenin, two proteins found in wheat grains, is commonly referred to as gluten.[8,9]

For the protection of sensitive people and to guarantee adherence to legal requirements, precise measurement of gluten (gliadin) in food products is crucial. The Enzyme-Linked Immunosorbent Assay (ELISA), one of the many gluten detection techniques available, is well known for its sensitivity, specificity, and user-friendliness.[10] Because ELISA-based techniques can measure traces of gluten in intricate food matrices, they are especially useful for routine analysis in food industry labs.[11] The development of effective methods for the analysis of fermented foods, such as supplementary enzymes utilizing the sandwich ELISA, has presented challenges for food laboratories.[12-14] In this work, we have developed a dependable ELISA method to detect gluten in an enzyme matrix. Finding a solution is crucial because the enzymes can hinder the detection of gluten in the enzyme matrix. The enzyme has the ability to hydrolyse the gluten in the solution as well as bind with the antibody on the ELISA plate. Our approach can solve these issues and provide the accurate gluten content. The amount of gluten in fermented foods and the amount of gluten broken down by protease enzymes in the enzyme matrix itself can both be measured using this technique in routine testing. The procedure has been thoroughly validated to guarantee its dependability, repeatability, and adherence to legal requirements. Additionally, we have investigated the kinetics of gluten hydrolysis using proteolytic enzymes, which is essential for comprehending how gluten breaks down in the gastrointestinal system and for creating gluten-free products.[15-17] Our research provides a thorough method for identifying gluten and its enzymatic hydrolysis, which advances food safety and aids in the creation of goods that are safe for people with gluten sensitivity.[18] The use of sandwich ELISA method has not been explored previously to study gluten degradation using protease enzymes.

DOI: 10.5530/ijper.20263324

Copyright Information :
Copyright Author (s) 2026 Distributed under Creative Commons CC-BY 4.0

Publishing Partner : Manuscript Technomedia. [www.mstechnomedia.com]

MATERIALS AND METHODS

All of the chemicals and reagents used in this work were purchased from Merck and were of food or reagent grade. A gift sample of Standard Gliadin was obtained from the Prolamin Working Group (PWG) in Germany. The supplier of the R5 ELISA RIDASCREEN (Sandwich) Gliadin Kit (Model-RIDASCREEN® Gliadin, Year-2024) was R-Biopharm AG, Germany. Prolamins from wheat (gliadin), rye (scalin), and barley (hordein) can be quantitatively analyzed in food using the sandwich enzyme immunoassay known as the RIDASCREEN Gliadin Kit.[19] Advanced Enzyme Technologies Limited in India supplied the enzymes, alkaline protease (EC 3.4.21.62), papain (EC 3.4.22.2) and bromelain (EC 3.4.22.32), as a gift sample. The absorbance was measured using the Alere Microplate Reader (Model-ALERE™ EASY READER, manufactured by Alere Medical Pvt. Ltd., India, Year-2017). We bought commercially available glucose biscuits from the market.

Preparation of standard solutions

Potassium phosphate Buffer at pH 6.8 (1M).

Preparation of Solution *A*

A volumetric flask with a capacity of 100 mL was filled with approximately 17.42 g of potassium phosphate dibasic after it had been weighed. About 50 mL of distilled water were added to this, and the flask's contents were shaken to change the solution. The flask's volume was created using distilled water.

Preparation of Solution *B*

A volumetric flask with a capacity of 100 mL was filled with 13.61 g of potassium dihydrogen phosphate that had been precisely weighed. To influence the solution, the contents of the flask were shaken and roughly 50 mL of distilled water was added. Phosphate buffer was made by precisely combining 38.1 mL of Solution A and 61.9 mL of Solution B in a 100 mL volumetric flask. The flask's volume was created using distilled water.

Gliadin solution (1000 µg/mL)

10 mg of Gliadin, precisely weighed, was added to a volumetric flask with a capacity of 10 mL. About 5 mL of 60% aqueous ethanol was added to this, and the flask's contents were shaken to influence the clear solution. The volume was adjusted to 10 mL using 60% aqueous ethanol.

Preparation of standard solution of bromelain (1000 µg/mL)

100 mg of precisely weighed bromelain was added to a 100 mL volumetric flask. About 50 milliliters of distilled water were added to this, and the flask's contents were shaken to influence the clear solution. The volume was increased to 100 mL using distilled water.

Preparation of standard solution of alkaline protease (1000 µg/mL)

A 100 mL volumetric flask was filled with precisely weighed 100 mg of alkaline protease. About 50 mL of distilled water were added to this, and the flask's contents were shaken to influence the clear solution. The volume was increased to 100 mL using distilled water.

Preparation of standard solution of papain (1000 µg/mL)

100 mL volumetric flask was filled with precisely weighed 100 mg of papain. About 50 mL of distilled water were added to this, and the flask's contents were shaken to influence the clear solution. The volume was increased to 100 mL using distilled water.

ELISA Analysis for quantification of Gluten Hydrolysis

Hydrolysis of Gluten using Enzymes

To achieve maximum hydrolysis, gliadin was hydrolyzed using each of the three enzymes (alkaline protease, papain, and bromelain) independently, following the guidelines in Table 1. In a 2 mL sample tube, 15 µL of gliadin solution (1000 µg/mL) was added. 15 µL of an enzyme solution containing 1000 µg/mL was added to this. Using phosphate buffer, the volume was diluted to 1 mL, and the mixture was vortexed for 2 min. Each of the three enzyme and gliadin reaction mixtures was incubated at 37°C for 75, 90, and 105 min. The mixtures were allowed to cool to room temperature following the reaction period. The ELISA kit was used to prepare the samples for analysis. After adding 3 mL of distilled water to 250 mg of reaction mixture in a centrifuge tube, the mixture was vortexed for 2 min. After adding 1 mL of 1M phosphate buffer, the mixture was vortexed for 2 min. 6 mL of absolute ethanol was added to this. The solution was vortexed for 2 min. For 15 min, the solution was submerged in a boiling water bath. After cooling to room temperature, the samples were examined. After that, the mixture was centrifuged for 10 min at 5000 rpm. After taking the tubes out of the centrifuge, 80 µL of the supernatant was transferred to a 1 mL sample tube. 920 µL of the ELISA kit's diluent buffer was added to this. For 2 min, a vortex mixture was used to mix this solution. For additional analysis, 100 µL of this solution was taken. As a negative control, the enzyme solutions (blank, without gliadin) for each of the three enzymes were also made using the above method. The gliadin concentration was measured using the gliadin standard solution from the ELISA kit.[19]

Analysis using R5 ELISA RIDASCREEN (Sandwich) Gliadin Kit

Separate wells were filled with 100 µL of each solution, which was made using three reaction mixtures, three enzyme blank solutions, and the gliadin standard solution from the ELISA

kit. After gently shaking the plate to combine the solutions, the plate was covered with aluminum foil and allowed to sit at room temperature (20° to 25°C) for 30 min. After 30 min the solution was poured out of the wells and the microwell holder was tapped upside down vigorously (three times) against absorbent paper to ensure complete removal of solution from the wells. 250 µL of diluted washing buffer was then added to each well, shaken gently for 30 sec, and then poured out once more. Two more times, this washing procedure was carried out. Each well was then filled with 100 µL of the diluted enzyme conjugate. After gently shaking the plates by hand, they were covered with aluminum foil and incubated for 30 min at room temperature (20° to 25°C). After 30 min, the liquid was poured out of the wells, and to make sure all of the liquid had been removed, the microwell holder was tapped firmly (three times) upside down against absorbent paper. 250 µL of diluted washing buffer was then added to each well, shaken gently for 30 sec, and then poured out once more. Two more times the washing procedure was carried out. Each well was then filled with 50 µL of the substrate solution and 50 µL of the chromogen solution. After gently shaking the plate to combine the solutions, the plate was covered with aluminum foil and allowed to sit at room temperature (20° to 25°C) for 30 min. Each well was then filled with 100 µL of the stop reagent. The microplate reader was used to measure the absorbance at 450 nm after the plates were gently shaken by hand. Within 30 min of the stop solution being added, the readings were obtained.

Estimation of Hydrolysis of gluten in biscuits using enzymes

Preparation of 0.1N HCl solution

About 5.1 mL of conc. HCl was taken and added to 600 mL distilled water to get 0.1*N* HCl solution.

Preparation of 0.1N NaOH solution

About 2.4 g of NaOH was taken and added to 600 mL distilled water to get 0.1*N* NaOH solution.

Preparation of phosphate Buffer at pH 6.8

About 28.8 g of Na_2HPO_4 and 11.45 g of KH_2PO_4 was weighed and added to 1000 mL distilled water to get Phosphate buffer (pH 6.8).

Preparation of Biscuit powder

About 25 g of glucose biscuits were weighed and triturated in mortor and pestle to get uniform powder.

Hydrolysis of gluten in biscuits

The biscuits were first exposed to 0.1 *N* HCl and then to phosphate buffer pH 6.8 in order to replicate the *in vivo* conditions. The enzymes were added straight to the buffer stage because they may denature in an acidic environment. In a conical flask, 50 mL of 0.1 *N* HCl was mixed with roughly 25 g of weighed glucose biscuit powder. For 2 hr, this solution was continuously shaken at 37°C in an incubator. The solution was removed after 2 hr, and 50 mL of 0.1 *N* NaOH were added to neutralize it. After adding 150 mL of phosphate buffer with a pH of 6 to 8, this solution was incubated for 90 min. A 50 mL volumetric flask containing approximately 0.67 g of this solution was used. Using a 60% ethanol solution, the solution was diluted to 50 mL and stirred for 10 min. This solution served as the control.

About 25 g of glucose biscuit powder was weighed and added to 50 mL 0.1 *N* HCl in a conical flask. This solution was kept in incubator at 37°C for 2 hr with continuous shaking. After 2 hr, the solution was taken out and 50 mL 0.1 *N* NaOH was added to neutralize it. 150 mL of phosphate buffer pH 6.8 was added to this solution. To this solution an enzyme mixture of bromelain and papain was added. The enzyme bromelain and papain were taken in a ratio of 3:1. This solution was kept in incubator for 90 min. About 0.67 g of this solution was taken in a 50 mL volumetric flask. The solution was diluted to 50 mL using 60% ethanol solution and mixed for 10 min. This solution was used as reaction solution.

The ELISA kit was used to prepare the samples for analysis. After adding 3 mL of distilled water to 250 mg of reaction mixture in a centrifuge tube, the mixture was vortexed for 2 min. After adding 1 mL of 1*M* phosphate buffer, the mixture was vortexed for 2 min. 6 mL of absolute ethanol was added to this. For 2 min, the solution was vortexed. The solution was kept in boiling water bath for 15 min. After cooling to room temperature, the samples were examined. After that, the mixture was centrifuged for 10 min at 5000 rpm. After taking the tubes out of the centrifuge, 80 µL of the supernatant was transferred to a 1 mL sample tube. 920 µL of the ELISA kit's diluent buffer was added to this. For 2 min, a vortex mixture was used to mix this solution. For additional analysis, 100 µL of this solution was taken. As a negative control, the enzyme solutions (blank, without gliadin) for each of the three enzymes were also made using the above method. The gliadin concentration was measured using the gliadin standard solution from the ELISA kit.[19]

Method Validation

The objective of validation of analytical method is to ensure that the method gives results with reproducible accuracy and is suitable for its intended purpose.[20-22]

Preparation of Enzyme solution (BLANK)

All three enzymes-alkaline protease, papain, and bromelain-were used to test and validate the analytical procedure. 3 mL of purified water were added to a centrifuge tube containing roughly 250 mg of enzyme. To create a uniform solution, the samples were vortexed for 15 min. To this solution, 1 mL of Phosphate buffer was added, followed by 6 mL of absolute ethanol. To create a

homogenous mixture, the solution was vortexed for 5 min. To stop any enzymatic activity, the solution was placed in a boiling water bath for 15 min. Following a 10-min centrifugation at 5000 rpm, the samples were allowed to cool to room temperature. Samples for ELISA analysis were prepared using the liquid supernatant. All three enzymes-alkaline protease, papain, and bromelain-were used in triplicate samples to create this solution. 920 µL of the test kit's buffer solution was added to a 2 mL tube containing 80 µL of enzyme solution (blank, without gliadin). For additional analysis, 100 µL of this solution was taken. The negative control was the enzyme solutions made for each of the three enzymes using the above method.

Preparation of Enzyme Solution Spiked with Gliadin

In a centrifuge tube, roughly 250 mg of enzyme was weighed, and then 3 mL of purified water was added. For 15 min, the samples were vortexed to create a uniform solution. 1 mL of phosphate buffer and 6 mL of absolute ethanol were added to this solution. For 5 min, the solution was vortexed to create a homogenous mixture. For 15 min, the solution was submerged in a boiling water bath to stop any enzymatic activity. After cooling to room temperature, the samples were examined. Different concentrations of gliadin (2.5, 20, 40 µg/g) were added to this solution in order to provide a control, which was equivalent to 5, 40, and 80 µg/g of gluten. For 10 min, the solution was vortexed to create a uniform mixture. The solution was then centrifuged at 5000 rpm, for 10 min Samples were prepared for ELISA analysis using the liquid supernatant. Bromelain, papain, and alkaline protease were all used in triplicate samples to prepare this solution. About 80 µL of gliadin-spiked enzyme solution was transferred to a 2 mL tube, and then 920 µL of test kit buffer solution was added. A 100 µL sample of this solution was collected for additional examination. The positive control for all three enzymes was the enzyme solutions made using the above method. The standard for gliadin concentration was the gliadin standard solution from the ELISA kit.

Parameters of Method Validation

This study was carried to prove that the analytical method is consistent with respect to Specificity, Linearity, Range, Accuracy, Precision, Limit of Quantitation and Robustness.

RESULTS

Enzymatic Hydrolysis of standard gliadin

The degree of hydrolysis was calculated using ELISA reader.[23-25] A standard curve of gliadin standard of different concentration was plotted against the absorbance. The absorbance of gliadin standard and the reaction mixture gives the concentration of the gliadin hydrolysed. The amount of gluten is considered doubled as that of gliadin i.e. 20 mg gliadin is equivalent to 40 mg gluten. Through the process of hydrolysis, the quantity of gliadin that has been hydrolysed down by each enzyme is depicted in Figure 1.

Hydrolysis of gluten in biscuits

The hydrolysis of gluten in biscuit samples was calculated using ELISA reader. A standard curve of gliadin standard of different concentration was plotted against the absorbance. The absorbance of gliadin standard and the reaction mixture gives the concentration of the gliadin hydrolysed. The amount of gluten is considered doubled as that of gliadin i.e. 20 mg gliadin is equivalent to 40 mg gluten. Through the process of hydrolysis, the quantity of gliadin that has been hydrolysed down enzyme mixture is given in Table 2.

Method Validation

Specificity

The specificity of the gluten detection technique in the enzyme matrix was assessed by incorporating a predetermined quantity of gliadin into the sample. The absorbance response of blank and spiked enzyme and its respective concentration was calculated.

Table 1: Hydrolysis reaction conditions.

Enzyme	Gliadin: Enzyme ratio	Reaction Temp (°C)	Digestion Time (min)
Papain	1.0	37	75
			90
			105
Alkaline Protease	1.0	37	75
			90
			105
Bromelain	1.0	37	75
			90
			105

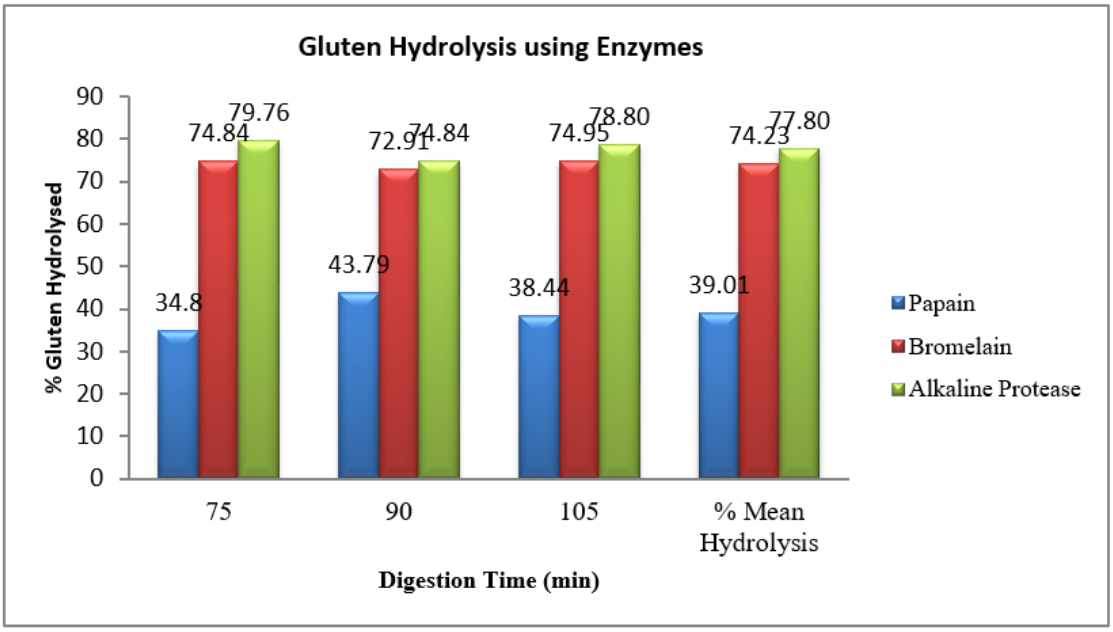


Figure 1: Hydrolysis of gluten using different enzymes in reaction time 75, 90 and 105 min.

Table 2: Hydrolysis of gluten in biscuit using enzyme mixture.

Sample	Reaction Temp (°C)	Reaction Time (min)	Gluten detected (µg/g)
Biscuit	37	NA	60
Biscuit + Enzyme Mixture	37	90	< 10

Linearity

The RIDASCREEN software was used to obtain a sigmoidal curve after getting the absorbance of the kit standards at doses of 0 µg/g, 5 µg/g, 10 µg/g, 20 µg/g, 40 µg/g, and 80 µg/g.

Range

To measure the amount of gluten in the sample matrix, samples were injected with gliadin concentrations of 2.5 µg/g, 20 µg/g and 40 µg/g corresponding to gluten concentrations 5 µg/g, 40 µg/g, and 80 µg/g. Therefore, it was verified that the concentration range is between 5 µg/g and 80 µg/g for gluten. The range results were derived from the accuracy analysis results and comply with the validation requirements.

Accuracy

To assess the accuracy of the method, we performed three sets of measurements at three different concentration levels and three samples of each concentration. We used bromelain as the enzyme matrix for this evaluation.

$$\% \textbf{ Recovery } = \frac{\text{Conc. Gluten detected}}{\text{Conc. Gluten spiked}} \times 100$$

The results of analysis showed more than 95% recovery for each concentration. The results of accuracy study are depicted in Figure 2.

Precision

The performed investigation involved two components: intermediate precision and repeatability.

Repeatability

To analyze the consistency of results, three different concentration levels of gliadin spiked in bromelain were tested. Each level was evaluated using triplicate samples. The data on accuracy and repeatability was obtained on same set of samples as one analysis. The standard deviation observed was less than 1.0%.

Intermediate Precision

Intermediate precision was assessed on a separate day using a different ELISA kit. To analyze the consistency of results, three different concentration levels of gliadin spiked in bromelain were tested. All the three different concentration samples were prepared in triplicate and analysed. The findings of intermediate precision showed standard deviation less than 2% and percentage relative standard deviation less than 3%. The results of intermediate precision study are depicted in Figure 3.

Limit of Quantitation (LOQ)

An assessment to determine the Limit of Quantitation (LOQ) of analytical method was carried out utilizing enzyme samples injected at a concentration of 5 µg/g. The precision and accuracy

results were considered satisfactory for validating the LOQ parameter at the 5 µg/g level.

Robustness

The method's robustness was examined by modifying variables such as buffer concentration, sample pH, ethanol content, and time of heating. A single concentration of gliadin was spiked to the samples. The samples were prepared in triplicate. The findings of the robustness analysis showed standard deviation less than 1% and percentage relative standard deviation less than 2%.

Verification of effect of heating on determination of gluten content

To examine the impact of heating on gluten level measurements, we created extra samples. After being spiked with gliadin, the samples were then subjected to boiling at a temperature of 100°C for 15 min. The samples were then cooled to room temperature, and subjected to centrifugation for 10 min. The supernatant portion was used to prepare the samples for the ELISA analysis. The findings of the heating effect for gluten analysis indicated there was no impact on results if samples were heated before and after spike as compare to heating only before spike.

DISCUSSION

The ELISA technique correctly detected gluten in the enzyme matrix without any interference from the enzyme matrix, based on the specificity test results. Since all three enzymes were used to establish specificity, only one enzyme, bromelain, was used to test the remaining validation parameters. A sigmoidal curve was created due to the linearity of the method, and this curve matches

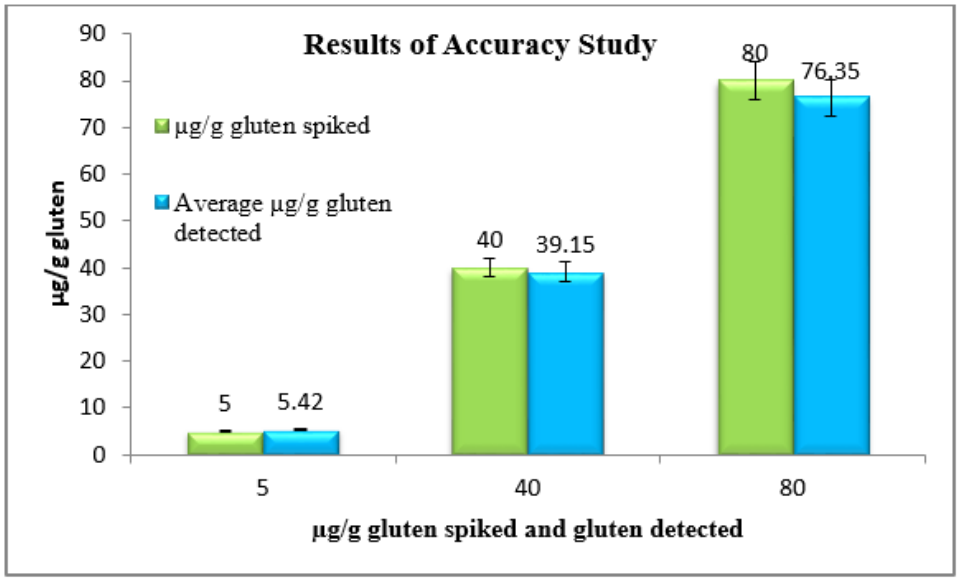


Figure 2: Results of Accuracy Study on ELISA using standards of different concentrations.

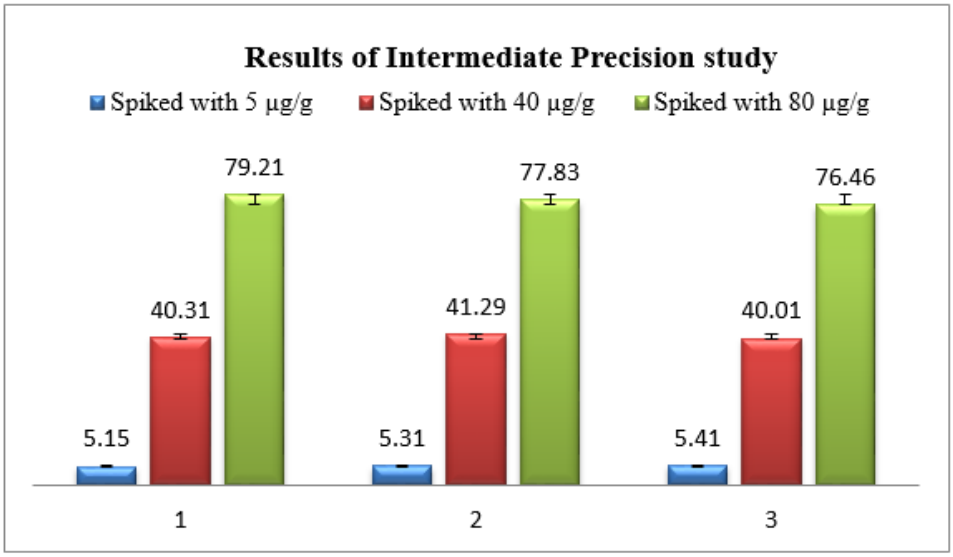


Figure 3: Results of Intermediate Precision Study.

the graph that the RIDASCREEN software showed. According to the results, the amount of gluten present can be precisely and consistently measured between 5 and 80 mg/kg. The recovery was greater than 95%, and the Relative Standard Deviation (RSD) was less than 3%. When the accuracy level was measured, the degree of variability in the results that were generated was deemed acceptable. The quantitation limit has been determined to be 5 mg/kg, and the results of the robustness analysis show that the variance is within an acceptable range, with a Relative Standard Deviation (RSD) of less than 2%. The study also discusses the ELISA technique for estimating the amount of gluten degradation in commercial glucose biscuit samples.

CONCLUSION

The accuracy of the ELISA-based technique for determining and quantifying gluten in the enzyme matrix is investigated in this manuscript. It also validates the use of an ELISA-based technique to measure the amount of gluten hydrolyzed by enzymes. There are no statistically significant variations in the results, and the validation complies with the acceptance criteria. The validation study's conclusions show that the method is reliable and that the outcomes are repeatable. Given that the gluten can be hydrolyzed by protease enzymes, determining and quantifying it in the presence of these enzymes may yield false-negative results. This drawback of the ELISA-based approach is eliminated by our approach. This approach can therefore be used for routine testing to determine the amount of gluten that has been broken down by protease enzymes as well as the amount of gluten that is present in the fermented food products such as the enzyme matrix. Our research on commercial glucose biscuits adds to application of ELISA for determination of gluten content. Additionally, this model can also be used to study the gluten degradation in commercially available products like biscuits, cookies using other moieties.

ACKNOWLEDGEMENT

The authors acknowledge sincere thanks to Principal and management of MET's Institute of pharmacy, Bhujbal Knowledge City, Nashik, India for providing necessary facility to carry out this work. The authors also Acknowledges sincere thanks to Advanced Enzyme Technologies Limited, India for providing gift samples of enzymes used in the study.

ABBREVIATIONS

ELISA: Enzyme-linked immunosorbent test; **RSD:** Relative standard deviation; **LOQ:** Limit of Quantitation; **EC:** Enzyme Commission (Number).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

CONSENT FOR PUBLICATION

Yes

SUMMARY

Gluten, a protein found in wheat, barley, and rye, triggers allergies in some individuals. A very sensitive and specific technique for identifying gluten in food additives and goods is the Enzyme-Linked Immunosorbent Test (ELISA). Without the enzyme matrix interfering, the ELISA technique correctly detected gluten in the enzyme matrix. The method's consistency and applicability were validated by the validation research. Gluten can be detected and measured using the ELISA method both unhydrolyzed and partially hydrolysed without the enzyme matrix interfering. The ELISA method for calculating the amount of gluten breakdown in commercial glucose biscuit samples was also examined in this investigation. Investigating the precision of the ELISA-based approach for identifying and measuring gluten in the enzyme matrix, the validation study's findings demonstrate the method's dependability and reproducibility. This method can be applied to regular testing to measure the amount of gluten in fermented food products and the amount of gluten broken down by protease enzymes.

REFERENCES

1. 21 CFR Part 101 Subpart F -- Specific Requirements for Descriptive Claims That Are Neither Nutrient Content Claims nor Health Claims. Ecfr.gov. https://www.ecfr.gov/current/title-21/part-101/subpart-F
2. Center for Food Safety and Applied Nutrition. Food Allergies. FDA. 2020. https://www.fda.gov/food/food-labeling-nutrition/food-allergies
3. EU Regulation No 1169/2011. https://eur-lex.europa.eu/legal-content/EN/TXT/?qid=1530180983115&uri=CELEX:02011R1169-20180101
4. Van Hengel AJ. Food allergen detection methods and the challenge to protect food-allergic consumers. Anal Bioanal Chem. 2007;389(1):111-8.
5. Gibert A, Kruizinga AG, Neuhold S, Houben GF, Canela MA, Fasano A, *et al.* Might gluten traces in wheat substitutes pose a risk in patients with celiac disease? A population-based probabilistic approach to risk estimation. The American Journal of Clinical Nutrition. 2013;97(1):109-16.
6. Mamone G, Picariello G, Addeo F, Ferranti P. Proteomic analysis in allergy and intolerance to wheat products. Expert Review of Proteomics. 2011;8(1):95-115.
7. Fasano A, Sapone A, Zevallos V, Schuppan D. Nonceliac gluten sensitivity. Gastroenterology. 2015;148(6):1195-204.
8. Wieser H. Chemistry of gluten proteins. Food Microbiology. 2007;24(2):115-9.
9. Dupont FM, Vensel WH, Tanaka CK, Hurkman WJ, Altenbach SB. Deciphering the complexities of the wheat flour proteome using quantitative two-dimensional electrophoresis, three proteases and tandem mass spectrometry. Proteome Sci. 2011;9(1):10.
10. Haas-Lauterbach S, Immer U, Richter M, Koehler P. Gluten fragment detection with a competitive ELISA. Journal of AOAC INTERNATIONAL. 2012;95(2):377-81.
11. Allred LK, Ritter BW. Recognition of gliadin and glutenin fractions in four commercial gluten assays. Journal of AOAC INTERNATIONAL. 2010;93(1):190-6.
12. Mendez E, Vela C, Immer U, Janssen FW. Report of a collaborative trial to investigate the performance of the R5 enzyme linked immunoassay to determine gliadin in gluten-free food: European Journal of Gastroenterology and Hepatology. 2005;17(10):1053-63.
13. Skerritt JH, Hill AS. Monoclonal antibody sandwich enzyme immunoassays for determination of gluten in foods. J Agric Food Chem. 1990;38(8):1771-8.
14. Panda R, Zoerb HF, Cho CY, Jackson LS, Garber EAE. Detection and quantification of gluten during the brewing and fermentation of beer using antibody-based technologies. Journal of Food Protection. 2015;78(6):1167-77.
15. Vojdani A. The evolution of food immune reactivity testing: why immunoglobulin G or immunoglobulin A antibody for food may not be reproducible from one lab to another. Altern Ther Health Med. 2015;21(Suppl 1):8-22
16. Comino I, Real A, De Lourdes Moreno M, Montes R, Cebolla Á, Sousa C. Immunological determination of gliadin 33-mer equivalent peptides in beers as a specific and

practical analytical method to assess safety for celiac patients. J Sci Food Agric. 2013;93(4):933-43.
17. Akiyama H, Isuzugawa K, Harikai N, Watanabe H, Iijima K, Yamakawa H, *et al.* Inter-laboratory evaluation studies for development of notified ELISA methods for allergic substances (Wheat). J Food Hyg Soc Jpn. 2004;45(3):128-34.
18. Briani C, Samaroo D, Alaedini A. Celiac disease: From gluten to autoimmunity. Autoimmunity Reviews. 2008;7(8):644-50.
19. Good ELISA Practice (GEP) manual, R-Biopharm. https://food.r-biopharm.com/technologies/elisa
20. ICH Guideline, Validation Of Analytical Procedures: Text And Methodology Q2(R1) https://database.ich.org/sites/default/files/Q2%28R1%29%20Guideline.pdf
21. Siebeneicher S, Deaton J, Cuentas A. Validation of a competitive ELISA method on supplemental enzyme matrices. J Nutr Food Sci. 2018;08(03).
22. Matić, J. et al. Analytical validation of an enzyme-linked immunosorbent assay for the determination of gluten, Food Processing, Quality and Safety. 2008;4:183-188.
23. Yu JM, Lee JH, Park JD, Choi YS, Sung JM, Jang HW. Analyzing gluten content in various food products using different types of ELISA test kits. Foods. 2021;10(1):108.
24. Jain S, Lamba BY, Dubey SK. Recent advancements in the sensors for food analysis to detect gluten: A mini-review [2019-2023]. Food Chemistry. 2024;449:139204.
25. Türköz Bakırcı G, Öncü Glaue Ş, Akcan T. Determination of gluten contamination in foods available on the turkish market via enzyme-linked immunosorbent assay (ELISA). Applied Sciences. 2023;13(10):6143.

Cite this article: Tonde AV, Chhajed SS. Hydrolysis of Gluten in Glucose Biscuits Using Protease Enzymes and Validation of Gluten Content Determination Using ELISA. Indian J of Pharmaceutical Education and Research. 2026;60(4):x-x.