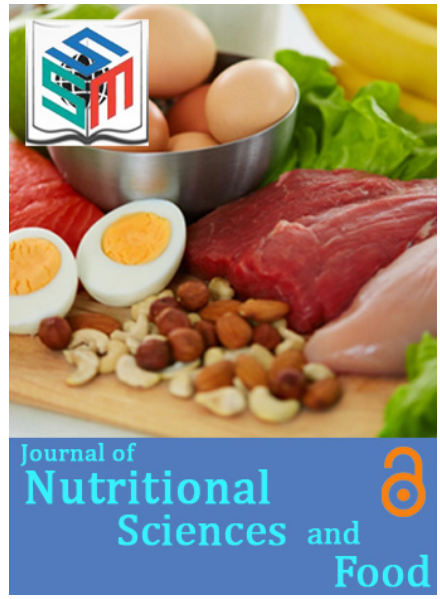
Mini Review

Identification of Gluten Peptide Using Mass Spectrometry: Is it a Better Choice?



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Abstract

Gluten contamination is a serious health issue for Celiac Disease (CD) patients. CD patients are highly sensitive to gluten traces. Only effective treatment for CD is life-long exclusion of gluten from the diet. However, complete removal of gluten from the diet is challenging. More than 10 mg/daily exposure of gluten in CD patients can cause inflammatory reaction. Hence, CD patients are dependent on a safe gluten-free food. Less than 20 mg/kg of gluten is considered a safe quantity of gluten to CD patients. To quantify this small quantity of gluten, gluten detection Enzyme-Linked Immunosorbent Assay (ELISA) method is a quicker and widely used method to identify gluten in the food. Apart from gluten ELISA, Mass Spectrometry (MS), a more sensitive analytical tool than ELISA, is used to identify peptides of gluten protein.

Despite being a highly sensitive and sophisticated analytical technique MS is less often used in laboratories because MS has multiple technical problems that require critical attention. However, with the use of suitable methodology and proper care, MS can be used as an efficient gluten detection tool. In this article, we presented the specific method to perform the MS and also discussed common technical difficulties and provided troubleshooting. This article could be very useful for researchers who remain puzzled while performing gluten identification. This article could also be helpful for beginners to establish MS in the laboratory.

Keyword: Celiac Disease; Gluten; Mass Spectrometry; Gluten Detection ELISA

Introduction

Celiac Disease (CD) is a chronic intestinal inflammation caused due to the ingestion of gluten, a wheat protein, in genetically susceptible individuals [1]. Worldwide more than 1 % individuals are affected with CD [2,3]. Gluten is found in wheat, rye, barley, and other related grains [1]. The only effective treatment for CD is following a life-long strict Gluten-Free Diet (GFD). However, gluten is a pervasive molecule hence it is used almost in every food and non-food industry [4-6]. Therefore, the complete removal of gluten is challenging. CD patients are critically sensitive to traces of gluten, exposure of >10 mg/day gluten is enough to initiate an immunological trigger in most of the CD patients [7]. Several government authorities i.e. Codex Alimentarius and the US Food and Drug Administration (FDA) approved a safe amount of gluten i.e. <20 ppm (mg/kg) of gluten [8,9]. Gluten-free products with <20 ppm of gluten are considered gluten-free [5]. Nevertheless, in recent years, studies have shown that commercially available 'labeled gluten-free food' are not actually gluten-free. Up to 9-30% of gluten contamination is reported in commercially available labeled gluten-free products. The authenticity of such products are under great suspicion [5,10,11].

Therefore, it is important to identify the gluten traces in gluten-free food products. There are two important methods to investigate gluten in food products. The first one is a widely used Enzyme-Linked Immunosorbent Assay (ELISA) method. R5 antibody-based ELISA method is an official method to quantify gluten in food products [5]. The second one is the Mass Spectrometry (MS) based method. MS is a highly sensitive method that can identify even a smaller fragment of gluten that may be missed from the conventional ELISA method [12]. MS is applied widely in sports doping, food authentication, and biomedical and pharmaceutical research [13-15].

However, MS is not a widely accepted method to identify gluten in food products because MS is an expensive and difficult analytical method to perform in the laboratory. It requires a trained and experienced researcher to perform and interpret the results. The protocol of performing MS is critical and requires several precautions and attention during the experiment. Therefore, due to these factors it is not standardized in every laboratory. Also being an expensive method, all laboratories do not have MS facility [12,16]. MS processing required diverse reagents and solutions and during its performance, there could be several handling and processing issues that need the full attention of the technician. In contrast, the ELISA method does not have such difficulties. That is why researchers give priority to the ELISA method. But, ELISA may provide false-positive results and it cannot distinguish the source of gluten among cereals due to cross-reactivity [17]. MS provides an authentic result (see Table 1 for the major differences between ELISA and MS). In this article, we have summarized the MS protocol and discussed the common difficulties during its performance and their troubleshooting.

S. No.	Factors	ELISA	Mass Spectrometry
1	Expense	Medium equipment cost	• Very high equipment cost • Low day-to-day reagent cost
2	Skilled and Trained staff	Less required	Highly required
3	Sample volume	High	less
4	Post translational modifications	Not identified	Identified
5	Automated	Semi-automatic	Almost completely automated
6	Range of analyte and antigen detected	limited	Wide range
7	Time	1-3 hours	2-5 hours
8	Sensitivity	High	High
9	Specificity	Poor (in terms of antibody batch)	High
11	Reproducible	Less	High
12	Cross reactivity	Less	No
13	Multiplexing	Limited multiplexing	High; multiple analyte can be screened
14	High throughput	High	Medium

Table 1: Major difference between gluten ELISA and MS.

Mass Spectrometry: Introduction and Principle

MS is highly sophisticated, precise and a high throughput analytical tool. It provides structural information of the analyte by measuring the mass-to-charge ratio (m/z) values [18]. MS has three major components (1) ionization source, (2) mass analyzer and (3) detector [15,18,19]. In the ionization source, the peptides get ionized (charged) by laser or inert gas. In mass analyzers, these ions are separated according to their mass-to-charge ratio and accelerated toward detector in the electric/magnetic field. The detector detects these ions and shows signals in the form of the mass spectrum. In a typical MS analysis, raw data comprises of mass values of the peptides that reached the detector. The target protein fraction is identified by matching experimentally observed peptide masses (protease digested sample) with the theoretical peptide masses present in the database [18-20]. In intact mass-analysis, the molecular weight of the undigested proteins gets identified. Despite of numerous advantages of MS there are some of the limitations too such as MS is expensive, require expertise, intolerance to contaminants and salts, low protein concentration, lack of protein databases and technical limitation of protein sequence coverage.

Gluten Isolation and Result Interpretation using Mass Spectrometry

For the extraction of gluten is based on the protocol already described by Schalk et al., [21]. To brief, for 100mg of flour, the floor is first treated with salt solution (2.0mL of 0.4M NaCl in 0.067M Na_2HPO_4/KH_2PO_4 buffer, pH 7.6 for 10min at 22°C to extract albumin and globulin content. Then flour is treated with 1.5mL of ethanol/water (60/40, v/v) for 10min at 22°C to extract prolamins and in the third step flour is treated with (2.0mL of 2-propanol/water (50/50, v/v) in 0.1M Tris-HCl, pH7.5, containing 2 M (w/v) urea and 0.06 M (w/v) Dithiothreitol (DTT) for 30 min at 60°C under nitrogen to extract glutelins. The prolamins (gliadins) and glutelins constitute gluten thus both second and third solution should be mixed and lyophilized [21].

Gluten protein exhibits several proteomics challenges as gluten protein has high molecular weight, contains high amount of glutamine, proline and hydrophobic amino acids. Hence trypsin is not a suitable enzyme for gluten digestion because it is very precise and specific for lysine and arginine residues which are present in very few in numbers. The enzymes of choice for gluten digestion are chymotrypsin and thermolysin. The digested sample is shown as mass spectrum along with peak list generated as raw data. Raw data can be searched on licensed software or freely available software. Software ease our search and neutral losses like H_2O and CH_3 etc. and post translational modification can be easily detected by the software. It gives all the information like contribution, confidence, score, sequence, species (source) of the peptide in the spectrum. It also provides information for the protein ID and determines sequence of peptides hence tells the sequence coverage of the protein [22].

Precautions in Processing Mass Spectrometry Sample

For MS experiments, MS grade high-quality reagents and chemicals/solvents must be used. This will reduce the contaminants that could suppress the ionization. All the instruments (gel apparatus, scanner) should be properly wiped with 70% ethanol to lessen contamination. Glasswares and spatula should be properly washed with mild laboratory detergent and rinsed with MiliQ to avoid contamination. Keratin contamination is the most notorious contamination in the protein sample that usually comes during gel handling and numerous steps in protein digestion. To avoid keratin contamination, wear gloves during the entire process and change them often. All the buffers should be made and gels handled wearing gloves. Ammonium bicarbonate solution should be made daily and DTT and Iodoacetamide solution should be made fresh shortly before use. Gel casting and running should be done in

properly washed apparatus wearing gloves. Gel bands should be cut on the clean glass plate with a clean razor. Gel band should be cut properly only stained area is chopped into pieces of 1 mm so that buffer/protease could enter the gel pieces and also not so small to be picked and removed during washing with a pipette. Gel pieces should be transferred to fresh tubes and washing and rinsing should be done with MiliQ twice/thrice with vortexing are recommendable to remove any keratin present on the surface of the gel. Gel pieces should be washed properly between each step to remove extra contamination of salts, buffer, detergents, etc. Also, one should take extra precautions to avoid the use of Triton X-100 and tween-20 for sample solubility as they will suppress the signals. Buffers like tris, phosphate, and HEPES should not be used. Use of Iodoacetamide (IAM) induced alkylation further improves the gluten digestion [12,19,23,24]. The samples should be speed vac dried and stored at -20°C until MS analysis.

Troubleshooting of Processed Sample in Mass Spectrometry

We discussed some common technical issues that arises during MS sample preparation, precautions to be taken care and in this section some important troubleshoots are highlighted to ensure good sample preparation/analysis.

Salt Contamination

Salts/denaturants contamination suppresses the ion signals. Ziptip can be used which binds only with the peptides and removes the salts, buffers, and detergents. Thus sample ionizes well and a clean spectrum is attained.

Keratin Contamination

If after taking all the precautions still, keratin contamination is coming in the results. Create a general list for contaminants and add this in the exclusion list while searching for raw data so that the peaks coming from contaminants will be excluded during data processing.

Fewer Peaks were Observed

The sample is not digested properly, 1% rapigest / 8 M urea can be introduced prior digestion for sample denaturation but there conc. need to be adjusted in the sample prior adding protease.

Conclusion

MS is establishing as a fundamental tool in identification, characterization and quantification of gluten in the gluten free foods. If suitable enzyme and digestion methods are followed in the supervision of experience operator, mass spectrometry can be used as a routine laboratory technique for gluten protein identification.

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Conflict of Interest

All authors declare no conflicts of interest.

Author's Contribution

P.D.; wrote the first version of the article and drafted the manuscript. **P.D. and A.K.V.;** conceived the idea. **P.D.;** Collection and acquisition of data. **A.K.V.;** critically revised the manuscript. **P.D. and A.K.V.;** final approval of the version to be published. **P.D.;** overall guarantor of the manuscript. All authors approved the manuscript.

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