A DISSERTATION ON

REAL TIME QUANTIFICATION OF SOYA ALLERGENS IN FOOD PRODUCTS

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BY

NEERAJ KUMAR PATEL

Roll No. 2001021024
M.Sc. Biotechnology (IV Semester)
Department Of Biosciences
Integral University, Lucknow

UNDER THE SUPERVISION OF

DR. ABHINAV PATHAK

Head Of Department, Biotechnology

INTEGRAL UNIVERSITY

Established Under U.P. Act No 09 of 2004 by state Legislation
Approved by University Grants Commission
Phone-No.: +91(0552)2890812,2890730,3296117,6451039
Kursi Road, Lucknow-226026 Uttar Pradesh (INDIA)

TO WHOM IT MAY CONCERN

This is to certify that **Mr. NEERAJ KUMAR PATEL** student of M. Sc. Biotechnology, (IV semester), Integral University has completed his four months dissertation work entitled **"REAL TIME QUANTIFICATION OF SOYA ALLERGENS IN FOOD PRODUCTS"** successfully. He has completed this work from the FARE Labs Pvt. Ltd., Gurgaon, under the supervision of **DR. ABHINAV PATHAK.** The dissertation was compulsory part of her M.Sc. degree.

I wish him good luck and a bright future.

Dr. SNOBER S.MIR

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FARE LABS Private Limited
L-17/3, DLF Phase-II, IFFCO Chowk, M.G. Road,
Gurgaon-122002, Haryana, INDIA
Phone : +91-124-4223207, 4034205
Fax : +91-124-4036038, Cell : +91-95992 21227
E-mail : farelabs@farelabs.com
Website : www.farelabs.com

30th May 2022

TRAINING CERTIFICATE

This is to certify that **Neeraj Kumar Patel,** S/o Hira Lal Patel from**, Integral University, Lucknow**, MSC in Biotechnology has successfully completed his dissertation in **"Real time quantification of soya allergens in food products "** from 31st Jan-2022 to 30th May 2022 at **FARE Labs Pvt. Ltd.** and has been awarded excellent grade on the basis of his performance.

He has accomplished the dissertation successfully. We have found him sincere and devoted during the training.

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DECLARATION

I, **NEERAJ KUMAR PATEL**, certify that the work embodied in the training report **"Real Time Quantification of Soya Allergens in Food Products" to** be submitted to the Master of Science in Biotechnology of Integral University, Lucknow, Uttar Pradesh, India is original and is the result of analysis carried out by me under the supervision of **Dr. Abhinav Pathak** Head of Biotechnology Department, FARE Labs Pvt. Ltd. for the time period of January, 2022 to June, 2022. The matter embodied in Master of Science thesis has not been submitted for the award of any other degree/ diploma.

I declare that I have faithfully acknowledged and referred to the research workers wherever their works have been cited in the text. I further certify that I have not willfully lifted up some other's work, paragraph, text data, results, etc. reported in journals, books, magazines, reports, dissertations thesis, etc., or available at web sites and included them in this M.Sc. thesis and cited as my own work. I have completed all pre submission requirement as per the University rules.

NEERAJ KUAMR PATEL

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Sincerely

Neeraj Kumar Patel

CONTENTS

1. INTRODUCTION

Humans require food to survive. In addition to basic nutrition, food consumption provides a sense of gratification pleasure and serves as a source of social entertainment. However, for a small percentage of the population, consumption of certain foods even in small quantities can result in life-threatening allergic reactions. In developed countries approximately 4-8% of young children and 2-4% of adults suffer from food allergies (Kenny et al. 2001, Munoz-Furlong et al. 2004, Sampson 2004, Sicherer et al. 2004, Breiteneder and Mills 2005). Recently nine foods (and their derived products), have been considered to be major allergens accounting for over 90% of all food allergic reactions, although over 170 foods are known to cause food allergies. FAAN (2001) reported that there are 8 major food or food groups which caused more than 90% of allergic reaction to the sensitive individual, these foods are egg, wheat, fish, shellfish, milk, soya, peanuts and tree nuts. The food containing protein may induce an allergic reaction too.

Food allergy is an immunological reaction, resulting from the ingestion, inhalation or contact of food. Immunological reactions can be mediated by IgE antibodies or other immune cells such as T cells. The foods contain several types of allergens, which trigger the immune response to allergic person. Recent studies have suggested that a small amount of allergen present in food can cause the allergic reaction but the extent can vary from individual to individual. Adverse response to foods can be defined as any unusual reaction to the food and component caused by the ingestion. Adverse response to foods is two type, one toxic and other is non-toxic reaction, toxic reactions may happen when an individual ingests certain amount of the toxic compound, while non-toxic response merely occur in susceptible individuals (Bruijnzeel et al. 1995). Food hypersensitivity is a type of adverse reaction recognized as food allergy. It may be due to immunological mechanisms; though, many of food reactions are not hypersensitive (non-allergic) (Johansson et al. 2001).

According to one report (Gupta et al., 2013) in the United States, $24.8 billion was the total annual economic impact of food allergy in the children. As the complete knowledge of molecular mechanisms involved in food allergy is still unknown, so cure of these allergies is not available. Avoidance of the offending food is therefore the best

option for sensitive individuals; however, this avoidance can occur only if the presence of the offending allergen is known.

There are food allergens known and my study is focused on Soya food allergen that is recognized as one of the richest sources of vegetable proteins that could cause adverse reactions in allergic individuals if consumed at even low concentrations.

Soya (*Glycine max*) is a legume belonging to the *Fabaceae* family and the *glycine* genus (**Fig 1**). Soya is recognized as valuable source of vegetable protein. On a dry basis, soya contain35–40% protein, 17–23%lipid, 31% carbohydrate and 4–5%minerals. Molecular weight of 16 IgE binding proteins of soya is 14 kDa to 70 kDa and major seed storage of proteins are to be expected the major allergens (Ballmer-Weber et al., 2007; Holzhauser et al., 2009; Ogawa et al., 1991; Ogawa et al., 1993). Soya is considered as one of the most nutritional plant sources of food providing a well-balanced amino acid profile and good supply of omega 3 and omega 6 fatty acids. Soya protein contains enough amount of vital amino acids; histidine, isoleucine, leucine, lysine, phenylalanine, tyrosine, threonine, tryptophan and valine. Soya is used by the food industry for the production of bakery products (e.g., bread and cookies), pastry, pasta, sauces, and vegetarian products (e.g., nonmeat sausages). Soya is treated into various fermented and non- fermented food packs in Asian countries such as soya sauce, yogurts, desserts, baby food, protein crisp, miso, natto, kinako and soya milk which is further treated into tofu, aburaage and yuba. Soya is listed in Canada, the United States, Australia and the European Union as a priority allergen, requiring labeling when it is used as an ingredient in food. The first allergic reaction to soya in humans was described in 1934. The Food and Agriculture Organization of the United Nations includes soya in its list of the 8 most significant food allergens. Anti-soya IgE antibodies have been identified but allergen specificity patterns are variable and complex. As many as 28 soya proteins bind to IgE from soya-allergic patients. Although the majority of allergic responses occur on ingestion, allergic reactions on inhalation of soya and soya byproducts has also been reported (Gonzalez et al. 1992, 1995, Codina et al. 1997).

Fig 1: Soya Seeds

Soya (Glycine max) is one of the "classic food allergens" which is responsible for causing reactions in children and adults. Soya allergy symptoms most commonly begin with prick in the mouth, swelling of the lips, face, throat, tongue, skin itching, runny nose, difficulty in breathing and also include digestive and respiratory disorders. These symptoms usually occur within seconds or minutes of exposure of the food. Sometimes it become life threatening anaphylactic reaction. Most important to think about exhaustion of soya food products does not cause a risk of allergy expect to those who are already sensitized (Goodman et al., 2008). Those who have allergy with soya are at risk if they consume soya and they should avoid the consumption of soya to avoid the risk of allergic reaction and non-allergic consumers can consume the soya without any risk.

The development of specific and sensitive method for the detection of declared, hidden or wrongly labeled allergen in food is a great challenge. The present study focuses on the qualitative rapid detection of soya allergen from different variety of food products by DNA based detection method that will provide valuable reinforcement tool in the ongoing effort to manage food allergens in food supply.

2. Review of Literature

2.1 Food

One of the basic necessities of our life is food. One can't survive without the consumption of food because it provides energy, various nutrients for the growth of our body. Food is necessary for our overall growth like physical, mental, social, etc. The food provides various nutrients like carbohydrates, proteins, minerals, vitamins, fats, etc. for our body to function properly.

2.2 Food Allergy

Food allergy is a chronic reaction of the immune system triggered by the consumption of a food protein antigen. Food allergy is reported as the common adverse immune response to food components, it affects approximately 5% of young children and 3% to 4% of adults in westernized countries, whose prevalence has increased in recent years (Scott H. Sicherer et al., 2010, Amadeo Sena-Torralba et al., 2020). Food allergies, causative factors are crustacean, egg and egg products, fish and fish products, milk and milk products, peanuts, soya and its products, tree nuts, sesame seed and its products (Hefle 1996; Bousquestwt al., 1998; Zarkadas et al., 1999; Sampson 2004). Food allergy reactions involve the immune system and IgE mediated reaction that occurs due to exposure of specific food proteins (Wang and Sampson 2011). Food induced allergic reactions are responsible for a variety of symptoms and disorders involving the skin and gastrointestinal, urticarial and respiratory tracts and some symptoms become a life-threatening anaphylactic reaction. Anaphylaxis is a serious and systematic reaction that occurs immediately after consumption of allergy causing substance (Sampson et al., 2006; Sampson 2008). Immunological reactions can be mediated by IgE antibodies or other immune cells such as T cells. In general, any protein-containing food can induce an allergic response in sensitive individuals. Allergic proteins in foods may be enzymes, enzyme inhibitors, structural proteins or binding proteins with varied biological functions (Stewart and Thompson 1996, Valenta et al. 1999, Chapman et al. 2000, Martin and Chapman 2001). Food allergies have become a health issue with various symptoms that range from mild to life threatening, especially in those countries which are industrialized (Patricia Schubert-Ullrich et al.,

2009, Florian Luber a et al., 2014). Food allergies trigger effect even at low concentration, patients are in risk due to food allergies i.e., gluten, milk, fish, egg, crustacean, soya, especially peanut allergy (Sasithon Temisaket al., 2019).

2.3 Soya

Soya is a crop native to China and Southeast Asia; it has a high nutritional value due to high concentration of oil (18-25%) and protein (38-50%). Soya is a popular food all over the world (Muller et al., 1998). Soya is most important technological support for the food industry and induces serious allergic reaction in food sensitized/allergic individuals (Caterina Villa et al., 2020). Soya crop is grown globally, 38% in US, Brazil (25%), Argentina (19%), China (7%), India (3%), Canada (2%) and Paraguay (2%), (Singh et al., 2008). Raw Soya are mainly used in soya milk, tofu, soya sprouts and edamame in western countries, while soya protein products are used in tofu burgers, soya sausages, chicken nuggets, soya ice cream, yogurt and various other products (Hammond and Jez, 2011). Various health benefits have been reported due to consumption of soya and soya products like lowering plasma cholesterol, preventing cancer build bone mineral density, diabetes, reduced risk of cardiovascular disease and safety in case of bowel and kidney disease (Anthony et al., 1996; Kennedy, 1998; Krejikamp-Kaspers et al., 2004; Friedman and Brandon, 2001; Hori et al. 2001; Chen et al. 2003; Zhang et al. 2003; Stephenson et al. 2005; Anderson 2008).

2.4 Soya Allergen

Soya is identified as a potential allergen, so presence of soya should be indicated in all food products (Montserrat Espineira & Franciosco J. Santaclara, 2017). Soya is listed as a preference allergen in Canada, United States, Australia and the European Union, labelling is required for use of soya as an ingredient in foods. Symptoms of soya allergy are similar to the other major allergens and include cutaneous, respiratory as well as gastrointestinal responses. Although the majority of allergic responses are ingestion and allergic reactions on inhalation of soya and soya by products which have also been reported (Gonzalez et al. 1992, 1995, Codina et al. 1997).

2.5 Major Soya Allergens

Over 17 different allergens have been identified in soya bean. These include soya bean glycinin (11S), β-conglycinin (7S), soya bean vacuolar protein (Gly m Bd 30K or P34), the Kunitz trypsin inhibitor (KTI), Gly m Bd 28K, soya bean profilin (Gly m 3), soya bean hull proteins (Gly m 1.0101, Gly m 1.0102, Gly m 2) and the pathogenesis-related (PR) soya bean protein SAM22 (Gly m 4) (Wilson et al. 2005, L'Hocine and Boye 2007, Boye et al. 2010). Glycinin and β-conglycinin have been studied extensively as they are the major soya bean storage proteins and represent over 70% of the proteins found in soya (Liu 1997).

2.6 Prevalence, Symptoms and Threshold

The prevalence rate of soya allergy is in between 0.3% and 0.4% (Becker et al. 2004, Sicherer and Sampson 2006). The threshold levels of soya protein, reported vary and are in between 0.0013 and 500 mg (Bindslev-Jensen et al. 2002, Ballmer-Weber et al. 2007).

2.7 Methods for detecting allergens

Food allergens can be detected either by the use of marker in the target food or by the presence of the target allergen itself. The detection technique will depend on factors such as food matrix interference, nature and quantity of the target allergen, the desired level of detection, specificity and time and resources required for running the assay (**Fig 2**).

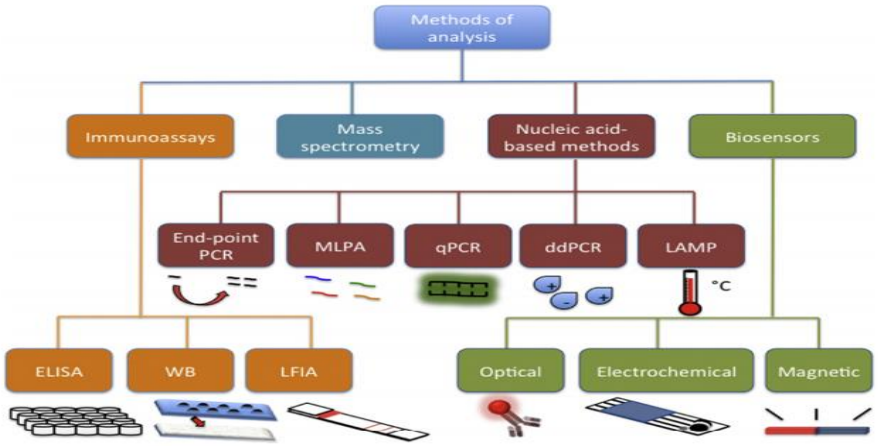

Fig 2: Outline of the current methods to analyse food-borne allergen detections.

Immunoassays are subcategorized into Western Blot, Enzyme Linked Immuno sorbent Assay and Lateral Flow Assay. Nucleic-acid based methods are subcategorized into Multiplex Ligation-Dependent Probe Amplification (MLPA), quantitative PCR (qPCR), end-point PCR, loop-mediated isothermal amplification (LAMP) and digital droplet PCR (ddPCR). According to the signal transduction, biosensors are categorized into magnetic, electrochemical and optical.

2.7.1 Immunoassays

For the detection of protein/peptide biomarkers immune analytical methods rely on using specific and high affinity antibodies that indicate the presence of allergenic ingredients in food samples. Antibodies can be either polyclonal or monoclonal, depending on their ability to bind with more than one epitope. The former is cheaper and faster to produce, but are more prone to batch-to-batch variability. Monoclonal antibodies have higher consistency but takes longer time to complete the experiment. Monoclonal antibodies are used in competitive assays while the combination of monoclonal and polyclonal antibodies is used in sandwich-based assays (N.S. Lipman et al., 2005; C.A. Ascoli &B. Aggeler 2018). The new generation of immune based bio receptors are used to detect food borne allergens, i.e., single-domain antibodies. These receptors give better results with improved characteristics.

2.7.2 ELISA (Enzyme Linked Immune sorbent assay)

ELISA has obtained importance and became a highly used technique in biomedical and biochemical field due to availability of automation, specificity, user friendliness and high sensitivity, it among the growing number of food allergen detection technique. Some ELISAs detect mixtures of proteins from the allergenic sources (e.g., peanut milk, almond-soluble proteins and total egg; Taylor et al., 2009), whereas other ELISAs are designed to detect specific allergens, e.g., Ara h1 (a major peanut allergen), BLG and shrimp tropomyosin). Rapid ELISA kits are commercially available for gluten, milk, soya, peanut, hazelnut, almond, egg and crustaceans, that produce qualitative or semi-quantitative results within 30 and 60 min (Patricia Schubert-Ullrich et al., 2008).

3.7.3 Western blot

Western blot combines techniques such as SDS-PAGE electrophoresis for the separation of protein allergens (based on molecular mass) which is followed by immunoassay on a membrane support for allergen detection. Western blot gains insight into the protein/peptide profile or differently processed foods, which is interesting for food allergen detection but sensitivity of western blot is low as compared to ELISA. This is useful, on the one hand for designing optimum antibodies based on the antigenic fragments present after food processing and other hand for distinguishing the allergen of interest from protein inhibitors that might lead to false-positive results in ELISA assay. Using HRP-conjugated gluten-specific antibodies (G12, R5, 2D4, MIoBS and Skerritt), from nine commercial gluten ELISA test kits, Panda and colleagues developed a Western blot assay for gluten detection. These authors analyzed 59 fermented-hydrolyzed foods from four food groups (beer, soya-based sauces, vinegar, and sourdough bread) and classified them into clusters based on differences in proteolytic fermentation processes. The assay proved to be highly specific, but unsuitable for allergen quantification. So, this analysis method should be combined with ELISA for proper allergen quantification when following appropriate calibration standards (R. Panda & E.A.E. Garber, 2019).

2.7.4 Lateral-flow assays (LFA) and Dipstick Tests

LFAs or strip tests are immune chromatographic tests with a mobile phase that allows movement of the allergen–antibody complex or sample along a test strip. This technique is based on an immune chromatographic procedure that uses antigen–antibody properties and allows rapid detection of the analyte (Rong-Hwa et al. 2010). Both of these tests are simplified versions of ELISA and are designed to meet the growing need of portable, reliable, user friendly and cost-effective methods that can detect trace number of allergens in suspected foods within a short period of time (Schubert-Ullrich et al. 2009). Dipstick tests are required incubation phase and principal of this test is based on the LFA principal, but without mobile phase. Oliver et al., (2002) described that the result of dipstick assay and the corresponding ELISA were in concordance when used for detection of peanut and hazelnut allergens. Food allergen detection and incubation time could vary from 10 minutes to 3 hours for dipstick assays (Schubert-Ullrich et al., 2009).

2.7.5 Mass spectrometry (MS)

Mass spectrometry (MS) has become an alternative to immunoassays for food-borne allergen detection in the last decade for offering interesting advantages from the analytical viewpoint. For instance, immunoassays show less sensitivity when food allergens undergo harsh transformation or processing (precipitation, fermentation, acidification, etc.), which also suggests false-negative results. They also tend to give false-positive results due to antibody cross-reactivity with homologous proteins. It is noteworthy that minor differences in sample preparation or antibody composition can dramatically affect assay repeatability, and immunoassays rely on complex strategies for multiplexing purposes, although this aspect has greatly improved in recent years. Indeed, MS has overcome these limitations because it does not require antibodies, offers simple multiplexing and is indifferent to denatured proteins, although modified proteins show allergic effects rarely (D, Croote & S.R. Quack, 2016). However, MS relies on expensive instrumentation and trained personnel. Therefore, LFIA is performed on-site and MS is restricted to specialized laboratories. The MS technique has four stages; peptide target selection, peptide specificity verification, running the targeted method and food allergen quantification. Enzymatic digestion of proteins and many more steps are included in last step, followed by HPLC separation and MS analyses. In the last decade, most widely used method for food allergen detection is MS with liquid chromatography, which is applied for the detection of fish allergen. (L. Sun et al., 2019; R. Stella et al., 2020), sesame (X. Ma et al., 2020), barley, corn, oats, rice, rye and wheat (W. Jira & S. Munch, 2019). Then, the triple quadrupole (QQQ) and quadrupole ion trap (Q-IT) systems draw attention because they enable food allergen quantification.

2.8 Nucleic acid-based methods

This is an indirect analytical method that relies on the detection of allergen coding genes. Even though it’s an indirect detection method, it presents several advantages over detection of the allergen itself. Firstly, DNA is much stable than proteins, which commonly denature on extreme conditions (temperature, acidity, etc.) are applied during food processing. The conformational changes in protein epitopes destroy antibodies targeting that gives false-negative results. On the other hand, allergen extraction methods are based on using acidic solutions and mechanical forces and

DNA is less affected by these methods. Therefore, nucleic acid-based detection methods are compatible with a wide range of allergen extraction protocols, and are typically related to higher recovery efficacies when challenged with commercial food products (L. Fu, 2019; M. Prado, 2016).

2.8.1 End-point PCR

End-point PCR is the simplest and oldest way to analyse PCR products, which are usually visualized by gel electrophoresis to determine their size and relative quantity (D. Fraga et al., 2008). This technique is often applied for cloning, sequencing, genotyping and sequence detection. For detection of food allergen, end-point PCR has been applied for multiplexing purposes in past few years. Multiplex PCR assay is based on using several primer pairs that are specific to multiple targets, and to enable their amplification in a single reaction, which thus lowers assay costs and time. However, differences in each target's amplification efficiency and issues related to primers competition mean that optimizing PCR conditions an essential requirement. Suh et al. developed a PCR assay for the synchronous detection of apple, kiwi, tomato and peach allergen-coding genes. In the processed foods, the DNA is usually degraded into small fragments; therefore, primers were designed in such a way that they amplify products not more than 200 bp (T. Yoshimura, 2005). The annealing conditions provide sensitivity and optimum specificity 62˚C for 20s. By using a gel electrophoresis, amplicons were analyzed.

2.8.2 Multiplex ligation-dependent probe amplification (MLPA)

Multiplex ligation-dependent probe amplification (MLPA) technique was introduced in 2002 which is a cost effective (as probes are cheaper), easy to use method with higher reproducibility that came as an alternative method of multiplex PCR (J.P. Schouten et al., 2002). The simultaneous detection of five food allergens: poppy, flaxseed, soya, sunflower and sesame was done by MLPA. Species specific ligation probes were designed for amplifying ribosomal DNA’s nuclear ITS1 region. Another set of probes was also designed to target 18S rRNA in order to serve the assay a positive control. Amplicons were analysed by capillary electrophoresis. The specificity of the designed probes was the first thing to be assessed by evaluating DNA extracts from 46 plant

species and four animal species. Non-specific peaks were not observed in capillary electrophoresis, so it was concluded that MLPA meets specificity requirements. It was revealed by the sensitivity assays that the lowest concentration detected by MLPA was 10 mg/kg that was 10-fold higher as compared to TaqMan Real Time PCR. So MLPA was not able to detect the non-declared foods but Real Time PCR was (I.M. Lopez-Calleja et al., 2017).

2.8.3 Real Time-PCR (RT-PCR)

Real-time PCR-based methods have been established and accepted in food analyses for many years now, and represent an indirect approach for food allergen analyses by measuring allergen coding genes, in that itself the allergenic protein is not the analytical target but gene sequence encoding sequence is used as the analytical target. In the food allergen detection, this technology provides a very specific, highly sensitive and quantitative result. As DNA is a very stable molecule, PCR based real-time methods can also be applied in highly processed food matrices, but this requires sample preparation for specific DNA extraction purpose. PCR gave reliable and consistent results for the detection of food allergens. After Prof. Higuchi introduced Real Time PCR and quantitative PCR (qPCR) in 1992, they are widely being used as DNA amplification tools. Its success lies in its ability to monitor the amplification of a target sequence in real-time using fluorescent labels (D. Fraga et al., 2008). Real time PCR quantifies nucleic acids even at very low concentrations of DNA accurately. This method is being used as the reference method to quantify and identify the allergen-coding genes in food products. Costa et al. developed a Real Time PCR method for the detection of soya bean allergen in processed meat products, which is coupled with a fluorescent hydrolysis probe. DNA was isolated by the Wizard Method while primers were designed by the Eurofins for the specific detection of lectin gene in soybean samples. Soybean in pork meat was assayed in both thermally processed and raw meat samples. A LOD of 10 mg/kg was estimated by that assay. 25 commercial meat samples were evaluated that gave reliable applicability. A good agreement was shown by about 90% meat samples in case of soybean labelled information (J. Costa et al., 2017.

2.8.4 Droplet Digital-PCR (dd-PCR)

Droplet digital PCR (dd-PCR) is also an interesting DNA-based method for determining allergenic ingredients in food. Its operation is based on partitioning target molecules into several thousands or millions of individual droplets in a water-oil emulsion. According to Poisson distribution, some droplets will contain target molecules, while others will contain no target molecules. The absolute quantification of the target molecule is done by counting the negative and positive droplets after completion of PCR cycling. The standard calibration curve is not needed in dd-PCR for the quantification of target genes as it is primarily required in Real Time PCR. This property of dd-PCR makes it more accurate and reliable (B.J. Hindson et al., 2011; C.M. Hindson et al., 2013). But the cost of dd-PCR system is way too high which is a major drawback of it. The most widespread instrument for dd-PCR is QX200 AutoDG (Bio-Rad) which costs around $15000. In the recent years, dd-PCR has gained popularity to detect food allergens as compared to real time PCR, as ddPCR gives high analytical sensitivity even at low concentrations of DNA. A dd-PCR assay was developed by Temisak et al. for the detection of biomarker of peanut, the Arachishypogaea allergen II gene.

2.8.5 Loop-mediated isothermal amplification (LAMP)

Under isothermal conditions LAMP assay is based on the amplification of target DNA, combined with the visual detection of amplicons by the naked eyes. The former is achieved by Bacillus stearothermophilus (Bst) DNA polymerase's strand displacement ability, which enables amplifications to be carried out at constant temperature (60-65˚C). The latter is accomplished by the turbidity-based detection of insoluble by-products (magnesium pyrophosphate) or the fluorescent-based detection of DNA-intercalating dye molecules (SYTO 9 or SYBR Green). While using dedicated readers, both of them confer LAMP with real-time quantification capabilities. No expensive laboratory equipment (thermocycler or electrophoresis) is required for carrying out the assay at constant temperature and producing naked eye evaluations enables LAMP to be applied at the point-of-care. This is probably the best advantage of LAMP over other DNA-based detection methods (N. Tomita et al., 2008; Y. Mori et al., 2001; T. Notomi et al., 2000). The LAMP assay however, does not have specific guidelines for optimization like other DNA-based detection methods and therefore, cannot be

appropriately compared to other methods. For instance, sensitivity in LAMP is often evaluated using 10-fold serial template dilutions, and the results are directly compared to the sensitivities of other PCR methods (E.M. Khorosheva et al., 2016).

2.9. Biosensors for food-borne allergen detection

Biosensors have been applied to a wide range of fields thanks to their excellent versatility in bio receptors, materials and transduction modes terms. Biosensors have appeared as an alternative to the laborious, expensive and time-consuming assays like spectroscopy and chromatography in the food processing industry, for the purpose of food authentication and safety monitoring. These include the detection of pathogens, pesticides, additives and allergens in food (P. Leonard et al., 2003; P. Poltronieri et al., 2014; M.S. Thakur & K.V. Ragavan, 2013). According to the signal transduction mode biosensors can be classified into five classes, optical, electrochemical, mass, magnetic, calorimetric or micro mechanical. Optical and electrochemical are most frequently used biosensors.

3.9.1 Optical biosensors

In this, the transduced light signal can be caused directly through the detection of labels coupled to the bio receptors (label-based biosensors) or by interaction of the target analyte with the transducer (label-free biosensors) (J. Svitel & J. Katrl, 2016). There are many optical phenomena that are useful for detection in biosensors. The most widely used can be classified upon the signal response, such as: phosphorescence, reflection, colorimetry, refraction, surface-enhanced Raman scattering, absorption, chemiluminescence, interferometry, fluorescence and dispersion (J.D.D. Habimana et al., 2018).

2.9.2 Electrochemical biosensors

Electrochemical biosensors use an electrochemical transducer to generate a signal in relation to a bio recognition event. The principle of this detection technique is the production or consumption of ions or electrons when the target analyte is recognized by immobilized bio receptors. Consequently, a change in the current, potential, conductivity or other electrical parameter is recorded (G.W. Daniel Thevenot & Richard

Durst Klara Toth, 1999). For the detection of food-borne allergen two electrochemical based biosensors have been developed in recent years. On the one hand, Pereira-Barros et al. designed a disposable ampere metric biosensor for the detection of the Sola I 7 tomato allergen-coding gene in real food samples. This biosensor bears a screen-printed carbon electrode (SPCE) which is associated with a magnetic bead-conjugated specific RNA capture probe (RNACp).

2.9.3 Magnetic Biosensors

Apart from optical and electrochemical classic bio sensing detectors, other types of signal transduction modes have been recently applied for food-borne allergen analyses. For instance, Ng and co-workers developed a giant magneto resistive (GMR) biosensor for the simultaneous detection of major peanut allergens Ara h1 and Ara h2, and wheat allergen Gliadin. The working of this biosensor is based on GMR biosensor containing capture antibodies which form a sandwich with detector antibodies after recognizing the target allergens. The latter were conjugated with magnetic Nano particles to generate a localized magnetic field, and consequently led to a change in the sensor's resistance.

2.10 DNA-Based Allergen Detection Methods

2.10.1 Polymerase Chain Reaction (PCR)

A new method has been developed for the specific and sensitive detection of soya (Glycine max) in processed meat products using polymerase chain reaction (PCR) technology (Rolf Meyer et al., 1996). The polymerase chain reaction involves these steps: isolation of DNA from the food, the amplification of specific genes by using specific primers that bind to the complementary strands of the target DNA and that is followed by agarose gel electrophoresis (Besler 2001). In principle a specific DNA fragment, flanked by 2 oligonucleotides serving as primers for the reaction, is amplified by a thermos stable DNA polymerase. PCR reaction contains 3 major parts, each part set on by a different temperature to allow melting of the double-stranded DNA, annealing of the primers and extension from the primers by the polymerase. Typically, 25-45 cycles of this temperature profile are run to produce a detectable quantify of the DNA fragment. Amplified product is visualized and identified by agarose gel electrophoresis (Roland Ernest & ElikeAnklam., 2004).

2.10.2 PCR-ELISA

PCR-ELISA is a combination of a highly specific DNA-based method with the rather simple and highly sensitive ELISA assay for semi-quantitative analysis (Poms et al., 2004). A specific DNA fragment of an allergenic food is amplified with PCR-ELISA, and amplified product is bound to the surface of a micro plate in post PCR step. After denaturation process DNA sequence specific probe is hybridized to the single strand PCR product. Probe is a protein or happen labelled DNA fragment, which is detected by an ELISA-like reaction (Poms et al., 2004). The concentration of DNA can be determined via absorbance obtained from an enzyme-substrate reaction.

3. MATERIALS AND METHODS

3.1 Isolation of genomic DNA

200 mg of homogenized sample was used for DNA Isolation. Isolation of DNA was based on CTAB method. Sample lysis with a detergent that solubilizes the cell components and inhibits the action of intracellular nucleases was done followed by protein denaturation by *Proteinase K* and elimination by salt precipitation. The DNA in solution is precipitated with isopropanol, washed with ethanol, and finally resuspended in a DNA-stabilizing buffer.

Steps of DNA isolation were as follows-

- Transfer 200 mg of a homogeneous sample in to a sterile 1.5 ml micro centrifuge tube.
- Add 300 µl of sterile deionised water, mix with a loop.
- Add 500 µl of CTAB- buffer, mix with a loop.
- Add 20 µl Proteinase K (20 mg/ml), shake and incubate at 65°C for 30-90 min.
- Add 20 µl RNase A (10 mg/ml), shake and incubate at 65°C for 5-10 min
- Centrifuge for 10 min at about 16,000xg
- Transfer supernatant to a micro centrifuge tube containing 500 µl chloroform and shake for 30 sec.
- Centrifuge for 10 min at 16,000xg until phase separation occurs
- Transfer 500 µl of upper layer in to micro centrifuge tube containing 500 µl chloroform, shake.
- Centrifuge for 5 min at 16, 000xg
- Transfer upper layer to a new micro centrifuge tube.
- Add 2 volumes of CTAB precipitation solution, mix by pipetting.
- Incubate for 60 min at room temperature
- Centrifuge for 5 min at 16,000xg
- Discard supernatant
- Dissolve precipitate in 350 µl NaCl (1.2M)
- Add 350 µl chloroform and shake for 30 sec

- Centrifuge for 10 min at 16,000xg until phase separation occurs
- Transfer upper layer to a new micro centrifuge tube
- Add 0.6 volumes of isopropanol, shake
- Centrifuge for 10 min at 16,000xg
- Discard the supernatant
- Add 500 µl of 70% ethanol solution and shake carefully
- Centrifuge for 10 min at 16,000xg
- Discard supernatant
- Dry pellets and re-dissolve in 100 µl of sterile T.E. buffer.

  *DNA solution may be stored in a refrigerator for maximum of two weeks, or in the freezer at -20°C for longer periods.

3.2 Quantification of gDNA

Qualitatively, the genomic DNA was assessed visually by agarose electrophoresis (Fig 3). 2µl DNA was combined with 0.5 µl of 5xbromophenol blue (loading dye) on a 0.8% agarose gel and the electrophoretic run was performed in 1x TAE buffer (1X= 40 mMTris acetate, 1mM EDTA). The visualization of DNA was done using SYBR safe DNA gel stain. The gel was run at 70V/cm and visualized under UV illumination. The dye intercalates within the molecule and gives off fluorescence. The intactness of band (without any shearing) shows good quality of gDNA. Quantification of DNA was done using a Qubit 4 Fluorometer and concentration of gDNA was adjusted to 50 ng and stored at 4°C.

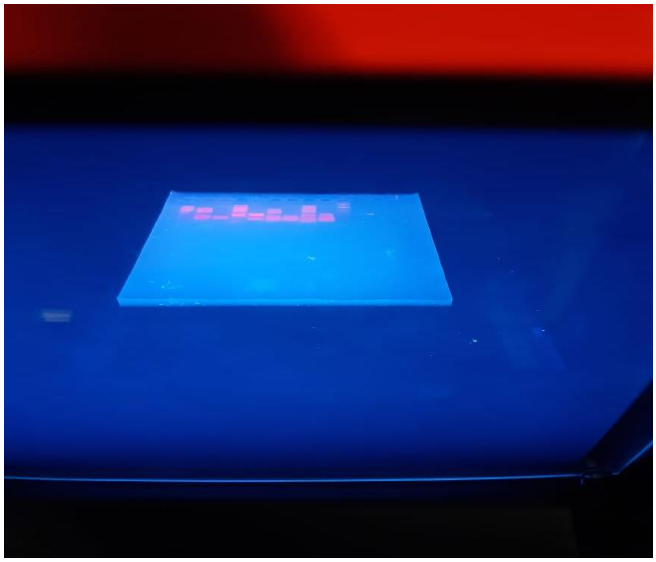

Fig 3: Genomic DNA Assessment through Agarose Gel Electrophoresis

3.3 Primer designing and custom synthesis

The soybean lectin *(Le1*) gene having accession no- K00821.1, was downloaded from the NCBI database as represented in Fig-1. Primers pairs were designed using online software, Primer3 (Rozen et al. 2000) as shown in Fig-2. The primer characteristics such as, hairpin loop, primer dimer formation, etc. were checked on Oligoanalyzer 3.1 (Integrated DNA technology) before custom synthesis.

>K00821.1 Soybean lectin (Le1) gene, complete CDs

CAATGCCATCGTATCGTGTCACAATGGAATACAGCAATGAACAAATGCTATCCTCTTGAGAAAAG
TGAAA
TGCAGCAGCAGCAGCAGACTAGAGTGCTACAAATGCTTATCCTCTTGAGAAAAGTGAAATGCAG
CGGCAG
CAGACCTGAGTGCTATATACAATTAGACACAGGGTCTATTAATTGAAATTGTCTTATTATTAAATAT
TTC
GTTTTATATTAATTTTTTAAATTTTAATTAAATTTATATATATTATATTTAAGACAGATATATTTATTTG
TGATTATAAATGTGTCACTTTTTCTTTTAGTCCATGTATTCTTCTATTTTTTCAATTTAACTTTTTATT
T
TTATTTTTAAGTCACTCTGATCAAGAAAACATTGTTGACATAAAACTATTAACATAAAATTATGTTAA
CA
TGTGATAACATCATATTTTACTAATATAACGTCGCATTTTAACGTTTTTTTAACAAATATCGACTGTA
AG
AGTAAAAATGAAATGTTTGAAAAGGTTAATTGCATACTAACTATTTTTTTTCCTATAAGTAATCTTTT
TT
GGGATCANNTGTATATCATTGAGATACGATATTAAATATGGGTACCTTTTCACAAAACCTACCCTT
GTTA
GTCAAACCACACATAAGAGAGGATGGATTTAAACCAGTCAGCACCGTAAGTATATAGTGAAGAAG
GCTGA
TAACACACTCTATTATTGTTAGTACGTACGTATTTCCTTTTTTGTTTAGTTTTTGAATTTAATTAATT
AA
AATATATATGCTAACAACATTAAATTTTAAATTTACGTCTAATTATATATTGTGATGTATAATAAATT
GT
CAACCTTTAAAAATTATAAAAGAAATATTAATTTTGATAAACAACTTTTGAAAAGTACCCAATAATG
CTA
GTATAAATAGGGGCATGACTCCCCATGCATCACAGTGCAATTTAGCTGAAGCAAAGCAATGGCTA
CTTCA
AAGTTGAAAACCCAGAATGTGGTTGTATCTCTCTCCCTAACCTTAACCTTGGTACTGGTGCTACT
GACCA
GCAAGGCAAACTCAGCGGAAACTGTTTCTTTCAGCTGGAACAAGTTCGTGCCGAAGCAACCAAA
CATGAT
CCTCCAAGGAGACGCTATTGTGACCTCCTCGGGAAAGTTACAACTCAATAAGGTTGACGAAAAC
GGCACC
CCAAAACCCTCGTCTCTTGGTCGCGCCCTCTACTCCACCCCCATCCACATTTGGGACAAAGAAAC
CGGTA
GCGTTG**CCAGCTTCGCCGCTTCCTTC**AACTTCACCTTCTATGCCCCTGACACAAAAA**GGCTTGCA
GATGG**
GCTTGCCTTCTTTCTCGCACCAATTGACACTAAGCCACAAACACATGCAGGTTATCTTGGTCTTTT
CAAC
GAAAACGAGTCTGGTGATCAAGTCGTCGCTGTTGAGTTTGACACTTTCCGGAACTCTTGGGATCC
ACCAA
ATCCACACATCGGAATTAACGTCAATTCTATCAGATCCATCAAAACGACGTCTTGGGATTTGGCC
AACAA

TAAAGTAGCCAAGGTTCTCATTACCTATGATGCCTCCACCAGCCTCTTGGTTGCTTCTTTGGTCTA
CCCT
TCACAGAGAACCAGCAATATCCTCTCCGATGTGGTCGATTTGAAGACTTCTCTTCCCGAGTGGGT
GAGGA
TAGGGTTCTCTGCTGCCACGGGACTCGACATACCTGGGGAATCGCATGACGTGCTTTCTTGGTC
TTTTGC
TTCCAATTTGCCACACGCTAGCAGTAACATTGATCCTTTGGATCTTACAAGCTTTGTGTTGCATGA
GGCC
ATCTAAATGTGACAGATCGAAGGAAGAAAGTGTAATAAGACGACTCTCACTACTCGATCGCTAGT
GATTG
TCATTGTTATATATAATAATGTTATCTTTCACAACTTATCGTAATGCATTGTGAAACTATAACACATT
TA
ATCCTACTTGTCATATGATAACACTCTCCCCATTTAAAACTCTTGTCAATTTAAAGATATAAGATTC
TTT
AAATGATTAAAAAAAATATATTATAAATTCAATCACTCCTACTAATAAATTATTAATTAATATTTATTG
A
TTAAAAAAATACTTATACTAATTTAGTCTGAATAGAATAATTAGATTCTAGA

GenBank: K00821.1downloaded from NCBI database, highlighted in red is Forward primer and yellow highlighted is Reverse primer.

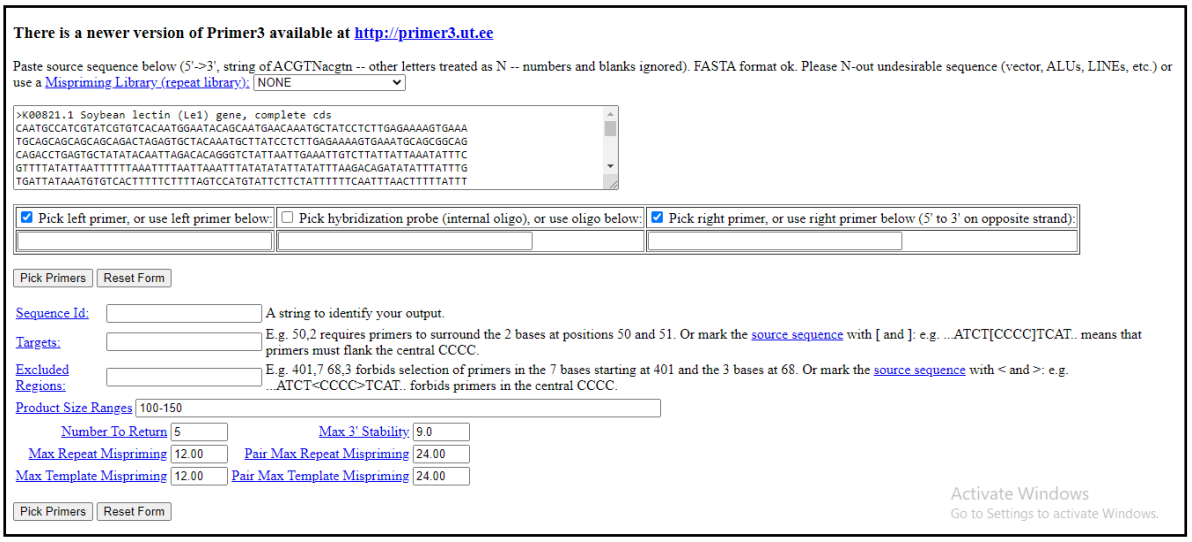

Fig 4: Snapshot of Primer-3 Software

Forward primer: - 5’ CCA GCT TCG CCG CTT CCT TC 3’

Reverse primer: - 5’ GAAGGCAAGCCCATCTGCAAGCC 3’

3.4 Preparation of primers

The primers were obtained in lyophilized form, which were dissolved in TE (pH 8.0; 10mM TrisHCl; 0.1 mM EDTA) to make stock solution (100 p.m.). The working primer solution (5pM) was prepared for use in PCR reactions.

3.5 Optimization of soya using conventional PCR

PCR amplification reaction was performed in a total volume of 15µl, which contained 50ng of DNA, 10x PCR buffer (10mM TrisHCl, pH 9.0, 50 mMKCl, 0.01% gelatin), 25Mm dNTP, 1.5mM $MgCl_2$, 5µM primers (forward and reverse) and 1.5U Taq polymerase (Genei). The amplification was performed in Bio-Rad C1000 thermal cycler. Cycling conditions were: initial denaturation at 94°C for 5 min, 25 cycles of 94°C for 30 sec, primer specific T_a for 30 sec, 72°C for 30 sec, and final extension at 72°C for 10 min. The PCR products were stored at 4°C. The success of amplification was checked on 1.5% agarose gel.

3.6 Detection of soya using real-time PCR

Detection of soya using real time PCR was based on Sybr Green assays for accurate, reliable detection and quantitation of soya in different food matrices (**Fig 5**). Mix all the reagents completely by quick vortexing and centrifuge briefly. The PCR conditions are mentioned in **Table1**. The PCR reactions were conducted in the Bio-Rad CFX 96 well, using the following programme: 10 minutes at 95 ºC, and 40 cycles of 15 seconds at 95 ºC and 45 seconds at 60 ºC.

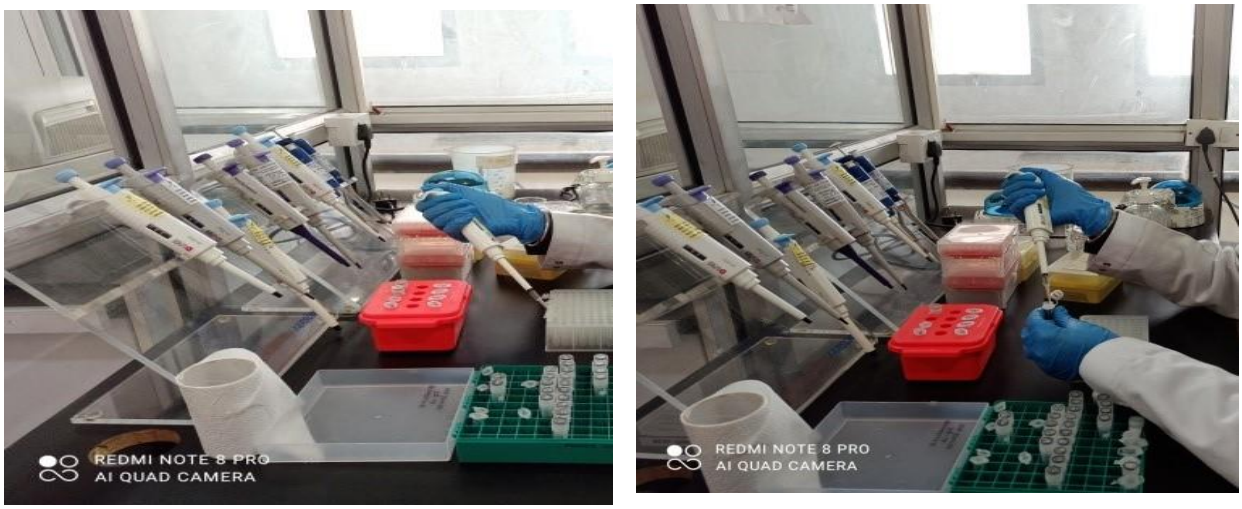

Fig 5: Performing Real Time PCR Assay

3.7 Data analysis

After completing real time amplification of soya, the Cq values were analyzed and melting curve were detected to know primer dimer and false amplification using Bio-Rad CFX manager software.

Table 1: PCR Constituents

Component	**Sample**	**Positive PCR Control**	**Positive PCR kit Control**	**Negative PCR control**
TB Green Premix EX-Taq	10 µl	10 µl	10 µl	10 µl
Primer Mix	0.6 µl	0.6 µl	0.6 µl	0.6 µl
Positive Control DNA	-	5 µl	5 µl	-
Negative Control DNA	-	-		2 µl
Sample DNA	5 µl	-		-
Nuclease Free Water	4.4 µl	4.4µl	4.4µl	4.4µl
Total Volume	**20 µl**	**20 µl**	**20 µl**	**20 µl**

4. Result and Discussion

4.1 Quantity and quality of isolated DNA

DNA from different eight samples was isolated using CTAB method. Quality and quantity of isolated DNA were determined using 0.8% agarose gel and Qubit 4 Fluorometer as shown in **Fig 6**. Thick and intact bands without smearing shows that the DNA is of good quality as seen in 0.8% agarose gel. Quantity of genomic DNA was determined through Qubit 4 Fluorometer and concentration of each sample is mentioned in **Table 2**.

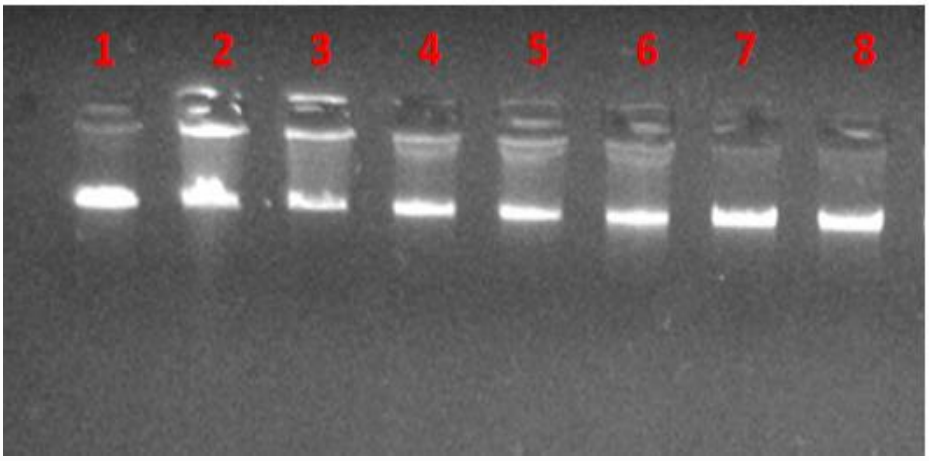

Fig 6: Quality of genomic DNA on 0.8% agarose gel

Table 2: Quantity of genomic DNA by Qubit 4 Fluorometer

Sample I.D.	Product name	Concentration (ng/µl)
Sample 1	Soya seed-2	80 ng/ul
Sample 2	Soya seed-1	74.4 ng/ul
Sample 3	Soya nut Butter	46 ng/ul
Sample 4	Whey protein	82.36 ng/ul
Sample 5	Soya yogurt	53 ng/ul
Sample 6	Sauce	78 ng/ul
Sample 7	Meat	93.6 ng/ul
Sample 8	Biscuit	89.7 ng/ul

4.2 Amplification of soya gene using conventional PCR

Genomic DNA of eight different samples was used to amplify lectin gene of soya having mol. wt. of 118bp. Out of eight different samples only six samples were amplified that shows the presence of soya constituents in samples i.e., Soya seed-2, Soya seed-1, Soya nut Butter, Whey protein, Soya yogurt and Sauce. Two samples sample7- meat and sample8- Biscuit did not amplify showing absence of soya ingredients in meat and biscuit food product (**Fig 7**).

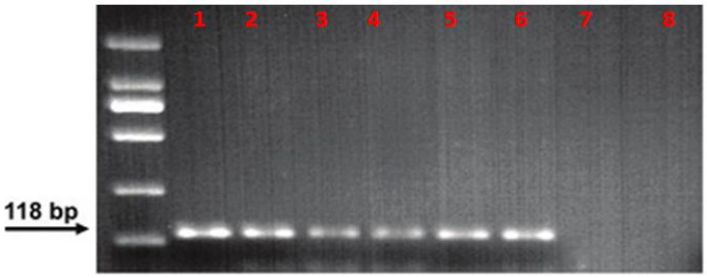

Fig 7: Agarose Gel (1.5%) showing amplification of lectin gene (118bp) by Conventional PCR

4.3 Real time amplification of soya gene

Real time amplification of lectin gene of soya was carried out for different food matrices, which are mentioned below in **Table 3**. Out of eight samples six samples show positive Ct value for the presence of lectin gene (**Fig 8**). In real time PCR we have used different controls like Environmental blank, Extraction blank, non-template control and Positive control respectively to verify authenticity of the experiment.

Post- amplification **melting curve** was analyzed and the results showed that six samples had real time amplification with single sharp peak at 78.50ºC, which assures PCR conditions were optimized with no primer-dimer artefacts (**Fig 9**).

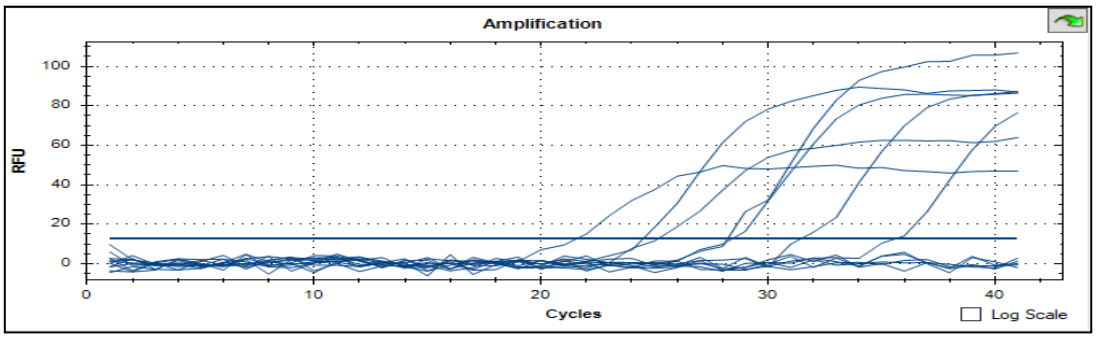

Fig 8: Real Time amplification with positive curves for 6 samples out of 8 food matrices

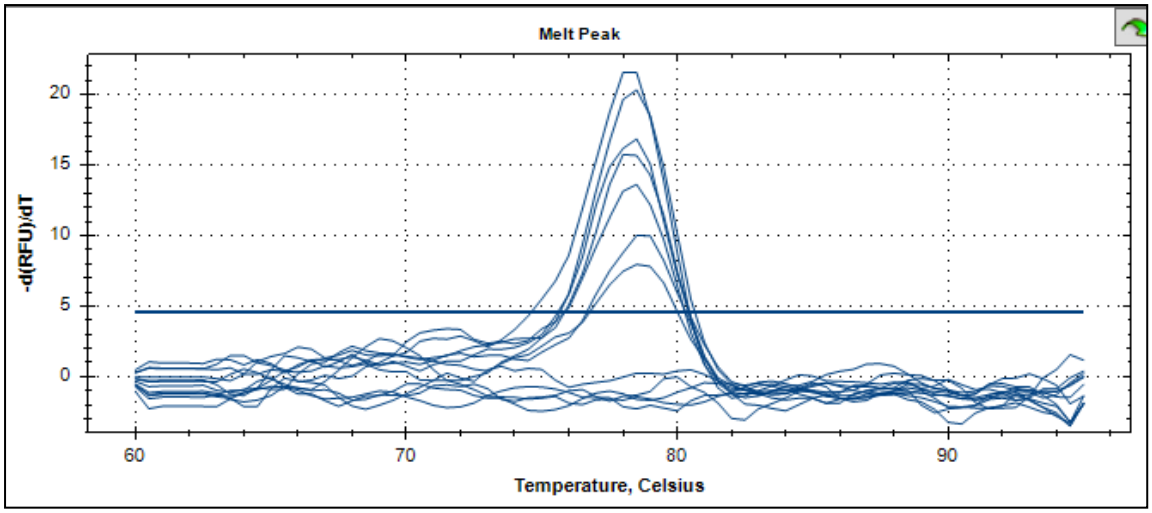

Fig 9: Melt Curve

Table 3: Quantification data for Real Time PCR

S. No	Sample ID	Ct Values	Melt Curve
1	Environmental Control	N/A	None
2	Extraction Blank	N/A	None
3	NTC	N/A	None
4	Positive Control	21.57	78.50
5	Soya seed 2	31.45	78.00
6	Soya seed 1	25.15	79.00
7	Soya Nut Butter	28.42	78.50
8	Whey Protein	35.61	78.50
9	Soya Yogurt	24.49	78.50
10	Sauce	28.22	78.00
11	Meat	N/A	None
12	Biscuit	N/A	None

5. Conclusion

The development of allergen detection methods is very crucial for our scientific community, especially as food allergy prevalence increases on a daily basis there is still no effective treatment for allergens. Thus, detection methods must be robust, specific, reproducible and sensitive. Besides, there is a need to develop cost-effective and simpler methods that can also enable the multiplexing of food-borne allergens. RT-PCR is a sensitive and robust technique and this work describes the presence of soy content in a wide range of foodstuffs by real time PCR. Among the advantages of these techniques, it is worth highlighting that this technique is reliable, allowing detecting even trace amounts of soybean in food products. Moreover, it is quick and cost saving. Therefore, it can be useful in questions regarding the presence of soy protein in food products, especially to verify the fulfilment of the labelling rules and to protect consumers' rights.

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7. APPENDICES

APPENDIX 1

1.1 5X TBE (Stock):

Tris base - 27.0 g

Boric Acid - 13.7 g

0.5 M EDTA (pH 8.0) - 12.5 ml

Makeup the final volume to 500 ml with distilled water.

1.2. Incubation Buffer:

Lysis Buffer:

Stock 0.5 M Tris-Cl (pH 8.3) - 2 ml

Stock 0.5 M EDTA (pH 8.0) - 0.2 ml

400 mMNaCl - 2.337 g

Make up the solution to 100 ml with distilled water

Autoclave it

Cool it down to room temperature.

Store at 4°C.

1.3 Proteinase K - 3µL

1.4. 3M Sodium acetate (pH 5.2):

Sodium acetate- 12.4 g

Distilled water - 20ml

Adjust the pH to 5.2 using glacial acetic acid

Makeup final volume to50ml.

Autoclave it.

Cool it down to room temperature.

Store at 4°C.

1.5. TE buffer:

Stock 0.5 M Tris-Cl (pH 8.0) - 2.0 ml

Stock 0.5 M EDTA (pH 8.0) - 0.02 ml

Make up the solution to 100 ml with distilled water.

Autoclave it.

Cool it down to room temperature.

Store at 4°C.

1.6. RNAaseA- 2µL

1.7. 1X TBE (Running buffer):

5x TBE	-	60 ml
Distilled water	-	240 ml

1.8. 5M NaCl (Sodium Chloride)

Sodium Chloride - 146.1g

Make up the final volume with distilled water.

1.9. 0.5 EDTA (ethylene diamine tetra acetic Acid)

EDTA - 93.4g
Distilled water - 500mL
Adjust the pH to 8.0 using glacial acetic acid

1.8. Chloroform-isoamyl alcohol (24:1)

1.9. Ethanol (70 and 99.9%)

1.10. SYBR Safe DNA Gel Stain

APPENDIX 2

2.1. Equipment and Instrument

- Weighing Balance
- Homogenizer
- Dry Bath Incubator
- Vortex Mixer
- Micro Centrifuge
- Micropipettes
- Qubit Fluorometer
- Autoclave
- Refrigerator
- Bio-Rad 96 well
- Mini Centrifuge
- Gel Electrophoresis
- Gel Doc System

2.2. List of software's used

- Primer3
- Oligoanalyzer 3.1 (Integrated DNA Technology)