COMPARATIVE DETECTION AND DETERMINATION OF AFLATOXIN IN RICE USING LC-MS/MS

M.Sc. (Ag.) THESIS

by

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DEPARTMENT OF PLANT MOLECULAR BIOLOGY AND BIOTECHNOLOGY

COLLEGE OF AGRICULTURE

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INDIRA GANDHI KRISHI VISHWAVIDYALAYA

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COMPARATIVE DETECTION AND DETERMINATION OF AFLATOXIN IN RICE USING LC-MS/MS

Thesis

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CERTIFICATE – I

This is to certify that the thesis entitled **"Comparative detection and determination of Aflatoxin in Rice using LC-MS/MS"** submitted in partial fulfilment of the requirements for the degree of **"Master of Science"** in Agriculture in the department of **Plant Molecular Biology and Biotechnology** of the Indira Gandhi Krishi Vishwavidyalaya, Raipur (Chhattisgarh) is a record of the bonafide research work carried out by **Shwetha D** under my/our guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee and the Director of Instructions.

No part of the thesis has been submitted for any other degree or diploma or has been published/published part has been fully acknowledged. All the assistance and help received during the course of the investigations have been duly acknowledged by her.

Date: 11-10-21

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CERTIFICATE – II

This is to certify that the thesis entitled **"Comparative detection and determination of aflatoxin in rice using LC-MS/MS"** submitted by **Shwetha D** to the Indira Gandhi Krishi Vishwavidyalaya, Raipur, in partial fulfillment of the requirements for the degree of **Master of Science in Agriculture** in the department of **Plant Molecular Biology and Biotechnology** has been approved by the external examiner and student's Advisory Committee after oral examination under the chairmanship of head of the Department/Dean (in case of out campii).

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Date: 27.10.2021

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Approved/Not approved

Director of Instructions ____________________

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Writing is a way to pen down our feelings which we can't express to everyone. While writing this thesis I want to pour down my feelings, my emotions, my experiences and my hidden sentiments with great deliberation

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Date: 11-10-21

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SYMBOL/NOTATION	INDICATES
µ	**Micro**
%	**Percent**
&	**And**
^{o}C	**Degree Celsius**
et.al.,	**And Others**
G	**Gram**
µg	**Microgram**
Ml	**Millilitre**
Mg	**Milligram**
PPM	**Parts per million**
PPB	**Parts per billion**
mM	**Millimolar**
µl	**Microlitre**
Cm	**Centimetre**
Min	**Minute**
Hr	**Hour**
No.	**Number**
Sec	**Seconds**
Nt.	**Nucleotide**
Conc.	**Concentration**

LIST OF ABBREVIATIONS

Abbreviations	Details
LC	Liquid Chromatography
MS	Mass Spectrometry
UHPLC	Ultra- High Performance Liquid Chromatography
HESI	Heated Electrospray Ionization
MRL	Maximum residue limit
RI	Retention Index
PTFE	Polytetrafluoroethylene
FLD	Fluorescence detection
RT	Retention time
SRM	Selected reaction monitoring
RSD	Relative Standard deviation
LOD	Limit of detection
LOQ	Limit of Quantification
CRMs	Certified Reference Materials
CRISPR/CAS9	Clustered regularly interspaced short palindromic repeats and CRISPR Associated protein-9
Bp	Base pair
RNA	Ribonucleic Acid
SgRNA	Single guide RNA
PAM	Protospacer adjacent motif
UTR	Untranslated region
CFD	Cutting frequency determination
NGS	Next generation sequencing

THESIS ABSTRACT

a) Title of the Thesis	:	"Comparative detection and determination of aflatoxin in rice using LC-MS/MS"
b) Full Name of the Student	:	Shwetha D
c) Major Subject	:	Plant Molecular Biology and Biotechnology
d) Name and Address of the Major Advisor	:	Dr.Archana.S.Prasad (Ph.D. in PMBB) Assistant Professor Dept. of Plant Molecular Biology and Biotechnology COA, Raipur, Indira Gandhi Krishi Vishwavidyalaya Raipur, Chhattisgarh.
e) Degree to be Awarded	:	M.Sc. (Ag.) in Plant Molecular Biology and Biotechnology

Signature of the Student

Shwetha D

Signature of the Major Advisor

17/10/21

Date- 11-10-21

Signature of Head of the Department

Rice, scientifically *O. sativa* has a place with the ubiquitous grass family; Poaceae perceived as "Millennium crop" and integral to the existences of billions of individuals all throughout the planet. It is one of the staple cereal harvests of the world and an essential wellspring of nourishment for more than a big part of the total populace. Organic cultivation of rice is quickly acquiring acknowledgement worldwide as a promising way to offer better food and to guarantee ecological sustainability. Right now, natural produce including organic rice is in colossal interest inferable from its capability to get exceptional cost in the worldwide market.

Aflatoxins are the sort of mycotoxins produced by Aspergillus types of growths, like *A. flavus* and *A. paraciticus*. Among different mycotoxins, aflatoxins have expected higher importance due to their cancer-causing consequences for individuals, poultry and domesticated animals. Accordingly, there is incredible interest for aflatoxins exploration to foster reasonable techniques for their evaluation, exact discovery and control to guarantee the security of buyer's wellbeing.

Virtually every food and feed product can be contaminated by fungal species and numerous of these growths are fit for producing one or on the other hand more mycotoxins. Because of the poisonous and cancer-causing capability of mycotoxins, there is an earnest need to foster an efficient mycotoxin detection and quantification strategies that are quick and profoundly specific. As of now, a wide scope of techniques are accessible to insightful researchers, going from recently depicted multi-toxin liquid chromatography coupled with mass spectrometry to rapid techniques based on immunological principles. This latter technique can provide quantitative data or the determination of contamination range above or below a pre-decided cut-off esteem.

This study comprises of detection of the aflatoxins (B1, B2, G1 and G2) in commercially procured white rice and organic rice and the method of quantification of these aflatoxins using an efficient analytical method Liquid Chromatography-(Ultimate 3000 UHPLC -Thermo Scientific) combined with tandem mass spectrometry – (Thermo Scientific^{TM} TSQ Endura) where the triple quadrupole is utilized for the detection of aflatoxins employing the Heated Electrospray Ionization as ion source. This technique of aflatoxin detection was carried out in Selected reaction monitoring (SRM) mode and the separation takes place using the Accucore aQ column of size 100 ×2.1 mm, 2.6 µm. The sample preparation for isolation and quantification of aflatoxins were carried out using the AOAC Official Method 2007.01 (Extraction by using Acetonitrile and partitioning with Magnesium sulfate). The organic rice showed great linearity with the R^2 value of 1.000 which is very rare and the aflatoxins AFB1, AFB2, AFG1, AFG2 were found in White rice whereas only AFG1 was detected in very trace amount which are present beneath the MRL with complying the %RSD Criteria (70-120%).

In this present study a small effort was made to exploit the technology of ever happening subject of interest in the discipline of genome editing i.e., CRISPR/Cas9 where *insilico* designing of sgRNA was carried out using the genome editing tool (CHOPCHOP) in rice genes for creating its novel alleles using 11 selected genes of rice aroma and quality. A total of 560 novel alleles were created from 11 selected genes of rice. More number of gRNA's were selected will reduce the false positive results and their efficiency was tested to select the ideal gRNA's. Three best gRNA's each for 11 selected genes were selected based on its efficiency score given by CRISPR Efficiency Prediction Tool.

शोध सार

ए) शोध का शीिषक: :"तुलनात्मक पता लगाने और के निर्धारण एलसी-एमएस/एमएस का उपयोग कर चावल में aflatoxin "

ब) छात्र का पूरा नाम : श्वेता डी

स) प्रमुख विषय : पौध आद्भिक जीवनवज्ञान और जैव प्रौद्योनवक

ड) प्रमुख सलाहकार का नाम और पता : डॉ. अर्चना प्रसाद
(पीएमबीबी में पीएचडी)
असिस्टेंट प्रोफेसर
पौध आद्भिक जीवनवज्ञान और जैव प्रौद्योनवक
सीओए, रायपुर,
इंदिरा गांधी कृषि विश्वविद्यालय
रायपुर, छत्तीसगढ़।

इ) उपानध से सम्मानित नकया जाएगा : एम.एससी. (कृ नि) पौध आद्भिक जीवनवज्ञान और जैव प्रौद्योनवक

छात्र के हस्ताक्षर

प्रमुख सलाहकार के हस्ताक्षर

तारीख: 11 - 10 - 21

विभाग के प्रमुख का हस्ताक्षर

चावल, वैज्ञानिक रूप से ओ सतीवा सर्वव्यापी घास परिवार के साथ एक जगह है; Poaceae "सहस्राब्दी फसल" के रूप में माना जाता है और सभी ग्रह भर में व्यक्तियों के अरबों के अस्तित्व के लिए अभिन्न । यह दुनिया के मुख्य अनाज फसलों में से एक है और कुल आबादी के एक बड़े हिस्से से अधिक के लिए पोषण का एक आवश्यक वेलस्प्रिंग है । चावल की जैविक खेती जल्दी से बेहतर भोजन की पेशकश करने और पारिस्थितिक स्थिरता की गारंटी देने के लिए एक आशाजनक तरीके के रूप में दुनिया भर में पावती प्राप्त कर रही है। अभी, कार्बनिक चावल सहित प्राकृतिक उत्पाद भारी ब्याज में अपनी क्षमता से अनुमानित करने के लिए दुनिया भर में बाजार में असाधारण लागत मिल रहा है ।

एफ्लावस और *ए पैरासिटिकस* जैसे एस्परगिलस प्रकार के विकास द्वारा उत्पादित माइकोटॉक्सिन की तरहहैं। विभिन्न माइकोटॉक्सिन के बीच, व्यक्तियों, पोल्ट्री और पालतू जानवरों के लिए उनके कैंसर पैदा करने वाले परिणामों के कारण एफ्लाटॉक्सिन ने अधिक महत्व की उम्मीद की है। तदनुसार, खरीदार की भलाई की सुरक्षा की गारंटी देने के लिए उनके मूल्यांकन, सटीक खोज और नियंत्रण के लिए उचित तकनीकों को बढ़ावा देने के लिए aflatoxins अन्वेषण के लिए अविश्वसनीय रुचि है।

लगभग हर भोजन और फ़ीड उत्पाद फंगल प्रजातियों से दूषित हो सकता है और इनमें से कई वृद्धिएं एक या दूसरी ओर अधिक माइकोटॉक्सिन के उत्पादन के लिए फिट हैं। माइकोटॉक्सिन की जहरीली और कैंसर पैदा करने की क्षमता के कारण, एककुशल माइकोटॉक्सिन डिटेक्शन और मात्राकरण रणनीतियों को एफओस्टर करने की गंभीर आवश्यकता है जो त्वरित और गहराई से विशिष्टहैं। अब तक, तकनीकों का एक विस्तृत दायरा व्यावहारिक शोधकर्ताओं के लिए सुलभ है, जो हाल ही में चित्रित बहु-विष तरल क्रोमेटोग्राफी से प्रतिरक्षा सिद्धांतों के आधार पर तेजी से तकनीकों के लिए बड़े पैमाने पर स्पेक्ट्रोमेट्री के साथ मिलकर किया जा रहा है। यह उत्तरार्द्ध तकनीक मात्रात्मक डेटा या संदूषण सीमा के निर्धारण को पूर्व-निर्धारित कट-ऑफ सम्मान के ऊपर या नीचे प्रदान कर सकती है।

इस अध्ययन में एफ्लाटॉक्सिन (बी 1, बी 2, जी 1 और जी-2) में व्यावसायिक रूप से खरीदे गए सफेद चावल और कार्बनिक चावल और एक कुशल विश्लेषणात्मक विधि तरल क्रोमेटोग्राफी का उपयोग करके इन एफ्लाटॉक्सिन के क्वांटिफिकेशन की विधि- (अंतिम 3000 यूएचपीएलसी-थर्मो साइंटिफिक) मिलकर मास स्पेक्ट्रोमेट्री के साथ संयुक्त - (थर्मो साइंटिफिक[टीएम] टीएसक्यू एंडुरा) जहां ट्रिपल क्वाडुपोल का उपयोग गर्म इलेक्ट्रोप्रेसी आयनीकरण स्रोत के रूप में गर्म विद्युतों को नियोजित करने वाले एवलोसेक्साविन का पता लगाने के लिए किया जाता है। एफ्लाटॉक्सिन डिटेक्शन की यह तकनीक चयनित प्रतिक्रिया निगरानी (एसआरएम) मोड में की गई थी और सेपरेशन 100 ×2.1 मिमी, 2.6 माइक्रोन के एक्यू कॉलम का उपयोग करके होता है। एएफएटॉक्सिन के अलगाव और मात्राकरण के लिए नमूना तैयारी एओएसी आधिकारिक विधि 2007.01 (एसिटोनिट्रिल का उपयोग करके और मैग्नीशियम सल्फेट के साथ विभाजन) का उपयोग करके किया गया था। जैविक चावल१.० के आर^2 मूल्य के साथ महान रैखिकता दिखाया जो बहुत दुर्लभ है और aflatoxins AFB1, AFB2, AFG1, AFG2 सफेद चावल में पाए गए, जबकि केवल AFG1 बहुत ट्रेस राशि है जो% RSD मापदंड (70-120%) का पालन करने के साथ MRL के नीचे मौजूद है में पाया गया था ।

इस वर्तमान अध्ययन में जीनोम संपादन के अनुशासन में रुचि के विषय का दोहन करने के लिए एक छोटा सा प्रयास किया गया था यानी, CRISPR/Cas9 जहां sgRNA के *insilico* डिजाइनिंग चावल की सुगंध और गुणवत्ता के 11 चयनित जीन का उपयोग कर अपने उपन्यास alleles बनाने के लिए चावल जीन में जीनोम संपादन उपकरण (CHOPCHOP) का उपयोग कर किया गया था । चावल के 11 चयनित जीन से कुल 560 उपन्यास एलील्स बनाए गए थे। GRNA के अधिक संख्या का चयन किया गया झूठे सकारात्मक परिणामों को कम करेगा और उनकी दक्षता आदर्श GRNA का चयन करने के लिए परीक्षण किया गया था । 11 चयनित जीन के लिए तीन सर्वश्रेष्ठ GRNA के प्रत्येक CRISPR दक्षता भविष्यवाणी उपकरण द्वारा दिए गए अपने दक्षता स्कोर के आधार पर चुना गया ।

CHAPTER I

INTRODUCTION

Rice (*O.sativa L.*) is the dominating food grain and a mainstay for the enormous segment of World's populace as well as the Chief wellspring of Subsistence for the rural sector (A.C. Sales & T. Yoshizawa 2007). It is the principal commodity to millions of small-scale farmers with land holdings of about 1-2 hectares & to Peasants who derive their income mainly from working on these lands. The Journey of this most popular staple food around the globe has been very gradual, but once it took root it stayed and became the life for the larger part of the human race.

Rice is generally the annual grass and this edible starchy cereal grain belongs to the family Poaceae. Long grained indica and short grained japonica are the 2 subspecies of *Oryza sativa*. It can also survive as perennial in areas of tropical climate & turnout as a ratoon crop for upto 30 years. India being the major centre of rice cultivation exported 15.5 million metric tons of rice (both Basmati and Non-Basmati) to various countries during the year 2020-2021, whereas Vietnam stands second in the export of rice with about 6.4 million metric tons.

In the marketing year 2020-21, the USDA forsees 120 million tonnes from 44.5 million hectares from India. Exports of rice from India are estimated to rise 18% to 12.3 million tonnes for 2019-20. On the report of/as stated by the USDA round about 500 million tonnes of milled rice have been produced during the year 2019-20 worldwide. The largest yielder/raiser China which supplies/totals 30% of the production, succeeded by India (24%), Bangladesh (7%), Indonesia (7%), Vietnam (5%) and Thailand (4%). With respect to consumption, the ranking is similar: China is the largest consumer (29%), followed by India (21% of global consumption), Bangladesh (7%), Indonesia (7%), Vietnam (4%) and the Philippines (3%).

Aflatoxins are a group of toxins produced by Aspergillus species of fungus (Principally A. flavus and A. parasiticus) that contaminate the grains and cereals including rice, maize and nuts (J. Bansal et.al.,2011). These fungi are broadly scattered in agriculture and profoundly common in tropical areas and regions having hot and humid climatic conditions which is most favourable for fungus growth. Aflatoxin B1[AFB1], Aflatoxin B2[AFB2], Aflatoxin G1[AFG1] and Aflatoxin G2 [AFG2] are the four significant aflatoxins which is a serious threat if present above the MRL (Hiroki Tanaka et.al.,2006).

Asia is the driving mainland for the creation and utilization of rice. In general, rice is grown in subtropical conditions with hot and damp environments that encourages the fungal growth and production of aflatoxins. Rice can be contaminated by the fungus at any stage when the climatic conditions become good for their development in the field (N. Ali, 2019). The high commonness of aflatoxins in rice and rice products highlight the significance of serious checking of this dietary staple around the globe. As per the World Health Organization (WHO), aflatoxin is a worldwide food security concern.

As indicated by Web of Science, there are almost 16,000 distributions since 1975 right up till today regarding aflatoxins, of which more than 7,000 have been distributed somewhat recently. These numbers and lawful limitations across the world with respect to the exceptionally cancer-causing aflatoxins show the significance of the topic.

Internationally around 120 nations have approved the administrative limits on permissible aflatoxin levels in human food and animal feed (Abdoulie jallow et al.,2021). A few nations put down certain boundaries for the four most conspicuous kinds of aflatoxins in food: AFB1, AFB2, AFG1 and AFG2. For instance, the United states and Kenyan guidelines specify a most extreme constraint of 20 and 10 ppb for the amount the four sorts of aflatoxins (Total aflatoxins), individually. Conversely, the EU that has distinct limits for different aflatoxin-food blends has a most extreme degree of 2 and 4 ppb for aflatoxin B1 and total aflatoxins in peanuts and maize, individually (European Commission, 2006). Furthermore, numerous nations have embraced maximum limits for milk and milk products (AFM1 and AFM2) (Abdoulie jallow et al.,2021).

In the present investigation, Liquid Chromatography with tandem Mass Spectrometry (LC-MS/MS), an incredible analytical technique was used for the comparative detection and determination of aflatoxins rice in which it combines with the separating capacity of LC with profoundly sensitive and selective mass analysis capacity of triple quadrupole mass spectrometry. The secondary metabolites of fungus (aflatoxins) in commercially procured organic and white rice were detected and determined quantitatively by using the LC-MS/MS instrument (Make – Thermofisher Scientific, Model no – TSQ Endura TQH-E1-0620). The samples are analysed by using Ultimate 3000 UHPLC (Ultra High-Performance Liquid Chromatography) combined with tandem mass spectrometry along with HESI (Heated Electrospray Ionization) with the use of Accucore aQ column, (100 × 2.1 mm 2.6 µm) capillary column.

Like Aflatoxin contamination, rice can be contaminated by many other substances like pesticides, herbicides etc., which decreases the quality of the rice where the quality plays an important role in international trading. So, to improve the quality of the rice, 11 genes of rice quality and aroma were selected and created its novel alleles using insilico web tool (CHOPCHOP) which are very useful in increasing the quality and yield parameters of rice.

By contemplating the most crucial requirement for aflatoxin determination in rice and other products, this study was undertaken with the following key objectives:

1. Extraction and Sample preparation for isolation of aflatoxin in commercially procured organic and white rice.
2. Quantitative estimation of aflatoxin in commercially procured organic and white rice.
3. *Insilico* designing/construction of novel alleles of selected genes of Rice quality and Aroma using gene editing tools.

CHAPTER II

REVIEW OF LITERATURE

Aflatoxin has high impact on human and animal health. Therefore, the large economy lost in international trade of cereal and crops could be attributed to legislation controlling the permissible exposure levels of this toxin to humans and animals. The rice worldwide has low levels of aflatoxin contamination which are mostly lower than locally produced rice and the international permissible levels.

In this chapter the work done related to my thesis entitled “Comparative detection and determination of Aflatoxin in rice using LC-MS/MS” in the world is reviewed and compiled.

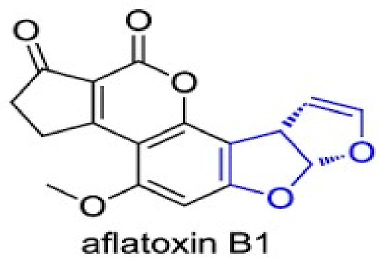
2.1 AFLATOXINS

Aflatoxins are noxious cancer-causing agents and mutagens that are created by specific molds (A. flavus and A. paraciticus) which grows in soil, rotting vegetation, roughage and grains (N. Arroyo-Manzanares et al.,2010, S. Firdous et al.,2012, M.I. Almeida et al.,2012, Turner et al.,2009). They are routinely found in inappropriately stored staple wares like corn (maize), chili peppers, cassava, millets, seeds of cotton, peanuts, sorghum and rice (Pittet 2001, J. Bansal et al.,2011, Kamkar et al.,2014, M. Mahammadi et al.,2012, A. Hussain et al.,2010, Manoochehri et al.,2014, M. Eslami.,2015). At the point when contaminated commodities are processed, aflatoxins enter the overall food supply both in human food and animal feedstock (X. Lai et al.,2014). These secondary fungal metabolites are most commonly consumed. In any case the most harmful kind of aflatoxin: B1, can prevade through skin (A.O. Elzupir et al.,2017, G.S. Toteja et al.,2005).

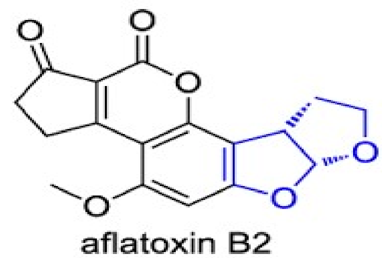
2.1.1 ORDER OF TOXICITY OF AFLATOXINS IN RICE.

The most hazardous secondary metabolites of molds present in rice; B1 and B2 (belonging to the Difurocoumarocyclopentenone series) and aflatoxin G1, G2 (belongs to the Difurocoumarolactone series) are grouped based on their chemical structure (Mukesh patel.,2016). Milk and milk products are highly vulnerable to the aflatoxin – M1 and M2 (Gholami-Shabani et al.,2018, WHO guidelines, 2018). The most toxic B1 is ranked first, followed by G1 being the second most toxic and G2 is being less toxic compared to B2 is ranked I based on the order of toxicity potential.

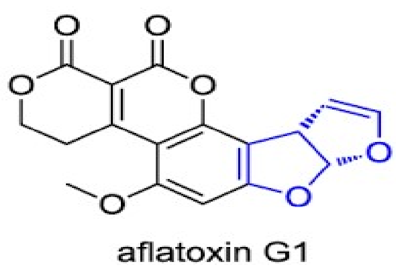
AFB1 > AFG1 > AFB2 > AFG2 (S. Firdous et al.,2013).



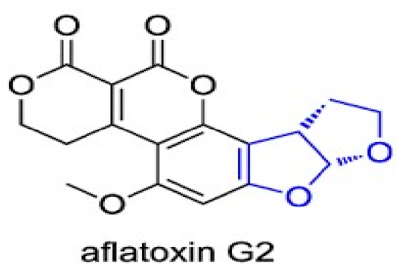
Chemical formula – $C_{17}H_{12}O_6$
Molecular mass – 312.277 g.mol^{-1}



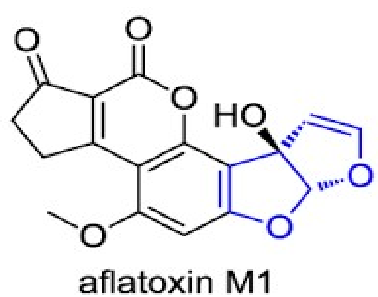
Chemical formula – $C_{17}H_{14}O_6$
Molecular mass – 314.29 g.mol^{-1}



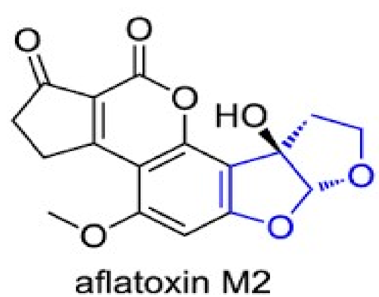
Chemical formula – $C_{17}H_{12}O_7$
Molecular mass – 328.27 g.mol^{-1}



Chemical formula – $C_{17}H_{14}O_7$
Molecular mass – 330.29 g.mol^{-1}



Chemical formula – $C_{17}H_{12}O_7$
Molecular mass – 328.27 g.mol^{-1}



Chemical formula – $C_{17}H_{14}O_7$
Molecular mass – 330.29 g.mol^{-1}

Fig 2.1: Chemical structure of Aflatoxins (F. Hemmerling et al.,2016)

2.1.2 Aflatoxins in Rice and Rice products

Rice has a potentially high risk of exposure to aflatoxin that can be easily controlled through proper post-harvest handling and storage of rice and its by-products (A.C. Sales et.al.,2005).

Table 2.1: Presence of Aspergillus section Flavi in Rice and its byproducts (A.C. Sales et al.,2005)

Food commodity	Nation	Number of analysed samples	Main Aspergillus section *Flavi* species isolated	Aflatoxin incidence in (%)	Aflatoxin Conc. in (µg/kg)	Method used/ detection limit
Rice (Boiled)	Philippines	15	_b	20	0.6 average	TLC[C]/5 µg/kg
Parboiled rice	Sri Lanka	_	_	_	185 $(AFB1)_{Max}$	_
White rice	Egypt	_	*A. flavus*	_	10	_
White rice	India	1	*A. flavus* and *A.paraciticus*	100	20	_
White rice	England	18	_	100	0.1-18	HPLC[d]/0.1 µg/kg
White rice	Thailand/Hong Kong/ Pakistan/Vietnam	22	*A. flavus*	25	0.1-0.3	IAC- HPLC[C]/ 0.1µg/kg
White rice	Nepal	12	*A. flavus*	75	15(AFB_1)	TLC/2.5 µg/kg
Rice & its products	Philippines	186	_	38	30.1 average	TLC/5µg/kg
Rice bran	India	58	*A. flavus*	21-30	10-100	ELISA[f]/0.1 µg/kg
Rough rice	India	170	*A. flavus*+	_	_	_
Rice meal	Columbia	22	_	36	1.0-52.8 (AFB_1)	HPLC/1µg/kg

2.2 Aflatoxicosis & other aflatoxin related diseases

The chronic and acute disease are the two kinds of diseases caused by intake of aflatoxin contaminated food where the chronic is more common (Razzaghi-Abyaneh 2013, Khoshpey et al.,2011). At instances of intense aflatoxicosis, a person has been presented to direct to undeniable degrees of aflatoxins; which frequently happen in Southeast Asia and Africa. Intense aflatoxicosis is portrayed by symptoms like fever, stomach ache, sickness, unconsciousness lastly demise. Aflatoxins acutate the changes particularly point mutations and change the message of DNA. The hereditary changes are prompting aggravations in the creation of DNA lastly related protein (Gholami-Shabani et al.,2017).

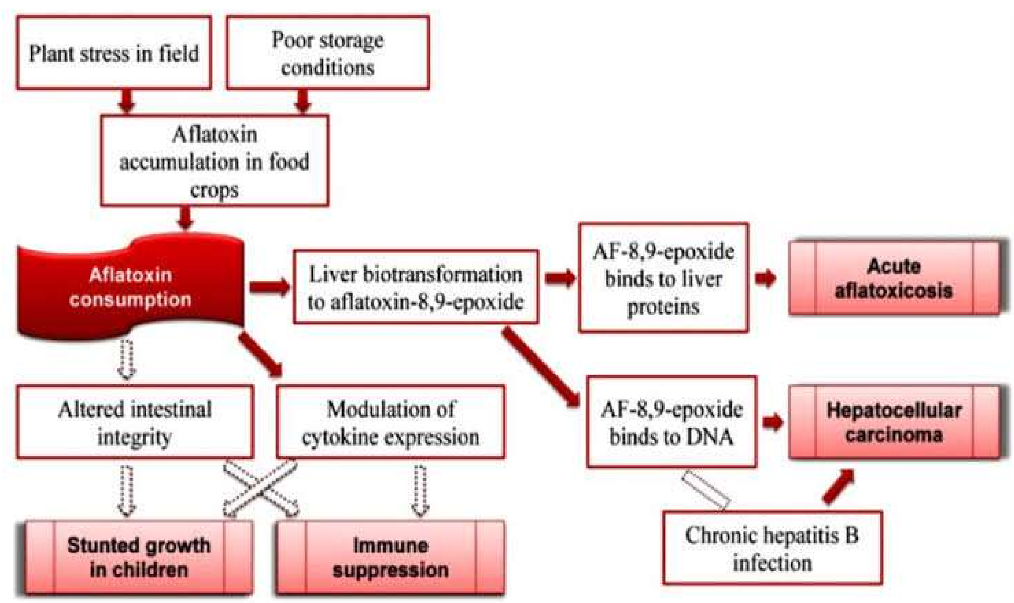


Fig 2.2: Aflatoxin pathway in causing diseases to humans (Source :Wu [33])

According to WHO guidelines, 2018 Continuous intake of aflatoxin contaminated food for long time can pose serious health issues : Aflatoxins are mutagenic in microbes, genotoxic and can possibly cause birth defects in kids (growth retardance). Aflatoxins cause immunosuppression, consequently may reduce resistance and being vulnerable to the attack by irresistible infectious agents (for example Tuberculosis, HIV).

2.2.1 Measures to Reduce the intake of Aflatoxins

Mouldy food products are conceivably defiled with aflatoxins and subsequently are potentially destructive when ingested. The molds don't simply develop on a superficial level yet enter profound into food. The primary prospects to stay away from any conceivable unsafe impacts of contamination of food and feed brought about by mycotoxins have been depicted by Halasz et al. (Halasz, Lasztity, Abonyi, Bata, 2009). To decreases exposure to these fungal metabolites, the customer is encouraged to:

- cautiously examine entire grains and nuts for presence of fungal mold and dispose of any that look rotten, discoloured or withered.
- Purchase only legitimate brands of nuts and nut spreads – aflatoxin molds are not altogether killed by preparing or cooking, so can appear in items for example peanut butter.
- Ensure that food varieties are thrown away appropriately and are not stored for longer timeframes previously being utilized; and attempt to guarantee his/her eating regimen is different; this assists with alleviating aflatoxin openness, yet additionally further develops wellbeing and nourishment. Buyers who need dietary variety need to give additional consideration to limit the danger of high openness to aflatoxins (A. Santini et al.,2013).

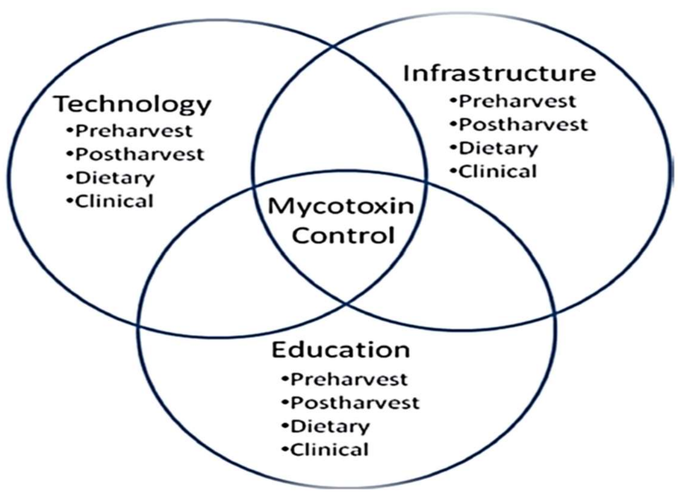


Fig 2.3: Mycotoxin control strategies in agricultural nations.

2.2.2 Regulations and limits

Since very long time, a need has consistently been felt for control on the standards of food commodities. With the revelation of aflatoxins in the mid-sixties, health organizations in many nations have become dynamic in setting up guidelines to secure their residents and animals for the potential damage brought about by aflatoxins (P.M. Mazumder et al.,2000, H.V. Egmond et al.,2002). In 1988, a first attempt was made to get an overview of worldwide regulations and limits for aflatoxins and other mycotoxins in food sources and feeds, resulting in a few publications. The decision of limits for mycotoxins relies upon accessibility of toxicological information, information on the occurrence of mycotoxins in different food items, accessibility of strategies for sampling methods and analysis.

Table 2.2: Regulatory limits for the major food items. (Source: FSSAI Manual, 2020)

Mycotoxins Regulatory limits given by Food Safety and Standards Authority of India (FSSAI), 2020.		
Mycotoxin	Commodities	Regulatory limit in µg/kg
Aflatoxin	Cereal and Cereal products	15
	Nuts (Ready to eat)	10
	Nuts (Dried- fig)	10
	Pulses	15
	Spices	30
	Arecanut	15
	Oil and Oil seeds	15
Aflatoxin – M1	Milk and dairy products	0.5
Patulin	Apple juice and ingredients of Apple juice in other beverages	50
Ochratoxin A	Wheat, Barley and Rye	20
Deoxynivalenol	Wheat	1000 (1 ppm)

Table 2.3: Country-wise limits for AFB1, AFB2, AFG1 and AFG2 in food and feed stuffs.

Countries	Food products	Mycotoxins	Limit (ppb)
India	All foods	B1	30
	Peanut meal	B1	120
China	Edible oils and rice	B1	10
	Peanut products, Maize	B1	20
	Oats, Barley, grains, Wheat, fermented foods	B1	5
	Cow's milk & its products	B1	0.5
EU	Cereals	B1	2-4
	Milk & its by-products	M1	0.05
	Infant food	B1	1-2
	Groundnut, cottonseed, maize, babassu & copra	B1	20-50
	Feedstuffs for Calves & lambs	B1	50
	Complementary feed for pigs and poultry except their young ones	B1	10
Australia	All food products	B1, B2, G1, G2	5
	Nuts & its products	B1, B2, G1, G2	15
United States of America	All foodstuffs	B1, B2, G1, G2	20
	Milk (Low fat, skimmed, whole)	M1	0.5
	Peanut & maize as feed for beef, cattle, swine or poultry	B1, B2, G1, G2	100-300
Israel	Nuts, fig & its products	B1, B2, G1, G2	20
	Milk	M1	0.05
Japan	All foodstuffs and fig	B1	10
	Peanut meal feed	B1	1000
German	All foods except enzymes	B1, B2, G1, G2	4
	Baby foods	B1, B2, G1, G2	0.05
France	All cereals	B1	5
	All vegetable oils	B1	5
	Milk powder for infants	M1	0.3

2.3 AFLATOXIN DETECTION METHODS

At Present the insightful strategies/methods used for the aflatoxin detection and determination incorporates:

1. Enzyme linked immunosorbent assay (ELISA) (Heber et al., 2001; Ware et al., 1999 and Thirumala- Devi et al., 2001).
2. Thin layer chromatography (TLC) (Krska et al., 2001 and Betina 1993).
3. LC-MS/MS– Liquid chromatography with tandem mass spectrometry (Thakur and Smith 1994; Young and Lafontaine 1993; Biselli et al., 2005).
4. Gas chromatography (GC) with electron capture (Langseth and Rundberget 1998) or mass spectrometric (Tanaka et al.,2000; Soleas et al., 2001; Langseth and Rundberget 1998; Schwadorf and muller 1992; Shephard 1998; Nielsen and Thrane 2001; Valenta 1998).
5. Liquid chromatography with fluorescence detection (LC-FLD) (Shephard 1998, Krska and Josephs 2001, Valenta 1998).

LC-MS/MS seems, by all accounts, to be the most promising as a profoundly specific, widely appropriate detection technique that gives both qualitative and quantitative data. Considering the conceivable staple grains contamination by few aflatoxins producing fungal species, a trend is to foster techniques reasonable for the assurance of few aflatoxins in a solitary run (Spanjer et al., 2009; Rundberget and Wilkins 2002; Kokkonen et al., 2005; Delmulle et al., 2006; Cavaliere et al., 2005; Monti et al., 2000; Sorensen and Elbaek 2005; Royer et al., 2004; Abbas et al., 2006; Berthiller et al., 2005; Monbaliu et al.,; Sulyok et al., 2006; Sewram et al., 1999)

J. Iqbal et al.,2014 conducted a study on aflatoxin detection in brown rice collected from Pakistan (B1, B2, G1 and G2) to compare the precision and accuracy of various aflatoxin detection methods like HPLC, ELISA, TLC and LC-MS/MS. Out of all these methods HPLC, TLC and LC-MS/MS methods were used for the quantification of aflatoxins whereas ELISA didn't offer this advantage. Besides, attributable to low-identification limit and sensitivity, LC-MS/MS and HPLC strategies have recognized more prominent number of aflatoxin contaminated samples in contrast with ELISA (Enzyme linked Immunosorbent assay) and Thin Layer Chromatography methods.

F. Soleimany, 2011 developed an HPLC method for the detection and partition of aflatoxins (AFB1, AFB2, AFG1 and AFG2), then a LC-MS/MS method was used to get an insight about the effect of liquid chromatography column (50mm and 150 mm) applying ESI method for the separation of aflatoxins. The LOD and LOQ values were got and at last this method was proved to be an efficient and fastest method for the simultaneous detection of aflatoxins in cereals.

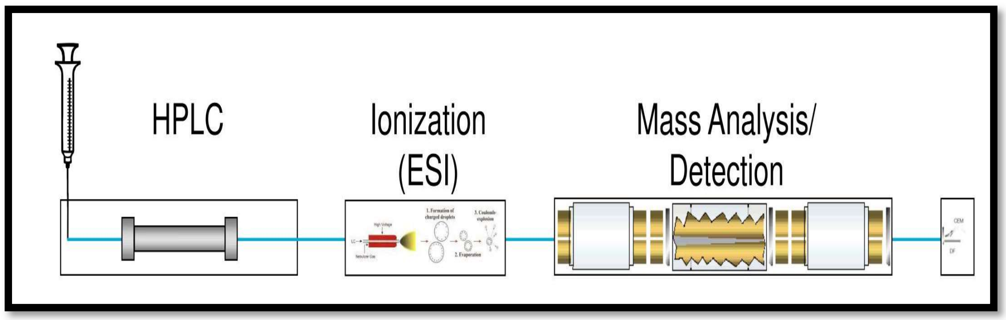


Fig 2.4: Schematic setup of LC-MS/MS System

T. Bessaire et al., 2019 conducted a inter-collaborative examination was co-ordinated to assess the efficiency of LC combined with MS system for the concurrent assurance of 12 mycotoxins in food which includes aflatoxin B1, B2, G1, G2, M1, zearalenone, T-2, HT-2, and deoxynivalenol. The strategy consolidated the straightforwardness of the QuEChERS approach with the immunoaffinity column clean-up efficiency. The RSD values for each aflatoxin complies with the values set in the EC 401/2006. The general arrangement of data shown that the strategy offered a special stage to guarantee consistence with EC 1881/2006.

A study was conducted to test the presence of aflatoxins (AB1, AB2, AG1 and AG2) in rice samples, applying a Quick, Easy, Cheap, Effective Rugged and Safe (QuEChERS) extraction followed by HPLC-FLD (High- performance liquid chromatography- fluorescence detection) using C18 column. Good linearity was observed in the calibration curve and the LOD and LOQ were ≤ 6 and ≤ 8 µg/kg. This proposed strategy was tried on rice samples bought from markets of which none showed the presence of aflatoxins (C.V. Garcia et al., 2018 and A. Gotah et al.,2018).

The Aflatoxin analysis has been carried out for the rice samples imported to Iran in the year 2006-07 using immunoaffinity column and High-Performance Liquid Chromatography is used for quantification of AFB1, AFB2, AFG1 and AFG2. Among 71 investigated rice samples, aflatoxin B1 was found in 59 samples. The mean of aflatoxin B1 was 1.89 ng/g for all the analysed samples and it is reported that only 9 samples had levels over the Maximum tolerance level of European Union in AFT (M. Mazaheri et al.,2009).

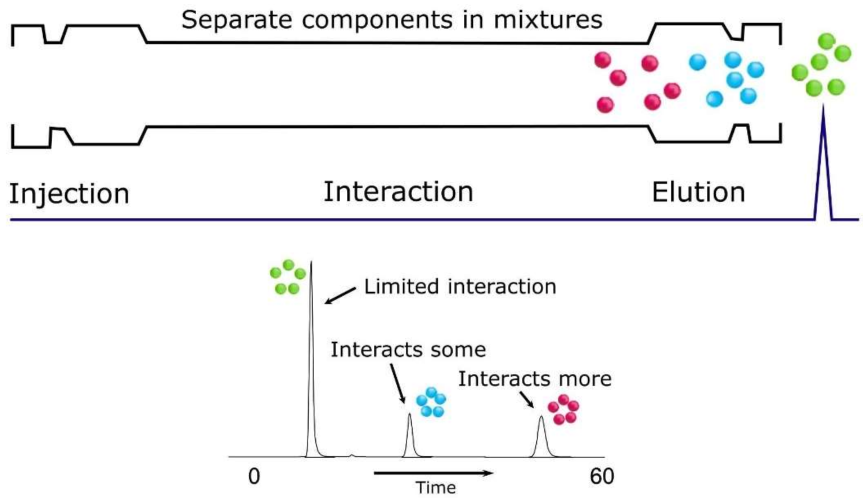


Fig 2.5: Separation of Analytes in High-Performance Liquid Chromatography.

Mukhtar et al.,2016 conducted a research on aflatoxin determination in Basmati rice (super kernel) consumed in various places of Punjab and Pakistan using the HPLC technique. The result showed the presence of B1, B2, G1, G2 aflatoxins in rice samples. A positive relationship was likewise seen between the aflatoxins and the moisture content of rice. The analysis showed a significant ($P<0.05$) result and it could add to different approaches prompting aflatoxin management in rice.

F. Qiu et al.,2019 used LC-ESI-MS/MS method to detect the aflatoxin B1, B2, G1, G2 and M1 in Semen Sojae Preparatum (SSP), a traditional medicine used in china) using C18 column (2.1 × 50 mm, 3.5 µm) and Electrospray ionization source in MRM mode and detected 5 aflatoxins in SSP.

M. Solfrizzo et al.,2018 used 12 different approaches for the synchronous LC tandem mass spectrometry detection of mycotoxins in food (cereals) and feed stocks. The approach resulting in maximum acceptable outcomes are selected for further analysis of mycotoxins in rice, maize and feed (spiked at high, medium and low mycotoxin levels). At low mycotoxin level in rice and corn, a significant matrix effect was noticed and it was compensated by utilizing ^{13}C Calibration whereas no significant matrix effect was noticed for the rice and maize spiked at high and medium mycotoxin levels.

Table 2.4: Methods of Aflatoxin detection; their advantages and drawbacks.

Class of Detection Methods	Methods	Advantages	Drawbacks	References
Chromato graphic-based methods	TLC	1. Good level of sensitivity 2. Capable of detecting multiple metabolites in a single test	1. Proneness to error 2. Require extensive sample treatments, 3. Skilled operator and expensive equipment	Mahfuz et al., (2018)
	HPLC	1. High accuracy 2. High sensitivity 3. Reliability	1. Extensive sample treatment 2. Tedious pre- and post-column derivation processes to improve sensitivity	Mahfuz et al., (2018)
	GC	1. Suitable for multi-toxin detection situation	1. Nonlinearity of calibration curves 2. High variation in terms of precision 3. Drifting responses 4. Effect from previous samples	Mahfuz et al., (2018)
	LC	1. High sensitivity 2. High versatility	1. Slow compared to other methods	Mahfuz et al., (2018)

	LC-MS/MS	1.High sensitivity 2. Reliable 3. No need of immune-affinity clean-up columns	1. Cumbersome sample preparation 2. Requires highly trained operator 3. Expensive	Ouakhssase et al., (2019)
	UHPLC-MS/MS	1. Suitable for multi-contaminant detection 2. Fast analysis 3. Sensitive and reliable 4. Less solvent consumption	1. Expensive 2. High matrix effects 3. Requires trained personnel	Rathod et al., (2019)
Immunochemical methods	ELISA	1. Simple, Cheap 2. Rapid and multiple samples can be tested at the same time.	1. Time consuming clean-up 2. Matrices dependent 3. Cross reactivity	Pal et al., (2004)
	Biosensors	1. Excellent sensitivity 2. Low LOD 3. Portable & suitable for on-site testing	1. High rates of false positive results 2. Matrices conditions affects the performance	Larou et al., (2013)
	Radio Immune assay	1. High sensitivity 2. Low LOD 3. Minimal matrix effect	1. False positive possibility 2. Requires pure anti-bodies 3.Safety concerns related to the use of radioactive elements in the assay	Matabaro et al., (2017)
	Lateral flow immuno-assay	1. Rapid 2. Straightforward in the field analysis 3. Suitable for multi-analyte detection	1. Expensive 2. Cross reactivity 3. Cumbersome to handle result and data management and interpretation	Ho and Wauchope (2002)

2.4 Gene editing in Rice

Gene editing and Genome editing are whole different kind of things where only a gene is edited in gene editing to carry out the site-specific changes (A.M. Khalil. 2020)

Kambale R et al.,2020 used the widely harnessed method of genome editing, CRISPR/Cas9 tool to generate the novel alternative forms of BADH2 gene in converting an elite non-aromatic variety of rice, ASD16 into an aromatic one by designing the synthetic guide RNA creating changes in the genome of rice leading to the Production of ASD16 rice lines with Aroma. The traditional breeding methods takes very long time in developing a variety rather than CRISPR/Cas9 technology.

S. Gaoneng et al.,2017 successfully edited the BADH2 (gene responsible for aroma) in Zhonghua 11 variety using CRISPR/Cas9 technology. A slight increase in the 2AP (2 Acetyl-1-pyrroline) content was observed (quantified by GC-MS) in the mutant and difference of 0.05 level can be seen in other yield related characteristics. This successful editing provides the theoretical insight about the genome editing system and accelerate the breeding process of aromatic rice.

CHEN Yuyu et al.,2020 reported that GS3 and GL3.1 (genes controlling grain size) are the negative regulators of grain size. Editing these genes (Knockout of GS3 and GL3.1) using CRISPR/Cas9 application improved the grain size but affected the other yield related traits.

F.T. Shiobara et al.,2011 reported that the loss of function mutation of DN1 (DENSE PANICLE 1) allele Dn1-1 gives the desired traits like semi-dwarfness and more number of spikelet formation which are required traits in Rice breeding. DN1 is allelic to DEP1 (DENSE & ERECT PANICLE 1).

Chunjue Xu et al.,2015 studied the differential expression of gene which regulates the size of grains in rice. GS5 is a gene which controls the grain size and has a positive association with grain width, grain filling and weight of grains.

2.4.1 CRISPR/CAS9 – a revolution in genome editing technology

Advancements of efficient techniques has consistently been one of the extraordinary viewpoints for biotechnologists. During the last decade, genome editing of various species has been a quick propelling field and hence has gotten a great deal of consideration from various specialists thoroughly checking on most recent accomplishments and offering conclusions on future bearings (A. M. Khalil, 2020).

Labun et al.,2021 has provided the protocols for various CRISPR/Cas9 applications like gene knockout, gene knock-in, effector targeting and given the considerations for carrying out these applications in different targeting modes.

The relevance of CRISPR/Cas9 system in reverse genetic investigations in various fields like Human, animal and also in microbes (curing viral disease like HIV) makes CRISPR/Cas9 system more adorable by providing the outcomes quickly.

Pawluk et al.,2016 reported three separate group of antiCRISPRs that exactly impede the CRISPR/Cas9 System of Neisseria meningitidis. These antiCRISPR proteins activates the "Off-switches" for CRISPR/Cas9 activity and provide a genetically encodable way to impede CRISPR/Cas9 system in Eukaryotes.

Jia-quing Liu et al.,2019 conducted loss of function mutations using CRISPR/Cas9 application and its compatibility in synthetic lethality and virus-host interactions.

S.Mehta et al.,2020 realized the need for increase in food production to satisfy the hunger of raising world population by exploiting CRISPR/Cas9 methodology which has enormous capacity to drastically increase the food production by using the cost effective insilico tools. The most edited food crop is rice (O.sativa) of poaceae family for increasing the yield and quality traits using various tools, softwares and databases of CRISPR/Cas9 technology.

Shreya et al.,2017 reported that the success of this CRISPR/Cas9 method is mainly subject to the requirement of PAM sequence present downstream the target site.

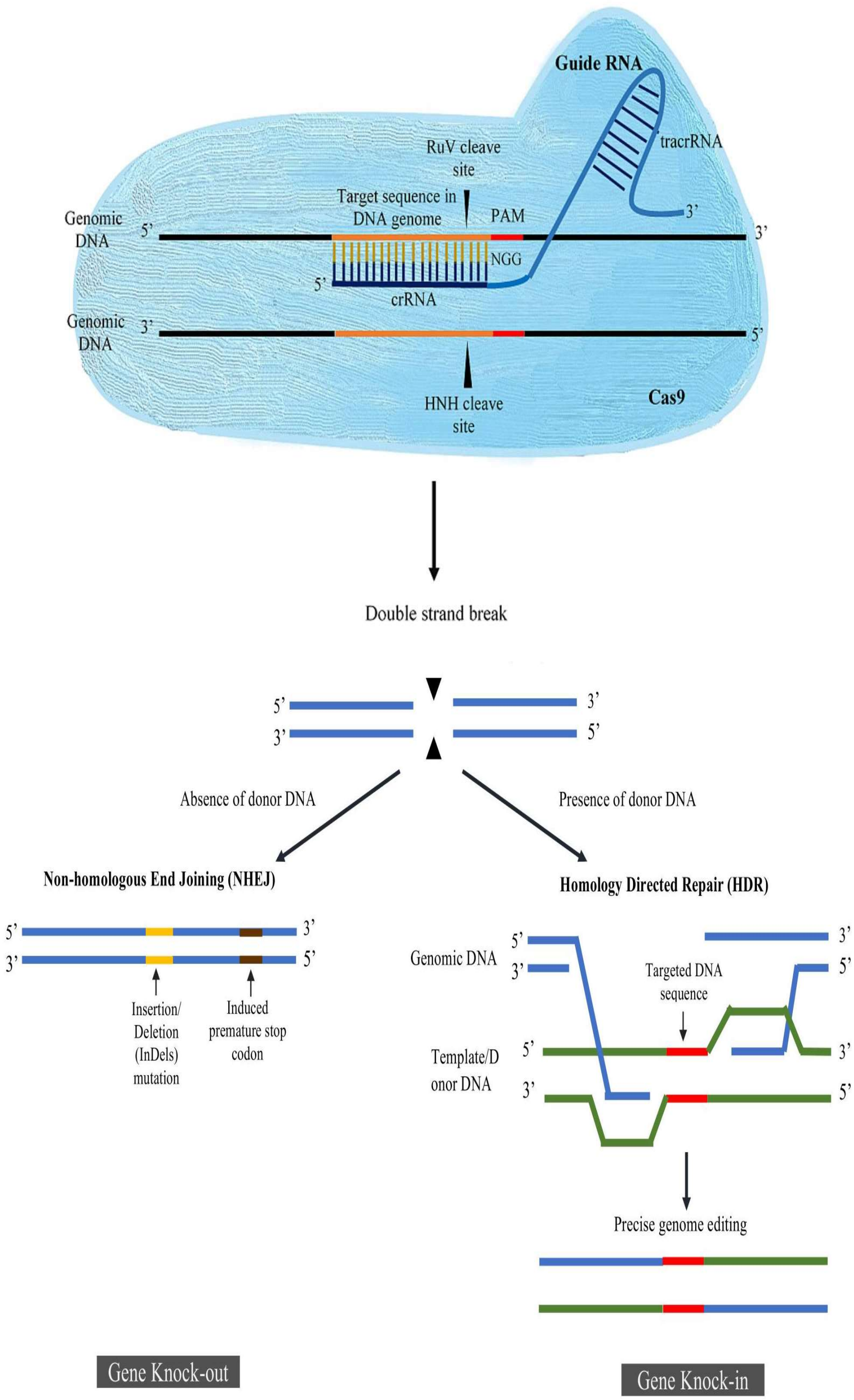


Fig 2.6 The Overview of CRISPR/Cas9 mechanism

2.4.2 CHOPCHOP a Versatile tool for Gene editing

G. Lie et al., 2020 suggested the scoring methods and computational tools used to find out the cleavage efficiency and cutting specificity in constructing effective gRNA. They also recommended the up-to-date version with refinements of 3 important web-based tools i.e., CHOPCHOP, CRISPOR and CRISPR RGEN tools for customized gene editing.

A.P. Karlapudi et al.,2018 reviewed the need to reduce the effect of most of the Acinetobacter strains forming biofilms which is responsible for causing virulence and play a role in Drug resistance. Taking Abal gene of A. baumanni as target, the gene knockout experiments were carried out insilico using various genome editing tools.

P. Sledzinski et al., 2020 presented a comprehensive and newly released versions of available genome editing tools. Among multitude of tools, there are tools which takes into account the Specificity and Efficiency for designing gRNA. Another group of tools exploits the visible biases in outcomes of repair profile to predict the result and some class of tools helps in evaluation of edited outcomes by Sequence data analysis.

CHOPCHOP being the most versatile and user-friendly toolbox has evolved with many updated versions with diverse modes of targeting the genome and transcriptome. The transcriptome and genome of many new species are added to upgrade the exploitability of CHOPCHOP (K. Labun et al.,2019)

The Performance of 17 selected genome editing tools are analysed. Among these tools only 5 tools (CHOPCHOP, SgRNA Scorer 2, mm10db, WU-CRISPR and CRISPR-DO) are worth exploitable for designing effective gRNA with limited overlap and analyse the entire genome which indicates the need for improvement in gRNA constructing tools by integrating multiple approaches (J. Bradford et al.,2018).

Despite the availability of multitude of genome editing tools, CHOPCHOP has emerged as a potent insilico tool accepting wide range of inputs and providing results within a flash of seconds (Montague et al.,2014)

Table 2.6: Insilico tools for sgRNA designing (Yingbo Cui et al.,2018).

SL NO	Name of the Tools	Inputs	Web Address	Scoring method for predicting Off targets
1	CHOPCHOP	RefSeq Gene ID Genomic region	http://chopchop.rc.fas.harvard.edu/	Cong et al.,2013 Hsu et al.,2013
2	CRISPOR	DNA Sequence Genomic region	http://crispor.tefor.net/	CFD Hsu et al.,2013
3	E-CRISP	Gene ID Gene Symbol DNA Sequence	http://www.e-crisp.org/ECRISP/	S-score
4	SgRNA Scorer 1.0	DNA Sequence	http://crispr.med.harvard.edu/sgRNAscorerV1/	No
5	SgRNA Scorer 2.0	DNA Sequence	http://crispr.med.harvard.edu/sgRNAscorerV2/	No
6	CRISPRdirect	DNA Sequence Genomic region Accession numbers	http://crispr.dbcls.jp/	Kmer+PAM
7	GuideScan	Genomic region Gene Symbol	http://www.guidescan.com/	CFD
8	Cas-OFFinder	CrRNA sequences	http://www.rgenome.net/cas-offinder/	Cas-OFFinder

9	CRISPRscan	DNA Sequence Gene ID Gene Symbol	http://www.crisprscan.org/	Cong et al.,2013
10	CASPER	DNA Sequence	http://github.com/TrinhLab/CASPER (Local Python Package)	CASPER
11	WU-CRISPR	DNA Sequence Gene ID Gene Symbol	http://crispr.wustl.edu	Wong et al.,2015
12	CRISPRseek	No	http://www.bioconductor.org/packeges/release/bioc/html/CRISPRseek.html (Local R Package)	CFD Hsu et al.,2013
13	CRISPR Multi-Targeter	DNA Sequence Gene ID RefSeq Sequence Gene Symbol	http://www.multicrispr.net/	GT-Scan Cas-OFFinder
14	CRISPR.mit	DNA Sequence	http://crisprmit.edu/	Hsu et al.,2013
15	CLD	Genomic region Gene ID	http://github.com/boutroslab/cld (Local Perl Package)	Heigwer et al.,2016
16	SSC	DNA Sequence	http://cristrome.org/SSC/	No
17	CROP-IT	Genomic region	http://cheetah.bioch.virginia.edu/AdliLab/CROP-IT/homepage.html	Singh et al.,2015

18	PROTOSPACER	Gene ID Genomic region DNA sequence	http://www.protospacer.com/	Hsu et al.,2013
19	CCTop	DNA Sequence	http://crispr.cos.uni-heidelberg.de/index.html	Stemmer et al.,2017
20	SgRNA designer	DNA Sequence Genomic region Transcript ID Gene Symbol	http://portals.broadinstitute.org/gpp/public/analysis-tools/sgrnadesign	CFD
Databases used for sgRNA designing				
1	Cas- Database		http://rgenome.net/cas-database/	
2	COSMID		http://crispr.bme.gatech.edu/	
3	WGE		http://www.sanger.ac.uk/htgt/wge/	
4	CrisprGE		http://crdd.osdd.net/servers/crisprge/.	
5	CRISPRdb		http://crispr.u-psud.fr/crispr	
6	Cpf1- Database		http://www.rgenome.net/cpf1-database/	
7	PGED (Plant Genome Editing Database)		http://plantcrispr.org/cgi-bin/crispr/index.cgi	
8	CRISPRInc		https://www.crisprinc.org/	

2.4.3 Efficiency of cleavage and Specificity of cutting

In principle, the CRISPR/Cas protein examines the PAM sequence and sgRNA perceives target loci and initiates endonuclease movement to divide particular site. In any case, cleavage efficiency fluctuates incredibly among various target sites and additionally cell lines proposing that several features can impact the binding and cutting ability of the sgRNA-Cas Complex. Many reseaches have uncovered that gRNA sequence features (GC Content, Sequence composition, nucleotide position), hereditary and epigenetic features (Gene expression, Chromatin accessibility) and also the energetic features like free energy, secondary structure and melting temperature all add to gRNA efficacy.

The principle hindrance for the utilization of CRISPR is Off-target effect. CRISPR nucleases may divide accidental genomic destinations what's more, cause sudden changes because of sgRNA's perceiving DNA sequences with a couple of wrong base matches or DNA/RNA bulges which is reffered as Off-target Cleavage. Off-target effects can be successfully solved by forseeing CRISPR Cutting Specificity and constructing ideal gRNA. Two principle techniques have been used to predict the CRISPR gRNA's specificity as depicted in the Fig. (Alignment based and Scoring based).

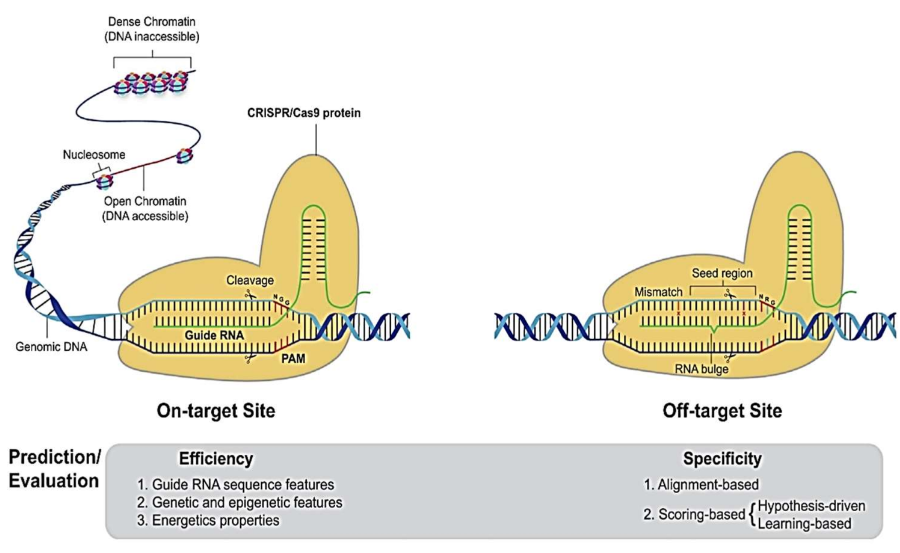


Fig 2.7: Schematic picture of CRISPR/Cas9 system at both Off target and On target site.

Jamal et al.,2017 emphasized the need for improvement of CRISPR/Cas9 On-target specificity which is of paramount importance in clinical research. There is a need to achieve the perfection in the specificity and the off target detection methods that determine the off targets below 0.1% is essential.

Mathur et al.,2019 compared the 3 models (Support Vector Machines- SVM, Convolutional Neural Networks- CNN and Logistic Regression) to predict the Off target potential and concluded that CNN and Logistic regression has greater accuracy compared to the existing CFD tool.

M.Haeussier et al.,2016 evaluated the efficiency of 8 scoring systems and collected the data. By comparing the data with other sites predicted by popular algorithms and found that sequence based Off target predictions are authentic with the maximum mutation rate of 0.1%. The integration of all the required advanced features from those 8 scoring systems into the new tool CRISPOR helps in the prediction of Off targets and selection of appropriate gRNA.

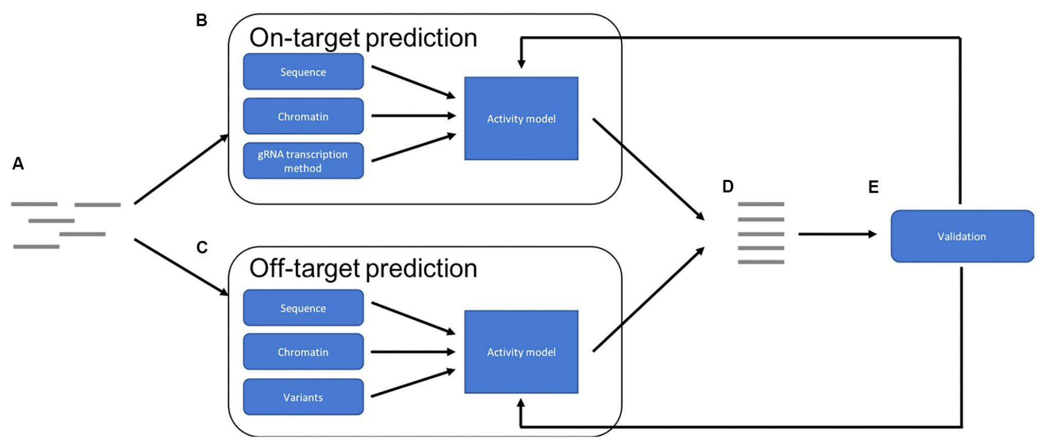


Fig 2.8: A flowline depicting the prediction of On-targets and Off-targets. A) Identified Off-target sites. B) A combination of Sequence, Chromatin and gRNA transcription methods are used for On-target prediction. C) Off targets are predicted using Sequence, chromatin and sequence variations. D) The outputs are combined and targets are ranked. E) The Predictions are experimentally validated and the accuracy is improved in the future experiments. (Wilson et al.,2018)

F. Ann Ran et al.,2013 demonstrated the minimization of Off target effect by using double nickase to reduce the effect by 50- 1500 fold in cell lines (knockout) without sacrificing on-target cleavage efficiency. This strategy enhances the Genome editing Specificity.

CHAPTER III

MATERIALS AND METHODS

Aflatoxins, on a worldwide scale, are important mycotoxins in human foods and animal feedstuffs. Aflatoxin contamination leads to significant economic losses of rice, sorghum, wheat and other commodities. When such contaminated food is consumed, the aflatoxins enter the general food supply. Aflatoxin poisoning most commonly results from ingestion, but the most toxic aflatoxin compound, B1, can permeate through skin. Even daily intake of low amount of aflatoxins is of health concern. Hence, it is necessary to detect and analyse the Aflatoxins in rice (AFB1, AFB2, AFG1 and AFG2).

This chapter includes the methodologies, Procedures or protocols used in conducting the experiment. The present study entitled "Comparative Detection and Determination of Aflatoxin in Rice using LC-MS/MS" was performed to detect and quantify the aflatoxins which are hazardous when present in high levels in rice.

The materials and methods adopted to carry out this experiment are specified under the corresponding title.

3.1 Experimental Material

In the present investigation, commercially procured organic and white rice samples were used as a material for detection and analysis of aflatoxins. The experiment was planned using laboratory available blank rice samples along with commercially procured organic and white rice in six replicates. The experiment was conducted in Kharif 2020-21 at the Phytosanitary Laboratory of Department of Plant Molecular Biology and Biotechnology, Indira Gandhi Krishi Vishwavidyalaya, Raipur, (C.G).

3.2 METHODS

Aflatoxin analysis in commercially procured organic and white rice were carried out using LC-MS/MS coupled with tandem mass spectrometry along with Heated Electrospray Ionization following the AOAC Official Method 2007.01.

3.2.1 Study I: Extraction and Sample Preparation for isolation of aflatoxin in commercially procured organic and white rice.

3.2.1.1 SAMPLE PREPARATION FOR ISOLATION OF AFLATOXINS IN COMMERCIALLY PROCURED ORGANIC AND WHITE RICE

Sampling plays the most pivotal role in detection and determination of aflatoxins in rice. Since the rice grains are the main organic tissue which is vulnerable to aflatoxin contamination hence it becomes very important to assess the level of aflatoxin contamination. In the present investigation four types of samples were prepared are as follows.

- Blank sample –The blank sample was prepared by taking 5g of laboratory available rice samples to which aflatoxin standards were not added, hence served as blank. A total of 16 each for organic and white rice samples were taken as blank. Running blank samples is necessary for cleaning the column and prevent the carrying over of previous samples.
- Sample Spiked – 5g each of organic and white rice samples were taken and it was spiked with 0.01 or 0.05 mg/kg of aflatoxin standards (Make – Restek, Lot no. - #120120) to prepare spiked samples. A total of 12 each for organic rice and white rice samples spiked at 2 concentrations (6 replicates @ 0.01 mg/kg and 6 replicates @ 0.05 mg/kg) were analysed to identify the matrix effects and determine the recovery of an analyte or selectivity of the method.
- Linearity Sample – Linearity samples were prepared by adding different concentration of standards (5-100 PPB) of aflatoxins in blank sample and LC-MS/MS grade water. A total of 6 each for organic and white rice samples were taken as linearity sample. Linearity samples are important because they define the range of the method within which the results are obtained accurately and precisely. Linearity means that a given change in an input variable gives the same change in output of an instrument.
- Test Sample - Rice samples (1kg each of organic and white rice) were commercially procured and 200g of subsample was homogenized using a homogenizer or electric mixer. A total of 2 each for organic and white rice were taken as test samples.

Table 3.1 Preparation of Linearity Standards

Sl No.	Volume of Aflatoxin Standard (mg/kg)	Volume of Blank Sample (µl)	Volume of LCMS grade Water (µl)	Volume of Stock solution (1 PPM)	Final volume (1 ml)
1	0.005 mg/kg	500 µl	495 µl	5 µl	1 ml
2	0.010 mg/kg	500 µl	490 µl	10 µl	1 ml
3	0.025 mg/kg	500 µl	475 µl	25 µl	1 ml
4	0.050 mg/kg	500 µl	450 µl	50 µl	1 ml
5	0.075 mg/kg	500 µl	425 µl	75 µl	1 ml
6	0.1 mg/kg	500 µl	400 µl	100 µl	1 ml

The stock solution of aflatoxin standards of concentration 1PPM was prepared and then dilutions were prepared to make a virtue standard of different concentration ranging from 5-100 PPB (0.005 -0.1 mg/kg).

3.2.1.2 Preparation of working Standards

The Standards were prepared in the concentration of 1PPM ,1ml and kept in refrigerator for further use and for making working standards. The working standards of aflatoxin was prepared from the commercially procured aflatoxin standards using the following formula:

$$N_1.V_1 = N_2.V_2 \qquad (\text{Where, } N_1 = 10 \text{ PPM}$$

$$10 \times V_1 = 1 \times 10 \text{ ml} \qquad N_2 = 1 \text{ PPM}$$

$$V_1 = 1 \text{ ml} \qquad V_2 = 10 \text{ ml})$$

The extraction of aflatoxins were carried out using previously prepared blank, spiked, test and linearity samples. The extraction was performed following the AOAC Official Method 2007.01 entitled " Aflatoxins in Food by Acetonitrile Extraction and Partitioning with Magnesium sulfate and Sodium acetate.

The details of the procedure used is as follows.

- 5g of Sample is weighed and transferred into 50 ml centrifuge tube. (Sample spiking is done at this step).

- To the sample, 15 ml LC-MS grade water is containing 1% Acetic acid is added and it is left soaking for 10 minutes. Then 15 ml Acetonitrile is added to it.
- After adding Acetonitrile, it is vigorously shaken for about 1 min with the use of vortex mixer at 2500 rpm.
- About 6g of $MgSO_4$ along with 1.5g NaOAc is added to the centrifuge tube containing the sample then it is vigorously shaken for 1 minute on a vortex mixer at 2500 rpm.
- Centrifugation is done at ambient temperature (18-20ºC) at >5000 rpm for 5 min. After centrifugation 3 layers are formed where the first is of supernatant which is filtered using PTFE membrane filter.
- The supernatant is diluted using the LC-MS grade water in the ratio of 1:1 in the 2ml LC-MS vial.

3.2.1.3 LC-MS/MS Conditions for Aflatoxin Analysis

The comparative detection and determination of Aflatoxins in Organic and white rice was carried out using the LC-MS/MS Instrument (Make-Thermo Scientific™, Model number – TSQ Endura TQH-E1-0620). Selected Reaction Monitoring along with triple quadrupole Mass Spectrometer (Fig 3.1) including Heated Electrospray Ionization (H-ESI) as ion source was used for quantitative estimation of aflatoxins. The HPLC Unit (for separation of Aflatoxins B1, B2, G1 and G2) contains 4 Compartments (Pump, Autosampler, Column compartment (column acts as a stationary phase) and MS Detector) was used. The above prepared sample is loaded to the 2ml vials inside the autosampler and the LC was optimized for the comparative and quantitative detection of aflatoxins. HESI is used as a ion source where it will convert the liquid sample into the form of droplets and was detected through MS for comparative and quantitative detection of aflatoxins.

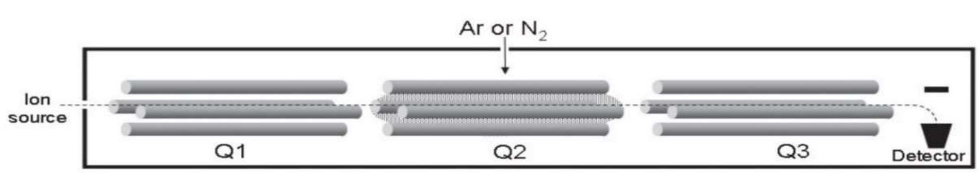


Fig 3.1: Triple Quadrupole system where Q1 and Q3 acts a mass filter, Q2 is a collision cell containing inert gas.

Table 3.2: The optimization of LC-MS/MS conditions

<table>
<tr><td colspan="5">Liquid Chromatography Method</td></tr>
<tr><td colspan="2">Instrumentation</td><td colspan="3">Ultimate 3000 UHPLC (Thermo Scientific™)</td></tr>
<tr><td colspan="2">Column</td><td colspan="3">Accucore aQ column, 100 × 2.1 mm, 2.6 µm</td></tr>
<tr><td colspan="2">Sample Compartment Temperature</td><td colspan="3">15ºC</td></tr>
<tr><td colspan="2">Column Oven Temperature</td><td colspan="3">30ºC</td></tr>
<tr><td colspan="2">Mobile Phase</td><td colspan="3">A: 5Mm Ammonium Formate in Water with 0.1% Formic Acid
B: 5Mm Ammonium Formate in Methanol with 0.1% Formic Acid</td></tr>
<tr><td colspan="2">Total Run Time</td><td colspan="3">15.0 Min.</td></tr>
<tr><td rowspan="9">LC Gradient Program</td><td>Time (min.)</td><td>Flow Rate (ml/min)</td><td>%B</td><td>Curve</td></tr>
<tr><td>0.000</td><td>0.300</td><td>0.0</td><td>0</td></tr>
<tr><td>0.000</td><td>0.300</td><td>0.0</td><td>0</td></tr>
<tr><td>0.500</td><td>0.300</td><td>0.0</td><td>0</td></tr>
<tr><td>7.000</td><td>0.300</td><td>70.0</td><td>0</td></tr>
<tr><td>9.000</td><td>0.300</td><td>100.0</td><td>0</td></tr>
<tr><td>12.000</td><td>0.300</td><td>100.0</td><td>0</td></tr>
<tr><td>12.100</td><td>0.300</td><td>0.0</td><td>0</td></tr>
<tr><td>15.000</td><td>0.300</td><td>0.0</td><td>0</td></tr>
<tr><td colspan="5">Optimization conditions used in Mass Spectrometry</td></tr>
<tr><td colspan="2">Instrumentation</td><td colspan="3">TSQ Endura Triple Quadrupole Mass Spectrometer (Thermo Scientific™)</td></tr>
<tr><td colspan="2">Method used</td><td colspan="3">Acquisition – Timed (SRM mode)</td></tr>
<tr><td colspan="2">Ion Source type</td><td colspan="3">Heated Electrospray ionization</td></tr>
<tr><td colspan="2">Spray voltage</td><td colspan="3">Static
Positive – 3500V
Negative – 2800V</td></tr>
<tr><td colspan="2">Sweep Gas</td><td colspan="3">1</td></tr>
<tr><td colspan="2">Auxiliary Gas</td><td colspan="3">5</td></tr>
<tr><td colspan="2">Sheath Gas</td><td colspan="3">60</td></tr>
<tr><td colspan="2">Vaporizer temperature</td><td colspan="3">450ºC</td></tr>
<tr><td colspan="2">Ion Transfer Tube Temperature</td><td colspan="3">200ºC</td></tr>
</table>

3.2.1.4 Estimation of Sample Preparation and Extraction of aflatoxins

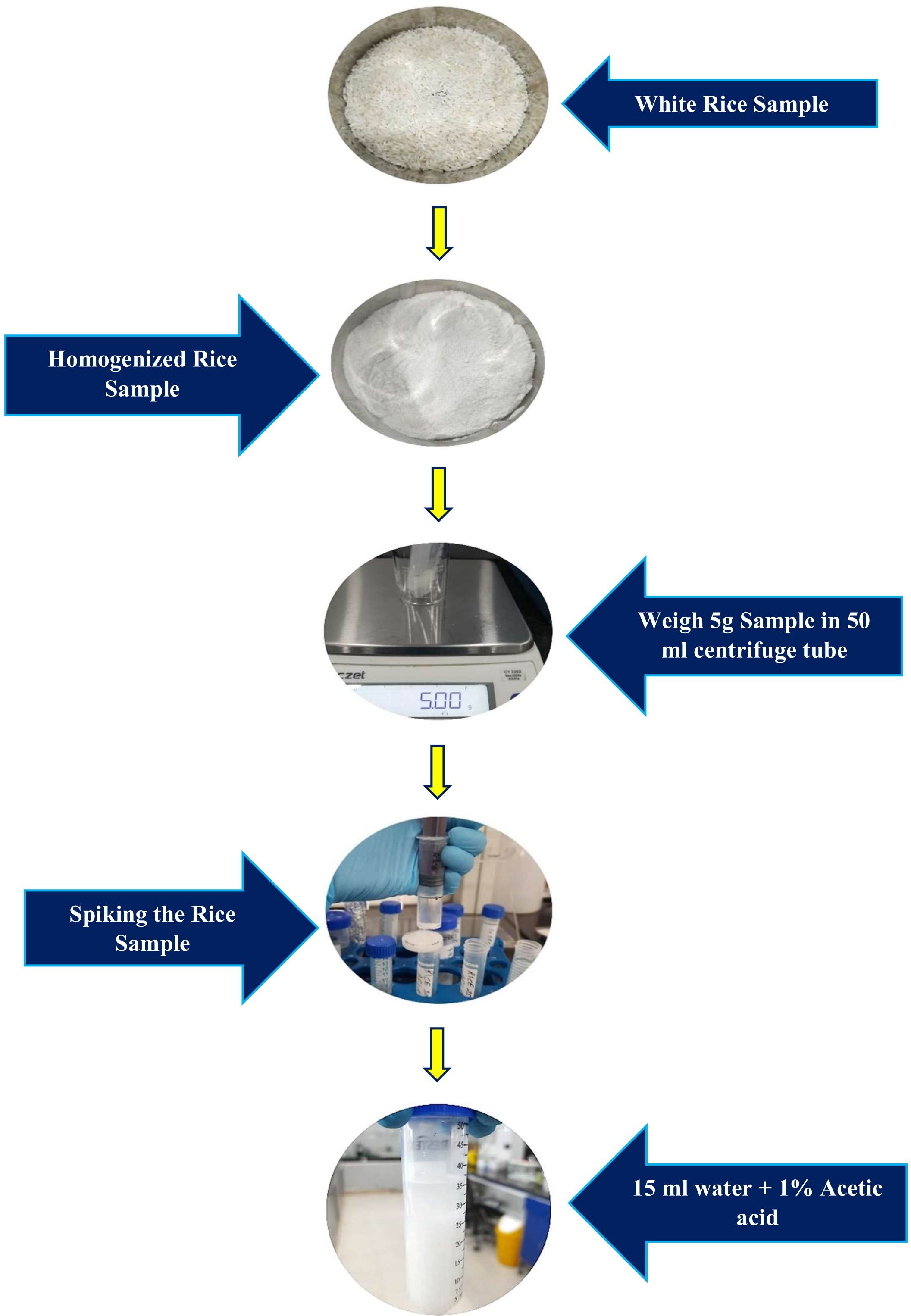
The accuracy of sample preparation and extraction of aflatoxin was estimated by estimation of recovery % in the range of 70-120% (SANTE's guideline 12682/2019) and linearity in the range ≥ 0.99 (SANTE's guideline 12682/2019)

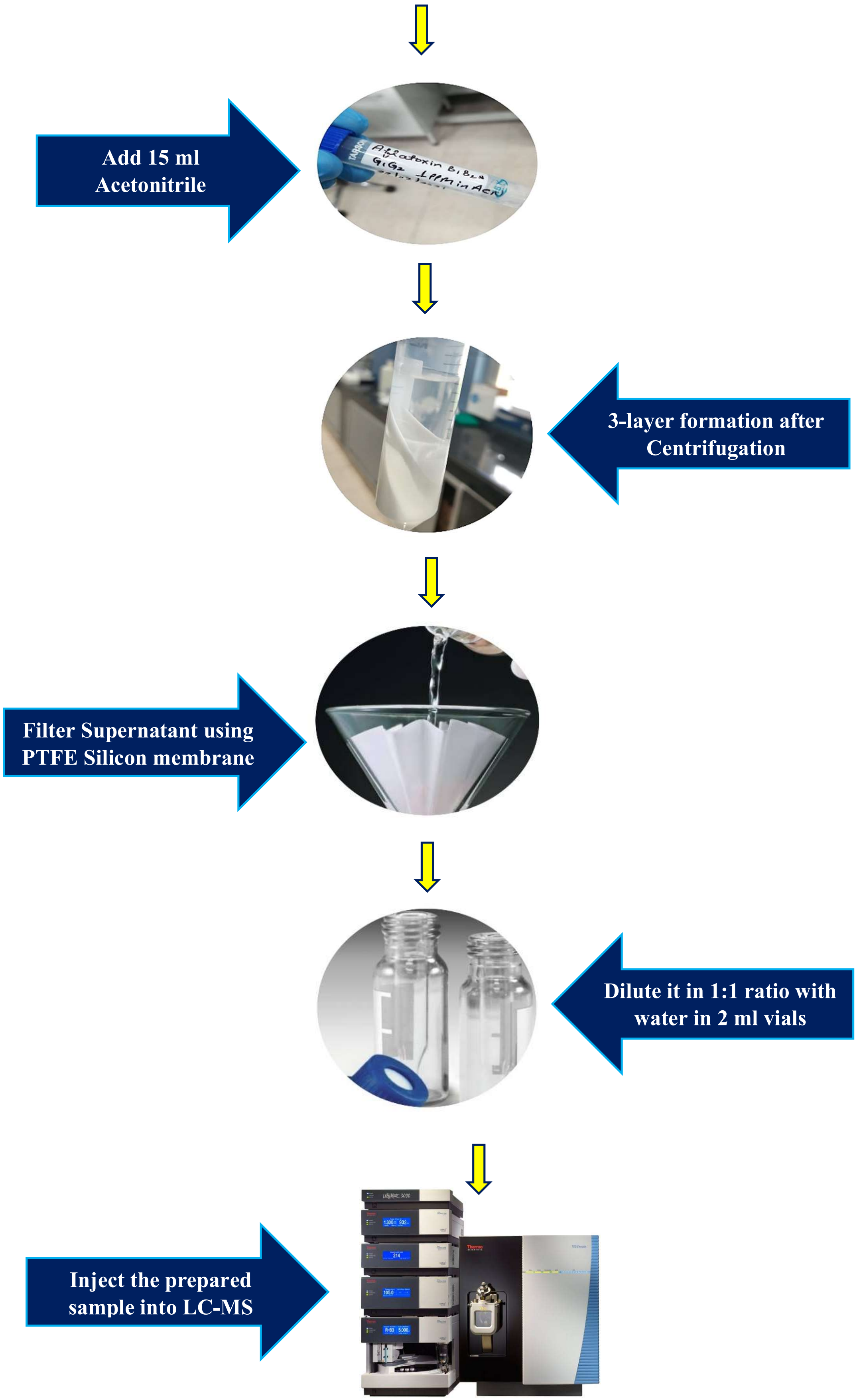
Table no 3.3: Conditions for Sample preparation and extraction of aflatoxins.

Sl No	Name of Sample	Linearity	Recovery %	Remark
1	Blank Sample	≥ 0.99	70-120 %	If Linearity ≥ 0.99, Recovery 70-120% = PASS
2	Spiked Sample	≥ 0.99	70-120 %	If Linearity ≥ 0.99, Recovery 70-120% = PASS
3	Linearity Sample	≥ 0.99	70- 120%	If Linearity ≥ 0.99, Recovery 70-120% = PASS

Fig 3.2: LC-MS/MS Instrument used for aflatoxin detection and determination.

Fig 3.3: Flowchart for Extration for isolation of Aflatoxins in rice.




Add 15 ml Acetonitrile
3-layer formation after Centrifugation
Filter Supernatant using PTFE Silicon membrane
Dilute it in 1:1 ratio with water in 2 ml vials
Inject the prepared sample into LC-MS

3.2.2 STUDY II: Quantitative estimation of aflatoxin in commercially procured Organic and White rice.

Aflatoxins are potent toxins and known carcinogens, so their levels in food should be limited to the practical level. Rice is one of the main agricultural produce which is vulnerable to aflatoxin contamination that ultimately harms human and animal health. The identification and quantitative estimation of aflatoxins in food and feed is a major challenge to guarantee food safety.

The quantitative steps followed for the determination and quantification of aflatoxins in commercially procured organic and white rice samples using previously prepared blank, spiked and linearity samples in six replicates are explained as follows:

Certified reference materials: Aflatoxins (B1, B2, G1 and G2) Standards purchased from Restek.

Lot No. #120120, Concentration: 10 µg/ml (10 PPM)

3.2.2.1 METHODS FOLLOWED FOR QUANTITATIVE STUDY

I. **Preparation of Working solution from Stock Standard Solution**

1 PPM aflatoxins standard is prepared from 10 PPM solution according to the formula $N_1V_1=N_2V_2$.

$N_1.V_1 = N_2.V_2$ (Where, N_1= 10 PPM

$11 \times V_1 = 1 \times 10$ ml N_2= 1 PPM

$V_1 = 1$ ml V_2= 10 ml)

So, 1ml aflatoxin standard is taken in 15ml tube + 9 ml Acetonitrile is added.

II. **Preparation of Matrix-Matched Linearity Standards**

The manual preparation of calibration standards for the analysis of Rice samples for contamination by aflatoxins are followed as mentioned in the Table 3.4.

Table 3.4: Preparation of Matrix Matched Linearity Standards.

SL No.	Concentration (PPB)	Matrix Amount (µl)	Stock Solution (1 ppm)	H2O (µl)	Total (ml)
1	5 PPB	500 µl	5 µl	495 ml	1 ml
2	10 PPB	500 µl	10 µl	490 ml	1 ml
3	25 PPB	500 µl	25 µl	475 ml	1 ml
4	50 PPB	500 µl	50 µl	450 ml	1 ml
5	75 PPB	500 µl	75 µl	425 ml	1 ml
6	100 PPB	500 µl	100 µl	400 ml	1 ml

III. Calculation of Reproducibility.

Reproducibility/repeatability of Organic and White rice Sample is calculated using the following steps:

1. Reproducibility (R1 to R6) values are obtained by multiplying the Sample amount with the dilution factor (i.e., 6)

2. Average of reproducibility is found out by summing the values from R1 to R6.

3. Standard deviation is calculated using the formula: $\mathbf{SD} = \sqrt{\frac{\sum(x-\bar{x})^2}{n-1}}$

4. Relative Standard deviation is calculated using the formula:

$$\%\mathbf{RSD} = \frac{\textbf{Standard deviation}}{\textbf{Average}}$$

IV. Quantitative Estimation of Aflatoxins.

Quantitative estimation of aflatoxins (AFG1, AFG2, AFB1, and AFB2) were carried out using 6 samples, 3 each of organic and white rice using the formula:

$$\text{Concentration of Aflatoxin} = \frac{\text{Detected concentration} \times \text{dilution factor}}{\text{Weight of sample taken}}$$

3.2.2 Some of the Apparatus, Euipments, Aflatoxin Standards, Chemicals and Reagents used in this study are listed below

Apparatus and Equipments

1. Spatula (stainless steel)
2. Micropipettes
3. Refrigerated Centrifuge
4. Homogenizer
5. Electronic weighing balance
6. Vortex shaker
7. 2 ml Autosampler vials
8. LC-MS/MS

Standards used: Analytical grade standards of aflatoxins (B1, B2, G1 and G2) are purchased from Sigma Aldrich, Germany.

Chemicals and Reagents

1. Methanol which is of LC-MS grade

2. Acetonitrile which is of LC-MS grade

3. Water which is of LC-MS grade

4. Acetic acid

5. Formic acid

6. Sodium acetate (NaOAc) – LR grade

7. Anhydrous Magnesium sulfate ($MgSO_4$) (Extra pure)

8. Certified reference materials

9. Distilled water

3.2.3 Study III: Insilico designing/construction of novel alleles of selected genes of Rice and Aroma using gene editing tools.

Creation of novel alleles of the genes are very important in crop genetic improvement to improve the quality of the produce, to increase the crop yield, to adapt crops to adverse climatic conditions, to induce resistance to pests and diseases in crop plants, to breed crops with uniform growth and maturity and to meet particular needs of growers and consumers.

Modern breeding techniques by employing the CRISPR/Cas9 genome editing method could improve the yield of rice. Successful breeding for crop improvement, however, depends on genetic variability in the parents, such that a lack of genetic variability would have the potential to significantly limit breeding progress and/or yield and quality crop improvements. Increased knowledge into the genetic diversity of any germplasm collection, therefore enhances the possibility of crop improvement and the development of superior cultivars.

3.2.3.1 Materials: A total of 11 genes of rice quality and aroma are selected for creating the novel alleles using JavaScript enabled easy to use web browser like Chrome, Firefox or Safari Computer/laptop was used for the current insilico designing of novel alleles of selected genes. The details of these 11 genes are listed in the Table 3.5.

3.2.3.2 METHODS

The creation of novel alleles of selected genes of rice and aroma was carried out using the very promising genome editing *insilico* system, Clustered regularly interspaced short palindromic repeats and CRISPR associated protein 9 (CRISPR-Cas).

The broadly exploited CRISPR/Cas9 genome editing tool CHOPCHOP (http://chopchop.cbu.uib.no) web tool having inbuilt database for designing sgRNA sequences was used to bring targeted changes in the genome of rice which can enhance the quality and yield parameters.

Table 3.5: **Genes of rice aroma and quality selected for creating novel alleles using gene editing tool.**

SL NO	GENES	LOCUS ID	POSITION	POPULATION / PARENT	EFFECT	EFFECTIVE REGION OF GENE	TYPE OF FUNCTION	REFERENCE
1	APO1	LOC_Os06g45460	Chr 6 27479769 – 27481075	Sasanishiki / Habataki	Increases number of grains per panicle.	Promoter	Gain of function	Nagata et al. (2002)
2.	OsBadh2	LOC_Os08g32870	Chr 8 20379794 – 20386061	ASD 16 (Non-Aromatic variety	Production of Aroma in rice	Exon 7	Loss of function	Kambale R et al. (2020)
3	DEP1	LOC_Os09g26999.1	Chr9 16411151 – 16415851	Balilla and Ardito	High yield with erect panicle.	Exon 5	Gain of function	Xu et al. (1995) Li et al., (2016)
4	GHD7	LOC_Os07g15770	Chr7 9155185 - 9152402	Zenshan 97 & Milyang 46	Delayed flowering – spikelet number & grain weight increased	Promoter	Gain of function	Zhen Hua et al. (2019)
5	GHD8	LOC_Os08g07740	Chr8 4335434 - 4333717	ZN & NN Transgenic lines	Delayed heading date & enhanced cold tolerance	Promoter	Gain of function	wang et al. (2019)
6	Gn1a	LOC_Os01g10110	Chr1 5275678 - 5270103	Habataki (Indian cultivar)	Enhanced grain production	5' UTR Exon 1 Exon 4	Loss of function	Ashikari et al., (2005)
7	GS5	LOC_Os05g06660	Chr5 3443769 - 3439259	NIL(H94) & NIL(ZS97)	Increased grain size & grain width	Promoter	Gain of function	Chunjue Xu et al. (2015)
8	GW2	LOC_Os02g14720	Chr2 8114961 – 8121925	Koshihikari	Increased seed width, length & weight	3' splicing site of 6th Intron.	Loss of function	Lee et al. Bot Stud (2018)
9	GW5	LOC_Os05g09520	Chr5 5365122 - 5366701	Japonica cv. Kitaake & indica cv. Kasalath	Increased Grain width and grain weight	Exon 1	Loss of function	Liu et al. 2017
10	MOC1	LOC_Os06g40780	Chr6 24311420 - 24316382	H89025 & Minghui 63	Increased number of tillers & reduced plant height.	Promoter	Gain of function	Cao Xu, Yonghong Wang et al. 2012
11	NAL1	LOC_Os04g52479	Chr4 31203525 - 31214741	Takanari & Koshihikari	Increased Photosynthesis rate	Exon 1	Loss of function	Toshiyuki Takai et al. (2013)

A total of 11 genes were characterised for enhancing the quality of yield parameters due to change caused by loss of function or gain of function in nature was selected for designing of novel alles.(Table 3.3). Fig: was used as a strategy to design a gRna construct.

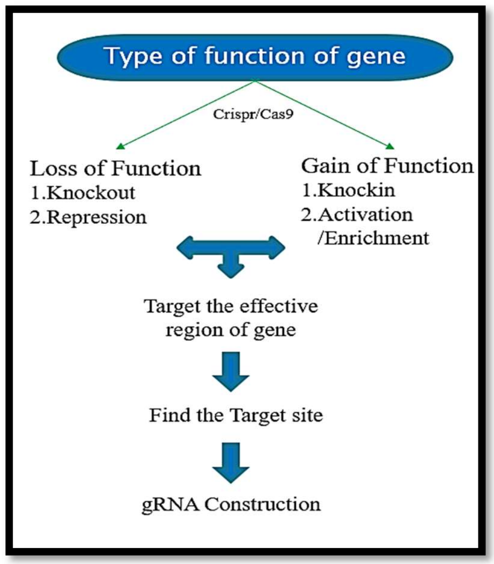


Fig3.4: Steps involved in insilico designing of gRNA

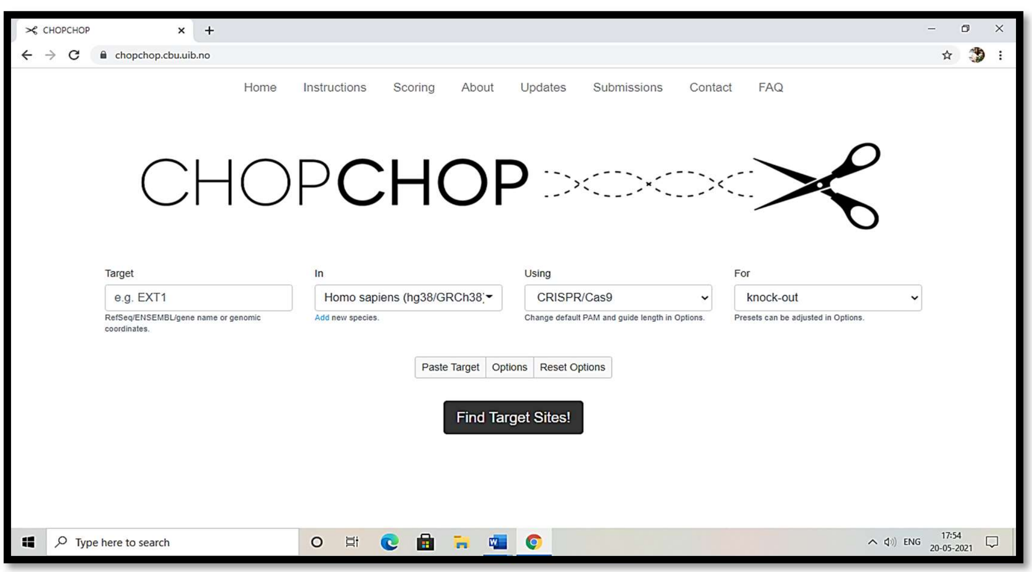


Fig 3.5: View of CHOPCHOP webtool used for creating the novel alleles of selected genes of rice aroma and quality

The Steps followed for sgRNA designing is as follows;

1.CHOPCHOP web tool was opened using the web address (http://chopchop.cbu.uib.no) using easy to use JavaScript enabled web browser (Chrome).

2. The Homepage of CHOPCHOP contains 4 main fields ; 1. 'Target',
2. 'In'
3. 'Using'
4. 'For'

- The Locus ID of the selected genes of rice was given as input in the 'Target field' tab.
- The type of organism i.e., Oryza sativa (MSU-version_7.0) was then selected in the 'In field' tab.
- Afterwards CRISPR/CAS9 mode was selected in the 'Using field' tab.
- 'For' field tab had options to carryout CRISPR/CAS9 applications like Knock-in (used for the gain of function genes) and Knock-out was employed (used for loss of function genes).

3. Optimization of the search parameters were carried out using the General and CRISPR Cas9 Advanced options (Cas9 and Primer options present in the Homepage of CHOPCHOP)

- Using the General option tab, the Target specific region of the gene is selected. The effective regions of the genes targeted for creating its novel alleles is mention in the below Table 3.6

Table 3.6: Effective regions of the genes to be targeted to create its novel alleles.

Sl. No	Name of the Gene	Effective region of the gene targeted
1	APO1	Promoter
2	OsBadh2	Exon 7
3	DEP1	Exon 5
4	GHD7	Promoter
5	GHD8	Promoter
6	Gn1a	5' UTR and Exon 1, Exon 4
7	GS5	Promoter
8	GW2	3' splicing site of 6^{th} Intron.
9	GW5	Exon 1
10	MOC1	Promoter
11	NAL1	Exon 1

4. Restrict targeting option is used to search exons and immediate short flanking regions.

- When searching for target sites in a region of interest, in the default mode CHOPCHOP allows the gRNA to bind just outside of that region so that the cur still occurs within the targeted region)

5. The whole gene, genes with multiple isoforms were targeted using the UNION Mode.

- Union Mode – searches for gRNAs in every exon of every isoform. This mode can be used to target one (or more) specific isoforms.

6. The Pre-filtering of gRNAs was done based on the GC content and self - complementarity score. Where the input for GC content is given between 40 to 70 % and the self-complementarity score is set for -1.

- sgRNA's with 40-70% GC Content are most effective.
- Self-complementarity if present in the gRNA affects the gRNA efficiency.

7. Some of the Optimization parameters like Restriction enzyme (New England Biolabs), color scoring in Fasta input and the displayed flanking sequence length in detailed view (300) was used in its default mode.

8. In 'Cas9' option the NGG PAM was selected.

9. Off-targets was set upto 1 mismatch and was followed according to method given by Hsu.et.,2013.

10. scoring method developed by Doench et al.2016 was employed to design the sgRNA's with high efficiency score.

11. Repair profile prediction and the cell type was selected according to the method given by Shen et.al.2018 model and the cell type was selected. In the present investigation the cell type was not known, hence the mESC option/tab was selected.

12. Using the standard backbone (AGGCTAGTCCGT) option, the Self-complementarity is checked (Thyme et.al.,2016).

13. The default parameters were used for primers size (18-25, optimal size-22), product size (150-290) and primer temperature (57-63, optimal temperature- 60). The minimum distance from primer to target site was 20 by default.

14. After optimizing the above mentioned parameters the target sites are found by submitting query (click the 'Find target sites!').

15. The query is processed in a matter of seconds and designed gRNA is displayed in the Result table which was downloaded for further selecting the ideal gRNA and testing its efficiency.

3.2.3.3 SELECTION OF IDEAL gRNA

Selection of ideal sgRNA's were carried out using some of the below mentioned criteria;

1. Once all the sgRNA's are annotated and ranked by the CHOPCHOP, the best ranked sgRNA was selected.
2. The sgRNA's with GC Content ranging from 40-70% was selected.
3. The sgRNA's with minimum Off-target effects with least mismatches were selected.
4. The sgRNA's with zero self-complementarity score was selected.
5. The sgRNA's with High Efficiency Score was selected.

3.2.3.4 INSILICO EFFICIENCY TESTING OF THE DESIGNED gRNA

Insilico efficiency testing was carried out for the selected designed gRNAs using the CRISPR Efficiency Prediction Tool (https://www.flyrnai.org/evaluateCrispr/)

The steps followed for predicting the sgRNA efficiency are:

Step I : The sequence of all the designed gRNA's was used as a query sequence.

Step II: Sequence information was mentioned by picking the choices underneath

→ PAM is avoided from input sequence (max. 20 bases)

Step III: Click Display Result

(Results were downloaded in excel format)

The results obtained were analysed based on its efficiency score, GC content and also self-complementarity.

Selecting only one sgRNA may lead to false positive result. So it is important to select more sgRNAs and their efficiency. sgRNAs with score above 7.5 is best and out of all the gRNA's designed the 3 best sgRNA's were mentioned

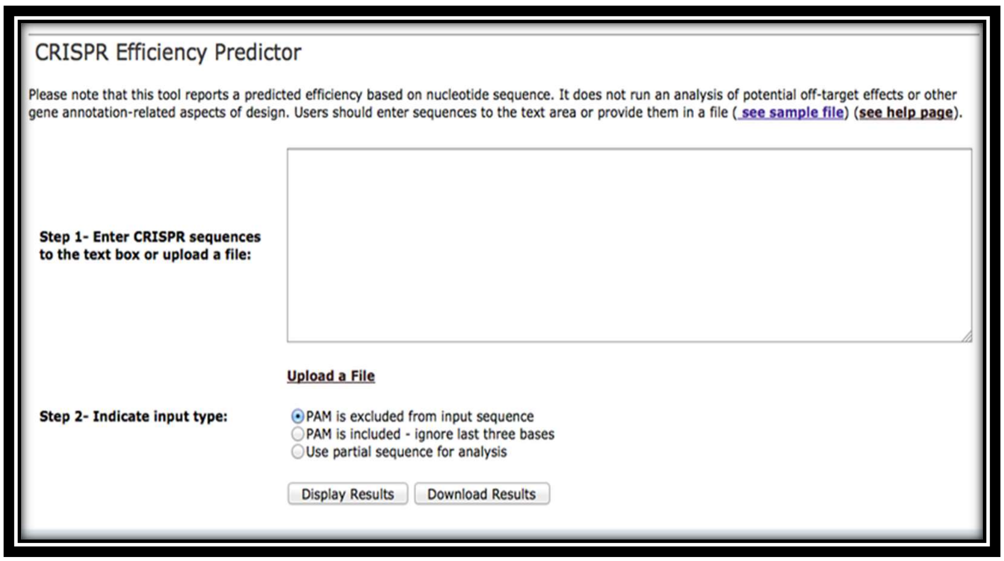


Fig 3.6 Homepage of CRISPR Efficiency Predictor web portal used for testing the efficiency of designed sgRNA's.

CHAPTER - IV

RESULT AND DISCUSSION

Aflatoxins are the secondary metabolites mainly produced by Aspergillus flavus. Out of 20 different types are known; four aflatoxins B1, B2, G1 and G2 could lead to some health problems as they are toxic in nature. They have been reported to lead to some health problems such as acute and chronic poisoning among animals and human beings; hepatotoxic, cirrhosis and some malignancies as well as immunodeficiency causing recurrent infections. Many of the committee and institutes therefore have determined standards for the acceptable amount of the mycotoxin in foods because of its harmful effects. Hence there is a need for quantitative estimation of aflatoxins in Rice and other food products. In this regard, international and national export control agencies have decided the maximum residue limits (MRL) of aflatoxin in food commodities and same has to be tested by the approved testing agencies for export across the globe.

The detection and quantification of aflatoxins in food samples require an efficient extraction step. Aflatoxins are generally soluble in polar solvents such as methanol, acetone, chloroform, and acetonitrile. The extraction of aflatoxins involves the use of these organic solvents. In the present investigation therefore, an experiment was carried out to quantitatively estimate aflatoxin in rice and the results are given below.

4.1 STUDY I : Extraction and Sample preparation for isolation of aflatoxin using LC-MS/MS

Commercially procured organic and white rice was used for the detection and determination of aflatoxins using LC-MS/MS. The prepared samples were run with the total runtime of 15 mins. The detection was carried out in Selected Reaction Monitoring (SRM) mode and the data processing was carried out using Thermo Scientific^{TM} Xcalibur^{TM} software inbuilt in LC-MS/MS.

A total of 72 samples, 36 each for organic and white rice samples were taken in the present investigation. Out of 36 samples, 16 samples were blank, 12 (6 spiked at

0.01 mg/kg and 6 spiked at 0.05 mg/kg) samples were spiked, 6 linearity samples and 2 test samples were analysed. The results obtained after the analysis is presented below.

4.1.1 Sample Preparation and Extraction for isolation of Aflatoxins in rice

4.1.1.1 Analysis of Blank Samples for Organic and White rice samples

The analysis of blank samples were carried out using LC-MS/MS in Selected reaction monitoring (SRM) mode for 15 minutes. Out of 16 blank samples analysed, all 4 types of aflatoxins (AFB1, AFB2, AFG1 and AFG2) were not detected. This indicated the efficiency of the method to be used for downstream analysis and further analysis of spiked and test samples for Aflatoxin detection (Table 4.1)

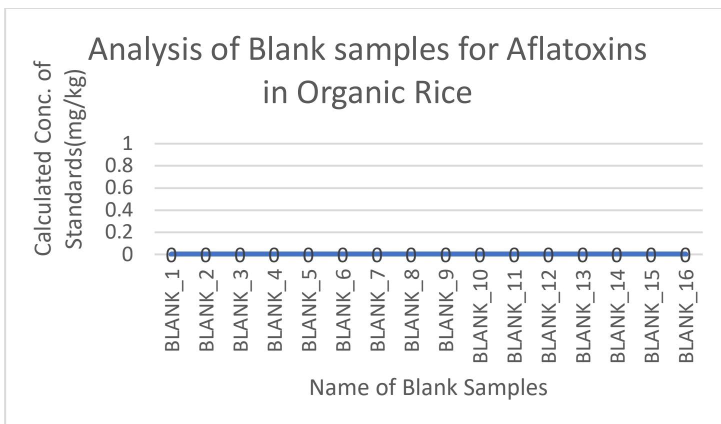


Fig 4.1: Analysis of Blank Samples for Aflatoxins in Organic rice.

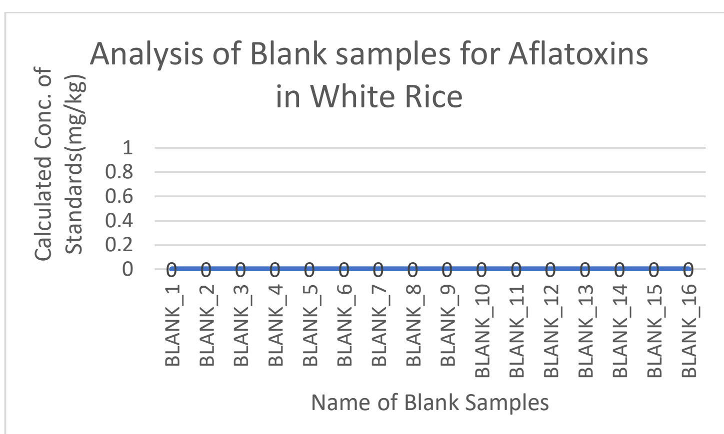


Fig 4.2: Analysis of Blank Samples for Aflatoxins in White rice.

4.1.1.2 Analysis of Linearity Samples for Organic and White rice.

A total of 6 each for organic and white rice, linearity samples were analysed in the present study. In both type of linearity samples the calibration standards were found to be equal or above accepted linearity score ($R^2 \geq 0.99$) which indicated the prepared samples are accurate, Method is fit for analysis and therefore it can be employed for further analysis of spiked and test samples (Table 4.1 and 4.2)

Table 4.1 Analysis of Linearity Samples for aflatoxins in Organic rice.

Sl No	Name of Linearity Sample	Concentration of Calibration Standard (mg/kg)	Compound Name	Calculated concentration of Standard (mg/kg)	Linearity Score (R^2 value - acceptable level $\geq$ 0.99)
1	*MM_1	0.005	AFG2	0.00516	0.9997
			AFG1	0.00509	0.9999
			AFB2	0.00507	1.0000
			AFB1	0.00503	1.0000
2	MM_2	0.01	AFG2	0.00977	0.9997
			AFG1	0.00977	0.9999
			AFB2	0.00988	1.0000
			AFB1	0.00985	1.0000
3	MM_3	0.025	AFG2	0.02496	0.9997
			AFG1	0.02518	0.9999
			AFB2	0.02503	1.0000
			AFB1	0.02530	1.0000
4	MM_4	0.05	AFG2	0.04889	0.9997
			AFG1	0.04964	0.9999
			AFB2	0.04966	1.0000
			AFB1	0.04983	1.0000
5	MM_5	0.075	AFG2	0.07567	0.9997
			AFG1	0.07581	0.9999
			AFB2	0.07540	1.0000
			AFB1	0.07522	1.0000
6	MM_6	0.1	AFG2	0.10056	0.9997
			AFG1	0.09952	0.9999
			AFB2	0.09997	1.0000
			AFB1	0.09977	1.0000

(*MM= Matrix matched linearity samples prepared from organic rice)

Table 4.2: Analysis of Linearity Samples for aflatoxins in White rice.

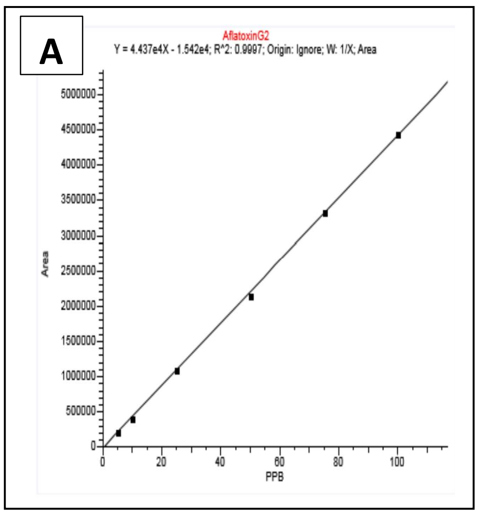
Sl No	Name of Linearity Sample	Concentration of Calibration Standard (mg/kg)	Compound Name	Calculated concentration of Standard (mg/kg)	Linearity Score (R^2 value - acceptable level ≥ 0.99)
1	MM_1	0.005	AFG2	0.00564	0.9912
			AFG1	0.00557	0.9910
			AFB2	0.00560	0.9989
			AFB1	0.00501	0.9944
2	MM_2	0.01	AFG2	0.00859	0.9912
			AFG1	0.00858	0.9910
			AFB2	0.00851	0.9989
			AFB1	0.00847	0.9944
3	MM_3	0.025	AFG2	0.02503	0.9912
			AFG1	0.02538	0.9910
			AFB2	0.02544	0.9989
			AFB1	0.02733	0.9944
4	MM_4	0.05	AFG2	0.05063	0.9912
			AFG1	0.05093	0.9910
			AFB2	0.05114	0.9989
			AFB1	0.05874	0.9944
5	MM_5	0.075	AFG2	0.07467	0.9912
			AFG1	0.07463	0.9910
			AFB2	0.07365	0.9989
			AFB1	0.06875	0.9944
6	MM_6	0.1	AFG2	0.10044	0.9912
			AFG1	0.09991	0.9910
			AFB2	0.10066	0.9989
			AFB1	0.09671	0.9944

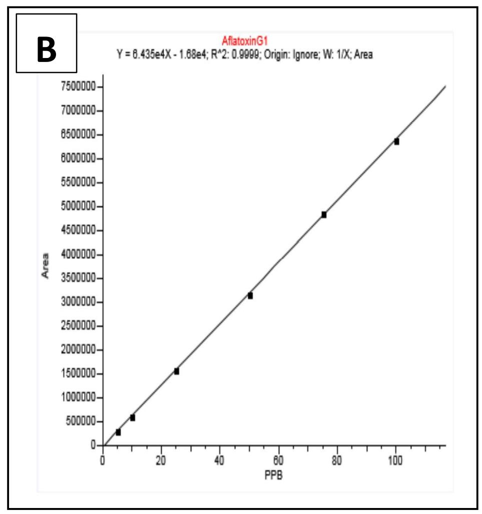
(*MM= Matrix matched linearity samples prepared from organic rice

In total for 4 types of aflatoxins AFG1, AFG2, AFB1 and AFB2, the correlation co-efficient calculated from 12 organic and white rice samples were found to be equal or more than R^2 Value 0.99 (Table 4.3)

Table 4.3: Analysis of 4 types of Aflatoxins from 12 organic and white rice linearity samples.

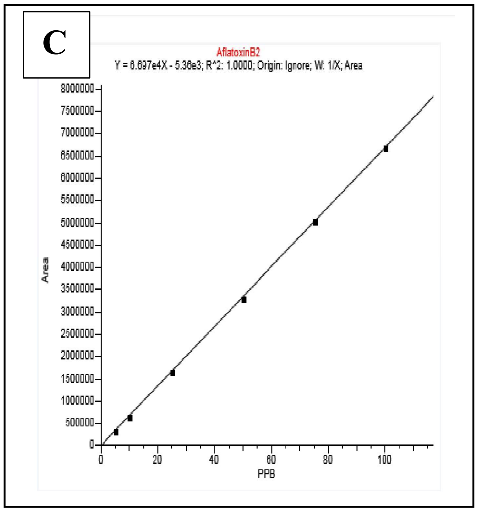
Sl No	Name of Aflatoxin	Correlation coefficient (R^2)	
		Organic rice	White rice
1	AFG2	0.9997	0.9912
2	AFG1	0.9999	0.9910
3	AFB2	1.0000	0.9989
4	AFB1	1.0000	0.9944

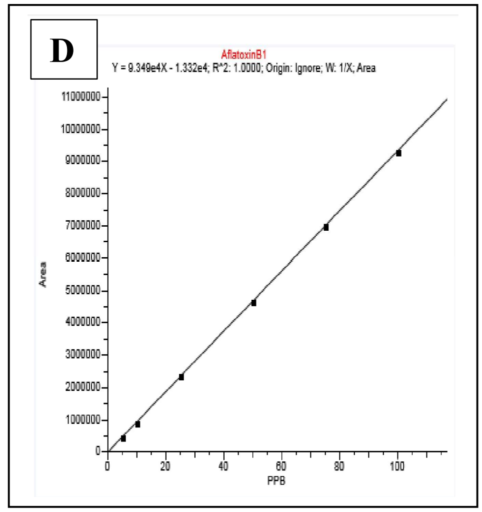




A Linearity curve obtained for Aflatoxin G2 in commercially procured organic rice (R2 = 0.9997)

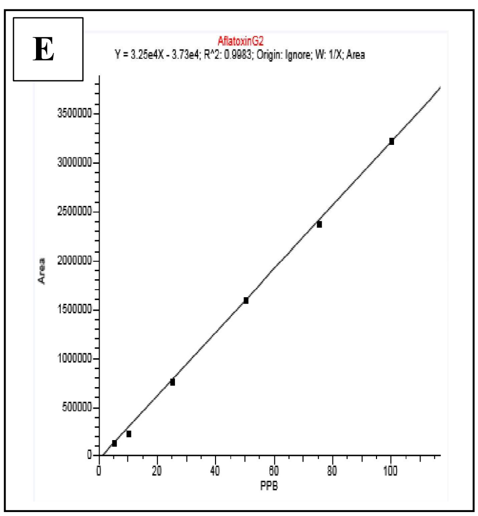
B Linearity curve obtained for Aflatoxin G1 in commercially procured organic rice (R2 = 0.9999)

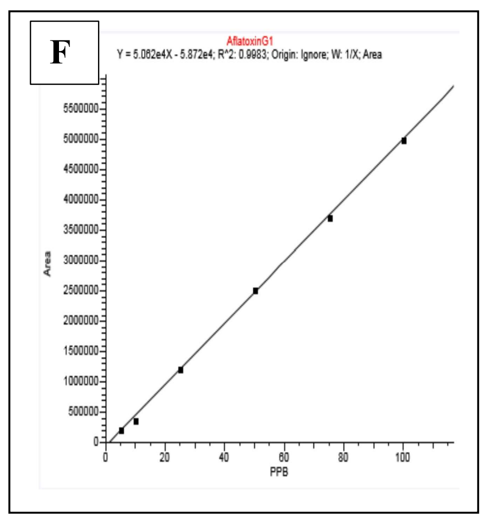




C Linearity curve obtained for Aflatoxin B2 in commercially procured organic rice (R2 = 1.0000)

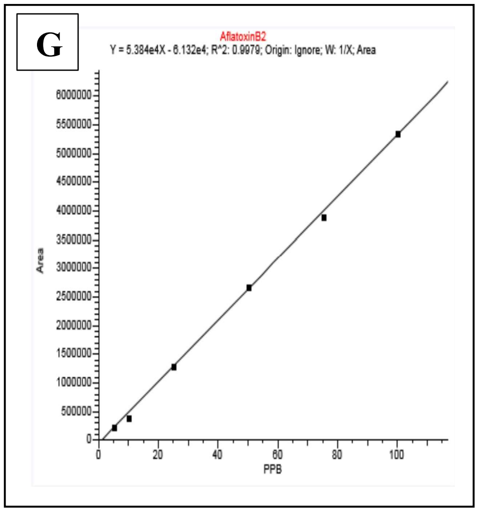
D Linearity curve obtained for Aflatoxin G2 in commercially procured organic rice (R2 = 1.0000)

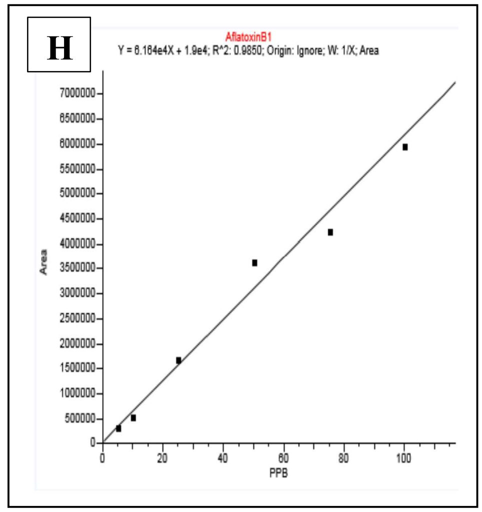




E Linearity curve obtained for Aflatoxin G2 in commercially procured White rice (R2 = 0.9983)

F Linearity curve obtained for Aflatoxin G1 in commercially procured White rice (R2 = 0.9983)





G Linearity curve obtained for Aflatoxin B2 in commercially procured White rice (R2 = 0.9979)

H Linearity curve obtained for Aflatoxin B1 in commercially procured White rice (R2 = 0.9850)

Fig 4.3: Linearity Curves of Aflatoxins G2, G1, B2, B1 in organic and white rice.

4.1.1.3 Estimation of recovery of Spiked samples

A total of 12 each for organic and white rice samples spiked at two concentrations (6 replicates spiked @ 0.01 mg/kg, 6 replicates spiked @ 0.05 mg/kg) were analysed to identify the matrix effects and determine the recovery of an analyte or selectivity of the method. The recovery was calculated as per SANTE's 12682/2019 guidelines. The recovery % should be between 70-120% in order to quantify aflatoxins in test samples. The recovery % if found below to this set limit indicates that the method is not fit for identification and analysis of aflatoxins.

In the present investigation, among 12 spiked samples, 6 each of organic and white rice for two concentrations *viz*. 10PPB and 50PPB, the recovery % was found between 70-120%. (Table 4.4). This indicated that the method is fit for analysis, Samples were prepared accurately and hence the same method can be employed for quantitative estimation of aflatoxins.

Table 4.4: Estimation of Recovery percentage using Organic and white rice through LC-MS/MS.

Type of Rice Sample	Aflatoxin	Recovery Sample Name	Sample amount	Recovery percentage
Organic Rice	AFG2	REC_10PPB	10.27	102.7
		REC_50PPB	43.18	86.36
	AFG1	REC_10PPB	9.82	98.28
		REC_50PPB	42.20	84.40
	AFB2	REC_10PPB	9.04	90.48
		REC_50PPB	42.20	84.4
	AFB1	REC_10PPB	9.09	90.91
		REC_50PPB	41.71	83.43
White rice	AFG2	REC_10PPB	10.91	109.1
		REC_50PPB	45.84	91.69
	AFG1	REC_10PPB	9.28	92.8
		REC_50PPB	46.03	92.06
	AFB2	REC_10PPB	11.53	115.35
		REC_50PPB	50.73	101.4
	AFB1	REC_10PPB	9.88	98.86
		REC_50PPB	47.67	95.35

In the present investigation the analysis of all the parameters showed that all the parameters including sample preparation (Blank, Spike & Linearity samples), instrument (Linearity and Spiked samples), Machine and method (Blank, Sample & Linearity samples) indicated that quantitative estimation of aflatoxins in the commercially procured organic and white rice can be carried out and further used for downstream analysis. Hence in the present investigation the quantitative estimation is carried out for aflatoxins in all the prepared samples using LC-MS/MS in SRM mode through triple quadrupole system.

Rice is the second largest quantity staple internationally traded cereal and is produces in climatic areas favourable to aflatoxin production, which affects the trading. In general, the contamination level is low and varies from country to country. However, the high daily intake of rice makes even these lower levels of concern, as aflatoxin B1 is carcinogenic and has been correlated with hepatocellular carcinoma incidence in some countries. Therefore, monitoring of aflatoxins in this important staple food cannot be neglected.

The presence of aflatoxins beyond limits has a serious bearing on the safety and ultimately can reduce the quality and marketability of food products. To monitor the severity of aflatoxin toxicity, some countries are endeavoring to determine the extent of their population's exposure to aflatoxins. Hence it is very significant to quantify the aflatoxins present in rice and other food products which is of major health concern and also affects trading.

4.2 STUDY II: Quantitative Estimation of Aflatoxin in commercially procured organic and white rice using LC-MS/MS

A total of 72 samples, 36 each for organic and white rice samples were taken in the present investigation. Out of 36 samples, 16 samples were blank, 12 (6 spiked at 0.01 mg/kg and 6 spiked at 0.05 mg/kg) samples were spiked, 6 linearity samples and 2 test samples were analysed.

The persual of the quantitative analysis, Out of all the 12 spiked samples the aflatoxins were present in organic and white rice. In test sample aflatoxin was found to be detected. However, all 4 aflatoxins (Conc.) were present in White rice whereas only one aflatoxin (AFB1) was detected in organic rice. Although the aflatoxins in white and organic rice was below the set MRL as per given by SANTE guideline N0.12682/2019

<u>Conclusive Remark</u>

The one and four aflatoxin detected in Organic and white rice respectively was below the set MRL limit therefore, safe to human consumption and fit for export to national and international market.

4.2.1 Quantitative Estimation of Aflatoxins in Organic and white rice

The final concentration of the aflatoxin was calculated using the formula:

$$\text{Concentration of aflatoxin} = \frac{\text{Observed value} \times \text{dilution factor}}{\text{Weight of sample taken}}$$

The comparative analysis of presence of aflatoxins for both organic and white rice samples is given in Table no 4.10

The comparative analysis of presence of aflatoxins was carried out for 4 types of aflatoxins between organic and white rice. Of all the 4 types of aflatoxins *viz* AFG2, AFG1, AFB1 & AFB2, it was present below the MRL in White rice whereas in organic rice only aflatoxin B1 is detected in very trace amount (0.265 mg/kg) though below set MRL (MRL for AFB1= 5µg/kg and MRL for Total Aflatoxins = 10µg/kg) which inturn consider to be safe under limit. From the present investigation it is therefore can be concluded that both rice used in analysis are safe for human consumption and hence can be safely exported to destination countries wherever the set MRL by APEDA is complied for export.

Table 4.5: Quantitative analysis of aflatoxins in commercially procured organic and white rice.

Type of Sample	Sample Name	Organic rice								White Rice							
		Retention Time				Detected concentration				Retention Time				Detected concentration			
		G2	G1	B2	B1	G2	G1	B2	B1	G2	G1	B2	B1	G2	G1	B2	B1
	BLANK_1	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F
	BLANL_2	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F
	BLANK_3	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F
	BLANK_4	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F
	BLANK_5	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F
	BLANK_6	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F
	BLANK_7	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F
Blank sample	BLANK_8	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F
	BLANK_9	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F
	BLANK_10	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F
	BLANK_11	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F
	BLANK_12	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F
	BLANK_13	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F
	BLANK_14	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F
	MM_5PPB	8.5	8.73	9.01	9.22	5.16	5.09	5.07	5.03	8.49	8.71	8.99	9.19	5.31	5.26	5.05	5.15
	MM_10PPB	8.5	8.73	9.01	9.22	9.77	9.77	9.88	9.85	8.49	8.71	8.99	9.19	8.54	8.75	9.70	9.16
	MM_25PPB	8.5	8.73	9.01	9.22	24.96	25.18	25.03	25.30	8.48	8.7	8.99	9.19	25.86	26.16	25.82	26.37
Linearity Sample	MM_50PPB	8.5	8.73	9.01	9.22	48.89	49.64	49.66	49.83	8.48	8.71	8.99	9.19	52.19	50.11	50.33	53.07
	MM_75PPB	8.5	8.73	9.01	9.22	75.67	75.81	75.40	75.22	8.48	8.71	8.99	9.19	74.88	76.91	73.07	70.74
	MM_100PPB	8.5	8.73	9.01	9.22	100.5	99.52	99.97	99.77	8.48	8.7	8.99	9.19	100.82	113.93	101.27	99.59

Table 4.6: Quantitative analysis of Recovery and test samples analysed for commercially procured organic and white rice.

Type of Sample	Sample Name	Organic rice								White Rice							
		Retention Time				Detected concentration				Retention Time				Detected concentration			
		G2	G1	B2	B1	G2	G1	B2	B1	G2	G1	B2	B1	G2	G1	B2	B1
Recovery Sample	REC_10PPB_1	8.5	8.73	9.01	9.22	1.69	1.65	1.52	1.53	9.27	9.55	9.91	10.17	1.82	1.57	1.76	1.62
	REC_10PPB_2	8.51	8.73	9.01	9.22	1.71	1.64	1.50	1.51	9.26	9.55	9.91	10.16	1.93	1.59	1.95	1.66
	REC_10PPB_3	8.51	8.73	9.02	9.22	1.69	1.63	1.48	1.52	9.25	9.53	9.9	10.15	1.78	1.53	1.95	1.64
	REC_10PPB_4	8.5	8.73	9.02	9.2	1.72	1.62	1.50	1.47	9.24	9.52	9.89	10.14	1.86	1.57	1.98	1.68
	REC_10PPB_5	8.51	8.73	9.02	9.22	1.73	1.62	1.52	1.55	9.22	9.51	9.87	10.12	1.74	1.50	1.95	1.61
	REC_10PPB_6	8.51	8.73	9.02	9.22	1.74	1.66	1.54	1.52	9.21	9.49	9.86	10.11	1.78	1.52	1.95	1.69
	REC_50PPB_1	8.51	8.74	9.03	9.23	6.85	6.72	6.87	6.72	8.93	9.24	9.62	9.89	7.73	7.75	8.37	7.90
	REC_50PPB_2	8.51	8.74	9.03	9.23	6.97	6.82	6.69	6.74	8.91	9.21	9.6	9.87	7.49	7.71	8.43	7.89
	REC_50PPB_3	8.51	8.74	9.02	9.23	6.93	6.81	6.76	6.70	8.87	9.18	9.57	9.85	7.58	7.62	8.62	8.07
	REC_50PPB_4	8.51	8.74	9.02	9.23	6.96	6.82	6.70	6.67	8.85	9.16	9.55	9.83	7.54	7.56	8.34	7.88
	REC_50PPB_5	8.51	8.74	9.03	9.23	7.47	7.33	7.50	7.29	8.82	9.13	9.53	9.81	7.76	7.69	8.44	7.99
	REC_50PPB_6	8.52	8.74	9.02	9.23	8.01	7.70	7.69	7.60	8.79	9.1	9.5	9.78	7.74	7.72	8.54	7.94
Test Samples	TEST_1	N/F	N/F	N/F	0.26	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F
	TEST_2	N/F	N/F	N/F	0.27	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F
	TEST_3	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	8.92	8.43	8.99	9.19	0.42	0.14	0.39	0.18
	TEST_4	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	8.9	N/F	8.98	9.2	0.43	N/F	0.39	0.18

Table 4.7: Organic and White Rice Reproducibility @ 0.025 mg/kg.

		Organic Rice Reproducibility @ 0.025 mg/kg								
		R1	**R2**	**R3**	**R4**	**R5**	**R6**	**AVG**	**SD**	**%RSD**
1	**Aflatoxin B1**	20.72	20.68	18.2	20.69	20.77	21.21	20.378	1.086	5.328
2	**Aflatoxin B2**	20.72	20.66	18	21.41	20.35	21	20.357	1.208	5.936
3	**Aflatoxin G1**	21.21	21.33	17.21	21.56	21.37	21.37	20.675	1.701	8.228
4	**Aflatoxin G2**	21.9	22.05	17.17	22.09	21.78	21.78	21.153	1.955	9.240
		White Rice Reproducibility @ 0.025 mg/kg								
1	**Aflatoxin B1**	20.835	20.217	20.651	20.821	20.482	20.293	20.550	0.263	1.281
2	**Aflatoxin B2**	25.837	25.642	25.712	26.032	25.885	25.405	25.752	0.218	0.846
3	**Aflatoxin G1**	26.278	25.782	25.835	26.211	25.87	25.676	25.942	0.244	0.941
4	**Aflatoxin G2**	27.137	26.924	26.147	26.588	26.345	26.485	26.485	0.506	1.910

Table 4.8: Comparative analysis of aflatoxins in organic and white rice

Aflatoxins	**Rice Sample**	**Observed value**	**Actual Concentration in mg/kg**
Aflatoxin B1	Organic rice	Not found	0
	White rice	0.183	0.22
Aflatoxin B2	Organic rice	Not found	0
	White rice	0.4	0.48
Aflatoxin G1	Organic rice	0.265	0.31
	White rice	0.143	0.171
Aflatoxin G2	Organic rice	Not found	0
	White rice	0.41	0.492

4.3 STUDY III: Insilico designing/construction of novel alleles of selected genes of rice quality and aroma using gene editing tools.

Gene editing- Which directly changes the "letters" of the genome is becoming more of a feature of gene therapy. This ground-breaking technology has the ability to alter a gene in a living cell offers many potential benefits, including treating inherited diseases, understanding what specific genes do, generating more resilient crops and even detecting species in the environment. CRISPR/Cas9 technology is very important because it allows scientists to rewrite the genetic code in almost any organism. It is simpler, cheaper, and more precise than previous gene editing techniques. Moreover it has a range of real world applications, including curing genetic disease and creating drought-resistant crops.

A total of 11 genes of rice quality and aroma are selected for creating its novel alleles The CHOPCHOP (https://chopchop.rc.fas.harvard.Edu) was employed for the investigation of gRNA designing. The Locus ID's of 11 genes of chosen quality and interest is given as input where it was explicitly used to target exon sequence, coding region, UTR region etc. CHOPCHOP examined the query sequence and showed all the conceivable 20bp sequences recognized right away after the PAM sequences (NGG), giving gRNA scores from best to most minimal.

4.3.1 IDENTIFICATION AND SELECTION OF sgRNA.

After the query has been successfully submitted CHOPCHOP analysed the input and displayed the result in a matter of seconds where the results were downloaded in the convenient format (Bed annotated file/Result table as. tsv format/GenBank annotated file/FASTA file of sequence).The sgRNA's were identified for all the 11 edited genes of rice quality and aroma. All the identified sgRNA's were tested for its efficiency using CRISPR Efficiency Prediction tool.

The sgRNA's are picked considering the scores given by CRISPR efficiency tool and also rank given by assessing the off-target effects. Selecting only one sgRNA may lead to false positive result. So it is important to select more sgRNAs and their efficiency. sgRNAs with score above 7.5 is best and out of all the gRNA's designed the 3 best sgRNA's were mentioned in the Table 4.9.

Out of all the 11 genes edited using CHOPCHOP, the number of possible sgRNA's with their efficiency range is mentioned in the graphical representation.

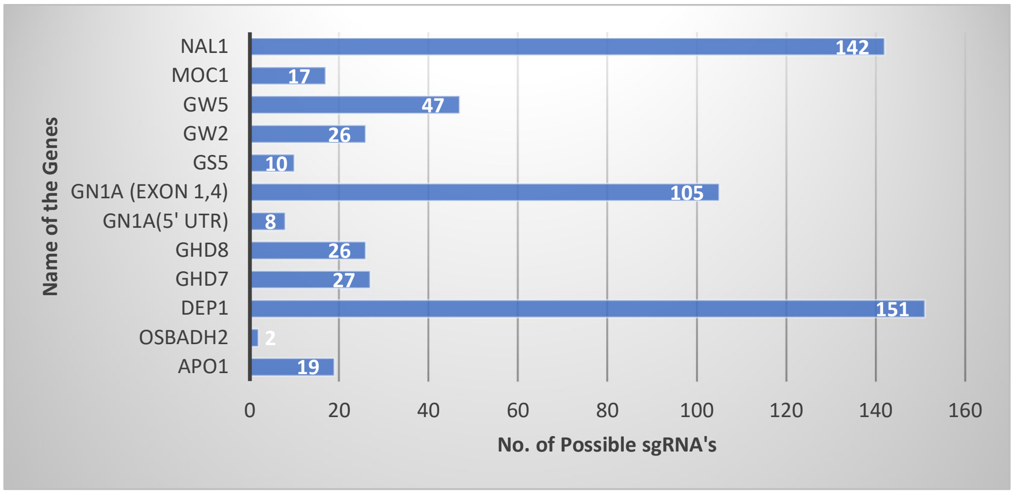
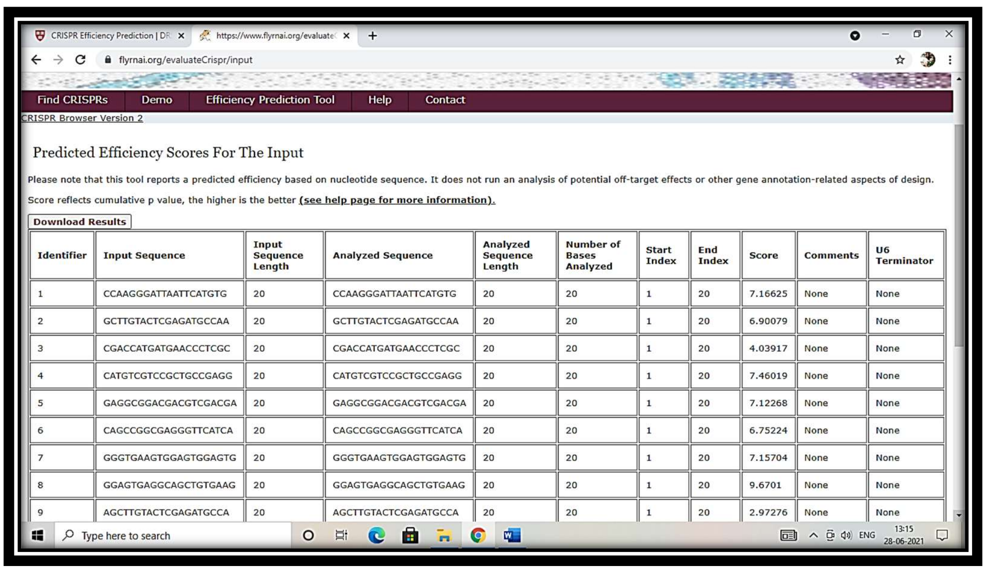


Fig 4.4: Number of Possible sgRNA's created using 11 genes of rice aroma & quality.



Identifier	Input Sequence	Input Sequence Length	Analyzed Sequence	Analyzed Sequence Length	Number of Bases Analyzed	Start Index	End Index	Score	Comments	U6 Terminator
1	CCAAGGGATTAATTCATGTG	20	CCAAGGGATTAATTCATGTG	20	20	1	20	7.16625	None	None
2	GCTTGTACTCGAGATGCCAA	20	GCTTGTACTCGAGATGCCAA	20	20	1	20	6.90079	None	None
3	CGACCATGATGAACCCTCGC	20	CGACCATGATGAACCCTCGC	20	20	1	20	4.03917	None	None
4	CATGTCGTCCGCTGCCGAGG	20	CATGTCGTCCGCTGCCGAGG	20	20	1	20	7.46019	None	None
5	GAGGCGGACGACGTCGACGA	20	GAGGCGGACGACGTCGACGA	20	20	1	20	7.12268	None	None
6	CAGCCGGCGAGGGTTCATCA	20	CAGCCGGCGAGGGTTCATCA	20	20	1	20	6.75224	None	None
7	GGGTGAAGTGGAGTGGAGTG	20	GGGTGAAGTGGAGTGGAGTG	20	20	1	20	7.15704	None	None
8	GGAGTGAGGCAGCTGTGAAG	20	GGAGTGAGGCAGCTGTGAAG	20	20	1	20	9.6701	None	None
9	AGCTTGTACTCGAGATGCCA	20	AGCTTGTACTCGAGATGCCA	20	20	1	20	2.97276	None	None

Fig 4.5: Output page showing the predicted efficiency of analysed sequence of APO1 gene.

The CRISPR Efficiency tool reported an anticipated efficiency score dependent on nucleotide sequences analysed. Scores above 7.5 show high productivity gRNAs. Remarks were added in the Results table for the presence of ambiguous bases or unlawful characters present in the input provided. The Results table likewise showed if the U6 terminator sequence (TTTT) is available.

Table 4.9: Top 3 Selected sgRNA with the use of genome editing web tools (CHOPCHOP – http://chopchop.cbu.uib.no and CRISPR Efficiency Prediction tool – http://www.flyrnai.org/evaluateCrispr/).

Name of the Gene		Ideal sgRNA sequence selected	Efficiency Score	GC Content	Self-Complementarity
APO1		GGAGTGAGGCAGCTGTGAAG	9.6701	60	0
		GGAGTGAGGCAGCTGTGAAG	8.1362	60	0
		CATGTCGTCCGCTGCCGAGG	7.46	70	0
OsBadh2		TATGGCTTCAGCTGCTCCTA	4.79	50	0
		TTCAGCTGCTCCTATGGTTA	3.80	45	0
DEP1		CAGGGCACGACGGTTTCGGG	7.6555	55	0
		TTGAAGCAGCTGGAGCAACG	7.60337	55	0
		AGCATGGGTTACGACACCGT	6.09234	55	0
GHD7		TGGGGGGATCTGGTTGCAAT	9.51489	55	0
		GACATTTTCCTAGTCTTGGG	8.13117	45	0
		CAGCCAGCGCAGAAGATCCT	8.0662	60	0
GHD8		ATATTCGAGCCCGTCAGCAA	10.0108	50	0
		AGTGGTGGTCATCCAGGCTG	7.82382	60	0
		GTGACATGGCTAGCTAGTGG	7.15225	55	0
Gn1a	5' UTR	GTTTGTGTGCTGACGATGGA	7.8840	50	0
		GTTCTTGCACCATTGTAGAA	7.3725	40	0
		TGTTCTTGCACCATTGTAGA	5.7515	40	0
	Exon 1,4	CGTGAAAATCCCCTGGCCAC	8.8525	60	0
		CTAGAGCTCGACGTCATCAC	7.9563	55	0
		GTTTGTGTGCTGACGATGGA	7.8840	50	0
GS5		AATTGACAGCATGAGAAGGA	8.8238	40	0
		TTTGGTGTTCTTGGAGGACT	7.9891	45	0
		GCGAAACTGATTGACATTAG	6.6400	40	0

GW2	**GGAGTGCCCCATCTGCTTCC**	**9.2736**	**65**	**0**
	GAGCAGGAGCAGGGAAGTAT	**7.9910**	**55**	**0**
	CAGCCTGTGCAGACCTGAAA	**7.7675**	**55**	**0**
GW5	**GGAGGCTTCCGGGGGCAATG**	**7.3391**	**70**	**0**
	TGGTTCCGCAACATGTGGGG	**7.3169**	**60**	**0**
	CGGTGGTTCCGCAACATGTG	**7.2951**	**60**	**0**
MOC1	**AGGTTTGAGAAGGTTTTGGG**	**8.2092**	**45**	**0**
	ACAATTGTTCCAGGCATATG	**7.4452**	**40**	**0**
	ATATGAGCTGAGGTTTGAGA	**6.6655**	**40**	**0**
	ATTGTTCCAGGCATATGGGG	**6.0955**	**50**	**0**
NAL1	**GATGGTTTTCCCGTCGCTTT**	**9.3620**	**50**	**0**
	GCGGCTCTTCACTTCTTCAG	**8.9493**	**50**	**0**
	GTGGAGTAGCATGATATTCT	**8.2264**	**40**	**0**

Even though if the sgRNAs are having highest efficiency score, it is rejected if the sequence contains TTTT, a terminator which makes the gRNA non-functional (as shown in the Table no.4.9 in case of MOC1 gene. The threshold was set where the sgRNAs below 7.5 efficiency score is rejected and zero self-complementarity is most preferred. The sgRNA having 8.20926 score is rejected because of the presence of the terminator sequence which is highlighted and the next high scored sgRNA is selected.

In the present investigation a total of 11 genes of rice aroma and quality was successfully edited to create 580 novel alleles where the highest number of possible sgRNA's were obtained for DEP1 gene (151 sgRNA's) and the least number of sgRNA's were obtained for OsBadh2 Gene. 142 sgRNA's were designed for NAL1 gene. The gene Gn1a targeting the Exon1,4 has got 105 possible sgRNA's. Out of 11 genes selected the exons were targeted for 5 genes (OsBadh2, DEP1, Gn1a, GW5, NAL1), Promoter region was targeted for 5 genes (APO1, GHD7, GHD8, GS5, MOC1) and 3' splicing site of 6th intron was targeted for one gene(GW2). These edited genes are very helpful in crop breeding programmes to enhance to quality and yield parameters of rice. Subsequent to planning and executing a Genome editing/CRISPR experiment, checking whether the gene/genome has been edited is a standard practice.

DISCUSSION

Aflatoxin issues are not certain to the developed or developing nation; these are the issues that influence the farming economies of numerous nations, meddle or indeed, even prevent exchange and influence human wellbeing. Aflatoxins are among the most powerful cancer- causing agents, naturally produced fungal secondary metabolites and cause ground-breaking health risks and intense toxicological impacts to people as well as animals. Rice contaminated with aflatoxins may harm the health to more prominent degree and if not appropriately decided; may leads to death. Around 120 countries are known to have implemented or proposed aflatoxin guidelines for food varieties intended for human consumption so as to avoid aflatoxin consumption.

Owing to the serious health issues caused by aflatoxin contamination in food, the present study was conducted to identify the aflatoxins present in rice and estimated quantitatively. Using AOAC Official method 2007.01 (G.S. Toteja et al.,2007) Commercially procured organic and white rice was analysed for the presence of aflatoxins using LC-MS/MS having Improved detection limits along with Heated Electrospray Ionization (G.S. Shephard.,2009). Acetonitrile Extraction was carried out for extraction and isolation of aflatoxins. The analysis of 4 types of samples; Blank, Spiked, Linearity and test samples were carried out and analysis of this sample showed that the method used is efficient, good recovery is obtained and the method is accurate and precise.

A total of 72 Samples, 36 each for organic and white rice was analysed for the presence of aflatoxins. Four types of aflatoxins AFB1, AFB2, AFG1 and AFG2 were found to be present in all the 36 samples of White rice whereas in organic rice, only two samples were found to be contaminated with aflatoxin G1 in a very trace quantity (0.265 mg/kg). The linearity curve obtained for all the aflatoxins showed great regression and the R2 value was found to be >0.99. The recovery and relative standard deviation were calculated which were found to be in the acceptable range (70-120% with %RSD $\pm$ 20%) (A. Gotah et.al.,2018). The aflatoxins detected were in the range below the set MRL which is safe for consumption (A. Gotah et. Al.,2018).

Apart from Aflatoxins, rice can be contaminated by many other contaminants like pesticides, herbicides etc., It is one of the major threat for the export of rice worldwide where quality of rice plays a major role in international trading. So, to enhance the quality parameters of the rice and to increase the quantity of rice production gene editing technique is used to create its novel alleles (Ashokkumar et.al.,2020) using CRISPR/Cas application (CHOPCHOP). Since its beginning, the CRISPR-Cas has become an amazing focusing on apparatus for silencing and activating both DNA and RNA in a scope of settings, each of which requires the use of explicit guidelines.

The *insilico* designing was carried out for the 11 selected genes if rice quality and aroma and designed a total of 560 novel alleles which are very helpful in crop genetic genetic improvement. The sgRNA's designed were checked for its efficiency using CRISPR Prediction tool and the best 3 sgRNA's were selected and the most targeted region was the promoter and Exons for the 11 edited genes of rice. The efficiency of the designed sgRNA is a major factor in the success of Cas9 gene editing applications, To predict the efficiency of designed sgRNA, scoring methods used (Doench et.al.,2016) were accurate. However, the predictions were not necessarily generalized to Cas9 applications in all cell types, organisms, and PAMs were not included in the efficiency scoring experimental data. Features like sgRNA self-complementarity, presence of homopolymers, and potential off-target effects which drastically affects the experimental outcomes and were often not considered by the scoring models. The sgRNA sequences that are able to form hairpins with themselves or with other regions of the RNA backbone have been shown to affect sgRNA activity and such sgRNA's with homopolymers were avoided(D. Beeber et al.,2019).

In the present investigation Commercially procured organic and white rice was used for the detection and determination of aflatoxins in rice using LC-MS/MS (Ultimate 3000 UHPLC- ThermoScientific^{TM}, Model- TSQ Endura TQH-E1-0620) with Heated Electrospray Ionization in Selected reaction monitoring (SRM)mode with a totat runtime of 15 minutes. From the analysis, it is found that the organic rice is very safe for consumption as the aflatoxins B1, B2,G2 were not found and G1 was found in very less amount(0.265 PPB) which is in acceptable range where as in white rice aflatoxins G1, G2, B1, B2 were found (Conc. – G2 – 0.492 mg/kg, G1 – 0.171 mg/kg, B1- 0.22 mg/kg, B2- 0.48 mg/kg,). In Organic rice, the linearity value (1.000) obtained by following the Extraction by Acetonitrile and Partitioning with Magnesium Sulfate shows that the Sample preparation was carefully done. The concentrations of detected aflatoxins in organic and white rice were below the set MRL and concluded that both the rice are safe for use by the consumers.

In the present study of creating novel alleles for the 11 selected genes of rice quality and aroma, the genes were studied for their functions and their correlation (+ve or -ve) with the growth and yield parameters of rice plant and classified accordingly. CRISPR/Cas9 applications (gene knock-out, knock-in, activation/repression) were used to edit the selected genes based on the type of function they perform (loss/gain of function) using the most widely used insilico tool : CHOPCHOP (http://chopchop.rc.fas.harvard.edu/) having highly exploitable advanced options. The parameters were optimized as per the requirement. CHOPCHOP uses Bowtie software for sequence alignment and analysis and listed the candidate guide RNA sequences as per the Scoring rule given by Doench et al.,2016. The sgRNAs were ranked taking into account the cleavage efficiency and cutting specificity and many other factors like GC content, mismatches in the Off-targets etc,. Best quality sgRNA is the one with the Rank 1, but to avoid the false positive results of the online tools some more gRNAs were selected and tested for its Efficiency in insilico using the CRISPR Efficiency Prediction tool for selecting the Ideal sgRNA.

Conclusion as per objectives:

Objective I and II –

- Because of improper stockpiling conditions, rice can be a great substrate for mycotoxin producing organisms and a large portion of the aflatoxigenic A. flavus strain isolated from rice showed a high capability with respect to aflatoxin production.
- LC-MS/MS is progressively utilized for the concurrent assurance and recognizable proof of huge quantities of mycotoxins.
- AOAC Official Method 2007.01 proved to be the best and widely used method for the detection and determination of Aflatoxins in rice where the sample preparation was carried out by Acetonitrile Extraction and Partitioning with Magnesium Sulfate.
- Recovery and repeatability (%RSD) which determines precision and accuracy are in acceptable range (70-120% with %RSD $\pm$20%)
- Subsequently the calibration/linearity curve obtained were linear and showed great regression. The linearity value (1.000 – extreme case) obtained from organic rice sample indicates that the steps involved in sample preparation is correct and carefully prepared.
- The Values obtained in both organic and white rice samples are beneath the MRLs set by various significant nations which is safe for consumption, and accordingly the technique could be utilized to screen the contamination of aflatoxins in this significant food staple.

Objective III –

- CRISPR/Cas9 technology mainly subject to the requirement of PAM sequence present downstream the target site.
- CHOPCHOP has proven to be the best insilico gRNA design tool because of its quite and uncomplicated operating procedure with the privilege of having advanced options for genome editing and giving outputs within a matter of seconds.

- Off-target effects can be successfully solved by forseeing CRISPR Cutting Specificity and constructing ideal gRNA. Furthermore, picking Cas variants is likewise important to decrease Off-target effects.
- On-target Duoble stranded break (DSB) Specificity is increased by double nickase approach to increase the overall number of bases that are specifically recognized in the target DNA.
- Genes of rice quality and aroma are successfully edited for creating its novel alleles using the CHOPCHOP and the sgRNA's efficiency is further checked by CRISPR Efficiency Prediction tool.

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RESUME

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B.Sc. (Ag)	GKVK(Gandhi Krishi Vigyan Kendra), Bangalore	University of Agricultural Sciences, Bangalore	8.63 /10	2015-19
Class XII	JSS PU College for Women, Chamarajanagara	Department of Pre- University Education	93.83	2013-15
Class X	St. Joseph's English High School, Chamarajanagara	Karnataka Secondary Education Examination Board	92.16	2013-14
Training				
Indira Gandhi Krishi Vishwavidyalaya	3 days training programme on Production of Bio-agents in Capacity building Programme under SC-SP ICAR EFC Scheme			2020
	2 days training programme on Commercial floriculture and dry flower Technology organized by IGKV, Raipur.			2020
Scholarships				
DBT Fellowship by The Department of Biotechnology (DBT) of the Ministry of Science and Technology				2019-21
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M.Sc. (Ag.)	Comparative detection and determination of Aflatoxins in Rice using LC-MS/MS			2020-21

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1 message

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