Western Australia School of Mines:
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Kafirin purification process development from *Sorghum bicolor*, Scale-up and application development

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Declaration

To the best of my knowledge and belief, this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature:

Date: 19/06/2020

Dedicated to

My Gurus

& Ashka

Abstract

Kafirin is a prolamin present in *Sorghum bicolor* and represents 50-60% of its total protein content. High hydrophobicity and plastifiable nature of this protein make it a unique candidate for application development in various fields ranging from food processing, pharmaceutical, and nutraceuticals to high-end applications such as scaffolding in tissue engineering.

This research focuses on the development and optimization of industrially viable batch and continuous processes from sorghum flour and DDGS as raw materials using different unit operations. QbD based approach was implemented for scalable and industrially viable process development. These operations were scaled up and validated to match the performance in terms of yield and quality of the kafirin produced. Column chromatography-based process was developed, scaled up and validated to produce high-quality kafirin. Process intensification approach using ultrasonic cavitation was implemented to increase productivity. Kafirin purified using different strategies was analysed to establish comparability.

Purified kafirin was then used for application development. Initially, plasticizers from different chemical families were screened which created a platform for targeted application development. Kafirin films with different plasticizers demonstrated a range of functional, mechanical and barrier properties ideal for unique applications such as films and coatings. Kafirin was formulated to develop and enteric coating for pharmaceutical applications. A similar coat was developed with food-grade materials that demonstrated increased shelf life of coated fruits. An integrated process was developed to manufacture kafirin microparticles and optimised to produce particles within the desired size distribution. Finally, these model applications were assessed for their suitability using an array of analytical tests.

Current research has produced the large scale kafirin purification process and identified the potential of kafirin as a natural and biodegradable replacement for synthetic polymers.

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Words do not always do justice to the feeling of gratitude they convey, especially when you want to say how grateful you are to someone, however, let's give it a try…

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List of abbreviations

2ME or BME	2 or beta-mercaptoethanol
ANOVA	Analysis of variance
CPP	Critical process parameter
CQA	Critical quality attributes
CV	column volume
DDGS	Distiller's dried grains with solubles
DOE	Design of experiments
DTT	Dithiothreitol
EAB	Elongation at break
FMEA	Failure mode effect analysis
GI	Gastrointestinal
HCl	Hydrochloric acid
HETP	height equivalent to a theoretical plate
HIC	Hydrophobic interaction chromatography
HMW	High molecular weight
ICH	International Conference on Harmonization
IPA	Isopropyl alcohol
K_d	Dissociation constant
LMW	Low molecular weight
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NR	Non-reduced/non-reducing
OA	Oleic acid
OFAT	One factor at a time
o-PA	Ortho phosphoric acid
OR	Operating range
PAR	proven acceptable range
PAT	process analytical technology
PCM	Paracetamol
PEG	Polyethylene glycol
pI	Isoelectric
PPG	Propylene glycol
q. s. to	Quantity sufficient to
QbD	Quality by design

QTPP	Quality Target Product Profile
R	Reduced/reducing
RH	Relative humidity
RPC	Reverse phase chromatography
RPN	Risk probability number
RT	Room temperature
S/L ratio	Solid to liquid ratio
SDS-PAGE	Sodium dodecyl sulphate Polyacrylamide gel electrophoresis
SEM	Scanning electron microscope
SMB	Sodium metabisulphite
TEA	Triethanolamine
TS	Tensile strength
WVP	water vapour permeability

List of symbols

α	alpha
β	beta
δ	delta
γ	gamma

List of units

°C	Degree centigrade
g	Gram
kDa	Kilo Dalton
L	Litre
mPa	Mega pascal
Mt	Megatonnes
µg	Microgram
µL	Microlitre
µm	Micrometer
mL	Millilitres
M	Molar
N	Normal
ppm	parts per million
%	Percentage
psi	pound-force per square inch
mbar	Pressure in millibar
RPM	Revolutions per minute
h	Time in hour
min	Time in minutes
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight

Chapter 1: Introduction

1.1. Introduction

Petroleum-based polymers such as polystyrene, polyethylene and polypropylene are non-compostable and low-density polymers occupy more space when disposed of in the landfills and are associated with environmental pollution. According to a report, more than 12% non-biodegradable plastic waste dumped in landfills that constitute 29% of total global waste disposal (1). These non-biodegradable and non-compostable materials have serious implications on environment and health, which lead to rising demand for environment-friendly products, promoting the development of compostable and biodegradable materials made of biopolymers.

Development of biodegradable biomaterial to replace petroleum-based non-biodegradable polymers is receiving increased attention since last few decades. As a result, researchers around the world exploring biomaterials as a viable alternative that offers durability, adaptability and economical equivalency to petroleum-based polymers. Nature-friendly biomaterials such as starch, cellulose, lipids and proteins investigated as a replacement for non-biodegradable polymers to address the issue of biodegradability. These biopolymers are carbon neutral, sustainable, renewable as they are derived from natural sources such as plants.

Zein, a maize protein has been extensively explored and established as a biomaterial for various applications that offer environmentally friendly traits such as degradability, compostability and edibility. Zein based edible films were tested for coating fruits and meat to reduce water loss, keep them fresh and increase their shelf life (2). Similarly, research for zein based non-food applications such as drug delivery, encapsulation and tissue engineering scaffolding were investigated and under consideration (3–8). Kafirin is a sorghum prolamin, homologous to zein in terms of structure, solubility, amino acid composition and molecular weight (9). Kafirin is more highly cross-linked, less digestible and hydrophobic than zein along with excellent gas, vapour and moisture barrier properties (10–12), hence under investigation since last few decades. Kafirin is being explored as a potential biomaterial for food, pharmaceutical and biomedical applications (13–15) and needs further

investigation in order to improve the mechanical and functional properties required for the targeted applications.

1.2. Motivation

Majority of the sorghum produced in developed countries is used as animal feed or raw material for ethanol production in biorefineries and distilleries. These distilleries produce distiller's dried grains with solubles (DDGS) as waste, a protein-rich residue, which is exported to countries like Japan, China and South America. Kafirin can be extracted from readily available sorghum grain/flour and DDGS. Kafirin extraction from sorghum and DDGS reported in the literature are at laboratory scale and produce a small amount of kafirin sufficient for research purpose. Application development and manufacturing requires a sustainable and economical process development at a larger scale that can accommodate both sorghum and DDGS as raw material. Kafirin produced at large scale can be used to develop applications and materialise them at production scale.

As a part of application development, kafirin needs to be investigated for its suitability with different additives to materialise various food and non-food applications. Plasticisers from various chemical families can be screened that impart and/or enhance the required mechanical and functional properties of kafirin-based applications. The matrix developed from screening can be used as a platform to design the target applications with specific attributes. Films, coatings and microparticles can be developed from this platform to demonstrate their attributes as food and non-food applications.

1.3. Research aim

The project is aimed at cost-effective and environment-friendly kafirin purification process development. The process developed for the kafirin purification needs to be optimised to accommodate both sorghum and DDGS as raw material followed by scale-up to meet the requirement for the intended purpose such as application development at a larger scale. The kafirin produced from the developed process needs to be tested for its suitability for various applications by varying their physicochemical, mechanical and functional properties by pairing with plasticizers from various chemical families. The design

space established from plasticizer screening provided the platform for application development.

1.4. Objectives

Objectives for the research are summarised and arranged considering the requirements of the project:

1) Development and optimisation of kafirin purification process from sorghum grain/flour. DDGS need to be tested as an economically feasible raw material for kafirin extraction and process optimization.
2) Scale-up of the individual unit operation from optimised process to achieve equivalent purity and yield. Validation of the scaled-up process.
3) Screening of plasticizers from various chemical families to test their suitability and properties imparted on freestanding films. To develop a matrix using these plasticizers for application development from the purified kafirin targeted towards industrial, food, nutraceutical and pharmaceutical applications.
4) Kafirin based spray development as an enteric coat for controlled and targeted drug release. Detailed analysis and i*n-vitro* study to demonstrate suitability and efficacy as an enteric coat.

1.5. Thesis outline

This thesis organised into 8 chapters that cover the research work details. Each chapter consists of a brief introduction related to background and research gaps followed by materials and methods used for the study and conclusion derived based on the discussion over the results obtained from the study. Thesis outline (*Figure 1*) and a brief description of the chapters and the work carried out is summarised as follows:

- Chapter 1 provides a general overview, base of research problems and inspiration followed by aims and objectives identified to carry out presented work.
- Chapter 2 reviews basic information for sorghum with reference to its global production scenario and its uses along with biochemistry of kafirin that helps design strategies to separate it from impurities such as starch,

oil and other proteins. A comprehensive literature review on extraction and purification strategies used for kafirin purification and scale-up. Quality by design (QbD) based process development/ optimization tactic was discussed, an approach that works on predefined objectives with emphasis over the product (kafirin), process understanding, and process control based on quality risk management.

It also provides an overview of applications developed using kafirin and the methods used to determine the mechanical and functional properties of these applications. Each section concluded with the summary of research gaps for existing extraction processes and applications developed thereby establishing the niche for this research.

- Chapter 3 presents preliminary extraction process development, optimization of individual steps/unit operations using QbD based approach to maximise kafirin yield and purity followed by scale-up and validation.
- Chapter 4 focuses on column chromatography-based process development, scale-up and validation.
- Chapter 5 presents the extraction process intensification using ultrasonic cavitation, its development and optimization.
- Chapter 6 focuses on the screening of plasticizer from various chemical families for kafirin-based applications and their mechanical and functional properties.
- Chapter 7 discusses the development of kafirin-based coat; investigate its mechanical, functional, barrier properties and *in-vitro* study for suitability as an enteric coat.
- Chapter 8 concludes the research work by summarising the major findings, important observations with recommendations towards prospective future research followed by the significance of outcomes and benefits.

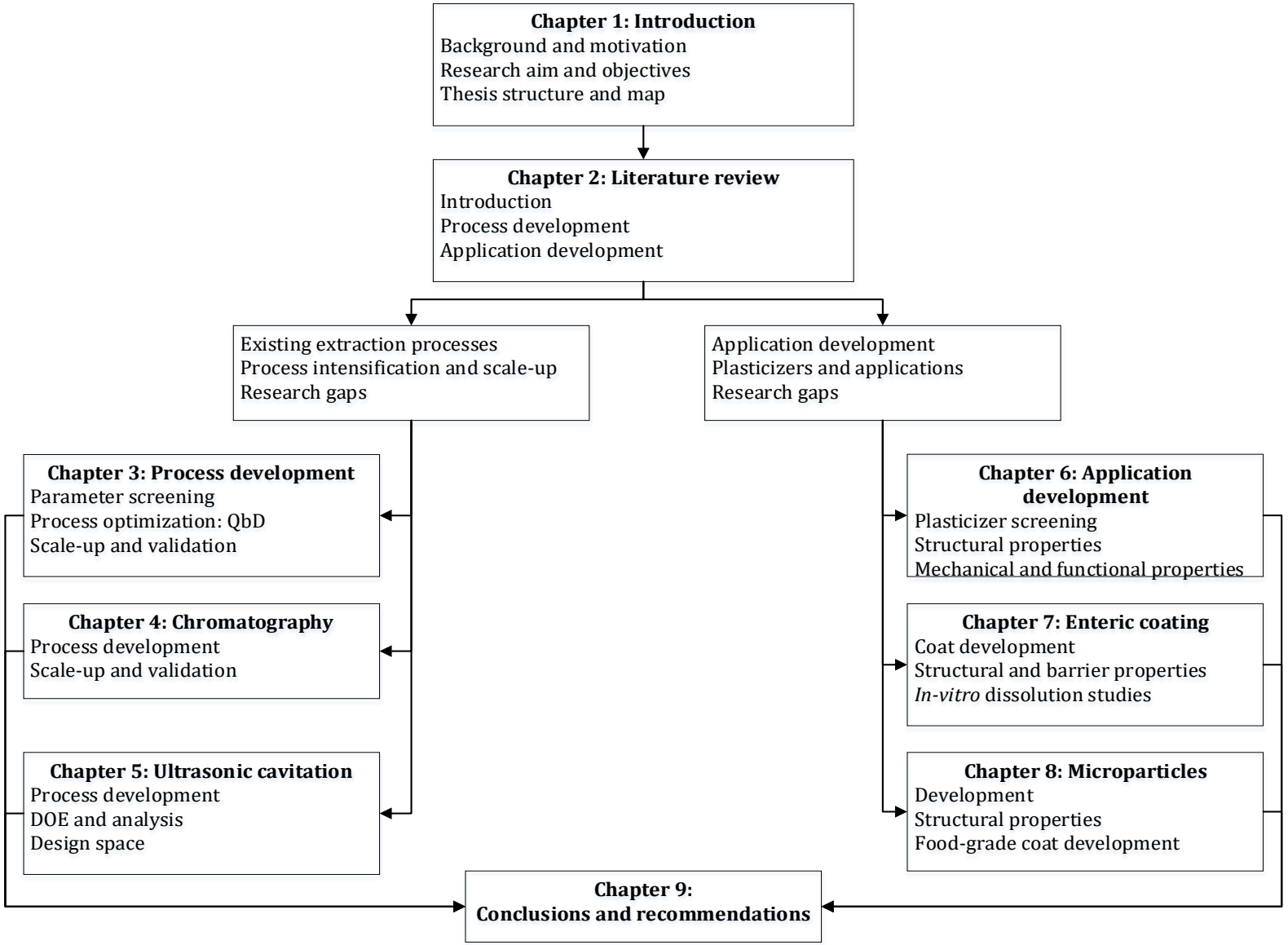

Figure 1: Thesis map/outline.

Chapter 2: Literature review

2.1. Introduction

This chapter describes sorghum (*Sorghum bicolor* (L.) Moench) with reference to taxonomy, agronomy, uses and biochemistry of sorghum grain, especially the main grain storage protein, a prolamin called kafirin. It enlists physicochemical properties of kafirin that help design and optimise efficient extraction process and application development. This chapter reviews reported history of kafirin extraction processes from different raw materials using various unit operations, their scale-up and applications developed from the purified Kafirin. The purpose of this chapter is to provide baseline information about sorghum, production (global scenario) and kafirin, classification, properties, reported extraction processes and applications developed in an effort to identify research gaps that need to be addressed to bridge the basic and applied research.

2.2. Sorghum taxonomy and properties

Sorghum or *Sorghum bicolor* (L.) Moench belongs to *Poaceae* (grass) family like other true cereal grains including wheat, oats, rice, corn (maize), barley, rye, and millet. (16,17) Sorghum has certain advantages over other crops such as resistance to fungi and mycotoxins, very high temperature and drought tolerance and can survive in relatively harsh climates. Sorghum can survive in areas receiving rainfall less than 350 mm whereas maize/corn requires up to 700 mm. This makes sorghum more economical to produce (18) hence production can be focused in the area where arid climatic conditions are prevailing, these qualities along with agronomic traits give sorghum an edge over other crops. Sorghum is an annual crop; however, some sorghum cultivars can grow throughout the year.

2.3. Production and uses

According to a recent report from the Food and Agriculture Organization of the United Nations (19), sorghum is the fifth most-produced cereal crop in the world, third in Australia after wheat and barley and the third-largest cereal grain in the United States. Global production of sorghum in 2018 estimated at approximately 60 Mt with the African continent being the lead sorghum producer in the world contributing almost 50% (*Figure 2*). The same report (19) also states that Australia and India produced ~1 and 4.57 Mt of sorghum respectively, most of it

used as fodder and raw material for ethanol refineries in the former and as a food in latter. It is a staple food for more than 500 million people in most African and Asian countries as reported by ICRISAT (2015).

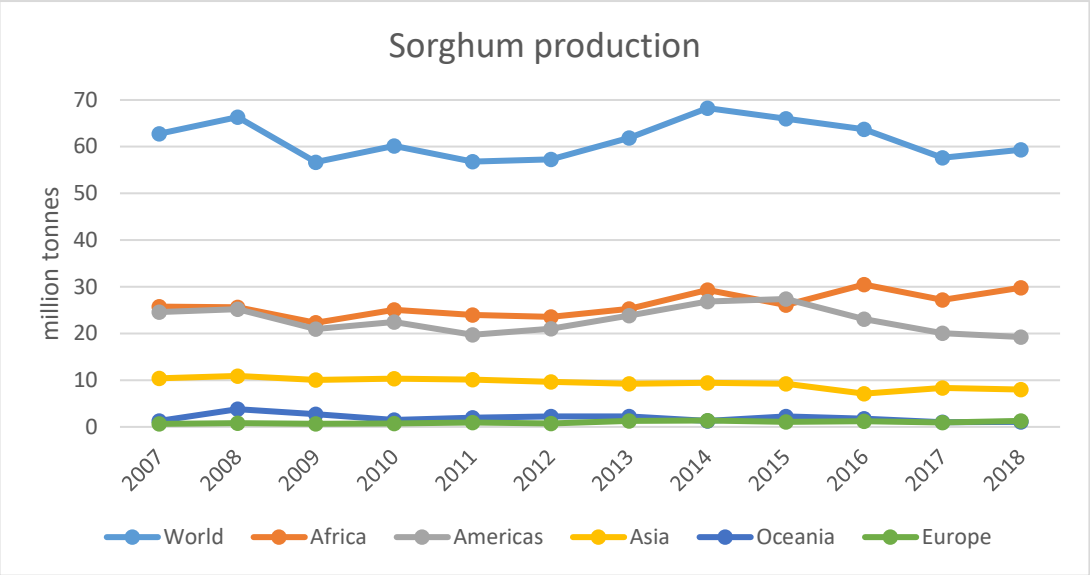

Figure 2: Sorghum production: a global scenario.

Source: FAOSTAT-2018 (19).

The Department of Agriculture and Fisheries, Queensland Government annual report suggests that almost all of the 1.4 Mt of sorghum produced was used exclusively as fodder for livestock and none for human consumption (20). Also, a substantial amount of sorghum produced is either exported to Japan, China and South America channelled towards the pet food industry (20). Most of the sorghum produced in Australia and the USA is used as fodder for livestock raised for meat and for the dairy industry, which indirectly contributes to human nutrition (10). Sorghum also used as a raw material in distilleries for ethanol production. According to Biofuels Association of Australia (2012), Dalby Bio-refinery, one of the three ethanol production plants in Queensland produced 80 million litres of fuel-grade ethanol per annum using 0.2 Mt of sorghum grain procured from local farmers. These distilleries produce distiller's dried grains with solubles (DDGS) as their main protein-rich byproduct at the end of the ethanol fermentation process, which then used as a feed for cattle or exported to countries like Japan and Mexico. Uses of sorghum including various food preparations and beer brewing which are preferable for coeliac and obese people have been thoroughly reviewed (21). Moreover, sorghum has been reviewed and

discussed by researchers in detail as a potential candidate for improvement of human health and in the prevention of health impairing conditions such as obesity, diabetes and cardiovascular disease owing to its slowly digestible starch, fibre, unsaturated fatty acids content along with high antioxidant activity (11,22–24).

2.4. Biochemistry of sorghum grain

Sorghum grain consists of three main parts namely pericarp (outer protective layer), endosperm (nutrition-rich) and germ (embryo). Chemical composition of these layers varies according to their role. Pericarp or bran consists of fibre, antioxidants, minerals, vitamins and other phytonutrients that help prevent the diseases and protect the germ and endosperm from external factors. The endosperm is the carbohydrate and protein-rich layer with minor quantities of fibre that surround germ and provides protection and required nutrition for germination (25).

2.5. Sorghum proteins: Prolamins and non-prolamins

The term prolamin was coined by Osborne which indicates high contents of proline and glutamine residues in this group of proteins (26). These two amino acids collectively contribute to more than 30% of the total amino acid residues. Johns and Brewster investigated prolamins from sorghum (dwarf kafir variety) and name it kafirin (27). The total protein content of sorghum grain may vary from 7% to 16% and classically is divided based on their solubility in different solvents; albumins (water), globulins (salt), kafirins (aqueous alcohol), cross-linked kafirins (aqueous alcohol with reducing agent), cross-linked glutelins (detergent and reducing agent at alkaline pH) and non-extracted protein residues (28,29). As per a recent and simplified approach, they can be classified into two classes, prolamins and non-prolamins, prolamins, kafirin in this case, represent 70–80% of total protein content (30–32). Where kafirin is a homogeneous storage protein (prolamin) and other non-prolamins (albumins, globulins and glutelins) are involved in cellular functions (32,33). Kafirin, the major prolamin protein from sorghum is thought to be the most hydrophobic of the cereal prolamins (34).

As summarised in *Table 1*, kafirins are classified into four groups based on differences in their solubility, amino acid content, molecular weight and reactivity with anti-zein sera; α, β, γ and δ-kafirins (35–37) equivalent to α, β, γ and δ-zeins from maize (36,37). There are some inconsistency within reported molecular weight/ranges and number of genes for different types of kafirin (35–39). Formation of oligomers and polymers because of polymerization where monomers act as chain extenders or chain terminators. For instance, oligomers and polymers can be formed from α and β kafirin monomers that are supported and stabilised by inter-chain and intra-chain disulphide bonds. Similarly, cysteine residues in β kafirins facilitate the formation of inter-chain disulphide linkages forming oligomers and polymers (35) leading to the difference in solubility of kafirin with or without reducing agents (38). Polymers formed by kafirin monomers separated based on their molecular weights by electrophoresis. SDS-PAGE profile of kafirin (Figure 3) suggest that kafirin under non-reducing conditions display monomers along with dimers, trimers and polymers where high molecular weight polymers fail to enter the gel. The same extract, in presence of reducing agent, reduced the dimers, trimer and polymers to monomers (α, β and γ-kafirins).

Table 1: Kafirin classification based on molecular weight and solubility.
(Adapted and modified from Belton et al (2006), da Silva (2012) and Cremer et al (2014)) (35,39,40)

Kafirin Type	**Reported Molecular weight/ranges (kDa)**	**No. of Amino Acid Residues**	**Amino Acid Composition**	**Polymerisation Behaviour**	**No. of genes**
α (α_1 and α_2)	26 – 27[21] 19 – 27[24] 22 – 26[25]	240 – 250[21]	Rich in non-polar amino acids, no Lys, one Trp, 10 blocks of repeated amino acids	Monomers, oligomers and polymers α_1: Chain extender α_2: Chain terminator	~ 20[21] 23[25]
β	18.745[21] 15 – 18[24] 18[25]	172[21]	Rich in Met and Cys, two Trp	Monomers (intra-chain disulphide bonds) Oligomers and polymers (intra-chain and inter-chain disulphide bonds) Chain extender	1[21]
γ	20.278[21] 16 – 28[24] 28[25]	193[21]	Rich in Pro, Cys, His. No Lys, Asn, Asp, Trp. Four repeats (consensus PPPVHL)	Oligomers and polymers (inter-chain disulphide bonds) Chain extender	1/2[21]
δ	12.961[21] 13[25]	114[21]	Rich in Met, no Lys, 1 Trp	Unknown	Unknown

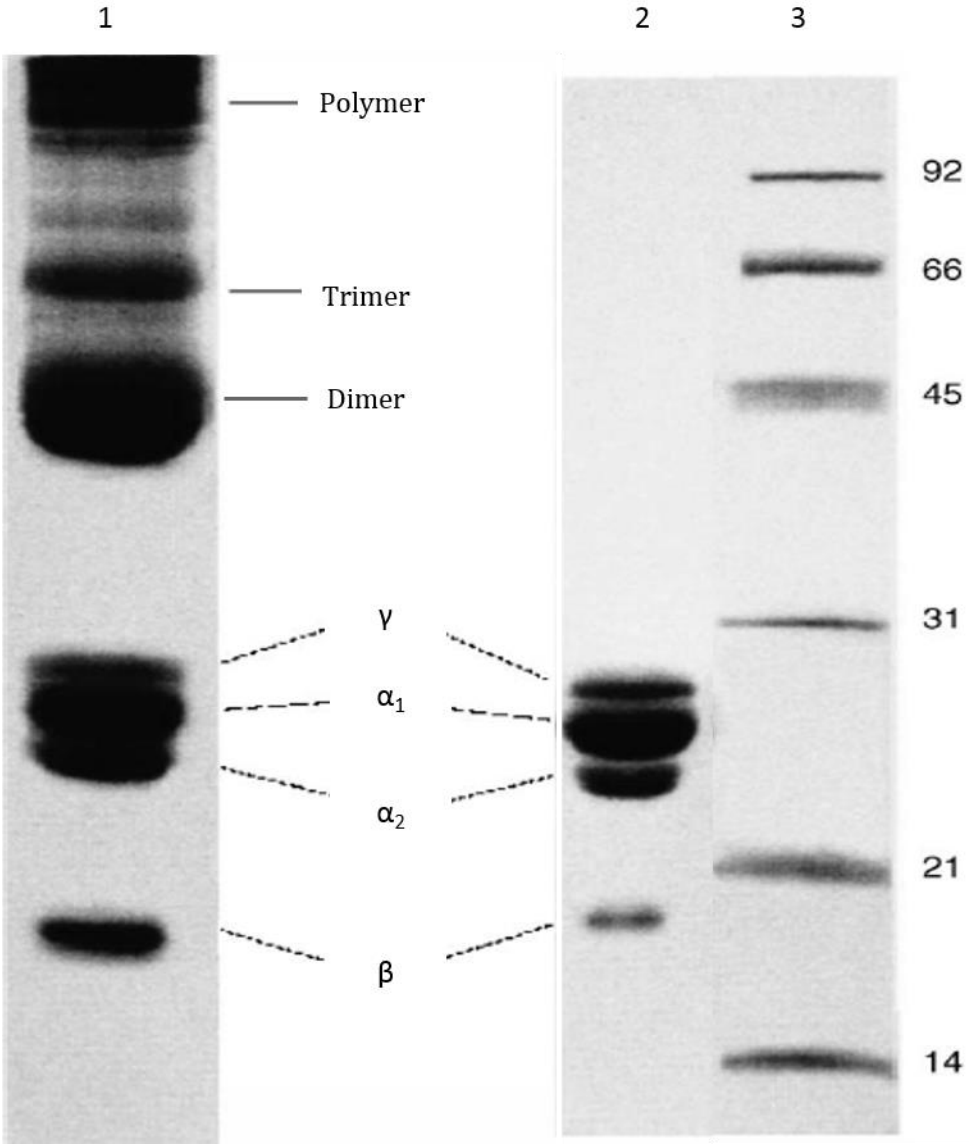

Figure 3: SDS-PAGE profile of sorghum kafirins.
Lane 1: kafirin extract (NR), lane 2: kafirin extract (R), lane 3: molecular weight marker for reference. Image adapted from El Nour et al. (1998) (38).

2.6. Extraction process development

2.6.1. Sources of kafirin

Purification of kafirin reported mainly from the sorghum seeds and protein content of different parts of the seeds was also evaluated (30,31,41). Seeds, natural storage structures anticipated to accumulate proteins in plants as they have a higher amount of "total stored protein (%)" as compared to leaves or any other part (31), however, different parts of grain harbour various challenges in terms of handling, extraction and purification of proteins (26). It was also

demonstrated that the age of the grain also affects the proportion of the type of kafirin; α-kafirins accumulating early followed by β and γ-kafirins and cross-linked to uncross-linked ratio increases with the maturation of the seeds (42). Kafirin purified from bran is of lower purity has lipids and polyphenols as impurities as compared to kafirin extracted from the endosperm (41) which indicates selection of source for kafirin extraction is essential. For instance, distilleries around the world produce DDGS as the byproduct of ethanol fermentation, which can be an ideal and potent source of kafirin as most of the starch has been converted to ethanol leaving a protein-rich residue. According to census obtained from U.S. grains council, ethanol plants in the U.S. produces more than 14 billion gallons of ethanol along with 39 million tonnes of nutrient-rich (energy and protein) DDGS as a byproduct (QAAFI annual report 2015). Similarly, as mentioned earlier, Dalby bio-refinery in Australia exclusively uses locally produced sorghum for ethanol production with 80 million litres of fuel-grade ethanol per annum capacity. However, there are few reported cases of kafirin extraction from DDGS which renders DDGS to be explored as a potent source for kafirin extraction (43).

2.6.2. Kafirin extraction

Kafirin extraction from sorghum grains/ flour and/or DDGS has been a difficult task due to the lack of solubility of kafirin in aqueous buffers. Sorghum proteins were originally separated using Osborne procedure (26) used for zein, which resulted in a mixture of different proteins in separated fractions, however, recovery was 3 times greater for zein (44). Variants of a similar process were also used with a reducing agent (29) and aqueous alcohol. Wet milling of grain sorghum performed for isolation of protein with varying steeping time (45) along with short steeping time (4 hours) attempted (46) for starch recovery where they reported 82.4 % overall protein (kafirin and other) recovery. Petroleum ether used to extract fat/oil from different process streams. In a separate study, the wet milling process without steeping was assessed (47) which result in high starch recovery and separate stream result in a protein-rich fraction. However, these streams were not assessed in terms of protein purity and need to be processed further to extract kafirin. Since these methods were designed for starch

extraction and have certain limitation such as low recovery and high wax content in purified protein fraction. Prolamins and non-prolamins were separated on two separate instances, the initial study reported low recovery (48), later, the process adapted from Wallace et al (49) resulted in higher yield compared to Landry-Moureaux method (29). However, the type of solvents, detergent and reducing agents used for purification render the process limited to non-food based applications (32). Later, Emmambux-Taylor (50) reported kafirin extraction using Carter and Reck process (51) for zein extraction where 46.1% yield was reported from whole-grain flour. In a similar study, bran was reported as a potential source of kafirin with extraction yield of 26.7% and 57.9% from bran and extracted flour respectively (41). Most of the processes discussed here reported lower recovery; these processes need optimization and replacing certain unit operations for cost-effective scale-up. Kafirin extraction using different combinations of acid, alkali and alcohol were evaluated (43), where, acetic acid extraction process from whole grain flour offered better purity, nonetheless, process time and recovery were shortcomings of the process (15). Protein solubility in the given solvent is the result of hydrophobic interactions and electrostatic repulsions between the protein molecules. Solubility in a given solution increases due to acquired charge because of change in pH leading to decreased hydrophobic interaction and increased electrostatic interactions (52,53). Protein-solvent interactions increases at higher or lower pH values than the pI as protein acquires a net positive or negative charge, which increases with changes in pH moved away from pI. Sorghum proteins were isolated at higher pH which resulted in low purity protein mixtures (54), which explains co-extraction of other protein and non-protein impurities. Similarly, kafirin was isolated at different alcohol concentration and pH, and with/without salt, with the aid of ultrasonication, however, it resulted in low protein recovery (55). Kafirin extraction processes discussed earlier and others reported in the literature are summarised in Table 2.

Distiller's dried grains with solubles (DDGS) is an alternative to sorghum grain/flour as a source of kafirin. DDGS High protein (especially kafirin) content of DDGS reduces process volumes and therefore reduces footprint and processing time and presents an economic alterative raw material for kafirin extraction.

Using DDGS would be preferable as extracting kafirin from sorghum grain/flour would produce starch as waste stream, discarding it would have negative implications on environment. On the other hand, first converting sorghum starch to glucose (for pharmaceutical and other uses) or ethanol (as fuel additive and industrial purpose) and then extracting kafirin from waste stream would ensure complete utilisation of sorghum as a raw material would be a beneficial considering waste handling and impact on environment.

DDGS being protein rich, it incorporates less process and product related impurities and hence efficient use of solvents can be achieved compared to sorghum grain/flour. Sorghum grain/flour have starch and other water-soluble proteins requires considerable high solvent load in extraction solution to solubilise these impurities. Also, grinding DDGS is less energy intensive process compared to sorghum grain grinding.

To summarise, kafirin extraction was attempted in reported processes using a combination of various unit operations that works on different principles, however, most of these processes developed at a small scale. These multistep processes employed some of the unit operations that were not feasible for scale-up considering several reasons such as operation time, recovery, resources and would require a larger footprint. Also, most of the reported processes used to extract kafirin at laboratory scale (in small volumes) for application development as 'proof of concept'; where optimum yield was not reported and within the scope of the research. Considering these gaps, kafirin extraction process needs to be developed and optimised without above-mentioned shortcomings followed by scale-up and validation.

Table 2: Accounts of reported kafirin extraction process development history.

Author/s (Year)	Yield (%)	Purity (%)	Miscellaneous details/ Pre-treatment	Extraction	Process flow	Ref.
Hamaker *et al.* (1986)	19	16.6	Albumin, globulin and LMW N2 fragments	1) 0.5 M NaCl for 60 min,	Sequential extraction in given order	(48)
	0.3	NA	Salt and traces removal	2) Double distilled water for 20 min,		
	11.6	17.3	Kafirin	3) 60% 2-propanol for 4 h,		
	20.8	24.5	Cross-linked kafirin	4) 60% 2-propanol + 1% dithiothreitol (DTT) for 4 h,		
	3.8	4.8	Glutelin like proteins	5) 0.1 M borate buffer, pH 10.8 for 4 h,		
	44.5	27.2	Glutelin	6) 0.1 M borate buffer, pH 10.8 + 1% DTT + 1% sodium dodecyl sulphate (SDS) for 18 h at 4 °C.		
		11.5	Non-extractable nitrogen			
Emmambux *et al.* (2003) and da Silva *et al.* (2004)	46.1 (WGF)	NA	Adaptation of Carter and Reck process (51)	70% (w/w) ethanol in distilled water, 0.35% (w/w) sodium hydroxide, and 0.5% (w/w) sodium metabisulphite at a ratio of 1:5 (w/w) flour-bran to extractant with vigorous stirring at 70 ± 0.1°C for 1 hr	Extraction, centrifugation, evaporation, pH precipitation, freeze drying, oil removal.	(50) (41)
Taylor *et al.* (2005)	54.3	89.3	Comparison of different extractants, flour screened through 800 µm opening screen	70% ethanol + 0.5% sodium metabisulphite + 0.35% NaOH at 70°C	Extraction, centrifugation, evaporation, pH precipitation, filtration, freeze drying, oil removal.	(15)

	55.3	91.2		55% isopropanol + 0.5% sodium metabisulphite+ 0.35% NaOH at 40°C		
	25	NA		Glacial acetic acid at 25°C		
	25	NA		Glacial acetic acid + 0.5% sodium metabisulphite at 25°C		
	52.8	NA		Presoak (1 hr) 0.5% sodium metabisulphite, glacial acetic acid at 25°C		
	59.3	NA		Presoak (16 hr) 0.5% sodium metabisulphite, glacial acetic acid at 25°C		
	61	92.9		Presoak (16 hr) 1.0% sodium metabisulphite, glacial acetic acid at 25°C		
Lau *et al.* (2015)	NA	NA	DDGS washed (3x) with hot water (50 °C), dry in hot air oven at 50 °C overnight before extraction	70% (w/v) ethanol + 0.5% (w/w) sodium metabisulphite + 0.35% (w/w) NaOH at 70°C for 1 h	Hot water wash, oil removal, extraction, centrifugation, dilution, centrifugation, de-ionised wash (3x), freeze-drying.	(56)
Xiao *et al.* (2015)	87 (total protein)	NA	Flour defatted with n-hexane (3x) (1:10 ratio), Washed with water (3x) (1:10 ratio) for 1 h and centrifuged at 8000 rpm for 10 min. The procedure repeated with 0.5 M NaCl and distilled water.	Extracted with 60% t-butanol (2x) for 2 h each and 10 min of ultrasonication using an FS- 28 solid-state ultrasonicator (bath type with sonic power, 225 W; sweep frequency, 40 kHz) at the interval of 30 min	Oil removal, saline wash, extraction, centrifugation, evaporation, pH precipitation, filtration, freeze-drying.	(57)

Giteru *et al.* (2017)	70 (total protein)	NA	Adapted from Emmambux and Taylor (50)	Sorghum flour (250 g) extracted using a mixture of 900 mL ethanol (70% w/w) in deionized water, 25 g/kg sodium metabisulphite and 17.5 g/kg (w/w) sodium hydroxide as a reducing agent. The mixture was heated and held at 70 °C with continuous stirring for 1 h.	Extraction, centrifugation, dilution, freeze-drying, milling, oil removal.	(58)
Muhiwa *et al.* (2017)	54.3	67.2	Percolation (liquid to solid ratio of 2.5:1)	70% (w/w) aqueous ethanol + 1.0% (w/w) sodium metabisulphite + 0.35% (w/w) glacial acetic acid or 0.35% (w/w) NaOH at 70°C for 1 h	Extraction, evaporation, pH precipitation, dilution, filtration, air-drying.	(59)
Sullivan *et al.* (2017)	NA	NA	Defatted flour with heptane for 2h at RT. Centrifuged at 2100 x g at 4°C for 20 min. Pellet dried overnight. Washed with 10x volumes of deionized (DI) water by stirring for 25 min at RT. After centrifugation, globulin extracted from the pellet with 10x volumes of 0.5 M NaCl solution by stirring for 1 h at RT and lyophilized.	10 g of pellet added to 100 mL of DI and treated at 0%, 20%, or 40% amplitude at either 5 min or 10 min, 4 °C using ½″ probe that resonates at a frequency of 20 kHz ± 50 Hz. Samples centrifuged and the pellet was extracted with 10x volume of 60% isopropanol with stirring for 4 h at RT.	Oil removal, centrifugation, air-drying, de-ionised wash, centrifugation, saline wash, centrifugation, ultrasonication, centrifugation, extraction, distillation, lyophilisation.	(60)

2.6.3. Process development and Quality by Design (QbD)

As per International Conference on Harmonization (ICH) guidelines, QbD can be defined as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management" (61). QbD is receiving increased interest from biopharmaceutical community following publication of process analytical technology (PAT) that provides a foundation for QbD implementation. PAT is "a system for designing, analysing, and controlling manufacturing through timely measurements (i.e., during processing) of critical quality and performance attributes of raw and in-process materials and processes with the goal of ensuring final product quality" (61). Inclusion of QbD in ICH guidelines for industry indicates the acceptability and effectiveness of the concept globally (61–64). As shown in Figure 4, traditional process development carried out considering the target product where process executed with defined parameters and if the resulting product meets specifications then the manufacturer locks the process and operates within narrow operating ranges (ORs) to maintain quality. In contrast, QbD paradigm employs key steps (65–68) that include defining Quality Target Product Profile (QTPP)[a] and identifying Critical Quality Attributes (CQAs)[b] (such as kafirin purity and solubility) followed by the risk assessment to identify critical process parameters. Process characterization studies carried out with defined ranges for potential CPPs to define the design space with proven acceptable ranges (PAR). This design space delivers wider ORs to ensure consistent process performance to produce the desired quality product. A process validation exercise demonstrates the effectiveness of the control strategy and continuous monitoring ensures consistent process performance and product quality.

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a: QTPP is "a prospective summary of the quality characteristics of a product that ideally will be achieved to ensure the desired quality, taking into account safety and efficacy of the product" (61).

b: CQA is "a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality. CQAs are generally associated with the product bulk, excipients, intermediates (in-process materials) and final product" (61).

Source: ICH Q8(R2) guidelines

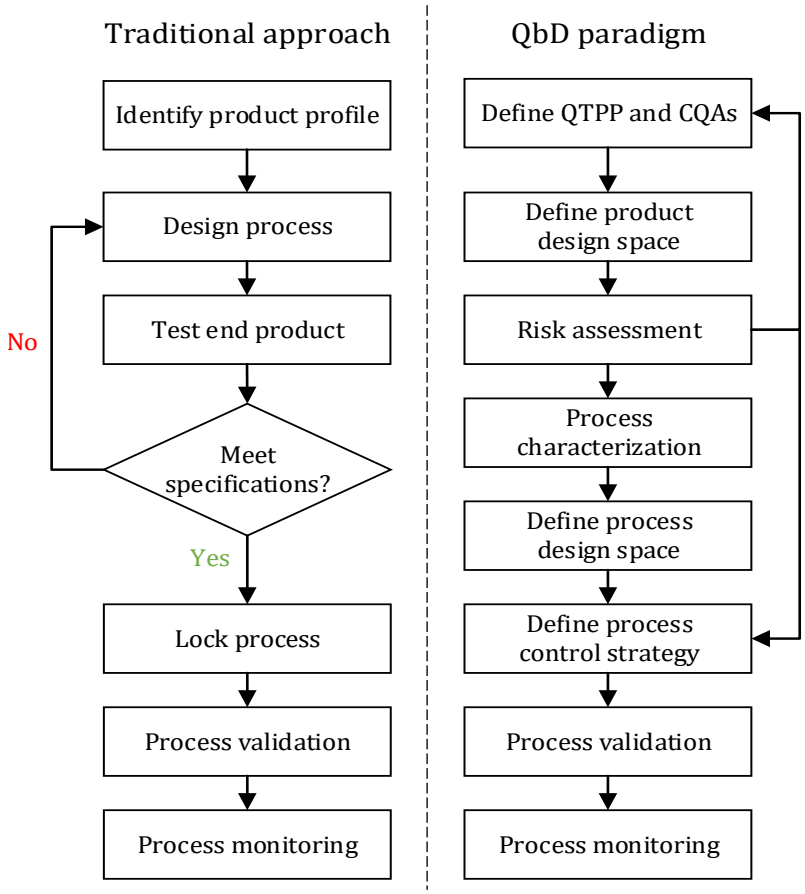


Figure 4: Process development strategy: Traditional v/s QbD.

Traditional process development approach requires testing of product generated from each process variant and revisit the process design if product attributes differ from specifications. ORs for the designed process is narrow making the entire process susceptible to variability such as in raw materials and this process will likely result in an increased number of incidences of failure. In the case of QbD, defined product design space, risk assessment followed by process characterization studies provides wider ORs for the process that result in a product with desired CQAs as illustrated in Figure 5. Characterisation range is the space explored during the process characterisation studies whereas acceptable range is the result of characterisation studies that define process design space (69).

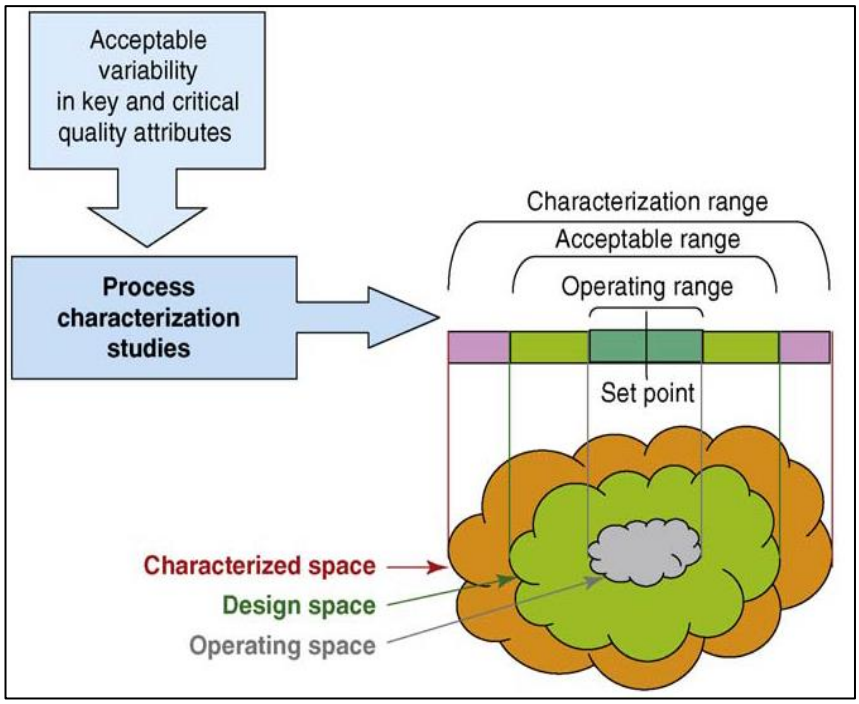


Figure 5: Design space created from characterization studies that define ORs and acceptable range that assures the desired product quality.
Image adapted from Rathore et al. (2009) (66).

PAT based dynamic control strategy allows a process to adapt and deal with variability that is either reduced or eliminated resulting in consistent product quality (70). Control strategy defined as 'a planned set of controls derived from current product and process understanding that assures process performance and product quality' (63). Figure 6 illustrates the concepts of a traditional versus PAT based dynamic control strategy. In traditional strategy, any variability in inputs results in variability in the product quality due to fixed set points and ORs. In contrast, dynamic control strategy offers adaptive set points and ORs that can be varied (within design space) to eliminate or reduce variability resulting in consistent product quality (71). The controls include but not limited to testing of raw material, in-process streams, product characterization, testing from process validation and continuous monitoring (66). Implementation of PAT increases process understanding, lowers failure rate, increases productivity and lowers overall cost (72–76).

Given these advantages, the QbD approach is worth exploring for process development that facilitates scale-up and assures consistent product quality.

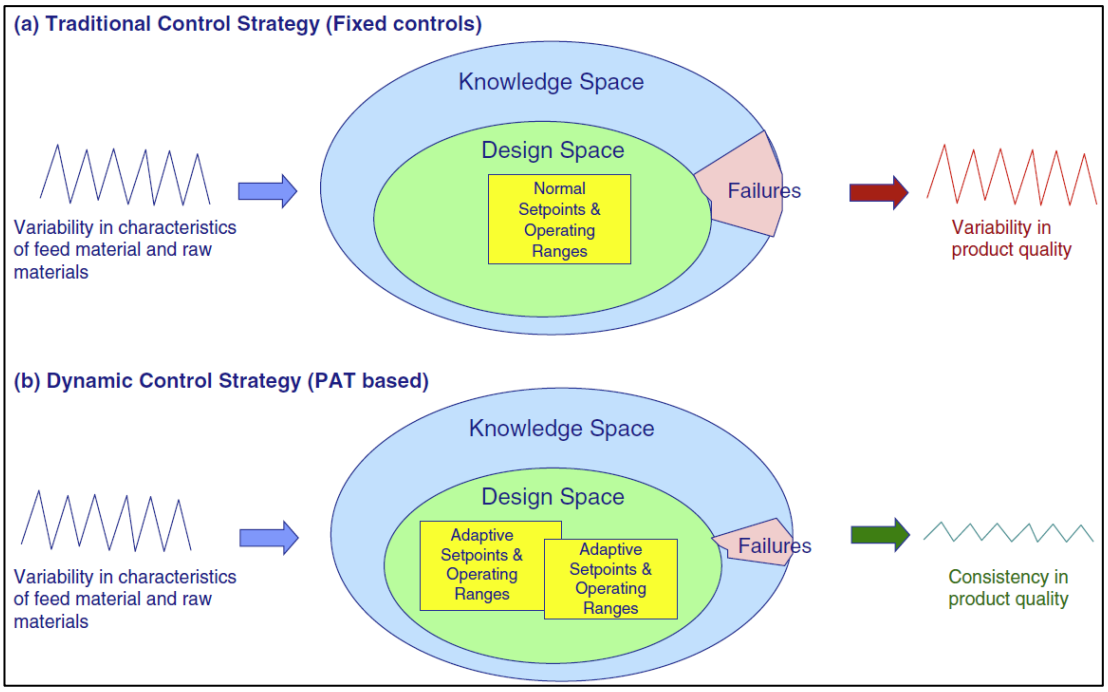


Figure 6: Effects of variability on the final product quality with traditional (a) and PAT based dynamic control strategy (b).
Image adapted from Rathore et al. (2009) (66).

2.6.4. Chromatography

Chromatography is an alternative to extraction processes for protein purification. Chromatography is the tool that uses physical, chemical and biological/biochemical properties of protein for separation. Different properties of the target protein or impurities are targeted to separate them from the rest of the sample. In the case of sorghum prolamins, it is difficult to maintain the solubility of kafirin in a wide range of buffers owing to their highly hydrophobic nature (77). For instance, Blackwell et al. demonstrated the separation of kafirin using non-porous cation exchange chromatography at analytical scale (78). They also evaluated certain additives that helped to maintain kafirin solubility during different stages of chromatography. Kumar et al. (2014) demonstrated kafirin adsorption on a range of ion-exchange resins concluding that dissociation constant (K_d) for various anion exchange resins is lower than cation exchange resins which indicate stronger binding and hence lesser recovery (79). There are no reported studies citing kafirin purification using chromatography at preparative scale, hence this needs exploring as a potential strategy for kafirin purification with easier scalability as an added advantage.

2.6.5. Ultrasonic cavitation

Ultrasonic cavitation one of the rapid, adaptable and emerging green technology being used in different industries. Industries implement ultrasonic cavitation for various purposes such as extraction (80,81), crystallization (82), protein determination (83), microbial inactivation (84) and many more. Ultrasonic cavitation can be used as a process intensification tool for kafirin extraction from sorghum flour or DDGS. The cavitation process can break the intermolecular linkage thereby separating target moiety from the bulk. It offers certain advantages over other processes such as it is non-toxic, environment-friendly and economical (85). It was demonstrated that ultrasonic cavitation based extraction resulted in increased recovery and produced a higher rate of extraction compared to traditional extraction processes (86). Based on the source, process parameters for the ultrasonic cavitation can be optimised form kafirin extraction. Feasibility with continuous process development and scale-up is another added advantage associated with the approach.

2.6.6. Scale-up

Once it has been developed, a kafirin purification process can be modified provided that the incorporation, modification or removal of purification step aids higher purity and recovery. It is also essential to evaluate individual unit operation in terms of scalability, process economy, footprint and feasibility of the operation. Solvent extraction is the first step in the kafirin extraction process that can be scaled-up keeping operating parameters constant in linear or not linear approach considering vessel geometry (87,88). Filtration and centrifugation are the two major separation processes, which are relatively easy to scale up, however, each has their limitations where the former needs higher filtration area at the higher scale and later is an energy-intensive process. Extraction procedures such as precipitation scaled up keeping all operating parameters constant. Certain factors such as vessel geometry and type of mixer affect the separation/ extraction and might need an amendment of operating parameters like mixing speed and operation time (89–92). Chromatographic techniques are scaled up for proteins according to the type of chromatography and requirement of the process. Initially, contact time at small scale is kept constant and the height

of the column increased to the maximum operational limit. Later, volumetric scale-up of chromatographic steps can be achieved by increasing the diameter of the column keeping other parameters constant such as column height and linear flow rate (93–96). Also, the purity of the process-intermediate from an individual step or the end product, step yield and overall recovery of the scaled-up process should be comparable to the laboratory-scale set-up and as such indicates efficient and successful scale-up (93).

2.7. Application development

Prolamins such as zein (from maize) and kafirin (from sorghum) have been assessed for their ability to be used as fibres, coatings, bioplastics, drug-carriers, antimicrobials, nutraceuticals and materials of construction for surgical implants (92,97–99). Both zein and kafirin can demonstrate excellent carrier and barrier properties which make them suitable raw materials for particle/ beads, films, fibres (97) and biomedical applications (100,101). Kafirin shares similarity with zein in terms of its molecular weight, solubility, structure, amino acid composition and polypeptide structure (37,102). However, kafirin is more hydrophobic, highly cross-linked and slowly digestible than zein and non-allergenic (48,101,103). Also, kafirin demonstrates higher hydrophobicity and less digestibility as compared to zein (10,11). These traits suggest kafirin as a better candidate than zein for the production of films with improved gas and vapour barrier properties and hence a more efficient coating agent (104) and source for film fabrication (9). Kafirin has more disulphide bonds than zein; this implies that it can be moulded into the better barrier and stronger films (12).

Kafirin purified from sorghum seeds has been used for both food and non-food based applications. Kafirin tested for limited pharmaceutical applications such as the primary excipient for sustained drug release (98) and microparticles or microspheres for food and pharmaceutical applications (105). Films made from kafirin were characterised for their physical, chemical and mechanical properties (9,106,107). Kafirin films were tested as a polymer for packaging material production (108). Freestanding kafirin films were also demonstrated as a carrier for bioactive materials such as essential oils and polyphenols and as a potential gas barrier with antimicrobial activity (58,109). However, production of both was

not possible partly because of the lack of commercially viable process and unavailability of compatibility data with the plasticizers and additives. Although kafirin has been examined for above-listed applications, it still needs to be extensively investigated for its use as a carrier for different pharmaceutical and non-pharmaceutical molecules, for use as films and for particles formed using different plasticizers (14,58,109). Food and non-food applications developed from kafirin using various plasticizers and solvents discussed earlier in this thesis and reported in the literature are summarised in *Table 3*. Most of the applications listed here report ethanol and glacial acetic acid as compatible solvents for kafirin extraction and demonstrated applications. Besides, plasticizers and/or additives used in these studies were Polyethylene glycol (PEG), lactic acid and glycerol adapted and modified from Buffo *et al.* (1997) with few studies reporting otherwise (13,110). It can be concluded that most of the applications demonstrated in various literature mostly used lactic acid, PEG and glycerol as plasticizers.

In light of the presented review of literature for kafirin-based applications, it can be inferred that the group of plasticizers tested for their compatibility with kafirin was very narrow compared zein. In order to fill the research gap, plasticizers screening needs to be carried out that provides a platform to develop kafirin based applications such as films, coating, spray and microparticles. Applications formed using various plasticizers of different chemistry should also be tested for their barrier properties against gas, moisture, water and oil respectively based on targeted applications.

Table 3: History of kafirin based application development.

Author/s (Year)	Application	Method	Plasticizers/ additives	Solvent	Reference
Buffo *et al.* (1997)	Film	Conventional casting	Glycerol and PEG 400	70% ethanol	(44)
Emmambux *et al.* (2004)	Film	Conventional casting	1:1:1 (w/w) mixture of glycerol, PEG 400 and lactic acid	70% ethanol	(111)
da Silva *et al.* (2005)	Film	Conventional casting	1:1:1 (w/w) mixture of glycerol, PEG 400 and lactic acid	70% ethanol	(9)
Taylor *et al.* (2005)	Film	Conventional casting	1:1:1 (w/w) mixture of glycerol, PEG 400 and lactic acid	70% (w/w) ethanol at 40 and 70 °C, lactic acid, glacial acetic acid at 25 °C, 55% (w/w) aqueous isopropanol	(104)
Gao *et al.* (2005)	Film	Conventional casting	Glycerol and PEG 400	70% (w/w) ethanol	(112)
Byaruhanga *et al*. (2005)	Film	Conventional casting	1:1:1 (w/w) mixture of glycerol, PEG 400 and lactic acid	Glacial acetic acid	(113)
Taylor, et al. (2009)	Microparticles	Phase separation	1:1:1 (w/w) mixture of glycerol, PEG 400 and lactic acid	Glacial acetic acid	(105)

Taylor *et al.* (2009)	Film from microparticles	Conventional casting	1:1:1 (w/w) mixture of glycerol, PEG 400 and lactic acid	Glacial acetic acid	(114)
Anyango *et al.* (2011)	Film	Conventional casting and microparticles	1:1:1 (w/w) mixture of glycerol, PEG 400 and lactic acid	Glacial acetic acid	(115)
Buchner *et al*. (2011)	Coat	Dip coating	1,2-propanediol and glucono-δ- lactone	70% ethanol	(13)
Lau *et al.* (2015)	Microparticles	Phase separation	None	70% ethanol	(56)
Giteru *et al*. (2015)	Film	Conventional casting	1:1:1 (w/w) mixture of glycerol, PEG 400 and lactic acid	96% ethanol	(109)
Xiao *et al.* (2016)	Fibre	Electrospinning	Polycaprolactone (PCL)	Acetic acid/dichloromethane mixture	(110)
Xiao *et al.* (2016)	Nanoparticles	anti-solvent precipitation with ultrasonication	None	Acetic acid	(116)
Lal *et al.* (2017)	Coat	Dip coating and conventional casting	PEG	70% ethanol	(14)
Muhiwa *et al.* (2017)	Film	Conventional casting	None	70% ethanol	(59)

Chapter 3: Process development and scale-up

3.1. Introduction

Preliminary kafirin extraction process developed was a multistep as shown in Figure 7, the process starts with steeping of sorghum grain followed by milling and fractionation by filtration to separate endosperm, bran and steep liquor. Sorghum grains (100 g) were soaked in steeping solution (200 mL) containing 1.4% v/v lactic acid and 0.3% w/v sodium metabisulphite at 50 °C for 48 h in stirring condition (200 RPM). After incubation, steep liquor was separated and the grains were washed with water to remove remaining traces of steeping solution. These steeped grains then added with 100 mL water and blended in the grinder to form a slurry. The process of steeping hardens the bran and softens the endosperm. This slurry was then filtered through a muslin cloth to separate endosperm (filtrate) and bran (residue). The filtrate centrifuged at 4700 RPM for 20 min to separate the pellet which was dried in a hot air oven at 60 °C for overnight to 24 h. Fractions obtained from the steeping and separation were analysed for their protein content. The dried endosperm (10 g) was extracted with extraction solution (50 mL) comprised of 70% ethanol (v/v), 0.35% (w/v) sodium hydroxide and 0.5% (w/v) sodium metabisulphite at 70 °C under stirring condition (180 RPM) for 1 h. The supernatant was separated by centrifugation at 4700 RPM for 20 min and kafirin was separated from the solution by diluting the solution with water to bring the final ethanol concentration below 20% and adjusting the pH of the solution to 4.5 – 5.0. These precipitates were recovered by centrifugation at 4700 RPM for 30 min. Precipitates were dried at 60 °C for overnight, milled to powdered kafirin.

In addition to being a multiple-step process, following aspects make the process discussed above less desirable for scale-up leading to commercialization and kafirin production at a higher scale. Steeping requires a considerable amount of time (48 h) and incubation at 50 °C temperature requires a setup such as a specially designed reactor with temperature controller. Washing followed by milling, filtration, and centrifugation separates bran and endosperm, the latter being rich in kafirin. However, the discarded bran also contains a significant amount of kafirin leads to loss of kafirin early in the initial stage (41). Dilution of

extract followed by isoelectric precipitation helps selectively isolate kafirin; however,

4x dilution of the process intermediate significantly increases the volume at a higher scale. In this case, dilution step was found highly inconsistent considering commercial-scale manufacturing as the quality of the process stream and recovery of isoelectric precipitation step governed by parameters such as volume, protein concentration (initial and post-dilution), pH, temperature, mixing speed and time within defined operating ranges.

The output stream of a unit operation affects the subsequent unit operations, which sometimes leads to loss of protein, hence reduced recovery and/or low purity. For instance, the extraction step followed by separation using centrifugation and filtration aimed towards maximum liquid recovery by clearance of particulate matter. Failure of one of these steps to produce the desired process stream may lead to failure of the subsequent unit operations i.e. distillation and isoelectric precipitation. After centrifugation or filtration, any unwanted particulate matter suspended in liquid provides nuclei for the precipitation of nonspecific proteins and thereby lowering the purity of the process intermediate and subsequently the final product although the ongoing unit operation operated with defined operating parameters.

These gaps in the process development and lack of reproducibility in certain unit operations that affect the next purification step and hence the inconsistent individual step recovery, overall process yield and product purity. All these gaps in the process were addressed and a defined process that consists of well-characterised unit operations developed for kafirin extraction.

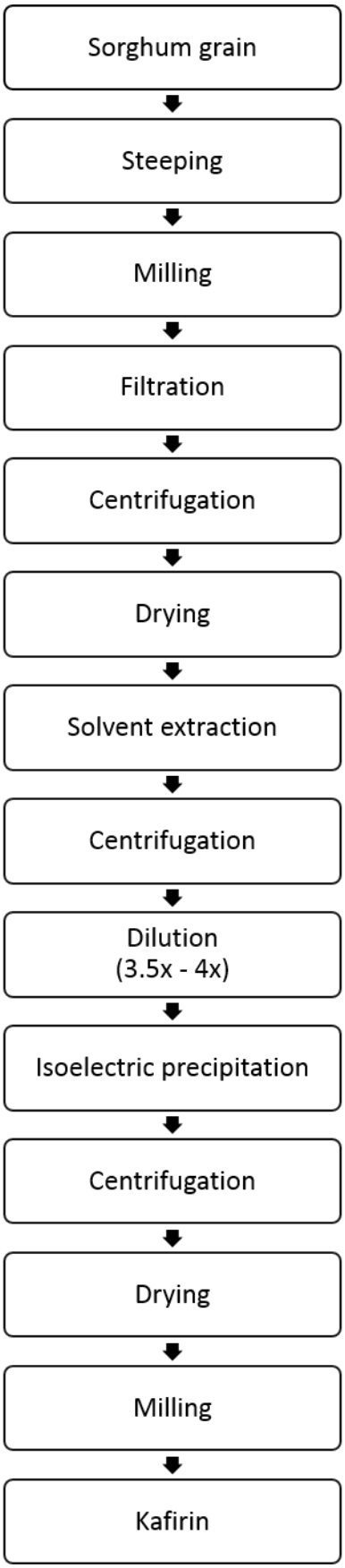


Figure 7: Kafirin extraction process (old) developed as preliminary work.

Several changes made to the existing purification process considering the shortcomings discussed above and with the scope of commercial scale-up. These changes were made to reduce time, the number of steps/unit operations, handling volumes and overall cost of the process. Major changes made to the existing process were as listed below.

1) Sorghum flour opted for extraction instead of dried sorghum endosperm obtained from steeping, milling, filtration followed by centrifugation. The purpose was twofold as several numbers of steps reduced to one (milling of sorghum flour) and prevention of loss of kafirin in bran discarded during steeping (41). The omission of steeping would save significant process time as sorghum grain steeping would require 48 hours followed by additional process time for subsequent unit operations (milling and separation using filtration and centrifugation).
2) Sorghum flour used for extraction was heated in hot air oven to respective extraction temperature to assure extraction solution, other ingredients and resulting slurry at the desired temperature.
3) Vacuum distillation was chosen for ethanol removal instead of dilution as former assured sustainability of the process by efficient ethanol recovery. Dilution step at higher scale would have increased the handling volumes at a higher scale and thus the manufacturing footprint. Recovery of ethanol from such large volumes would be more energy-intensive and/or increase a load of recycling plant thereby rendering the process less environment friendly.
4) Dilution step before isoelectric precipitation increased the process volumes as the water was added (up to 3.5 to 4 folds) to process stream in order to reduce alcohol concentration resulting in kafirin precipitation. However, low protein concentration resulted in low recovery or failure of the process as the diluted process stream would not have the critical protein concentration required for efficient precipitation. Isoelectric precipitation after dilution in the existing process required more than 12 hours whereas distillation followed by precipitation requires less time with high protein concentration and low system volume stream.
5) Kafirin extracted at lab scale dried in hot air oven whereas spray dryer was used at higher scales.

Initially, one factor at a time (OFAT) strategy was implemented considering two points, first was to determine the operating parameter ranges for different unit operations and second was assuring kafirin production at a small scale to initiate the second major objective of the research i.e. application development from the purified protein. Purpose of the OFAT studies was to identify the preliminary operating ranges for different parameters of the individual unit operations. Data generated from these batches provided the platform for risk assessment using FMEA to identify the critical process parameters (CPPs) as an initial step for QbD based process development. DOE, as part of QbD exercise, implemented to screen the CPPs for their acceptable ranges within the characterization space and determine the operating ranges to define the process design space. Another purpose of this exercise was to identify the potential failure modes and causes leading to definite failure of the critical quality attributes (CQAs) due to the parameter/s which when operated within certain range or excursion and extremes.

3.2. Materials and methods

3.2.1. Sorghum and chemicals

Sorghum grains obtained from the Department of Agriculture and Fisheries, Queensland, Australia milled to flour and screened using sieves of different sizes to obtain fractions of different particle size ranges. All reagents and chemicals used in the study were of analytical grade or equivalent. Ethanol, hydrochloric acid, sodium hydroxide, t-butanol, isopropyl alcohol (IPA), acetic acid, orthophosphoric acid (OPA), sodium metabisulphite, dithiothreitol (DTT), silver nitrate, glycerol, sodium thiosulphate, sodium carbonate and ammonium persulphate (APS) obtained from Sigma-Aldrich, Australia. SDS-PAGE gels (10%), 2-mercaptoethanol (2ME), SDS-PAGE (Tris-glycine) running buffer, Laemmli buffer, broad range protein markers procured from Bio-Rad, Australia. Water used throughout the study was deionised or RO grade else specified otherwise. DDGS was produced in-house for preliminary experiments and acquired from Dalby Bio-Refinery, Australia for laboratory and scale-up batches.

3.2.2. Analytical methods

3.2.2.1. Protein content

Samples were analysed for their nitrogen content using elemental analyser, 2400 CHNS/O Series II system from Perkin Elmer. Kafirin content was calculated using Eq. 1 given below.

$$Protein\ (\%)(w/w) = 6.25\ x\ Nitrogen\ (\%) \qquad \text{Eq. 1}$$

3.2.2.2. Extractable kafirin content

Samples were extracted (3x) using extraction solution to ensure complete extraction, these extracts were pooled. Ethanol removal from the extract followed by isoelectric precipitation with 1.0 N HCl. Precipitates obtained as a pellet after centrifugation was washed with hexane and dried in the hot air oven at 60 °C. Dried kafirin weighed and analysed to calculate the extractable kafirin content of the sample.

3.2.2.3. SDS-PAGE and staining

SDS-PAGE is a modified version of native PAGE created by Laemmli (117) to overcome limitations of later by incorporating sodium dodecyl sulphate (SDS), a detergent to the discontinuous buffer system. Under reducing conditions, Proteins are denatured in the presence of denaturing agents and SDS. Once in denatured state, SDS bind to entire molecules and masks the intrinsic charge of the protein. The negative charge from the SDS repels the proteins away from each other preventing aggregation. It also imparts a similar charge to mass ratio as SDS binds to proteins at the consistent rate of 1.4 g of SDS per gram of protein for all proteins in the mixture. As a result, protein migrates in the gel and separation achieved primarily based on its size.

Samples preparation:

Samples from respective streams were prepared by mixing the calculated amount of samples and (reducing or non-reducing) Laemmli buffer (2X) in 0.5 mL polypropylene tubes. Samples were mixed and incubated in boiling water bath or heating block for 5 minutes. Samples were centrifuged at maximum speed for 1-

2 minutes and calculated volume (5 µg or 10 µg) of protein was loaded in the gel. 4X Laemmli buffer was used for low protein concentration samples.

Assembly, sample loading and gel running:

- Precast gels removed from the storage pouch and the comb by pulling it upward in one smooth motion. Green tapes were removed from the bottom of the cassette. 100 mL of 10X stock tris-glycine running buffer diluted using 900 mL of deionised water in a graduated measuring cylinder to make 1L of 1X working gel running buffer.
- Gel wells were rinsed with running buffer. Gels were assembled in the cassette by placing the plates in the gel holder. Cassette was placed in gel running assembly and gel running buffer added to the inner and outer chambers of the unit.
- Respective wells were loaded with the calculated volume of reducing/non-reducing samples. 7 µL of molecular weight marker loaded to one of the wells per gel for reference purpose. Headcover was placed on the assembly and cables were connected to the power pack. Voltage was set to 100, the unit was switched on and allowed to run until the dye front reached the reference line.
- After the completion of the run, power supply was switched off and cables were disconnected. Headcover was detached from the assembly and cassettes were removed. Cassettes were opened the using an appropriate tool provided with the kit as per the directions provided in the manual to retrieve the gel.
- Gel removed from the cassette and processed with the silver staining to visualize the protein bands.

Silver staining:

Silver staining procedure and the solution preparation must be carried out in fume hood or under fume extraction device. The staining procedure performed on rocker shaker. Solutions were prepared as given in Table 4.

Table 4: Silver staining solution recipes.

Solution	Ingredients	Amount/ Volume
Fixing solution	Methanol	50 mL
	Glacial acetic acid	12 mL
	Formaldehyde (37%)	50 µL
	DI water (q. s. to)	100 mL
Washing solution	Methanol	150 mL
	DI water (q. s. to)	300 mL
Pre-treatment solution	sodium thiosulphate	20 mg
	DI water (q. s. to)	100 mL
Impregnation solution	Silver nitrate	200 mg
	Formaldehyde (37%)	75 µL
	DI water (q. s. to)	100 mL
Developing solution	Sodium carbonate	6 g
	Sodium thiosulphate	1 mg
	Formaldehyde (37%)	50 µL
	DI water (q. s. to)	100 mL
Stopping solution	Methanol	50 mL
	Glacial acetic acid	12 mL
	DI water (q. s. to)	100 mL

Staining procedure:

- Gels were placed in trays and incubate in the fixing solution for one hour. The fixing solution was removed and gels were washed with the washing solution for 10 minutes. The washing step was repeated twice.
- The washing solution was decanted and gels were rinsed with deionised water for 20 seconds each.
- Pre-treatment solution was poured over gels in the tray and incubated for 1 minute. The solution was decanted and gels washed with 50 mL of water for 20 seconds.
- Gels were incubated in impregnation solution for 20 min. The solution was decanted and gels washed twice with 50 mL of water.
- Developing solution pour over the gel and allowed to incubate till bands develop.
- The staining procedure was terminated by decanting the developing solution and washing the gels with stopping solution.

Coomassie staining:

Staining procedure and the solution preparation must be carried out in fume hood or under fume extraction device. The procedure was performed on rocker shaker. Prepare the solutions using ingredients listed in Table 5.

Table 5: Coomassie staining solution recipes.

Solution	Ingredients	Amount/Volume
Staining solution	Methanol	50 mL
	Glacial acetic acid	10 mL
	Coomassie Blue R250	1.0 g
	DI water (q. s. to)	40 mL
Add glacial acetic acid to water. Dissolve dye in methanol. Mix the solutions and filter before use.		
Destaining solution	Methanol	50 mL
	Glacial acetic acid	10 mL
	DI water (q. s. to)	40 mL

Coomassie staining procedure:

- Staining solution poured over gels in trays and incubated for a minimum time (one hour to overnight) necessary to visualize the protein bands.
- The staining solution was recovered and gels were washed with the destaining solution for an hour. The process was repeated until the background is clear.

3.2.3. Extraction

Kafirin extraction from sorghum flour was performed by sequentially optimising process parameter for the extraction step. Initially, sorghum flour was extracted using different solvents for different time interval and later with additives. Particle size, solid to liquid ratio and temperature were optimised with selected solvent and additive. Extraction solvent and additives screened prior to particle size optimisation with the aim that extraction solution should be able to extract kafirin considering heterogeneity in the feed material. The sequence of parameter optimisation for extraction is as shown in *Figure 8*.

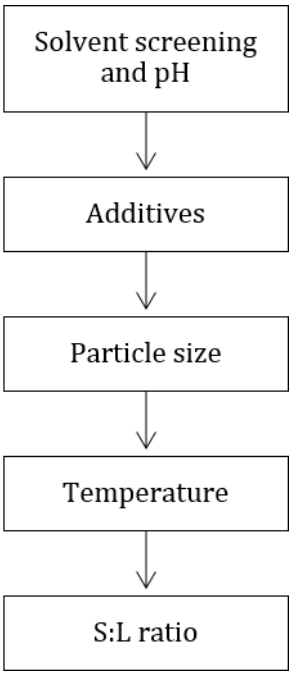


Figure 8: Extraction parameter optimisation sequence for kafirin extraction from sorghum flour.

3.2.3.1. Solvent screening and pH

Sorghum flour (50 g) was extracted with different solvents at room temperature under stirring condition (200 RPM) for 180 minutes. Samples were collected at different time intervals to analyse the amount of protein in the bulk at a given time point. Samples were analysed for their protein content to calculate recovery and yield for the given range of extraction solvents. 6.0 N HCl was used to adjust pH 3.0 and 0.5 M NaOH for pH 12.

3.2.3.2. Effect of additives

Sodium metabisulphite (SMB) (1 % w/w), dithiothreitol (DDT) (0.05 % w/w) and 2-mercaptoethanol (BME) (0.5 % w/w) were added to extraction solution as reducing agents to assess their effect on kafirin extraction yield. Additive selected was further assessed at pH 2.0 and 12.0, where extraction solution pH was adjusted using 6.0 N HCl and 0.5 M NaOH.

3.2.3.3. Effect of particle size

Sorghum flour was sieved and fractionated in different particle size groups to assess the effect on the kafirin extraction yield. Sorghum flour was sieved in <53

µm, 53 µm to 100 µm, 100 µm to 250 µm, 250 µm to 500 µm and >500 µm. These fractions were extracted with ethanol, SMB at pH 12.0.

3.2.3.4. Temperature optimization

Sorghum flour was extracted with ethanol, SMB at pH 12.0 as mentioned earlier at different temperatures (25, 40, 50, 60, 70 and 80). Samples were analysed for their kafirin content to assess the impact of temperature on the extraction efficiency.

3.2.3.5. Effect of Solid to liquid ratio

Sorghum flour was extracted with increasing amounts of extraction solvents to evaluate the influence of solid to liquid ratio. The ratios considered for the study were 1:5, 1:10, 1:15 and 1:20.

3.2.4. Separation

Centrifugation followed by filtration was used to separate solid mass from the mixture. Details of the optimisation of these unit operations are as follows.

3.2.4.1. Centrifugation

Sorghum flour and extraction solution were mixed on a weight basis for kafirin extraction. After incubation, the extraction mixture was poured into centrifuge container; the weight of the container was recorded with and without mixture. The mixture was centrifuged at 2700, 3700 and 4700 RPM for different time intervals from 10 to 30 minutes with 10 minutes time difference. The supernatant was decanted and filtered with a 1.0 µm filter in a separate pre-weighed container and raffinate was retained in the container. Both supernatant and raffinate were weighed to assess the amount of liquid retained in the sediments and quality of the supernatant in terms of clarity/ turbidity due to suspended solids.

3.2.4.2. Filtration

Kafirin extracts obtained after centrifugation was filtered through filters to assess the filtration area required per unit volume of extract. Different pore size filters (0.2 µm, 0.45 µm and 0.8/0.2 µm) were used to filter the extract. Samples obtained from centrifugation were filtered using given size filters to assess the

flow decay until the filter is clogged or essentially exhausted otherwise. All streams available at the end of filtration were measured for output volume.

Centrifugation output samples were pooled and 500 mL of sample was taken in a glass bottle with regulated pressure. The outlet of the bottle connected to filter using silicon tubing. Collection vessel placed below filter outlet, a graduated cylinder or glass bottle on a balance. Pinch was removed to start the filtration and filtered sample was collected to measure the output with increase in time.

3.2.5. Distillation

Clarified supernatant obtained from centrifugation and filtration were subjected to distillation to recover ethanol. Bath temperature (50 °C) and chiller temperature (10 °C) were set considering the vacuum applied (102 mbar) to achieve desired vapour temperature (30 °C) for ethanol. Distillation was carried out using Rotavap R-300 (Buchi) with the vacuum pump and recirculating chiller.

3.2.6. Isoelectric precipitation

Ethanol-free stream obtained from distillation was brought to room temperature and 100 mL of solution kept in stirring condition (150 RPM) in glass bottles. The pH of the stream in individual bottles adjusted to 3.5 to 6.5 with the increment of 0.5 units. Samples centrifuged after 2 hours, the supernatant decanted and solids were recovered, both supernatant and solids were analysed for their protein content.

3.2.7. Oil removal

Isoelectric precipitation output samples were treated using different solvents Methanol, ethanol, ethyl ether, ethyl acetate and hexane were screened based on the literature available for oil extraction (118,120,121). Precipitates from last unit operation were extracted using individual solvents with 1:5 solid to liquid ratio at 60 °C (RT for ethyl ether) under stirring condition (200 RPM) for one hour. Supernatant separated from the solids and the process was repeated until clear supernatant obtained.

Oil determination: Solvents were recovered from these supernatants by distillation to obtain solvent-free oil. Oil extracted by individual solvent washes were weighed to assess the amount of oil extracted with each solvent wash.

3.2.8. Drying

After oil removal, solids were separated in a glass petri dish and kept in a hot air oven for drying at 60 °C for 8 to 10 hours or more (overnight) for drying.

Kafirin extracted at higher scale was spray-dried using a pilot-scale spray dryer (Mini spray dryer B-290 Advanced, Buchi) with a dehumidifier (B-296) and inert loop (B-295). Parameters such as inlet temperature, outlet temperature, flow rate and aspirator speed were optimised to reduce operation time and cost. Spray dryer operations were conducted as follows:

- Instrument and the aspirator were switched on. The temperature of the inert loop was set to -20 °C.
- Main nitrogen gas valve was opened and gas flow adjusted to 40 mm using the needle valve in the gas flow meter. The aspirator was turned on to 80% to start feeding the gas to the system until the oxygen level in the system reached less than 6% (on inert loop indicator).
- Inlet temperature set at 120 °C and the heater was switched on.
- Tubing was placed in the feed valve and pump setpoint adjusted to 30% (60% for solvents).
- Once the inlet temperature reached 120 °C, the end of the tube placed in a container with distilled water/solvent and pump was switched on.
- Inlet temperature and feed flow rate were adjusted to obtain the desired outlet temperature for the product.
- Feed tubing was placed in the solution containing the product. Solution placed on magnetic stirrer to homogenise the feed during the entire operation. Inlet temperature and feed flow were adjusted accordingly to obtain the desired outlet temperature.
- Once the feed is over, feed tube was placed in solvent/distilled water to flush the tube and nozzle and ensure maximum recovery.
- Product was recovered from the collection vessel and weighed.

- Cylinder, cyclone, coupler, outlet temperature sensor and other removable parts were detached and cleaned thoroughly. All parts were air-dried before reassembly.
- Solvent recovered from the receiving bottle in the inert loop. Water collected in the humidifier collection container was decanted.

3.3. Results and discussion

3.3.1. Extraction

The solubility of kafirin in any solvent is the function of the interaction of kafirin molecules with the solvent molecules. Solubility is governed by solvent type, additives, pH and temperature, where increased solubility reflected in terms of higher extraction rate. Particle size, solid to liquid ratio, mixing also influence the extraction rate and their effect on overall yield and purity discussed in subsequent sections.

3.3.1.1. Solvent screening and pH

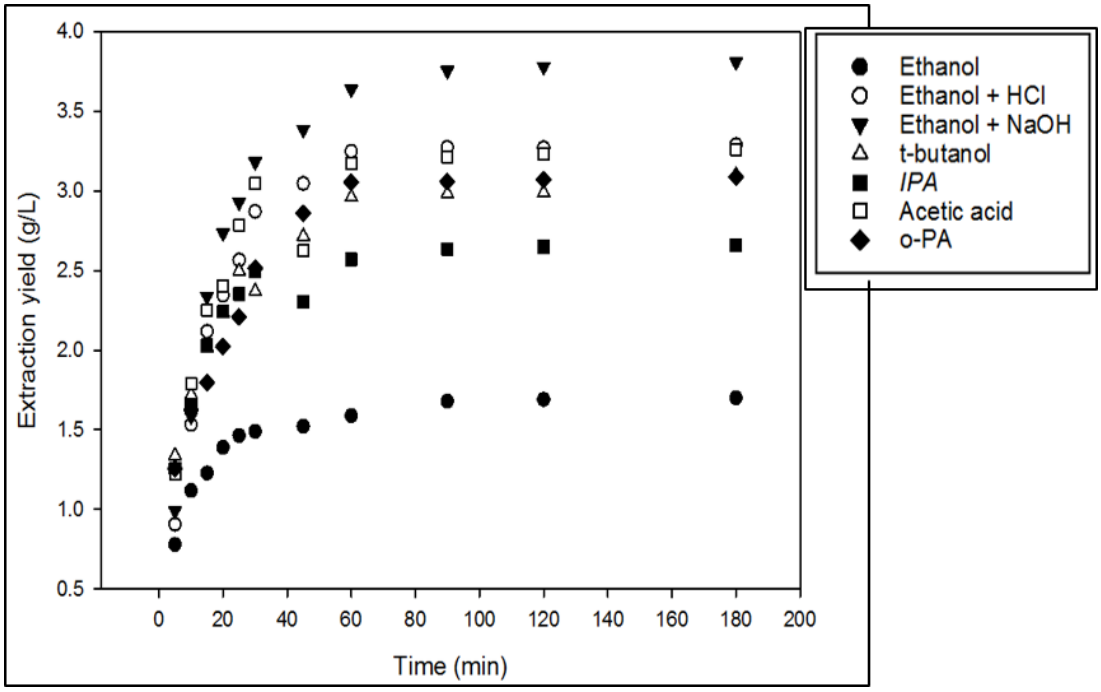


Figure 9: Solvent screening for kafirin extraction from sorghum flour.

Kafirin extraction was carried out from sorghum flour with different solvents at room temperature under stirring condition (200 RPM) for 180 minutes and samples were collected at different time intervals to analyse the amount of

protein in the bulk at a given time point. As evident from Figure 9, ethanol without any additive was the least effective whereas ethanol at higher pH demonstrated highest extraction rate followed by acetic acid and ethanol at low pH. Orthophosphoric acid (o-PA) and t-butanol demonstrated better extraction compared to IPA and ethanol without additive after ethanol with NaOH, ethanol with HCl and acetic acid. Difference between kafirin solubility in different solvents may lead to different extraction rates.

It was observed that the pH of the extraction solution played a vital role when it comes to extraction efficiency as ethanol without additive found less effective than the ethanol at either high or low pH. Similarly, the extraction efficiency of acetic acid and OPA were comparable to other solvents at both pH extremes, whereas IPA and t-butanol with pH values 8 and 7 respectively, had comparatively low extraction efficiency. Protein solubility in a given solution mainly depends on the isoelectric point of the protein molecule and pH of the extraction solution. Solution pH close to pI of the protein renders no net charge on the molecule, in this state, protein is no longer soluble in the solution. However, solubility increase with the increase in the difference between the pH of the solution and pI of the molecule (5.0 for kafirin). In the case of ethanol, IPA and t-butanol, pH of the solution is close pI of kafirin so the solubility is limited. Acids and other ethanol combinations have pH away from molecule pI demonstrate higher solubility and hence higher extraction rate (15,31,38).

For all solvents, the maximum extraction rate was observed during the initial 30 minutes and a gradual decrease in extraction rate in next 30 minutes i.e. up to 60 minutes. After 60 minutes, there was no significant increase in the rate of extraction for up to 180 minutes. Kafirin extraction rate for acetic acid was higher than ethanol+HCl (pH 2.0) for the initial 30 minutes, however, extraction rates thereafter were comparable. Similarly, the extraction rate for t-butanol and OPA were comparable throughout the experiment.

Ethanol+NaOH (pH 12.0) maintained a higher extraction rate among all solvent candidates. As evident from Figure 9, extraction with ethanol+NaOH can be divided into three different extraction rates/phases throughout the experiment. Initial 30 minutes comprised the first rapid extraction phase, evident from the stiff slope followed by the second phase where a gradual decline in extraction rate

was observed from 30 minutes onwards up to 60 minutes. 60 minute onwards up to 180 minutes represents the last phase where the extraction rate was close to zero, which means there was no further extraction occurred with increase in time.

Ethanol + NaOH was the most effective among solvents screened for kafirin extraction in terms of higher solubility and better extraction rate, an observation consistent with the reported studies (27,38,122). Ethanol at pH 12.0 selected for further studies considering its availability and process economy.

High Solid to liquid ratio (1:20) was chosen to avoid saturation of extraction solution in order to avoid any effect on the rate of extraction. Other parameters such as temperature, additives, particle size might affect the rate of extraction and subsequently the extraction yield. These parameters were studied individually and their effects on the kafirin extraction discussed in subsequent sections.

3.3.1.2. Effect of additives

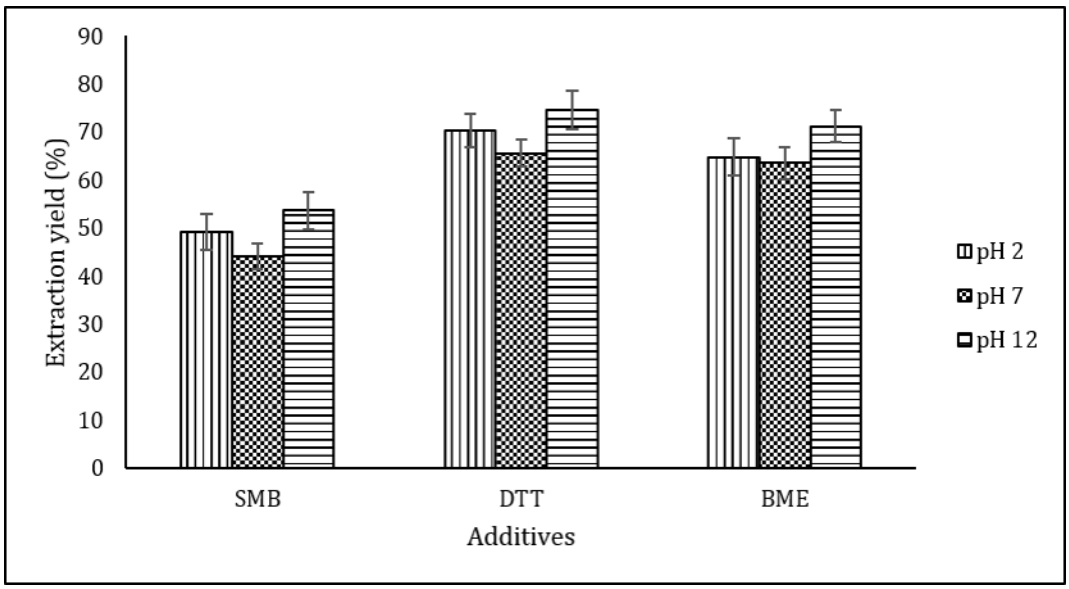


Figure 10: Effect of additives on kafirin extraction yield from sorghum flour.

Ethanol + NaOH was selected as the extraction solvent with the highest extraction yield. However, the extraction efficiency of the solvent system was further enhanced using reducing agents as additives. SMB, DDT and BME were tested for extraction with ethanol at neutral and both pH extremes (pH 12 and 2) where yield with SMB was significantly lower than DTT and BME. It was observed that

addition of DTT and BME resulted in higher kafirin extraction yield at pH 12 compared to pH 2 (Figure 10), as both strong reducing agents efficiently cleaved intermolecular and intramolecular disulphide linkages that aid formation of protein-protein complexes within the seed. This finding was contradictory to the expected outcome as they act efficiently at lower pH but have limited reducing capability over pH 7. DTT and BME are powerful reducing agent with a redox potential of -0.33 V and -0.26 V at pH 7 (123,124). Reducing efficiency of the DTT is higher at low pH as the thiolate form, the negatively charged species responsible for reducing reaction and not the protonated thiol form generated at high pH (123). It was found that the low yield for the strong reducing agents was apparently due to limited solubility of kafirin in low pH conditions. Kafirin demonstrated higher solubility at pH 12 compared to pH 2 and the observation was consistent even in the presence of reducing agents (79). These observations implied that strong reducing conditions were not necessary for higher yield and extraction at higher pH significantly reduced solvent requirements.

Although, with higher extraction yield, both DTT and BME imparted a strong smell to subsequent process streams, final product, and required the introduction of additional process steps and suitable analytical methods to assure their removal. Essentially, use of kafirin purified using these additives for food-related applications was a questionable approach. The addition of sodium metabisulphite in extraction solution at pH 12.0 significantly improved the extraction yield, finding of this study was in agreement with the reports of Gao *et al.* (112). However, findings of Taylor *et al.* (122) suggests otherwise, which is apparently due to differences in extraction process and other operating parameters such as the number of extractions, temperature, extraction time and drying conditions. Although extraction yield was significantly higher, extraction at high pH also facilitated co-extraction of oil from feed compared to extraction at neutral or low pH conditions, an observation consistent with the findings reported for zein extraction process (31). This might increase the impurity load of the inlet stream for distillation followed by higher hexane requirement at the subsequent stage.

Addition of sodium metabisulphite increased solubility of kafirin in the extraction solution at high pH as it cleaved existing inter and intra-molecular disulphide

linkages reducing polymer aggregates to monomers. These disulphide linkages are strong forces that facilitate the protein-protein interaction and formation of protein bodies embedded within the protein-starch matrix in the endosperm of the sorghum seeds (38,42,92). Presence of single prominent band in SDS-PAGE profile of the kafirin extracted without reducing agent at high pH supports the obtained higher kafirin yield whereas kafirin extracted with just solvent result in high molecular weight product-related impurities such as dimers, trimers and polymers in the form of protein aggregates and a relatively small band of kafirin monomers (38).

Given these reasons, the addition of sodium metabisulphite as reducing agent at pH 12 to the extraction solution significantly increased kafirin yield compared to solvent without any additives, hence considered an additive of choice and an ideal candidate to proceed with for process optimization.

3.3.1.3. Effect of particle size

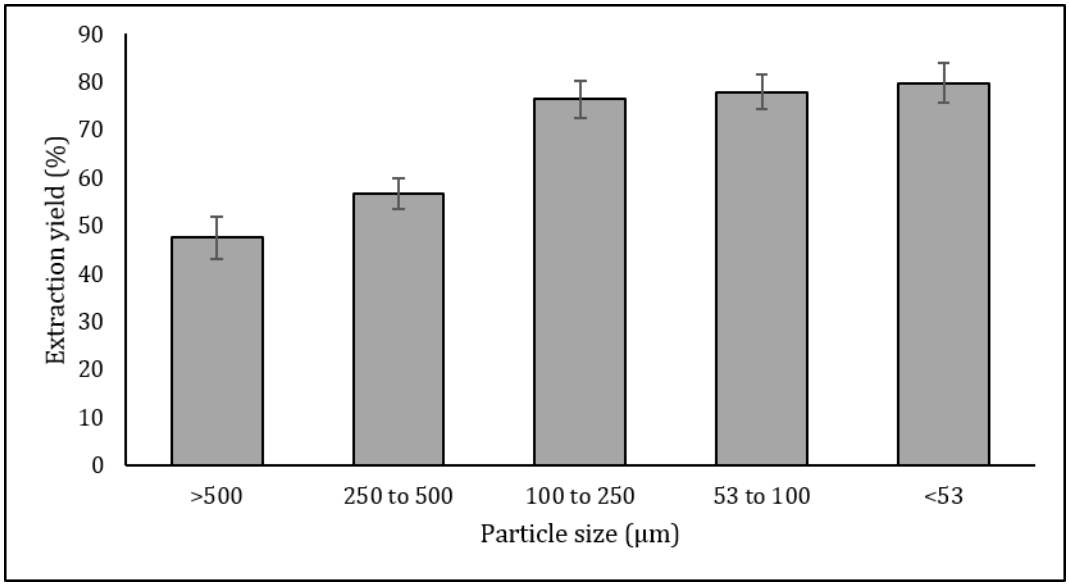


Figure 11: Effect of particle size on extraction efficiency.

To assess the effect of particle size on kafirin extraction, sorghum flour sieved through a set of different size mesh screens. This resulted in flour portions of specific particle size distributed over certain range i.e. <53 µm, 53 to 100 µm, 100 to 250 µm, 250 to 500 µm and >500 µm. Smaller particle size offers a higher surface area for extraction compared to larger particles for the given amount of solid and hence higher kafirin yield under similar extraction conditions. These

fractions were analysed and it was found that their protein content was comparable irrespective of the particle size difference. As evident from Figure 11, larger particle size (up to 250 μm) resulted in low extraction yield and particle size smaller than 250 μm resulted in significantly higher extraction yield.

In case of larger particle size, limited diffusion and inaccessibility of kafirin molecules at the core a feed particle would limit the rate of extraction. Diffusion of solvent occurs from bulk to the external surface of the feed particle. Since the sorghum flour particles are porous, further solvent diffusion occurs within pores towards the core. Once at the core, kafirin molecule dissolved by reactants leads to desorption from the surface within the pores. Diffusion of dissolved kafirin molecules from the interior of the pores towards the pore mouth at external surface occurs. Mass transfer of kafirin molecules from the external surface to the bulk occurs as the final step. Diffusion of solvent on the external surface of the particle and kafirin from the external surface to bulk can be categorised as external diffusion whereas mass transfer within pores can be categorised as internal diffusion (125). Larger particles would limit the internal diffusion i.e. movement of solvent from bulk to the core and subsequently movement of dissolved kafirin molecules from the core to the external surface and finally to bulk fluid. As a result, kafirin molecules near the external surface were extracted and inaccessible molecules at the core lost in raffinate leading to inefficient recovery. Smaller particle size allows faster internal diffusion and extraction from the core and no longer limits the rate of extraction (125).

As the mass transfer coefficient is a function of hydrodynamic conditions such as fluid velocity (rpm) and particle diameter (μm). Higher mixing rates/fluid velocity reduces the external boundary layer surrounding the particle through which the kafirin molecules migrate to the bulk, consequently eliminating the possibility of extraction rate limitation due to external diffusion. Increase in fluid velocity (rpm) and/or lower particle diameter (μm) decreases external resistance which leads to increase in mass transfer coefficient until saturation or kafirin exhaustion in the feed material, whoever occurs first (125,126).

It can be concluded that feed particle size smaller than 250 μm would result in the efficient and higher extraction yield. Since process optimization and scale-up

to commercial scale were primary objectives of the study, particle size ranges were considered, however, particle size distribution within the given ranges needs to be investigated to understand the significant difference in the yield. Study with smaller particle size/range needs to be carried out to understand extraction kinetics and other rate limiting factors.

3.3.1.4. Effect of temperature

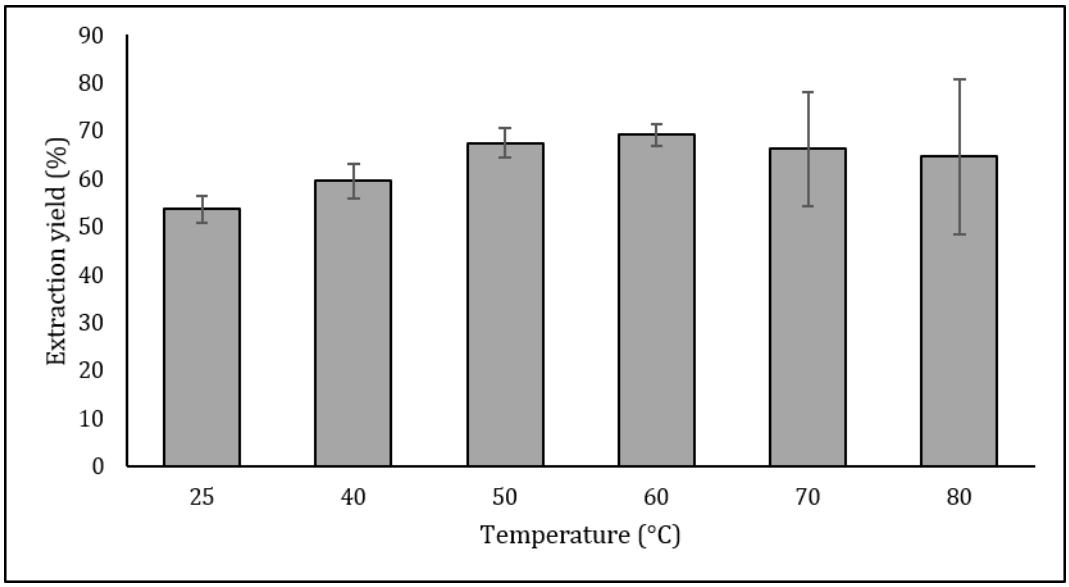


Figure 12: Effect of temperature on extraction efficiency.

Kafirin was extracted using ethanol with sodium metabisulphite, pH 12.0 at different temperatures to assess the effect of temperature. It was observed that extraction efficiency increased with increase in temperature up to 60 °C (Figure 12), and decreased with further increase in the temperature. At temperature 70 °C and higher, there were inconsistent incidences of gelling after centrifugation which resulted in a lower kafirin yield. Kafirin yield at temperatures higher than 60 °C was inconsistent compared to lower temperatures and varied up to 16%. Analysis of supernatant, gel layer and raffinate for the nitrogen content confirmed that the gel layer formed at high temperatures trapped extracted kafirin from the bulk upon centrifugation and further reduced yield by retaining the kafirin within the raffinate. Extraction from separated gel layer, supernatant and raffinate confirmed the presence of kafirin in these process streams.

It was also found that kafirin solubility increased with increase in temperature up to 60 °C. Increased temperature and high pH in the presence of reducing

agents leads to deamidation of glutamine and asparagine resulting in glutamate and aspartate. Deamidation leads to decrease in interchain and intrachain Glu-Glu (glutamic acid) interactions that induce electrostatic repulsion of charged glutamate residues and a decrease in hydrophobicity (127). Synergistic effect of high temperature, pH and reducing agent increased the solubility of kafirin in extraction solution and increase the yield. The findings of this study are consistent with the zein extraction process at commercial scale (128).

3.3.1.5. Solid to liquid ratio

Initially, an experiment was performed (section 3.2.3.1) to assess the suitable solvent for the kafirin extraction where the highest solid to liquid (S/L) ratio was considered to avoid bulk saturation to maintain significant mass transfer. Solid to liquid ratio optimised to assess the minimum required solvent volume to achieve the optimum kafirin yield.

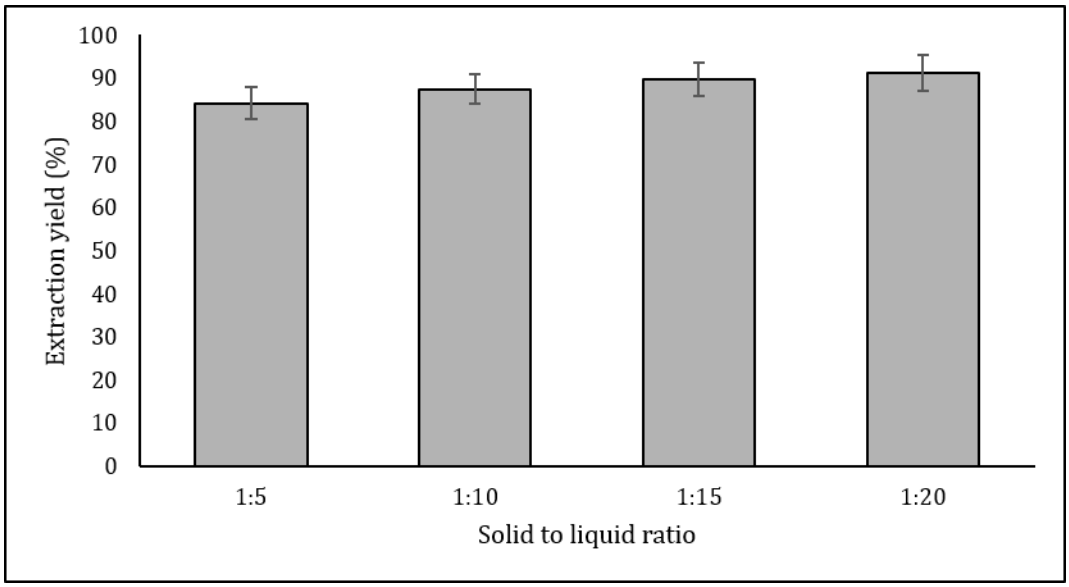


Figure 13: Effect of solid to liquid ratio on Kafirin extraction.

As obvious from Figure 13, difference between the increase in kafirin yield with increasing liquid ratios was not significant. For instance, there was a significant difference (~6 %) between the yield from the S/L ratio of 1:5 and 1: 20. However, subsequent S/L ratio throughout the range showed no significant increase (~2 % to 3%). Another major observation was increase is kafirin yield was not proportional to the additional volumes of solvent used. Although the volume of solvent was 1.5x and 2x higher, there was no comparative increase in the yield

which indicates that there was no extractable kafirin in the raffinate as higher S/L ratio would provide conditions far from saturation and solvent volume required at higher S/L ratio were statistically significant. According to reported studies, a high concentration of solids in the extraction mixture affect the diffusion of both solvent and kafirin in the bulk (126,129). It was demonstrated that such shortcoming could be eliminated by increasing the liquid component. Besides, considering variability in the raw material i.e. higher protein/kafirin load similar yield can be achieved by using slightly higher (1:6) S/L ratio.

3.3.2. Separation

Removal of raffinate from the mixture done by different unit operations that work on different principles. Both centrifugation and/or filtration can be used to separate solids from the extract at a laboratory and higher scale, which are scalable in both linear and non-linear fashion. These unit operations used separately and in tandem to clarify the supernatant to reduce total suspended solids and turbidity.

3.3.2.1. Centrifugation

Data obtained from the preliminary studies suggest that the output streams produced at low centrifugation speed and operation time lead to the formation of loose pellets as well as higher solvent retention. Loss of extraction solution trapped in these loose pellets leads to the loss of kafirin in the early stages of the extraction process. Process streams obtained from low RPM and 10 minutes of centrifugation were relatively more turbid and with a significant amount of solids suspended in the liquid.

The amount of suspended solids in the supernatant was critical as it affects the performance of the subsequent unit operation i.e. filtration and quality of the process intermediate from distillation. Liquid trapped in these pellets retrieved by vacuum filtration; however, overall filtration area required was several folds higher compared to supernatant alone. In other words, the filter area requirement increases by several folds to recover the retained liquid, which ultimately increases overall operation costs.

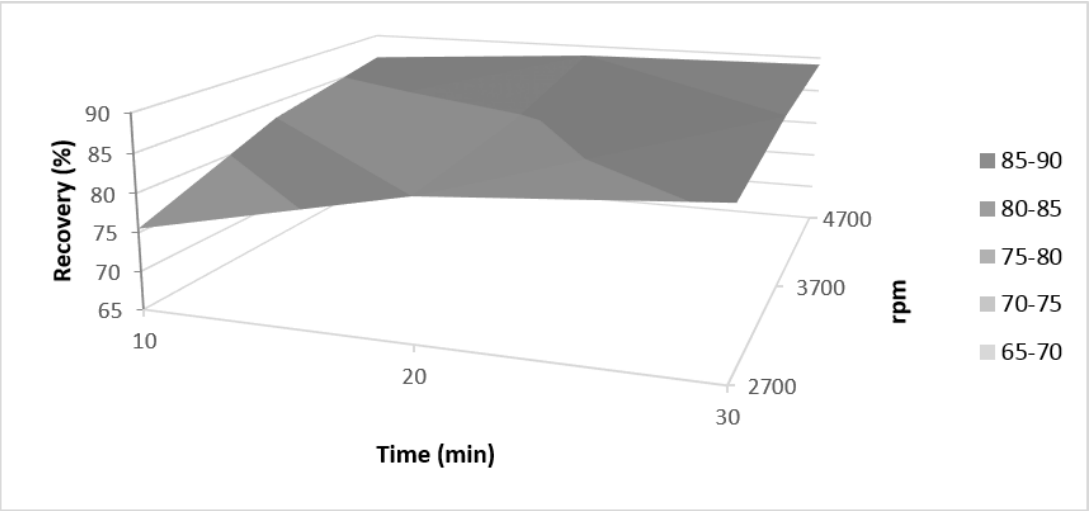


Figure 14: Post centrifugation recovery (%) of extraction solution at different centrifugation speed (rpm) and time (min).

Extraction slurry composed of the coarse sorghum flour (particle size >800 µm) produced an unstable pellet and supernatant separation was not possible by decanting. Initially, the mixture was filtered through 100 µm mesh to retain the raffinate resulting in low solvent retention. Resulting filtrate was turbid and particulate, which when processed further resulted in low kafirin purity and yield (18.27%). Extract volume recovery after centrifugation for single extraction (87%) was lower than the subsequent extraction (93%) as the same pellet after the first extraction was re-extracted resulted in reduced liquid retention.

3.3.2.2. Filtration

Filtration is any physical, mechanical or biological process where solids are separated from the liquid or gas using a filter media that allows the liquid or gas to flow through but retains the solids. Filter sizing was performed using constant pressure test where the challenge solution is passed through the filter at a selected differential pressure and the filtered volume is recorded as a function of time (usually for 10 minutes). The experiment was set up as shown in Figure 15 where 500 mL Schott bottle was used dispensing vessel with regulated air pressure (5 psi). All experiments were conducted in triplicates. Details of filters and their specifications are in Table 6.

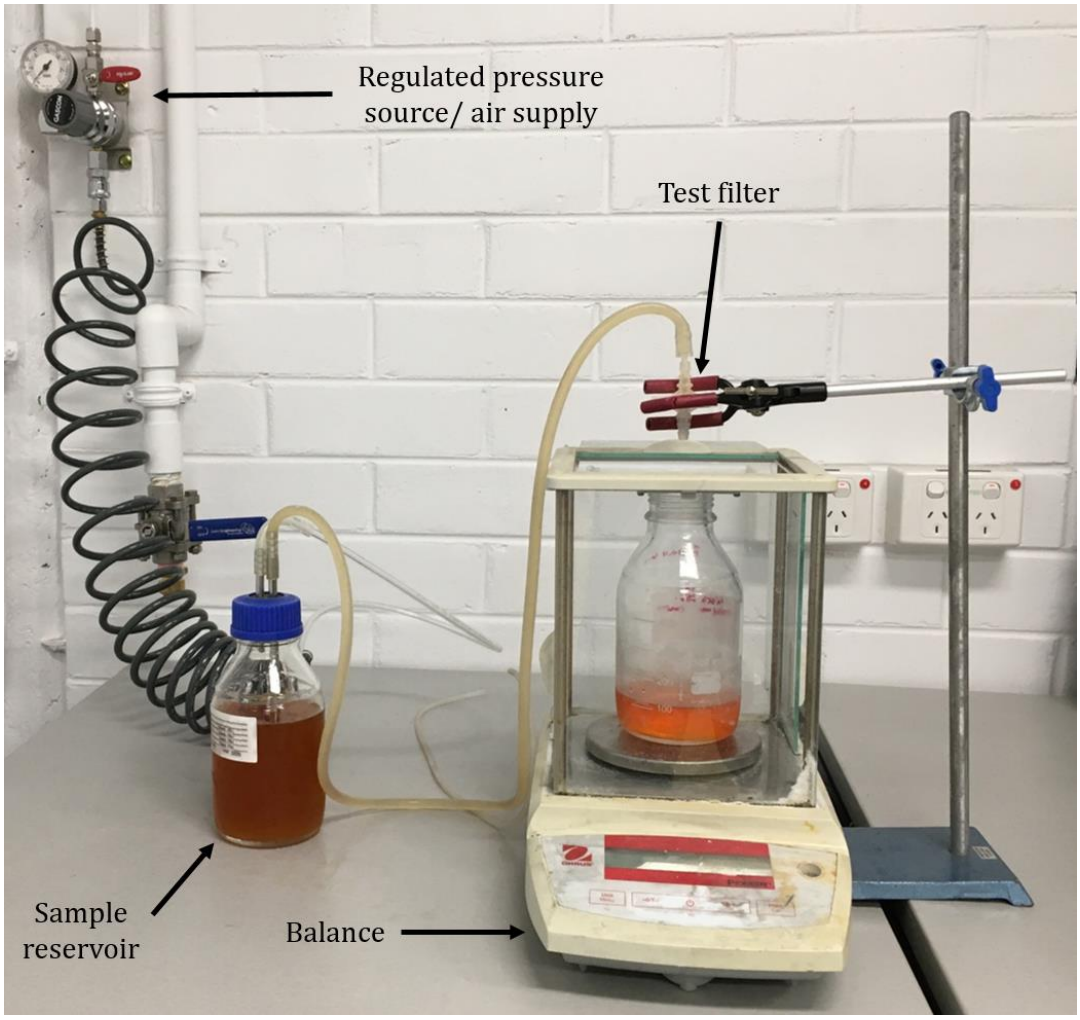


Figure 15: Test assembly setup for filter sizing study.

The supernatant obtained from 20 minutes of centrifugation at 4700 RPM considered as an ideal input for filtration, as it was relatively clear of suspended particles. Centrifuge output was passed through the filters until there was no more flow from the outlet. These volumes were recorded to assess the filtration capacity of a filter (Figure 16).

Table 6: Filters under evaluation and their specifications.

Pore size (µm)	Size (mm)	Effective filtration area (cm^2)
0.2	32	5.8
0.45	32	5.8
0.8 + 0.2	32	5.8

Quality of the filtration output not only affect the performance of subsequent unit operations but also the quality of the kafirin extracted as the end-product. Filtration plays a vital role in terms of producing a clear feed for the subsequent unit operation, which is distillation. The resulting volume in conjunction with the effective filtration area will be used to estimate the size of the filter required at the proposed manufacturing scale and discussed with scale-up.

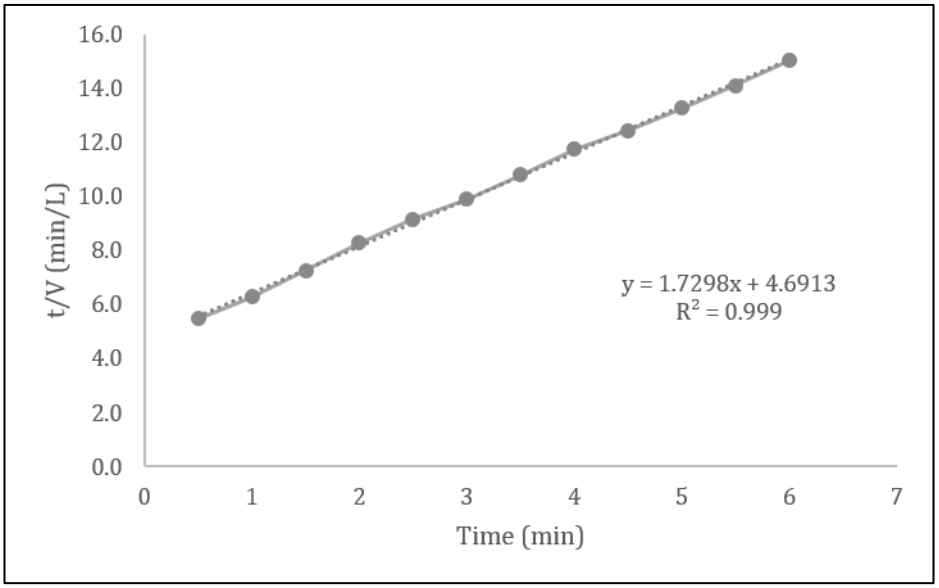


Figure 16: Filter sizing study for centrifugation output clarification.

An experiment was carried out to assess the filter size required at both laboratory and higher scale batches. The time required to filter per unit volume (min/L) was plotted against time (min) where R^2 value was greater than 0.99, which indicates that Vmax model can be applied to this filter for which the main fouling mechanism is gradual pore plugging. Maximum process stream volume (L) that can be filtered using a given filter calculated using the following formula.

$$V_{max} = 1/slope$$

For the selected filter (0.8 + 0.2 µm), the slope was 1.7298 resulting in 0.578 L maximum filterable volume with 58 cm^2 total filtration area. At higher scale, additional filters or a single filter with a larger area will be used for higher process volumes.

3.3.3. Distillation

Distillation is a process of separation of liquids from the mixture based on their boiling points and condensation. In other terms, separation of components from

mixtures based on dissimilarities in the conditions required to change the phase of individual components. The clarified extract was further processed with distillation to recover ethanol. The efficiency of this unit operation capacity depends on different parameters such as heating bath temperature, vacuum applied, size and rotation speed of the evaporating flask. Above listed process parameters were optimised for the process at lab scale and provide a customisable platform and assembly design at a higher scale. During vacuum distillation, lower pressure reduces the boiling point of the solvent making it less energy-intensive and faster than the processes carried out under normal ambient conditions.

Distillation parameters were optimised to reduce process time and make the process less energy-intensive. Heating water bath and vapour temperature can be adjusted as per the boiling point of the solvent at a given pressure. Vapour pressure (P) increases gradually with an increase in temperature (T) and the relation can be expressed as in equation 2. Pressure-temperature nomograph based on Clausius-Clapeyron equation 3 which describes the relationship between vapour pressure and the temperature was used to calculate the boiling point of the solvent at the given pressure. This equation allows us to calculate the vapour pressure at another temperature provided enthalpy of vaporization and vapour pressure at any temperature are known. The approach used for the current study was adapted from the literature where enthalpies of vaporization of binary mixture were estimated by the Clausius-Clapeyron equation (130,131).

$$P \propto e^{-\Delta H_{vap}/RT} \qquad \textit{Eq. 2}$$

$$\ln\left(\frac{P_1}{P_2}\right) = \frac{\Delta H_{vap}}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \qquad \textit{Eq. 3}$$

Where, ΔH_{vap} is the enthalpy of Vaporization, R is the gas constant (8.3145 J mol^{-1} K^{-1}), P_1 and P_2 are the vapour pressures at temperatures T_1 (actual boiling point) and T_2 (target boiling point).

McCabe-Thiele method (132) should be considered to determine the number of theoretical plates required to achieve the desired degree of separation for the given binary mixture. At higher scale, continuous distillation can be implemented with varying reflux ratio to achieve desired separation, i.e. >60 % ethanol concentration in present study considering solvent recycling.

The temperature difference between heating bath, evaporating flask and condenser should be 20 °C. The pressure in the evaporating flask set accordingly so that the boiling point of the solvent was around 40 °C. Based on the calculations (enthalpy of vaporization for ethanol 38.6 KJ mol^{-1})(133), the vacuum was set at 200 mbar to reduce the boiling point of ethanol to 40 °C from 78 °C (134). Heating bath temperature was set to 60 °C to ensure reduced water evaporation rate, efficient energy utilization and safety. Condenser temperature was set at 20 °C to absorb the energy from the solvent gained from the heating bath. The flow of the coolant from circulating chiller can be adjusted (based on the type of coolant used) to achieve desired temperature.

Input stream for distillation played vital role considering the yield and purity of the kafirin. It was observed that the extract obtained after centrifugation at low RPM for lesser time (and without filtration), when subjected to distillation, yield higher mass in terms of weight but results in low purity and kafirin yield (18.27%). The stream generated at the end of distillation with such input material was turbid, a non-homogenous suspension with other protein and non-protein impurities. As the solvent concentration gradually reduces with the progress of distillation, kafirin no longer remains soluble and precipitate out to forms an aqueous suspension. In the case of kafirin extraction, the quality of the input stream does not affect the performance of the distillation, as the sole purpose of this unit operation is the recovery of the solvent. Amount of ethanol recovered from 500 mL feed varied from 280 mL to 310 mL and ethanol concentration in the recovered stream ranged from 80 to 85%. Solvent recovery at the end of distillation was ranging from 74.67 to 87.83%.

The distillation process was further optimized based on the type of solvent used by assuring precise process conditions discussed as follows. Condensate should not cover more than 3/4 of the condenser and balanced by adjusting the vacuum as an increase in vacuum resulted in reducing the boiling point, which generated more vapours and vice versa. Immersion angle of the evaporating flask in a heating bath maintained such heat transfer is efficient along with optimum turbulence inside the flask. Stiffer immersion angle leads to inefficient heat transfer and low turbulence whereas flat flasks increased the contact surface for heat transfer but created higher turbulence leading to foaming and bumping.

Optimum evaporation flask rotation speed produced maximum turbulence inside the flask and prevented spillage of heating media at the same time. Size of the evaporation flask, as a larger surface area, resulted in greater turbulence and increased evaporation rate along with reduced bumping and foaming. However, larger flasks were more difficult to handle and are less flexible with changing immersion angles. Also, the thickness of the flask should not only allow efficient heat transfer from heat media through the glass to the solvent but also not too fragile.

Vacuum distillation was used as it offers several advantages over other types of distillation when it comes to ethanol recovery. As a closed system prohibits the release of ethanol vapours in the lab environment and assures safety. The process is less energy-intensive compared to simple distillation as the vacuum applied reduces the boiling point of the solvent needs to be recovered. The time required for the vacuum distillation is less than that of other types of distillation to recover the equivalent volume of solvent.

3.3.4. Isoelectric precipitation

The pI of most of the proteins lies within the pH range of 4 to 6 so the range for optimum pH scanning was restricted from 3.5 to 6.5 for selective kafirin precipitation. Ethanol-free distillation output stream distributed in seven aliquots (200 mL each) and pH of solutions adjusted to the desired pH (3.5 to 6.5 with an increment of 0.5 units). The samples were analysed for total protein and kafirin content where extractable kafirin (%) expressed in a percentage calculated from the total protein in precipitates, however, quantitative analysis for supernatant for kafirin was not possible as the stream was diluted with a very low concentration of kafirin. Total protein yield obtained at pH 3.5 after 120 minutes incubation was comparatively higher and decreased with further increase in pH up to 5.5 Figure 17. Data for pH 6.0 and 6.5 were not as significant as the amount of protein precipitates was low and inconsistent. Low protein yields at higher pH range also supported by the data for protein content of the respective supernatants.

Unlike total protein, kafirin yield was relatively low at pH 3.5 and increased gradually with further increase in pH up to 5.0 as pH was closing in towards pI.

However, a sharp decrease observed at pH 5.5 as the pH moved away from kafirin pI. Total protein and kafirin content of samples at different pH suggest that the maximum kafirin recovered at pH 4.5 and 5.0. Although the total protein recovery was maximum at pH 3.5, it is evident that other major proteins (such as albumin, globulin, glutelins and other) also co-precipitated along with kafirin.

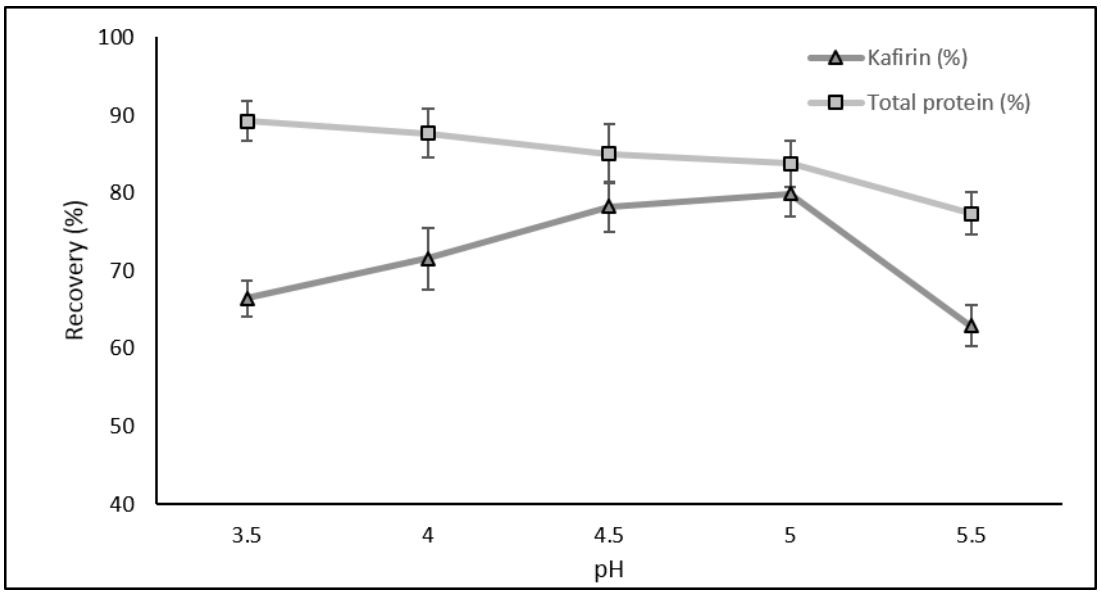


Figure 17: Total protein and Kafirin yield at different pH.

Pellets and supernatants samples from each pH set point were analysed for protein content in individual samples. pH 5.5 samples were not included as both protein recovery and kafirin yield were relatively low compared to other pH set points.

Supernatant and pellets samples from isoelectric precipitation studies analysed with SDS-PAGE (Figure 18) confirmed that maximum kafirin was recovered at pH 5.0. However, at lower pH set points more kafirin was retained in the supernatant, which was confirmed by increasing concentrations of kafirin at lower pH set points. On the other hand, kafirin pellet sample analysis from pH 5.0 setpoint suggests that kafirin purity was higher (Figure 18 gel a) but further decrease in pH attracted more protein impurities. The amount and type of protein impurities varied as pI for different protein impurities close to kafirin also precipitated along with kafirin.

It was observed that protein precipitates increased in the first 90 minutes and there was no significant increase in precipitates with further increase in time up

to 120 minutes. Similarly, kafirin yield also increased with increase in time up to 60 minutes, however, incubation for more than 60 minutes leads to precipitation of other protein impurities but no significant increase in kafirin yield. Also, it was found that mixing for prolonged durations (up to 120 minutes) disintegrated the precipitate leading to loss of kafirin and total protein in the supernatant, an observation later confirmed during process characterization studies for isoelectric precipitation step (Figure 33).

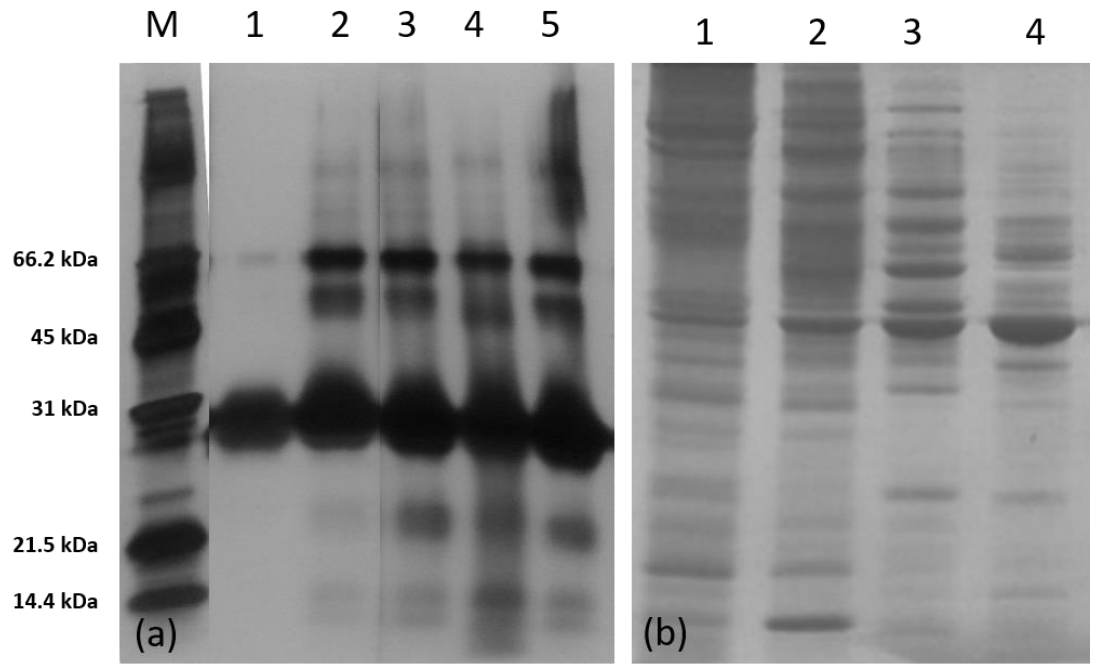


Figure 18: SDS-PAGE profile for samples from isoelectric precipitation study.
Gel a: lane M- Broad range protein marker, 1- Purified kafirin, 2- Kafirin pellet pH 5.0 (NR), 3- pellet pH 4.5 (NR), 4- pellet pH 4.0 (NR), pellet pH 3.5 (NR).
Gel b: lane 1- Supernatant pH 5.0 (NR), 2-Supernatant pH 4.5 (NR), 3- Supernatant pH 4.0 (NR), 4-Supernatant pH 3.5 (NR).

In general, when protein refolds, hydrophobic amino acids form the core and the hydrophilic amino acids are on the surface of the protein with scattered hydrophobic patches. The solubility of the protein in any solution depends on the net charge of these hydrophobic and hydrophilic amino acids present on the surface. Kafirin has a high number of hydrophobic amino acids and hence water-insoluble, but soluble in organic solvents such as ethanol. Isoelectric point (pI) of the protein, pH at which net charge of a protein becomes zero in a given solution, is another factor that plays an important role in the solubility. Protein acquires positive charge with a decrease in pH of the solution and gains negative charge

when the pH of the solution is below its pI. For instance, proteins with high hydrophilic amino acids on the surface have a higher solubility in an aqueous buffer; however, higher hydrophobic amino acid content on the surface decreases the solubility. The similar (positive or negative) charges generated on the surface of the adjacent protein molecules at pH of a solution other than its pI exhibit the repulsive forces and repel the protein molecules away from each other. On the contrary, when the pH of the solution is the same as the pI, the net charge on the surface becomes zero and there are no repulsive forces. This leads to loss of water monolayer on the surface of the protein, which in turn facilitates the interaction of intermolecular hydrophobic amino acids and formation of precipitates. Owing to this property of proteins, precipitation is a widely used technique in downstream purification processing of protein-based products in food, dairy, plasma fractionation and biopharmaceuticals.

3.3.5. Oil removal

Protein precipitates obtained as a pellet from the isoelectric precipitation were homogenised and divided into equal parts. These precipitates were treated with different solvents at 60 °C for one hour in stirring condition (200 RPM) to assess their oil extraction capacity. At the end of incubation, the mixture was centrifuged, the supernatant was collected separately and the pellet was resuspended in the same solvent for re-extraction, this process was repeated twice. Oil recovered from individual solvent wash were separated and weighed.

Table 7: Oil removal from precipitates using different solvents.

Solvents	Number of washes	Oil recovery (g/100 g feed)	Oil recovery (%)
Hexane	3	6.87	86.85 ± 3.71
Ethyl ether	5	6.32	79.90 ± 7.93
Ethyl acetate	4	6.51	82.30 ± 3.24

It can be observed from the data (Table 7) that the total amount of oil extracted by all solvents were comparative, however, overall oil recovery with hexane was higher compared to ethyl ether and ethyl acetate. To remove 7.91 g of oil from 100 g of feed material, 3 washes of hexane required compare to 5 and 4 for ethyl ether and ethyl acetate. Hence, the total volume of solvent required to extract

maximum extractable oil from the same feed was significantly less in the case of hexane.

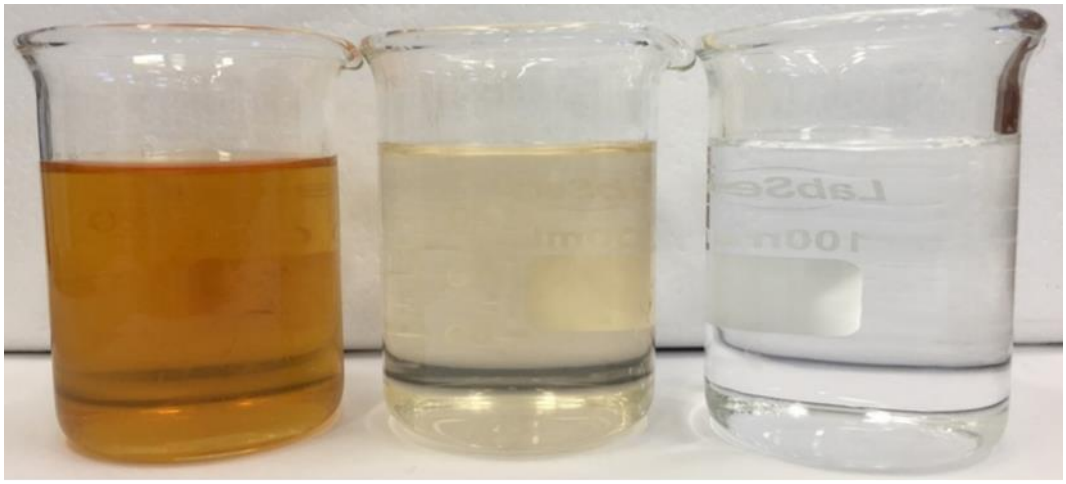


Figure 19: Oil removal with hexane, first wash (a), second wash (b) and third wash (c).

Initial hexane wash removed a significant amount of oil compared to the second and third wash, where the amount of oil removed with third wash was insignificant or unquantifiable (Figure 19). On the other hand, Ethyl ether and ethyl acetate took 5 and 4 washes respectively i.e. significantly higher volumes of solvents. Oil removal carried out at room temperature using ethyl ether as it has a low boiling point (34.4 °C) and highly volatile, a probable reason for the variability in oil recovery.

From a safety perspective, the residual solvent in the final product was taken into consideration. As per ICH guidelines, solvents are classified according to their Permissible Daily Exposure (PDE) (mg/day) or concentration limit (ppm) based on available toxicity data (135). Solvents used throughout the process are summarized below in Table 8 along with their class and residual limit.

Table 8: Solvents used for kafirin purification and their classification.

Solvent	Class	PDE (mg/day)	Concentration limit (ppm)
Hexane	2	2.9	290
Ethyl ether	3	50	5000
Ethyl acetate	3	50	5000

Ethanol	3	50	5000

3.3.6. Drying

Oil-free precipitates obtained after hexane wash at laboratory scale were incubated in a hot air oven for drying to remove hexane and residual water. Pellets obtained after drying were crushed to powder using mortar-pestle and stored at 2 °C to 8 °C. At higher scale, kafirin obtained after hexane wash was homogenised and spray dried using a mini spray dryer (B-290 advanced, Buchi). Residual solvent and water were recovered using inert loop and dehumidifier respectively. Kafirin dried using both procedures weighed to calculate recovery and assessed for the solubility of the final product.

Kafirin dried using the hot-air oven was flakier and non-uniform (Figure 20, a) whereas spray-dried kafirin was uniform and powdery (Figure 20, b) in terms of particle size and texture. Kafirin purified kafirin with an oven and spray drying tested for solubility, it was observed that the spray-dried kafirin showed higher solubility compared to oven-dried kafirin. Scanning electron microscopy (SEM) examination of spray-dried kafirin revealed that the size of particles generated was in microns. Lower particle size generated offered larger surface area and hence increased solubility compared to oven-dried kafirin. Optimization of the spray drying process parameters to manufacture of kafirin particles within the desired size range and discussed in chapter 8.

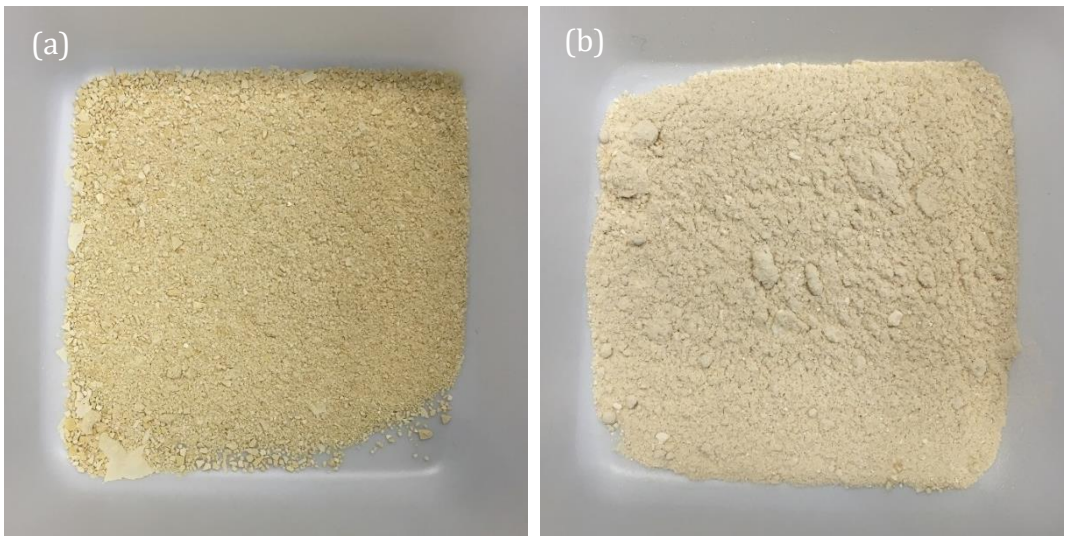


Figure 20: Final product oven-dried (a) and spray-dried (b) kafirin powder.

Data from Table 9 provides the comparative analysis between different drying processes in terms of recovery (%) and solubility (%) of the kafirin produced as a final product. Kafirin drying using hot air oven offered 100% recovery as the solvent evaporated at a higher temperature for prolonged duration leaving the kafirin cake, which then pulverised in fine powder whereas kafirin recovery with spray drying was up to 97 %. Since kafirin purified from developed process dissolved in an appropriate solvent system to materialise application, the solubility of the kafirin produced from both processes assessed, which varied significantly. Spray-dried kafirin showed 100 % solubility whereas solubility of oven-dried kafirin was around 74%.

Table 9: Comparative data for recovery and solubility from different drying processes.

Drying process	Recovery (%)	Solubility (%)
Oven	100	74.41 ± 6.29
Spray dryer	94.28 ± 2.57	100

Difference between the solubility of kafirin from different drying processes can be attributed to properties of the kafirin produced and/or nature of the purification and/or drying process. For instance, kafirin dried in hot air oven forms cake, which then powdered resulting in particle size varying over a broad range whereas spray dryer produced kafirin particle size was restricted in micron. Consequently, microparticles generated with spray drying offered desired particle size (in microns) and higher surface area for solubilisation and hence faster and increased solubility. Also, oven drying exposes kafirin molecules at higher temperatures for prolonged duration i.e. approximately 8-10 hours to overnight whereas during spray drying, kafirin molecules exposed to a higher temperature for few seconds or less based on flowrate and protein concentration in the feed solution. The probable reason behind the Exposure to higher temperature might have altered the molecular structure of kafirin or induced higher degree polymerization leading to the generation of higher molecular weight aggregates with limited solubility in aqueous alcohols (38,42). However, the mechanism behind the difference in solubility needs to be investigated at the molecular level for kafirin produced from different drying processes.

The drying process also ensured the removal of solvents used throughout the process. As the boiling point of both solvents, ethanol (78 °C) and hexane (68 °C) was less than the drying temperature (120 °C); these residual solvents recovered using inert loop in a spray dryer. It was confirmed when the final product analysed for moisture content as the weight difference was <1%.

3.4. Quality by design

QbD was implemented to identify the design space for the kafirin extraction process. QTPP and CQAs were identified and product design space was defined. A risk assessment carried out using FMEA to identify critical process parameter (CPPs). Identified CPPs were examined using DOE to determine the effect of individual parameter and their interactions on CQAs. process design space for the individual step was established based on characterisation studies, PARs for CPPs were established and validated.

3.4.1. QTPP and CQAs

QTPP and CQAs were identified considering optimum recovery and purity of the kafirin produced. Another important desirable trait of the kafirin being solubility of the final product as kafirin needs to be solubilised using appropriate solvent for application development.

3.4.2. Product design space

Recovery, purity and solubility were identified as CQAs. Essentially, each CQA needs defined and acceptable variability range to define the product design space. These ranges ultimately govern the process development as narrow ranges would require processes/ unit operations to be operated within narrow ranges i.e. small design space whereas broad ranges would allow processes to be operated over a wide range, hence significantly larger design space that offers flexibility with the operation. For instance, scaffolding in tissue engineering or similar applications would require high purity kafirin (>99%) which changes the entire QTPP of the kafirin to be produced. Here, we have discussed kafirin QTPP and CQAs considering films and spray coat as primary applications. CQAs with

acceptable ranges that defines QTPP that defines product design space are summarised in Table 10.

Table 10: CQAs with acceptable ranges for the kafirin as a final product.

CQAs	Specifications
Purity (%)	>95%
Recovery (%)	>70%
Solubility (%)	>95%

3.4.3. Risk assessment

Risk analysis was carried out using the failure modes and effects analysis (FMEA) tool (136) to identify the parameters and their operating ranges that are likely to affect the overall process performance and product quality. Risk analysis was carried out based on three criteria, severity (S), occurrence or likelihood (O) and detection (D). Severity score assesses the impact of a failure mode due to a parameter and is estimated for an excursion three times the operating range for that particular parameter in certain unit operation. Occurrence score assigned based on how often the failure may occur or its likelihood, whereas, detection score specifies the probability of detection and possibility of correction of the excursion (Table 11). Product of all these scores provides the risk priority number (RPN) for individual parameters, which then ranked to identify the potential parameters with high risk for further analysis using DOE.

Risk analysis carried out using FMEA as discussed above for the individual process parameter for each unit operation. Parameters considered for these unit operations are listed and discussed for their impact in terms of potential effects of failure and possible causes leading to failure of one or more CQAs.

Table 11: Risk analysis tool used for performing FMEA for the proposed process to define the design space. RPN score derived from Severity, likelihood, and detectability for individual parameter were examined using this tool.

Risk score	Severity (S)	Occurrence/ Likelihood (O)	Detectability (D)
	(Assume excursion ~ 3× outside prescribed range)	(Assume excursion outside normal operating range)	(Assume excursion outside normal operating range)
5	Fails final product specs >50% of the time, or product unrecoverable.	>20%	No way to detect the impact
4	Fails in-process performance parameters >50% of time or 25% yield loss.	5–20%	Unit sampling and inspection. Defect not detected before process impact.
3	Runs on edge of in-process acceptance limits or approximately 10% yield loss.	1–5%	Auto-detection/ inspection. Defect not detected before process impact.
2	A slight effect on in-process quality attributes and yield.	<1%	Auto detection/ inspection. Defect detected before process impact.
1	No effect on performance.	Never	Obvious defect. Always detected.

Table 12: FMEA of operating parameters for the extraction step.

Parameters	Potential failure mode	Potential effect(s) of failure	Potential cause(s) of failure	S	O	D	RPN
Ethanol	Weighing or operator error	Low protein recovery	Extraction solution saturation leading to loss of protein in the raffinate	5	2	5	50
Sodium hydroxide	Weighing or operator error probe malfunction	Low protein recovery Low purity	Low may result in the incomplete dissolution of protein and high may lead to higher impurity levels.	5	2	5	50
Sodium metabisulphite	Weighing or operator error	Low protein recovery	Less may reduce protein dissolution and high may not have any impact.	5	2	5	50
Extraction temperature	Instrument malfunction or operator error	Low protein recovery Low purity	Kafirin trapped in starch gelling at high temperatures and higher non-protein impurities. Loss of protein in raffinate due to incomplete extraction at lower temperatures.	5	2	2	20
S/L ratio	Weighing or operator error	Low protein recovery Low purity	Low may lead to loss of protein in raffinate due to saturation of extraction solution and high may not have any impact on recovery but attract higher impurities.	5	2	2	20
RPM	Instrument malfunction or operator error	Low protein recovery	Low may lead to improper mixing and sedimentation leads to incomplete extraction and hence poor yield. High may not have any impact.	5	2	1	10
Extraction time	Operator error	Low protein recovery Low purity	Less time leads to loss of protein in raffinate due to incomplete extraction and high in higher impurity levels.	5	1	2	10

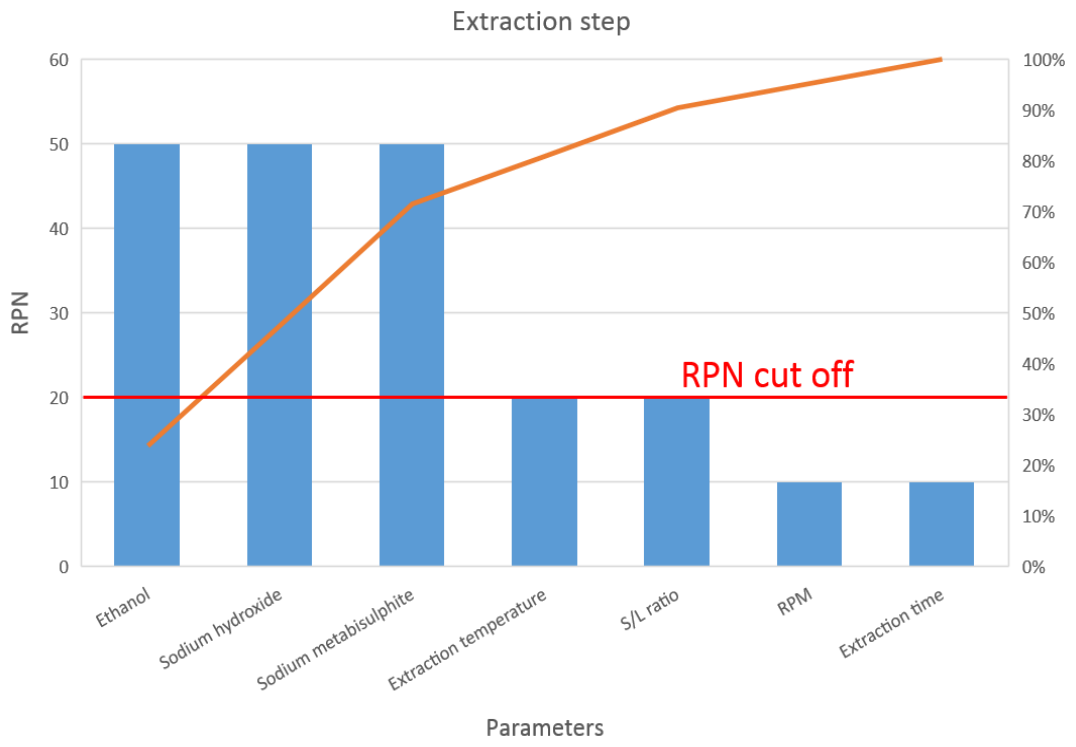


Figure 21: Pareto chart for parameter screening from FMEA analysis for the extraction step.

Ethanol concentration, sodium hydroxide (NaOH) concentration, sodium metabisulphite ($Na_2S_2O_5$) concentration, extraction temperature, S/L ratio, mixing speed (RPM) and extraction time were the parameters considered for extraction step. The outcome of FMEA analysis (Table 12) and Pareto chart was plotted based on the RPN score obtain from the potential excursion for a particular parameter. Some of the parameters might adversely affect when operated beyond higher or lower range while some may affect when operated beyond one of the extremes. For instance, high concentration of NaOH may lead to extraction of both protein and non-protein impurities such as oil whereas low concentration may lead to incomplete extraction leading to loss of protein in raffinate and hence low recovery. Similarly, low mixing speed may result in sedimentation and incomplete extraction whereas high may not have any impact. From FMEA analysis, ethanol, NaOH and $Na_2S_2O_5$ concentrations identified as the potential parameters with high RPN scores (Figure 21) that might significantly affect the performance of extraction step as unit operation and subsequently the overall process. These parameters will be screened with DOE to identify the design space for the extraction step.

Table 13: FMEA of operating parameters for the separation step.

Centrifugation

Parameters	Potential failure mode	Potential effect(s) of failure	Potential cause(s) of failure	S	O	D	RPN
RPM	Instrument malfunction or operator error	Low recovery and low purity	Low may lead to loose pellet formation, Higher liquid retention in pellet, Particulate supernatant. High may not have any impact.	5	2	1	10
Time	Instrument malfunction or operator error	Low recovery and low purity	Low may lead to loose pellet formation, Higher liquid retention in pellet, Particulate supernatant. High may not have any impact.	5	2	1	10

Filtration

Parameters	Potential failure mode	Potential effect(s) of failure	Potential cause(s) of failure	S	O	D	RPN
Filter area	Operator error	Low recovery and low purity	Less may result in filter choking and high may not have any impact.	5	2	1	10
Volume	Operator error	Low recovery and low purity	Less may not have any impact and high may result in filter choking.	5	2	1	10

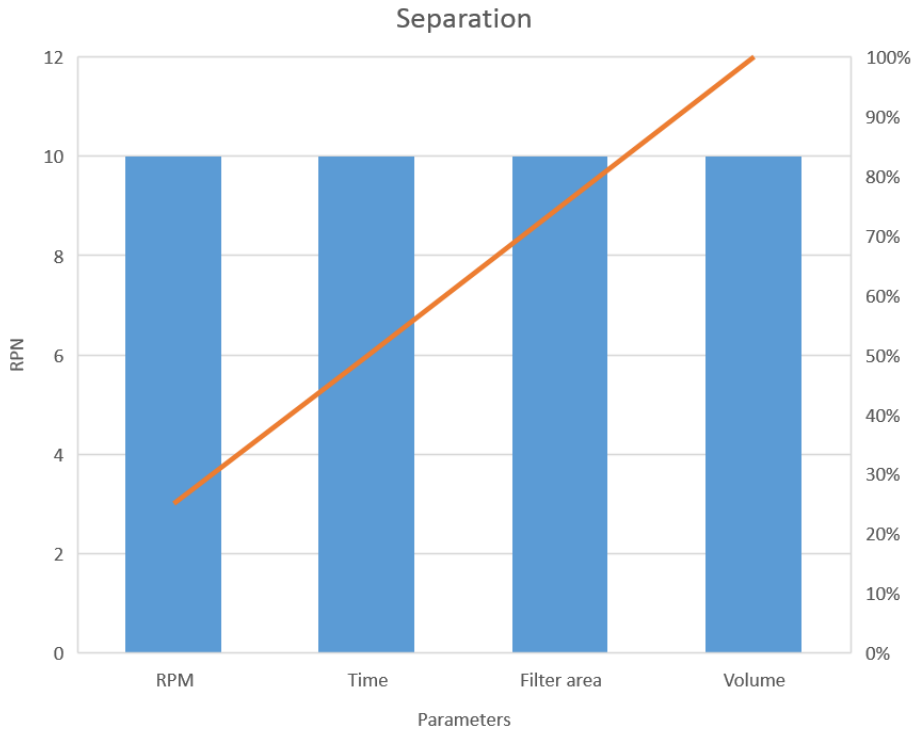


Figure 22: Pareto chart for parameter screening from FMEA analysis for the separation step.

For separation steps, centrifugation speed (RPM) and time in centrifugation and filtration area and volume for filtration were considered for FMEA analysis. The outcome of the analysis and their RPN scores are listed in Table 13. Pareto chart was plotted with obtained RPN score based on their excursion, however, all the parameters have the same score. Most of these parameters, when operated at one of their extremes, might affect the recovery and subsequently purity and overall process yield. In case of centrifugation, both low speed and low time may result in loose pellet formation, higher liquid retention in the pellet with particulate supernatant, which leads to low purity and yield whereas high, may not have any impact. Similarly, low filtration area may result in filter choking; high may not have any impact and high filtration volume may lead to filter choking whereas low may not affect the process.

From FMEA analysis, it can be concluded that all the parameters, centrifugation speed (RPM) and time for centrifugation and filtration area and volume for filtration have low RPN score (Figure 22) and excursion at one end might affect the process. Hence, these can be screened for their impact at one of their extremes.

Table 14: FMEA of operating parameters for the distillation step.

Parameters	Potential failure mode	Potential effect(s) of failure	Potential cause(s) of failure	S	O	D	RPN
Temperature	Instrument malfunction or operator error	Low recovery and low purity	Low may increase operation time and high may lead to loss of protein due to frequent bubbling.	5	4	5	100
RPM	Instrument malfunction or operator error	Low recovery low purity	Low may increase operation time and high may lead to loss of protein due to frequent bubbling and excessive agitation.	5	2	2	20
Vacuum	Instrument malfunction or operator error	Low recovery and low purity	Low may increase operation time and high may lead to loss of protein due to frequent bubbling.	5	2	2	20

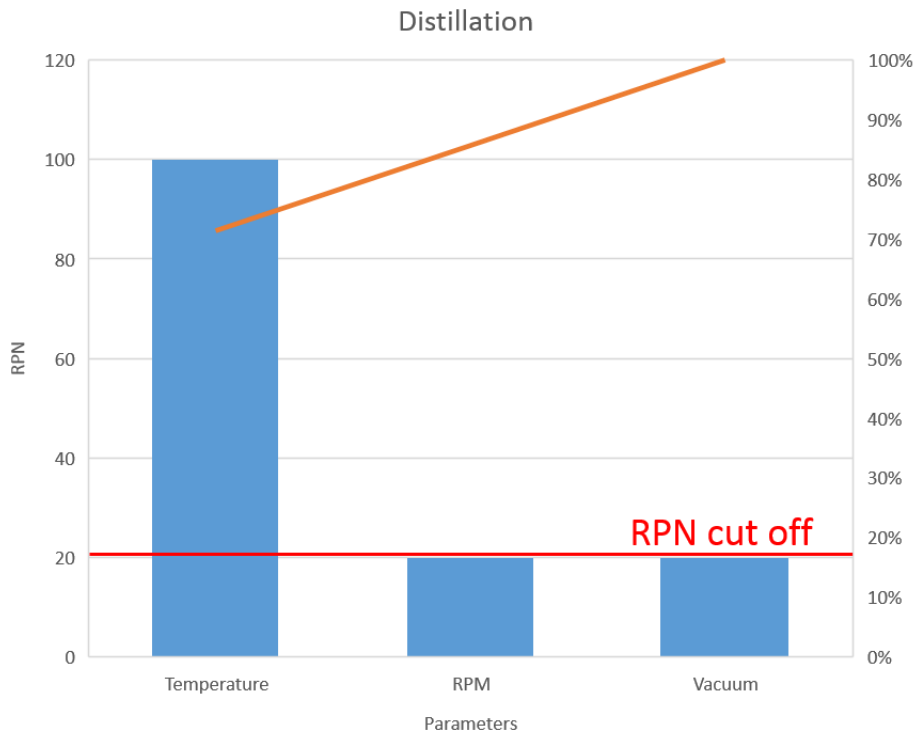


Figure 23: Pareto chart for parameter screening from FMEA analysis for the distillation step.

Temperature, mixing speed and vacuum applied were the parameters considered for FMEA analysis for distillation step. The outcome of the analysis and their RPN scores are listed in Table 14. Pareto chart was plotted with obtained RPN score based on their excursion, the only temperature has high RPN score whereas the other two have low scores. High temperature may lead to frequent bubbling and protein denaturation, which affects both purity and yield. Mixing speed and vacuum applied, their excursion from higher range end may lead to loss of protein only observed at higher temperatures. Both these parameters at a lower range may not impact the process.

From FMEA analysis, it can be concluded that the parameters, Mixing speed and vacuum have low RPN score (Figure 23) and can be screened for their impact at one of their extremes. The temperature was the only parameter with high RPN score that needs to be screened for the ranges to obtain optimum yield.

Table 15: FMEA of operating parameters for the isoelectric precipitation step.

Parameters	Potential failure mode	Potential effect(s) of failure	Potential cause(s) of failure	S	O	D	RPN
pH	Probe malfunction or calibration error	Low recovery and low purity	Low and high, both may lead to incomplete precipitation and higher protein impurities.	5	5	3	75
Time	Operator error	Low recovery and low purity	Low may lead to loss of protein due to incomplete precipitation and high may attract nonspecific precipitation of impurities.	5	3	1	15
RPM	Instrument malfunction or operator error	Low recovery and low purity	Low may decrease the precipitation and hence poor yield, high may lead to excessive agitation, precipitate disintegration and protein loss in the supernatant.	5	2	1	10

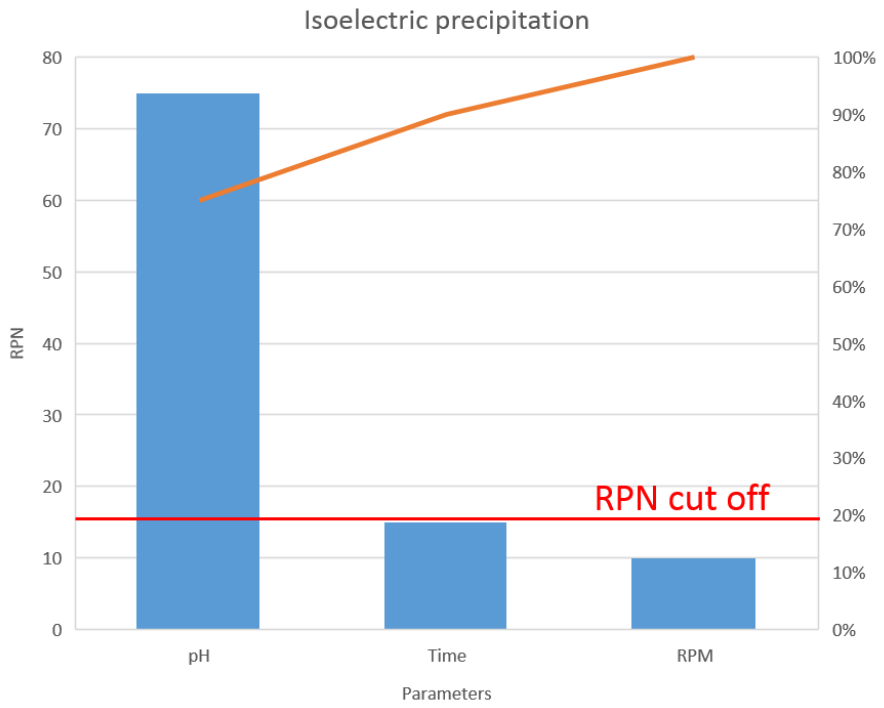


Figure 24: Pareto chart for parameter screening from FMEA analysis for isoelectric precipitation step.

FMEA analysis for isoelectric precipitation step was carried out using three parameters namely pH, time and mixing speed. The outcome of the analysis and their RPN scores are listed in Table 15. Pareto chart was plotted with obtained RPN score based on their excursions, where pH was the only process parameter with high RPN score whereas time (min) and mixing speed (RPM) have low RPN score. Excursions at both ends for pH will adversely affect the process, as operation at both ends will lead to incomplete precipitation and incorporation of protein impurities, which significantly affect the process yield and purity. Excursions for time (min) and mixing (RPM) may affect the CQAs, however, the impact may not be significant and hence the low RPN score.

From FMEA analysis, it can be concluded that the pH is the only process parameters with high RPN score and needs to be screened for the ranges to obtain the optimum yield (Figure 24).

Table 16: FMEA of operating parameters for the oil removal step.

Parameters	Potential failure mode	Potential effect(s) of failure	Potential cause(s) of failure	S	O	D	RPN
Temperature	Probe malfunction or calibration error	Low recovery and low purity	Low may lead to inefficient oil removal and high may lead to loss of solvent due to evaporation.	3	3	2	18
RPM	Instrument malfunction or operator error	Low recovery and low purity	Low may increase impurities and high may not have any impact.	3	2	1	6
Time	Operator error	Low recovery and low purity	Low may increase impurities and high may not have any impact	3	1	1	3
No. of washes	Operator error	Low purity	Low may increase impurities and high may not have any impact	3	1	1	3

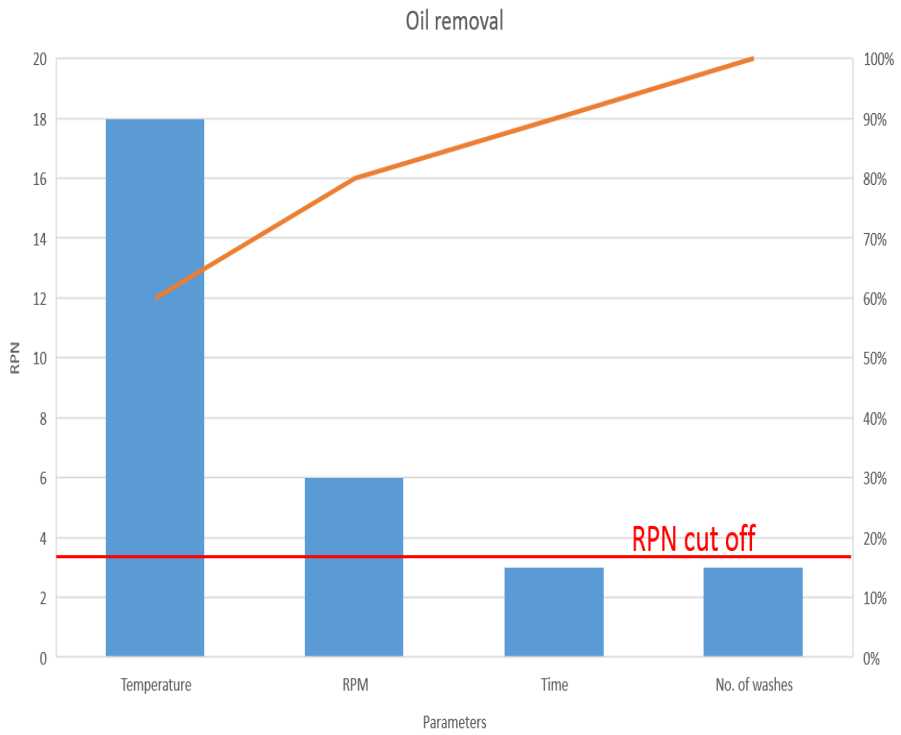


Figure 25: Pareto chart for parameter screening from FMEA analysis for oil removal step.

Temperature, mixing speed (RPM), time and number of washes were considered for FMEA analysis for oil removal step. The outcome of the analysis and their RPN scores are listed in Table 16. Pareto chart (Figure 25) was plotted with obtained RPN score based on their excursions, where temperature with relatively high RPN score and others were listed with low RPN scores. Excursions at lower ends for all parameters may affect the oil removal efficiency whereas higher range may not effect.

From FMEA analysis, it can be concluded that temperature and mixing speed (RPM) are the parameters identified with relatively high RPN score, however, no further exploration required considering low individual RPN score.

Table 17: FMEA of operating parameters for the drying step.

Parameters	Potential failure mode	Potential effect(s) of failure	Potential cause(s) of failure	S	O	D	RPN
Inlet temperature	Instrument malfunction or operator error	Low recovery	Low may lead to increased operation time and protein loss due to high liquid/ moisture content and high may degrade/ denature the protein and hence poor recovery.	5	3	4	60
Flow rate	Instrument malfunction or operator error	Low recovery	Low may not have any impact and high may lead to loss of protein due to high liquid/ moisture content.	5	2	4	40
N_2 flow	Instrument malfunction or operator error	Low recovery	Low may lead to increased operation time and protein loss due to high liquid/ moisture content and high may not have any impact.	5	2	2	20

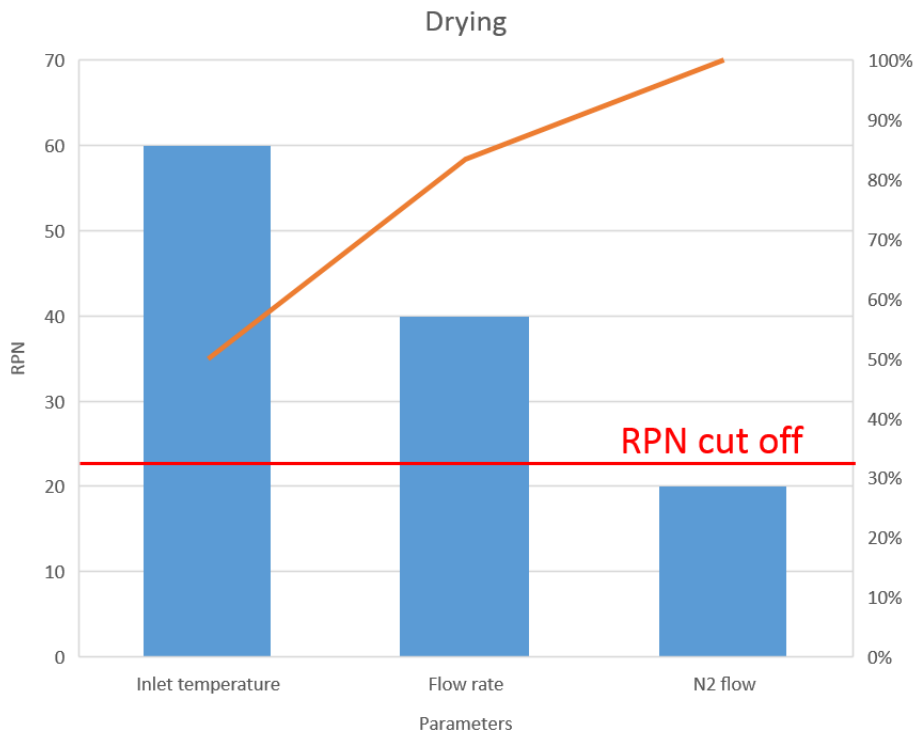


Figure 26: Pareto chart for parameter screening from FMEA analysis for the drying step.

For drying step, inlet temperature, flow rate and N_2 flow were the parameters considered for analysis. The outcome of FMEA analysis is as shown in Table 17 and Pareto chart (Figure 26) was plotted based on the RPN score obtain from the potential excursions for a particular parameter. High inlet temperature may degrade/ denature the protein and lead to poor recovery whereas low may lead to protein loss due to high liquid/ moisture content and to increased operation time and hence the high RPN score. Similarly, high flow rate and low N_2 flow may lead to loss of protein due to high liquid/ moisture content resulting in low yield whereas low flow rate and high N_2 flow may not have any impact.

From FMEA analysis, inlet temperature and flow rate were identified as the potential parameters with high RPN scores (Figure 26) that might significantly affect the performance of drying step as unit operation and subsequently the overall process. These parameters will be screened with DOE to identify the design space for the drying step.

3.4.4. Process characterization and process improvement

3.4.4.1. Extraction

This study was carried out to determine the effect of identified potential parameters and their interactions on CQAs. These parameters were screened at three levels, ethanol (45, 60 and 75%), NaOH (0.3, 0.6 and 0.9 g/100mL) and SMB (0.1, 0.5 and 1 g/100mL), experimental design and results are listed in Table 18.

Table 18: Experimental design for the extraction step for yield (%).

Sr. No.	Ethanol	NaOH	SMB	Yield (%)
1	45	0.3	0.1	36.45
2	45	0.3	0.5	41.09
3	45	0.3	1	46.06
4	45	0.6	0.1	52.50
5	45	0.6	0.5	57.46
6	45	0.6	1	61.54
7	45	0.9	0.1	63.55
8	45	0.9	0.5	67.83
9	45	0.9	1	71.48
10	60	0.3	0.1	51.70
11	60	0.3	0.5	54.56
12	60	0.3	1	61.55
13	60	0.6	0.1	66.56
14	60	0.6	0.5	71.28
15	60	0.6	1	76.02
16	60	0.9	0.1	74.13
17	60	0.9	0.5	81.19
18	60	0.9	1	84.48
19	75	0.3	0.1	28.49
20	75	0.3	0.5	32.86
21	75	0.3	1	38.47
22	75	0.6	0.1	34.46
23	75	0.6	0.5	41.20
24	75	0.6	1	49.17
25	75	0.9	0.1	44.63
26	75	0.9	0.5	49.95
27	75	0.9	1	54.60

The R^2 value and p-value for the model are listed in Table 19 and Table 20 respectively. The p-value (<.0001) for the model is less than the significance level of 0.05, which indicates that at 0.05 significance level, the coefficient is not zero. The R-square value is the coefficient of determination that shows the proportion of the variance in the response (yield) variable, which is explained by the given model. R-square can range from 0 to 1. A model with an R-square value of 0 has no prediction power whereas a model with an R-square of 1 can perfectly predict the response. Here, the R-square value of 0.995 for the given model indicates that the model explains about 99.5% of the variability in kafirin yield for the extraction step. These small p-values and R-square value indicate that the model is statistically significant.

Table 19: Summary of fit for kafirin yield (%) from the extraction step.

RSquare	0.995
RSquare Adj	0.992
Root Mean Square Error	1.39
Mean of Response	55.31
Observations (or Sum Wgts)	27

The data obtained from the input parameters were analysed using analysis of variance (ANOVA). The ANOVA summary obtained from the model for extraction yield is given in Table 20.

Table 20: ANOVA summary for a full factorial design for kafirin yield from the extraction step.

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	9	6207.90	689.77	359.44
Error	17	32.62	1.92	**Prob > F**
C. Total	26	6240.52		<.0001*

The value obtained for the model for F-ratio and p-value were 359.44 and <.0001 respectively which suggest that the model is significant. Effect summary in Table 21 lists the model effects based on their p-values. Here, the effects are listed in decreasing order as per their significance.

Table 21: Effect summary for kafirin yield for the extraction step.

Source	LogWorth	PValue
NaOH (0.3,0.9)	16.365	0.00000
Ethanol (45,75)	12.878	0.00000
SMB (0.1,1)	10.721	0.00000
Ethanol*NaOH	5.039	0.00001
NaOH*NaOH	2.717	0.00192
Ethanol*SMB	0.972	0.10673
SMB*SMB	0.415	0.38479
NaOH*SMB	0.128	0.74512

Higher values of LogWorth indicates the smaller p-values and greater significance. Ethanol, SMB and NaOH concentrations were significant effects as they have low p-value, the same is true for ethanol x SMB interaction.

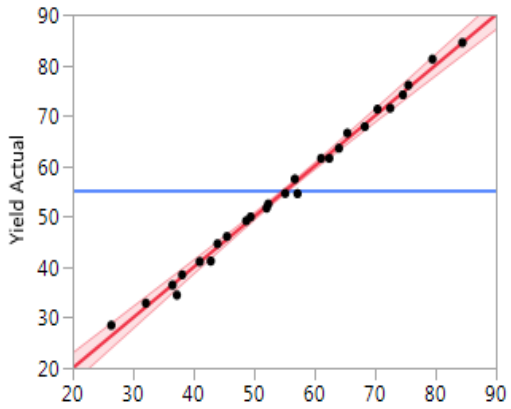


Figure 27: Actual v/s predicted plot for kafirin yield from the extraction step.

The actual by predicted plot (Figure 27) shows that there is no obvious evidence of lack of fit. Model is significant as indicated by the p-value mentioned and the actual by the predicted plot. Effect test report (Table 22) suggests that three of the model's terms, ethanol, SMB, NaOH and ethanol x NaOH interaction considered significant at 0.05 level, other interactions with higher values were not significant.

Table 22: Effect tests details for kafirin yield for the extraction step.

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Ethanol (45,75)	1	1	848.038	441.911	<.0001*
NaOH (0.3,0.9)	1	1	2229.271	1161.669	<.0001*

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
SMB (0.1,1)	1	1	459.045	239.208	<.0001*
Ethanol*NaOH	1	1	74.501	38.822	<.0001*
Ethanol*SMB	1	1	5.567	2.901	0.107
NaOH*SMB	1	1	0.210	0.109	0.745

Figure 28 shows the effect of individual process parameter on extraction yield. Curvature in the graph for ethanol shows that both extremes of ethanol concentration range under screening resulted in low yield whereas maximum yield obtained at 57% concentration. In contrast, an increase in NaOH and SMB concentration resulted in higher extraction yield.

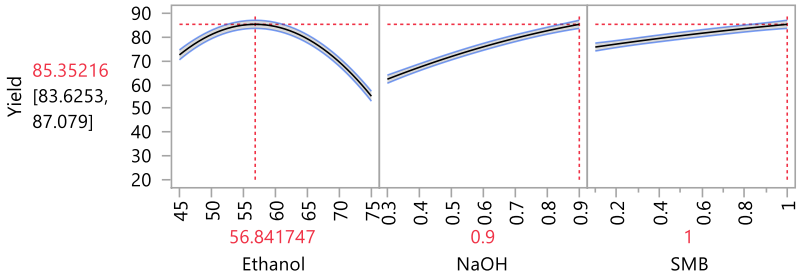


Figure 28: Effect of individual process parameter on extraction yield.

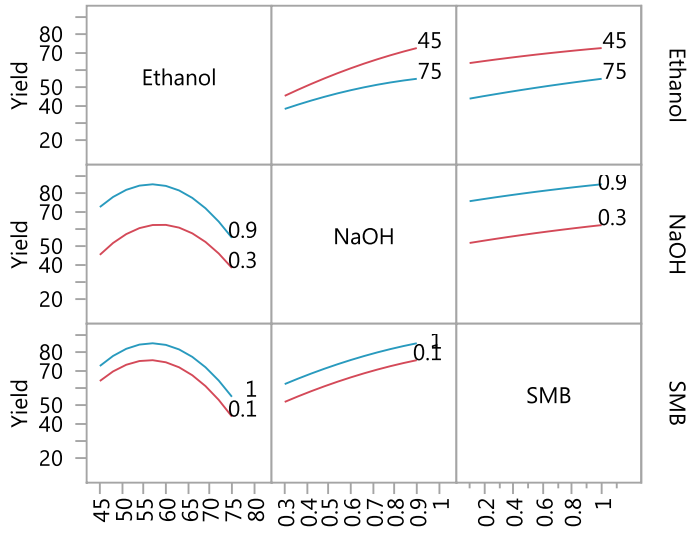


Figure 29: Interaction profiles of process parameters for the extraction step.

Interaction profiles of selected process parameters indicate the effect their interactions at various levels on extraction yield (Figure 29). Curvature in the graph for ethanol (first column) demonstrates the variation in yield because of increasing ethanol concentration at various concentrations of other ingredients.

Similarly, the second and third columns show the influence of the interaction of different concentrations NaOH and SMB respectively with other ingredients on extraction yield.

While performing DOE experiments, it was observed that both SMB (1 %) and NaOH (0.9 %) were not completely dissolved and tend to crystallise as sediments when mixed at higher concentrations of ethanol (75 %). Incomplete dissolution of SMB and NaOH might be the reason for low recovery with high ethanol concertation mix and reflected in the results from experimental data (Table 18). In contrast, kafirin yield was relatively higher at low ethanol concentration (45 %), which indicates that NaOH and SMB concentrations are important CPPs.

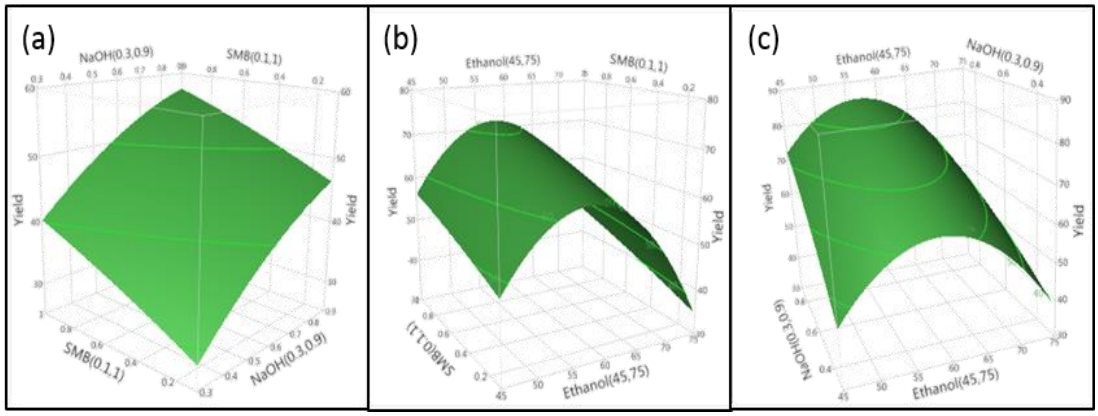


Figure 30: Surface profiles for the interaction of process parameter interaction and their effect on extraction yield. Surface profilers for (a) SMB x NaOH, (b) SMB x ethanol and (c) NaOH x ethanol.

Surface profilers show the impact on extraction yield from the interaction at various levels of different process parameters (Figure 30). Surface profilers for SMB x NaOH (Figure 30a) show that with an increase in concentrations of both ingredients, extraction yield also increases. However, SMB x ethanol (Figure 30b) and NaOH x ethanol (Figure 30c) depicts that extraction yield increased with an increase in concentrations of ingredients but limited by a further increase in ethanol concentrations. Curvature in both the surface profilers resembles the profile for the effect of ethanol on extraction yield (Figure 28). Surface profilers (Figure 30b and c) also shows that ethanol concentration as a process parameter governs the extraction yield whereas SMB and NaOH assist with further increase in the yield.

Contour profilers from different process parameters give the idea about design space to achieve >70% process yield (Figure 31a and b) when operated within operating ranges for interacting process parameters. It can be gathered from the contour profilers that minimum 70% extraction yield can be achieved with ethanol concentrations (52 to 62 %) keeping SMB and NaOH concentrations >0.6 g/100 mL of extraction solution.

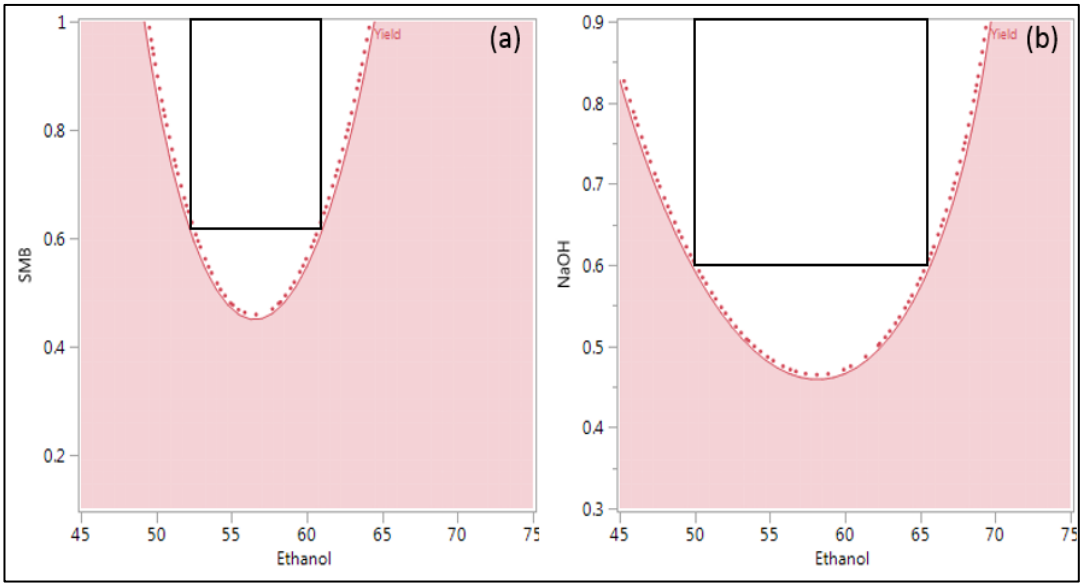


Figure 31: Contour profilers depicting design space to achieve >70% extraction yield with operating ranges for interacting process parameters. Contour profiles for (a) SMB x ethanol and (b) NaOH x ethanol.

Based on design space derived for extraction step from characterisation studies, it was concluded that 57 ± 3.2% (w/w) ethanol concentration with SMB and NaOH concentrations 0.8 ± 0.2 % (w/w) and 0.75 ± 0.15 % (w/w) respectively considered ideal for kafirin extraction. However, contour profilers and surface plot (Figure 30a) for SMB and NaOH indicate that excursion towards higher concentrations would result in higher yield. As discussed earlier, complete dissolution of SMB and NaOH was not possible at high ethanol concentrations, however, low ethanol concentration/increased aqueous component permitted dissolution of SMB and NaOH even at higher concentrations. Excursion studies were carried out to validate the observation. The design space derived from these excursion studies resulted in kafirin yield (>80%) from the extraction step whenever operated within the PARs given in Table 23.

Table 23: PARs derived from process characterisation and excursion studies for the extraction step.

CPPs	PAR	Old set point
Ethanol (%) (w/w)	56 ± 3	60 %
SMB (%) (w/w)	1.4 ± 0.3	1.0 g/100 mL
NaOH (%) (w/w)	1.3 ± 0.2	0.9 g/100 mL

3.4.4.2. Separation

Centrifugation and filtration opted for separation of kafirin rich extract from solid raffinate. For centrifugation, maximum recovery obtained at 4700 rpm and 30 minutes centrifugation time. Data obtained from the preliminary study (Section 3.3.2.1) suggests that higher centrifugation speed and time result in higher recovery. At lab scale, centrifugation was a prerequisite for filtration to generate particulate-free material for distillation step.

At higher scale, a 100 µm mesh was used as an initial filter and the resulting filtrate was again filtered with 0.8/0.2 µm filter. Introduction of mesh instead of centrifugation increased the volume recovery as extract retention volume in pellet significantly reduced. Hence, the separation step does not need to be characterised at this stage.

3.4.4.3. Distillation

Distillation was used to remove and recover ethanol from extraction solution with kafirin. Risk assessment of the distillation step identified temperature as an important process parameter and assigned high RPN score. The temperature was the only process parameter identified as high risk, however, distillation at higher scale can be regulated with automated temperature controls along with other parameters such as vacuum and mixing. Hence, the distillation step does not need to be characterised.

3.4.4.4. Isoelectric precipitation

Isoelectric precipitation removes protein impurities present in ethanol-free stream generated from distillation. Risk assessment for isoelectric precipitation as unit operation indicated pH as a high-risk process parameter with high RPN number. Optimum pH range for the optimum recovery and purity was screened

through OFAT studies conducted earlier along with excursion on both extremes. It was found that the low pH extreme resulted in significant kafirin yield but also increased protein impurities. On the other hand, excursion towards high pH range resulted in low total protein and consequently low kafirin yield. Data from these excursions indicate that screening required within pH range 4.0 to 5.0.

As an additional measure to increase the purity, precipitates were washed with water. Pellets obtained after centrifugation were resuspended in water, homogenised and incubated in stirring condition for 15 minutes. The mixture was centrifuged, supernatant and pellets were separated. The washing step repeated three times where water-soluble proteins, impurities and residual chemicals used throughout the process were solubilised and retained in the supernatant. Purified kafirin obtained as firm pellet assessed for total protein and kafirin content.

Characterisation study was carried out to determine the effect of identified potential parameters and their possible interactions on CQAs. These parameters were screened at three levels for process parameters pH (4.0, 4.5 and 6.0) and precipitation time (45, 60 and 75 minutes), experimental design and the results obtained are listed in Table 24.

Table 24: Experimental design for protein precipitation step for kafirin yield (%).

Sr. No.	pH	Time (min)	Kafirin yield (%)
1	4	45	74.38
2	4	60	81.54
3	4	75	82.49
4	4.5	45	89.37
5	4.5	60	94.87
6	4.5	75	96.16
7	5	45	91.24
8	5	60	96.87
9	5	75	97.28

Significance of both R^2 value and p-value explained earlier (in section 3.4.4.1) and are listed in Table 25 and Table 26 respectively for isoelectric precipitation step. The p-value (<.0001) for the model is less than the significance level of 0.05

indicates that the coefficient is not zero. Similarly, the R-square value of 0.999 for the given model indicates that the model explains about 99.9% of the variability in kafirin yield for the isoelectric precipitation step. This small p-value and R-square value indicate that the model is statistically significant.

Table 25: Summary of fit for kafirin yield (%) from the isoelectric precipitation step.

RSquare	0.999
RSquare Adj	0.998
Root Mean Square Error	0.347
Mean of Response	89.356
Observations (or Sum Wgts)	9

The data obtained from the input parameters (pH and operation time) were analysed using analysis of variance (ANOVA). The ANOVA summary obtained from the model for kafirin yield from isoelectric precipitation is given in Table 26.

Table 26: ANOVA summary for the full factorial design for kafirin yield from isoelectric precipitation step.

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	5	531.650	106.330	885.053
Error	3	0.360	0.120	**Prob > F**
C. Total	8	532.011		<.0001*

The value obtained for the model for F-ratio and p-value were 885.05 and <.0001 respectively which suggest that the model is significant. Effect summary in Table 27 lists the model effects based on their p-values and effects are listed in decreasing order as per their significance.

Table 27: Effect summary for kafirin yield for isoelectric precipitation step.

Source	**LogWorth**		**PValue**
pH (4,5)	4.886		0.00001
pH*pH	3.861		0.00014
Time (45,75)	3.835		0.00015
Time*Time	2.750		0.00178
pH*Time	1.234		0.05831

As mentioned earlier in section 3.4.4.1, higher LogWorth value indicates the smaller p-values and greater significance. pH is the only parameter with significant effects as it has low p-value, an observation supports the assigned high RPN score during the risk assessment.

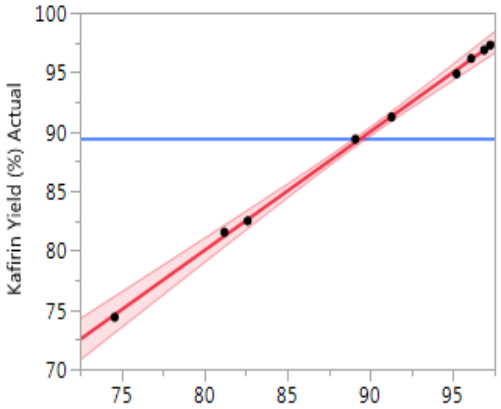


Figure 32: Actual v/s predicted plot for kafirin yield from isoelectric precipitation step.

The actual by predicted plot (Figure 32) shows that there is no obvious evidence of lack of fit. Model is significant as indicated by the p-value mentioned and the actual by predicted plot. As evident from the effect test report (Table 28), only pH was considered significant at 0.05 level, operation time and interactions with higher values were not significant.

Table 28: Effect tests details for kafirin yield from isoelectric precipitation step.

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
pH (4,5)	1	1	367.853	3061.878	<.0001*
Time (45,75)	1	1	73.081	608.296	0.0001*
pH*Time	1	1	1.071	8.917	0.0583
pH*pH	1	1	76.056	633.059	0.0001*
Time*Time	1	1	13.589	113.113	0.0018*

Figure 33 shows the effect of pH and operation time as an individual process parameter on kafirin yield from isoelectric precipitation step. Curvature in both the graphs shows that both extremes of ranges for pH and operation time under screening resulted in low yield and maximum yield obtained at pH 4.8 and 65 minutes of operation time. Interaction profiler also suggests that a decrease in pH

results in low yield as pH of the system moves away from the kafirin pI and hence lower rate and amount of precipitation due to increasing unfavourable conditions. A decline in kafirin yield with further increase in time confirmed the observation from OFAT studies.

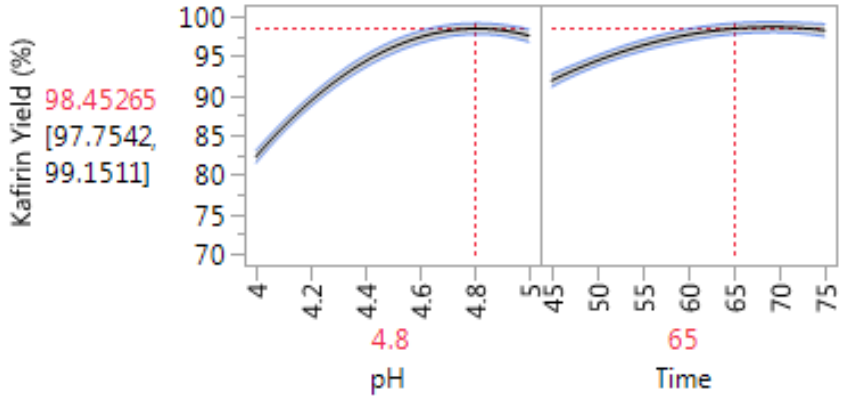


Figure 33: Effect of individual process parameter on kafirin yield from isoelectric precipitation step.

Interaction profiles of screened process parameters indicate the effect their interactions at various levels on kafirin yield from isoelectric precipitation step (Figure 34). Curvature in the graph for pH (first column) demonstrates the variation in yield at a varying range of pH for different operation time. Similarly, the second column shows the influence of the interaction of different pH within the given operation time range on kafirin yield. Kafirin yield was relatively low for pH 4 with an increase in time compared to pH 5. However, curvature in the graphs from interaction profiler and individual effect (Figure 33) indicates that optimum pH and operation time for maximum yield rests between the screened ranges for both parameters.

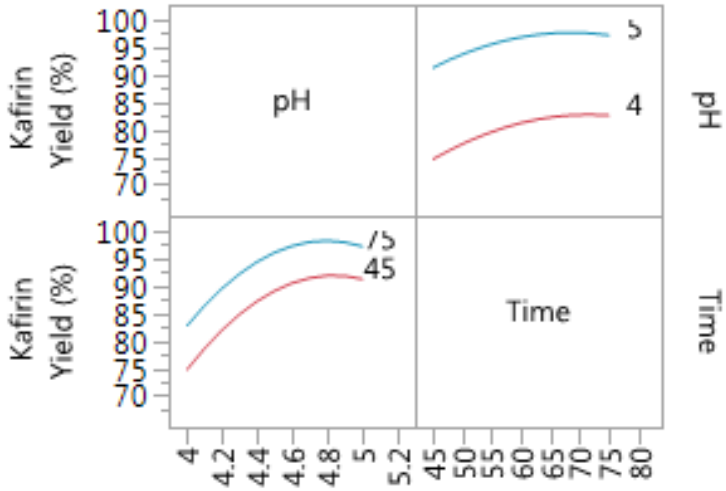


Figure 34: Interaction profiles of process parameters for the isoelectric precipitation step.

Surface profilers demonstrate the effect of the interaction of different process parameters at various levels on kafirin yield (Figure 35). Profile depicts that at kafirin yield increase at a given pH with an increase in time up to 70 minutes and decrease with further increase in time, an observation consistent throughout the characterised pH range. Similarly, kafirin yield increase for a given operation time point and pH up to 4.8 and decreases with further increase in pH.

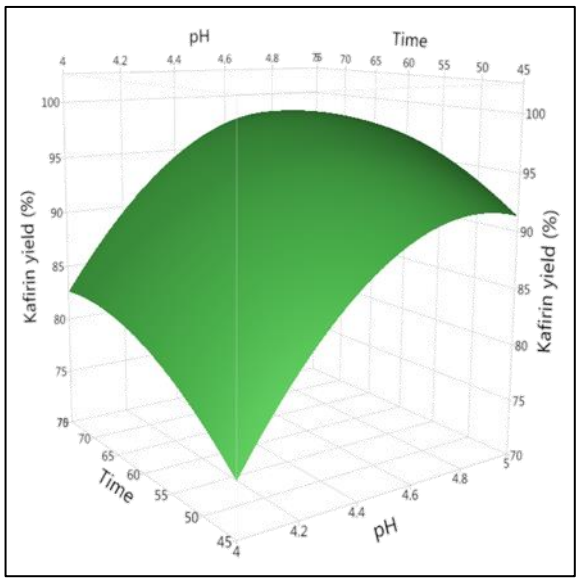


Figure 35: Surface profiles for process parameter interaction and their effect on kafirin yield from isoelectric precipitation step.

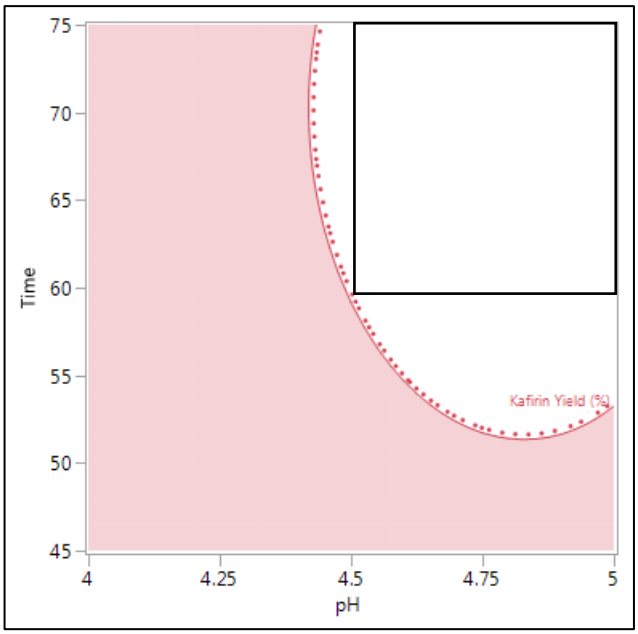


Figure 36: Contour profilers depicting design space for isoelectric precipitation step.

Contour profiler for isoelectric precipitation step gives the idea about design space to achieve >90% kafirin yield (Figure 36) when operated within operating ranges for interacting process parameters. It can be gathered from the contour profilers that minimum 90% extraction yield can be achieved with pH ranging from 4.5 to 5.0 and operation time 60 to 75 minutes.

Based on characterisation studies for kafirin extraction, a design space derived for isoelectric precipitation step and experiments were carried out to validate the PARs. Based on validation studies, it was concluded that minimum kafirin yield (>90%) can be achieved when operated within the PARs given in Table 29.

Table 29: PARs derived from process characterisation studies for the extraction step.

Parameter	PAR	Old set points
pH	4.8 ± 0.1	5.0
Operation time (min)	65 ± 5	60

3.4.4.5. Oil removal

Temperature and mixing speed (RPM) were identified as process parameters with high risk, however, as discussed earlier during risk assessment (section

3.4.3); low individual RPN score (Table 16) of these parameters suggests that oil removal step does not need to be characterized at this stage.

3.4.4.6. Drying

Inlet temperature and flow rate were identified as the potential process parameters with high RPN scores. Automated spray dryer will be used for drying operations at higher scale where inlet temperature and flow rate controlled using automated controllers. Considering automated controls for these parameters, the drying step does not need to be characterized at this stage.

3.4.5. Process design space for kafirin extraction

Process characterization exercise established base and aided with defining process design space for kafirin extraction. Characterisation studies carried out using DoE based approach highlighted the main effects of individual CPPs as well as their interactions on CQAs. CPPs for different unit operations throughout the process assigned PARs from the characterisation studies. The resulting matrix from the outcome of these studies forms the design space for different CQAs that defines QTPP. The design space derived from the characterisation studies met the criteria decided earlier (Table 10) for CQAs. The designed process resulted in >70 % recovery with > 95 % solubility and purity for the purified kafirin.

A comparative summary of processes developed with traditional (OFAT) and QbD based approach compared in terms of process parameters, step yield and overall process yield presented in Table 30. Unit operations with major changes in the identified CPPs (in bold letters) with their PARs derived from design space. Also, individual step recoveries and overall process recovery were compared to indicate the significant increase in the overall recovery from a process developed with QbD based approach.

Table 30: Comparative summary of processes developed using traditional and QbD based approaches.

Process step	Parameters	OFAT setpoints	OFAT based process recovery (%)	QbD PARs	QbD based process recovery (%)
Extraction	**Ethanol (%)**	60	87.49	**56 ± 2.8**	**92.48**
	SMB (%)	1		**2.6 ± 0.6**	
	NaOH (%)	0.9		**1.5 ± 0.2**	
	Temperature (°C)	60		60	
	S/L ratio	1:6		1:6	
	Time (min)	45		45 ± 10	
	Particle size (µm)	100 to 250		100 to 250	
	RPM	200		200	
Separation (Filtration)	Coarse filter (µm)	NA	89#	100	96.88
	Filter (µm)	0.8/0.2		0.8/0.2	
Isoelectric precipitation	**pH**	5	68.41	**4.8 ± 0.1**	**93.25**
	Time (min)	60		**65 ± 5**	
Defatting	Temperature (°C)	60	98	60	97.69
	Time (min)	60		60	
	S/L ratio	1:5			
	Number of washes	3		3	
	RPM	200		200	
Drying (Spray dryer)	Temperature (°C)	120	96.93	120	95.11
	Time (min)	Varies*		Varies*	
Overall recovery			50.60		77.63

CPPs for the characterised unit operations highlighted with their PARs.
*: Operation time depends on the process stream volume.
#: Separation step recovery from centrifugation followed by filtration.

3.5. Process adaptation for DDGS

DDGS was explored as an economic alternative for kafirin extraction instead of sorghum grain or flour. DDGS is a solid waste generated from the distilleries and ethanol bio-refineries after fermentation of grain followed by ethanol recovery. The starch from the seeds converted to glucose and finally to ethanol with the help of yeast and/or added enzymes during fermentation leaving the protein-rich residue. Ethanol removed as distillate and the stillage is processed to separated syrup and cake, latter milled to DDGS. Process adaptation exercise was carried out to accommodate the DDGS instead of sorghum flour as feed material for kafirin extraction. Since both raw materials vary in terms of their physicochemical and biochemical properties such as kafirin/total protein content and impurity distribution, process parameters of individual unit operations were modified accordingly.

3.5.1. DDGS pre-treatment

Initially, DDGS was extracted for kafirin with the same process parameters used for sorghum flour; however, the attempt resulted in a loss of the significant amount of protein in the raffinate. During separation of extract from raffinate, higher particle size of DDGS lead to the formation of loose pellet resulting in the supernatant with high particulate matter. In other words, it was difficult to separate the supernatant by decanting. Turbid and high-particulate matter supernatant led to increased filtration area required for supernatant clarification. DDGS was milled to finer particle size (< 500 µm) to address the issue and to achieve higher extraction rates. Finer particle size resulted in firm pellet formation and reduction in the filter area required post centrifugation. Furthermore, a coloured extract obtained after initial extraction suggests that initial DDGS washes need to be introduced in the existing process to get rid of colour and other water-soluble impurities. Initial water wash followed by 0.5 M NaCl wash and another water wash was introduced, which partially removed these impurities. The first water wash removed water-soluble coloured impurities, low molecular weight impurities such as albumins generated during milling, fermentation and distillation process and water-soluble protein such as globulins that are intrinsic to the raw material (Figure 37a). Apart from kafirin,

albumins, globulins and low molecular weight nitrogenous impurities constitute ~26 % of the total protein in the sorghum seeds (137). Saline wash removed proteins that are insoluble in water but soluble in saline solutions and residual colour (Figure 37b). Second water wash aimed at the removal of residual salt from the saline wash also removed residual coloured impurities along with remaining low molecular weight nitrogenous impurities (Figure 37c).

(a) (b) (c)

Figure 37: DDGS pre-treatment supernatant: (a) first water wash, (b) Saline wash and (c) second water wash.

3.5.2. Extraction and separation

After initial extraction from DDGS (Figure 38a), the supernatant was separated and analysed for protein and kafirin content. Extraction yield from the first extraction (54.16 %) from DDGS was significantly low. It was later confirmed by higher protein and kafirin content of raffinate, which indicated that re-extraction was required as the protein content of DDGS (35-40 %) was significantly higher than sorghum flour (12.4 %). Similarly, higher solid to liquid ratio (1:6) was considered for DDGS extraction. Re-extraction of residual proteins from DDGS raffinate significantly improved the yield of extraction (96.41 %). Extraction mixture was passed through 100 µm mesh and later filtered with 0.8/0.2 µm filter.

3.5.3. Distillation and isoelectric precipitation

The clarified supernatant (Figure 38b) was then subjected to distillation to recover ethanol and pH of the remaining aqueous solution was adjusted to 4.8 ± 0.2. Precipitates were separated from the supernatant by centrifugation and recovered as a pellet. It was observed that the precipitation pellet and supernatant were moderately coloured. Pellets were resuspended in water and kept on stirring condition for 15 minutes; the procedure repeated 3 times. Supernatant from the initial wash was coloured (Figure 38c), the third water wash was relatively clear. Precipitates obtained after last wash was weighed to calculate the step recovery.

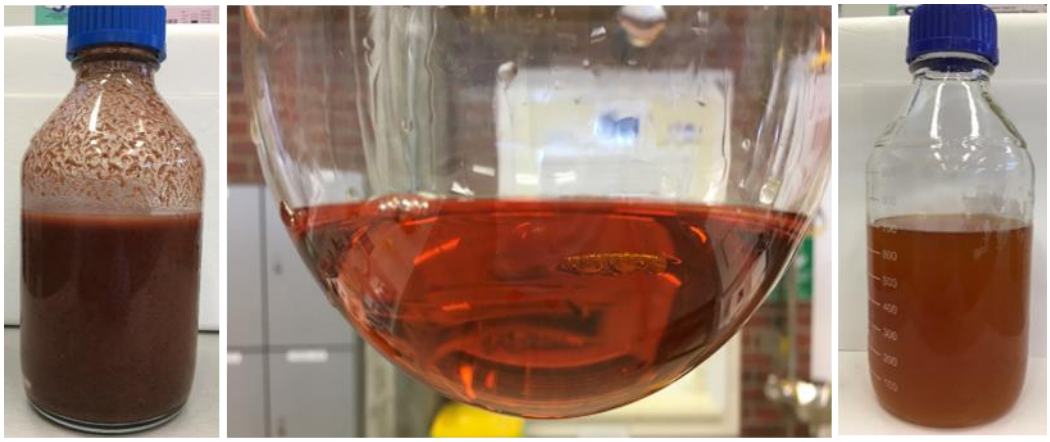


Figure 38: Process streams from DDGS extraction. Post-extraction mix (a) before centrifugation and filtration, (b) after filtration and (c) post pI precipitation water wash.

3.5.4. Oil removal and drying

Precipitates obtained after the last water wash were treated with hexane to remove oil. The solid to liquid ratio and number of hexane washes for the oil removal were same i.e. 1:5 and 3 respectively considering variability in the raw material. First two washes removed coloured impurities whereas the third wash was relatively clear. Kafirin from laboratory scale batches dried using hot air oven and the spray dryer was used at a higher scale.

Unit operations across the entire extraction process and the process parameters were modified/optimised to accommodate the DDGS. Process steps/unit operations along with process parameters modified are summarised in Table 31.

Table 31: Summary of process adaptations to accommodate DDGS as raw material for kafirin extraction.

Process steps	**Parameters**	**Details**	**Purpose**
Pre-treatment	Water and saline wash	Water wash	Process related impurity removal (albumin, globulin and other water-soluble impurities)
		0.5 M NaCl wash	Remove globulins and colour
		Water wash	Remove residual salt and colour
Extraction	Particle size	100-250 µm	The higher surface area offers higher mass transfer and increased rate of extraction
	S/L ratio	1:6	Higher protein content (35-40 %) in feed material
	Number of extractions	2	Higher protein content (35-40 %) in the feed material
Distillation	Runs	2	Increased solvent load from higher S/L ratio and additional extraction
pI precipitation	Water wash	3	Removal of water-soluble impurities, residual salts and colour
Defatting	S/L ratio	1:5	Colour and oil removal
	Number of washes	3	Colour and oil removal

Table 32 summarises data from a representative batch for individual step recovery and overall process yield for kafirin extraction from DDGS. Kafirin yield for most of the steps for DDGS was comparable to extraction from flour. Isoelectric step increased by 3.57 %, which might be due to high kafirin concentration in the input stream for this step and reduced water-soluble protein impurities as a result of additional water washes.

Comparative data from the representative batches for kafirin extraction from sorghum flour (Table 30) and DDGS (Table 32) confirmed the fact that the process developed with QbD based approach can produce a consistent quality product. Protein to extraction solution ration was the key factor while accommodating different raw materials for this process. DDGS and sorghum flour vary greatly in terms of their protein and kafirin content. In order to accommodate DDGS, rational changes were made, based on the difference between kafirin content of flour (7.89 %) and DDGS (26.9 %) as latter would require 3 times more solvent to extract the equivalent amount of kafirin. However, the comparable yield was achieved with the same S/L ratio and re-extraction of raffinate from initial extraction with fresh extraction solution. It was demonstrated that by keeping the protein content to solvent ratio constant, the extraction process resulted in comparable yield even with variability in the raw materials. It was assumed that ethanol fermentation and prolonged heat treatment are part of the process that produces DDGS might have generated highly cross-linked non-extractable kafirins. The observation was confirmed upon analysis when it was found that amount of extractable kafirin in raffinate was not significant although it has high protein content.

3.6. Scale-up

Kafirin extraction process developed at bench/lab-scale was upscaled to 20 fold in order to demonstrate scalability. Scale-up was carried out considering infrastructure available in the laboratory. Process parameters for unit operations were kept constant for both the scales.

3.6.1. Extraction and separation

The extraction process was carried out in a 7.5 L glass reactor with stainless steel headplate with six-bladed Rushton turbine impellers attached to the motor with speed range form 25 – 1200 rpm for mixing. The temperature was sensed by a Resistance Temperature Detector (RTD) inserted through thermowell submerged in the mixture and controlled by process control software throughout the process. The temperature of the reactor maintained with detachable stainless steel jacketed bottom-dished head for the temperature-controlled water circulation.

Preheated DDGS was added to the extraction solution maintained at 60° C in the glass reactor. Agitation speed was set to 200 rpm and mixed for 45 ± 10 minutes. The slurry was removed and passed through 100 µm mesh followed by 0.8/0.2 µm filter. The raffinate retained using mesh was added with fresh extraction solution. The process was repeated and filtered/clarified streams were pooled.

Initial kafirin content of DDGS (26.9 %) was extracted in a given condition, where, 96 % of kafirin was extracted indicating that ~11 g was lost in raffinate, which as discussed earlier might be non-extractable kafirin. Filtration of the extract resulted in 97.78% recovery, where 5.74 g of kafirin was lost in extraction solution retained in the raffinate.

3.6.2. Distillation and isoelectric precipitation

The clarified extract was added to distillation flask and ethanol was recovered. Process parameter for distillation was kept constant as the same setup was used with the larger capacity distillation flask (4.0 L). Kafirin rich aqueous solution obtained at the end of each run were pooled. Aqueous solution pool obtained from distillation collected in a glass bottle kept on continuous stirring condition. The pH of the solution was adjusted to 4.8 ± 0.1 with 6.0 N HCl and incubated for 65 ± 5 minutes. The mixture was centrifuged and pellets were water washed for 3 times.

96.41 % kafirin was retained as precipitates where almost 9 g of kafirin lost during the water washes, which can be considered as this step increases the purity of stream by removing water-soluble impurities.

3.6.3. Oil removal and drying

Pellets were washed with hexane (3x) to remove oil and coloured impurities. Kafirin was dried and obtained in powdered form using a spray dryer.

Both these steps, oil removal and drying resulted in 97.68 % and 95.81% kafirin yield respectively. Drying step recorded loss of ~10 g of Kafirin, however, higher batch sizes would result in reduced loss.

Table 32: Comparative data summary of step recovery and overall process yield for kafirin extraction from DDGS at laboratory scale and scale-up batch.

Process steps	Laboratory scale		20x scale-up	
	Protein (g)	Kafirin yield (%)	Protein (g)	Kafirin yield (%)
DDGS	26.9	--	269	--
Extraction	24.06	89.46	258.3	96.02
Filtration	23.07	95.88	249.53	96.60
Isoelectric precipitation	22.34	96.82	241.54	96.80
Oil removal	21.92	98.11	236.69	97.99
Drying	21.38	97.56	227.96	96.31
Overall recovery		79.48		84.74

Individual step recovery and overall process yield from both laboratory and scale-up batches are summarised in Table 32. Kafirin yield for individual steps was comparable, where, overall process yield for the scale-up batch was higher than laboratory-scale batch. Certain factors lead to the difference between the recoveries such as sampling at individual step, process stream loss during operation and transfer of intermediates. At laboratory scale, since the volumes and quantities of process intermediates are significantly smaller compare to scale-up, removal of a fixed amount of sample matters at a small scale. Similarly, transfer and processing lead to loss due to incomplete recovery from vessels and reactors. These factors add to the decrease in overall recovery, however, it can be gathered from these observations that loss of kafirin at the pilot and commercial scale will be low and may not have a significant impact on step yield and overall recovery.

Kafirin purified at both scales analysed with SDS-PAGE for quality and comparability purpose. Non-reduced (NR) kafirin sample with HMW and LMW

was also loaded on the gel for comparison. It was observed that the quality of kafirin extracted from DDGS at both scales was comparable. Both HMW and LMW impurities were removed. However, there were some faint bands observed below the main kafirin band. These bands might be the second most abundant form (β-kafirin), next to α-kafirin. There were no dimers or HMW aggregates present above the main band and LMW fragments below (probable β-kafirin and) main kafirin band.

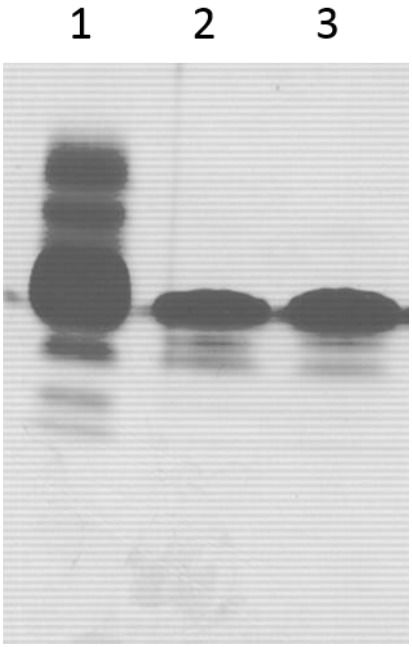


Figure 39: SDS-PAGE analysis of kafirin extracted from DDGS at laboratory scale and scale-up batch.
Lane 1- Kafirin (NR), 2-Kafirin-laboratory scale (R) and 3-Kafirin- scale-up batch (R).

3.7. Conclusions

Kafirin purification process was developed from sorghum flour using traditional OFAT approach where individual unit operations were optimised by scanning different process parameters within a given range. Unit operations were optimised considering kafirin purity, recovery and to minimize the raw material usage. Kafirin purification process developed using OFAT approach provided set points for individual parameters from each unit operation, which resulted in 50.60% kafirin recovery from the process.

QbD based approach was implemented to characterise the process. Kafirin purity, yield and solubility were considered as important CQAs. A risk assessment

carried using FMEA identified potential critical process parameters for individual unit operation. These process parameters varied within characterisation ranges considering excursions the possibly affect CQAs. DOE based experimental designs were used to assess the effect of individual process parameters and their interactions on kafirin yield. A design space derived for individual unit operation using CPPs from the characterization studies data. PARs were assigned to CPP's and validated to assure consistent kafirin recovery and purity from the process. Kafirin purification process resulted in 77.63 % process recovery when operated within assigned PARs. Ethanol usage for the developed process is 56 %, which is significantly less than earlier reported in-house and (70 %) other processes (≥ 70 %) discussed in the literature review (15,138,139).

DDGS opted as an economical alternative native to sorghum flour. Kafirin purification process design using QbD based approach was able to accommodate DDGS with minor changes in the process considering high protein content. Overall, process recovery for kafirin extraction from DDGS at laboratory-scale was 79.48 %.

A 20-fold scale-up was planned for the designed process using DDGS, where, individual step recovery for each unit operation was comparable. Scale-up batch using DDGS reported at 84.74 % process recovery. Qualitative analysis of kafirin purified from laboratory-scale and scale-up batch suggest that they were comparable.

It can be concluded that QbD based approach for process development helped design a process at laboratory-scale with defined operating ranges that produce kafirin with consistent purity and recovery. Define PARs derived from characterisation studies provides the flexibility with the operation and laid down a platform for easier scale-up.

Chapter 4: Column chromatography and scale-up

4.1. Introduction

Kafirin being highly hydrophobic and celiac safe makes it a subject of interest for various food, pharmaceutical and biomedical applications. The growing interest in the food processing applications, micro and nanoparticles for drug delivery and scaffolding for tissue engineering using kafirin has raised the prerequisite of high purity kafirin at a larger scale. Moreover, like other prolamins, kafirin has attracted a lot of attention in recent years as they satisfy the demand of being eco-friendly, cost-effective and ample availability for proposed and ongoing research to develop targeted applications. Earlier, kafirin has been extracted using organic solvents and acids such as ethanol, isopropanol, t-butanol, acetic acid with other additives such as reducing agents (15,32,122,140). Generous use of these solvents ensure the higher yield, however, lack of selectivity of these solvents results in lower purity. Kafirin extract is then processed with a series of unit operations that are energy-intensive and again with a lack of selectivity for kafirin. Unlike these unit operations, chromatography offers higher recovery and selectivity as the conditions to purify a targeted moiety and can be customised to achieve desired purity, recovery, or both.

Chromatographic separation has been reported earlier for separation of prolamins, mostly for zein, at both analytical and preparative scales (141–144). Similarly, separation using chromatography has been reported for kafirin on a few occasions, however, all these accounts are at analytical scale (77,78,138) and none at the laboratory, pilot or production scale. To develop a kafirin purification process, hydrophobic interaction (HIC) or reverse phase chromatography (RPC) cannot be used, which makes ion exchange resins more suitable for kafirin purification at the preparative scale. An earlier report has shown the capability of ion exchange resins for kafirin purification (79). Oil, the major impurity co-extracted with kafirin is a neutral moiety. Since there is no charge on oil, it will flow through the column and only kafirin molecules will bind which makes the purification easier as compared to the other existing multistep processes.

In the present study, next-generation resins were assessed for their binding capacities through adsorption isotherm and uptake kinetics. To identify the resin candidate bench-scale experiments were performed to evaluate the effects of various operating parameters such as pH, buffer composition, the solvent content

of the mobile phase, and the gradient. Mobile phase conditions and gradient with optimum purity and recovery were chosen from the experimental data. Effect of flow rate and protein loading was evaluated based on resin performance to obtain a better separation. Initial experiments conducted to screen the optimum conditions such as mobile phases, binding capacity, gradient design were conducted using a HiTrap™ column (1 mL). Once the optimum conditions were finalised, an XK16 column (5 mL) was used maintaining both kinetic and dynamic equivalence between the columns. Both these columns were successfully tested for their equivalence by operating them at identical bed height, mobile phases, linear flow rate, protein load (mg protein/ mL of resin) and run conditions.

To process higher volumes, the usual approach is to maintain plate count and proportional increase of the feed volume and the column volume by keeping the bed height the same with a proportional increase in the diameter, which keeps the contact/residence time constant when operated at same linear flow rates. The XK 16 column was packed with the Capto Q resin with an increased bed height of 10 cm and operated keeping the same residence/contact time. The bed height to be used at the pilot and/or commercial scale should be kept constant with the bench scale to achieve comparable column performance.

4.2. Materials

Chromatography resins Capto Q and SP Sepharose Fast Flow purchased from GE Healthcare (Australia), Toyopearl QAE-550C, Toyopearl SuperQ-650M and Toyopearl SP-650M from Tosoh Bioscience (USA). XK16 and prepacked HiTrap™ columns purchased from GE Healthcare (Australia). 12% Mini-PROTEAN® TGX™ Precast Protein Gels, 2x Laemmli sample buffer, Precision Plus Protein™ Unstained Standards (Broad-Range SDS-PAGE Standards), PowerPac™ Universal Power Supply, Mini-PROTEAN® Tetra Vertical Electrophoresis Cell and NGC™ Medium-Pressure Liquid Chromatography Systems were from Bio-Rad Laboratories, CA, USA. All chemicals, namely, 2-mercaptoethanol, absolute ethanol, sodium metabisulphite, sodium hydroxide, hydrochloric acid, methanol, acetic acid, formaldehyde, sodium thiosulphate, silver nitrate, sodium carbonate were purchased from Sigma-Aldrich (Australia). Analytical instruments used

were 2400 CHNS/O Series II System and UV-VIS spectrophotometer (both from Perkin Elmer).

4.3. Methods

4.3.1. Kafirin extraction

Kafirin was extracted from sorghum flour using the process developed and explained in chapter 3. Briefly, sorghum flour was mixed with extraction solution and stirred at 200 rpm for 1 hour at 60 °C. The mixture was centrifuged; the supernatant was separated and filtered with a 1.0 µm prefilter followed by a 0.45 µm filter. The extraction step was repeated with the remaining sediments to ensure maximum recovery of kafirin. Filtered supernatants were pooled and ethanol was recovered by distillation. The pH of the remaining aqueous solution was adjusted to 4.8 ± 0.2 using 1.0 N HCl. The solution was stirred for 65 ± 5 minutes at room temperature. The mixture was centrifuged at 4700 rpm for 20 minutes, the supernatant was discarded and the sediments were washed three times with 10 volumes of hexane to remove the oil. Sediments obtained were dried in a hot air oven overnight and the kafirin solids obtained after drying were ground to a powder and stored at room temperature until further analysis and studies.

4.3.2. Kafirin solubilisation

Kafirin extracted from the sorghum flour was solubilised in 60 % (w/w) ethanol for anion exchange and cation exchange chromatography at pH 9.0 and 1.5 respectively. Solutions were slowly stirred at 60 °C for 20 minutes. Then, solutions were brought to room temperature and the ethanol vaporised during dissolution was made up with absolute ethanol. Solutions were filtered through pre-weighed filter paper to separate the insoluble residue. These filter papers were dried and weighed and the weight of insoluble residue after filtration was calculated. These insoluble residues were assessed with CHN analysis to determine loss of kafirin and their identity was confirmed with SDS-PAGE. The weight of these insolubles was also considered when calculating the concentration of kafirin in the solution.

Table 33: Physicochemical properties of the ion-exchange resins selected for kafirin adsorption studies.

Description	**Toyopearl QAE-550C[a]**	**Toyopearl SuperQ-650M[a]**	**Capto Q[b]**	**Toyopearl SP-650M[a]**	**SP FF[b]**
Functional group	Quaternary ammonium	Quaternary ammonium	Quaternary ammonium	Sulphonate	Sulphonate
Base matrix	Methacrylate	Methacrylate	Agarose	Methacrylate	Agarose
Particle size (µm)	100	65	90	65	90
Pore size (Å)	500	1000	–	1000	–
Ion capacity (meq/mL)	0.33	0.25	0.16 – 0.22	0.15	0.18 – 0.25
Dynamic capacity (mg/mL)	60 – 80[c]	105 – 155[c]	> 100[c]	40 – 60[c]	70[d]
Working pH range	1 – 13	1 – 13	2 – 14	1 – 13	3 – 14

a: Manufactured by Tosoh Biosciences
b: Manufactured by GE Healthcare
c: Dynamic capacity with BSA
d: Dynamic capacity with RNase A

4.3.3. Adsorption isotherm studies

Kafirin adsorption upon three anion-exchange and two cation-exchange resins were studied and adsorption isotherms determined in batch systems. All resins had variations in physiochemical properties such as ligand chemistry, particle size, pore size, ion and dynamic binding capacity (Table 33) and were studied to observe the effects of these parameters on adsorption. Ion exchange resins were washed with deionised water to remove the storage solution and then equilibrated with 60 % (v/v) aqueous ethanol at pH 9.0 for anion exchangers and at pH 1.5 for cation exchangers on a rocker shaker for 30 minutes in separate tubes. 3 mL of kafirin solution of different concentrations ranging from 3 mg/mL to 20 mg/mL were contacted to the resins in respective tubes and kept on the rocker shaker for 3 hours at room temperature. At the end of incubation, tubes were centrifuged at 3000 rpm for 5 minutes and the supernatant was collected from each tube. 3 mL of equilibration buffer was added and the sample incubated for 20 minutes on the rocker shaker to wash off any unbound or loosely bound protein from the resin. Later, elution of bound kafirin was undertaken with 3 mL of elution buffer composed of 60 % (v/v) aqueous ethanol with 0.7 M sodium chloride at pH 5.5 for 20 minutes on the rocker shaker. This elution step was repeated three times and eluates were pooled to ensure stripping-off of maximum possible kafirin under given elution conditions. Resins were regenerated using 60 % (v/v) aqueous ethanol with 1.0 M sodium chloride at pH 4.5 for 30 minutes on the rocker shaker. All batch adsorption experiments were performed in triplicates.

4.3.4. Uptake kinetic study

Anion and cation exchange resins (5.0 mL each) were incubated with the 25 mL kafirin solutions at pH 1.5 and 9.0 respectively for 80 minutes on a rocker shaker. Samples were taken at appropriate time intervals. One sample per minute for the first five minutes, five-minute intervals until 30 minutes elapsed followed by ten-minute intervals until 80 minutes.

4.3.5. Column packing and qualification

XK 16 column packing was done in a way slightly different from the manufacturer's recommendations. Column parts were cleaned and rinsed with DI water and assembled in the following order. Column bottom adapter was connected to the column tube ensuring that there was no air bubble/pockets between the nylon ring and support screen. 5 mL of 20% ethanol was poured in the column and the level was marked for the final bed height (2.5 cm). Resin slurry was poured on the glass wall by tilting the column to avoid air bubble trapping followed by 20% ethanol to wash the walls. Once the resin bed was settled, the top adapter was inserted and fixed at around 2 to 5 mm above the resin layer. 20 % ethanol followed by water was passed through the resin bed and the flow rate of the water was gradually increased to 300 cm/h to ensure uniform bed packing. The packed column was then stored in 20% ethanol at room temperature until further use. The same column was packed with a 10 cm bed height for scale-up experiments.

Column qualification to evaluate packing quality was performed by the pulse test. The column was equilibrated with water at 20 cm/h and injected with tracer (2% v/v acetone - 1% of the column volume). Water was passed at the mentioned flow rate until the tracer elutes and the chromatogram was analysed for plate number (N), asymmetry (A_S) and height equivalent to a theoretical plate (HETP) using formula given below.

$$N = 5.545 \times \left(\frac{V_R}{W_h}\right)^2$$

Where, V_R = volume eluted from the start of a sample application to the maximum peak height and W_h= peak width measured at half of the peak height

$$HETP = L/N$$

Where, L is bed height and N is plate number

$$Asymmetry\ factor\ (A_s) = b/a$$

Where, a = first half peak width at 10 % peak height and b = second half peak width at 10 % peak height.

4.3.6. Chromatography conditions

The column was washed with water to remove the storage solution and equilibrated with equilibration buffer. Kafirin extract was loaded on the column and equilibration buffer was passed to wash excess or loosely bound proteins and impurities. Elution buffer was passed through the column to elute the bound protein and collected as fractions. The column was regenerated using extraction solution with 1.0 M NaCl solution to remove tightly bound fractions such as aggregates and other impurities. The column was sanitised with 0.5 M NaOH solution and stored in 0.1 M NaOH solution until further use, or in 20% ethanol for long-term storage.

4.3.7. Scale-up

The XK16 chromatography column was packed with the resin (5 mL) to 2.5 cm bed height to keep the linear flow rate (150 cm/h) consistent with the process developed at HiTrap™ column scale. Volumetric flow rates (mL/min) for column regeneration, sanitisation and storage were adjusted accordingly. Process steps at this scale were kept consistent with the process at HiTrap™ column scale starting from equilibration followed by protein loading and post-load wash, finally elution, column regeneration and storage keeping the same CVs at each step.

For further scale-up the XK16 chromatography column was packed with the same resin (20 mL) with increased bed height to 10 cm; which enables higher protein loading (20X). Flow rates were adjusted accordingly to maintain the residence time (i.e. 1 min). CVs for each step were kept the same to maintain the process volumes constant and hence the concentrations for individual process streams.

All process stream outputs were collected for required qualitative and quantitative analysis to calculate the step yield, recovery and impurity profile among the processes from different scales. Total protein and kafirin recovery will be considered as parameters for the process validation.

4.3.8. Analysis

Samples generated during the studies were subjected to qualitative and quantitative analysis using spectrophotometry, CHN analysis and SDS-PAGE. Data obtained from the quantitative analysis were used to determine the step yield and overall recovery of the process. The qualitative analysis indicated the impurity profile for the individual process streams.

4.3.9. Spectrophotometry

Samples generated during adsorption studies were quantitated for their concentrations and mass balance calculations. Kafirin concentration of purified fractions was determined using UV-VIS spectrophotometry by measuring absorbance at 280 nm. Standard curves were generated for both pH 9.0 and pH 1.5 kafirin solutions to determine the unknown concentrations based on absorbance.

4.3.10. SDS-PAGE

Samples generated from the study were analysed as mentioned earlier in chapter 3 (section 3.2.2.3) for the presence of kafirin (monomer), kafirin related impurities (such as low molecular weight fragments, dimers or aggregates) and other protein impurities. Samples were loaded on 12% SDS-PAGE gels and allowed to run at 100 V. Gels were processed with silver staining for detection of protein bands.

4.3.11. Protein recovery

Samples were quantitated using elemental analysis for their protein content based on their nitrogen content. Acetanilide was used as a standard to calibrate the elemental analyser. The factor of 6.25 was used for nitrogen-to-protein conversion (145). Protein recovery was calculated as follows.

$$Protein\ recovery\ (\%) = \frac{Total\ protein\ in\ output\ streams}{Total\ protein\ in\ load} \times 100$$

4.3.12. Oil removal

Oil was recovered from the process intermediates (solids) as explained earlier (refer section 3.2.7 for oil removal). FT and wash were pooled and subjected to

distillation to remove organic solvents and water. The remaining fraction was extracted with hexane to recover the oil.

4.3.13. SE-HPLC

SE-HPLC of purified kafirin samples was carried out using Agilent Bio SEC-3 with 3 µm particle size and 100 Å pore size. This column can separate proteins of molecular weight ranging from 0.1 to 100 kDa. Column dimensions were 4.6 mm (inner diameter) and 300 mm length. Samples were analysed using the Agilent 1260 Infinity II series HPLC system (Agilent Technologies Inc.) at 214 nm. 50 % ethanol with 1% sodium metabisulphite at pH 8.0 was used as the mobile phase. Purified kafirin samples were dissolved in the mobile phase to achieve 4 mg/mL sample concentration and filtered with 0.1 µm syringe filters before injection. 5 µL sample was injected on the equilibrated column and 0.3 mL/min flow rate was maintained throughout 40 minutes run time.

4.4. Results and discussion

4.4.1. Insolubles from kafirin solubilisation

To confirm the presence of kafirin and determine the concentration of kafirin in solutions prepared for adsorption studies, SDS-PAGE and CHN analysis of the insoluble solids were performed. Kafirin lost as insoluble fractions at pH 1.5 and 9.0 were found to be < 2.0 % and 8.74 % respectively, which indicated that dissolution at pH 9.0 leads to comparatively higher kafirin loss. Furthermore, loss of kafirin as insoluble residues was confirmed by SDS-PAGE (Figure 40). Prolonged exposure to heat and other operating parameters during extraction might be one of the reasons behind the lack of solubility of kafirin under the given conditions.

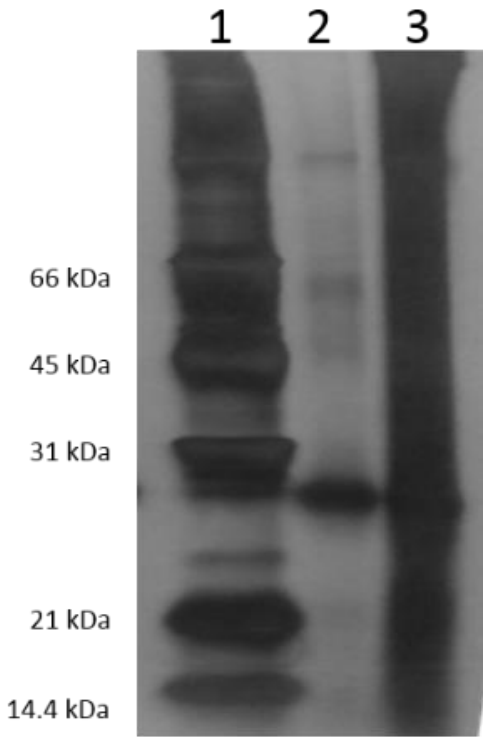


Figure 40: Kafirin insoluble residues detection by non-reducing SDS-PAGE. Lane 1: Broad range marker, lane 2: pH 1.5 insoluble solids, lane 3: pH 9.0 insoluble solids.

4.4.2. Adsorption studies

Kafirin adsorption was studied on strong ion exchange resins with different physicochemical properties as listed in (Table 33). Langmuir or Freundlich adsorption isotherm models can explain adsorption of kafirin on the resin. Langmuir isotherm model (1) considers monolayer adsorption (146) whereas the Freundlich isotherm model (2) is a multilayer adsorption process (147). These isotherms give an empirical relation between concentrations of protein on the surface of resin with respect to the concentration of protein in the solution in contact with the resin.

Adsorption isotherms are classified into four main groups (S, L, H and C) based on their slope and curvatures (148). This classification was purely based on observations, which determines the type of isotherm from the shape of a curve, in the present case, the convex curvature of the isotherm supports Langmuir isotherm. Langmuir isotherm is considered an indicator of monolayer adsorption on the homogeneous adsorbent surface. q_{max} and K_d values from the Langmuir equation defines the maximum adsorption capacity and interaction strength of

the protein with the resin respectively. Moreover, the value of the Langmuir equilibrium constant (R_L) indicates the type of isotherm for the given protein with a given resin candidate, such as R_L =0 indicates that the isotherm is irreversible whereas values 1 and >1 indicates that it is linear and unfavourable respectively. An ideal scenario, 0 < RL < 1 confirms that the isotherm is favourable.

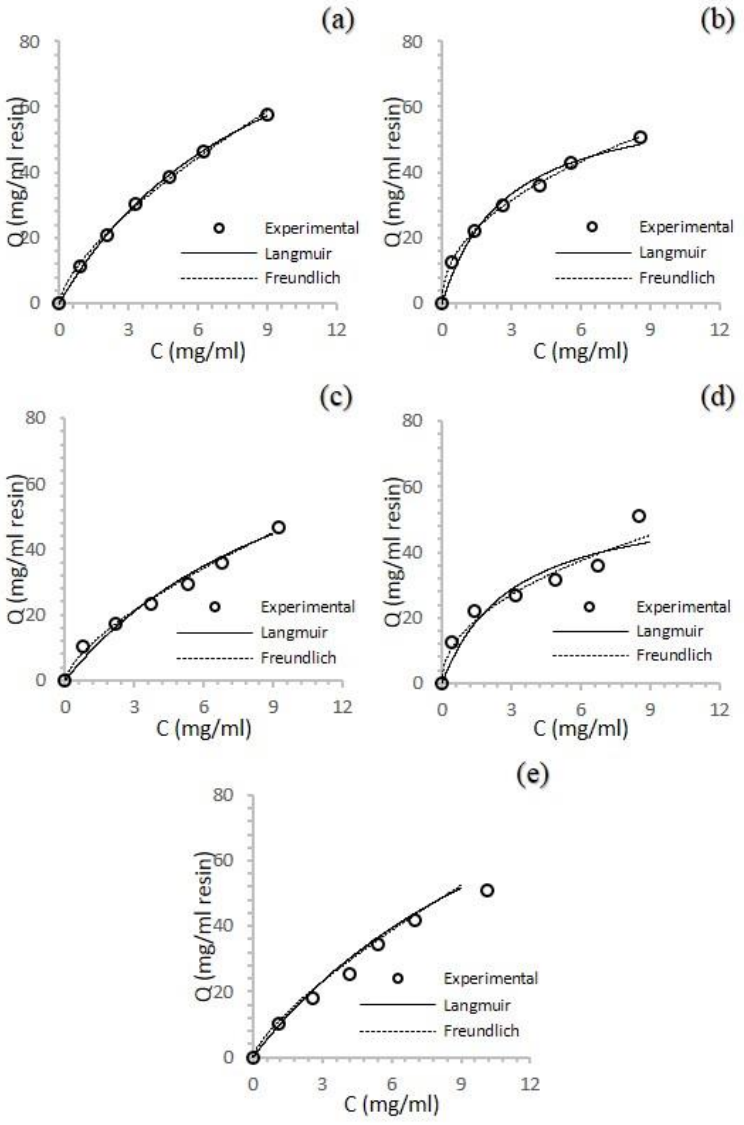


Figure 41: *Adsorption isotherm data of kafirin adsorption for resins. Toyopearl QAE-550C (a), Toyopearl SuperQ-650M (b), Capto Q (c), Toyopearl SP-650M (d) and SP FF (e).*

Langmuir isotherm $$q_e = \frac{q_{max} C_e}{K_d + C_e}$$ (eq. 1)

Langmuir equilibrium constant $$R_L = \frac{1}{1+(C_0/K_d)}$$ (eq. 2)

Freundlich isotherm $$q_e = q_f {C_e}^{1/n}$$ (eq. 3)

Where, q_e: equilibrium concentration in solid (mg/mL resin), q_{max}: Langmuirian adsorption capacity (mg/mL resin), C_e: equilibrium concentration in solution (mg/mL), C_0: initial bulk-liquid phase concentration (mg/ml), K_d: Langmuirian dissociation constant (mg/mL), q_f: Freundlich adsorption capacity (mg/mL), n: Freundlich adsorption intensity.

The values of adsorption constants such as maximum adsorption capacity (q_{max}) and dissociation coefficient (K_d) values for all ion exchange resins were determined initially by doing linear regression using Hanes-wolf (149) and Lineweaver-Burk plots (150) but they did not give optimised fit. Hence, Curve fitting application in MATLAB 2014b was utilised for non-linear regression to get a better fit to the experimental data (Table 34). Similar approach was reported earlier to obtain fit using non-linear regression (151–156). As mentioned earlier, Langmuir isotherm supports monolayer adsorption whereas Freundlich isotherm, which is an extension to the former, adopts multilayered adsorption. Also, Freundlich isotherm is usually used whenever the Langmuir isotherm fails to address the protein adsorption behaviour with the particular resin. According to a study, it was observed that kafirin adsorption on ion-exchange resins follows both Langmuir and Freundlich adsorption isotherm models (79). However, the present study confirmed that Langmuir, which is monolayer adsorption, is a favourable mechanism involved in the kafirin adsorption process. Though Freundlich isotherm showed a precise fit, it was rejected. The reason being, the value of intensity factor (n) that dictates the adsorption was greater than 1 which underestimated adsorption capacity for the resins. Furthermore, data obtained from the Langmuir isotherm equation (Table 34) gave a fair idea about the adsorption capacity of the resins which was closer to the literature values for kafirin (79). To further support the applicability of Langmuir isotherm, the value of Langmuir isotherm equilibrium constant, R_L was found to be less than 1 for all inlet concentrations of adsorption experiment, which as explained earlier, suggesting reversible binding kinetics making it favourable for the chromatographic separation. Equilibrium adsorption data from batch studies of kafirin adsorption with isotherm fits at varying initial concentrations are shown in Figure 41.

The maximum binding capacity (q_{max}) gives an idea about the amount of kafirin required to saturate all possible binding sites. Both cation exchangers, Toyopearl SP-650M (135.40 mg/mL) and SP FF (118.60 mg/mL) have the highest binding capacity as compare to their anion counterparts (Table 34). Among the anion exchangers, Toyopearl SuperQ-650M has the highest binding capacity (102.30 mg/mL) followed by Capto Q (63.99 mg/mL) while Toyopearl QAE-550C (57.60 mg/mL) with similar lower values. These differences are probably due to differences in particle size and resulting surface area available for adsorption. Other parameters such as higher pore size and low ionic capacity also facilitate adsorption capacity. Experimental data were compared with literature values from findings of Kumar et at. The value of q_{max} for Toyopearl QAE-550C was comparable to literature whereas Toyopearl SuperQ-650M and Toyopearl SP-650M showed 49.7 % and 130 % higher values respectively. The selected resin in the research reported was UNOsphere Q (q_{max} = 78.26) which is not considered in this work. In the present study, CaptoQ provided comparative binding capacity with higher binding strength (~ $1/3^{rd}$ K_d value) than UNOsphere Q, making it a better option to use at high flow rates. In extension to this, Capto Q has proved to be an efficient resin in the current research paradigm because of its unique design such as porosity, high bead rigidity and operation flexibility at higher bed heights (157).

The dissociation constant (K_d) is equally important which indicates the degree of interaction between kafirin and the resin candidate under screening. K_d values for ion exchangers lie between 10^{-8}M and 10^{-4}M (158). Higher K_d value indicates weaker interaction whereas low K_d value denotes strong binding (Milner 1999) which leads to lower yield. Both the cation exchangers have K_d values much higher which indicate weaker binding compared to anion exchangers, which means loss of protein and hence low recovery during elution. Toyopearl SuperQ-650M, the anion exchanger with the higher binding capacity also demonstrated high K_d value which again falls into the same category as evaluated cation exchangers. Capto Q is the next suitable candidate with fair binding capacity and low K_d value that assures strong binding and hence better recovery which is evident from the experimental data (Table 35). Also, binding capacity for Capto

Q and Toyopearl QAE-550C derived from Langmuir isotherm is comparable to the experimental values whereas the values for other resins are not comparable.

Table 34: Results for Langmuir and Freundlich isotherm models from adsorption studies.

Resin	Langmuir isotherm					Freundlich isotherm		
	q_{max}	K_d ($\times 10^{-5}$)	R^2	R_L at C_1	R_L at C_6	q_{max}	K_d ($\times 10^{-5}$)	R^2
SP FF	118.60	9.62	0.9995	0.71	0.26	13.17	1.47	0.9986
Toyopearl SP-650M	135.40	16.43	0.9946	0.27	0.05	9.29	1.34	0.9940
Capto Q	63.99	2.73	0.9835	0.32	0.07	19.04	2.18	0.9989
Toyopearl SuperQ-650M	102.30	11.89	0.9798	0.52	0.14	9.95	1.47	0.9931
Toyopearl QAE-550C	57.60	2.87	0.9080	0.27	0.05	16.60	2.14	0.9545

q_{max}: Adsorption capacity in mg of protein/mL of resin, K_d: Dissociation constant

[1]Molecular weight of kafirin as 27 KDa (40)

Table 35: Average kafirin adsorbed (%) on different ion exchange resins.

Resin	Kafirin Adsorbed (%)
Toyopearl QAE-550C	56.80
Toyopearl SuperQ-650M	47.10
Capto Q	60.50
Toyopearl SP-650M	36.80
SP FF	47.30

4.4.3. Uptake kinetics

Initial concentrations of the kafirin solutions loaded on each anion and cation exchange resin was 16.99 mg/mL and 18.62 mg/mL respectively, determined by analysis of the insoluble solids formed during dissolution. Uptake kinetic data obtained for kafirin adsorption on each ion-exchange resin is shown in Figure 42. Adsorption rates of kafirin on cation exchange resins were slightly slower than those for anion-exchange, which was reflected by the higher K_d values calculated in the equilibrium adsorption experiments. It can also be observed that equilibrium was reached at approximately 30 minutes mark for both cation-exchange resins in comparison to the 10 minutes with anion exchangers. Among cation exchangers, Toyopearl SP-650M saturated earlier compared to SP FF, however amount of protein bound to later was higher. Anion exchanger took the similar time (3-4 minutes) to achieve saturation; Capto Q and Toyopearl QAE-550C were comparable in terms of total bound protein whereas least protein adsorption was observed on Toyopearl SuperQ-650M (Table 35).

Owing to the low q_{max} for Toyopearl QAE-550C, equilibrium concentration was higher than that of Toyopearl Super Q-650M and Capto Q. Besides, adsorption on Capto Q was found to be slightly faster than Toyopearl SuperQ-650M which is again reflected by lower K_d value. However, it is evident from the experimental values (Table 35) amount of protein adsorbed on Capto Q was high compared to other ion exchange resins. Furthermore, Capto Q also offers a lower pressure drop across the resin bed which is an added advantage for upscale of chromatographic processes (GE LifeSciences 2006).

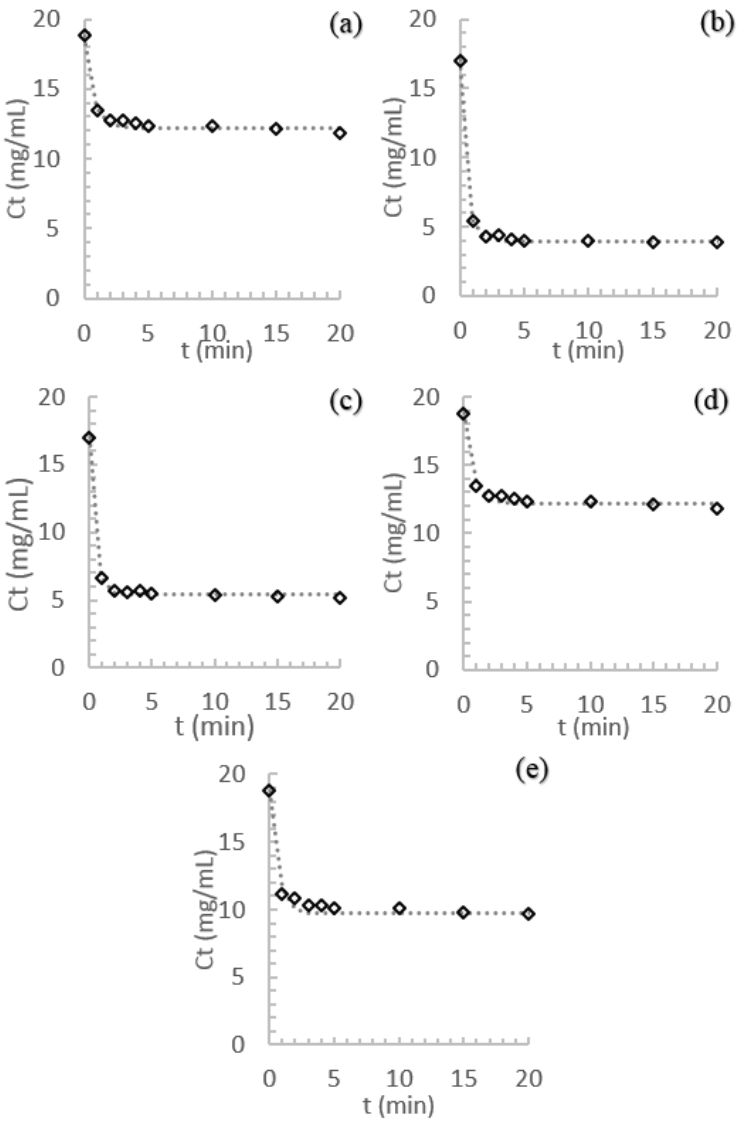


Figure 42: *Uptake kinetics data of kafirin adsorption for resins. Toyopearl QAE-550C (a), Toyopearl SuperQ-650M (b), Capto Q (c), Toyopearl SP-650M (d) and SP FF (e).*

4.4.4. Column chromatography

Capto Q was finalised as an ideal resin candidate from uptake kinetics and adsorption isotherm studies. Its binding capacity was tested at a small scale in bind and elution mode as mentioned earlier in section 4.3.6.

Column qualification was carried out to ensure efficiency or performance of the column as it assures robustness and desired quality and safety of the final product. It can be expressed in terms of the height equivalent to a theoretical plate (HETP) and asymmetry factor (A_S). Representative chromatogram (Figure 43) and column qualification parameters (Table 36) derived from column

efficiency testing exercise for Capto Q informed about the packed column and its performance during separation. Peak asymmetry value obtained as the column qualification parameter was 1.09, based on that, the column considered qualified for the chromatography as the asymmetry value should fall within the range of 1.0 ± 0.2.

Column efficiency also expressed as the number of plates per meter of column bed height (N) or as HETP (H). The number of plates are hypothetical stages where molecules come into equilibrium. Greater the number of theoretical plates for a column, the more efficient the separation. HETP is used to normalise the N across columns of different dimensions. A lower HETP value for a particular column signifies better separation and is usually measured over time to monitor the column performance. Increase in HETP value points out decay in column performance and indicates the requirement for column repacking.

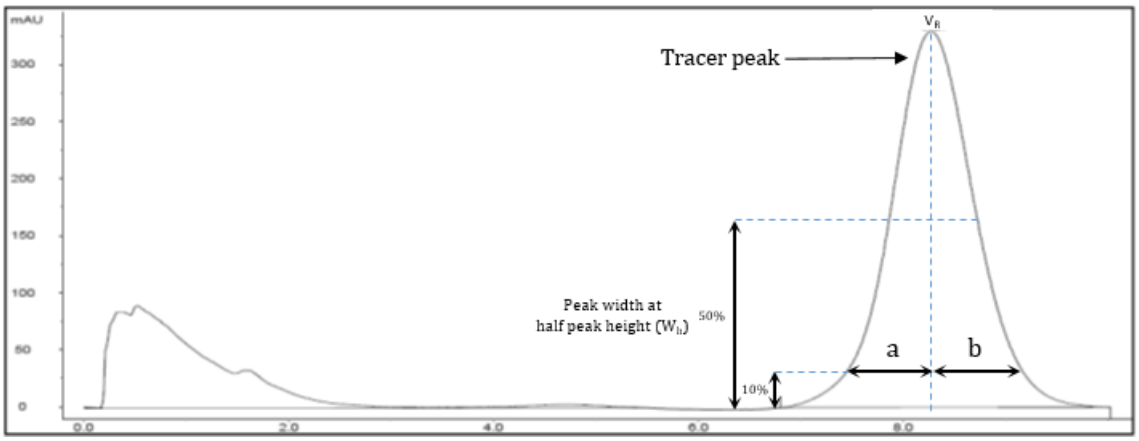


Figure 43: Capto Q column chromatogram from column efficiency testing.

Table 36: Column qualification parameters.

No	Retention (ml)	Area (mAU*ml)	Height (mAU)	Plates/ Meter (N/m)	10 % Left (ml)	10 % Right (ml)	Asymmetry (A_S)
1	8.27	314.17	329.91	**5104**	7.46	9.16	**1.09**

Although HETP and asymmetry cannot be used to predict the recovery of the process or purity of the product, it is the quickest way to test the performance of the column and chromatography system. This test was repeated regularly to monitor the state of the bed throughout the working life of the column and assess its performance over time.

4.4.5. Anion exchange chromatography: Capto Q HiTrap

Anion exchange chromatography was carried out using Capto Q HiTrap column. The column was flushed with water to remove the storage solution and equilibrated with the buffer consists of 60 % ethanol at pH 9.0. Kafirin dissolved in equilibration buffer was loaded and FT was collected to assess any unbound kafirin. The column was washed with equilibration buffer to remove any loosely bound material. As observed in the chromatogram (Figure 44), kafirin elutes out in a single peak (eluate peak) when the elution buffer was passed. A small peak (high molecular weight (HMW) aggregate peak) emerged on the tailing end of the main peak. The column was stripped off any tightly bound impurities using regeneration buffer. Another peak (regeneration peak) was observed when the regeneration buffer was passed which was collected separately.

These collected fractions were analysed using SDS-PAGE to identify the product and process-related protein impurities in different streams. Electrophoresis profile of these samples revealed that the eluate peak contains kafirin with faint bands of LMW and HMW impurities. The HMW peak eluted later contains a prominent kafirin band with HMW impurity band of relatively higher intensity than in the eluate peak. These HMW impurities might have eluted because of loose binding to the column due to the strength of the interaction between these HMW and ligand may be similar to that of kafirin molecule and ligand. These loose interactions may occur due to several reasons such as unavailability of ligand due to the hydrodynamic radius of the molecule bound to the adjacent ligand. Similarly, high concentration of the protein during elution is likely to result in the higher intermolecular interactions resulting in the generation of high molecular weight aggregates. The regeneration peak at the end contains the majority of tightly bound HMW that elutes under strong elution conditions, which is at high salt concentrations.

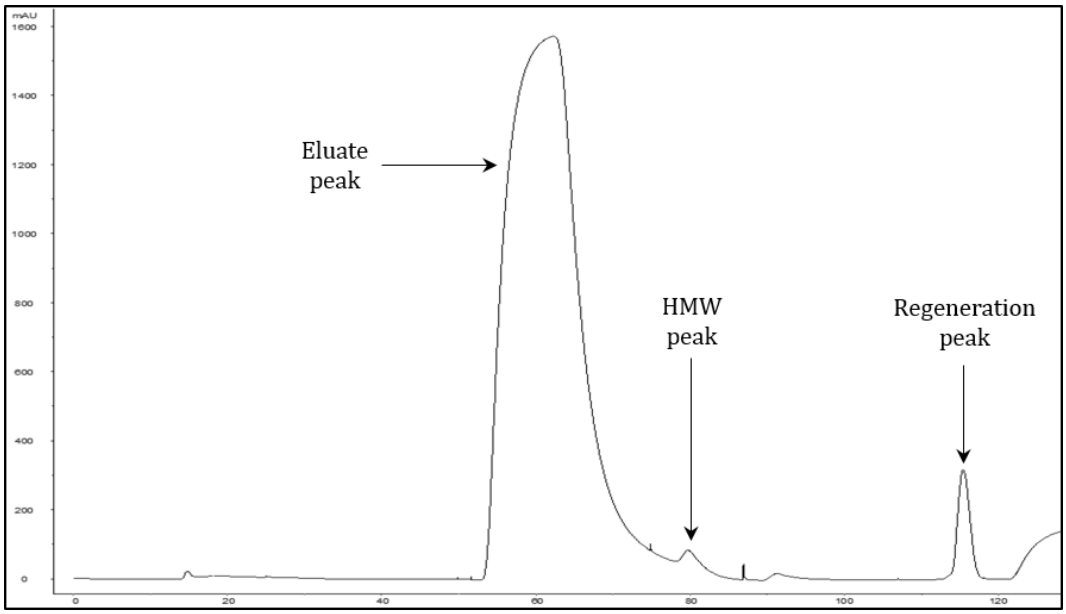


Figure 44: Anion exchange chromatogram for kafirin purification with Capto Q Hitrap column.

4.4.6. Anion exchange chromatography: Capto Q in XK16 column

Considering higher aggregate content and loss of kafirin in HMW and regeneration peak (Figure 44), 1% sodium metabisulphite was added to buffers as a reducing agent to avoid intermolecular interactions and generation of HMW. As evident from the chromatogram (Figure 45), anion exchange chromatography performed with the buffers containing reducing agent resulted in absence of HMW peak and a significant reduction in regeneration peak, which are nothing but tightly bound HMW aggregates. Addition of reducing agent resulted in better yield 86% (earlier ~80%) and higher purity (>98 %).

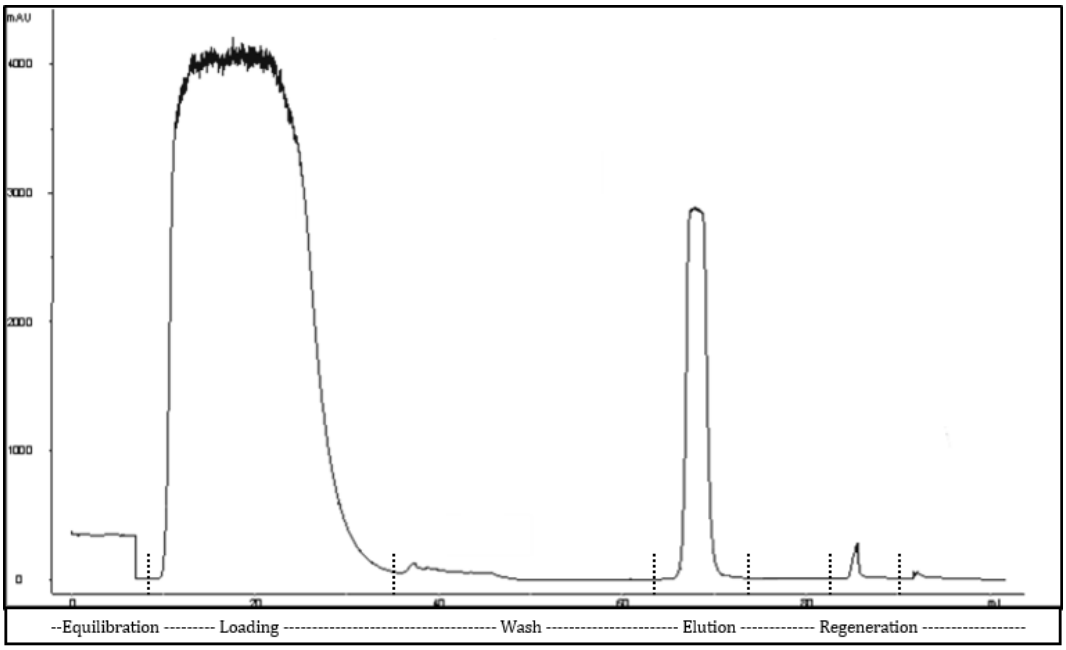


Figure 45: Anion exchange chromatogram for kafirin purification with Capto Q at a higher scale.

Unlike earlier trial (Figure 44), a peak was observed (in loading region) as crude kafirin extract was loaded that contains both protein and non-protein impurities. The peak was collected and labelled as flow-through (FT) along with the post-load equilibration buffer wash as it contains all protein and non-protein impurities that do not bind to the column in a given condition. FT analysis by SDS-PAGE confirmed that there was no kafirin, which assures the binding of desired protein at given conditions. Presence of other protein bands in the FT stream suggesting that most protein impurities did not bind to the column. It was observed that oil did not bind to the column, as it has no charge and was collected in FT. After the post-load wash, elution buffer was passed to elute the protein of interest, which resulted in a peak. SDS-PAGE profile confirmed the stream was a purified kafirin as there were no other bands observed other than kafirin. There were some faint bands, which were product-related impurities such as high molecular weight aggregates above the main band and possibly low molecular weight truncated kafirin below the main band. Regeneration solution was passed to strip the column of any tightly bound entities. The peak was later confirmed as the high molecular weight aggregates of kafirin. It was confirmed that there were no traces of oil in the dried purified protein stream after elution and extracted using hexane.

4.4.7. Scale-up and validation

The scale-up strategy of the chromatography step was two-fold. Initially, the column was scaled-up by a factor of five by keeping the height constant and increasing the diameter of the column. Later, the same column was used with the increased bed height, which was again a 4-fold leading to overall 20-fold scale-up. Chromatography conditions optimised at bench scale using HiTrap™ column for kafirin purification were same at the higher scale.

Chromatography was carried out (1 mL and 20 mL) and recovery compared at both the scales (Table 37). B1, B2 and B3 were executed at 1 mL scale whereas S1, S2 and S3 represent the 20 mL scale. As defined in ICH guidelines, validation is the exercise carried out to establish a documented evidence that the process can perform effectively and will reproduce the product or process intermediates with similar specification when operated within the established parameters (64). Kafirin was purified at two different scales and the purified kafirin was analysed for its purity and yield. Process related impurity (oil) and product-related impurities (HMW and LMW) compared along with purified kafirin to validate the process.

Total protein recovery (Table 37) for the process refers to mass balance i.e. the total protein recovered in various output streams such as FT, wash, eluate and regeneration with respect to the protein content in the input stream. Data for protein recovery from batches from both scales were comparable and averaging 97.55% and 97.69% respectively. Kafirin yield (Table 37) was calculated in terms of extractable kafirin in purified fraction with respect to the total kafirin content in load material. Bench-scale batches resulted in an average yield of 85.26 % whereas higher scale batches were with mean of 88.03%, which are comparable.

Table 37: Comparative qualitative and quantitative data from chromatography at bench and higher scale batches.

Parameters	Bench-scale			Scale-up (20 x)		
	B1	B2	B3	S1	S2	S3
Kafirin yield (%)	86.53	86.02	83.23	87.07	87.44	89.58
Protein recovery (%)	98.68	97.52	96.45	96.45	98.74	97.88
Purity by SE-HPLC (%)	98.42	99.30	98.87	99.21	99.49	99.26
HMW impurity by SE-HPLC (%)	1.37	0.56	0.92	0.65	0.43	0.69
LMW impurity by SE-HPLC (%)	0.21	0.14	0.21	0.14	0.08	0.05
Oil in FT (%)	4.23	7.79	5.02	8.23	8.79	9.02
SDS-PAGE profile	Comparable					
HMW*	ND	ND	ND	ND	ND	ND
LMW*	ND	ND	ND	ND	ND	ND
FTIR profile	Comparable					

*ND: Not detected (trace or less than 2 % of main kafirin band).

Purified kafirin obtained from batches at both scales were analysed by SE-HPLC to determine the purity and impurity content (Figure 44). HMW observed at RT of 11.5 min followed by main kafirin peak at 12.9 min. There were few minor peaks observed at RT 16.2, 17.6 and 20.4 min, which were identified as LMW. These HMW and LMW impurity peaks were integrated and their percentage contribution to the total area under the curve was calculated (Table 37). SE-HPLC purity of the kafirin purified at different scales was comparable along with the impurity content. Kafirin from bench-scale batches was >98% pure whereas >99% purity was recorded for higher scale batches (Table 37).

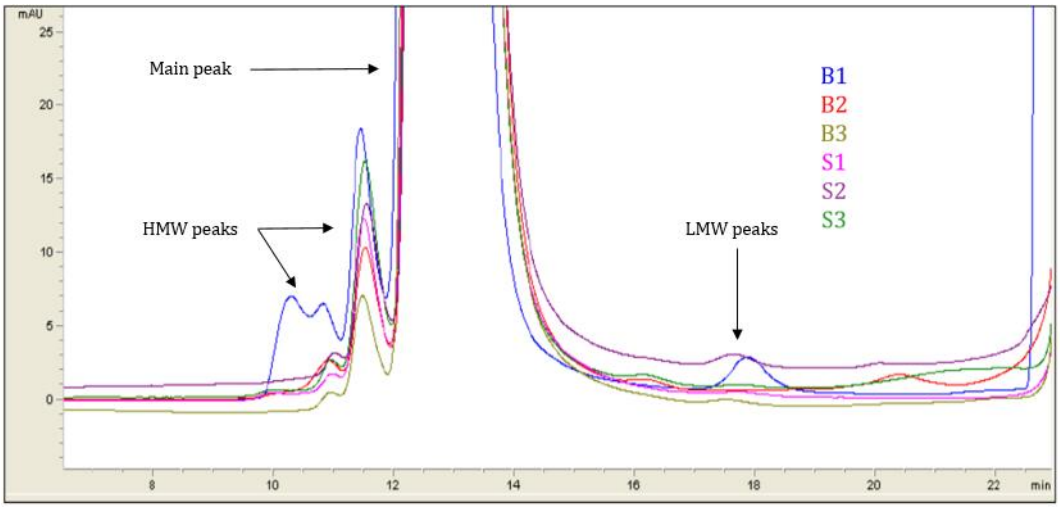


Figure 46: SE-HPLC chromatogram for purified kafirin at bench (B1, B2 and B3) and higher scale (S1, S2 and S3) batches.

FT and wash were pooled to analyse oil removal. Oil extraction data in Table 37 obtained from the higher scale batches indicated that the step was efficiently removing the oil present in the load material (~ 10%). As discussed earlier, the remainder of oil might be removed in later stages of sample handling. It was confirmed that there was no oil in the purified kafirin when extracted with hexane. Data obtained from the small-scale batches were inconsistent due to low handling volumes.

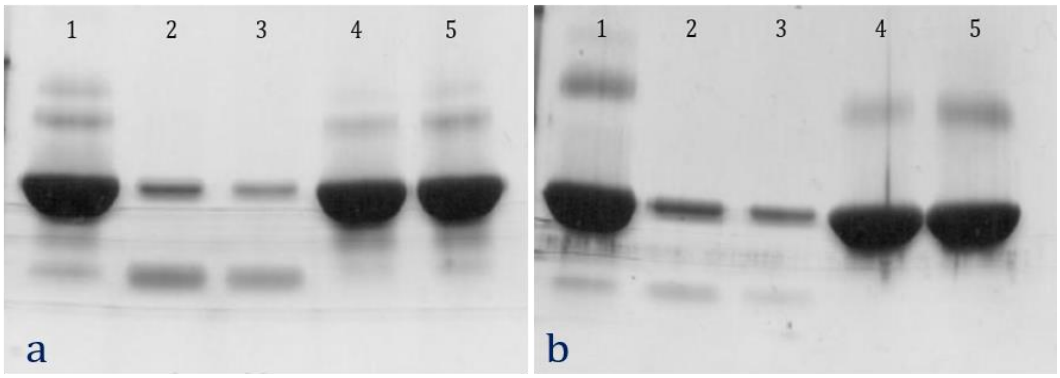


Figure 47: SDS-PAGE profile of kafirin purified from the bench (a) and higher scales (b) batches.
Gel a: Purified kafirin from batches B1, B2 and B3 in lane 1, 4 and 5 respectively.
Gel b: Purified kafirin from batches S1, S2 and S3 in lane 1, 4 and 5 respectively.
Lane 2 and 3 in both gels contain 2% and 1% kafirin respectively for reference purpose.

SDS-PAGE profiles of kafirin extracted at bench scale were consistent as the band intensity of the kafirin in each lane was comparable (Figure 47a). It is evident from the SDS-PAGE profile that HMW and LMW impurities in all samples were consistent and less than 1%. Similarly, purity of the kafirin from higher scale batches was comparable and their impurity profile suggests that both HMW and LMW were below 1% (Figure 47b). The observations were consistent with and supported by the data obtained from SE-HPLC. Similarly, FTIR spectra of the kafirin purified from both bench-scale and scaled up batches aligned and were comparable.

Another important observation from the SDS-PAGE profile is the LMW impurity band below the main kafirin band (α-kafirin, molecular weight ~27 kDa) from batches at different scales might be the β-kafirin (molecular weight ~18 kDa). However, to identify and confirm the presence other types of kafirin/isoforms such as β-kafirin, further investigation using specific analytical techniques such as isoelectric focusing (IEF) or 2D SDS-PAGE coupled with immunoblotting (western blotting), peptide mass fingerprinting and sequencing, which was beyond the scope of the current study.

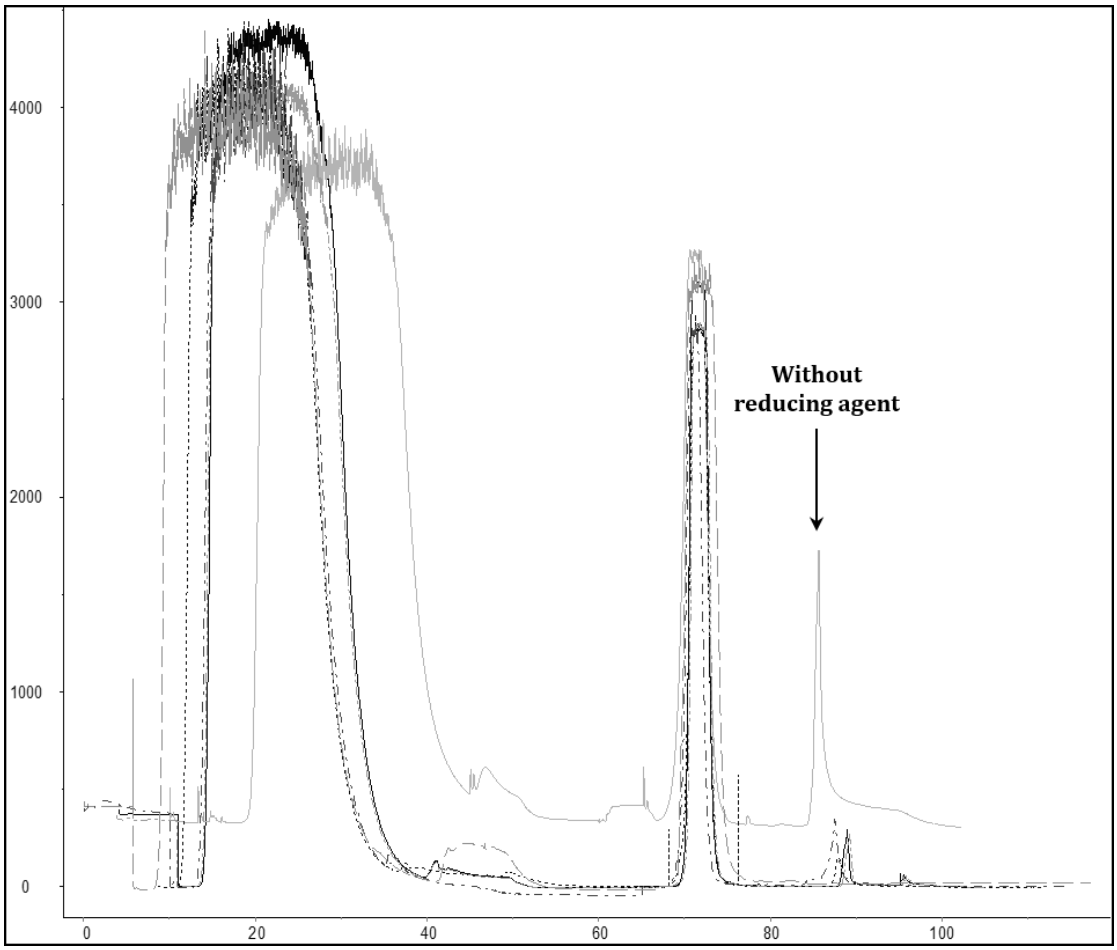


Figure 48: Ion exchange chromatograms for purification of kafirin.

As shown in Figure 48, representative (normalised) chromatograms from both scales indicate that the chromatographic purification of kafirin using ion-exchange chromatography was scaled up successfully. Initial peak appeared during the loading mainly composed of the impurities that do not bind to the column and collected as FT and wash. Spikes appeared at the top of FT peak during loading may appear because of absorbance saturation around 4000 mAU. Baseline after wash assured that there was no loosely bound material left in the column. A sharp peak appeared when elution buffer was passed which contained the product followed by the smaller peak containing tightly bound impurities such as HMW aggregates. Initially, amount of HMW kafirin aggregates was significantly higher which lead to reduced step yield (Figure 48). Chromatogram (solid line) in Figure 48 aligned with other batches to compare the effect of reducing agent on the kafirin purification using anion exchange chromatography. Addition of sodium metabisulphite increased the kafirin recovery and reduced the HMW impurities, which can be explained by the fact that addition of reducing

agents cleaves the disulphide linkages and reduces intermolecular and intramolecular interactions and aids in the extraction which is in agreement with the observations reported by Gao et.al (112).

Operating throughput for any given chromatography process depends on the linear flowrate for the entire unit operation. In present study, XK16 column was packed with the resin column volume (5 mL) to 2.5 cm bed height and operated at the linear flow rate (150 cm/h) consistent with the process developed at HiTrapTM column (1 mL) scale. To increase the operating throughput, loading capacity of the available column was increased by increasing the bed height to 10 cm that accommodates 20 mL resin. Flow rate at this scale was 5 mL/min for the entire unit operation. It was demonstrated that the recovery and purity of the purified kafirin were consistent with the small-scale process. However, scale up to pilot and industrial scale would give clear idea about the actual throughput of the process. Also, continuous chromatography or counter current extraction would provide better platform to determine the LHSV of the process.

4.5. Conclusion

In the present study, various ion exchange resins with different physicochemical properties were investigated for adsorption isotherm and uptake kinetics for kafirin. The binding study determined R_L values less than 1, which indicates monolayer adsorption that in turn is best described by Langmuir adsorption isotherm. Adsorptions studies indicated that cation exchange resins have higher binding capacities compared to anion exchange resins, however, higher K_d values for former suggests weaker binding and hence poor yield. Among the screened anion exchange resins, Capto Q showed the highest binding capacity with lowest K_d value signifying higher yield (86.97%) and efficient separation. As compared to resins screened earlier in the literature and among the candidates in the current study for separation of Kafirin, Capto Q presents a prospective alternative, which can ensure lower inventory of resin and higher binding capacity. This can further translate into higher productivity for similar process scales. Furthermore, with added advantages such as less backpressure and higher salt tolerance that Capto Q offers, it can be a potential candidate for large scale purification of kafirin (157). Uptake kinetics suggested that anion exchange

resins attained equilibrium faster as compared to cation exchange contenders. Pre-treatment of raw material (as discussed in section 3.5.1 for DDGS pre-treatment) before extraction significantly reduced both protein and non-protein impurities in the load material thereby increasing the loading capacity of the column. Addition of sodium metabisulphite to the mobile phases is highly recommended as it reduces the amount of aggregates in the elution thereby increasing the yield and reducing the HMW product-related impurities. To conclude, the study presents an efficient way of purifying kafirin with a limited number of steps resulting in the reduction of overall processing time (Figure 49).

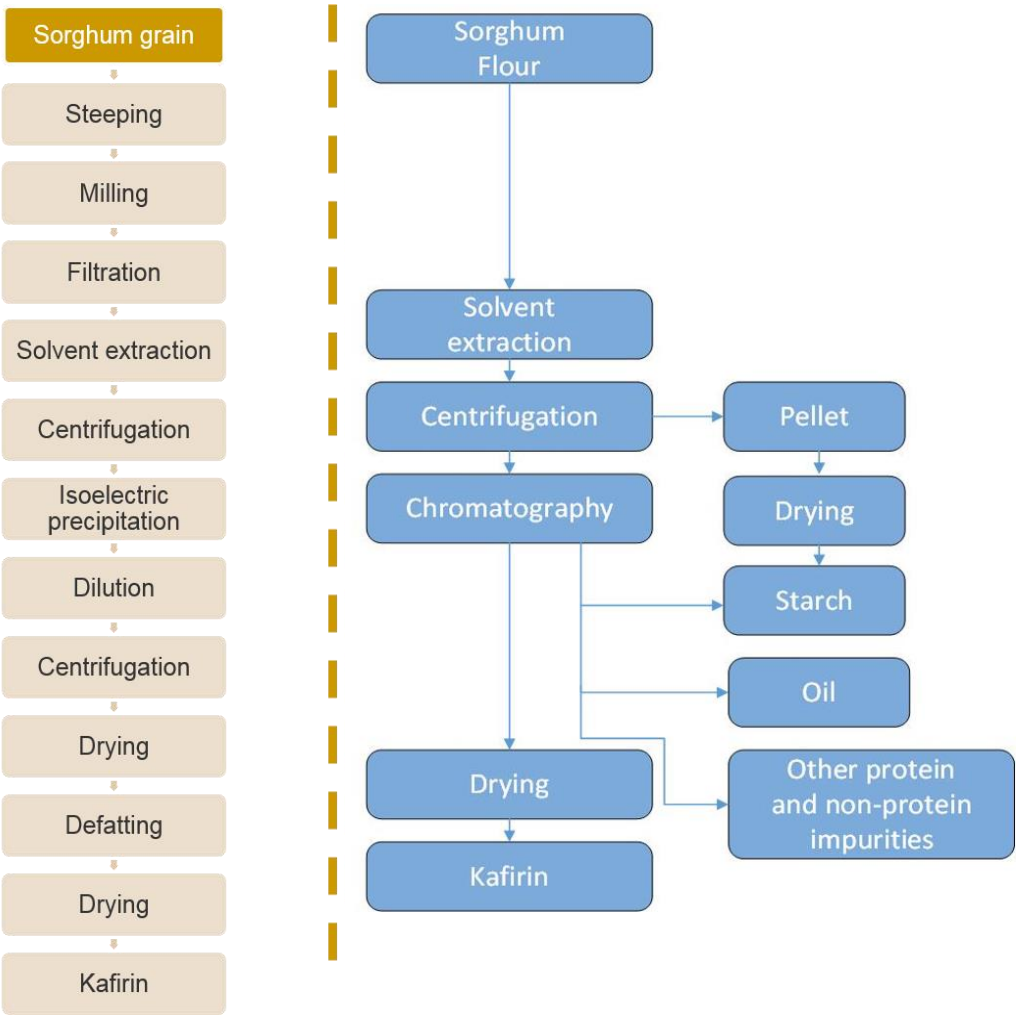


Figure 49: Comparative representation of preliminary extraction process v/s chromatography-based purification process.

Chapter 5: Ultrasonic cavitation

5.1. Introduction

Distiller's dried grains with solubles (DDGS) is the major byproduct of the distilleries and bio-refineries the produces alcohol from grains. DDGS is a high protein content (~ 40 %) solid waste generated after brewing. DDGS has been used as a protein-rich and low-cost animal feed for a long time. Other than that, DDGS has been tested and used for both food and non-food applications such as to induce bricks porosity instead of hay (160), metal ion removal (161), brew inoculum (162,163), and added to snacks like its source, sorghum in "Weetbix" (Sanitarium, Australia). These qualities and proven application make DDGS suitable for food and other applications. Dalby refinery in Australia uses sorghum as raw material for ethanol production and produces kafirin rich DDGS, which is again, used as fodder and exported to other countries such as china, japan and USA. Using DDGS as an alternative to sorghum grain/flour as a source of kafirin offers several advantages such as it presents economic alterative raw material for kafirin extraction. DDGS is a protein rich solid waste stream with less impurities compared to sorghum flour/grain. High protein (especially kafirin) content of DDGS reduces process volumes and therefore further reduces footprint and processing time. Use of DDGS incorporated less process and product related impurities and hence efficient use of solvents achieved compared to sorghum grain/flour. Using DDGS would be preferable as extracting kafirin from sorghum grain/flour would produce starch as waste. So, first converting starch to glucose (for pharmaceutical and other uses) or ethanol (for industrial and as fuel additive) and then extracting kafirin from waste stream (DDGS) would ensure complete utilisation of sorghum as a raw material, which makes process environment friendly. Grinding grains to flour is energy intensive process compared to grinding DDGS.

Ultrasonic cavitation has been widely used in different fields including food processing and preservation (164), non-destructive testing (165), water purification (166), medical imaging, mining, nanotechnology and as process intensification tool (167). Kafirin extraction from sorghum flour using ultrasonication has been reported earlier, however, it was carried out at a smaller scale in bath type ultrasonicator which is considered less effective (57). Also, different fractions were collected during the study, however, there were no

reported accounts of recovery and purity of different fractions. Kafirin analysed using combustion method with a nitrogen analyser that determine total protein content, not the kafirin content. SDS-PAGE or other specific method needs to be employed to confirm purity of the kafirin. Another study reports extraction using probe, however, used of heptane for kafirin extraction limits the food related application development due to acute toxicity of the solvent used (60). In present study, ultrasonic cavitation was employed using ultrasonic probe for the extraction of kafirin from DDGS using DOE based approach. Initially, preliminary experiments were carried out to define the boundary conditions for the process parameters under screening. Operating process parameters beyond these boundary conditions have negative implications on the process yield and product quality. For instance, lower amplitude and time range resulted in lower extraction recovery whereas higher extremes resulted in protein denaturation followed by degradation. A full factorial design was used to assess the optimal conditions for kafirin extraction. Parameters considered for the study were extraction time (min), amplitude and solid to liquid (S/L) ratio. DOE based approach was instead of traditional OFAT to understand the effect of individual parameters and their interactions on kafirin extraction.

5.2. Materials and methods

5.2.1. Materials

DDGS (27.34 % moisture, 37.82 % protein/26.9 % kafirin) was obtained from Dalby Bio-refinery, Australia. The dry DDGS washed (as explained earlier in chapter 3), dried and ground to fine particle size. Ground DDGS passed through 250 µm mesh sieves and stored in airtight bags at 2 to 8 °C. Ethanol, sodium metabisulphite and sodium hydroxide (analytical grade) procured from Sigma Aldrich, Australia. Ultrasonic probe diameter 19 mm (3/4”) was used with Sonics Vibra cell controller (VCX-500, Sonics and Materials, Newtown, USA).

5.2.2. DOE design

Three independent variables were selected with their optimization ranges (Table 38) to assess the effect off individual parameters and their interactions using full factorial design. There are numerous reports that adopted similar designs to

screen process parameters at 3 levels, derived model and data presented with interaction profiles, surface profilers and contour profilers (136,168–170).

Table 38: Ultrasonic cavitation parameters with ranges.

Parameters	Range		
Time (min)	4	7	10
Amplitude	35	45	55
S/L ratio	5	10	15

5.2.3. Kafirin extraction procedure

The extraction of kafirin was performed by adding required amount DDGS (based on S/L ratio) into 200 mL of extraction solution in a jacketed container. Mixture was sonicated using an ultrasonic processor with a ¾" probe, which was operated at set amplitude for given time. The sonication was carried out in a jacketed container with circulating coolant bath to control the temperature. After ultrasonic treatment, the stream was processed as extraction output stream as described in chapter 3. Powdered kafirin was analysed for its purity and quantity.

5.2.4. Kafirin analysis

The extracted kafirin in the sample was quantitatively analysed using elemental analyser based on their nitrogen content. Acetanilide was used as a standard to calibrate the elemental analyser.

5.2.5. SDS-PAGE

Kafirin sample was analysed with SDS-PAGE (chapter 3) for the purity of the protein and presence and HMW and LMW generated during the process and other protein impurities.

5.3. Result and discussion

5.3.1. Design and analysis

DOE based full factorial design was used to determine the effect of potential process parameters for ultrasonic cavitation and the interactions on Kafirin yield. These parameters were screened at three different levels and the results from the experiments are listed in Table 39.

Table 39: Full factorial design for ultrasonic cavitation.

Sr. No.	Pattern	Time (min)	Amplitude	S/L ratio	Kafirin yield (%)
1	331	10	55	5	48.41
2	111	4	35	5	49.45
3	213	7	35	15	57.9
4	000	7	45	10	61.51
5	132	4	55	10	60.47
6	313	10	35	15	61.89
7	131	4	55	5	56.12
8	223	7	45	15	63.58
9	000	7	45	10	61.78
10	233	7	55	15	65.41
11	222	7	45	10	60.49
12	212	7	35	10	54.58
13	321	10	45	5	51.82
14	121	4	45	5	54.85
15	123	4	45	15	57.94
16	333	10	55	15	67.73
17	231	7	55	5	53.94
18	221	7	45	5	54.56
19	232	7	55	10	61.97
20	113	4	35	15	47.11
21	133	4	55	15	61.62
22	323	10	45	15	67.78
23	311	10	35	5	46.32
24	000	7	45	10	60.64
25	211	7	35	5	50.4
26	322	10	45	10	59.53
27	312	10	35	10	56.2
28	112	4	35	10	50.55
29	332	10	55	10	60.25
30	122	4	45	10	56.97

The experiments carried out with combination of different parameters where energy intensities were different leading to difference in the kafirin yield (%). The energy intensity varied from 13.41 to 21.53 watts/cm^2. Also, temperature of the extraction solution sonicated at 55 % amplitude for 10 minutes at (higher) pulse ratio of 5:1, reached to 60 °C (without cooling). Ethanol from extraction solution started evaporating in this condition with increase in time up to 10 minutes. Sonication was carried out in a jacketed container with circulating coolant bath to control the temperature and pulse ration of 1:1 was used to avoid over heating of the sample.

The experimental result obtained from full factorial design were analysed. The R^2 value (Table 19) and p-value (Table 20) for the model were calculated to assess the statistical significance of the model. The R-square value is the coefficient of determination that shows the proportion of the variance in the kafirin yield (%), which is explained by the given model. R-square can range from 0 to 1 where the value of 0 has no prediction power whereas a model with an R-square of 1 can perfectly predict the response. Here, the R-square value of 0.988 for the given model indicates that the model explains about 98.8 % of the variability in kafirin yield for the extraction step. The p-value (<.0001) for the model is less than the significance level of 0.05, which indicates that at 0.05 significance level, the coefficient is not zero. This small p-value and R-square value indicate that the model is statistically significant.

Table 40: Summary of fit for kafirin yield (%) from ultrasonic cavitation.

RSquare	0.988
RSquare Adj	0.983
Root Mean Square Error	0.775
Mean of Response	57.392
Observations (or Sum Wgts)	30

The data obtained from the input parameters were analysed using analysis of variance (ANOVA). The ANOVA summary obtained from the model for Kafirin yield (%) by ultrasonic cavitation is given in Table 41.

Table 41: ANOVA summary for the full factorial design for kafirin yield from ultrasonic cavitation.

Source	DF	Sum Squares	of Mean Square	F Ratio
Model	9	979.888	108.876	181.382
Error	20	12.005	0.600	Prob > F
C. Total	29	991.893		<.0001*

The value obtained for the model for F-ratio and p-value were 181.38 and <.0001 respectively which suggest that the model is significant.

Figure 50 shows the effect of individual process parameter on kafirin yield (%). Curvature in the graphs for amplitude shows that both extremes of ranges under screening resulted in relatively low yield whereas the optimum value for the amplitude was closer to the upper extreme of the range. For time and S/L ratio, the upper extreme of the range resulted in maximum kafirin yield (%).

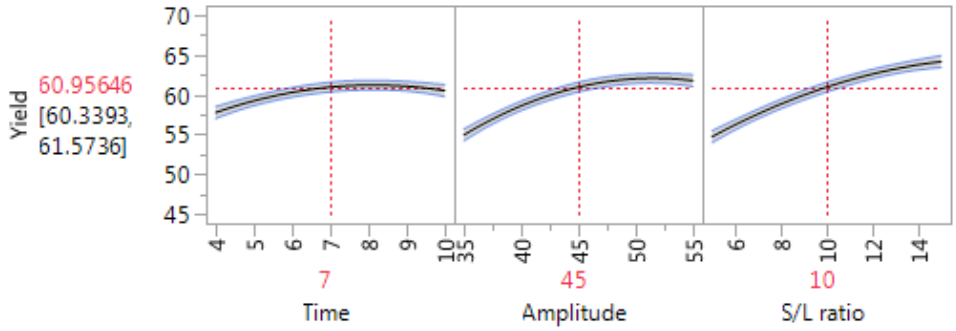


Figure 50: Effect of individual process parameter (time, amplitude and S/L ratio) on kafirin yield.

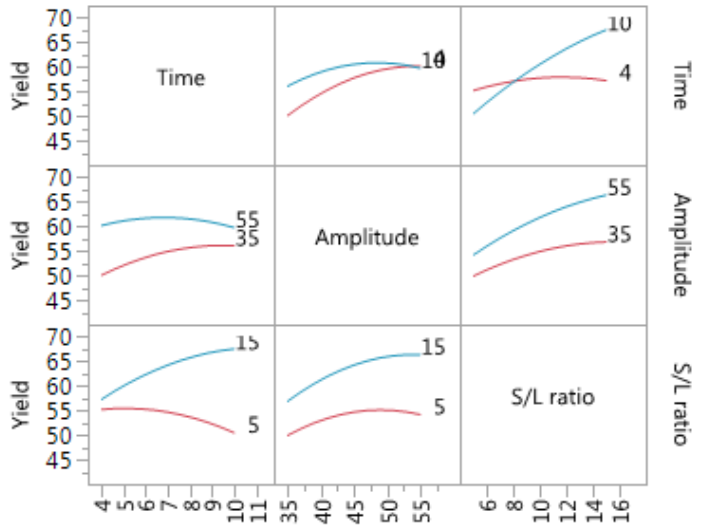


Figure 51: Interaction profiles of process parameters for ultrasonic cavitation.

Interaction profiles of selected process parameters indicate the effect their interactions at various levels on extraction yield (Figure 51). Curvature in the graph for time (first column) demonstrates the variation in yield with an increase in time at different levels of amplitude and S/L ratio. Similarly, the second and third columns show the influence of the interaction of amplitude and S/L ratio respectively with parameters on kafirin yield (%).

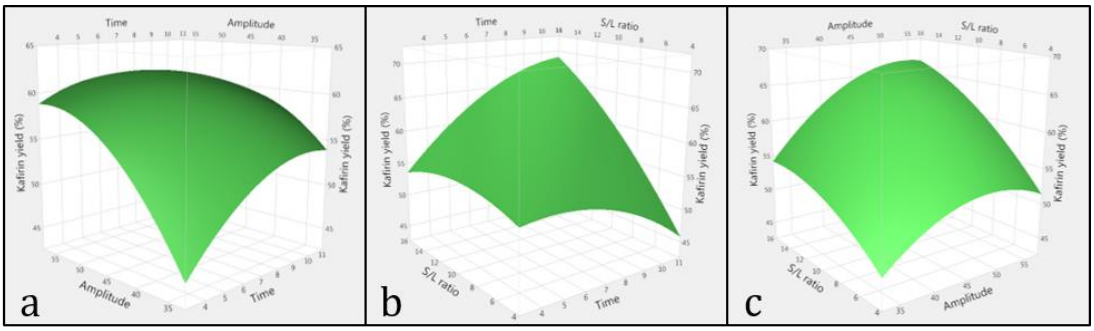


Figure 52: Surface profiles for process parameter interaction and their effect on kafirin yield. Surface profilers for (a) amplitude x time, (b) S/L ratio x time and (c) S/L ratio x amplitude.

Surface profilers show the impact on kafirin yield from the interaction at various levels of all process parameters (Figure 52). Surface profilers for amplitude x time (Figure 52a) show that kafirin yield also increases with an increase in amplitude and time up to a certain limit and a further increase in both resulted in a decrease in yield. S/L ratio x time (Figure 52b) depicts that kafirin yield increased with an increase in both the parameters and maximum yield was obtained at upper extremes for both parameters. S/L ratio x amplitude (Figure 52c) shows that kafirin yield would increase with an increase in S/L ratio, however, the yield is limited by a further increase in amplitude.

Contour profilers from different process parameters give the idea about design space to achieve a minimum 60% process yield (Figure 53) when operated within operating ranges for interacting process parameters. It can be gathered from the contour plots that minimum 60% kafirin can be extracted from DDGS whenever operated within given ranges. Operation time less than 7 minutes would result in kafirin yield < 60 %. In order to keep operation time 7-minute, amplitude and S/L ratio need to be on their higher extremes of the screened ranges. Similarly, the

minimum S/L ratio of 1:10 would be needed when operated at 35 amplitude with a minimum 10-minute operation time.

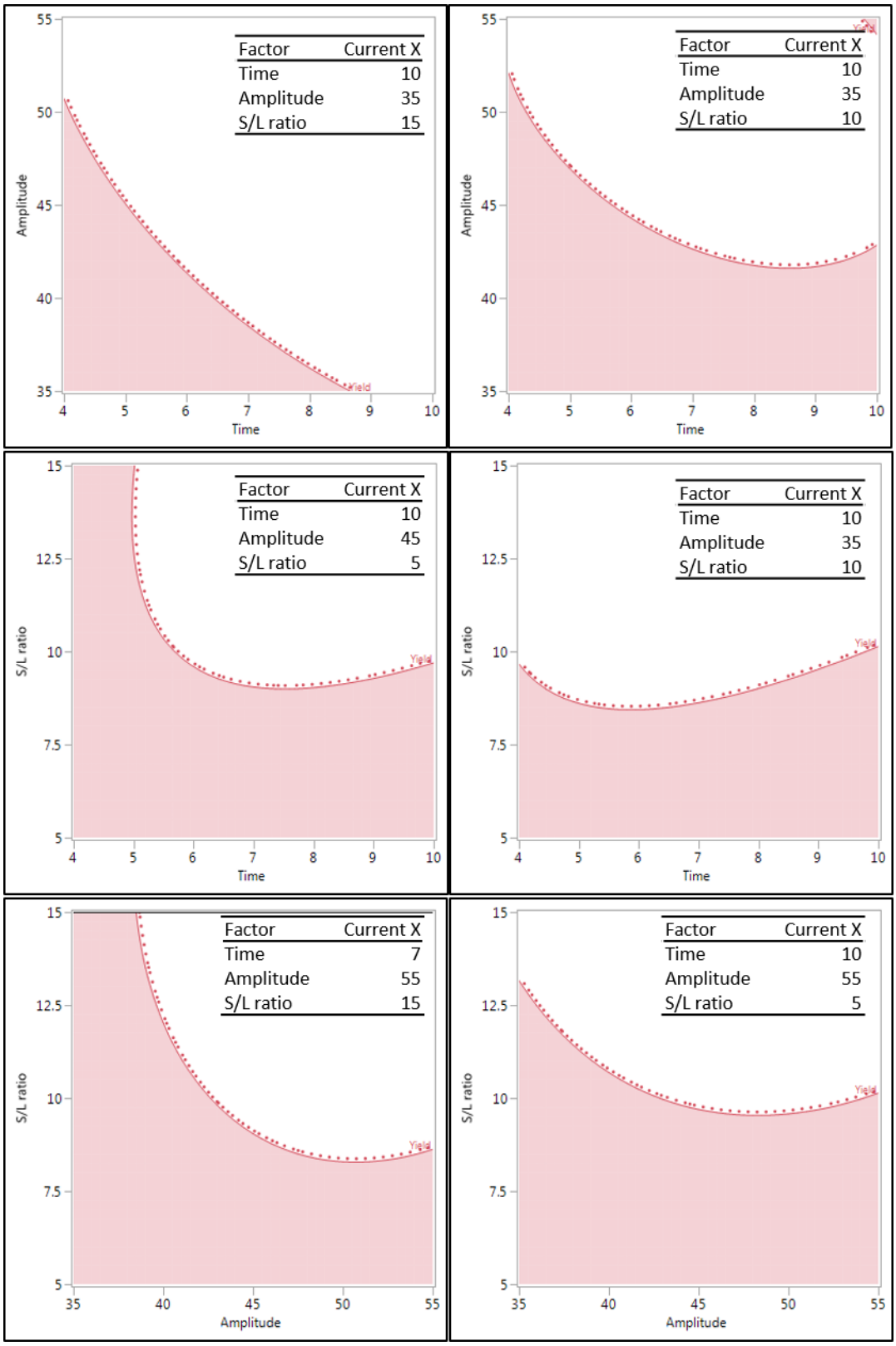


Figure 53: Contour profilers depicting design space to achieve a minimum 60 % kafirin yield with operating ranges for interacting process parameters.

5.3.2. Kafirin purity

Kafirin purified from DDGS using ultrasonic cavitation was analysed with SDS-PAGE (Figure 54) for quality and comparability purpose. Non-reduced (NR) kafirin sample with HMW and LMW, kafirin purified using the developed extraction process and chromatography was also loaded on the gel for comparison. As explained earlier, the quality of kafirin extracted from DDGS at both scales was comparable. Absence of both HMW and LMW impurities suggests that these were efficiently removed with the developed process. However, there were some faint bands observed below the main kafirin band. These bands might be the second most abundant form (β-kafirin), next to α-kafirin. There were no dimers or HMW aggregates present above the main band and LMW fragments below (probable β-kafirin and) main kafirin band. The profile is consistent with the earlier reports on purity of kafirin purified using ultrasonication (60), where trace forms of kafirin (γ-kafirin) not detected apparently due to difference between the feed material.

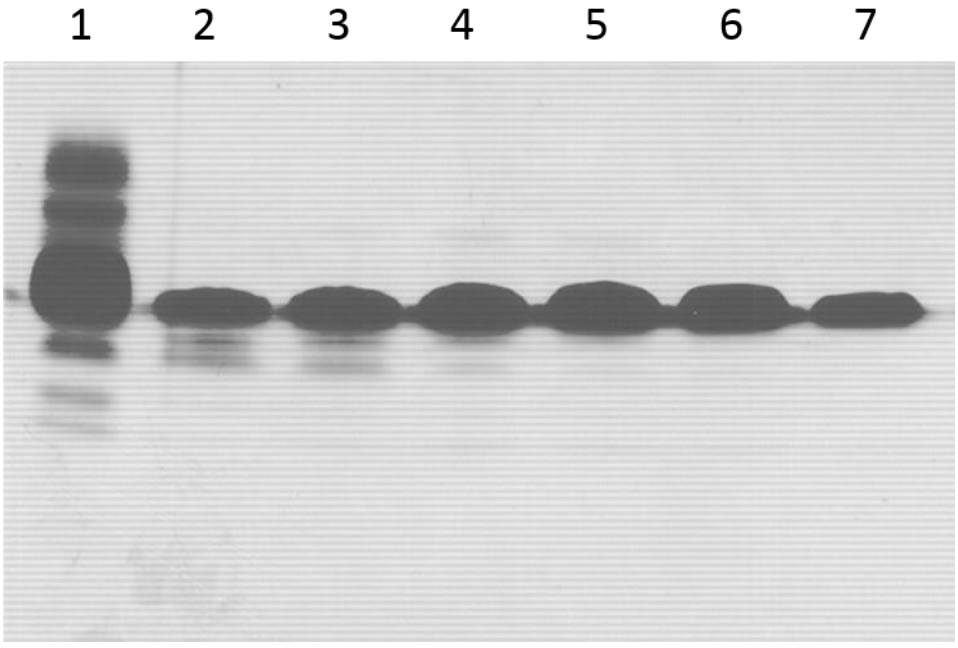


Figure 54: Comparative SDS-PAGE profile of kafirin extracted using various methods.
Lane 1- Kafirin with HMW and LMW (NR), 2- laboratory scale (DDGS) (R), 3- scale-up batch (DDGS) (R), 4- Chromatography (lab-scale-from DDGS), 5- Chromatography (scale-up batch-from DDGS), 6- Ultrasonic cavitation (DDGS), 7- Ultrasonic cavitation (sorghum flour).

5.3.3. Model validation

The screened process parameters were operated within the ranges to validate the model. An amount of kafirin obtained from validation exercise under different operation conditions is as in Table 42.

Table 42: Validation experiments for ultrasonic cavitation.

Time (min)	Amplitude	S/L ratio	Kafirin yield (%)
7	50	10	61.14
8	45	15	63.37
10	45	15	64.89

5.4. Conclusion

Kafirin extracted from DDGS using ultrasonic cavitation and the process was optimised using DOE based approach with full factorial design. Ultrasonic cavitation operation was optimised for three process parameters (time, amplitude and S/L ratio) in order to get minimum 60% of kafirin yield. Kafirin purified with ultrasonic cavitation was compared with kafirin purified using other strategies such as traditional solid-liquid extraction and chromatography. Kafirin was also extracted from sorghum flour using the optimised process, however, it is it resulted in lower Kafirin yield as both the raw materials vary greatly in terms of their total protein content. Purity from SDS-PAGE profile suggests that Kafirin purified using ultrasonic cavitation was comparable to Kafirin purified using other strategies. Ultrasonic cavitation can be further optimised to design a continuous extraction process.

Chapter 6: Plasticizer screening

6.1. Introduction

Flexibility is not an inherent property of the protein films as they are too brittle. These proteins need to be mixed with plasticizers to increase their flexibility whenever used as freestanding films or any other application where strength and elasticity both are needed. Plasticizer molecules when mixed, intercalate between the polymer chains, reduces the intermolecular interactions between these polymer chains and thereby increasing the flexibility of the films (112). However, the increased flexibility also reduces the tensile strength of the film and hence needs to be balanced accordingly based on the desired properties of the target application. Solvents also play an important role in the making of a solvent-plasticizer system that results in films with desired quality. Usually, the solvent is selected considering the solubility of the polymer in it, it is expected that the chosen plasticizer is also readily soluble in the same solvent system.

In the current study, ethanol was chosen as solvent of choice as it can solubilise kafirin along with a majority of plasticizers compared to other solvents reported for kafirin dissolution such as glacial acetic acid, lactic acid, glycerol and isopropanol (104). Plasticizers from different chemical families were assessed for their suitability with kafirin in terms of their effect on various physicochemical, mechanical and functional properties of the films.

6.2. Materials and methods

6.2.1. Materials

Kafirin was extracted from sorghum flour using the process developed in chapter 3. Glucose (Glu), galactose (Gal), glycerol (Gly), polyethylene glycol (PEG), propylene glycol (PPG), diethanolamine (DEA), triethanolamine (TEA), oleic acid (OA), ethanol and other chemicals used in this study were analytical grade and purchased from Sigma Aldrich, Australia.

6.2.2. Methods

6.2.2.1. Film casting

Films were prepared by dissolving kafirin in 70% (w/w) ethanol at 70 °C in stirring condition (100 rpm) until completely dissolved, the mixture was weighed before heating. Once the kafirin is dissolved, plasticiser was added and mixed

well to obtain a uniform solution. The mixture was held at 45 °C and topped up with absolute ethanol for the lost weight due to evaporation. The solution was mixed at slow speed to avoid air entrapment, which was then poured on a levelled plate kept in the oven at 50 °C for drying. After drying, the films were peeled from the plate and stored at 25 ± 5 °C and 50 ± 3% RH for further analysis.

6.2.2.2. Film thickness

Thickness was measured along the length of the film at five random locations using a digital micrometre. Mean thickness of each film specimen was used to calculate tensile strength and water vapour permeability (WVP) for respective films.

6.2.2.3. Moisture content

AACC method 44-15.02 was modified to analyse the moisture content of the films. 0.5 g of films (n=3) were cut and placed in pre-dried aluminium weighing tubs. The total weight was recorded before and after drying the films at 110 °C for 60 minutes. Samples were cooled to room temperature before recording final weight. Moisture content was determined by comparing the weight of the samples before and after heating. Moisture content was calculated using the following formula.

$$Moisture\ content\ (\%) = \frac{Initial\ weight\ (g) - Final\ weight\ (g)}{Initial\ weight\ (g)} \times 100$$

Individual plasticizers and films manufactured with these plasticizers were also analysed to assess their individual contribution to overall moisture content.

6.2.2.4. Swelling

The films were tested for their medium uptake ability at room temperature. Films were weighed and soaked in 50 mM phosphate buffer, pH 7.4 ± 0.2. Samples removed from media after 24 hours and excess of water removed by gently pressing the films between the tissue paper leaves. Weights of the wet films were recorded and change in weight was calculates using the formula given below.

$$Weight\ change\ (\%) = \frac{(W_t - W_0)}{W_0} \times 100$$

Where, Wt = final weight and W_0 = initial weight of the kafirin coat.

6.2.2.5. Scanning electron microscopy

Samples were cut into small pieces from the kafirin films using a surgical blade and mounted on aluminium stubs using double-sided carbon tape. These samples were then coated with a carbon layer using evaporative coater (model: 208C, Cressington) and examined for their microstructural analysis using SEM (model: Evo 40XVP, Zeiss).

6.2.2.6. Tensile properties of the films

Films were assessed for their tensile strength (TS) and elongation at break (EAB) using a modified method based on ASTM D882-12 (American Society for Testing and Materials, 2012). Three strips were cut from each film with 60 mm length and 10 mm width with SD < ± 0.03 mm. Films were measured at five random places for thickness and mounted one at a time in grips covered with abrasive paper to ensure a better grip on the film. Initial grip separation distance was 40 mm and the test speed was 0.4 mm/s. Maximum force at break and distance (mm) at the break for each film were recorded, stress and strain were calculated using the following formula.

$$Stress\ (\text{MPa}) = F/A$$

$$Strain\ (\%) = \Delta L/L_0 \times 100$$

Where, stress or TS is given in N/mm^2 or MPa, F is the force at break (N),
A is the area of the cross-section of the film (m^2),
Elongation at break (EAB) or strain is given in %,
L_0 is the initial length (m) and
ΔL is the elongated length (m).

6.2.2.7. Water vapour permeability (WVP)

WVP of the films were determined gravimetrically using ASTM method E96. The test assembly was prepared with films cut to 40 mm diameter circles (n=3) and measured for their thickness at five random points. These circles were mounted on the 100 mL Schott glass bottles containing 90 mL of deionised water with the help of screw cap GL 45 PBT red open-topped with 34 mm central aperture. Films were secured at a place with silicone sealing gasket to ensure a watertight seal. The assembly was weighed every 12 hours for 5 days and the changes in their

weight over time were plotted. Bottle kept without any film covering mouth was kept as a positive control. Airtightly sealed bottle with cap covered with silicon tape was kept as a negative control. All films were placed with the side of the film in contact with the glass during the casting process facing the water (i.e. higher vapour pressure) in bottles. Silicon gaskets and paraffin films were used as a sealant to keep the film in place and watertight during the entire experiment duration. WVT and WVP were calculated using the following formula.

$$WVT = \frac{G}{tA} = \frac{G/t}{A}$$

Where, WVT = rate of water vapour transmission, (g/h·m^2),
G = weight change (from straight line) (g),
t = time (h),
G/t = slope (g/h),
A = test area (m^2).

$$WVP = \frac{WVT \times X}{P_0 \times (R_1 - R_2)/100}$$

Where, WVP = water vapour permeability, (g mm/h·m^2 kPa),
X = specimen thickness (mm)
P_0 = vapour pressure difference at 25 °C (3.167 kPa)
R_1 = relative humidity at the source/inside the bottle (it was assumed that the relative humidity (R_1) in the bottle was 100 %),
R_2 = relative humidity at the vapour sink/outside the bottle (measured using the digital temperature and humidity logger).

6.3. Results and discussion

6.3.1. Film thickness

The thickness of the film specimens was measured along the length at five random locations and not greater than 0.25 mm as a requirement for a film (as defined in terminology ASTM D883) in terms of thickness (171). Kafirin film thickness recorded (in Table 43) is a mean thickness of specimens used for tensile strength and WVT testing.

Table 43: Average thickness and moisture content of the kafirin films formulated using different plasticizers.

Plasticizer	%	Thickness (µm)	Moisture content (%)
Glucose	20	84 ± 12	14.57 ± 2.12
	30	89 ± 9	16.43 ± 2..32
	40	81 ± 8	17.75 ± 1.93
Galactose	20	80 ± 12	12.12 ± 1.23
	30	84 ± 7	12.16 ± 1.75
	40	93 ± 5	13.72 ± 3.19
Glycerol	10	112 ± 10	13.09 ± 2.07
	20	103 ± 9	14.94 ± 2.08
	30	104 ± 5	18.02 ± 2.09
PEG	15	109 ± 12	7.55 ± 2.48
	25	104 ± 9	8.69 ± 1.94
	35	112 ± 11	10.22 ± 2.17
PPG	10	97 ± 6	4.48 ± 2.35
	20	95 ± 7	5.14 ± 1.96
	30	91 ± 11	6.48 ± 2.07
Diethanolamine	10	102 ± 14	10.1 ± 2.52
	15	107 ± 11	12.1 ± 2.36
	20	108 ± 16	14.6 ± 3.08
Triethanolamine	10	105 ± 8	5.24 ± 3.17
	15	101 ± 12	6.96 ± 3.32
	20	108 ± 14	7.81 ± 2.99
Oleic acid	10	97 ± 10	4.01 ± 1.36
	20	98 ± 8	4.79 ± 0.91
	30	96 ± 9	5.63 ± 1.23

6.3.2. Moisture content

The moisture content of the kafirin films with different plasticizers was determined for their innate moisture content. Glucose, galactose and glycerol containing films had the highest moisture content respectively. It was observed that increasing concentration of plasticizer also increased overall moisture

content. For instance, kafirin films with 30 % glycerol had more moisture compared to 10 % glycerol content films. Films plasticized with oleic acid has the lowest moisture content among the lot as oleic acid is a relatively hydrophobic compared to other plasticizers. The amount of moisture absorbed by the films and given conditions was ranked based on the average moisture content was glucose > galactose > glycerol > diethanolamine > polyethylene glycol > triethanolamine > propylene glycol > oleic acid. The average moisture content of kafirin films with varying amounts of plasticizers is as in Table 43. It is also worth noting that the moisture content of the given film depends on various factors such as base material type and concentration (kafirin in present study), plasticizer concentration (%), film preparation and casting method, drying and storage conditions (2,172–175). Hence, moisture content of the films can be directly compared with reported literatures provided the above mentioned and other factors that might affect the moisture content of the films were same.

6.3.3. Swelling

Kafirin films with different plasticizer content were placed in water and subjected to swell for 24 hours. Results were analysed and reported as an average weight of the film with standard deviation values. Figure 55 indicates that swelling of kafirin films for all the plasticizers was relatively low. It was observed that kafirin films with hydrophilic plasticizers such as glucose, galactose and glycerol demonstrated relatively low swelling, whereas, films with diethanolamine, polyethylene glycol and triethanolamine were relatively higher. Propylene glycol and oleic acid plasticized kafirin films showed swelling with no significant difference even with increasing amounts.

In the case of sugars (Figure 55a), swelling increases with an increase in concentrations of both glucose and galactose. There was no significant difference in swelling of films at 20 % and 30 % concentrations, however, at 40% sugar concentrations; swelling was relatively higher for galactose. For polyols, Gly, PPG (Figure 55b) and PEG (Figure 55d) showed similar behaviour, i.e. swelling increased with increasing concentrations of plasticizers. Swelling for PPG was significantly higher for relative plasticizer concentrations of other polyols. Like sugars, polyols are relatively hygroscopic compared to other plasticizers tested

in this study. As evident from Figure 55b, oleic acid is a relatively hydrophobic plasticizer as a relative increase in swelling was less compared to other plasticizers. Both DEA and TEA exhibited similar behaviour as increase in concentration lead to a relative increase in swelling of the films. Swelling for both DEA and TEA was higher compared to other plasticizers.

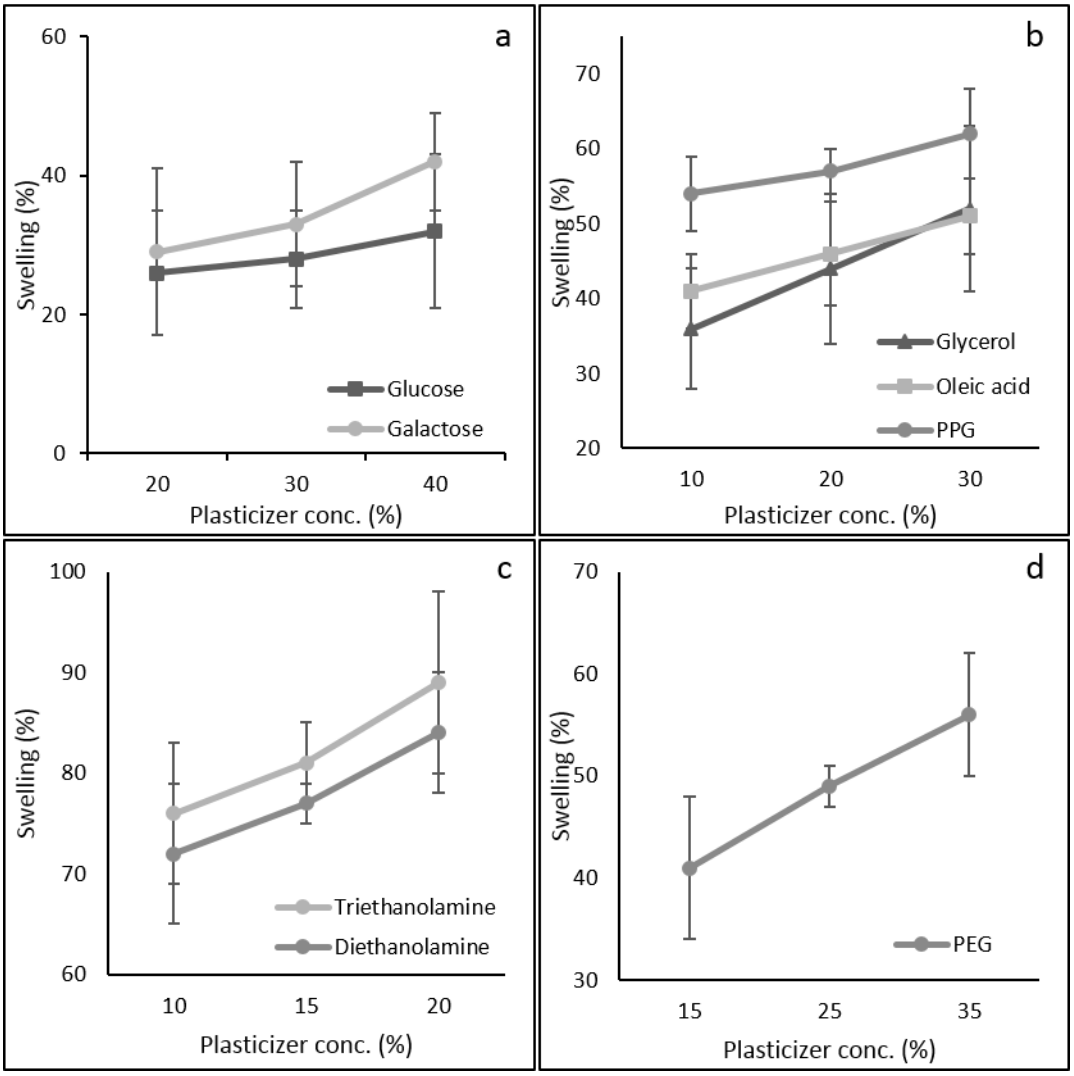


Figure 55: Swelling behaviour of kafirin films with varying concentrations of plasticizers.

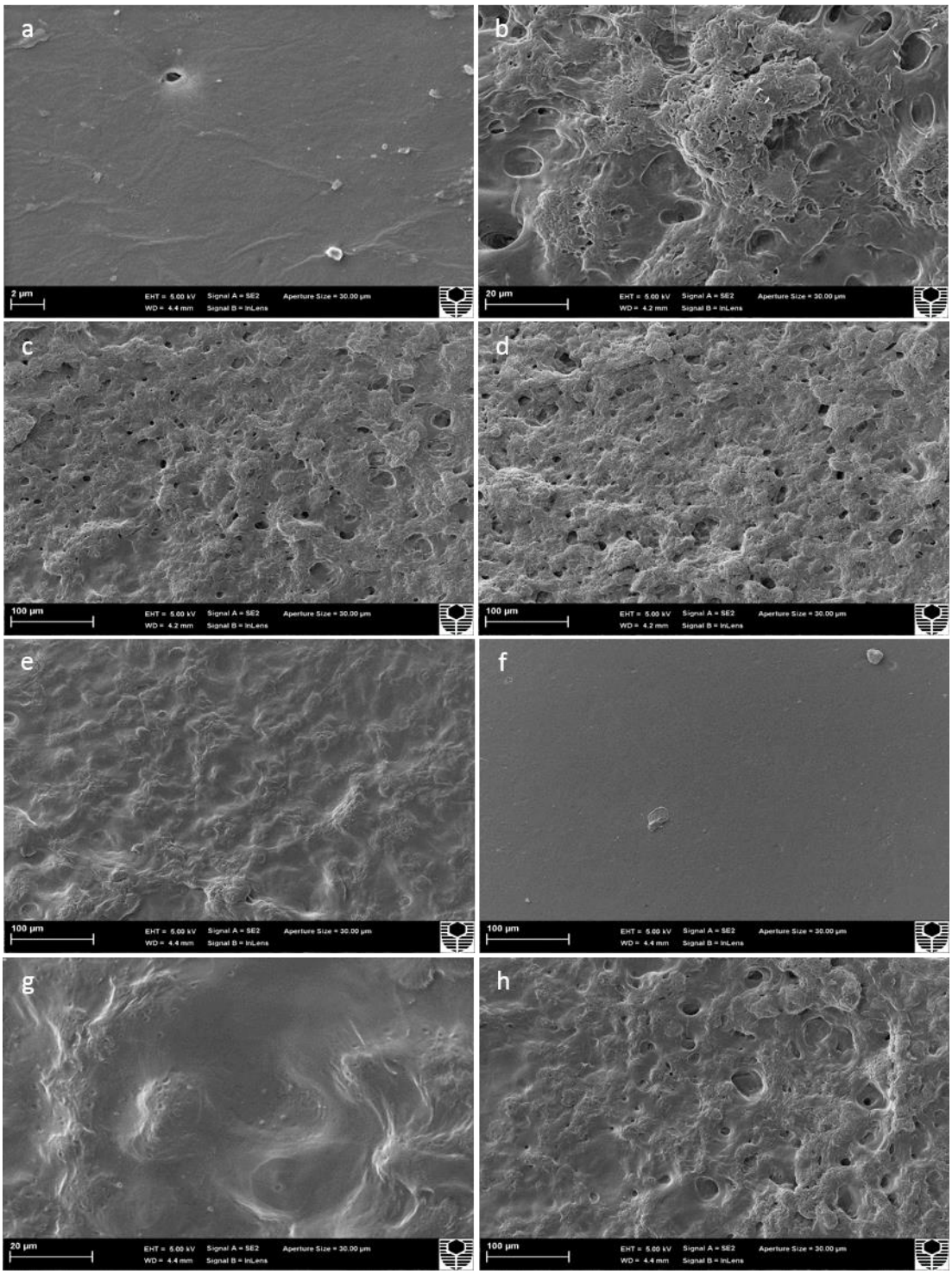
The swelling is property of the material to assess of spontaneous uptake of water, which also defines initial stages of the dissolution process (176). Water absorption in by polymer leads to swelling and increases the distance between the polymer chains that are responsible for the coherent structure of the material (177). Plasticizers interact with the polypeptides, increases cross-linking and stabilises the structure, behaviour that coincide with the reported study for

protein films (178,179), which supports the findings that these plasticizers in the current study maintained the kafirin film integrity. The same study also reported that increased crosslinking also reduces the free volume between the polymer chains resulting in decreased swelling, the behaviour corresponds to the plasticizing effect from low molecular weight plasticizers that intercalate between chains and reduce the free volume (177,178). Plasticizers like glycerol forms a weak hydrogen bond with protein and removed easily, which leads to increased swelling (180). The water uptake for any biomaterial, for this instance kafirin films, depends on protein and the processing conditions during its purification. The hydration capacity of kafirin decreases due to heat exposure during processing that leads to incomplete denaturation of kafirin molecule. The denaturation leads to exposure of hydrophobic amino acids embedded at the core, which increases the hydrophobicity of the resulting molecule and hence material produced from it. As the kafirin is highly hydrophobic protein, water absorption capacity of the kafirin films is low compared to other protein films (106,107). The rate of absorption also depends on temperature, pH and ionic strength of the media (181–183). Similarly, swelling capacity of protein-based biomaterials also depends on steric hindrance because of intermolecular and protein-plasticizer interactions (176).

6.3.4. Microstructural properties

Microstructural properties of the films were analysed using SEM. Micrographs of the films formulated using different plasticizers displayed an array of topographical and morphological characteristics as shown in Figure 56. Kafirin films plasticized with different plasticizers produced smooth and rough, porous and non-porous surfaces. SEM images at different resolutions give an idea about the finer details of the supramolecular structure of the films. Based on SEM micrographs of the kafirin films (Figure 56) plasticized with glucose (c) and galactose (d) were rough and porous whereas glycerol (e), PPG (g) and oleic acid (i) were relatively smoother, with striations and non-porous. SEM examination of kafirin films plasticized with DEA (f) and TEA (j) shows that surfaces are smooth and non-porous. The relatively smoother surface of these films supports the enhanced tensile strength (Figure 57) with low WVP (Figure 58) of the films.

Similarly, high porosity and rough surface of the glucose and galactose plasticized films indicate their high WVP and low TS. The difference between morphology and topography of the films suggests the effect of different plasticizers on the structure, however, it also indicates that the different mechanism of plasticization. Oleic acid and PPG are low molecular weight plasticizers that enter the space between polymer chains and stabilises the structure, also known as internal plasticization (184,185).



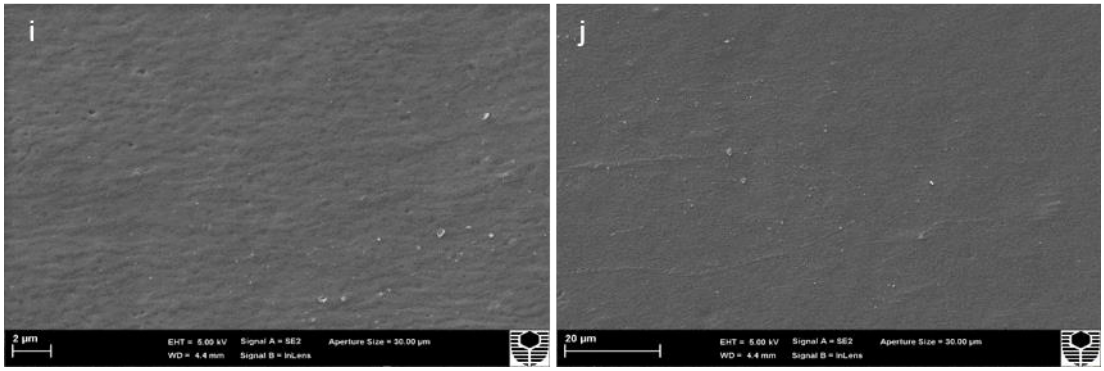


Figure 56: Scanning electron micrograph of kafirin plasticized with different plasticizers.

On the other hand, high molecular weight plasticizers such as glycerol and PEG could not enter the interchain spaces. However, both these hydrophilic plasticizers interact with hydrophilic amino acids on the surface of the protein forming weak hydrogen bonds (186,187). Visual examination of films with high glycerol concentrations stored at high relative humidity forms a sticky glycerol layer on the surface.

A study on films from kafirin has reported similar findings in terms of film surface smoothness, porosity and striations (wavy texture) (111). These reports were similar to the findings of the present study where films were porous with a scaly and rough surface and addition of plasticizer improved the texture to smoother with less porosity. Another report from the same research group where they proposed that starch contamination in purified kafirin is one of the reasons of the high porosity of films, which increased when films were soaked in water as starch dislodged from films (59). It was also proposed that the heat-induced crosslinking and polymerization resulting in poor kafirin solubility during film casting would have resulted in films with poor quality (188). They have also reported films of kafirin extracted from treated (washed) DDGS resulted in a smooth surface with fewer defect (59) similar to the findings of the present study (Figure 56a, f, i and j) where kafirin films with a smoother surface without striations and pores were produced.

6.3.5. Tensile properties

Tensile strength and EAB were assessed to understand the effect of different plasticizers on tensile properties of the kafirin films. Data obtained from the analysis of tensile strength and elongation at break are as shown in Figure 57. Aim of plasticizer addition to any polymer is to reduce brittleness, enhance flexibility and improve processability. Addition of plasticizer reduces intermolecular interactions thereby reducing tensile strength and form plasticizer-polymer hydrogen bonds that add flexibility to the material. Films containing oleic acid and glycerol had the highest tensile strength and EAB respectively than sugars and ethanolamines. However, third after glycerol and oleic acid, low concentrations of TEA showed relatively higher TS compared to others. Glucose and galactose have comparative EAB but galactose produced films with relatively higher TS. Among polyols, glycerol films had higher EAB with higher concentrations whereas PEG showed higher TS at lower concentrations. Films with PPG showed relatively low TS and EAB. TEA showed higher TS compared to DEA at low plasticizer concentrations whereas both of them demonstrated comparable EAB for all concentrations.

These differences between TS and EAB could be attributed to their plasticizing efficiencies. However, moisture content also plays an important role when it comes to tensile properties of the protein films. A study reports difference between TS and EAB for similar films at different relative humidity suggest that at films tends to absorb moisture at higher humidity and tends to lose the strength because of increased moisture content (189). In the current study, the difference between TS and EAB for glycerol and oleic acid films showed significant difference as the former being hygroscopic attracts moisture and increase the water content of the film, which is evident from the moisture content data (Table 43). On the other hand, oleic acid is relatively hydrophobic and absorb three times less moisture compared to glycerol. The difference between the moisture content of these two films leads to the difference between their mechanical properties as glycerol films with high moisture content were more flexible whereas films with oleic acid were strong but with relatively low flexibility. These findings were in agreement with a similar study where various plasticizers tested at a different relative humidity (RH) concluded that ST of the

films was consistent and comparable up to 60 % RH but decrease sharply with further increase in RH. They also concluded that EAB for the same set of plasticizer was significantly similar and low up to 50% RH and observed > 300 % maximum increase in EAB between 70 % to 80 % RH. These findings in support of the present study suggest that film preparation and storage conditions greatly influence the mechanical properties of the film.

As reported in research earlier, heat-induced crosslinking also improve the tensile properties of the kafirin films (188) and other prolamins such as zein (190). In similar studies, zein was crosslinked to improve the tensile properties (191), stability (192)and cytocompatibility (193) of the films and fibres.

The higher standard deviations for TS and EAB in case of glycerol, PPG, TEA and oleic acid could be attributed variables that influence the tensile properties of the films (44). The same study also suggests that difference between film casting methods, conditioning and storage, specimen dimensions have an impact on the strength and elasticity of the films. A similar study reported as high as 47.9% standard deviation for tensile properties citing the same reasons (104).

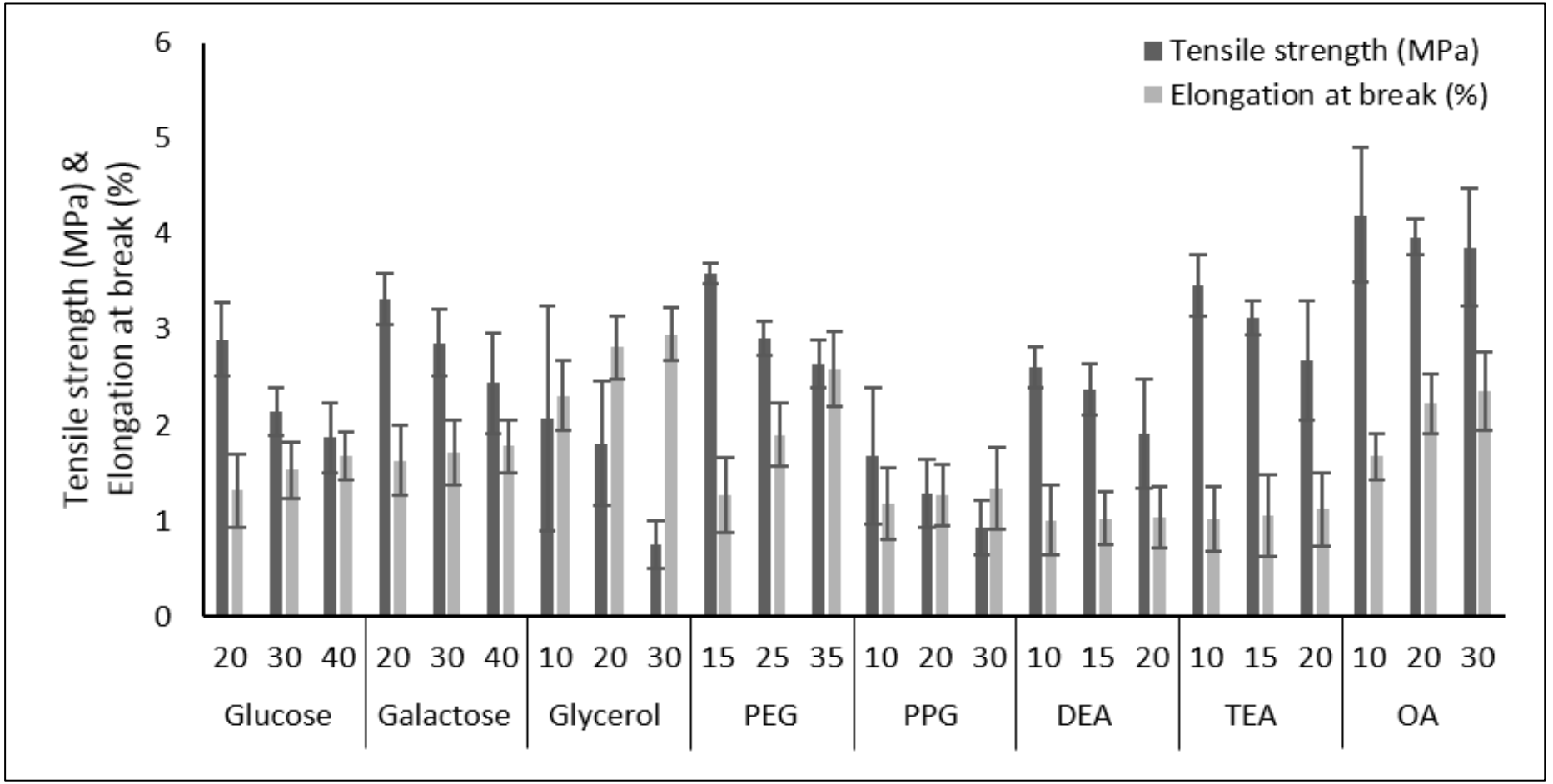


Figure 57: Tensile strength (MPa) and elongation at break (%) for kafirin films at different concentrations of plasticizers.
PEG: Polyethylene glycol, PPG: Propylene glycol, DEA: Diethanolamine, TEA: Triethanolamine, OA: Oleic acid.

6.3.6. Water vapour permeability

Water vapour transmission rate and water vapour permeability testing were done to evaluate the possibility of using films as an effective moisture barrier.

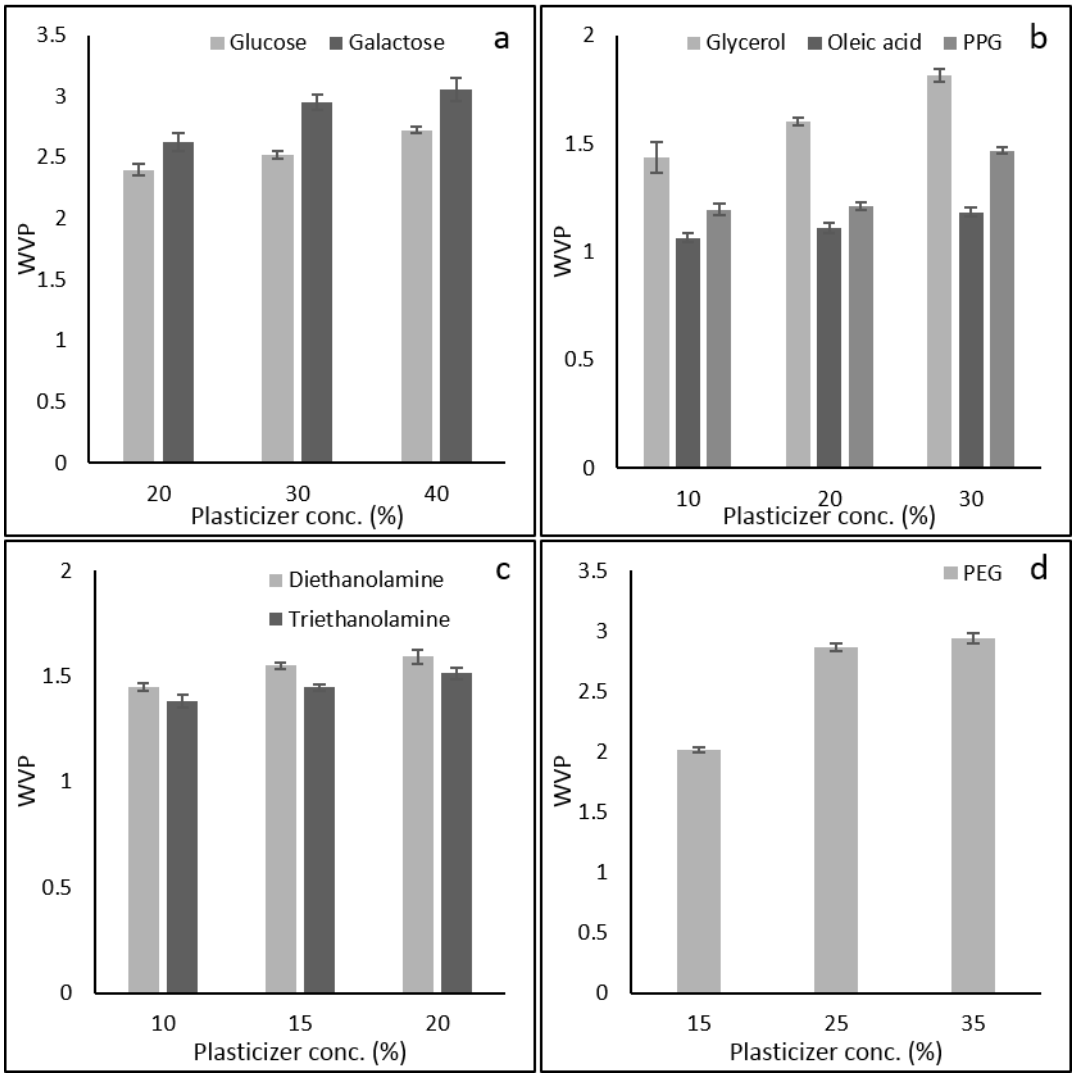


Figure 58: Water vapour permeability (g mm/m² h kPa) for kafirin films at different concentrations of plasticizers.

The water vapour permeability (WVP) of kafirin films plasticized with different plasticizers plotted as shown in Figure 58. The WVP for glucose, galactose and PEG plasticized films were relatively higher compared kafirin films with DEA, TEA, PPG and oleic acid as plasticizers. There was no significant difference between WVP obtained for DEA and TEA plasticized films even at different concentrations. However, PEG and glycerol plasticized films showed a remarkable difference at different concentrations. These findings were consistent with the reports of the effects of glycerol on WVTR on the films (194).

It has been reported that the plasticizers, mainly with external plasticizing mechanism plays a significant role in the migration of moisture through the protein films (58,106,186). In the current study, glucose, galactose, glycerol and PEG had a higher WVP as they are hydrophilic and latter being highly hygroscopic with a tendency to capture moisture. These hydrophilic plasticizers capture water with an increase in time and increase water within the films, which ultimately facilitates the diffusion through the films. Kafirin films plasticized with more than one plasticizers (glycerol, lactic acid and PEG in equal proportions) reported a mean WVP of 0.43 (104) and 0.66 (g mm/m^{2}h kPa) (109) on two separate instances that were relatively lower than the WVP for films from individual plasticizers in the present study. These differences in the WVP of the films from different studies could be due to several reasons such as kafirin source, its extraction and drying process, ingredients and film casting process. Plasticized kafirin films with low water transmission rate can be further developed and used as an edible film for food packaging that prevents both moisture loss and gain.

6.4. Conclusion

Kafirin films plasticized with different individual plasticizers showed competitive tensile properties where oleic acid and glycerol films demonstrated the highest TS and EAB respectively. Oleic acid and PPG plasticized films showed competitive WVP values among the screened plasticizers. Swelling behaviour suggests water uptake capacity of the films where DEA and ETA adsorbed most water. Sugar and glycerol films demonstrated least water absorption with a possibility that being hydrophilic; these were extracted from the film leading to lower water retention. However, these plasticizer needs to be paired with other plasticizers with complementary attributes in order to design applications with desired properties. For instance, a combination of oleic acid with glycerol would produce material with optimum TS, EAB, and barrier properties. The present study reports plasticizers that demonstrated an array of mechanical and barrier properties and can be used based on suitability for targeted applications.

Chapter 7: Kafirin spray coat development

7.1. Introduction

The enteric coating can be defined as the coating that can resist low pH in the stomach but easily dissolve/disintegrate at high pH in the intestine. Enteric coating applied to any pharmaceutical and nutraceutical ingredients is meant to protect the drug from gastric pH and/or drug delivery to the intestine and/or to avoid gastric irritation.

Most of the enteric coating materials used to coat are either synthetic (mostly cellulose derivatives) such as cellulose acetate succinate, cellulose acetate phthalate, hydroxypropyl methylcellulose (HPMC) phthalate and HPMC acetate succinate, polyvinyl acetate phthalate (PVAP), or shellac and zein that are of natural origin. The zein and kafirin have close similarity in their physicochemical properties. However, kafirin is more hydrophobic than zein due to high glutamine, proline and alanine content which makes it a better candidate for protein-based coating application.

Here, kafirin was mixed with suitable plasticizer was used to spray coat the drug-loaded capsules to assess the suitability of kafirin as an enteric coat. Effect of various concentrations of plasticizer on the secondary structure of kafirin assessed with FTIR. The swelling behaviour of kafirin coat was studied at both acidic and alkaline pH. Kafirin coated capsules were analysed for their drug release profile in acidic and alkaline media. The findings of the current study suggest that kafirin can be developed into an effective enteric coat and should be further investigated for other application development such as drug-loaded microparticles and food packaging.

7.2. Materials

Kafirin was extracted from sorghum flour using the extraction process explained earlier in chapter 3. Glycerol, polyethylene glycol, hydrochloric acid, disodium hydrogen phosphate, sodium dihydrogen phosphate, methanol and ethanol were purchased from Sigma Aldrich, Australia. Paracetamol powder and gelatine clear capsules (size 0) received from Valaenza Pharmaceuticals Pvt Ltd.

7.3. Methods

7.3.1. Capsule filling

Clear gelatine capsules filled with 400 mg of paracetamol were sealed and weighed. Sealed capsules were spray-coated as per the procedure mentioned below until the uniform coating achieved.

7.3.2. Kafirin coating

Kafirin coating solutions with respective plasticizers were prepared by dissolving kafirin in 70 % (w/w) ethanol and kept on a magnetic stirrer at 70 °C in stirring condition (100 rpm) until complete dissolution, the mixture was weighed before heating. Plasticisers (15, 25 and 35 %) added to the dissolved kafirin and mixed well to obtain a uniform solution. The mixture was held at 45 °C and weighed to calculate the mass loss, which was topped up with absolute ethanol for the lost weight due to evaporation. The solution was mixed at slow speed to avoid air entrapment, filled in the glass canister and loaded on the airbrush/spray pen with modified size 5 head (1.06 mm) to handle viscous solutions. Operating air pressure was 30-35 PSI, higher in case of thicker slurry. The width of the spray depends on the distance between the tip and the application surface (capsule); closer distance resulted in the fine narrow lined thicker coat whereas higher distance leads to a thin broad lined coat. Paracetamol filled clear gelatine capsules sprayed with kafirin solution and several coats were applied to achieve a uniform coating and air-dried before testing. Similarly, kafirin coats for the testing purpose were prepared by spraying the kafirin solutions on the glass plate and air-dried. Multiple coats were applied to obtain the coat similar to that of the capsules. The dried coat/film was then peeled off for further testing.

7.3.3. FTIR

FTIR spectra were recorded on Perkin Elmer FT-IR spectrometer (model: Spectrum 100) with Spectrum V10 software. Samples were carefully pressed on the crystal to ensure good contact and scanned within range of 4000 cm^{-1} to 400 cm^{-1}, 100 spectra were averaged at 4 cm^{-1} resolution. The empty crystal served as a reference.

7.3.4. Swelling

The films were tested for their medium uptake ability at room temperature. Films were weighed and soaked separately in acidic and alkaline solutions. Samples were taken out at a defined time interval and excess of water removed by gently pressing the films between the tissue paper leaves. Change in weights of the wet films was recorded up to 72 hours. Change in weight was calculates using the formula given below.

$$Weight\ change\ (\%) = \frac{(W_t - W_0)}{W_0} \times 100$$

Where, Wt = final weight and Wo = initial weight of the kafirin coat.

7.3.5. Water vapour permeability (WVP)

WVP of the spray coat films were determined gravimetrically as explained earlier (section 6.2.2.7) using ASTM method E96. The test assembly was prepared with films cut to 40 mm diameter circles (n=3) and measured for their thickness at five random points. These circles were mounted on the 100 mL Schott glass bottles containing 90 mL of deionised water with the help of screw cap GL 45 PBT red open-topped with 34 mm central aperture. Films were secured at a place with silicone sealing gasket to ensure a watertight seal. The assembly was weighed every 12 hours for 5 days and the changes in their weight over time were plotted. Bottle kept without any film covering mouth was kept as a positive control. Airtightly sealed bottle with cap covered with silicon tape was kept as a negative control. All films were placed with the side of the film in contact with the glass during the casting process facing the water (i.e. higher vapour pressure) in bottles. Silicon gaskets and paraffin films were used as a sealant to keep the film in place and watertight during the entire experiment duration. WVT and WVP were calculated using the following formula.

$$WVT = \frac{G}{tA} = \frac{G/t}{A}$$

Where, WVT = rate of water vapour transmission, (g/h·m^2),

G = weight change (from straight line) (g),

t = time (h),

G/t = slope (g/h),

A = test area (m^2).

$$WVP = \frac{WVT \times X}{P_0 \times (R_1 - R_2)/100}$$

Where, WVP = water vapour permeability, (g mm/h·m^2 kPa),

X = specimen thickness (mm)

P_0 = vapour pressure difference at 25 °C (3.167 kPa)

R_1 = relative humidity at the source/inside the bottle (it was assumed that the relative humidity (R_1) in the bottle was 100 %),

R2 = relative humidity at the vapour sink/outside the bottle (measured using the digital temperature and humidity logger).

7.3.6. Solubility study

Kafirin coated gelatine capsules subjected to dissolution study using USP 1 dissolution (basket) apparatus (Erweka DT6). The coated capsules were placed in respective baskets and immersed in the 900 mL of acidic media i.e. 0.1 N HCl maintained at 37 °C ± 0.5 °C for 2 h at 50 rpm. Samples were withdrawn from acidic media in each container at 15, 30, 60, 90 and 120 minutes for coated capsules and every 5 minutes for uncoated capsules up to 1 h. After 2 h, baskets were transferred to another container with alkaline media i.e. 50 mM phosphate buffer, pH 7.4 ± 0.2 maintained at 37 °C ± 0.5°C at 50 rpm. 2 mL of samples were withdrawn from each container at every 30 minutes up to 6 hours and at 24, 48 and 72 h from alkaline media. Samples were filtered with 0.2 µm filters before analysis. Paracetamol concentration in samples determined as described as follows in section 7.3.7.

7.3.7. Paracetamol quantification

Paracetamol (PCM) calibration curve was prepared by adding required amount of PCM in 15 mL methanol and mixed well to dissolve; 85 mL of water was added to make up 100 mL final volume in a volumetric flask. 1 mL from the prepared solution was withdrawn and added to another 100 mL volumetric flask. Volume was adjusted to 100 mL using diluent i.e. 15 % methanol (v/v) in water. Calibration curve was prepared with 25, 50, 75, 100, 125 and 150 mg/L

concentrations. Samples were withdrawn and diluted with 15 % methanol (v/v) in water accordingly to measure absorbance to determine the final concentration.

7.4. Results and discussion

The kafirin coat and capsules were analysed for their mechanical, structural/ conformational, swelling behaviour, WVT, dissolution properties.

7.4.1. Coated capsules

Bare gelatine capsules filled with drug and spray-coated with plasticized kafirin. Weight and dimension of these capsules were recorded (Table 44).

Table 44: Weight and dimension of empty, filled and coated capsules.

Empty gelatine capsule (mg)	Filled gelatine capsule (mg)	Coated capsule (mg)	Length (mm)
99 ± 6	502 ± 4	523 ± 11	21.6 ± 0.3

7.4.2. Kafirin secondary structures

Kafirin coat, both native and with plasticizers were analysed for structural changes in the kafirin molecules with FTIR. The analysis of spectra of the kafirin coat with varying plasticizer content shows that there were two prominent peaks within the frequency ranges where amide I and amide II are highly likely due to C = O and N – H stretching respectively (195). The peak in the frequency range from 1630 cm^{-1} to 1620 cm^{-1} were allocated to β-sheets and the peak within the frequency range from 1650 cm^{-1} to 1635 cm^{-1} allocated to α–helices within the amide I region (which is 1690 cm^{-1} to 1600 cm^{-1}) (196–198). However, other secondary structures such as random coils are known to contribute towards the intensity of the peak in these regions (199). Similarly, for amide II region (1575 cm^{-1} to 1480 cm^{-1}), a peak near 1530 cm^{-1} was assigned to α–helices whereas β-sheets were located near 1520 cm^{-1}. It was observed that the intensity of the peaks assigned to β-sheets, however, it cannot be said for the peaks allocated to α–helices. These observations were consistent with the reported results for plasticized kafirin films with varying content of plasticizers (14).

Secondary structure of protein varies with the solvent, where, the solubility of the protein depends on the solvent used, temperature, pH and other excipients

where the structure of protein changes aided by cleavage and formation of inter and intrachain bonds which stabilises the native protein structure (200). Both proteins and peptides are least soluble at the pH close to their pI as they have net charge zero and solubility increases when moved away from the pI (201), these factors aids to the solubility of the protein by altering the native structure of the protein. Kafirin solubilised in ethanol at alkaline pH with reducing agents and plasticizers leads to alteration in its molecular structures and subsequently mechanical and functional properties of the target applications (112).

7.4.3. Swelling

Kafirin spray coat from each formulation was spray dried on the glass sheets and bare gelatine capsules were soaked in solutions at pH 1.2 and 7.4 (202,203) that mimic the gastrointestinal (GI) tract conditions to assess the suitability of kafirin as an enteric coat. Intact gelatine capsules swelled, started disintegrating after 35 minutes and completely disintegrated after 2 hours.

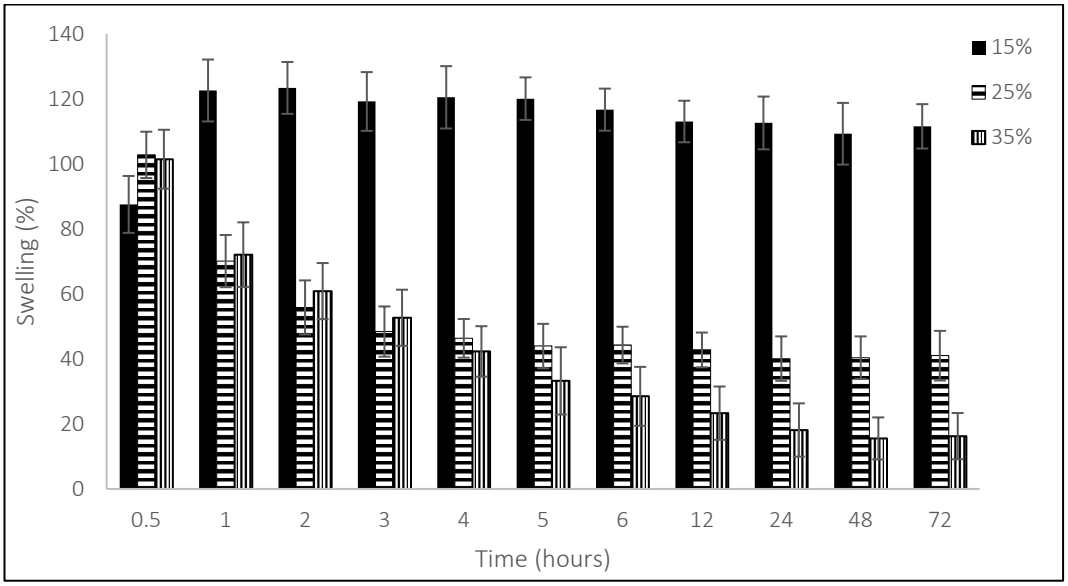


Figure 59: Swelling behaviour of kafirin spray coat in acidic media.

In acidic media, kafirin spray coat with lowest plasticizer content swelled up to 90% in the first 30 minutes, which increased to maximum and maintained the swelling at 117 ± 5 % for the next 72 hours. Initially, higher plasticizer content films swelled more compared to low plasticizer films; however, swelling gradually reduced with increase in time to 41 ± 8 % and 16 ± 7 % for 25% and 35% plasticizer content respectively (Figure 59).

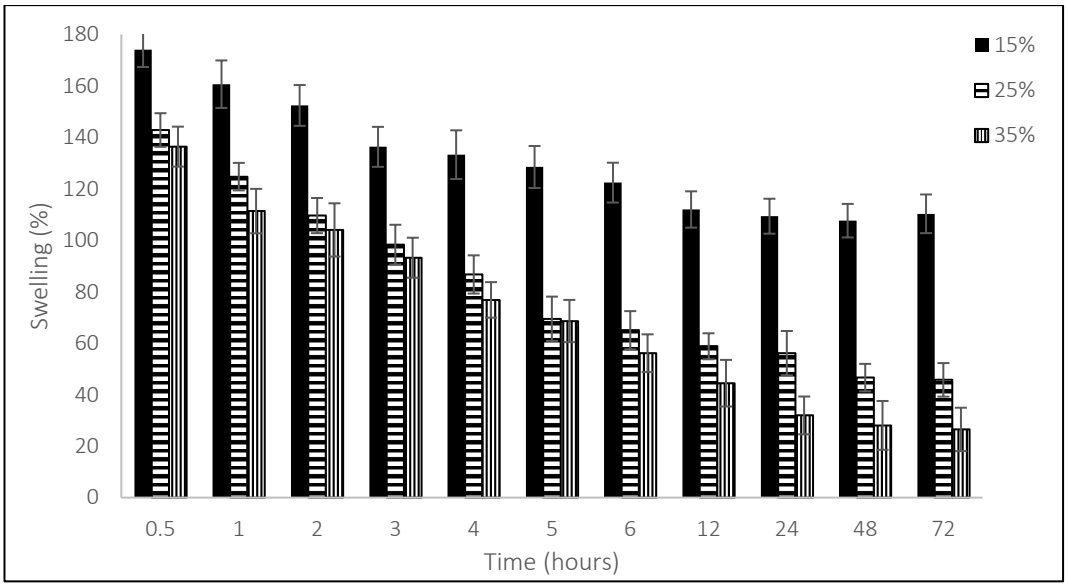


Figure 60: Swelling behaviour of kafirin spray coat in alkaline media.

Kafirin spray coats soaked in alkaline media demonstrated a similar profile with the difference in swelling for different plasticizer content. Coat with 15% plasticizer swelled to ~170 % in the first 30 minutes and gradually decreased to 110 % at the end of 72 hours. High plasticizer content coats (25% and 35%) exhibited similar behaviour but with less swelling (~140%), which gradually reduced to 46% and 26% for 25% and 35% plasticizer content coats (Figure 60). Alkaline media uptake for the coat was faster compared to acidic and can be due to hydrophilic plasticizer within the coat. Glutamine residue in kafirin, upon deamidation, convert to glutamic acid in acidic conditions and glutamate in alkaline condition. Glutamate is charged and protonated at alkaline pH leads to increased affinity for protons, which decreases hydrophobicity and increased water permeability (127).

Swelling is an important parameter investigated to understand the behaviour of kafirin coat with various plasticizers in different environments. The compatibility of the solvent and polymer and the degree of crosslinking drive swelling factors such as medium uptake capacity and expansion of the coat (204). The ionisation of the functional groups of amino acids in the polypeptides that constitute the backbone of the coat results in the inflow of ions of opposite charge from the solution to neutralise electrostatic repulsions. This inflow of ions induces an osmotic pressure that is equalised by the movement of solvent into the polymer coat, which leads to swelling. The swelling caused by the inflow of the solvent

then counteracted by strain in the backbone and when these forces are in balance, swelling equilibrium is reached (204,205) which explains the medium uptake by the kafirin coat with time. Degree of cross-linking also play an important role in the swelling, a lower degree of cross-linking increases swellability whereas higher cross-linking decreases the swelling of the film (206). Kafirin films with hydrophobic amino acid-rich backbone and relatively higher cross-linking demonstrated lower swelling compared to bare gelatine capsules.

The kinetics of swelling depends primarily on the solvent's rate of diffusion and arrangement of protein structure (205). The phenomenon of swelling occurs in two stages, first, a rapid inflow of water from the buffer and second, a slow movement of water from the buffer in later stages facilitated by rearrangements in the backbone over time until equilibrium is reached. This supports the results obtained in this study where an initial rapid uptake of the medium was observed at the end of first 30 minutes followed by slow increase with further incubation up to 72 hours in case of kafirin coat incubate at acidic pH. However, in the case of kafirin coat incubated at alkaline pH, initial rapid uptake phase was observed with a gradual decrease in the retention of the medium until the end of the experiment. The kafirin coat in the alkaline medium starts disintegrating and lose the integrity after 24 hours whereas; the coat was intact until the end of the experiment in acidic medium.

7.4.4. Paracetamol quantification

Samples were withdrawn at a regular time interval from the system and diluted accordingly. Drug released from capsule in the media at different time points were determined using the calibration curve given below in Figure 61.

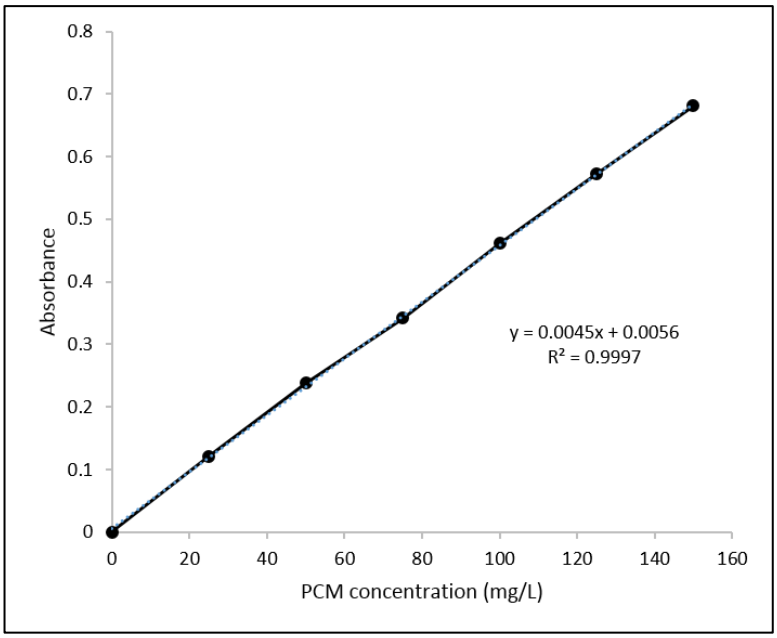


Figure 61: Calibration curve for PCM concentration determination.

7.4.5. Solubility study

Solubility studies were undertaken to determine the kafirin coat resistance to the acidic environment (in the stomach) by *in-vitro* testing the coated capsules and their dissolution behaviour as an enteric coat. Both uncoated and kafirin spray-coated capsules incubated in acidic condition for 2 hours followed by incubation in phosphate buffer at alkaline pH. These acidic and alkaline media were analysed for drug release at regular time intervals using UV spectrophotometer at 248 nm wavelength. For uncoated capsules, instant drug release (27%) observed within the first 5 minutes, more than 99% of the drug released in the media at the end of 45 minutes (Figure 62). Gelatine capsules are soluble in hot water at temperatures above 40 °C. This observation supported by swelling data from the current study where uncoated gelatine capsules swelled and absorbed water up to ~7-8 times of its weight and formed gel (207). Gelatine (sterile solutions) are stable for a prolonged time at lower temperatures but at higher temperatures, they are prone to hydrolysis/ peptide bond cleavage leading to the generation of free amino acids and decrease in gel strength. Similar behaviour observed when degradation accelerated by extremes in pH, proteolytic enzymes and bacterial action.

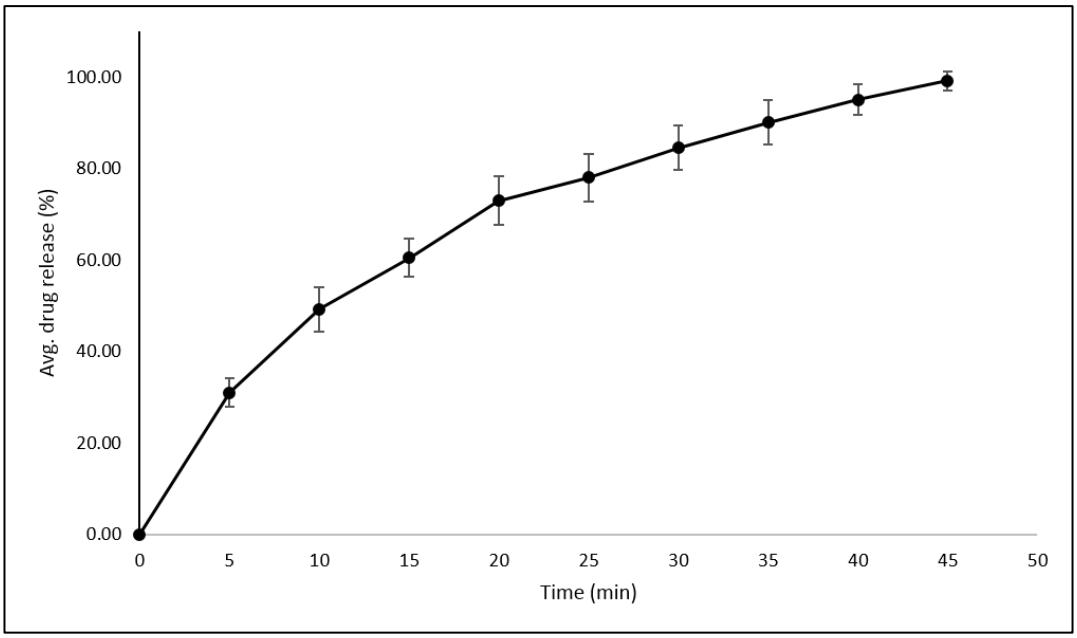


Figure 62: Drug release profile for bare gelatine capsules in acidic media.

In the case of coated capsules, no drug release observed in acidic media for the first 2 hours (Figure 63). However, when incubated at alkaline pH in phosphate buffer, instant drug release (4 %) detected after 5 minutes, which gradually increased with time for all formulations (Figure 63). Drug release for low concentration plasticizer coats was significantly less than increasing concentration plasticizer formulations. More than 50 % of drug release detected within first 12 hours for high plasticizer concentration formulation, whereas low concentration formulation took more than 48 hours to release the same amount of drug.

As discussed earlier in section 7.4.3, an inflow of solvent mainly governed by the structural composition of the material. Higher (hydrophilic) plasticizer content makes films less hydrophobic, which facilitates the flow of solvent within the film, which leads to swelling and loss of plasticizer solubilized in media with time. Although kafirin holds the structural integrity, washed-out plasticizer weakens the strength and permeability of the coat, which leads to the release of drug in the media.

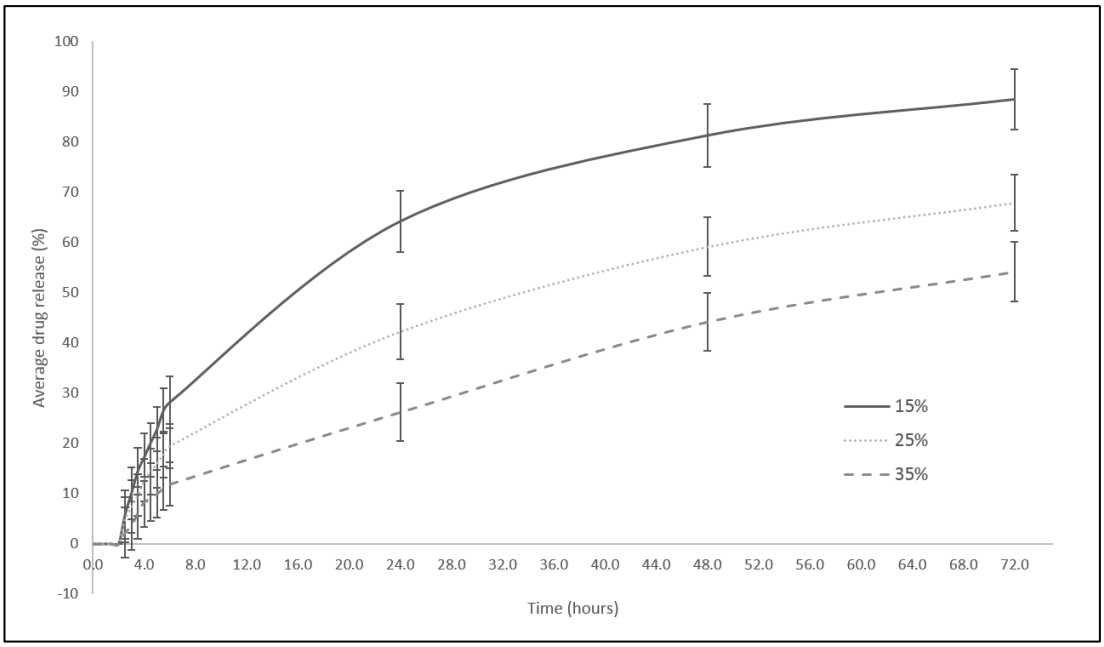


Figure 63: Drug release profile for coated capsules in acidic and alkaline media.

Covalent (disulphide bonding) and non-covalent interactions (hydrogen bonding and hydrophobic interactions) between the molecules within the coat determines the integrity, strength and solubility of the coat. Presence of strong reducing or oxidising agents, ionic and non-ionic detergents in coat forming solutions are capable of breaking covalent and non-covalent interaction which further reduces the strength of the coat (208).

7.4.6. Water vapour permeability (WVP)

The change in WVP (g mm/m^2 h kPa) for the kafirin coat formulated using different plasticizer concentrations plotted and presented in Figure 64. It was observed that the WVP increases with increasing concentration of plasticizer. This tendency could be explained by the nature of coating material, i.e. protein to plasticizer ratio in terms hydrophobicity of the mixture. Hydrophobicity of kafirin is significantly higher due to high contents of amino acids with hydrophobic side chains such as proline and alanine. These hydrophobic amino acids have very small dipole movements and tend to repel the water. Whereas PEG is a relatively less hydrophobic molecule know to be soluble in both polar and non-polar solvents. PEG is essentially a non-polar molecule with hydroxyl groups at the end of its chain. The ether oxygen in the chain, which is slightly polar, binds with water rendering PEG a soluble moiety. This hydrophilic nature of PEG attracts

water and increase WVP of the coat with a further increase in plasticizer concentration.

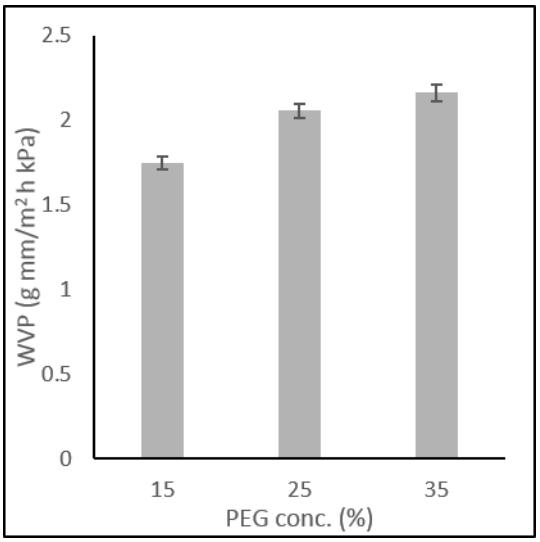


Figure 64: Water vapour permeability of kafirin spray coat at different plasticizer concentrations.

As compared to PEG plasticized films (chapter 6), kafirin spray coat with similar plasticizer content showed lower WVP which is beneficial given the desired properties of the application under investigation. The difference between the WVP of films and coat can be attributed to casting methods. Films were produced by traditional pour and dry method whereas the spray coat was the result of multiple coats applied over the other. Also, increased WVP can be attributed to the structure and porosity of the films as excess ethanol evaporates during drying, which might leave pore throughout the structure. On the other hand, spray coating creates fine aerosol like particles with very low ethanol content. Besides, multiple coats applied would create non-continuous pores leads to lower WVP. Usually, plasticizers intercalate themselves between protein chains thereby altering mechanical properties of the material; however, PEG is a relatively larger molecule that does not get inserted between the chains but more likely to cross-link them. Another reason for not able to intercalate is steric hindrance due to adjacent side chains. These interactions are results of reduced internal hydrogen bonding creating intermolecular spaces within protein molecules and overall structure. The increased spacing allows more water molecules within these spaces resulting in swelling and further penetration in protein network as observed in the coat with higher plasticizer concentration.

7.5. Conclusion

Kafirin films prepared with different formulations demonstrated variation in their mechanical, functional and barrier properties. Kafirin solubilised in a solvent mixed with plasticizers alters the molecular structure of kafirin, which leads to modifications in its functional properties. Kafirin spray coat tested for swelling exhibited resistance to dissolution at low pH but solubilise at alkaline pH, conditions similar to GI tract. Drug release profiles from *in-vitro* studies at both acidic and alkaline pH indicate similar as coat holds the integrity at low pH but drug release was observed as soon as it is placed in alkaline media. Low WVTR indicates excellent barrier properties of the coat. These studies assure that kafirin-based formulation can be used as an effective enteric coat that needs to be instigated at a commercial scale.

Chapter 8: Kafirin microparticles and food-grade coating

8.1. Introduction

Microparticles are efficient and proven drug delivery systems with improved pharmacokinetic benefits in terms of adsorption, bioavailability and distribution of the drugs. The size distribution of microparticles varies within the range of 1 to 1000 μm. Microparticle offers an advantage over nanoparticles that they do not enter interstitial space and time transported to other organs that enable targeted drug delivery and hence act locally (209). This feature reduces if the risk of possible toxicity due to accidentally encapsulated material. Microparticles can also be formulated into solids (capsule or tablets), semi-solids (gels and paste) or liquid formulations such as solutions thereby offering a choice of the dosage form with desired the delivery (210). Spray drying is one of the few techniques widely used in industry for microparticle manufacturing as it offers certain advantages such as uniform particle size, single-step operation, organic solvent and heat-sensitive material compatibility, hence explored for kafirin microparticle development.

This chapter discusses kafirin microparticle production with spray drying at the end of the kafirin purification process. Spray drying opted as the drying step as it can handle high volumes process intermediates and efficient at commercial scale. Spray drying was optimised as a production step that manufactures kafirin microparticles with uniform size distribution to integrate the kafirin purification process with the microparticle production process. Microparticles formed by varying parameters were analysed and characterise in terms of their solubility, morphology, microstructural properties, and particle size distribution. The outcome of the current study suggests that kafirin microparticles can be developed into an effective drug carrier and should be further investigated for other application development such as drug-loaded microparticles. These microparticles were dissolved and mixed with plasticizer and applied on fruit as a preservative coat where it was demonstrated that the shelf life of the fruit can be significantly extended.

8.2. Materials

Kafirin used for microparticle formation was extracted from sorghum flour/DDGS using the extraction process explained earlier in chapter 3.

8.3. Methods

8.3.1. Spray drying

Kafirin solution was spray-dried using a pilot-scale spray dryer (Mini spray dryer B-290 Advanced, Buchi) with a dehumidifier (B-296) and inert loop (B-295). Input protein concentration was varied (0.2 mg/mL to 1.0 mg/mL) and other parameters such as inlet temperature, outlet temperature, flow rate and aspirator speed were optimised to produce microparticles within desired particle size distribution range.

Spray dryer operations were conducted as follows:

- Instrument and the aspirator were switched on. The temperature of the inert loop was set to -20 °C.
- Main nitrogen gas valve was opened and gas flow adjusted to 40 mm using the needle valve in the gas flow meter. The aspirator was turned on to 80% to start feeding the gas to the system until the oxygen level in the system reached less than 6% (on inert loop indicator).
- Inlet temperature set at 120 °C and the heater was switched on.
- Tubing was placed in the feed valve and pump setpoint adjusted to 30% (60% for solvents).
- Once the inlet temperature reached 120 °C, the end of the tube placed in a container with distilled water/solvent and the pump was switched on.
- Inlet temperature and feed flow rate were adjusted to obtain the desired outlet temperature for the product.
- Feed tubing was placed in the solution containing the product. Solution placed on magnetic stirrer to homogenise the feed during the entire operation. Inlet temperature and feed flow were adjusted accordingly to obtain the desired outlet temperature.
- Once the feed is over, feed tube was placed in solvent/distilled water to flush the tube and nozzle and ensure maximum recovery.
- Product was recovered from the collection vessel and weighed.
- Cylinder, cyclone, coupler, outlet temperature sensor and other removable parts were detached and cleaned thoroughly. All parts were air-dried before reassembly.

- Solvent recovered from the receiving bottle in the inert loop. Water collected in the humidifier collection container was decanted.

8.3.2. Solubility study

Kafirin samples were dissolved in 70% ethanol and kept on a magnetic stirrer at 70 °C in stirring condition (100 rpm) until complete dissolution (maximum 30 minutes). The mixture was cooled to room temperature and weighed to calculate the mass loss, which was topped up with absolute ethanol for the lost weight due to evaporation. Solutions were then filtered using preweighed 1.0 µm filter papers in separate containers followed by 70% ethanol (20 mL) to wash retained kafirin solution within the filter paper. These filter papers were dried in a hot air oven at 80° C and weighed for insoluble kafirin. Change in weight was calculates using the formula given below.

$$Weight\ change\ (\%) = \frac{(W_t - W_0)}{W_0} \times 100$$

Where, Wt = final weight and Wo = initial weight of the filter paper.

8.3.3. Particle size distribution

Kafirin microparticles produced by varying process parameters were tested for the particle size distribution using Malvern Mastersizer (Malvern Panalytical Australia).

8.3.4. Scanning electron microscopy

Samples were sprinkled on double-sided carbon tape mounted on aluminium stubs, excess ad loosely bound powder blown using pressurised air. These samples were then coated with a carbon layer using evaporative coater (model: 208C, Cressington) and examined for their microstructural analysis using SEM (model: Evo 40XVP, Zeiss).

8.3.5. Coating

Kafirin microparticles were dissolved is explained earlier (in section 8.3.2) and mixed with 10 % plasticizer (1:2 mixture of glycerol and propylene glycol). The mixture was cooled to room temperature and weighed to calculate the mass loss,

which was topped up with solvent for the lost weight due to evaporation. The coating solution was rested for 2 hours before use. The solution was fed into a glass canister and sprayed on the fruits (pears from a local orchard with an average weight of 186 ± 16.2). The coat was allowed to air dry and fruits (with uncoated pears as control) were incubated at room temperature (21 ± 2° C).

8.3.6. Weight loss and visual inspection

Both coated and uncoated pears were weighed every 24 hours to assess the weight change. These fruits were visually inspected for any changes in skin colour with respect to ripening and gloss.

8.4. Results and discussion

The kafirin microparticles were analysed for their morphology, microstructural properties, solubility and particle size distribution.

8.4.1. Solubility

The solubility of Kafirin samples dried using hot air oven spray-dryer was analysed. As evident from the data obtained and discussed in chapter 3 (Table 3.6), the solubility of the Kafirin produced using spray-dryer was significantly higher (100 %) than the hot air oven-dried kafirin (< 80 %). Solubility is one of the CQAs considered while developing the kafirin purification process as the final product would be solubilized in a suitable solvent system in order to develop/manufacture an application. As discussed earlier, the slow and incomplete dissolution of oven-dried kafirin could be the result of exposure to a higher temperature for longer duration might have altered molecular structure of kafirin and induced higher degree polymerisation leading to the formation of higher molecular weight aggregates (38,42). On the other hand, an increased rate of solubility of spray-dried kafirin can be attributed to a smaller particle size that offers a larger surface area resulting in faster dissolution.

8.4.2. Particle size distribution

Kafirin microparticles produced using process stream with different protein concentrations were analysed for their particle size distribution. Initially, process intermediate generated after oil removal step, a high protein concentration

stream, was spray-dried which resulted into microparticles with a wider particle size distribution (Figure 65), an observation supported by micrographs for the sample (Figure 66b and d). Upon closer examination, it was found that smaller microparticles (< 100 µm) were also generated, however, in lesser proportions (Figure 65d and f). Similar process stream, when diluted (5x) resulted in smaller microparticles (Figure 66c), however, with size distribution within a very narrow range (Figure 65). It was observed that the undiluted/higher protein concentration stream (~1.0 mg/mL) formed larger microparticles (Figure 65), whereas, low protein concentration (0.2 mg/mL) resulted in smaller microparticles (Figure 65) with similar process parameters/conditions.

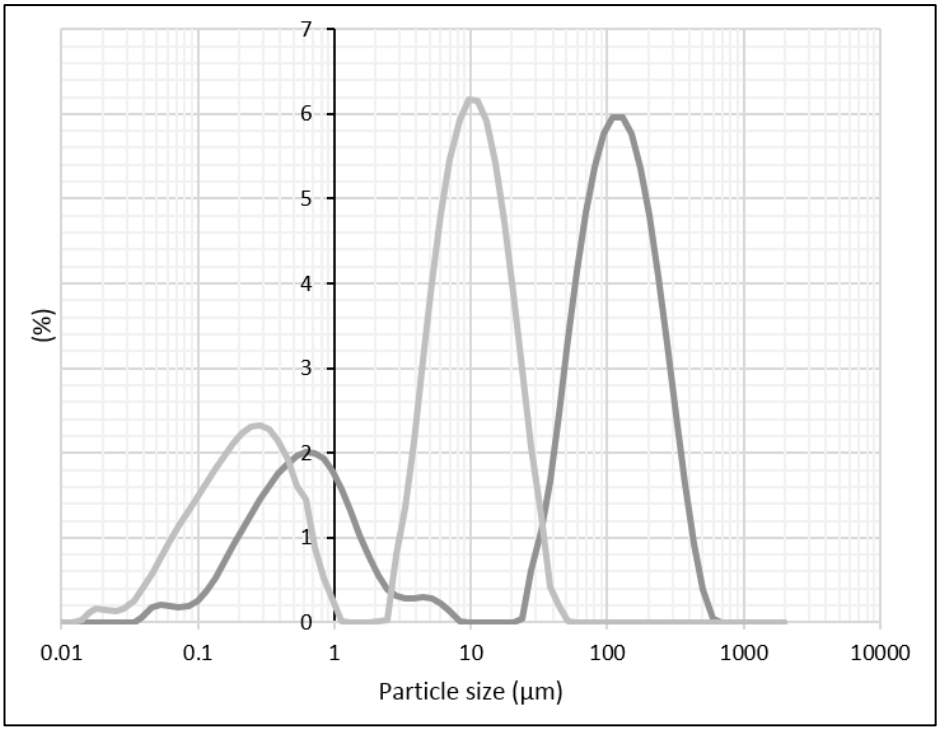


Figure 65: Particle size distribution for Kafirin microparticles produced at different protein concentrations. High protein concentration/native stream (black) and low protein concentration/diluted stream (grey).

8.4.3. Microstructural properties

Kafirin microparticles were examined for their morphological characterization and microstructural properties using SEM. Scanning electron micrographs of kafirin microparticles (Figure 66) produced by varying protein concentration of the input stream resulted in the formation of particles within narrow size distribution ranges. Other than particle size, scanning electron micrographs displays morphology of these microparticles. It was observed that the surface of

the microparticles was smooth irrespective of their size. Kafirin microparticles with size < 100 µm were mostly non-uniform spherical structures (Figure 66c). The surface of these smaller microparticles was relatively small and non-porous. On the other hand, microparticles generated with a high protein concentration stream were relatively larger (~200 µm), both spherical and elliptical in shape and uniform structures. These larger microparticles have a smooth surface and macroporous (with pore size > 50nm).

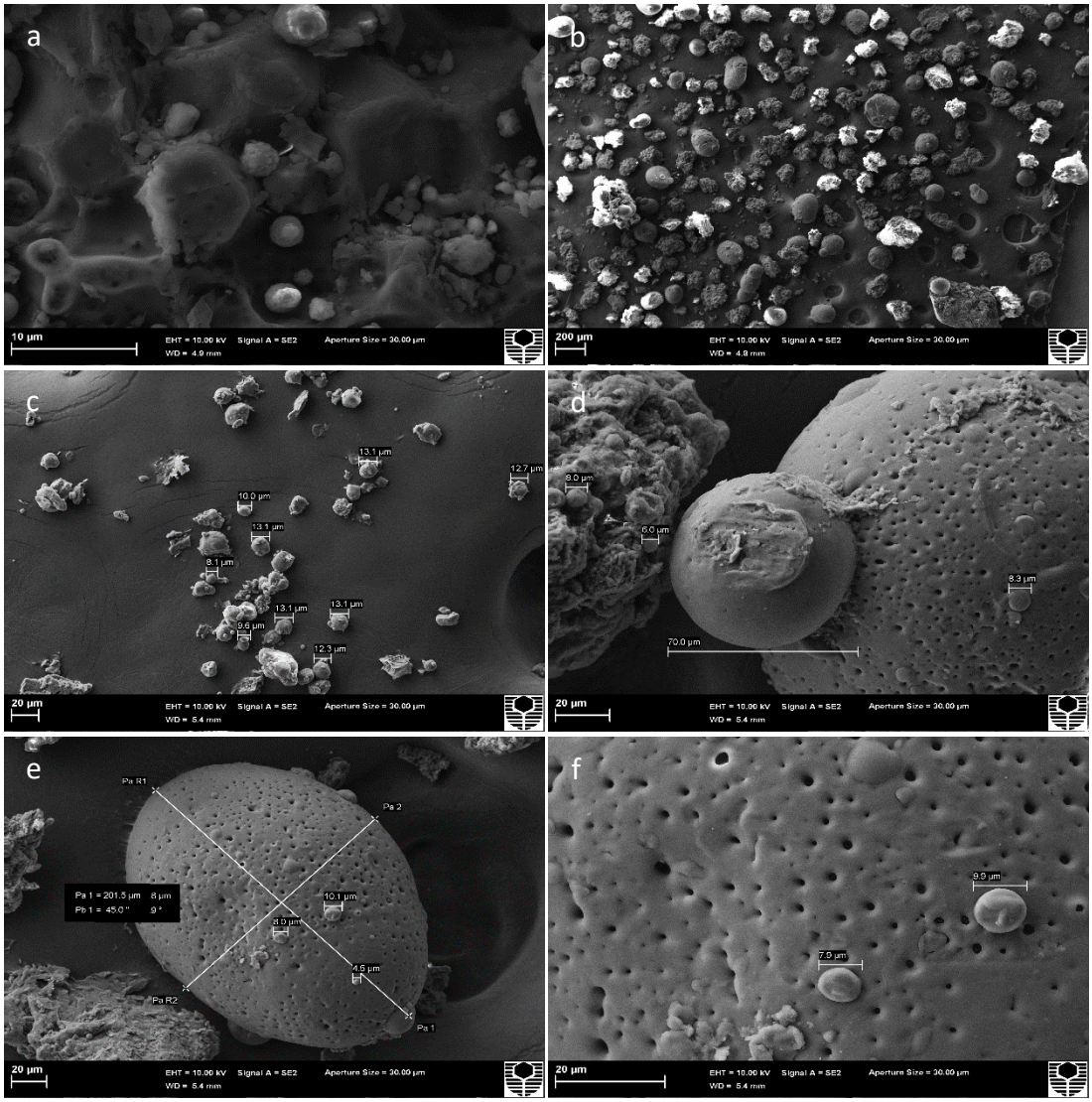


Figure 66: Scanning electron micrographs of Kafirin microparticles.

8.4.4. Fruit coating: visual inspection and weight loss

Initial visual inspection (day 0) of the fruits (both coated and uncoated) suggests that both were with green skin background with black and brown spots on the outer surface where the spray coat was able to retain the gloss on the surface.

The skin background colour and the (black and brown) spots were the indicators of fruit ripening. In the case of uncoated fruits, the gloss faded and the skin background colour of the fruits changed to yellow and the majority of the spots faded to light brown colour with the increase in time. However, coated fruit retained much of the gloss up with green background where the majority of the spots were black with few faded to brown.

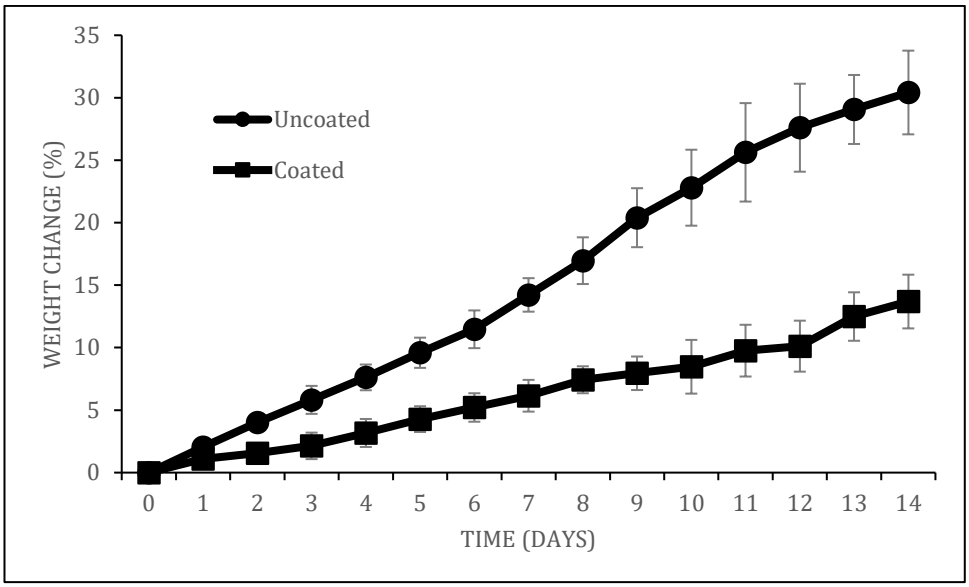


Figure 67: Weight change (%) for coated and uncoated pear over 14 days.

As shown in Figure 68, both coated and uncoated pears have a similar appearance in terms of background colour and spots on the surface on day 0. The colour change in background and spots were observed every day with the increase in time. On day 14, the background skin colour of the uncoated pear changed to yellow and the spots on entire skin faded to brown. Kafirin coated pear, on the other hand, was able to retain the green skin colour (with slight yellowish shade) with very few of the spots faded to brown.

Ideally, fresh pears take 7 to 8 days to enter the ripening phase also known as climacteric phase (211,212). The climacteric phase can be characterised as ethylene production and increased respiration of the fruits that lead to weight change (213,214). The ripening followed by senescence where the fruit is at the peak of it's ripened state and might decay with further increase in time governed by several internal and external factors. External factors such as oxygen, temperature, relative humidity and other volatile compounds and internal factors such as ethylene production and moisture loss play a key role in fruit

ripening (215). The controlled weight change in the case of kafirin coated pear suggests that the moisture loss was minimal compared to uncoated fruits, which demonstrates that the kafirin coat can act as an efficient moisture barrier.

It was also observed that the coating of post-climacteric pears leads to faster maturation of the fruit, an observation in agreement with the reported study (13). This is probably due to the fact that mature pears produce bursts of ethylene which induces ripening where coating would allow the partial or incomplete release of gas from the fruit leading to faster ripening (211).

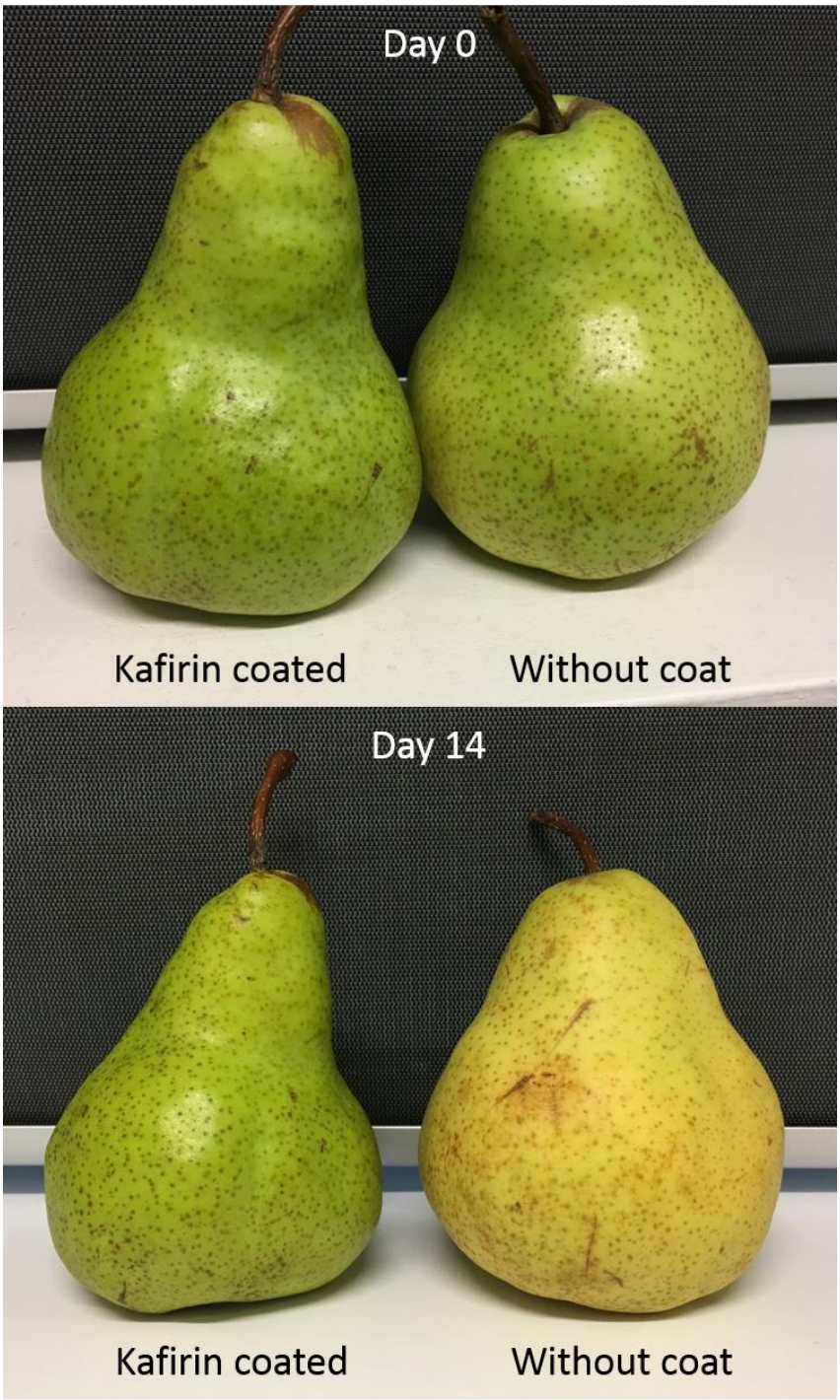


Figure 68: Kafirin coated and uncoated pears on day 0 and day 14.

The observations of this study were in agreement with reports for pear coating up to a certain extent due to the difference in study design (13). For instance, pears were stored at low temperatures for a week before coating and testing. Besides, pears were dip-coated whereas, in the present study, spray coating has opted. The (coated) fruits under testing developed wrinkles over time resulting in the unacceptable appearance of the fruit. This is probably due to ethanol

exposure for a prolonged time during dip coating. However, in the present study, pears did not develop wrinkles and maintained gloss after 14 days.

8.5. Conclusion

Kafirin microparticles produced with different particle size distribution ranges. These microparticles were characterised in terms of their solubility, morphology, microstructural properties and size distribution. Microparticles formed with different process parameters resulted in particles with different morphological and microstructural properties with excellent solubility. It was demonstrated that kafirin microparticles can be produced within the desired size distribution range. It was demonstrated that the kafirin coat can prolong the shelf life of the coated fruit. Also, this was a preliminary study conducted to assess the effect of kafirin coat on ripening considering colour and weight change. The ripening of the fruits regulated by regulating temperature, humidity and gas content of the vicinity/atmosphere and reflected in terms of colour, texture, flavour, aroma and it's sugar, acid and phenolic content depending on the type of fruit. In order to develop and commercialise the kafirin coat as a protective coat, detailed study needs to be carried out with different types of fruits with test conditions designed considering above listed parameters. The current study suggests that kafirin biomaterials has the potential and can be developed into product food spray coat and pharmaceutical applications such as drug-loaded microparticles for targeted drug delivery.

Chapter 9: Conclusion and future work

9.1. Conclusion

This thesis investigates the suitability of different raw material for kafirin purification and bio-material based application development from purified kafirin. Sorghum flour and distiller's dried grains with solubles (DDGS), an industrial waste from distilleries and bio-refineries were used to extract kafirin. Kafirin purification process developed with "Quality by Design" (QbD) based approach was able to accommodate both sorghum and DDGS, the approach assured consistent product quality and facilitate scale-up to commercial scale and technology transfer. The process has been optimised, scaled-up and validated to ensure the adequate supply of kafirin. The purified protein was used to design food grade applications such as films, coatings, and microparticles with targeted properties. A spray coat was developed for perishable foods using kafirin. Kafirin was also formulated to coat pharmaceuticals and nutraceutical active ingredients, where the protein layer acts as an efficient enteric barrier. Following sections concludes and summarise the work from individual chapters from this thesis.

9.1.1. Kafirin extraction process development and scale-up

A preliminary purification process was developed to extract kafirin from sorghum flour. Traditional OFAT approach was implemented to optimise individual process parameters for different unit operations. Later, QBD a risk-based approach was implemented to optimise the process in order to produce high purity kafirin with increased recovery. Critical quality attributes (purity, solubility and recovery) were identified to define the quality target product profile and product design space. Risk assessment was carried out for individual unit operation using FMEA. Potential critical process parameters were identified and process characterisation studies were carried out with identified process parameters that were characterised in a broader range using design of experiments. Data analysis from these studies identified critical process parameters and their interactions that have a significant impact on critical quality attributes. Operating ranges for individual process parameter (PARs) were defined from characterization studies.

DDGS opted as an economical source for Kafirin extraction. Process adaptation approach was implemented to accommodate DDGS into the existing process with obvious modifications considering high protein content. The designed extraction process was scaled-up and validated. Quality of the kafirin produced from different raw material and at different scales were comparable.

9.1.2. Column chromatography and scale-up

The column chromatography was used to produce high purity kafirin considering the development of biomedical applications such as implants and scaffold for tissue engineering. Various cation exchange and anion exchange resins with different physicochemical properties available in the market were screened. Adsorption isotherm and uptake kinetic studies were carried out to identify resin candidate that offers better purity and higher recovery. Capto Q was selected and the laboratory-scale process was developed. 20 fold scale-up was carried out to assure scalability of the developed process. Quality of the kafirin purified at laboratory scale and scale-up studies were comparable.

9.1.3. Ultrasonic cavitation

Ultrasonic cavitation was used and the process intensification tool in order to reduce operation time for the Kafirin purification process. Full factorial screening design was used to optimise the process parameter for kafirin extraction using ultrasonic cavitation. Process time, amplitude and S/L ratio were considered and optimised to achieve a minimum 60% Kafirin yield. Ultrasonic cavitation was implemented considering the development of a continuous process.

9.1.4. Application development: Plasticizer screening and films

Purified kafirin was paired with suitable plasticizers to enhance functional properties for desired applications. Different plasticizers were screened to produce films and tested for their mechanical, functional and barrier properties. Kafirin films plasticized with different plasticizers were tested for their tensile strength and flexibility. They were also tested for their water uptake and moisture content. Scanning electron micrographs revealed structural changes imparted by different plasticizers, findings were complementary to the other

analysis. Finally, Kafirin films were tested for their moisture barrier properties. Plasticizers screened during the studies produced an array of functional properties they provided a platform for the kafirin based application development.

9.1.5. Enteric Spray coat development

kafirin based coating was formulated for pharmaceutical and/or nutraceutical products for targeted drug delivery. Characterization and *in-vitro* studies carried out confirmed that the spray coat act as an excellent enteric coat that can reduce drug loss in the stomach, especially for low pH sensitive drugs and may decrease the overall dosage. This assures that kafirin coat can withstand low pH environment and enzymatic digestibility, which are desirable criteria for enteric coating.

9.1.6. Kafirin microparticles and food-grade coat development

To develop an integrated kafirin purification and application development process, spray drying operation was optimised to produce microparticles. Operating process parameters were optimised to produce microparticles within the desired particle size distribution. These particles were analysed and characterised.

A kafirin spray coat was developed to preserve perishable food. Kafirin was paired with edible plasticizers to design the spray coat with required elasticity and viscosity to assure proper coating for fruits. It was demonstrated that a kafirin coat could increase the shelf life of fruits up to 14 days. The increased shelf life of these food articles is due to effective water and gas barrier properties of the coat, which are indispensable characteristics of packaging material.

9.2. Innovative and Unique contribution from the current research

1. Kafirin purification process: First reported scale-up and validated kafirin purification process optimised using QbD based approach.
2. Process adaptation approach to include DDGS as an economic alternative to produce consistent quality kafirin.

3. Chromatography based process developed and scale-up to produce high purity kafirin for development of biomedical applications such as tissue engineering scaffolds and implants.
4. Ultrasonic cavitation-based process developed to purify kafirin from DDGS that paved the way to further develop continuous extraction process.
5. Plasticizer screening study provided platform for further application development as material generated displayed array of physicochemical, mechanical and function properties.
6. Pharmaceutical application 1: kafirin based spray coat was developed with enteric coating properties and can be used for targeted drug delivery.
7. Pharmaceutical application 2: Kafirin microparticles and their characterisation.
8. Spray coat for perishable food articles to increase their shelf-life.

9.3. Significance

Exploring kafirin as a viable and sustainable source for various applications has gained a lot of attention. Commercial-scale development and implementation of different applications developed from kafirin will have a major impact on several sectors of the community.

- Sorghum, being the raw material for kafirin, demand at higher purification scale will require a recurring source. The increase in demand for sorghum for higher kafirin production will encourage the farmers and agriculture industry to ensure its sustainability, which helps them owing to the increase in demand and returns on kafirin rich sorghum production. It will directly benefit the farmers (producers) rather than revenue being channelized to intermediaries.
- Prerequisite of sorghum will encourage the researchers to come up with innovative ideas to develop cultivars, which can survive extreme climatic condition as well as resistance to the diseases. Also, research will be promoted for perennial cultivars of sorghum with higher protein content and improved nutritional value.
- Purified kafirin can be cast into film or coating for pharmaceutical, biopharmaceutical and nutraceutical drugs not only as an encapsulation but as it

also increases its nutritional value. Encapsulation material used by most pharmaceutical industries are synthetic; kafirin can be the alternative derived from natural sources.

- Packaging industry uses synthetic materials such as plastic, which is non-biodegradable and is a potent environment hazard. Kafirin has the traits to be developed as a packaging material because of its unique properties, which enable it to be a better replacement being biodegradable and environment-friendly. Similarly, It also ensures survival and flourishes other industries, which are connected directly or indirectly such as fertiliser, farming tools and logistics.
- Applications developed from kafirin will encourage the researchers to materialise cost-efficient and scalable purification processes to fulfil the increasing demand of kafirin. Also, it will replace the products manufactured from non-biodegradable and hazardous contenders.
- Research and manufacturing facilities will lend opportunities for the public with different specialisations and expertise.
- These socio-economic benefits associated with sorghum industry will improve the quality of life for farmers and other tradespeople along with their families related to this trade.

9.4. Future recommendations

9.4.1. Process development

- The process developed in the present study can be scaled up to pilot scale and later to commercial scale to increase the productivity of kafirin for application development and mass production. Counter-current extraction can be developed and implemented to develop a continuous purification process.
- Chromatography was used as a process intensification tool to obtain kafirin suitable for high-end applications such as scaffolding for tissue engineering. Other commercially available and upcoming resins with higher selectivity and binding capacities can be tested as a suitable replacement to the candidate from the present study to improve purity, increase the yield and economy of the process. Developed chromatography process can be characterised, optimised and scaled up to pilot and commercial production scale to increase the productivity and yield.

- Process intensification using ultrasonic cavitation can be scaled up to increase productivity and characterised to improve the efficiency of the process. A continuous process can be designed at a larger scale to assure the constant production of purified kafirin. Also, hydrodynamic cavitation-based purification process design development can be considered followed by scale-up to make the process energy efficient.
- The final stage of the purification process i.e. spray drying, at higher scale, can be integrated with the suitable application development platform such as enteric spray coating and microparticle synthesis (pharmaceutical and/or nutraceutical products) to minimise the process intermediate or final product hold-time. Process integration will ensure an increase in productivity by reducing solvent and energy consumption required for re-dissolution for application development and production.

9.4.2. Application development

- Kafirin produced from processes working on diverse principles exhibited a difference within their properties. This demands further characterization of the molecules using various techniques to understand their structure and properties at the molecular level. Efforts have been initiated in collaboration with researchers at ICL, London, UK to study the effects of extraction and purification operating parameters on the native structure of the kafirin molecule. The data obtained from these studies will aid the design and development of new applications and open doors for kafirin to other unexplored sectors as a biodegradable alternative to the synthetics.
- Kafirin spray coating was successfully tested for its suitability as an efficient enteric coat in the present study. It can be further optimised, characterised and *In-vivo* studies of the kafirin-based enteric coating can be carried out to establish kafirin as a natural and biodegradable candidate for its suitability compared to other commercially available synthetic equivalents.
- Microparticles manufactured at large scale can be extensively tested for their properties such as enteric coating stability, sustained-release profiles and other mechanical and functional properties. Bought In-vivo and in-Vitro studies

can be carried out for drug-loaded microparticles to assess their suitability as their drug carrier and targeted drug delivery.

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Figure 5: Design space created from characterization studies that define ORs and acceptable range that assures the desired product quality.

Reference: Rathore *et al*. (2009) *(66)*

Figure 6: Effects of variability on the final product quality with traditional (a) and PAT based dynamic control strategy (b).

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