Department of Chemical Engineering

Isolation and Purification of Protein (Conglutin-γ) from Lupin Seeds

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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: 02- 08- 2017

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Abstract

Lupin, the largest legume crop in Australia, is gaining global attention because of its unique protein γ-conglutin, which has shown promise as a nutraceutical for controlling blood glucose level and thus reducing the risk of type II diabetes development. Despite its bioactivity, a commercial formulation of γ-conglutin is still not available. Previously, several research groups have reported trials on extracting and purifying proteins from lupin seeds. However, most of these methods have focused on protein isolates as food ingredients. Very few reports have aimed to purify γ-conglutin from total proteins, but the methods reported are time-consuming and unsuitable for commercial scale production of high purity γ-conglutin due to the involvement of many processing steps for nutraceutical and pharmaceutical application. Developing a scalable process to obtain high purity γ-conglutin will open up a new avenue for lupin seed product in nutraceutical and pharmaceutical market. Therefore, this research work was aimed at the development of an industrially viable process for purification of γ-conglutin from lupin seeds.

In this study, a hybrid process approach by integrating selective extraction and adsorptive chromatographic purification was used to achieve more than 90% purity of γ-conglutin with least possible process steps. The selective extraction of γ-conglutin was optimized to acidic pH 4, which reduced the major protein impurities in the extraction stage itself, unlike conventional alkaline extraction. The key parameters for γ-conglutin extraction were investigated and optimized to yield ~15% w/w γ-conglutin enriched fraction. Furthermore, four cation exchange adsorbents were screened for adsorption behaviour of γ-conglutin enriched extract depending on pH of the medium and distribution of surface charges on γ-conglutin. An intermediate pH modulation step was introduced to impart neutral charges on the surface of protein impurities. As a result, the impurities did not bind to the charged adsorbent, which otherwise would increase the number of chromatographic steps to obtain pure γ-conglutin.

The batch adsorption experiments showed maximum binding on Capto S, which was further studied for its adsorption behaviour in dynamic conditions. The adsorption of γ-conglutin on Capto S was observed to follow Langmuir isotherm pattern, with a

maximum binding capacity of 91.75 mg/ml and dissociation constant of 4.47×10^{-5} M. Cation exchange column chromatography process was then optimized by studying the effect of various process parameters on the purity of γ-conglutin. Flow rate (0.5 ml/min) and elution gradient were optimized to obtain the maximum possible resolution (Rs= 1.59) between impurities and γ-conglutin peak.

The obtained γ-conglutin fraction (~2.38% w/w of total globulins) with 95% purity was further successfully confirmed for its identity by a range of analytical techniques such as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), scanning densitometry, western blotting, Fourier transform infrared (FTIR) spectroscopy and mass spectrometry (MS). A reverse phase high-performance liquid chromatographic (RP-HPLC) method was also developed and validated for detection and quantitation of γ-conglutin from lupin seed extracts.

Publications and Patent

Peer reviewed journals

Mane S, Johnson S, Duranti M, Pareek V and Utikar R (2018). Lupin seed γ-conglutin: extraction and purification methods-A review. *Trends in Food Science and Technology*, 73, 1-11.

Mane S, Bringans S, Johnson S, Pareek V and Utikar R (2017). Reverse phase HPLC method for detection and quantification of lupin seed γ-conglutin. *Journal of Chromatography B*, 1063, 123-129.

Mane S, Hackett M, Johnson S, Pareek V and Utikar R (2018). FTIR spectroscopic characterization of lupin seed γ-conglutin. *Biochimica et Biophysica Acta (BBA)-Protein Structure* (to be submitted, June 2018).

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Conference proceedings

Mane S P, Agrawal S A, Johnson S K, Utikar R P and Kale S B (2014). Extraction of conglutins from lupin seeds. *Proceedings of Chemeca Conference*, Perth, Western Australia. 1503- 1508.

Mane S P, Agrawal S A, Johnson S K, Utikar R P and Kale S B (2015). γ-Conglutin: extraction kinetics and adsorption isotherm studies. *Proceedings of XIV International Lupin Conference,* Milan, Italy.

Patent application

A patent application is being filed based on the content in the Chapter 4.

Awards/Recognition

Curtin Commercial Innovation Award in Health Sciences 2017- Winner

Harnessing Australia's most grown legume to combat world's costliest disease

JCEC (WA) Postgraduate Research Excellence Award 2016- Finalist

Purification of an antidiabetic bioactive (γ-conglutin) from lupin seeds

Table of contents

Nomenclature

Acronyms

ASL	Australian sweet lupin
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
AUC	Area under curve
BCA	Bicinchoninic acid analysis
BSA	Bovine serum albumin
CCD	Central composite design
CV	Column volume
CV	Coefficient of variation
DAD	Diode-array detector
DAFWA	Department of Agriculture and Food Western Australia
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
FTIR	Fourier transform infrared spectroscopy
GI	Glycemic index
HMW	High molecular weight
ICH	International Conference on Harmonisation
IMW	Intermediate molecular weight
kDa	Kilodalton
LMW	Low molecular weight
LOD	Limit of detection
LOQ	Limit of quantitation
M	Molar
m/z	Mass to charge ratio
MRM	Multiple reaction monitoring
MS	Mass spectroscopy
NaCl	Sodium chloride
NGC	Next generation chromatography
OFAT	One factor at atime
pI	Isoelectric point

PVDF	Polyvinylidene difluoride
RFO	Raffinose family oligosaccharides
RP-HPLC	Reverse phase high-pressure liquid chromatography
RSD	Relative standard deviation
RSD	Relative standard deviation
RSM	Response surface methodology
RT	Retention time
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacryl amide gel electrophoresis
TBS	Tris-buffered saline
TFA	Trifluoroacetic acid
Tris	Tris(hydroxymethyl)aminomethane

Symbols

As	Peak asymmetry
C	Liquid phase concentration (mg/ml)
C*	Equilibrium solid phase concentation (mg/ml)
C_0	Initial liquid phase concentration (mg/ml)
K_d	Langmuir dissociation constant (M)
N	Adsorption intensity
q	Solid phase concentration (mg/ml)
q*	Equilibrium liquid phase concentation (mg/ml)
q_F	Freundlich adsorption capacity (mg/ml adsorbent)
q_{max}	Langmuir adsorption capacity (mg/ml adsorbent)
R_L	Langmuir equilibrium constant
Rs	Resolution
α	Alpha
β	Beta
γ	Gamma
δ	Delta

List of Figures

List of Tables

Chapter 1

Introduction

1.1 INTRODUCTION

Diabetes is one of the largest global health emergencies of the 21st century with type II diabetes accounting 91% of all diabetes cases (WHO, 2014) in part due to lack of healthy diet and physical exercise. According to International Diabetes Federation (WHO, 2014), one in 10 adults will have diabetes by 2040, which requires 12% of global health expenditure to be dedicated to diabetes treatment. With this tremendous cost of diabetes, both in terms of economy and burden of disease, alternative and/or supplementary approaches to existing preventative and control strategies to those currently in use are required. The use of bioactives from plant sources may be of value in assisting to curb this 'pandemic'.

Grain legumes effectively are the source of a balanced diet, and thus can prevent diseases, including type II diabetes and cardiovascular diseases (Marcello Duranti, 2006; Leterme, 2002). Legumes are known to control blood glucose level, as they have a low glycemic index (GI) on account of the specific composition of starch present in them (Jenkins, Wolever, & Jenkins, 1988). Lupin, the largest legume crop in Australia has long been known for assisting in controlling symptoms of diabetes (Marcello Duranti, 2006). However, this 'glucose modulating activity' in lupin is credited to some specific component other than starch, as lupin seed has no starch and thus very low glycemic index (Marcello Duranti, 2006). This specific component in lupin seeds responsible for controlling blood glucose level is its unique protein, γ-conglutin, as reported from cellular, animal model and human post-prandial studies (Bertoglio et al., 2011; Lovati et al., 2012; Magni et al., 2004; Terruzzi et al., 2011).

In spite of this 'glycemic modulating property' of γ-conglutin, lupin seed is still not commercially utilised in the nutraceutical and pharmaceutical industries. This undervaluation of γ-conglutin as an 'antidiabetic agent' is due to its unavailability on a commercial scale, as there is a lack of any effective larger scale technology for the purification of γ-conglutin at a grade suitable for human consumption (Mane, Johnson,

Duranti, Pareek, & Utikar, 2018). The low abundance of γ-conglutin in lupin seed (~2-3% w/w of total seed protein) is one of the challenges for the development of an industrial scale process for its purification.

1.2 MOTIVATION OF THIS RESEARCH

The major storage proteins of the lupin seeds are globulins, which are also termed as conglutins (Ritthausen, 1882), and consist of α and β-conglutin (major conglutins), γ and δ-conglutin (minor conglutins) (Blagrove & Gillespie, 1975; Casey, 1999). α and β-Conglutins are the most studied lupin proteins, due to their high abundance and techno-functional properties, mainly emulsification and oil binding properties, and thus application as a replacement for animal or soy proteins in food products (D'Agostina et al., 2006). Therefore, the literature on lupin protein purification (Bez et al., 2005; D'Agostina et al., 2006; El-Adawy, Rahma, El-Bedawey, & Gafar, 2001; Lqari, Vioque, Pedroche, & Millan, 2002; Wasche, Muller, & Knauf, 2001) reveals that the primary focus has been on the development of methodologies for isolation of the major conglutins, α and β-conglutins.

α and β-Conglutins have their isoelectric point (pI) in the acidic range, similar to most other legume proteins (Derbyshire, Wright, & Boulter, 1976). As a result, lupin proteins, till date, have most commonly been extracted by alkaline extraction followed by isoelectric precipitation at acidic pH. This conventional extraction approach yields the major conglutins (α and β-conglutins) in the precipitate, whereas γ-conglutin remains in the supernatant after acidic isoelectric precipitation due to its basic pI. Few reports (Czubinski, Dwiecki, Siger, Neunert, & Lampart-Szczapa, 2014; M Duranti, Scarafoni, Di Cataldo, & Sessa, 2001; Melo, Ferreira, & Teixeira, 1994) have further processed this acid soluble fraction (supernatant) for isolating γ-conglutin by a combination of different purification techniques. However, these reported methods are time-consuming and unsuitable for commercial scale production of nutraceutical and pharmaceutical grade γ-conglutin due to the use of potentially toxic reagents such as zinc and the involvement of many processing steps. In addition, the reported methods for γ-conglutin purification are based on the traditional knowledge of extraction and purification strategies for legume proteins (Derbyshire et al., 1976). This heuristic approach of process design is less likely to be effective in the case of γ-conglutin due to its peculiar pI amongst legume proteins. Hence there is unfulfilled need to develop

a novel process for cost-effective scalable purification of γ-conglutin specifically tailored to its use in nutraceutical and pharmaceutical applications.

The purification of γ-conglutin from lupin seeds will open up a new avenue for high-value products from lupin seeds as nutraceuticals, the market for which is predicted to reach US$ 250 billion by 2018 (Dutta, Mahabir, & Pathak, 2013). The availability of pure γ-conglutin will open the doors of new dimensions in diabetic research sectors where the source of the protein, its refining and formulation has always been a stringent process, in terms of its quality and regulatory concerns. Further, purifying this 'glucose modulating protein' from lupin seeds will likely benefit the agriculture sector of Western Australia through greater demand and returns on its major rotation crop.

1.3 RESEARCH AIMS AND OBJECTIVES

The project is aimed at developing a novel and industrially viable process for isolation and purification of γ-conglutin for nutraceutical/pharmaceutical applications. To achieve this aim, the following specific objectives are set:

- Designing and developing an efficient and selective extraction process for obtaining γ-conglutin enriched extract. The design of experiment (DoE) approach is used to identify the optimal combination of key parameters for conglutin extraction in order to maximize product purity and yield.
- Screening of different cation exchange adsorbents to determine the most suitable adsorbent for selective capture of γ-conglutin from the extract by performing equilibrium adsorption experiments.
- Developing an efficient cation exchange chromatography process for producing high purity γ-conglutin.
- Optimizing column chromatography process for obtaining maximum resolution between γ-conglutin and impurities.
- Analytical characterization of purified γ-conglutin by a range of techniques such as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, Fourier transform infrared (FTIR) spectrometry, reverse phase high-pressure liquid chromatography (RP-HPLC) and mass spectrometry (MS) to confirm its identity.

1.4 THESIS STRUCTURE

Chapter 2 will briefly explain the comprehensive literature review, in particular, the information on lupin protein fractions, their applications and their physico-chemical properties will be presented. Further, the available purification approaches and limitations in their scale-up potential for isolating pure γ-conglutin fraction will be highlighted. Chapter 2 will also provide the research process paradigm-shift required for developing a novel process for γ-conglutin purification.

In Chapter 3, the experimentation on the extraction of total lupin proteins for food applications and its statistical optimization will be discussed. Initially, the key parameters for conglutin extraction will be identified by one factor at a time approach, followed by use of DoE approach to maximize the extraction yield and purity. Further, the extraction optimization by three different extraction approaches (magnetic stirring, ultrasonication and hydrodynamic cavitation) will be discussed to study the effect of extraction process on protein yield and purity.

In Chapter 4, experimentation on the stages in developing a cation exchange chromatography process for purification of γ-conglutin will be presented. The impurities in the extract will be identified and reduced before loading on adsorbent by introducing an intermediary pH modulation step. Batch equilibrium experiments will be performed for screening different cation exchange adsorbents, to find the suitable adsorbent for selective capture of γ-conglutin. Further, the experimentation on column chromatography with the selected adsorbent will be discussed and optimized with respect to different process parameters, in order to obtain highest possible resolution between γ-conglutin and an impurity peak. This Chapter will provide the first report on selective extraction and purification of γ-conglutin with minimum process steps.

In Chapter 5, experiments on the conformational characterization of purified γ-conglutin by different analytical techniques such as SDS-PAGE, scanning densitometry, FTIR, Western blotting and MS will be discussed. A simple, accurate and sensitive RP-HPLC method for quantitation of γ-conglutin will also be developed and validated.

Finally, Chapter 6 will summarize the major findings of this thesis and provides suggestions for future work.

Chapter 2

Literature review

Information in this Chapter has been published as follows:

Mane S, Johnson S, Duranti M, Pareek V and Utikar R (2018). Lupin seed γ-conglutin: extraction and purification methods - A review. *Trends in Food Science and Technology*, 73, 1-11.

2.1 INTRODUCTION

The need for legume-derived bioactive is growing expectedly on account of their high nutritional value and lower production costs as compared to animal proteins. Recently, it is been revealed that legume proteins not only serve as the source of nutrients in the diet but also play important bio-active roles in various physiological functions (Duranti, 2006). Soybean is the most studied legume in terms of the health benefits of its proteins (Xiao, 2008). However, there remains some controversy over the widespread use of soy protein due to the potential toxicity of its high levels of phytoestrogenic compounds (Sirtori, Arnoldi, & Johnson, 2005). In recent years, increasing global interest has been shown for legume lupin due to its comparable protein content, non-genetically modified status, low cost and low levels of potentially hazardous phytoestrogenic compounds compared to soy (Sirtori et al., 2005).

2.1.1 Lupins

Lupin is an ancient leguminous, economically and agriculturally valuable plant (Lupins.org, 2015). This leguminous crop is able to grow in harsh climates with low rainfall and poor soils, which makes it an ideal crop for drier and warmer climatic conditions (Lupins.org, 2015). Lupins are mainly cultivated for as a ruminant feed, as a green manure, for human nutrition and for fixing atmospheric nitrogen to the soil (Uzun, Arslan, Karhan, & Toker, 2007). Earlier, the role of lupins in modern agricultural and food system was limited, due to its toxic alkaloid content (Mohamed & Rayas-Duarte, 1995). Later in the 19th century, the breeding program of low-alkaloid (sweet) varieties enabled this species to transform from only use as green manure to

that of more demanded grain legume by food and modern agricultural system (Sweetingham & Kingwell, 2008).

In recent years, there is been increasing interest for legume lupin as it has a unique combination of high-protein content, high-dietary fiber content, no starch and thus very low glycemic index (Sipsas, 2008), low levels of potentially toxic phytoestrogenic components and non-genetically modified status as compared to soybean (Sirtori et al., 2005). In addition, an important attribute of lupin seeds is that it does not contain gluten, therefore it has been widely used in gluten-free foods (Scarafoni, Ronchi, & Duranti, 2009).

Lupin taxonomy, species and agricultural production

Lupins (*Lupinus spp*) belong to the Genisteae tribe of Fabaceae or Leguminosae family, which has more than 400 known species, of them only four are of agronomic interest, including *L. albus*, *L. angustifolius*, *L. luteus* and *L. mutabilis* (Uzun et al., 2007). *Lupinus albus*, which is termed as white lupin is extensively grown in Europe, while *Lupinus luteus* (yellow lupin) is native to Mediterranean region (Lupins.org, 2015). *Lupinus angustifolius*, which is well known as a blue or narrow-leafed lupin or sweet lupin, is a major rotation crop in Western Australia, whereas *Lupinus mutabilis* (pearl or Tarrwi lupin) belongs to South America (Reinhard, Rupp, Sager, Streule, & Zoller, 2006). Due to low levels (0.02%) of bitter and toxic alkaloids, these species are known as 'sweet lupins' (Wasche, Muller, & Knauf, 2001).

Lupins have occupied ~3% of global pulse production with top 5 lupin producing countries in 2014 were Australia, Poland, Russian Federation, Germany and Belarus (FAO-STAT, 2017). Australia is responsible for producing 80% of the total global supply of lupin seeds, with Western Australia is the largest global exporter (DAFWA, 2017) of lupin grain, the majority of which is Australian sweet lupin (ASL).

Component analysis of lupin seeds

In this Chapter, the term 'seed' means total/intact seed, 'hulls' means seed coat/bran, and 'kernels' means cotyledons. Lupin seed, in general, is composed of ~25% hulls and 75% kernels (Lupins.org, 2015). The lupin hulls are high in cellulose or hemicellulose, but, low in lignin (Jean-Marc & Carre, 1989), while kernels are mainly composed of galactan. Lupin seed has a number of bioactive molecules and functional

compounds with notable properties. The percentage of these components vary between different lupin species and hence ranges of values are generally provided when mentioning compositional analysis. Broadly, 36-52% protein, 5-20% oil, and 30-40% fibre are present in whole lupin seed (Gross et al., 1988).

Unlike other legumes, lupins are low in undesirable constituents, such as phytic acids, alkaloids, oligosaccharides, lectins, trypsin inhibitor and saponins (Lupins.org, 2015). The major contribute of lupin seed is a protein, with biological value as comparable to egg proteins (Kohajdova, Karovicova, & Schmidt, 2011). Lupin seed proteins also represent a good source of essential amino acids (Drakos, Doxastakis, & Kiosseoglou, 2007) as similar to soybean proteins (Sipsas S, 2004). On account of easier handling and high denaturation temperature, lupin protein isolates have an additional advantage in the food industry over animal proteins (Chapleau & de Lamballerie-Anton, 2003). The detailed characteristics of lupin proteins are discussed further in the section 2.1.2.

The oil extracted from lupin has shown free radical scavenging and antioxidant activity. In general, lupin oil is characterised by a balanced fatty acid composition with total 10% of saturated fatty acids and 90% of unsaturated fatty acids, of which 32-50% is oleic acid, 20-40% is linoleic acid, 2-10% is linolenic acid, 0.5-10% palmitic acid and 1-5% stearic acid (Uzun et al., 2007). The presence of high percentage of oleic acid has enabled the use of lupin oil in cosmetics industry (Rona, Vailati, & Berardesca, 2004).

Lupin seeds have also shown to have considerable amounts of prominent carotenoids such as lutein, zeaxanthin, β-carotene and others (α-carotene, violaxanthin, lycopene cis isomer and lycopene) in small amounts, which can be conditioned to prevent the development of some chronic diseases in humans including cancer and cardiovascular diseases (Wang, Errington, & Yap, 2008). Lupin seeds are also found to have the presence of antioxidants in the form of polyphenolics, flavonoids and tannins (Oomah, Tiger, Olson, & Balasubramanian, 2006). Minerals such as manganese, zinc and iron (Hove, 1974), and vitamins such as niacin, riboflavin and thiamine (Caballero, Trugo, & Finglas, 2003) are also present in lupin seeds.

Lupin seeds are a rich source of dietary fibres (~40% w/w), out of which 10% is soluble and 30% is insoluble dietary fibre (Hall, Johnson, Baxter, & Ball, 2005). The dietary fibres in lupin seed are composed of non-starch polysaccharides and raffinose family

oligosaccharides, which are involved in the nutritional and techno-functional properties of lupin seeds (Evans, Cheung, & Cheetham, 1993).

2.1.2 Lupin proteins

The main proteins of the lupin seeds are the storage proteins, which are located in the storage vacuoles of cotyledonary tissues (Duranti, Consonni, Magni, Sessa, & Scarafoni, 2008). Lupin proteins can be fractionated according to Osborne classification into water soluble albumins, salt soluble globulins, alcohol-soluble prolamins and acid/alkali soluble glutelins (Mandal & Mandal, 2000). The major storage proteins of lupin seeds are globulins (~87% of total lupin protein), which are also termed as conglutins (Ritthausen, 1882). The albumins are ~13% of total protein, while glutelins and prolamins are found only in very small amounts (Duranti, Cerletti, Postiglione, & Lanza, 1990). The further separation of lupin globulins was carried out by Blagrove and Gillespie (1975) into four main fractions as α and β-conglutin (major conglutins), γ and δ-conglutin (minor conglutins), based on their electrophoretic mobility (Blagrove & Gillespie, 1975).

Like other legume proteins, lupin proteins have also been traditionally extracted with conventional alkaline extraction on account of their acidic pI, followed by isoelectric precipitation at acidic pH (Bez et al., 2005; Blagrove & Gillespie, 1975; Derbyshire, Wright, & Boulter, 1976; Wasche et al., 2001). This conventional extraction process yields major conglutins (α and β-conglutin) in the precipitate, however, γ-conglutin remains in the acid soluble fraction (supernatant) due to its basic pI. The major conglutins (α and β-conglutin) are known to have excellent emulsifying properties (Sironi, Sessa, & Duranti, 2005), whereas acid soluble fraction containing bioactive peptide γ-conglutin was observed to have good foaming properties (Wong, Pitts, Jayasena, & Johnson, 2013). Therefore, earlier lupin proteins were majorly known in food industries, due to their favourable nutritional and techno-functional characteristic role in human diet and health (Johnson, in press). Recently, lupin proteins have also been investigated for their nutraceutical properties, including hypoglycemic in rats (Magni et al., 2004), hypotensive in mice (Pilvi et al., 2006), hypocholesteromic in rats (Sirtori et al., 2004) and anti-atherosclerotic in rabbits (Marchesi et al., 2008).

The most studied lupin proteins are, α-conglutin and β-conglutin, due to their high abundance in lupin seeds and their massive applications in food industries as a

replacement for animal or soy products (Arnoldi, 2008). Therefore, up till now, most of the reported methods for lupin protein purification (Bez et al., 2005; D'Agostina et al., 2006; El-Adawy, Rahma, El-Bedawey, & Gafar, 2001; Lqari, Vioque, Pedroche, & Millan, 2002; Wasche et al., 2001) were targeted for the purification of major conglutins (α and β-conglutin) by traditional alkaline extraction. Further, separation of γ-conglutin from acid soluble stream has been reported by few research groups (Blagrove & Gillespie, 1975; Czubinski, Dwiecki, Siger, Neunert, & Lampart-Szczapa, 2014; Duranti, Scarafoni, Di Cataldo, & Sessa, 2001; Melo, Ferreira, & Teixeira, 1994) by combination of different chromatographic techniques. However, these studies were performed on lab scale as the focus was on protein characterization and bioactivity measurements. As such, there is no scalable process which will produce pure γ-conglutin, specially tailored for its use in nutraceutical and pharmaceutical applications. Hence there is unfulfilled need to develop a novel process for scalable purification of γ-conglutin.

The following section describes the physico-chemical characteristics of γ-conglutin and other conglutins, and how these properties have been used as a tool for designing its purification process. An overview of reported purification processes for γ-conglutin to date is also given. The comparison of the positive and negative aspects of these processing approaches is provided, followed by a discussion of obstacles in their scaling up, with the aim of envisaging and developing new enrichment strategies.

Lupin conglutins

The most abundant lupin seed protein is β-conglutin (~44% w/w of total conglutins) (Duranti, Restani, Poniatowska, & Cerletti, 1981). Its native molecular weight is in the range of 143-260 kDa, thus suggesting a trimeric quaternary structure, as most vicilin-like globulins (Derbyshire et al., 1976) and consists of precursor and proteolytically cleaved polypeptides of molecular weights ~15-72 kDa. It is distinguished from other types of lupin conglutins by the absence of disulphide bond which holds monomeric units together (Glenng, 1999).

The second most abundant globulin, which is ~33% w/w of total conglutins, is α-conglutin, a legumin-like globulin (Duranti et al., 1981) and is very similar in terms of its solubility, amino acid composition, sedimentation behaviour and charge in all lupin species (Glenng, 1999). α-Conglutin is also known for its characteristic glycosylation

(Duranti, Guerrieri, Takahashi, & Cerletti, 1988). It is a hexamer with the native molecular weight of 300-430 kDa, made up of subunits ranging from 53-74 kDa and consisting of acidic and basic chains which are bound together by disulphide linkage (Melo et al., 1994). The third conglutin is δ-conglutin (~12% w/w of total conglutins). It has been described variously as a globulin and as an albumin-globulin complex, but often as an albumin (Glenng, 1999).

The minor seed globulin is γ-conglutin which is ~3-4% w/w of total globulins (Duranti et al., 1981). γ-Conglutin has unique physico-chemical properties, which makes it different amongst lupin proteins. Table 2.1 summarizes the characteristics of γ-conglutin reported in the literature. The values for these characteristic properties may vary between species of lupin and varieties within the species, and hence ranges of values are provided.

γ-Conglutin exists as an oligomeric protein consisting of four subunits at neutral pH (Blagrove, Gillespie, Lilley, & Woods, 1980; Duranti et al., 1981). At pH 5 and lower, the tetramer dissociates into monomers with molecular weights of about 47-48 kDa consisting of two polypeptide chains of 27-30 kDa and 16-18 kDa respectively (Restani, Duranti, Cerletti, & Simonetti, 1981), of which large chain (27-30 kDa) undergoes glycosylation (Eaton-Mordas & Moore, 1979). γ-Conglutin has higher levels of the essential amino acids such as methionine, cysteine and lysine than α-conglutin and β-conglutin, indicating a higher nutritional value (Glenng, 1999). The most peculiar characteristic of γ-conglutin is its pI in the basic range unlike other conglutins, which have an acidic pI. These physico-chemical characteristics of γ-conglutin should be carefully considered while selecting an efficient process for purification of γ-conglutin from lupin seeds. The remainder of this review will focus on available extraction and purification methods for γ-conglutin.

Table 2.1 Physico-chemical characteristics of lupin seed γ-conglutin

Characteristic	Technique used	Parameter Value
% w/w of total conglutins[a]	Micro-Kjeldahl	3-4% w/w
Quaternary structure	Sedimentation[b], crystal structure[c]	Tetramer[b], Hexamer[c]
pH based transition[b,d]	Gel filtration, circular dichroism	Monomer- pH 4.5 Dimer- pH 5.5 Tetramer- pH 6.5
Native molecular weight	Native PAGE, gel filtration[b] and light scattering[d]	280-320[b], 240[d] kDa
Monomer molecular weight[b,e]	SDS-PAGE and gel filtration	47-48kDa
Monomer composition[b,e]	SDS-PAGE and gel filtration	Large chain- 27-30 kDa Small chain- 16-18 kDa
Isoelectric point[b,e]	Isoelectric focussing	Large chain- 7.9-8.0 Small chain- 6.1-6.9
Glycosylation[f,g]	Glycopeptide analysis	Large chain- Yes Small chain- No
3D structure[b,c]	Optical rotary dispersion and circular dichroism, crystal structure[c]	β-structure (predominant) α-helix β-turns

[a](Duranti et al., 1981)

[b](Blagrove et al., 1980)

[c](Czubinski et al., 2015)

[d](Capraro, Spotti, Magni, Scarafoni, & Duranti, 2010)

[e](Restani et al., 1981)

[f](Eaton-Mordas & Moore, 1979)

[g](Schiarea, Arnoldi, Fanelli, De Combarieu, & Chiabrando, 2013)

2.2 EXTRACTION AND PURIFICATION OF γ-CONGLUTIN

Most processes for lupin protein extraction and fractionation into individual conglutins feature four major steps, namely seed pre-treatment, extraction of total conglutins,

membrane filtration and chromatographic purification as represented in Figure 2.1. Similar procedures for protein extraction are shared for most protein-rich legume seeds (Derbyshire et al., 1976). Lupin seeds are initially pre-treated for the effective separation. This step includes a physical separation of seed into its components, namely hulls and kernels, followed by milling and defatting of the flours. Total proteins are then extracted from the defatted flour using various extraction conditions such as aqueous, alkaline or salt-assisted protocols. Some processes additionally involve membrane filtration to remove extraction additives such as salts and low molecular weight non-protein materials. The total proteins are further purified and fractionated into individual conglutins using a combination of chromatographic purification techniques depending on the necessity of the specified application. A detailed description of these process steps and difficulties associated with their scale up potential for isolating pure γ-conglutin is described in the following section.

2.2.1 Seed pre-treatment methods

Protein bodies in lupin seeds are concentrated in the kernels, and therefore most reported studies on lupin protein purification involve preliminary fractionation of seeds into fibre rich hulls and protein rich kernels so that all the seed fractions can be used for value-added applications. The process of dehulling reduces the possibility of phytochemical extraction from hulls, which otherwise could contaminate the extracted protein fraction (Derbyshire et al., 1976).

Isolated kernels are then milled to the desired particle size, followed by defatting of the resulting kernel flour with hexane by using Soxhlet apparatus (solvent recycle) (Sironi et al., 2005) or by agitation (Melo et al., 1994) or by soaking (El-Adawy et al., 2001). Defatting leads to protein isolates with improved purity as it minimizes the risk of co-extraction of saturated and unsaturated aldehydes and carboxylic acids which might impair protein extraction (Bader, Czerny, Eisner, & Buettner, 2009; Bader, Oviedo, Pickardt, & Eisner, 2011). However, few studies have reported aqueous extraction of proteins from kernel flour without defatting the starting material (Aguilera, Gerngross, & Lusas, 1983; Berghout, Boom, & van der Goot, 2014; Chew, Casey, & Johnson, 2003). Though aqueous extraction was performed to omit the use of organic solvent during defatting, co-extraction of oil components and long chain carbohydrates was observed (Berghout et al., 2014), impairing protein separation.

Therefore, aqueous fractionation is not suitable when the focus on protein purity is necessary. Moreover, the removal of lipid fraction improves the stability of the flour and decrease the risk of off-flavour generation.

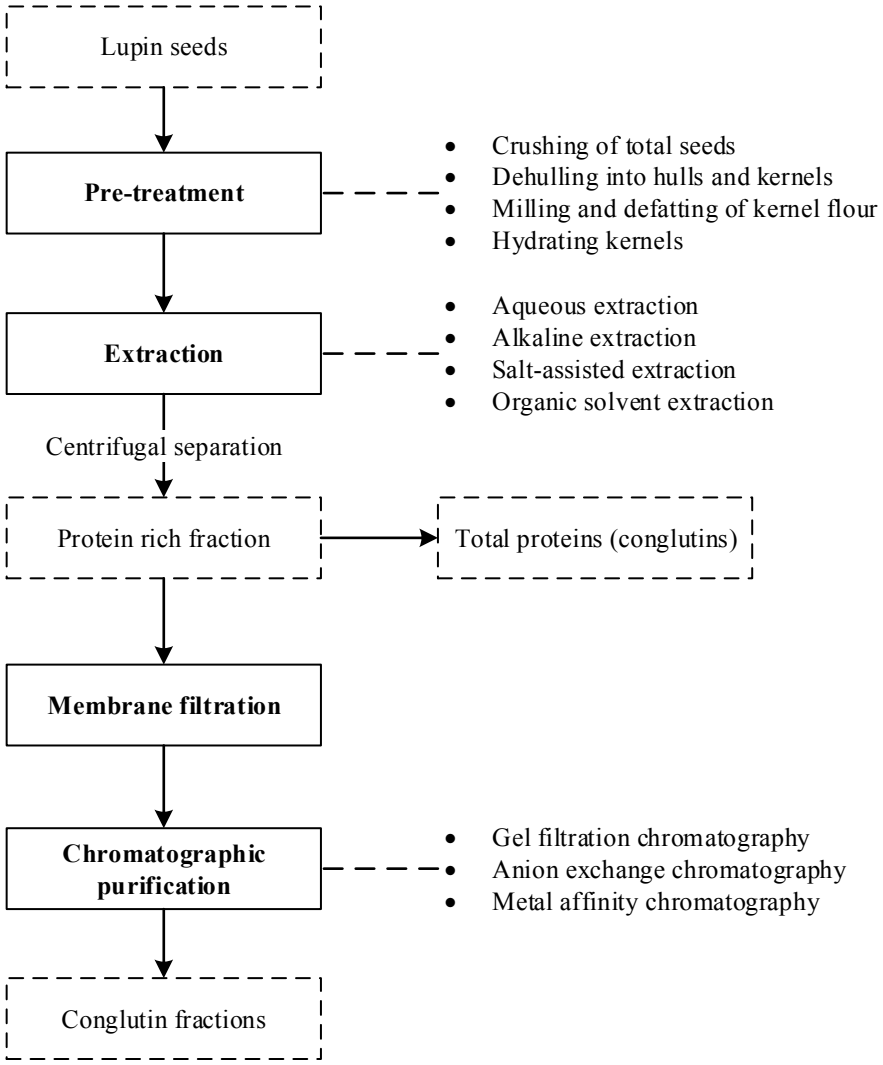

Figure 2.1 Schematics of process steps used for purification of lupin conglutins (Continuous boxes represent process steps, whereas dotted boxes indicate fractions)

Hydrating the intact lupin kernels in excess water has been reported (Wong et al., 2013). This pre-treatment was performed to reduce the levels of low molecular weight compounds, including raffinose family oligosaccharides (RFO) which can later contaminate the protein fraction. However, during the hydration process some proteins are lost as a result, no increase in protein extraction yield was observed (Wong et al., 2013).

2.2.2 Extraction methods

The extraction of proteins from legume seeds is enhanced by different chemical treatments depending on the type of the protein to be extracted (Derbyshire et al., 1976). Conventionally alkaline pH has been used for the extraction of major proteins of legume seeds due to their acidic pI. Some legume proteins, however, have been extracted by adding reducing agent such as 2-mercaptoethanol, which increases protein solubility (Derbyshire et al., 1976). 'Salting-out' of legume proteins with ammonium sulphate was seen to be more specific in avoiding co-extraction of non-protein materials, thus increasing protein extraction yields (Derbyshire et al., 1976).

Main reported applications of lupin proteins are in food industries due to the emulsification properties of major conglutins (α and β-conglutins) (Kohajdova et al., 2011). Therefore, studies on lupin protein extraction are focussed more on the extraction of major conglutin fraction, while very few methodologies have been reported for extraction of minor conglutin (γ-conglutin), which will be discussed further with more details in the following section.

Aqueous/alkaline extraction

Lupin conglutins (α, β and δ-conglutins) have acidic pI in the range of pH 4-5, while unlike other lupin proteins, γ-conglutin has pI in the basic range of 6.9-8.0 (Restani et al., 1981). Proteins show minimum solubility at the pH of their isoelectric region, due to almost zero net surface charge (Widmann, Trodler, & Pleiss, 2010). Therefore, the solubility of major fractions of lupin proteins (α, β and δ-conglutins) is minimum at acidic pH range of 4-5, whereas maximum solubility is exhibited at alkaline pH of 8 and above (Lqari et al., 2002; Ruiz & Hove, 1976). Hence, most of the reports on lupin protein extraction use alkaline extraction followed by isoelectric precipitation at acidic pH, yielding the major conglutin fractions, while γ-conglutin remains in the acid soluble fraction.

During conventional alkaline extraction followed by acidic precipitation, some proteins (such as γ-conglutin) remain in the residue and are not extracted which leads to incomplete recovery of proteins (Millan, Alaiz, Hernandez-Pinzon, Sanchez, & Bautista, 1994; Wong et al., 2013). Moreover, alkaline extraction has shown to co-extract oil components with proteins, impairing the purity of the proteins extracted, unless effective de-oiling step has been performed (Derbyshire et al., 1976). The

efficiency of protein separation process can also be hindered by the formation of complexes promoted at higher pH due to association/dissociation reaction of proteins (Derbyshire et al., 1976). In addition, one study (Sipsas S) has reported enrichment of fibre residue with γ-conglutin after conventional alkaline extraction and acidic precipitation, indicating binding of γ-conglutin to fibre residue. Therefore, the conventional approach of alkaline extraction followed by acidic precipitation is not an ideal choice for selective extraction of γ-conglutin.

Attempts on purifying further γ-conglutin from the acid soluble fraction obtained by conventional alkaline extraction have been reported by its precipitation with zinc chloride (Sironi et al., 2005). This zinc precipitation step increased the purity of γ-conglutin, however, SDS-PAGE of the resulting precipitate demonstrated the presence of other protein fractions contaminating γ-conglutin. Also, the use of metal ions is not suitable for purifying γ-conglutin as a nutraceutical, since complete removal of metal ions is a prerequisite in nutraceutical applications (Cheung, Wong, & Ng, 2012).

Salt-assisted extraction

A major fraction of lupin proteins is classified as globulins (salt soluble proteins) (Mandal & Mandal, 2000). Therefore, there have been attempts for extracting lupin proteins quantitatively using salts such as sodium chloride or ammonium sulphate (Casey, 1999), which will be discussed in the following section.

The use of salts for extracting lupin proteins was first reported by adding 10% w/v sodium chloride to water at neutral pH, followed by precipitation of the lupin protein extract with 85% w/v ammonium sulphate (Joubert, 1956). This study yielded two major fractions, albumin (supernatant) and globulin (precipitate), further separation of which was not reported. Also, salt extraction was observed to co-extract yellow coloured components along with the proteins, removal of which using solvent partitioning likely led to denaturation of proteins (Joubert, 1956). Modifications to this study were performed by using series of precipitation processes with different percentage of ammonium sulphate to obtain conglutins as individual fractions (α, β and γ-conglutins) (Blagrove & Gillespie, 1975). This was the first report on separation of conglutins into individual fractions. However, the scale-up potential of this process is questionable due to a large number of precipitation steps in series making the process lengthy. Also, high use of salts during precipitation steps can make the process less

cost effective as complete removal of salts at each stage is necessary by dialysis or by using desalting column or ultrafiltration processes.

The effect of salt on protein solubility is described by a 'salting-in' region at low salt concentrations and a 'salting-out' region at high salt concentrations (Curtis, Prausnitz, & Blanch, 1998). 'Salting-in' is a favourable electrostatic interaction between salt molecules and charged protein surfaces, whereas high salt concentrations lead to precipitation of proteins ('salting-out') due to increased protein-protein hydrophobic interactions (Curtis et al., 1998). The effect of salt concentration on lupin protein extraction was also studied (El-Adawy et al., 2001). This study reported that the percentage solubility of lupin proteins increased with increase in sodium chloride concentration from 0.2 M to 1 M (salting-in). At higher salt concentrations, solubility was seen to be decreased due to the 'salting-out' effect, indicating 1 M sodium chloride as an optimum salt concentration for lupin protein extraction (El-Adawy et al., 2001).

Salt-assisted extractions of lupin proteins have been compared with conventional alkaline extraction by studying the microscopic structure of the extracted proteins (Muranyi, Otto, Pickardt, Koehler, & Schweiggert-Weisz, 2013). The microstructure characterization indicated that alkaline extraction followed by isoelectric precipitation resulted in irreversible unfolding/denaturation of proteins due to the formation of disulphide bridged polymers (Derbyshire et al., 1976), whereas salt-assisted extraction maintained the globular structure of the extracted proteins. Therefore salt-assisted extraction is preferred over conventional alkaline extraction when the native structure of a protein is of interest (Muranyi et al., 2013).

Extracting lupin proteins with aqueous alcohols have also been attempted (Blaicher, Nolte, & Mukherjee, 1981) using consecutive extractions with 70% v/v ethanol or methanol. The aim of this work was to selectively extract proteins without extracting the bitter potentially toxic alkaloid compounds found in some varieties of lupins. The results of this study showed enrichment in protein extraction by reducing co-extraction of alkaloids. However, extracting lupin proteins with alcohol can cause undesirable changes in their structure due to hydrophobic interactions, leading to their denaturation (Hron, Koltun, & Graci, 1982).

2.2.3 Extraction and membrane filtration

Membrane filtration of lupin protein extract has been reported by only few research groups, as a step for removing lower molecular weight non-protein materials. Fraunhofer IVV researchers (Fraunhofer Institute, Germany) were the first to report the use membrane filtration for lupin protein separation at pilot scale to improve their techno-functional properties for specific food applications (Bez et al., 2005). This technology yielded lupin proteins in two major fractions type E (39% w/w yield with 92% protein content) and type F (10% w/w yield with 83% protein content). Initially, acid extraction at pH 4.5 was performed to produce a fraction with good foaming ability, type F (fraction enriched in γ-conglutin). They followed this by ultrafiltration through a 10 kDa membrane to reduce any oligosaccharides or lower molecular weight contaminants. The residue after the acidic extraction was then extracted twice at neutral pH to obtain a fraction with good emulsifying ability, type E (enriched in α and β-conglutin). Although the type F fraction was reported to be enriched in γ-conglutin, SDS-PAGE analysis of this fraction produced on lab scale has shown many protein impurities (Figure 2.2) (Mane, Agrawal, Utikar, & Kale, 2014). Therefore, this process defined by Fraunhofer research group (D'Agostina et al., 2006) appears ideal for obtaining protein isolates on pilot scale for specific food applications, where the isolation of individual conglutins is not a pre-requisite.

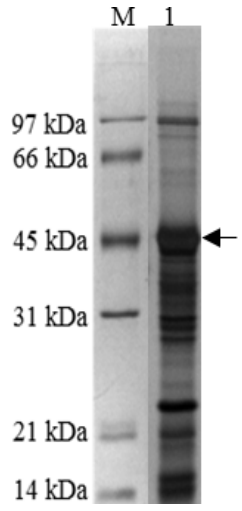

Figure 2.2 SDS-PAGE (non-reducing) of lupin protein acidic extraction at pH 4.5 (M-molecular weight marker, L1- pH 4.5 protein extract)
(Arrow represents γ-conglutin at 48 kDa)

The protein profile of the acid soluble γ-conglutin enriched fraction (ultrafiltered) obtained by conventional alkaline extraction followed by isoelectric precipitation was compared with that of direct acid extraction followed by ultrafiltration (Wong et al., 2013). Though a very little difference in their protein profile was observed as analyzed by SDS-PAGE, direct acid extraction followed by ultrafiltration gave improved foaming ability for γ-conglutin enriched fraction (Wong et al., 2013).

An alternative approach has been used for enrichment of γ-conglutin from acid soluble fraction obtained by conventional alkaline extraction followed by isoelectric precipitation (Wasche et al., 2001). This method reported separation based on surface charges on the membrane (zirconium oxide) and charges on the proteins during the process. The methodology involved passing the acid soluble fraction through a 15 kDa zirconium oxide membrane at pH 7-8 as they assumed the pI of γ-conglutin in the range of pH 6-7. This process improved the foaming properties of the extract, possibly due to the removal of salts. However, no evidence of the purity of γ-conglutin fraction was presented. It is possible that the membrane filtration had no impact on the purity of γ-conglutin as its pI is not between pH 6-7. Also, successive runs of filtration on the membrane can lead to the deposition of γ-conglutin cake on the membrane, complete removal of which is necessary to maintain the efficiency of the process. In addition, recovery of bound γ-conglutin might involve the use of solvents, high pH buffers or high salt concentration, which can cause changes in the structure of proteins, leading to denaturation and/or bioactivity loss.

The use of membrane filtration for lupin protein extract appeared to be beneficial in reducing lower molecular weight non-protein contaminants effectively to improve their specific functional properties. However, the complete separation of lupin conglutins into individual fractions (α, β and γ-conglutins) by membrane filtration has not been reported yet. The key points for using membrane filtration for complete conglutin fractionation can be, either selection of specific cut-off size membranes or by using series of membrane filtration steps due to the broad molecular weight distribution of the conglutins.

In contrast to membrane filtration, there is more evidence in the literature of the efficacy of chromatographic techniques such as gel filtration chromatography, ion exchange chromatography, metal affinity chromatography or their combinations to

isolate pure fractions of the individual conglutins, as will be explained in the following section.

2.2.4 Chromatographic methods

Gel filtration chromatography

Gel filtration chromatography achieves separation of molecules based on their size and shape, in which larger molecules cannot enter the pores of the adsorbent and travel down faster as compared to smaller molecules (Biotech, 1998). In the case of lupin proteins, gel filtration has been used for removing low molecular weight non-protein contaminants during the protein purification step (Blagrove et al., 1980). It has also been used as an analytical tool to predict the molecular weight of conglutins and their oligomers (Duranti et al., 1981).

Gel filtration has also been used for the separation and purification of the major conglutin (α and β) fractions (Lqari et al., 2002). Firstly, the conventional alkaline extraction followed by isoelectric precipitation was used to yield the major conglutins (α and β) in the precipitate. The resulting precipitate was then solubilized and loaded on a gel filtration column (Superose 12HR 10/30) in fast protein liquid chromatography system. Using this technique, different oligomeric forms of α-conglutin and β-conglutin were isolated. However, the complete separation of all lupin conglutins into individual fractions by gel filtration has not been reported yet. This molecular size separation technique is likely to be less effective (Derbyshire et al., 1976) in purifying γ-conglutin from other conglutin fractions because of the similar range molecular sized oligomers of α-conglutin and β-conglutin with those of γ-conglutin (Melo et al., 1994). Therefore, some other separation methods, for example, based on charge differences, would be more useful and one approach that can do this is ion exchange chromatography.

Anion exchange chromatography

Ion exchange chromatography separates molecules based on the surface charge present on them and their interactions with oppositely charged ion exchange adsorbent (Biosciences, 2002). Positively charged molecules bind to negatively charged ion exchange adsorbents (cation exchange), whereas negatively charged molecules bind to positively charged ion exchange adsorbent (anion exchange). Ion exchange

chromatography has provided better separation of legume seed proteins due to its high specificity (Derbyshire et al., 1976). Ion exchange chromatography can be operated in two ways, positive mode (target molecule will bind to the adsorbent) and negative mode (target molecule will not bind to the adsorbent). Most of the reported chromatographic purification processes for lupin proteins have used anion exchange chromatography (Duranti et al., 1981; Melo et al., 1994). For instance, γ-conglutin has been purified in the negative separation mode at neutral pH, under which condition it will not be captured on the column and elute rapidly.

The use of weak anion exchanger, Whatman DE 52 DEAE-cellulose to achieve the separation of γ-conglutin from the major α-conglutin and β-conglutin fractions was described (Duranti et al., 1981). Lupin proteins were extracted at neutral to slightly alkaline pH values in a buffer containing sodium chloride, followed by desalting of the extract and then loading on an anion exchange DEAE-cellulose column at pH 7.5. At this pH, γ-conglutin will have positive surface charges due to its basic pI, whereas α-conglutin and β-conglutin will have negative surface charges because of their acidic pI. Therefore, under the process conditions of pH 7.5, positively charged γ-conglutin did not bind to positively charged adsorbent (anion exchange) and eluted rapidly with less molarity of sodium chloride. However, negatively charged α-conglutin and β-conglutin were captured by anion exchange adsorbent and were eluted later with increased molarity of sodium chloride. Similar methodology has also been reported using strong anion exchange adsorbent, Mono Q (Melo et al., 1994).

From the reports in the literature, anion exchange chromatography has shown a purer form of γ-conglutin as compared to the previously reported methods (Blagrove & Gillespie, 1975; D'Agostina et al., 2006; Sironi et al., 2005). However, γ-conglutin fraction obtained from the anion exchange studies still demonstrated contaminating proteins as analyzed by SDS-PAGE, especially if albumin removal is not achieved prior to globulin extraction (Duranti, personal communication). Also, other non-protein contaminants from the alkaline extract might co-elute along with γ-conglutin fraction due to their non-specific interactions with the adsorbent. To prevent this contamination of γ-conglutin fraction with other proteins, selective extraction of γ-conglutin instead of alkaline extraction might be viable.

There have been attempts to increase the purity of γ-conglutin based on its affinity towards metal ions, which will be described in the further section.

Metal affinity chromatography

Metal affinity chromatography is based on the coordinate covalent interaction between amino acid residues (particularly histidine) and metal ions (such as zinc) (Hunt, Ahmed, & Fierke, 1999). In solution, this specific interaction may lead to aggregation (metal-induced aggregation) and precipitation of proteins (Hughes & Klotz, 1956). This approach has been used (Duranti et al., 2001) to study the effect of various divalent and trivalent metal ions on aggregation of purified γ-conglutin obtained by anion exchange chromatography. Of the various metal ions studied in the form of chloride salt, Zn^{2+} showed most striking effects with almost complete aggregation of γ-conglutin. The loss of solubility of γ-conglutin was observed to be strictly dependent on the concentration of metal ion and pH, with maximum effects at three-fold molar excess of the protein and pH of 6.5 respectively (Duranti et al., 2001). This study also reported complete reversibility of the aggregation process, since 90% resolubilization of precipitated γ-conglutin occurred in the presence of a metal chelating agent such as Ethylenediaminetetraacetic acid (EDTA), implicating no denaturation of γ-conglutin (Duranti et al., 2001). The occurrence of specific interaction between metal ion and γ-conglutin was further studied by metal affinity chromatography by coupling various divalent metal ions on a stationary phase in a column (Duranti et al., 2001).

In the same study, similar results were reported for metal affinity chromatography (Duranti et al., 2001) where a Zn^{2+} coupled matrix showed the selective quantitative capture of γ-conglutin. The binding of γ-conglutin to Zn^{2+} coupled matrix was performed at neutral pH, while desorption from the matrix was carried out at acidic pH in the presence of EDTA and imidazole. This transition of γ-conglutin was observed to take place around pH 6 which corresponds to pKa of histidine side chains, confirming that the interaction between Zn^{2+} ions and γ-conglutin could have involved histidine side chains of γ-conglutin (Duranti et al., 2001).

This metal (Zn^{2+}) affinity approach resulted in a purer γ-conglutin fraction compared to that obtained from other previous methods. However, to use γ-conglutin for human consumption, complete removal of Zn^{2+} ions from the final preparation is essential (Cheung et al., 2012) due to the toxic nature of Zn^{2+} ions. Also, the chelating agents

used such as EDTA can cause leaching of metal ions from the chromatography resins, which can further contaminate the γ-conglutin fraction. Some amino acids, especially histidine residues are susceptible to metal catalyzed oxidation reaction resulting in free radicle formation which can cause site-specific cleavage to a protein that might alter its potential bioactivity (Gaberc-Porekar & Menart, 2001). Therefore, despite showing promise in the purification of γ-conglutin, metal affinity chromatography would not be recommended for nutraceutical and biopharmaceutical applications.

To date, the combination of chromatography processes appears to be the most effective process to purify γ-conglutin. However, these multistep processes likely have limited potential for cost-effective production of γ-conglutin on an industrial scale. The summary of these purification methods and barriers to their scale up are described in Table 2.2. Thus, it can be said that this traditional approach for extraction and purification of legume proteins is not effective for γ-conglutin, which provokes the development of an industrially viable purification process for producing nutraceutical and pharmaceutical grade γ-conglutin. The following section describes the strategies for protein purification in general, which can be implemented to design a novel process for purification of γ-conglutin.

2.3 STRATEGIES FOR PROTEIN PURIFICATION

The purification of proteins is technically challenging and remains a bottleneck in the pathway from basic research to production, especially when the purified protein has pharmaceutical applications. The complexity of the process increases when purifying the low abundant protein from its original source, necessitating handling of large volumes which can create additional problems associated with fouling of the equipment (Amersham, 2004). The bioseparation of protein is often regarded as the critical factor in the successful commercialization of protein based products. Therefore, the logical selection of purification process needs to be done in order to overcome all the hurdled posed by the nature of the protein and requirement of desired purity.

Table 2.2 Summary of methods used for γ-conglutin extraction and purification

Purification method used	Type of method	Rationale of protein purification	Limitation of the method
Extraction	Conventional alkaline extraction and isoelectric precipitation	Major conglutins are solubilised at alkaline pH due to their acidic pI, which is then recovered from the extract by acidic isoelectric precipitation.	• Mainly targeted for extracting total conglutins, non-selective for γ-conglutin purification. • Alkaline conditions can also extract low molecular weight pigments, which require extra process steps for their removal (Derbyshire et al., 1976). • Alkaline solubilisation can cause irreversible structural damage to the proteins (Muranyi et al., 2013). • Loss of essential amino acids can be triggered by alkaline pH (Rodrigues, Coelho, & Carvalho, 2012).
	Salt extraction/ ammonium sulphate precipitation	Lupin conglutins are salt-soluble globulins, Nonetheless, the addition of salts above a threshold concentration provokes aggregation and precipitation of proteins.	• Salt-assisted extraction targets total conglutin extraction and are non-selective for γ-conglutin. • Use of excessive salts can denature the protein, which can reduce its nutritional quality and potential bioactivity. Therefore, removal of salts by dialysis or by using desalting columns is essential, which will increase the cost of the process.
Extraction and membrane filtration	Conventional extraction and ultrafiltration	Removal of non-protein contaminants from the purified fraction.	• Ultrafiltration can be an intermediate process to remove low molecular weight non-protein contaminants/salts, however, is not suitably selective as a final purification step for γ-conglutin.
Chromatographic method	Gel filtration	Fractionation of conglutins and their oligomers based on molecular size	• It is not an effective separation tool for lupin conglutins because different conglutin fractions have similar molecular size.
	Anion exchange	Fractionation of lupin conglutins based on surface charge on protein driven by their isoelectric region	• Anion exchange chromatography is selective in the negative mode of separation for γ-conglutin purification. However, some non-protein contaminants may also co-elute with a γ-conglutin fraction. • The purity of γ-conglutin is not nutraceutical grade when using anion exchange chromatography.
	Metal affinity	Separation based on affinity of proteins for metal ions	• Repeated runs in metal affinity chromatography column can lead to leaching of metal ions in protein solution, which can damage protein structure and function, and also can give potentially toxic contamination.

Table 2.3 Separation processes for protein purification

Protein property	**Technique**
Size	Membrane separation, filtration, centrifugation
Density	Centrifugation, sedimentation
Shape	Centrifugation, sedimentation, filtration
Diffusivity	Membrane separation
Solubility	Extraction, precipitation, crystallization
Charge	Ion exchange chromatography
Hydrophobicity	Hydrophobic interaction chromatography
	Reverse phase chromatography
Specific interaction with ligand	Affinity chromatography
Metal ion binding	Metal affinity chromatography
Mixed interactions	Multimodal chromatography
pH gradient	Chromatofocussing
Net charge and size	Electrophoresis

The selection of purification process for proteins depends on its physico-chemical properties such as density, distribution coefficient, molecular weight, charge distribution, electrophoretic mobility and hydrophobicity (Roe, 2001) as described in Table 2.3. The choice of separation technology is a function of protein stability, its application, recovery and economics. Based on selectivity and productivity these techniques can be categorized as low resolution-high throughput techniques and high resolution low-throughput techniques (Ghosh, 2006). Bioprocess aims at high resolution (or selectivity) and high throughput (or productivity) and in overall process multiple techniques are sequenced/integrated to obtain high-resolution and high throughput. Designing of high resolution-high throughput techniques often requires a balance between purity, productivity and process economics.

Amongst various unit operations, chromatography is the only unit operation which gives high purity standards dictated by the regulatory authorities for commercial bioproducts (Sofer, 1995). Chromatography is a high-resolution separation technique often employed as packed-bed chromatography and is almost a ubiquitously found in most biomolecules separation processes. In-fact chromatographic techniques are of singular importance and unassailable especially for therapeutic protein purification

where purity is strictly monitored. Chromatography is probably the most versatile, powerful and scalable technique, where the mixture of components can be separated into individual ones in a single step process. The rapid growth in biotechnology had provided additional impetus to establish rational design and scale-up criteria in chromatographic techniques. The importance of preparative chromatography for the large scale purification of high-value proteins has been increasingly recognized over the past decade (Jagschies, Sofer, & Hagel, 2007; Sofer, 1995).

Preparative chromatography aims at maximum throughput and recovery of purified product. As interest expands in the application of preparative and process chromatography from high-value low volume therapeutic products towards the low-value high volume quantity food grade products, the material requirements, the selectivity of separation, recovery of active product and overall process economy becomes an important and essential constraint for large scale manufacturing. Hence there is need of alternate bioprocess development technologies for handling such requirements. Since the stationary phase or adsorbent is the heart of separation in chromatography, the objective of alternative bioprocess development is achieved by (a) selectivity designing or selectivity engineering, i.e. the adsorbent under conditions employed, should show selectivity for target compound and (b) Process engineering i.e the method should be selective and scalable to production level.

In addition to selecting suitable technique, following basic guidelines needs to be considered for protein isolation and purification (Amersham, 2004).

- The objective with respect to purity, quantity and activity of final product should be defined.
- The physico-chemical properties of the target protein and expected impurities should be studied in order to simplify technique selection and its optimization.
- Analytical methods for detection of target protein and impurities should be identified/developed.
- Sample handling at every stage should be minimized to avoid lengthy procedures which might risk in losing activity or reducing recovery.
- The use of additives such as salts or solvents should be minimized as they might interfere in purification process and their removal might add extra purification step in the process.

- The number of process steps should be kept as minimum as possible in order to increase process yield and reduce process time.
- Final scale of purification required should be defined.
- Effect of selected technique on process economics should be studied.

Considering all the above requirements for process design, the following processing paradigm was designed to develop the novel process for purification of γ-conglutin.

2.4 PROCESSING PARADIGM FOR PURIFICATION OF γ-CONGLUTIN

In order to develop a novel and industrially viable process for purification of γ-conglutin with minimum possible process steps, a hybrid technique that harnesses a logical combination of optimization algorithms and high throughput experimentation for designing cost-effective process for desired purification (Nfor et al., 2008), will be implemented. For this purpose, a systematic approach based on hybrid technique will be designed by integrating the process for selective extraction and adsorptive chromatography for the capture of γ-conglutin (Figure 2.3).

While previous studies have identified key parameters that govern the extraction process, how they affect γ-conglutin purity has not been carefully investigated. Therefore, the study of key parameters that govern the selective extraction of γ-conglutin and how they affect the purity of γ-conglutin, will be carried out, in order to design selective extraction of γ-conglutin from lupin seeds. The design of experiment (DoE) approach will be used to investigate the interactions between the parameters and to find the optimal conditions for maximizing the product purity at extraction stage. The optimized extraction process will be simultaneously guided by characterizing extracted proteins using analytical techniques so as to reveal how processing conditions affect the purity and secondary structure of extracted γ-conglutin fraction.

Low concentration of γ-conglutin in the lupin seed requires the purification process to be more selective for its capture and to provide high throughput with least possible process steps. Adsorptive chromatographic processes are promising in this regard as they are able to handle the demands of selectivity and high throughputs during the process (Janson, 2012). At present, two-stage chromatographic process (anion exchange followed by cation exchange) is traditionally employed for purification of γ-

conglutin from the extract. The number of chromatographic operations in the developed process will be reduced by understanding the interaction of γ-conglutin with various adsorbents under different process conditions. For this, different chromatographic adsorbents will be screened for their selective binding capacity by batch uptake experiments and adsorption isotherm analysis. Adequate attention to scalability and viability will be given while selecting the adsorbent in order to make the process cost-effective. For effective scale up, the parameters affecting chromatographic purification process will be investigated and optimized. Like extraction, γ-conglutin quality in terms of its purity, structure, and bioactivity at each process stage will be continuously monitored during process development stage to ascertain the integrity of its structure and the bioactivity.

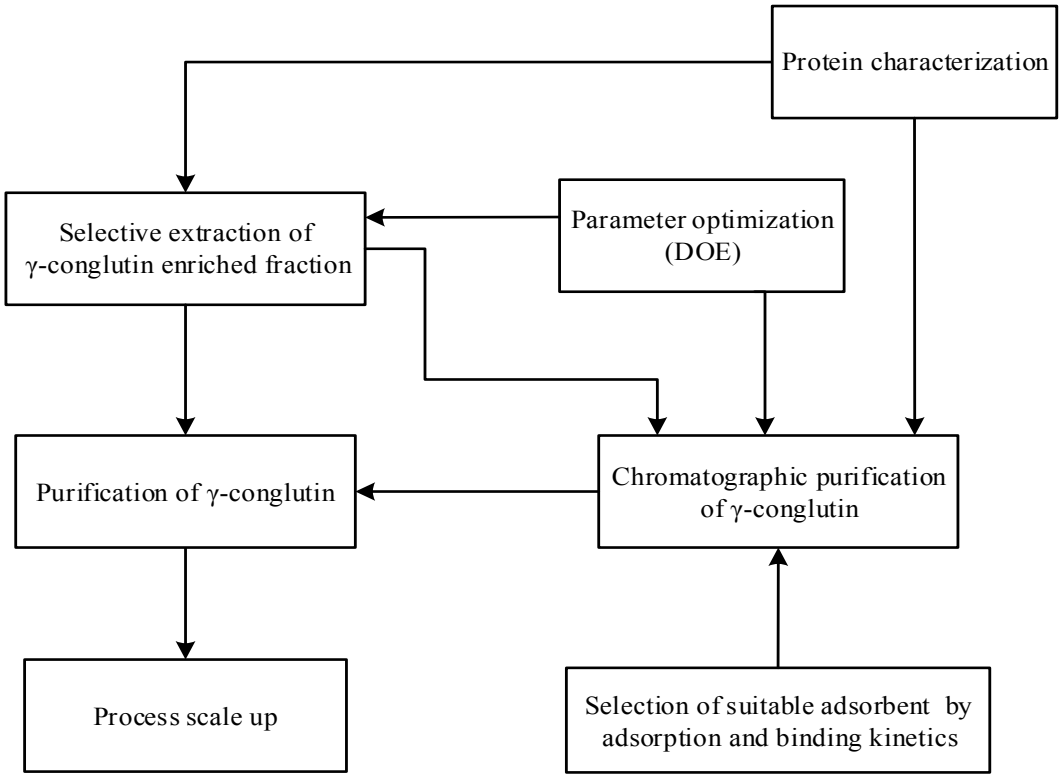

Figure 2.3 Research process paradigm for purification of γ-conglutin

The success of developing a scalable purification process for γ-conglutin ultimately depends on the optimal selection of appropriate extraction conditions and selective adsorptive capture. The major obstacles in the scale up such as increased back pressure, loss of product and lack of reproducibility will be addressed by carefully

selecting high throughput chromatographic adsorbent and optimizing process parameters.

2.5 SELECTION OF LUPIN VARIETY FOR THIS RESEARCH WORK

Six commercially grown varieties of Australian sweet lupin (*Lupinus angustifolius*) seeds namely, Belara, Coromup, Gungurru, Jenabillup, Mandelup and Tanjil were kindly provided by Department of Agriculture and Food Western Australia (DAFWA) for this research work. As discussed in the section 2.2.1, these seeds were dehulled and fractionated into respective hulls and kernels. Preliminary analysis on the protein content of kernels was performed, so as to select a variety for further studies. The protein content of Coromup variety was found to be maximum (~43% w/w of kernel flour), which was selected for further extraction and purification process. The same Coromup kernel flour is reported to have the presence of 39.3% dietary fibre, 7.8% fat, 7.4% moisture and 2.9% ash (Villarino, 2014).

2.6 CONCLUSION

Lupin seed protein, γ-conglutin has a great potential to be used as 'glucose controlling bioactive' in nutraceutical and biopharmaceutical applications. However, the commercial production of γ-conglutin is still a long way due to cumbersome processing steps in reported methods. This provokes clear understanding of purification processes available for γ-conglutin, their scalability and purity as demanded by nutraceutical and pharmaceutical applications. This Chapter forms a basis for understanding processing aspects of the reported methods for purification of γ-conglutin. It underlines a new paradigm for designing and developing industrially viable purification process for γ-conglutin. Any such development should be guided by continuous monitoring of bioactivity of γ-conglutin during various processing steps. Such an approach will enable researchers and industry to come up with a cost-effective process which is vital for the manufacture of pure γ-conglutin for its potent applications.

Chapter 3

Extraction optimization of lupin conglutins

Information in this Chapter has been published as follows:

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3.1 INTRODUCTION

As discussed in Chapter 2, the major proteins of the lupin seed (α and β-conglutin) have potential applications in the food industry on account of their oil binding and emulsification properties. Therefore, most of the reported work to date on lupin proteins (Blagrove & Gillespie, 1975; Duranti, Consonni, Magni, Sessa, & Scarafoni, 2008; Duranti, Restani, Poniatowska, & Cerletti, 1981; Melo, Ferreira, & Teixeira, 1994) is based on extraction of total proteins by conventional alkaline extraction followed by acidic precipitation at isoelectric point, yielding these major conglutins. Despite this, there are very few reports on extraction studies of these conglutins from lupin seed (Ruiz & Hove, 1976). This Chapter focuses on the statistical optimization of conglutin extraction from *Lupinus angustifolius* seeds (Coromup variety).

Many factors such as pH of extraction, the volume of solvent, extraction time, temperature, and a number of stages, may affect the extraction process significantly. Up till now, one factor at a time approach has been reported to study lupin conglutin extraction process (Ruiz & Hove, 1976). However, this approach does not consider the interaction between parameters which directly affects the response. The optimization of any process, in general, is achieved by empirical or statistical methods, of which statistical method leads to complete optimization (Liyana-Pathirana & Shahidi, 2005). Unlike conventional optimization, statistical optimization allows consideration of the interaction between the parameters and its effect on the response.

Response surface methodology (RSM), which has been successfully utilised for developing and optimising processes (Myers, Montgomery, Vining, Borror, & Kowalski, 2004), allows the study of effects of various process variables and their

interactions with the response. This methodology has been successfully used for biochemical and biotechnological processes (Liyana-Pathirana & Shahidi, 2005). Therefore, RSM was applied to study the effect of key parameters for extraction, their interaction and their effect on conglutin extraction (% w/w). Further, the conditions for extractions were optimized to obtain a response with maximum desirability.

Different extraction ways such as magnetic stirring, ultrasonication and hydrodynamic cavitation-assisted extraction were investigated in order to increase conglutin extraction efficiency. Ultrasonication and hydrodynamic cavitation-assisted extraction are often used to increase the extraction rate by increasing partitioning between the solute and solvent (Waksmundzka-Hajnos, Petruczynik, Dragan, Wianowska, & Dawidowicz, 2004). Therefore, the effect of extraction techniques on conglutin extraction was studied and optimized using RSM. The conglutins extracted with RSM optimized conditions by three different processes of extraction were characterised by FTIR spectrometry to identify any structural changes to the proteins and were profiled with SDS-PAGE. This work provided the valuable new data on conglutin extraction optimization to assist in future industrial scale process design.

3.2 MATERIALS

Mature seeds of *Lupinus angustifolius* (Coromup variety) were provided by DAFWA. All the chemicals and reagents purchased were of analytical grades. n-Hexane, hydrochloric acid and sodium hydroxide were used for the extraction process. Bovine serum albumin (BSA) was used as standard protein for analysis of protein content. Ethanol, methanol, sodium carbonate, silver nitrate, sodium thiosulphate and acetic acid were used for silver staining process. All the chemicals were purchased from Sigma-Aldrich Pty Ltd (Perth, Western Australia). Bicinchoninic acid (BCA) protein assay reagent kit was obtained from ThermoFisher Scientific (Perth, Western Australia). Aqueous solutions were made using ultrapure water (Ariumpro ultrapure water system, Sartorius Australia Pty Ltd, Perth, Western Australia).

3.3 EXPERIMENTAL METHODS

3.3.1 Preparation of samples

Lupin seeds were dehulled (LH 5095 dehuller, DAFWA) followed by air induced and manual separation of kernels from the seed coat (hull). The resulting lupin kernels were milled (Retsch ZM200 mill, DAFWA) and sieved through 300 µm sieve (Labtech Essa, Perth, Western Australia), followed by vacuum packing in the plastic bags and storage at 4ºC until use.

Lupinus angustifolius kernels (Coromup variety) have been reported to contain ~7-8% w/w of oil components (Villarino, 2014). Defatting of kernel flour has therefore been recommended as a pre-treatment step (Derbyshire, Wright, & Boulter, 1976) for removing oil and pigmented materials from flour which may otherwise interfere in the protein isolation process. Therefore, the milled lupin kernel flour was defatted three times with n-hexane (1:3 w/v) at room temperature for 1 hour under constant shaking conditions of 300 rpm, followed by centrifugation (Heraeus Multifuge 1S, ThermoFisher Scientific, Perth, Western Australia) at 4700 rpm for 10 min to separate n-hexane containing oil fraction and defatted kernel flour residue. The obtained residue was then dried at room temperature and the resulting defatted lupin kernel flour was used as starting material for extraction optimization studies

n-Hexane fraction (generated during defatting process) containing oil extracted from lupin kernel was then evaporated using vacuum distillation unit (Labrota 4002 control, Heidolph Instruments, John Morris Scientific Pty Ltd, Perth, Western Australia) at 60^{o}C to separate n-hexane from oil fraction. The remaining oil fraction was weighed accurately and stored at -20ºC until further analysis.

3.3.2 Identifying key parameters of extraction by one factor at a time approach

It is necessary to design an efficient extraction process for major conglutins (α and β-conglutin), so as to have advantage of their specified emulsification property in food industries (Kohajdova, Karovicova, & Schmidt, 2011). Lupin major proteins are alkali soluble proteins (Casey, 1999) and thus are commonly extracted in the alkaline conditions. There have been many reports stating use of different extracting media for lupin conglutins such as salt-assisted extraction (Blagrove & Gillespie, 1975), aqueous extraction at neutral pH (Aguilera, Gerngross, & Lusas, 1983), alkaline extraction

(Ruiz & Hove, 1976), alcoholic extraction (Blaicher, Nolte, & Mukherjee, 1981). Many authors have reported increased conglutin extraction at alkaline pH and hence it is a conventionally used method of extraction. Along with the alkaline pH, various process operating parameters such as solid to solvent ratio, extraction time and temperature can significantly contribute in increasing the solubility of conglutins in the extraction media. Therefore, the key parameters for conglutin extraction were investigated by one factor at a time (OFAT) approach, as described in the following section. These key parameters were then statistically optimized using Design of Experiment (DoE) approach as described in the section 3.3.5.

Effect of extraction process parameters

The effect of process parameters on conglutin extraction was studied by extracting defatted flour (2 gm) with ultrapure water at different operating conditions. Initial experiments of conglutin extractions by OFAT were performed using magnetic stirring. The obtained extract was centrifuged at 4700 rpm for 10 min and filtered through Polyvinylidene difluoride (PVDF) syringe filters (0.45 μm). All the extraction experiments were repeated in duplicate. The protein content of the residues and extracts were analysed by an elemental analyser (as described in the section 3.4.1), whereas extraction yield was calculated on a dry weight basis of the residue obtained after extraction. The amount of protein in the residue and starting material (defatted kernel flour) was determined by multiplying the yield obtained with the respective protein content. Conglutin extraction (% w/w) was then calculated by subtracting the amount of protein in the residue from the amount of protein in the defatted flour.

pH of extraction medium

The effect of pH of extraction medium (solvent) on conglutin extraction (% w/w) was studied by performing extractions with ultrapure water at different pH conditions varying from 2 to 12. Acidic pH was maintained by slowly adding 1N hydrochloric acid while basic side pH was maintained with 1N sodium hydroxide. All other extraction conditions were kept constant as described above.

Solid to solvent ratio

The volume of water required for effective extraction of conglutins was studied by extracting the defatted flour with different volumes of ultrapure water ranging from 10 ml/gm to 70 ml/gm at optimized pH keeping all other parameters constant.

Extraction time

The time required for maximum extraction of conglutins was obtained by extracting the flour with optimized pH and solid to solvent ratio, at different time intervals ranging from 5 min to 60 min. Sampling was done at an interval of every 5 min to study reaction kinetics.

Extraction temperature

The temperature for effective extraction of conglutins was optimized by carrying out extraction with all optimized conditions at different temperatures in a range suitable for protein extraction (10ºC to 60ºC).

Number of extraction stages

The optimization of a number of extraction stages required for complete extraction of conglutin using optimized pH, solid to solvent ratio, time and temperature was carried out up to four extraction cycles. Extractions were carried out as described above while replacing the old extractant with a fresh one every time.

3.3.3 Extraction kinetics

Extraction of conglutins was performed at optimized conditions as mentioned above and samples were withdrawn at every 5 minutes interval for analysis. The kinetics of conglutin extraction was studied using Peleg's model, as it has been extensively used to explain the extraction of biological materials from plant sources (Planinic, Velic, Tomas, Bilic, & Bucic, 2005). The Peleg's semi-empirical kinetic model equation for extraction can be expressed (Karacabey, Bayindirli, Artik, & Mazza, 2013) as given in an equation (3.1),

$$C_t = C_0 + \frac{t}{K_1 \times + K_2 \times t} \tag{3.1}$$

Where, C_t is concentration of target solute (conglutin) in extract at extraction time 't' (mg/ml), K_1 is Peleg's rate constant (min ml/mg) and K_2 is Peleg's capacity constant

(ml/mg), C_0 is concentration of solute in the extract at the very beginning, which is 0 mg/ml. The kinetic parameters (K_1 and K_2) can be determined experimentally by plotting t/C_t against t.

3.3.4 Extraction methods

Once the key parameters were identified, statistical optimization of extraction was performed by three different processes namely magnetic stirring, ultrasonication and hydrodynamic cavitation to study the effect of extraction process on extraction yield and efficiency.

Magnetic stirring experiments were carried out using a magnetic stirrer (IKA RCT basic stirrer, Sigma-Aldrich, Perth, Western Australia) and a magnetic bead at fixed rpm of 300 at room temperature.

Ultrasound-assisted extraction was carried out using sonication probe (Standard probe, Autotune Series, John Morris Scientific Pty Ltd, Perth, Western Australia) with fixed frequency of 20 KHz operated at a power of 250 W. Extraction was carried out with pulsed sonication of 55/5 (55 sec sonication and 5 sec rest).

Cavitation-assisted extraction was carried out using cavitation device and dosing pump (ProMinent and Fluids Control Pty Ltd, Sydney, Australia) with regular cleaning of the device after each experiment. In typical cavitation-assisted extraction process, the cavitation device is attached to the dosing pump to pump the slurry through the device and the process is kept continuous until the desired extraction time is reached. The flow rate to pump the slurry was maintained at 120 ml/min with 20 strokes/min.

3.3.5 Statistical optimization of conglutin extraction

Statistical optimization of conglutin extraction was performed by RSM as described further in this section.

Experimental design

Statistical optimization of conglutin extraction from the defatted flour was carried out using response surface methodology central composite design (RSM CCD). An axial value of 2 was used and the design consisted of seventeen experiments with three replicates at the centre point. All the seventeen experiments were repeated in two sets. From one factor at a time study, the design variables which were selected for

optimization were pH of extraction medium, solid to solvent ratio and extraction time while response variable was % w/w conglutin extraction (amount of conglutin extracted from total conglutins).

Data analysis and verification of model

The experimental results were analysed using statistical software JMP 11 (SAS Institute Inc., USA). Regression analysis procedure was used to fit the experimental data to the quadratic model to determine regression coefficients. The obtained response data was also studied using response surface plots and contour plots. Prediction profilers were used to obtain optimized values with maximum desirability. The experimental and predicted values were compared to determine the validity of the model.

3.4 ANALYTICAL METHODS

3.4.1 Proximate component analysis

Proximate composition analysis of lupin seed flour and fractions generated during the process was carried out using the protocol mentioned below. All analyses were conducted in at least duplicates and expressed in terms of % dry weight basis unless otherwise stated.

Quantification of total moisture content

Moisture was determined by oven drying in accordance with AOAC method, 925.10 (Horwitz, 2002). 2 gm (W_1) of the sample was kept in an oven at 105ºC for 3 hr with continuous monitoring of weight every 15 min until stable weight (W_2) was obtained. Moisture was calculated as represented by an equation (3.2),

$$\%\, Moisture\ = \frac{(W_1 - W_2)\times 100}{W_1} \tag{3.2}$$

Quantification of total protein content by CHN analyser

The total protein content in whole seed flour, kernel flour and fractions generated during the process was determined by multiplying the nitrogen content of sample with the factor of 6.25 (Duranti et al., 1981). The nitrogen content of the respective samples was determined by the elemental analyser in CHN mode (Perkin Elmer Series II analyser 2400).

In CHN operating mode, Perkin Elmer Series II analyser uses a combustion method to convert the sample elements to simple gases (CO_2, H_2O and N_2). The sample is first oxidized in a pure oxygen environment using classical reagents. Elements such as halogen and sulphur are removed by scrubbing reagents in the combustion zone. The resulting gases are homogenized and controlled to exact conditions of pressure, temperature and volume. The homogenized gases are allowed to de-pressurize through a column where they are separated in a stepwise steady-state manner and detected as a function of their thermal conductivities.

CHN operating mode procedure include selecting CHN mode, weighing calibration standards and samples, running blanks, calibrating the analyser, and running samples. The samples were freeze-dried, crushed and homogenized using mortar and pestle, and weighed as per the procedures mentioned in the manual. The samples were weighed using a Perkin Elmer AD-4 Ultramicrobalance and the sample size varied from 1.5-2.5 mg as recommended for CHN analysis. The instrument was calibrated as mentioned in the manual prior running the samples for CHN analysis.

3.4.2 Measurement of protein concentration by BCA analysis

The protein concentration in the sample was determined by bicinchoninic acid (BCA) assay by using Pierce BCA protein assay kit (Scientific, 2013) (ThermoFisher Scientific, Perth, Western Australia) at 562 nm using bovine serum albumin as the standard protein.

3.4.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of extracted conglutins under non-reducing and reducing conditions was performed according to Laemmli (Laemmli 1970) using 12% precast polyacrylamide gel (Bio-Rad Laboratories Pty Ltd, Perth, Western Australia). Conglutin extracts were diluted with Laemmli sample buffer (Bio-Rad Laboratories Pty Ltd, Perth, Western Australia) for non-reducing conditions, whereas reducing samples were prepared by adding reducing buffer (β-mercaptoethanol added to the Lamemeli sample buffer) followed by heating the resulting sample at 95ºC for 10 min. Electrophoresis was performed with TGX (tris-glycine) running buffer using Mini-PROTEAN Tetra Cell (Bio-Rad Laboratories Pty Ltd, Perth, Western Australia) at 150 mV for 1 hr.

Proteins were fixed and gels were stained using a silver staining protocol. The molecular weights of major peptides in the samples were estimated by comparing their migration with broad range molecular weight SDS-PAGE markers (Bio-Rad Laboratories Pty Ltd, Perth, Western Australia).

3.4.4 Fourier transform infrared (FTIR) spectrometry

FTIR spectra were recorded using an attenuated total reflectance (ATR)-FTIR spectrometer (Perkin Elmer Spectrum 100 FTIR spectrometer) at room temperature. All spectra were recorded over accumulative 32 scans with a resolution of 4 cm^{-1} in the range of 4000-400 cm^{-1}. Spectra were scanned against a background spectrum recorded from the clean empty cell at room temperature. The analysis of spectra was performed using 'Spectrum' software associated with the instrument.

3.5 RESULTS AND DISCUSSION

The majority of the proteins of lupin seeds are concentrated in the kernels and therefore preliminary fractionation of lupin seed into protein rich kernels and fibre rich hulls was performed to enable the possible use of each fraction of the lupin seed (Rowe & Hargreave, 1988). Dehulling before protein extraction also reduces the risk of phytochemical extraction from hulls, which otherwise could contaminate the purity of extracted protein fraction (Derbyshire et al., 1976). *Lupinus angustifolius* seeds (Coromup variety) were dehulled as described in the section 3.3.1, which resulted in 25% (w/w) of hulls and 75% (w/w) of kernels. The results of dehulling are in agreement with the study of Glencross (Glencross, 2008) which reported approximately 24% (w/w) hulls being present in mature *Lupinus angustifolius* seed. The isolated kernels were then milled to the desired particle size. Ruiz and Hove (Ruiz & Hove, 1976) reported a significant increase in lupin protein extraction at smaller particle size with a maximum extraction at 300 μm, therefore the kernel flour was passed through 300 μm sieve prior to extraction. The milled kernel flour was then defatted, which showed 7.3% w/w oil extracted from lupin kernel flour. The extracted lupin kernel oil was then stored at -20ºC until further analysis.

Defatted kernel flour was then used for designing an efficient extraction process for conglutin. The following section describes the effect of process parameters on conglutin extraction yield.

3.5.1 Selection of key parameters of conglutin extraction

pH of extraction medium

The study of the effect of pH on the extraction of proteins gives the solubility curve for proteins which decides the first step of protein purification process. Lupin major conglutins have an acidic pI in the range of pH 4-5 (Restani, Duranti, Cerletti, & Simonetti, 1981) and proteins are reported to show minimum solubility at the pH of their isoelectric region (Widmann, Trodler, & Pleiss, 2010). Therefore, the solubility of lupin major conglutins is minimum in their isoelectric region (pH 4-5), whereas pH away from the isoelectric region in both acidic and basic side show considerable extractability, with maximum at alkaline pH of 9 and above (Lqari, Vioque, Pedroche, & Millan, 2002; Ruiz & Hove, 1976).

The experiments to study the effect of pH on lupin protein solubility were performed as described in the section 3.3.2. The resulting solubility curve for lupin conglutins as shown in Figure 3.1, is a 'U' shaped curve over a broad range of pH. As seen from Figure 3.1, acidic pH of 2 exhibited ~70% w/w conglutin extraction, which was found to decrease as pH was shifted from from the value of 3 to 5. Less than 20% w/w conglutins were extracted at acidic pH range of 4-5, whereas basic pH of 9 and above showed more than 80% w/w conglutin extraction, with maximum extractability (90% w/w) at pH 12 (Figure 3.1). Similar results of conglutin solubility have been reported by Ruiz and Hove (Ruiz & Hove, 1976), and Lqari et al (Lqari et al., 2002).

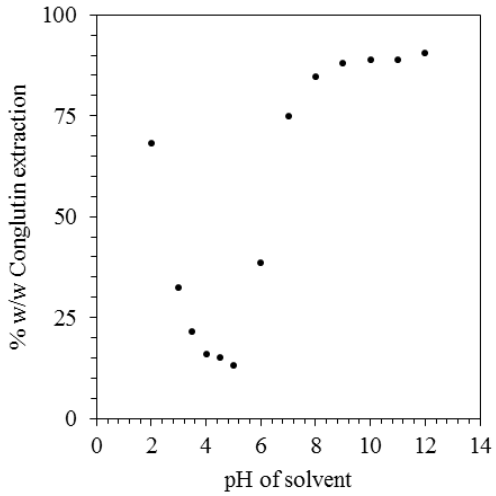

Figure 3.1 Solubility curve for lupin conglutins

Though % w/w conglutin extraction was observed to be maximum at pH 12, harsh alkaline conditions can lead to denaturation of proteins and formation of lower molecular weight peptides. Also, alkaline solubilization of proteins (pH ≥10) can cause irreversible structural damage to the proteins (Muranyi, Otto, Pickardt, Koehler, & Schweiggert-Weisz, 2013) and loss of essential amino acids (Rodrigues, Coelho, & Carvalho, 2012). Therefore, the suitable alkaline pH for maximum conglutin extraction with desired integrity of proteins was selected by studying the profile of lupin conglutins extracted at different pH values by SDS-PAGE (Figure 3.2).

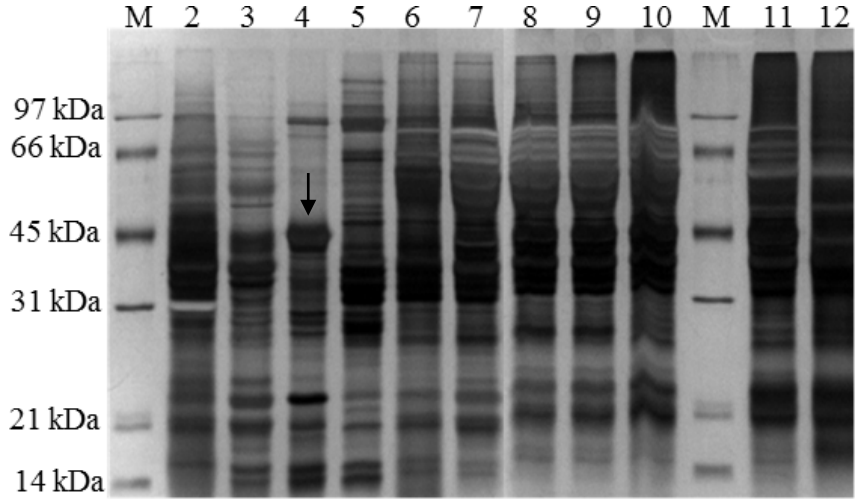

Figure 3.2 SDS-PAGE (non-reducing) of conglutins extracted at respective pH (M is molecular weight marker and number on the lane represents respective pH) (Arrow represents γ-conglutin at 48 kDa)

SDS-PAGE profile of lupin protein solubility study clearly indicated the increase in protein band intensity as pH shifts from their isoelectric region (Figure 3.2). At the isoelectric region of major conglutins (pH 4-5), the presence of 48 kDa was seen predominantly, representing γ-conglutin (Blagrove & Gillespie, 1975) as it is acid soluble protein and it has a unique pI in the basic range of pH 6.9-8.0 (Blagrove, Gillespie, Lilley, & Woods, 1980). As pH is shifted from neutral to the basic range and also at acidic pH of 2, the presence of intermediate and high molecular weight proteins ranging from 20 to 100 kDa was observed indicating the presence of major conglutins (α and β) (Melo et al., 1994). The profile of proteins extracted at pH 9 and above was observed to be same from SDS-PAGE analysis. Also, no significant difference in % w/w conglutin extraction was observed at pH 9 and pH 12. Therefore

pH 9 was selected as optimum pH (~89% w/w extractability) for conglutin extraction and further parameters were optimized as discussed in the following section.

Effect of solid to solvent ratio

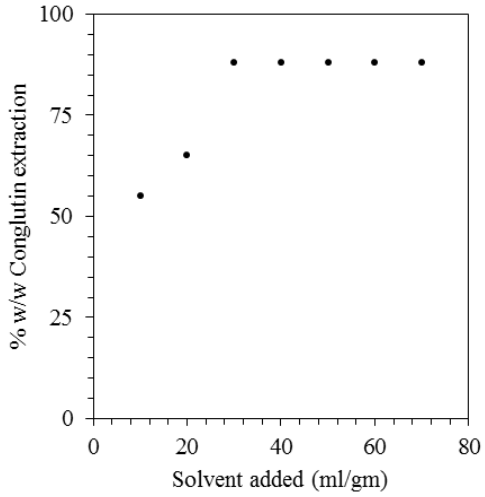

Figure 3.3 Effect of solid to solvent ratio on extraction of conglutins

The volume of water required for extraction depends on the solute concentration attaining equilibrium in the solvent. The effect of different volumes of water added per gm of defatted kernel flour on % w/w conglutin extraction is shown in Figure 3.3. By increasing the volume of water added from 10 ml/gm to 30 ml/gm, conglutin extraction (% w/w) was found to increase from ~50% to 89% due to good mass transfer favoured by solid and liquid phase concentration gradient, beyond 30 ml/gm no significant increase in conglutin extraction was observed. Therefore, 30 ml/gm was selected as the optimum volume of the solvent required for effective extraction of lupin conglutins, so as to avoid solvent usage and difficulties in handling larger solvent volumes.

The data obtained from the study of the effect of solid to solvent ratio on conglutin extraction is in contradiction with the study by Ruiz and Hove (Ruiz & Hove, 1976) which stated no significant change in conglutin extraction as the amount of solvent added was varied from 10 ml/gm to 40 ml/gm. One of the reasons behind this observation could be interference of pigmented components in protein extraction as the author (Ruiz & Hove, 1976) did not use defatted kernel flour sample.

Effect of extraction time

The effect of extraction time on conglutin extraction is shown in Figure 3.4. Approximately 1.5 times increase in % extractability was seen with the increase in extraction time from 5 min to 30 min. As seen from Figure 3.4, the beginning rate of extraction was very fast, followed by a slower rate for the remaining of the extraction period until it reached the plateau after ~30 minutes of extraction. At the end of 30 min, almost 89% w/w conglutins were extracted, beyond which no significant change in conglutin extraction was observed.

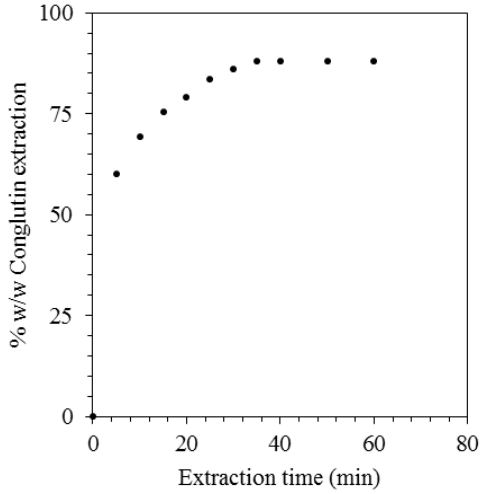

Figure 3.4 Effect of extraction time on extraction of conglutins

Due to the driving force of the fresh solvent, initially, the major part of the solute gets extracted quickly, and then the extraction process gets much slower due to the external diffusion of the remainder solute into the solution. This pattern of extraction rate is explained by the second order rate kinetics which was analysed by Peleg's model (Planinic et al., 2005). To determine rate constants K_1 and K_2 of the Peleg model, the experimental data was plotted as t/C_t against t (Figure 3.5). The value of regression coefficient (R^2=0.9953) implied good agreement of the experimental data of conglutin extraction with Peleg model. The value of Peleg's rate constant (K_1) was found to 0.17 mg/ml min, which indicates maximum extraction per minute. The lowest amount of conglutin extracted can be represented by Peleg's capacity constant (K_2) which was found to be 0.0682 mg/ml.

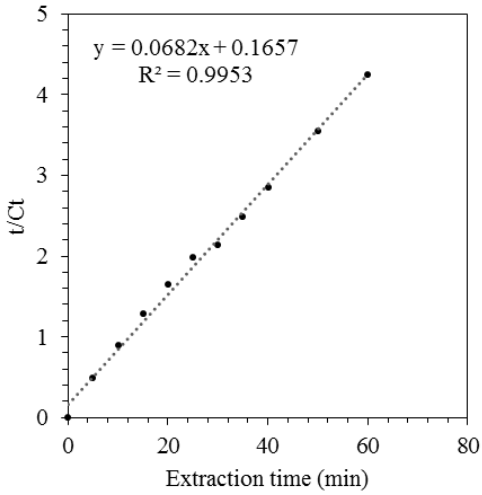

Figure 3.5 Second order kinetics of conglutin extraction

Effect of extraction stages

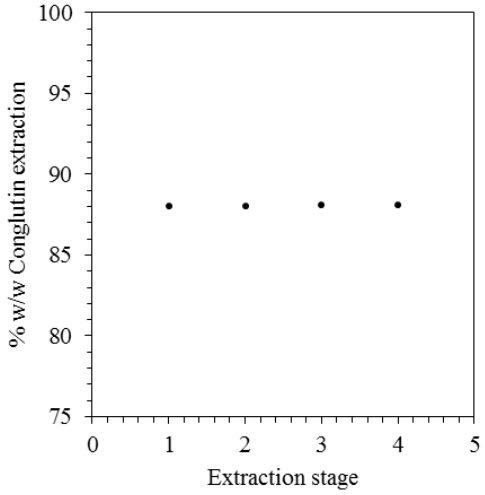

Figure 3.6 Effect of extraction stages on extraction of conglutins

The number of extraction stages required for complete extraction of lupin conglutins was determined by plotting extraction stages against conglutin extraction (% w/w). As seen from Figure 3.6, conglutin extraction (% w/w) was not significantly varied as the number of extraction stages was increased. In the very first stage itself, almost 89% w/w lupin conglutin extraction was achieved. Hence, extraction was optimized to its first stage only. Ruiz and Hove (Ruiz & Hove, 1976) have suggested the use of two-stage extraction as the amount of solvent used in that case was lower at 10 ml/gm for each stage.

Effect of extraction temperature

Lupin conglutins when extracted at different temperatures (Figure 3.7) showed no significant change in conglutin extraction as the temperature was increased from 10ºC to 60ºC. The results indicated that use of any temperature in the given range extracts the maximum amount of conglutins (89% w/w). Hence, room temperature (~25ºC) was selected for further extraction studies as it is beneficial for protein structural integrity (higher temperatures can cause structural damage to the protein) and for process cost (no need to maintain temperature during extraction process).

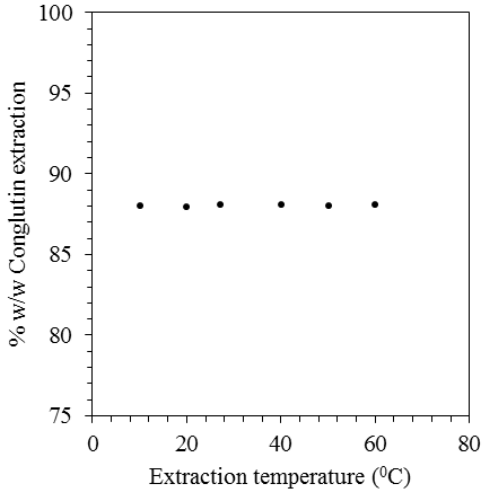

Figure 3.7 Effect of extraction temperature on extraction of conglutins

The results obtained with above extraction experiments indicated that pH of water (9), the volume of water added to the flour (30 ml/gm), extraction time (30 min) were key parameters for effective extraction of conglutins (~89% w/w), whereas extraction stages (one stage) and extraction temperature (room temperature) did not affect the conglutin extraction significantly.

3.5.2 Global optimization by response surface methodology (RSM)

Further enhancement of the extraction efficiency was achieved by optimizing key parameters by global optimization tools. Statistical designs such as RSM CCD are beneficial in process development as they can give optimized results in a limited number of experiments, thereby saving time and resources. They also help in understanding the interactions between key factors. The optimization was also performed for three different extraction processes (magnetic stirring, ultrasonication

and hydrodynamic cavitation-assisted extraction) to check the effect of extraction method on conglutin extraction.

Selection of design variables and experimental design

In RSM, experimental variables are converted into coded values (Myers et al., 2004) which are dimensionless and with same standard deviation. Central composite design (CCD) was used to create experimental design matrix of independent variables as represented in Table 3.1. The RSM CCD matrix of the three independent variables namely pH of solvent, the volume of solvent added (ml/gm) and extraction time (min), and their corresponding experimental results for optimization of conglutin extraction are given in Table 3.2. The same set of experiments were repeated for magnetic stirring, ultrasonication and hydrodynamic cavitation-assisted extraction.

Table 3.1 Coded and actual values of independent variables used for optimization

Independent variables	**Symbol**	**Coded value**				
		a	**-**	**0**	+	**A**
		-2	**-1**	**0**	**+1**	**+2**
pH of solvent	X_1	8	8.5	9	9.5	10
Solvent added (ml/gm)	X_2	10	20	30	40	50
Extraction time (min)	X_3	10	20	30	40	50

Fitting the model

The quadratic model for the relation between response and independent variables was predicted using the least squares technique. With the model fitted, JMP generated model coefficients, regression coefficient values and F-values, which justify the significance of each experimental variable. On the basis of seventeen experiments generated by CCD (Table 3.2), maximum response (% w/w conglutin extraction) was predicted by JMP for each set of experiment. The adequate fit of the model with that of experimental results is necessary, which otherwise would mislead the optimization studies. Therefore, experimental response data was plotted against predicted response as shown in Figure 3.8.

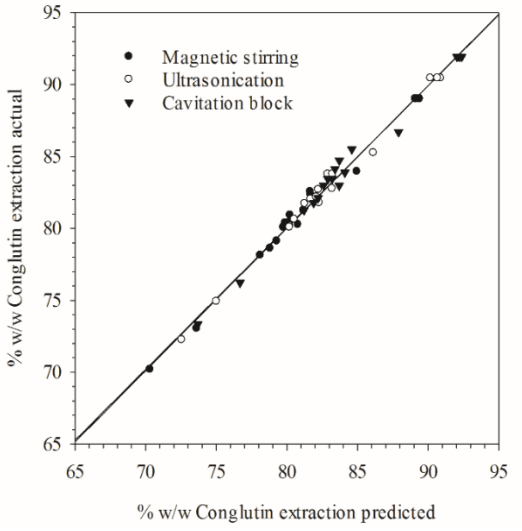

Figure 3.8 Actual versus predicted plot for % w/w conglutin extraction

The actual versus predicted plot showed an excellent fit with R^2 value of 0.990 for magnetic stirring, 0.991 for ultrasonication and 0.990 for hydrodynamic cavitation. R^2 value closer to 1 indicates that the predicted model is fit to the experimental data and is reliable to optimize and maximize the response desired. Adj R^2 is also an important regression parameter, which measures the amount of variation around the mean explained by the model and adjusted for the number of terms on the model. The value of Adj R^2 for all the three extraction processes was greater than 0.97, which indicates that the variables selected in the model add value to the model significantly.

Statistical analysis of the obtained response was done by ANOVA, which subdivides the total variation of a set of data into component parts associated with specific sources of variation for the purpose of testing a hypothesis on the parameters of a model (Institute, 2000). ANOVA summary for conglutin extraction by three different processes of extraction is as shown in Table 3.3. The model 'F-value' in the ANOVA table is an indication of a test of comparison of model predicted with all the models available in the software. If this value is higher than expected by chance alone, then the model predicted has a significant effect.

Table 3.2 CCD matrix of independent variables and their corresponding response for optimization of conglutin extraction by three extraction processes

Run order[a]	Pattern	X_1	X_2	X_3	% w/w conglutin extraction yield[b]		
					MS[c]	US[d]	HC[e]
1	a00	8	30	30	70.10±0.30	72.56±0.31	73.69±0.45
2	−−−	8.5	20	20	78.03±0.13	80.21±0.13	81.21±0.11
3	−−+	8.5	20	40	81.76±0.08	82.90±0.43	83.72±0.09
4	−+−	8.5	40	20	78.88±0.10	80.19±0.08	81.89±0.20
5	−++	8.5	40	40	79.63±0.37	81.70±0.18	82.90±0.06
6	0a0	9	10	30	80.90±0.16	82.29±0.33	83.70±0.11
7	00a	9	30	10	79.81±0.07	81.26±0.19	82.60±0.23
8	000	9	30	30	89.29±0.13	90.90±0.24	92.20±0.08
9	000	9	30	30	89.46±0.08	90.68±0.04	92.04±0.26
10	000	9	30	30	89.16±0.09	90.18±0.42	92.39±0.06
11	00A	9	30	50	84.98±0.04	86.13±0.11	87.90±0.09
12	0A0	9	50	30	79.37±0.11	80.53±0.03	82.20±0.18
13	+−−	9.5	20	20	80.46±0.33	82.09±0.06	83.20±0.01
14	+−+	9.5	20	40	81.71±0.09	83.23±0.11	84.60±0.06
15	++−	9.5	40	20	81.22±0.06	83.20±0.13	84.10±0.06
16	+++	9.5	40	40	80.43±0.29	82.23±0.01	83.40±0.20
17	A00	10	30	30	73.58±0.06	75.01±0.16	76.22±0.17

[a]Non randomized
[b]Mean ± standard deviation of duplicate experiments
[c]MS- magnetic stirring
[d]US- ultrasonication
[e]HC- hydrodynamic cavitation

Table 3.3 ANOVA for response surface quadratic model of conglutin extraction

Factor	Sum of squares	DF[a]	F ratio	Prob>F[b]
Magnetic stirring				
Model	404.84	9	78.27	< 0.0001*
X_1	8.10	1	14.11	0.0071*
X_2	1.31	1	2.29	0.1731
X_3	15.07	1	26.23	0.0014*
$X_{1*} X_2$	0.05	1	0.09	0.7706
$X_1* X_3$	2.19	1	3.82	0.0916
$X_{2*} X_3$	2.96	1	5.16	0.0574
X_1^2	366.06	1	636.86	< 0.0001*
X_2^2	105.01	1	182.71	< 0.0001*
X_3^2	59.21	1	103.02	< 0.0001*
Ultrasonication				
Model	378.92	9	90.47	< 0.0001*
X_1	7.08	1	15.23	< 0.0059*
X_2	1.34	1	2.88	0.1333
X_3	12.43	1	26.72	0.0013*
$X_{1*} X_2$	0.22	1	0.47	0.5128
$X_1* X_3$	2.03	1	4.36	0.0751
$X_{2*} X_3$	1.35	1	2.90	0.1319
X_1^2	344.56	1	740.44	< 0.0001*
X_2^2	103.51	1	223.43	< 0.0001*
X_3^2	58.67	1	126.08	< 0.0001*
Hydrodynamic cavitation				
Model	391.97	9	54.07	< 0.0001*
X_1	8.36	1	10.51	0.0142*
X_2	0.73	1	0.92	0.3685
X_3	13.70	1	17.21	0.0043*
$X_{1*} X_2$	0.003	1	0.0045	0.9482
$X_1* X_3$	0.98	1	1.24	0.3023
$X_{2*} X_3$	1.61	1	2.03	0.1979
X_1^2	356.43	1	447.70	< 0.0001*
X_2^2	106.83	1	134.19	< 0.0001*
X_3^2	60.92	1	76.52	< 0.0001*

[a]Degree of freedom

[b,]*Significant term

The model 'F-value' of 78.27 for magnetic stirring, 90.47 for ultrasonication and 54.07 for hydrodynamic cavitation-assisted extraction, imply that the model is significant and there is only a 0.01% chance that model 'F-value' can deviate from the mean value which could occur due to noise. 'Prob>F' value is the observed significance probability of obtaining greater 'F-value' by chance alone if the specified model fits no better than the overall response mean. In a simple language, if the 'Prob>F' value is less than 0.05, then the individual term in the model has a significant effect on the response. As observed from 'Prob>F' value in Table 3.3, pH of solvent (X_1) and extraction time (X_3) were seen to be significant parameters for conglutin extraction in case of all the extraction processes. The least significant parameter was the volume of solvent added (X_2), which was also observed during OFAT studies.

The model described the relationship between the independent variables and response in the form of quadratic polynomial equation as represented in equations (3.3), (3.4) and (3.5) for magnetic stirring, ultrasonication and hydrodynamic cavitation-assisted extraction respectively.

For magnetic stirring assisted extraction,

$$Y = 89.01 + 0.71\times X_1 - 0.29\times X_2 + 0.97\times X_3 + 0.08\times X_1\times X_2 - 0.52\times X_1\times X_3 - 0.61\times X_2\times X_3 - 4.34\times {X_1}^2 - 2.32\times {X_2}^2 - 1.75\times {X_3}^2 \quad (3.3)$$

For ultrasonication assisted extraction,

$$Y = 90.45 + 0.66\times X_1 - 0.28\times X_2 + 0.88\times X_3 + 0.16\times X_1\times X_2 - 0.50\times X_1\times X_3 - 0.41\times X_2\times X_3 - 4.21\times {X_1}^2 - 2.31\times {X_2}^2 - 1.74\times {X_3}^2 \quad (3.4)$$

For hydrodynamic cavitation-assisted extraction,

$$Y = 91.94 + 0.72\times X_1 - 0.21\times X_2 + 0.92\times X_3 - 0.022\times X_1\times X_2 - 0.35\times X_1\times X_3 - 0.45\times X_2\times X_3 - 4.29\times {X_1}^2 - 2.35\times {X_2}^2 - 1.77\times {X_3}^2 \quad (3.5)$$

Where, Y= conglutin extraction (% w/w), X_1= pH of solvent, X_2= the volume of solvent added (ml/gm) and X_3= extraction time (min).

Analysis of response surfaces

The lack of fit for all the three models was observed to be insignificant, the responses were sufficiently explained by the regression equations. According to the regression

equations, three-dimensional surface plots and contour plots were constructed to determine the optimal level of variables and response (Figure 3.9, 3.10 and 3.11). The nature and extent of the interaction between different variables were predicted by the shapes of response surfaces and contour plots.

Figure 3.9 (a and d) shows the 3D surface plot and contour plot respectively of magnetic stirring assisted extraction for the effect of pH of solvent and extraction time on conglutin extraction (% w/w). As stated earlier, both of these parameters have a significant effect on conglutin extraction which was easily seen from the surface plots. The graphs suggested that the area for maximum conglutin extraction was observed when pH of extraction reached the value of 9 and extraction time of 30 min. After 30 min of extraction time, the trend was found to be same for conglutin extraction, whereas an increase of pH over 9 slightly reduced the response value. Similar graphs were observed for ultrasonication (Figure 3.10 a and d) and hydrodynamic cavitation-assisted extraction (Figure 3.11 a and d). Similarly, the surface plot and contour plot for the effect of pH of solvent and extraction time (min) on conglutin extraction for magnetic stirring (Figure 3.9 b and e), ultrasonication (Figure 3.10 b and e) and hydrodynamic cavitation (Figure 3.11 b and e), indicated that desired response was achieved when extraction was performed with pH 9 of solvent and the volume of solvent added was more than 30ml/gm.

The factor ‘solvent added (ml/gm) was observed to have less significance on conglutin extraction as compared to other two factors and therefore its interaction with other two factors was also seen to be less significant when quadratic terms of other two factors were considered.

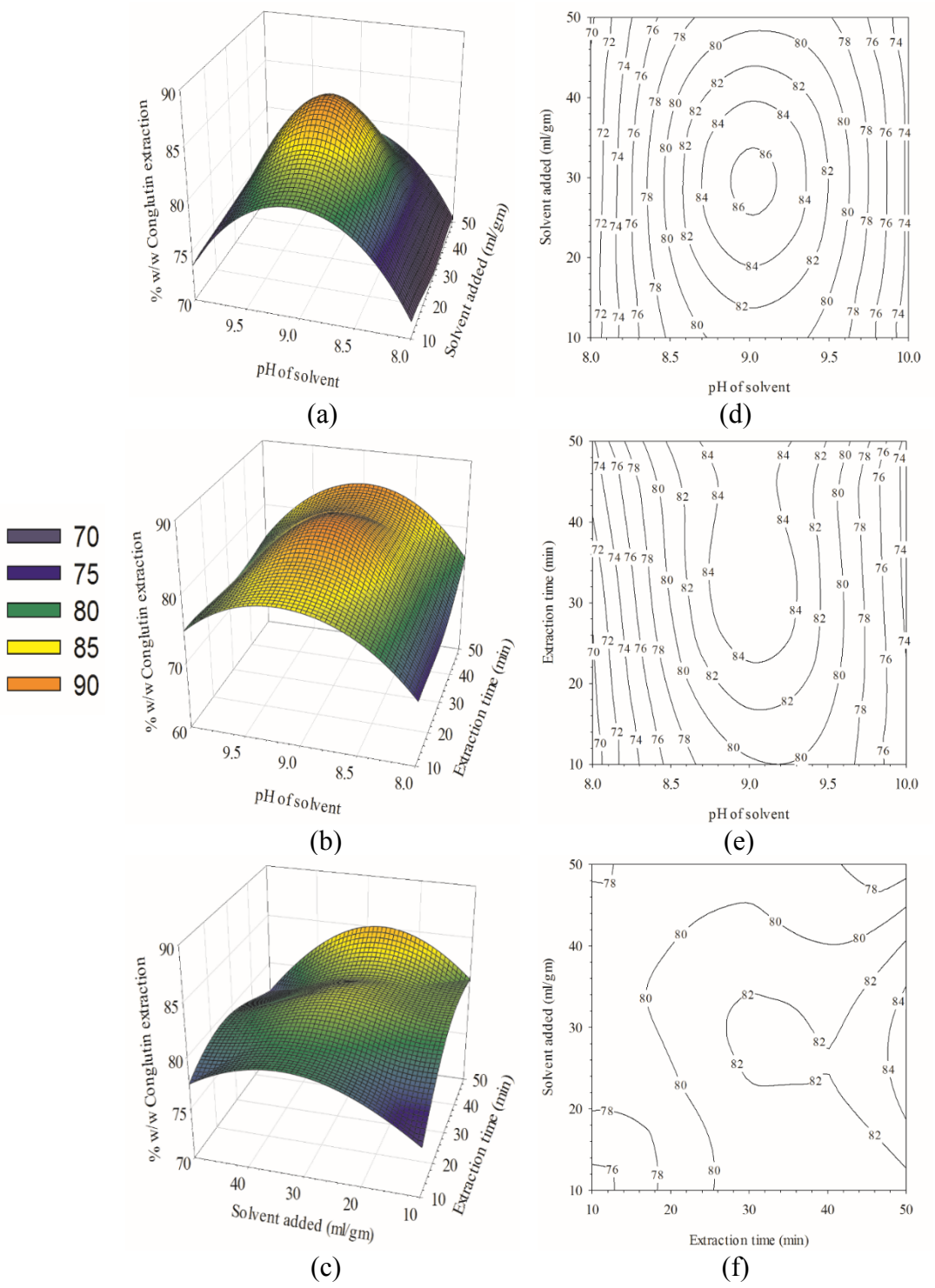

Figure 3.9 Response surfaces (a, b, c) and contour plots (d, e and f) for effect of extraction parameters on conglutin extraction by magnetic stirring

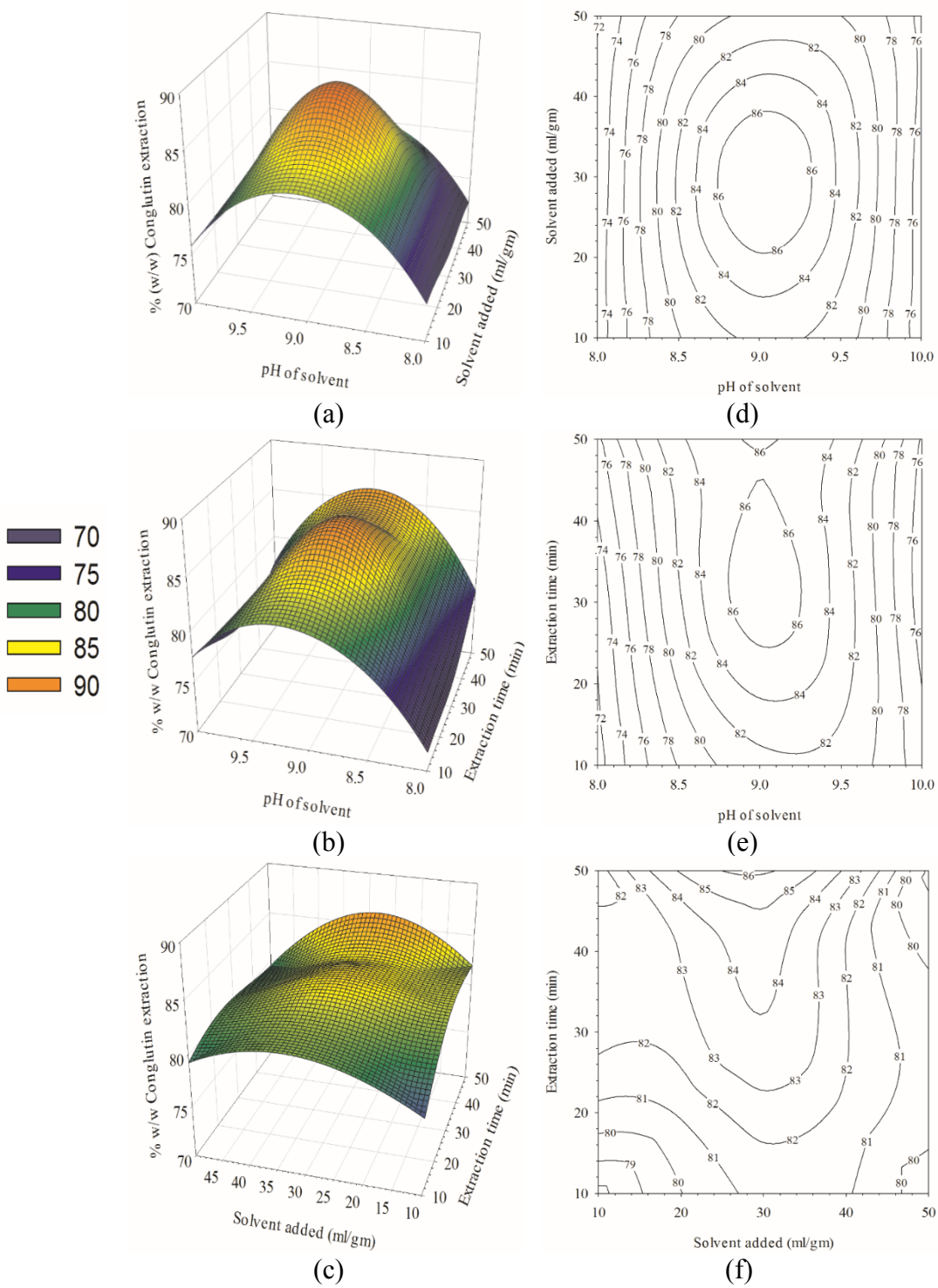

Figure 3.10 Response surfaces (a, b, c) and contour plots (d, e and f) for effect of extraction parameters on conglutin extraction by ultrasonication

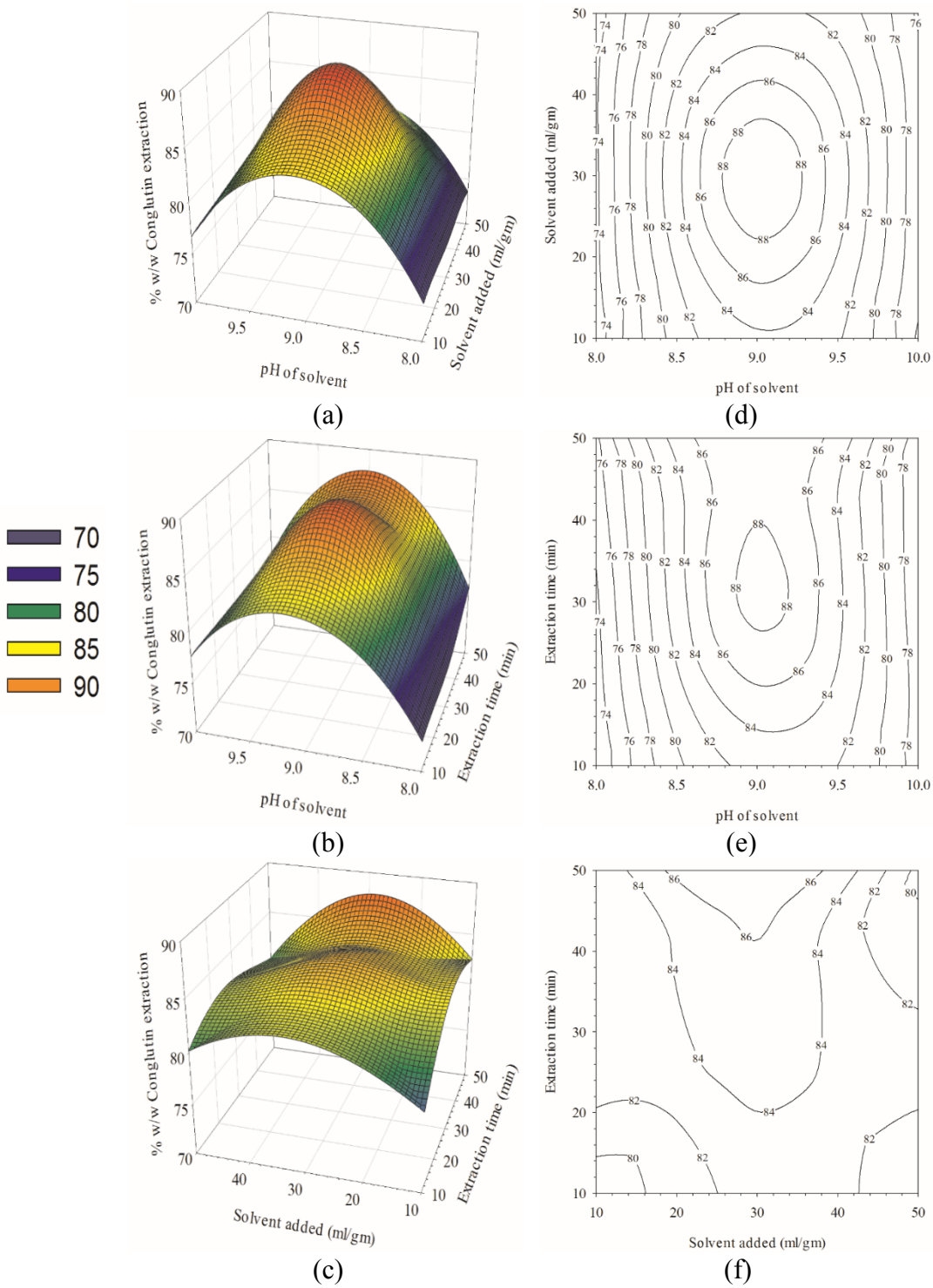

Figure 3.11 Response surfaces (a, b, c) and contour plots (d, e and f) for effect of extraction parameters on conglutin extraction by hydrodynamic cavitation

Verification experiments

From the prediction profiler of RSM analysis, the predicted experimental conditions were obtained with maximum desirability. These predicted set of experiments were

performed and the obtained responses were compared with JMP predicted responses (Table 3.4), which were observed to be almost similar confirming the validity and adequacy of the model predicted. Also, the verification experiment results proved that the predicted values for conglutin extraction with each extraction process could be satisfactorily achieved within 95% confidence interval of experimental values.

Table 3.4 Predicted conditions and verification experiments for response surface quadratic model

Parameters	X_1	X_2	X_3	**% w/w conglutin extraction**	
				Experimental[a]	**Predicted**
Magnetic stirring	9.03	29.12	30.15	89.54±0.13	89.60
Ultrasonication	9.02	29.16	30.13	90.28±0.17	90.98
Hydrodynamic cavitation	9.03	29.18	30.02	91.98±0.08	92.42

[a]Mean ± standard deviation of duplicate experiments

As seen from Table 3.4, there is no significant difference in the range of independent variables and responses for all the three extraction processes. Maximum conglutin extraction (~92% w/w) was observed with hydrodynamic cavitation-assisted extraction process. Studies on the intensification of protein extraction by hydrodynamic cavitation phenomenon have already been reported by Preece et al (Preece, Hooshyar, Krijgsman, Fryer, & Zuidam, 2017). The success in increasing extraction yields by hydrodynamic cavitation is mainly due to the formation of air bubbles, their growth and their potential collapse near the solid surface (Gogate, Tayal, & Pandit, 2006). This collapse leads to the formation of high shear near solid surfaces, which might have resulted in cell disruption and solubilization of proteins from kernel cells, increasing the extractability of the process.

3.5.3 Characterization of conglutins extracted by RSM optimized experiments

The conglutins extracted with RSM optimized conditions by three different extraction processes were characterised to study the effect of extraction process on protein structure. The protein content of the conglutin extract obtained by RSM optimized conditions was found to more than 90% w/w in all the three cases. These obtained

extracts were freeze-dried (Alpha 1-4 LDplus freeze dryer, John Morris Scientific, Australia) and stored at -20ºC until analysis.

FTIR spectroscopic analysis of extracted conglutins

FTIR spectra of conglutins extracted from defatted lupin kernel flour by three different extraction processes (magnetic stirring, sonication and hydrodynamic cavitation) were observed to show a similar profile of amide I and II bands (Figure 3.12). Bands at 3280 cm^{-1} (merged amide A and A' bands due to NH stretch strongly overlapped with the OH stretching band of hydrating H2O molecules), 2972-2864 cm^{-1} (CH stretching vibrations), 1634 cm^{-1} (amide I band due to C=O stretching), 1538 cm^{-1} (amide II band due to out-of-phase combination of NH in-plane bending and CN stretching) and 1399 cm^{-1} (amide III band due to in-phase combination of NH in-plane bending and CH stretching).

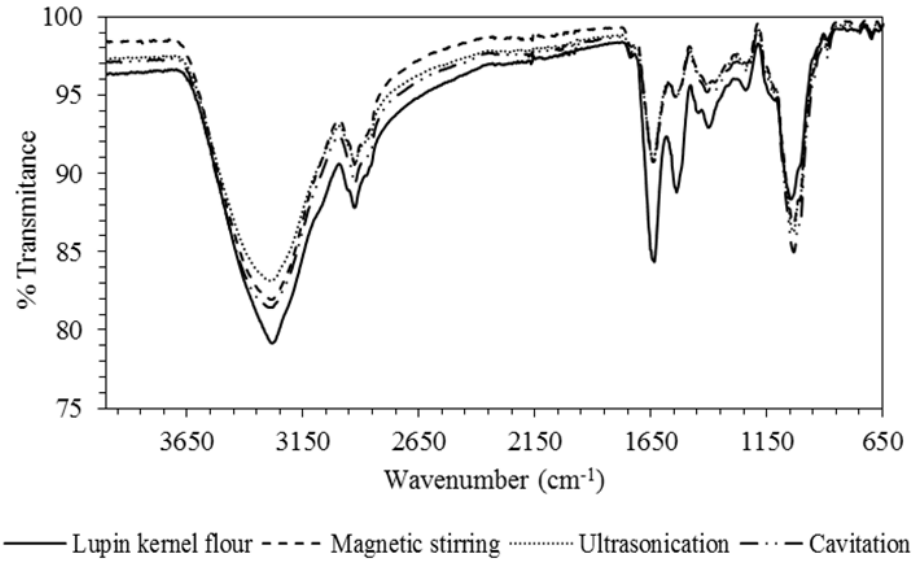

Figure 3.12 Overlay of FTIR spectra of defatted lupin kernel flour with conglutins extracted by magnetic stirring, sonication and hydrodynamic cavitation

The amide I, II, III and amide A, A' are bands typically visible in IR spectrum of proteins and structure of these bands depends on the population of secondary structure elements in a protein. The peak at 1634 cm^{-1} indicates the presence of α-helix, peak at 1538 cm^{-1} indicates the presence of conglutins in β-sheeted form. Splitting at amide I band indicates that presence of antiparallel strand in β-sheeted protein. The peak of amide III band at 1399 cm^{-1} has very less significance in identifying the protein

structure due to its small size. No structural changes in the proteins extracted were evident from FTIR spectra during magnetic stirring, ultrasonication and hydrodynamic cavitation-assisted extraction.

SDS-PAGE of extracted conglutins

SDS-PAGE (non-reducing) of conglutins extracted with RSM optimized conditions by different extraction processes was performed to study the changes in the profile of proteins as affected by extraction processes (Figure 3.13). As seen from Figure 3.13, the molecular weight profile of the proteins was observed to be similar in all the lanes of the gel. Occurrence of proteins at molecular weights of 9, 14, 21, 32, 35, 38, 40, 45, 48, 53, 72, 92, 200 kDa was observed. The gel bands in the range of 17-64 kDa represent β-conglutin of low molecular weight to high molecular weight range, whereas the bands in the range of 70-200 kDa represent α-conglutin. No structural changes or denaturation of protein into low molecular weight peptides was observed for SDS-PAGE analysis in all the three types of extraction processes.

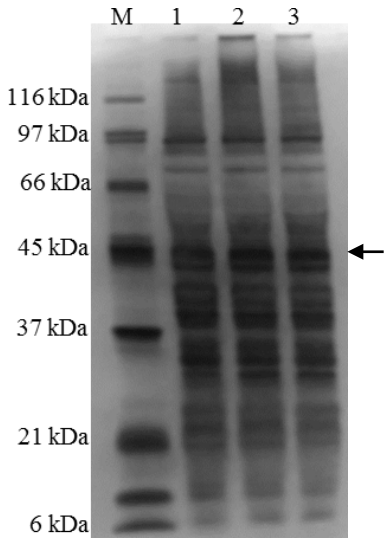

Figure 3.13 SDS-PAGE of conglutin extracted by three extraction processes (M: marker, L1: magnetic stirring, L2: ultra-sonication, L3: cavitation) (Arrow represents γ-conglutin at 48 kDa)

FTIR and SDS-PAGE characterization showed that the extraction performed by magnetic stirring, ultrasonication and hydrodynamic cavitation did not modify the protein structure. Though RSM optimized parameters and conglutin extraction (% w/w) did not show a significant difference for these three extraction methods, magnetic

stirring assisted extraction would be an ideal choice for conglutin extraction. The preference for magnetic stirring over cavitation-assisted extraction is to preserve the emulsification property of the major conglutins, as cavitation might apply more pressure during operation which can disrupt protein bodies and hence can cause variations in emulsification properties (Mujumdar, Kumar, & Pandit, 1997).

3.6 CONCLUSION

This study provides the detailed analysis of key factors affecting the extraction of the lupin conglutins from the defatted kernel flour. It is the first report on utilisation and comparison of magnetic stirring, ultrasonication and hydrodynamic cavitation-assisted extraction for conglutin extraction. Central composite design combined with response surface methodology was successfully implemented for optimization of conglutin extraction by these three extraction processes. The key parameters for extraction and their optimized values by RSM were found to be pH of ultrapure water (9), the volume of water added (30 ml/gm) and extraction time (30 min), yielding almost 89% w/w conglutins. The interaction between these key factors and conglutin extraction was studied by regression analysis and a quadratic equation was obtained to predict the response. Conglutin extraction was fitted to second-order reaction kinetics as analysed by Peleg's model. The characteristic analysis of the proteins extracted with optimized extraction factors by three different processes of extraction did not show significant structural change in protein by FTIR and SDS-PAGE analysis.

Chapter 4

Development of cation exchange chromatography process for purification of γ-conglutin

A patent application is being filed based on the content in this Chapter.

4.1 INTRODUCTION

Based on the physiochemical properties of γ-conglutin (discussed in Chapter 2), many research groups (Blagrove & Gillespie, 1975; D'Agostina et al., 2006; Marcello Duranti, Restani, Poniatowska, & Cerletti, 1981) have attempted its extraction and purification from lupin seeds. However, while those attempts were successful in the separation of α and β-conglutin, they did not achieve good separation for γ-conglutin. The previous methods are also time-consuming and involve many processing steps, making them unsuitable for commercial scale production of high purity γ-conglutin. Different combinations of chromatographic processes such as anion exchange with gel filtration chromatography (Melo, Ferreira, & Teixeira, 1994) or with cation exchange and metal affinity chromatography (M Duranti, Scarafoni, Di Cataldo, & Sessa, 2001) have been practised for purification of γ-conglutin. However, no justified data on the purity of obtained fractions is reported. These multistep chromatographic processes also have many hurdles in their scale up (Mane, Johnson, Duranti, Pareek, & Utikar, 2018), making γ-conglutin unavailable on a commercial scale.

This study describes an approach for selective extraction and purification of γ-conglutin from *Lupinus angustifolius* seeds (Coromup variety) with a minimum number of process steps and highest possible purity. The solubility of γ-conglutin, depending on the pH of the medium was studied in order to optimize selective extraction of γ-conglutin enriched fraction. Further, the obtained extract was investigated for its binding on four cation exchange adsorbents by batch adsorption experiments. Based on batch adsorption results, an intermediate pH modulation step was introduced to reduce the load of impurities which were competing with γ-conglutin for binding to the adsorbents and then the batch adsorption studies were repeated to validate better binding performance.

Column experiments were further performed to find an approach to preparative scale purification using column packed with selected adsorbent. The effect of various process parameters such as conductivity, flow rate and elution gradient in column purification of γ-conglutin was studied and optimized for maximum possible resolution between impurities and γ-conglutin peak. The data presented in this study will be valuable in designing and scaling up of preparative cation exchange chromatography process for purification of γ-conglutin from lupin seeds and similar proteins from other legume seeds.

4.2 MATERIALS

4.2.1 Chemicals and reagents

Sodium chloride, sodium phosphate (monobasic), sodium phosphate (dibasic) and Tris (tris-aminomethane) were used for batch adsorption studies. All the chemicals purchased were of analytical grades from Sigma-Aldrich Pty Ltd (Perth, Western Australia).

4.2.2 Adsorbents, chromatographic column and chromatographic system

Adsorption studies were performed on four cation exchange adsorbents (listed in Table 4.1) depending on the pH of the extraction medium and the surface charges present on γ-conglutin. Capto S and SPFF were purchased from GE Healthcare (Perth, Western Australia). Toyopearl adsorbents were purchased from Tosoh Bioscience (Redland Bay, Queensland, Australia).

Table 4.1 Physical properties of ion exchange adsorbents

Description	**Capto S**	**SPFF**	**SP-550 C**	**SP-650 M**
Functional group	Sulphonate	Sulphopropyl	Sulphonate	Sulphonate
Base matrix	Agarose	Agarose	Methacrylate	Methacrylate
Particle size (μm)	90	90	100	65
Pore size (Å)	500	500	500	1000
Ion capacity (μeq/ml)	110	180	130	120
Dynamic capacity (mg/ml)	120	110	120	140
Working pH range	4-12	4-13	1-13	1-13

The preparative scale chromatographic purification experiments were performed using NGC chromatographic system (Bio-Rad Laboratories Pty Ltd, Perth, Western Australia) equipped with sample pump 100, multi-wavelength UV/Vis detector, pH detector, conductivity monitor, temperature monitor and fraction collector. The instrument was controlled by software ChromLab associated with the system.

Pre-packed columns used for optimising cation exchange chromatographic process were HiTrap Capto S and HiScreen Capto S (Table 4.2) (GE Healthcare, Perth, Western Australia). The developed process for purification of γ-conglutin was scaled up by manually packing 20 ml of Capto S slurry in a XK 16/20 glass column (16 mm internal diameter) (GE Healthcare, Perth, Western Australia). The uniform distribution of the buffer and sample in the packed column was provided with the plunger adapter associated with the column. The adaptor QuickLock mechanism facilitated the packing of the adsorbent to the desired bed height (20 ml)

Table 4.2 Details of pre-packed Capto S columns

Column	**Column volume (ml)**	**Particle size (µm)**	**Bed dimension (mm X mm)**
HiTrap Capto S	0.96	90	7 X 25
HiScreen Capto S	4.66	90	7.7 X 100

4.3 EXPERIMENTAL METHODS

4.3.1 Selective extraction of γ-conglutin enriched fraction

Lupinus angustifolius (Coromup variety) kernels were milled to 300 µm size and defatted with n-hexane as described in Chapter 3 (section 3.3.1). pH for the selective extraction of γ-conglutin enriched fraction was optimized from the results obtained with lupin conglutin solubility curve (discussed in Chapter 3, section 3.5.1). pH based solubility of lupin conglutins was studied with respect to maximize extraction of γ-conglutin with none or lesser impurities of other conglutins.

4.3.2 Preparation of cation exchange adsorbents

All adsorbents were pretreated prior to experiments by washing them with ultrapure water to remove all traces of preservative agents. All washed cation exchange adsorbents were degassed using a vacuum and equilibrated using an equilibration

buffer. Initial studies of adsorption were carried out using 10 mM sodium acetate buffer (pH 4.40) as an equilibration buffer under mild shaking on rocker shaker (John Morris Scientific Pty Ltd, Perth, Western Australia) for 15 min at room temperature. The equilibrated adsorbents were kept in the respective equilibration solutions until further use. The stepwise elution of proteins bound to the adsorbent was carried out using elution buffers, which were prepared by increasing concentration of sodium chloride (0.1 M to 1 M) in the same equilibration buffer (10 mM sodium acetate buffer, pH 4.40). Based on the purity of the target protein, binding conditions were changed and buffer solutions respective to those binding conditions were used.

4.3.3 Screening of cation exchange adsorbents

The screening of adsorbents was carried out to investigate optimum binding and elution of γ-conglutin on respective adsorbent by using batch adsorption method. The analysis of this experiment was done on the basis of SDS-PAGE (non-reducing) and by measuring protein concentration of all samples by BCA analysis. In a typical experiment, fixed amount (1 ml) of pre-equilibrated ion exchange adsorbents were transferred into the respective falcon tubes and contacted with 5 ml of γ-conglutin enriched extract (protein content 2 mg/ml). To attain the equilibrium, the falcon tubes were kept on rocker shaker for 3 hr at room temperature. The adsorbent was then pelleted by centrifugation (4700 rpm, 5 min) and the supernatants from each tube were sampled for analysis of unbound protein fraction.

The remained adsorbent pellet was further contacted with 5 ml of equilibration buffer for 15 min on rocker shaker at room temperature. The obtained supernatant after re-centrifugation (4700 rpm, 5 min) was sampled and described as a wash fraction which contained loosely bound proteins. Similarly, different elution buffers with increasing concentrations of sodium chloride were added and respective elution fractions were sampled for analysis.

4.3.4 Intermediary pH modulation step

The step of modulating the pH of γ-conglutin enriched extract was introduced as an intermediate step between extraction and chromatographic purification so as to reduce the load of impurities and number of process steps. The impurities in γ-conglutin enriched extract were analysed by SDS-PAGE and the identified impurities were carefully studied for their isoelectric regions from the available literature (Marcello

Duranti et al., 1981; Melo et al., 1994). The pH corresponding to the isoelectric region of the identified impurities was selected to precipitate them.

α and β-Conglutin subunits having pI in the range of pH 5-7, were observed to be the major impurities as identified by SDS-PAGE analysis. Hence, pH range of 5-7 will precipitate these impurities (α and β-conglutin), whereas γ-conglutin will remain in the solution without precipitation, due to its basic pI. Therefore, experiments for isoelectric precipitation of impurities were conducted, where pH of γ-conglutin enriched extract was modulated to pH 5, pH 6 and pH 7.

According to the buffer table (Speight, 2005), Tris buffer was used for modulating pH in the required range. The desired pH was obtained by adding 0.1 M Tris buffer slowly in the extract under mild stirring conditions. The resulting solution was then centrifuged (4700 rpm, 15 min) followed by filtration through PVDF syringe filter (0.45 μm) and analysed for its protein profile by SDS-PAGE. Based on the results of SDS-PAGE (non-reducing) analysis, the optimum pH at which most of the impurities were either precipitated or charged neutral, was selected for further studies of the adsorption process.

4.3.5 Optimization of cation exchange chromatography process

In chromatographic processes, the efficacy of separation is enhanced by selection of the most appropriate column characteristics, binding capacity and optimized process conditions (Janson & Hedman, 1987). Therefore, experiments were performed to optimize the column chromatography process conditions such as conductivity of equilibration, flow rate and elution gradient by varying one factor at a time for effective purification of γ-conglutin from the extract. All the optimization experiments were performed on HiScreen Capto S column. 10 mM sodium phosphate (pH 6.5) was used as an equilibration buffer (buffer A) whereas 1 M sodium chloride in buffer A was used as an elution buffer (buffer B). The flow rate of the method was maintained at 2 ml/min. 5 ml of sample (pH modulated γ-conglutin extract) was loaded on a column with sample pump at the flow rate of 1 ml/min at room temperature. All the experiments were performed in duplicate to confirm the results. These experiments were analysed through evaluation of the obtained chromatograms and SDS-PAGE (non-reducing) analysis of collected fractions.

Effect of conductivity of process

The molarity of equilibration buffer was varied to match the conductivity of sample to be loaded on the column (pH modulated γ-conglutin extract). Two trials were performed, one with10 mM sodium phosphate (pH 6.5) as an equilibration buffer and other with 25 mM sodium phosphate (pH 6.5) as an equilibration buffer, with 1 M sodium chloride in their respective equilibration buffer as an elution buffer. All other method parameters were kept constant as described above in this section.

Effect of flow rate

The optimum flow rate for purification of γ-conglutin was obtained by performing experiments at different flow rates in the range of 0.5 ml/min to 2.5 ml/min. The experiments were performed with selected conductivity of equilibration buffer, whereas other parameters were kept same as stated above.

Effect of elution gradient

Based on the chromatogram and SDS-PAGE results of initial method tried, the gradient was varied in linear as well as in a step-wise manner to obtain the optimum elution protocol for bound γ-conglutin. Also, the molar concentration of sodium chloride was reduced from 1 M to 0.5 M based on the concentration of sodium chloride required to elute γ-conglutin peak.

4.3.6 Adsorption isotherm experiments

Adsorption isotherm experiments were performed to determine the static binding capacity of purified γ-conglutin per ml of Capto S. The adsorptive chromatographic process is based on the distribution of the solute between a stationary phase (adsorbent) and a mobile phase (buffer). The adsorption equilibrium is determined by isotherm, which gives correlation between loadings of solute on the adsorbent ‘q’ at different fluid phase concentrations ‘C’ under equilibrium.

Adsorption isotherm experiments for binding of pure γ-conglutin on Capto S were carried out by batch adsorption method in 10 mM sodium phosphate buffer at pH 6.5. For this study, 0.1 ml of pre-equilibrated Capto S was transferred in each of 2 ml ependroff tubes and contacted with 1 ml of standard γ-conglutin solution (prepared by an optimized process as discussed further in this Chapter) with different initial fluid phase concentrations (0.1-10 mg/ml). The eppendroff tubes were then kept on rocker

shaker for 3 hr at room temperature. Upon attaining the equilibrium, the resulting mixtures were centrifuged at 10,000 rpm for 5 min, and then respective supernatants were analysed for protein content by BCA analysis. The amount of γ-conglutin adsorbed per ml (solid phase concentration) of Capto S was calculated by subtracting final liquid phase concentration from initial liquid phase concentration. The isotherms of γ-conglutin adsorption on Capto S were analysed by using Langmuir and Freundlich isotherm models (Kale & Lali, 2011). The equations for adsorption isotherm models are represented in (4.1) and (4.3),

Langmuir isotherm model,

$$q^* = \frac{q_{max} \times C^*}{K_d + C^*} \tag{4.1}$$

Langmuir equilibrium constant,

$$R_L = \frac{1}{1 + (C_0 / K_d)} \tag{4.2}$$

Freundlich isotherm model,

$$q^* = q_F \times {C^*}^{1/n} \tag{4.3}$$

Where, q* is the equilibrium concentration of γ-conglutin in solid (mg/ml adsorbent), q_{max} is Langmuir adsorption capacity of γ-conglutin (mg/ml adsorbent), C* is the equilibrium concentration of γ-conglutin in liquid (mg/ml), K_d is Langmuirian dissociation constant (mg/ml), q_F is Freundlich adsorption capacity (mg/ml) and n is Freundlich adsorption intensity.

4.4 ANALYTICAL METHODS

The quantification of protein content (by CHN analyser and BCA analysis) and SDS-PAGE analysis (reducing and non-reducing) of fractions generated during purification process were performed as explained in Chapter 3 (section 3.4).

4.5 RESULTS AND DISCUSSION

Most of the reported methods on the extraction of lupin conglutins (Blagrove & Gillespie, 1975) involve alkaline extraction of total lupin proteins followed by isoelectric precipitation, yielding major conglutins (α and β), while γ-conglutin

remains soluble in the acidic media, which then may be recovered later. This conventional process of alkaline extraction is not selective for γ-conglutin (Mane, Johnson, Duranti, Pareek, & Utikar, 2018), also it involves a number of processing steps and the use of toxic chemicals such as zinc, to recover γ-conglutin from the acid soluble fraction. Therefore, there is need to design and optimize extraction process which selectively extracts γ-conglutin with least impurities in minimum process steps using food grade non-toxic reagents.

4.5.1 Selective extraction of γ-conglutin

The solubility curve for lupin conglutins and SDS-PAGE analysis of conglutins extracted at different pH have been previously presented and discussed earlier in this thesis in Figure 3.1 and 3.2 respectively (Chapter 3). From Figure 3.2 (Chapter 3), it can be observed that, as pH of the extraction was shifted from neutral to acidic side, the intensity of major conglutin extraction was decreased, leading to enrichment of extract with γ-conglutin at 48 kDa. Therefore, although the solubility curve (Figure 3.1, Chapter 3) exhibited less percentage of extraction at the acidic side, pH 4 was found to selectively extract γ-conglutin. Therefore, extraction was optimized at pH 4. The rationale behind optimising pH 4 for selective extraction of γ-conglutin was to make pH of extraction medium equal to the isoelectric region of major conglutins (impurities). As a result, major conglutins will be neutral at that pH and will not tend to solubilize in the extraction medium.

The selective extraction of γ-conglutin was further optimized by varying one parameter at a time and it was observed that ~15% γ-conglutin enriched fraction (w/w of kernel total proteins) can be obtained by extracting defatted kernel flour at pH 4 for 30 min with solid to solvent ratio (1:30) at room temperature. Initially, all the extraction studies were performed in ultrapure water as extracting medium, but later in order to maintain the stability of the extracted proteins and for chromatographic process purpose, extraction was performed in 10 mM sodium acetate buffer (pH 4).

SDS-PAGE profile of γ-conglutin enriched fraction (pH 4, Figure 3.2) predominantly shows the presence of its monomer at 48 kDa, its single polypeptides at 30 kDa and 18 kDa, also a dimeric form of γ-conglutin at ~92 kDa. Thus, conventional alkaline extraction followed by acidic precipitation was replaced with single step acidic

extraction for γ-conglutin. As a result, all the hurdles associated with the conventional approach can be overcome by the proposed selective acidic extraction.

Few protein impurities of molecular weight ~14 kDa, 20 kDa, 22 kDa, 27 kDa, 34 kDa, 38 kDa and 42 kDa were also present in γ-conglutin enriched fraction. These impurities may be low and intermediate molecular weight peptides of β-conglutin (Melo et al., 1994), having their pI in the basic range (Marcello Duranti, Consonni, Magni, Sessa, & Scarafoni, 2008). The mentioned protein impurities in γ-conglutin enriched fraction were further removed by optimizing and developing adsorptive chromatographic purification process as discussed in the following section.

4.5.2 Adsorptive chromatographic purification of γ-conglutin

Ion exchange chromatography is one of the best ways to achieve selective purification of proteins at pH values carefully selected to maximize the differences in the net surface charge on the component of interest and the impurities. Each protein has its unique net charge versus pH relationship, which is visualised as a titration curve and shows how the net charge on protein varies according to the pH of the surrounding. No data on the titration curve for lupin conglutins is available, and hence pI of these conglutins (Blagrove, Gillespie, Lilley, & Woods, 1980) was used to predict the net surface charges on them at respective pH.

γ-Conglutin has a basic pI in the range of 6.9-8.0 (Blagrove et al., 1980), therefore a positive or negative charge on it can be developed easily for its purification based on pH of the medium. At a pH value less than its pI, a protein of interest will generally have positive charges on the surface and will bind to negatively charged matrix or cation exchange solid phase and vice versa (Komkova, 2010). This principle was considered in the present study as the basis for selecting cation exchange chromatography for purification of γ-conglutin, as at the pH 4 of extraction, γ-conglutin will have positive charges on the surface (Capraro, Spotti, Magni, Scarafoni, & Duranti, 2010) which can be easily captured by negatively charged strong cation exchange adsorbents. Therefore, a successful cation exchange chromatography can be developed by carefully selecting the adsorbent and binding conditions.

Selection of binding conditions

The extraction of γ-conglutin enriched fraction from lupin flour was observed to increase the pH of the obtained extract (Table 4.3) from pH 4 to 4.40, which might be due to the extraction of ions present in the lupin flour (Hove, 1974) or due to some interaction of the buffer ions with the proteins in γ-conglutin enriched fraction. The conductivity and pH of the buffer used for extraction, the obtained extract, equilibration and elution buffers are given in Table 4.3.

Table 4.3 Conditions of buffers and extract obtained

Process	Buffer	pH	Conductivity (mS)
Extraction buffer	10 mM sodium acetate	4.00	0.90
γ-conglutin enriched extract	Extract in buffer	4.40	2.20
Equilibration buffer (A)	10 mM sodium acetate	4.40	0.92

As pH of the extract (sample load) was 4.40, the same pH was selected for the initial adsorption studies, based on principles of ion exchange chromatography process (Komkova, 2010) and pI of γ-conglutin. Sodium acetate was selected as an equilibration buffer as it has a better buffering capacity in the same pH range. For the displacement of a target protein from the charged group of adsorbent, increasing concentrations of sodium chloride was used as an elution buffer based on Hofmeister series (Komkova, 2010) and its displacing activity (Kopaciewicz & Regnier, 1983).

Screening of cation exchange adsorbents

Depending on the molecular weight of γ-conglutin and the surface charges present on it at extraction and binding conditions, different cation exchange adsorbents were screened (Table 4.1) based on their physiochemical properties such as functional group, base matrix, ligand density and pore size. Strong cation exchange adsorbents were selected so that development and optimization of the separation process is fast and easy since the charge characteristics of the medium do not change with pH. Also, for better accessibility of binding sites, the adsorbents with a higher pore size (≥500 Å) were selected.

The separation between the target protein and the impurities not only depends on the nature of functional group on the adsorbent but also on the experimental conditions of the solvent phase such as pH and ionic strength. Hence, batch adsorption studies were

performed (as detailed in the section 4.3.3) for the screening of the suitable adsorbent and binding conditions.

Batch adsorption study

SDS-PAGE (non-reducing) analysis of batch adsorption study for binding of γ-conglutin on SP-550 C and SP-650 M are shown in Figure 4.1, whereas Capto S and SPFF binding results are shown in Figure 4.2. In both SP-550 C and SP-650 M, γ-conglutin was observed to have non-specific interactions (elution along with the impurities at each salt concentration), which might be due to the hydrophobic methacrylate base matrices of SP-550 C and SP-650 M. As a result, γ-conglutin might not have selectively bound to the hydrophobic charged matrices, on account of being charged and hydrophilic at the pH of the extraction.

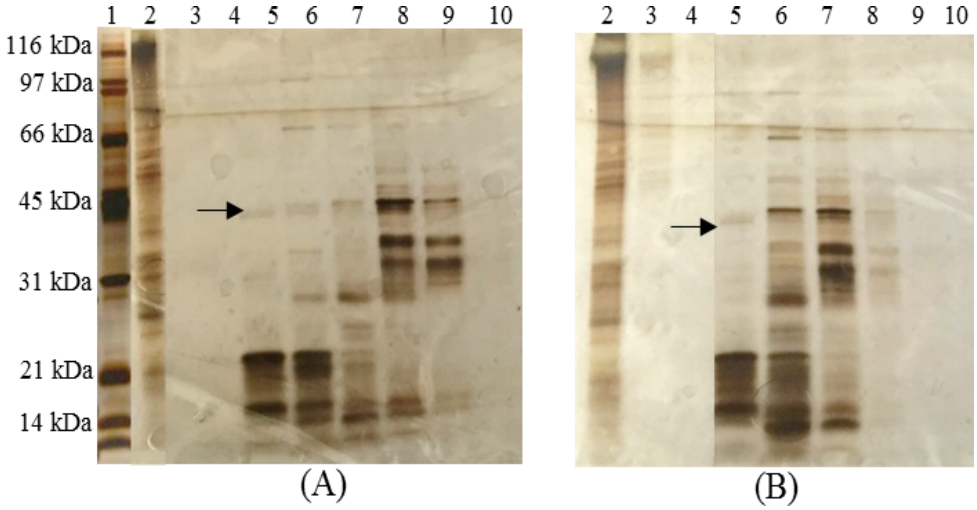

Figure 4.1 SDS-PAGE of fractions from batch studies of SP-550 C (A) and SP-650 M (B),

(Arrow represents γ-conglutin at 48 kDa

(L1: marker, L2: unbound, L3: wash, L4: 0.1 M NaCl elution, L5: 0.2 M NaCl elution, L6: 0.3 M NaCl elution, L7: 0.4 M NaCl elution, L8: 0.5 M NaCl elution, L9: 0.7 M NaCl elution, L10: 1 M NaCl elution)

Capto S and SPFF exhibited better binding performance than SP-550 C and SP-650 M as analysed by SDS-PAGE (Figure 4.2). Lane 1 of both the gels (Figure 4.2 A and B) shows molecular marker with known molecular weight of proteins. Lane 2 represents γ-conglutin enriched extract which was loaded on Capto S and SPFF for batch adsorption study. After loading for 3 hr, the fraction which did not bind to the adsorbent (unbound fraction) is shown in lane 3 of both gels. This unbound fraction

consisted of other protein impurities from the extract without any γ-conglutin (48 kDa), which shows the selective binding of positively charged γ-conglutin to the negatively charged functional groups (sulphonate in the case of Capto S and sulphopropyl in the case of SPFF) of adsorbents. Also, there was no loose interaction seen between bound proteins and adsorbent, as a result, no proteins were observed in the wash fraction (lane 4).

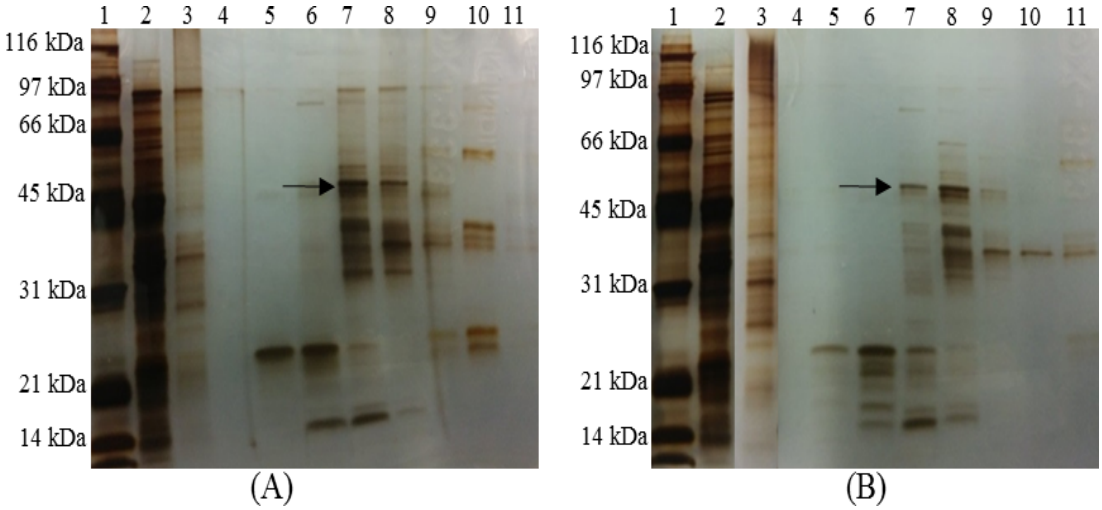

Figure 4.2 SDS-PAGE of fractions from batch studies of Capto S (A) and SPFF (B) (Arrow represents γ-conglutin at 48 kDa)
(L1: marker, L2: γ-conglutin enriched extract, L3: unbound, L4: wash, L5: 0.1 M NaCl elution, L6: 0.2 M NaCl elution, L7: 0.3 M NaCl elution, L8: 0.4 M NaCl elution, L9: 0.5 M NaCl elution, L10: 0.7 M NaCl elution, L11: 1 M NaCl elution)

In earlier elutions of 0.1 M and 0.2 M sodium chloride (lane 5 and 6), lower molecular weight proteins ranging from 10-22 kDa were eluted. These lower molecular weight proteins could be proteins from low molecular weight (LMW) peptides of β-conglutin or δ-conglutin (Marcello Duranti et al., 2008) or smaller subunit of γ-conglutin (Blagrove et al., 1980) with a pI in the range of 5-6. As there was no significant difference between pH of binding (4.4) and their pI (5-6), these peptides might have bound loosely, resulting in early elutions with lower concentrations of sodium chloride.

As the concentration of sodium chloride was increased from 0.3 to 0.4 M, γ-conglutin (48 kDa) was observed to elute along with lesser impurities (lane 7 and 8) ranging from 35-40 kDa. These protein impurities ranging from 35-40 kDa could be

intermediate molecular weight (IMW) peptides of β-conglutin or basic units of α-conglutin, whose pI can vary in the range of 5.2-8.4 (Marcello Duranti et al., 2008). Due to their pI, some of these impurities with a molecular weight of 35-37 kDa (lane 9, 10 and 11) were seen to bind tightly, which required increased concentrations of sodium chloride until 1 M for elution from the adsorbent matrix. Though γ-conglutin was observed to bind to the charged functional group of Capto S and SPFF, it was seen to have interference/competition by other proteins in the extract preventing its selective binding and elutions.

Therefore, many screening experiments were then performed in an attempt to selectively bind and elute γ-conglutin on Capto S and SPFF. pH gradient elution (elution at respective pI) is reported as an important tool in ion exchange chromatography to desorb the bound proteins from the matrix (Ahamed et al., 2007). This technique was also implemented in the present study for selective binding and elution of γ-conglutin, by performing elutions with different pH buffers (ranging from pH 5 to 9 at the interval of 0.5) corresponding to the pI of impurities observed. Ideally, proteins should start to elute from ion exchange media about 0.5 units from their pI (Biosciences, 2002). However, SDS-PAGE results of this study demonstrated that this pH approach did not improve the performance for selective binding. γ-Conglutin was seen to elute with pH 6 buffer along with the same impurities as seen in NaCl elution study and continued eluting until pH 9. Also, denaturation of the proteins was observed when the elution gradient was increased beyond pH 7. In all the trials, the common protein impurities competing with γ-conglutin for binding sites on the adsorbent were observed to be of the molecular weights matching to that of previously reported for intermediate molecular weight (IMW) β-conglutins in the range 25-48 kDa and pI in the range of 5.3-8.4 (Marcello Duranti et al., 2008).

Hence, complete separation of IMW β-conglutins from γ-conglutin was not possible if pH 4 extract was loaded on the adsorbent and adsorption was carried out at pH 4.40. Also, from the conglutin solubility curve (Figure 3.2, Chapter 3), it was observed that changing pH of initial extraction to any value other than pH 4 did not contribute in reducing impurities. Thus, there was a need for an approach, by which the protein impurities (IMW β-conglutins) could be reduced in the pH 4 extract itself before loading on the adsorbent

Intermediary pH modulation step

A better way to achieve the separation between impurities and γ-conglutin fraction could be by altering the surface charges on proteins, where only γ-conglutin will have positive surface charges and most of the protein impurities will carry either neutral or negative surface charge. As a result, the impurities will not compete with γ-conglutin for binding to the adsorbent.

This phenomenon could be achieved by modulating the pH of γ-conglutin extract (obtained as described in the section 4.5.1) in the range of pI of the impurities (pH 5-7). Therefore, experiments were performed as reported in the section 4.3.4, where the pH of γ-conglutin enriched extract was modulated from pH 4.4 to pH 5, 6, 6.5 and 7 slowly by adding 0.1 M Tris buffer under mild stirring conditions at room temperature. The obtained extracts (with different pH) were then loaded on Capto S and SPFF separately, followed by batch adsorption studies on the selective capture of γ-conglutin. The adsorption studies of these pH modulated extracts were performed with different equilibration buffers corresponding to the respective pH and increasing concentrations of sodium chloride in those buffers as elution buffers. SDS-PAGE (non-reducing) results of the batch adsorption studies of pH modulated extracts (not reported here) did not show any significant change in binding and elution of γ-conglutin when pH was modulated to 5 or 6. Also, with pH 7 modulated extract, the loss of γ-conglutin in unbound and wash fractions was observed to be higher. Therefore, pH 6.5 was selected as an optimum pH for modulating the pH of γ-conglutin enriched extract before loading on the equilibrated adsorbent.

SDS-PAGE (non-reducing) profile of the fractions obtained from the batch adsorption studies of pH 6.5 modulated extract on Capto S and SPFF are shown in Figure 4.3. Lane 2 of both the gels shows γ-conglutin enriched extract, which was initially extracted at pH 4 and later modulated to pH 6.5 before loading on the equilibrated adsorbents. When the SDS-PAGE profile of pH 6.5 modulated extract was compared with pH 4 extract, it was observed that the concentration of lower molecular weight impurities in the range of 10-22 kDa (LMW β-conglutin and δ-conglutin) was reduced (as analysed by BCA), which might be due to partial neutral surface charges imparted on them at pH 6.5 due to their pI, leading to their precipitation.

Lane 3 (Figure 4.3) represents the unbound fraction, which shows the protein impurities in the range of 30-40 kDa (IMW β-conglutin). These protein impurities being the major hindrance in γ-conglutin purification, did not opt to bind to the negatively charged adsorbent at pH 6.5, due to the neutral or negative charge developed on them. Some of these IMW impurities were observed to bind loosely to the adsorbent which were washed off in the wash fraction (lane 4).

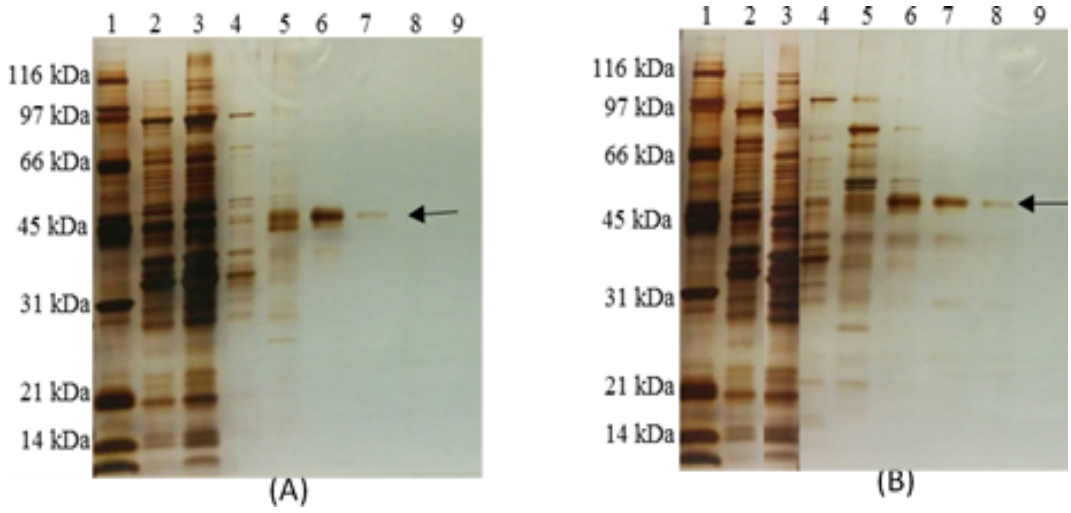

Figure 4.3 SDS-PAGE of fractions from batch studies (pH modulated to 6.5) on Capto S (A) and SPFF (B)

(Arrow represents γ-conglutin at 48 kDa)

(L1: marker, L2: pH 6.5 modulated extract, L3: unbound, L4: wash, L5:0.1 M NaCl elution, L6: 0.2 M NaCl elution, L7: 0.3 M NaCl elution, L8: 0.5 M NaCl elution, L9: 1 M NaCl elution)

The elutions with increasing concentrations of sodium chloride are represented lane 5 onwards, which exhibited lesser impurities along with γ-conglutin as compared to pH 4 binding studies (Figure 4.2). γ-Conglutin started eluting early at 0.1-0.2 M sodium chloride concentration, due to less positive charge on it at pH 6.5 as compared to pH 4. Thus, modulating the pH of γ-conglutin enriched extract to 6.5 before loading on the adsorbent provided the selective binding and elution of γ-conglutin from the extract. This intermediate pH modulation step was observed to precipitate the major impurities (~17% w/w protein precipitation from γ-conglutin enriched extract as analysed by BCA) before adsorption studies, leading to a reduction in a number of chromatographic processes in series from the conventional method.

Thus, the batch adsorption process for the purification of γ-conglutin was optimized by extracting γ-conglutin enriched fraction from the lupin flour at pH 4, followed by an intermediary step of modulating the pH of the extract to 6.5 before loading on cation exchange adsorbent. Though the same binding and elution profile was obtained with both Capto S and SPFF, Capto S was selected for further studies due to the reasonable cost of the adsorbent and back pressure issues associated with SPFF during scale up studies (as per manufacturer's recommendation). The dynamic behaviour between the target solute (γ-conglutin) and solid phase (adsorbent) generally varies from batch studies (Harrison, Todd, Todd, Petrides, & Rudge, 2015) and hence, a basic understanding of the process in dynamic conditions (column chromatography) is essential for providing design information for future large scale process. The following section describes the development and optimization of cation exchange chromatography for purification of γ-conglutin from the obtained extract, by studying the effect of various process parameters on a resolution between impurities and γ-conglutin.

4.5.3 Optimization of cation exchange column process

Cation exchange column studies were performed using pre-packed Capto S column to avoid channelling and zone broadening (observed in manual column packing) so that the efficiency of the process will be maintained throughout the optimization studies. There are different pre-packed Capto S columns available (GE Healthcare) with varying specifications, amongst which HiScreen Capto S (Table 2) was selected as it is an excellent choice for a method optimization and parameter screening study (GE_Healthcare, 2017). HiScreen Capto S column was connected to a BioRad NGC chromatography system as described in the section 4.2.2. The column was initially washed with ultrapure water to remove the storage solution and then equilibrated with equilibration buffer before loading the extract.

10 mM sodium phosphate (pH 6.5) was used as an equilibration buffer (buffer A) which was enough to equilibrate the column (Biosciences, 2002), whereas 1 M sodium chloride in buffer A was used as an elution buffer (buffer B). The bound proteins are generally eluted either by linear or step gradient or by a combination of both. Preparative chromatographic practice suggests the use of linear gradient at early stages of process development so as to predict the elution profile of bound proteins

(Biosciences, 2002). Therefore, simple linear gradient (Table 4.4) was implemented initially, followed by its optimization to achieve the maximum possible resolution between impurities and γ-conglutin. The gradient method is described here in terms of column volumes (CV), which facilitates the transfer of methods to different dimension columns while scaling up. The flow rate of the method was maintained at 2 ml/min. For the first trial, 5 ml of sample (pH 6.5 modulated extract) was loaded on a column with sample pump at the flow rate of 1 ml/min. All the experiments were performed in duplicates to confirm the results. The experiments were analysed from the results of the obtained chromatograms and SDS-PAGE of the collected fractions.

Table 4.4 Chromatographic method steps (trial 1- linear gradient)

Method step	Initial % B	Final % B	Column volume
Equilibration	0	0	2
Sample application	0	0	1
Column wash	0	0	5
Elution 1- linear	0	50	5
Elution 2- linear	50	100	1
Elution 3- linear	100	0	1

The chromatogram obtained with the method trial 1 (Figure 4.4) clearly showed two peaks which were collected in four different fractions and then analysed by SDS-PAGE (non-reducing) (Figure 4.5). The first peak was collected in two different fractions as flowthrough and column wash. Once the extract (lane 2) was loaded on the column, absorbance at 280 nm started increasing as unbound proteins started eluting in the flowthrough fraction (lane 3). SDS-PAGE (non-reducing) profile of load and flowthrough fraction (lane 2 and 3 respectively) exhibited the similar profile as batch adsorption studies (Figure 4.3).

The pigmented components in the extract were removed in flowthrough step itself, which led to decrease in contamination of the final product. As per the previous report (Villarino, 2014), the pigmented components could be carotenoids, flavonoids, alkaloids and phenolics present in the coromup kernel flour, which could otherwise interfere protein extraction. Washing the column with the equilibration buffer before elution step removed few of the protein impurities which were loosely bound to the adsorbent (lane 4). Both the fractions of flowthrough and column wash showed the

minor concentration of γ-conglutin at 48 kDa, which might have eluted from the column due to the high flow rates (which will be discussed in a later section).

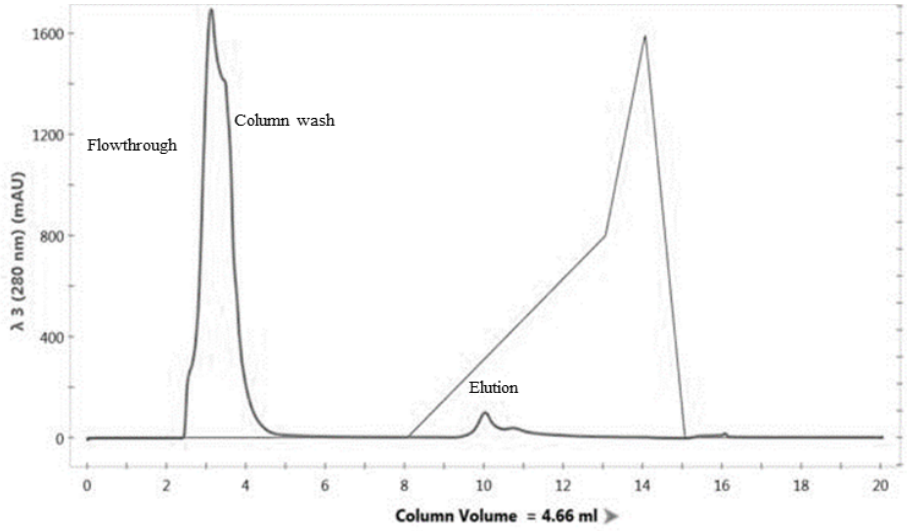

Figure 4.4 Chromatogram obtained with trial method 1

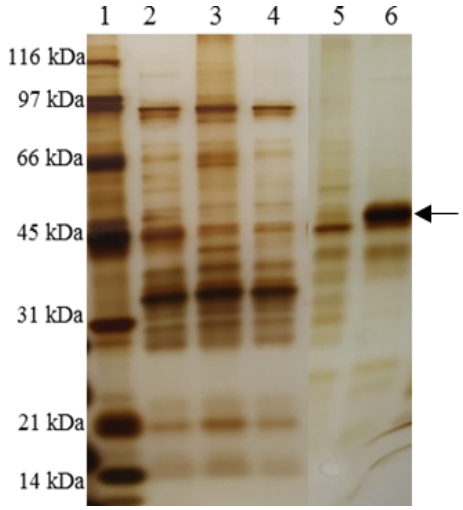

Figure 4.5 SDS-PAGE of fractions obtained with method 1
(L1: marker, L2: pH modulated extract load, L3: flowthrough fraction, L4: column wash fraction, L5: Elution 13-20% B, L6: Elution 20-30% B,
(Arrow represents γ-conglutin at 48 kDa)

From the results of batch studies (described in the section 4.5.2), the concentration of sodium chloride required for elution of γ-conglutin was in the range of 0.1-0.3 M. Therefore, the linear gradient of 0-50% B was given to predict the concentration of sodium chloride required for elution of γ-conglutin peak in dynamic conditions. The second peak (Figure 4.4) represents the elution of bound proteins, which started when % B (1M NaCl in buffer A) reached 13% and continued until 30%. The second peak

had a sharp front and pronounced tailing, which might be due to the elution of more than one component and therefore it was collected in two different fractions (one in 13-20% B and other in 20-30% B) for SDS-PAGE analysis.

The elution fraction between 13-20% B (lane 5) exhibited the same profile of impurities in the range of 25-55 kDa as previously reported for the batch adsorption study (section 4.5.2). The elution of γ-conglutin was seen in between 20-30% B (lane 6) when the concentration of sodium chloride reached from 0.2-0.3 M (conductivity 10.5-16 mS). This fraction when concentrated through 1 kDa centrifugal filter, observed to have interference by LMW and IMW β-conglutin impurities. Therefore, the elution peak was further resolved in two different peaks for better separation.

The success of a chromatographic separation is judged by the ability of the system to resolve one analyte peak from another. Resolution (*Rs*) is generally defined as the ratio of the difference in retention time between the two peaks to the mean of their base widths. Resolution between peaks in chromatography depends on the efficiency of the column, capacity factor and selectivity. Ideally, the value of *Rs* should be greater than 1 to achieve good separation between two analytes. Trial method 1 had very poor resolution factor (*Rs*= 0.39) between the peaks of interest. Therefore, further trials to optimize the gradient were performed so as to increase the resolution to a maximum possible value for the development of a more effective method for purification of γ-conglutin.

Different reasons can be responsible for low resolution between impurities and γ-conglutin peak. One of the reason could be the occurrence of other types of non-specific bindings such as van der Waal's forces and non-polar interactions in addition to ion exchange interactions. Also, retention in ion exchange chromatography sometimes depends on the effective charge on one part of protein rather than net charge (Stout, Sivakoff, Ricker, & Snyder, 1986). In general, the chromatographic process depends on various parameters such as conductivity, ionic strength, flow rate, elution gradient, sample loading and these parameters in a combined way affect the resolution between peaks. Hence, the cation exchange column process for purification of γ-conglutin needed to be studied and optimized with respect to all these parameters, in order to increase the resolution. The following section describes the effect of process parameters on a resolution between impurities and γ-conglutin peak. All these

parameters were studied and optimized by 'one variable at a time' approach in a column process and results were analysed by the findings of the chromatograms obtained and SDS- PAGE of the resulting fractions. Both the parameters (SDS-PAGE and chromatogram) in this study were qualitative and not quantitative to be used as a response for DoE optimization. The lack of a quantitative response (e.g. % purity of γ-conglutin on HPLC basis) for DoE optimization of chromatographic process was due to commercially unavailability of γ-conglutin standard. Therefore, γ-conglutin standard in bulk was produced through optimized chromatographic process and, then used as a standard for developing HPLC method for its analysis (as described in section 5.4.5, Chapter 5).

Effect of the conductivity of the process

Initially, the effect of the conductivity of the process on a resolution between impurities and γ-conglutin peak was studied, because increase in the conductivity of equilibration as compared to sample load can decrease the binding capacity of ion exchange adsorbent (Johansson et al., 2003) (Tek, Turhan, & Etzel, 2005). This was assumed to be one of the reasons for the interference caused by the impurities with the elution of γ-conglutin, as the conductivity of sample loaded (2.7 mS) was three times higher than the conductivity at which the column was equilibrated (0.9 mS). Therefore, the protein impurities from the extract might have replaced the equilibration counter ions bound to the functional group of the adsorbent (non-specific binding of impurities to the adsorbent).

Hence, experiments were performed in two ways to match the conductivity of equilibration and conductivity of sample load. The first way was to reduce the conductivity of sample load from 2.7 mS to 0.9 mS by desalting it with a 1 kDa centrifugal filter. And the second way was to increase the conductivity of the equilibration from 0.9 mS to 2.7 mS by using 25 mM sodium phosphate buffer at pH 6.5 as an equilibration buffer. In both the trials, the resolution between impurities and γ-conglutin peak was found to be the same (Rs = 0.39). This indicates that increase or decrease in conductivity did not aid in selective capture and elution of γ-conglutin from the extract. Therefore, the conductivity of the sample load or the equilibration buffer was not changed in the process development to reduce the process steps (first case- use of membrane filtration to reduce the conductivity of load) or to reduce salt

utilisation (second case- use of 25 mM sodium phosphate buffer to increase the conductivity of equilibration buffer). Further parameter studies were thus performed by keeping the conductivity same (sample load- 2.7 mS and equilibration buffer- 0.9 mS) as the initial process.

Effect of the method flow rate

The use of high flow rates in trial method 1 (method flow rate 2 ml/min and sample loading flow rate 1 ml/min, as described above in this section) was considered to be one of the reasons for the loss of γ-conglutin in flowthrough and column wash step. Therefore, the study of the effect of method flow rate on binding and elution of γ-conglutin was required in order to minimize its loss in earlier fractions of the process. The optimum/lower flow rates are reported to allow enough time for sample components to diffuse in and out of the pores as they bind to or dissociate from the functional groups (Biosciences, 2002). As a result, lower flow rates are recommended during sample application and elution steps, whereas higher flow rates in a working range can be used for equilibration and regeneration steps. This principle was used as a basis for selecting a suitable flow rate for the sample loading and elution steps in the present study.

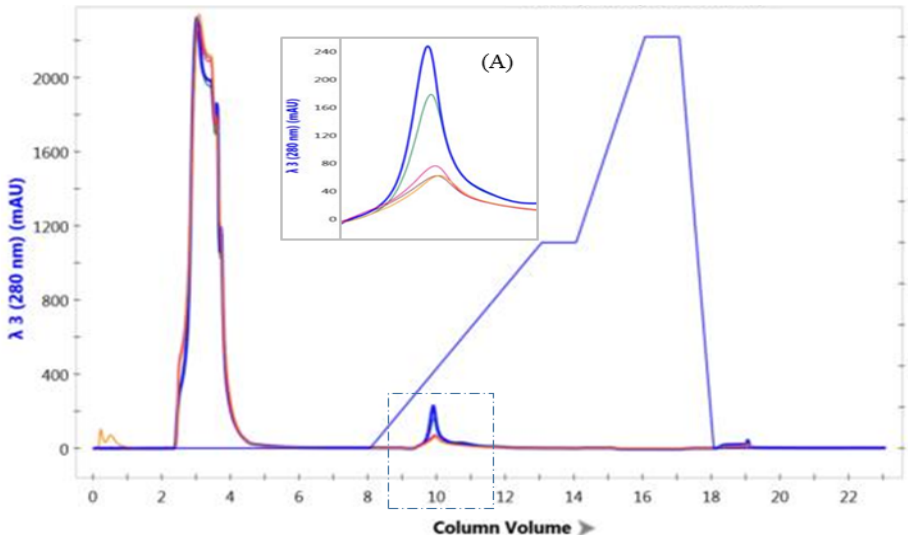

Figure 4.6 Overlay of chromatograms at different flow rates
(Blue: 0.5, green: 1, pink: 1.5, brown: 2, orange: 2.5 ml/min)

The effect of flow rate on the complete capture of γ-conglutin was studied as stated in section 4.3.5, by varying the flow rate of sample loading and elution steps between 0.5 ml/min to 2.5 ml/min. The results were analysed based on chromatograms obtained,

area under curve (AUC) of both the peaks and SDS-PAGE (non-reducing) of the fractions. The overlay of chromatograms obtained by trial method 1 at different flow rates is represented in Figure 4.6. It was clearly observed from Figure 4.6 and area under curve of the peaks, that the reduction in the flow rate from 2.5 ml/min to 0.5 ml/min led to decrease in the intensity of the first peak (flowthrough and column wash fraction) and increase in the intensity of the second peak (γ-conglutin) (Figure 4.6 A). This suggests that the lower flow rates were assisting γ-conglutin to bind to the adsorbent by allowing sufficient time to diffuse through the pores and interact with the ligand molecules.

These results were further confirmed by SDS-PAGE (non-reducing) analysis of the fractions (Figure 4.7). Lane 1 (Figure 4.7) in all the gels represent pH 6.5 modulated extract which was loaded on the column at different flow rates. This fraction majorly represented γ-conglutin along with IMW and LMW units of β-conglutin and basic units of α-conglutin (having pI in the range of γ-conglutin). SDS-PAGE profile of flowthrough fractions at different flow rates (lane 2 of respective gels) exhibited the absence of γ-conglutin (band at 48 kDa) at the lowest flow rate of 0.5 ml/min. This confirms complete binding of γ-conglutin to the functional groups of the adsorbent at the flow rate of 0.5 ml/min. Minor quantities of γ-conglutin were observed in the flowthrough fraction when the sample was loaded at the flow rate of 1 ml/min. Beyond 1 ml/min, at all the flow rates, γ-conglutin was seen clearly in flowthrough fractions, indicating its only partial binding to the adsorbent at higher flow rates. Therefore, the flow rate of 0.5 ml/min was selected for loading the sample on the column.

The flow rate of 0.5 ml/min (linear velocity 64.20 cm/hr) was observed to be suitable for column washing step also, as it did not wash out the bound γ-conglutin (lane 3, Figure 4.7). The elution fractions at different flow rates are shown in lane 4 and 5 of all gels. The second peak when observed on chromatogram and SDS-PAGE showed similar impurity profile as the previous experiments. This indicates that the lower flow rates could increase the overall binding of γ-conglutin, but did not increase the resolution between impurities and γ-conglutin peak (Rs= 0.39).

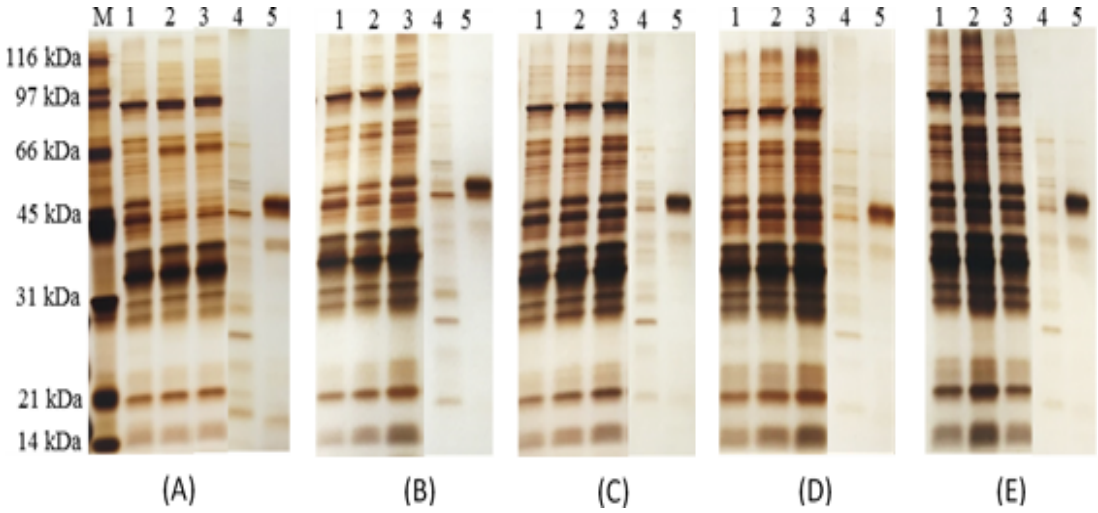

Figure 4.7 SDS-PAGE of fractions of the flow rate study
(A: 0.5 ml/min, B: 1 ml/min, C: 1.5 ml/min, D: 2, E: 2.5 ml/min)
(M: marker, L1: extract, L2: flowthrough fraction, L3: column wash fraction, L4: Elution 13-20% B, L5: Elution 20-35% B)

Effect of elution gradient

In ion exchange chromatography, the retention of the target molecule on the column majorly depends on the ionic composition of mobile phase and the way in which gradient of the ionic solution is implemented (Gibbs & Lightfoot, 1986). The elution gradient in the chromatographic process can be performed either as linear or step elution or a combination of both, according to the aim of separation. The optimum gradient is generally designed in a way to get high resolution, reduced buffer/salt consumption and faster separation times (Biosciences, 2002). Therefore, following gradients were executed to design the best possible elution gradient, confirming the optimum gradient characteristics.

Step gradient

Stepwise elution of bound proteins was carried out to check the group elution of impurities and to predict the conductivity at the respective percentage of buffer B, so as to optimize the range of gradient suitable for the selective elution of γ-conglutin. Based on the results of a linear gradient (trial method 1), a step gradient was performed at every 10% B as represented in Table 4.5. The chromatogram obtained (Figure 4.8) when observed on zoomed scale, indicated the splitting of the elution peak (Figure 4.8 A) in different peaks at every step of the gradient. SDS-PAGE (not shown here) profile

of the elution until 20% B exhibited impurities in different groups corresponding to IMW units of β-congltuin.

The peak eluting at the start of 30% B (Figure 4.8 A) was collected in two different fractions as it was observed to split into two merged peaks. SDS-PAGE analysis of both of these fractions showed similar profile consisting majorly of γ-conglutin (48 kDa) along with some minor impurities (IMW units of β-conglutin). The resolution between impurities and γ-conglutin peak (*Rs*= 0.44) was observed to be improved compared to trial method 1 but still less than that required to achieve the complete resolution between the impurity and γ-conglutin peak. The step gradient results indicated that the elution of γ-conglutin enriched peak was in between 18-30% B and therefore this particular gradient was made shallower in next trial, with the aim of achieving even better resolution.

Table 4.5 Chromatographic method steps (trial 2- step gradient)

Method step	**Initial % B**	**Final % B**	**Column volume**
Equilibration	0	0	2
Sample application	0	0	1
Column wash	0	0	5
Elution 1- step	5	5	3
Elution 2- step	10	10	3
Elution 3- step	20	20	5
Elution 4- step	30	30	5
Elution 5- step	40	40	5
Elution 6-linear	40	100	1
Regeneration	100	100	2

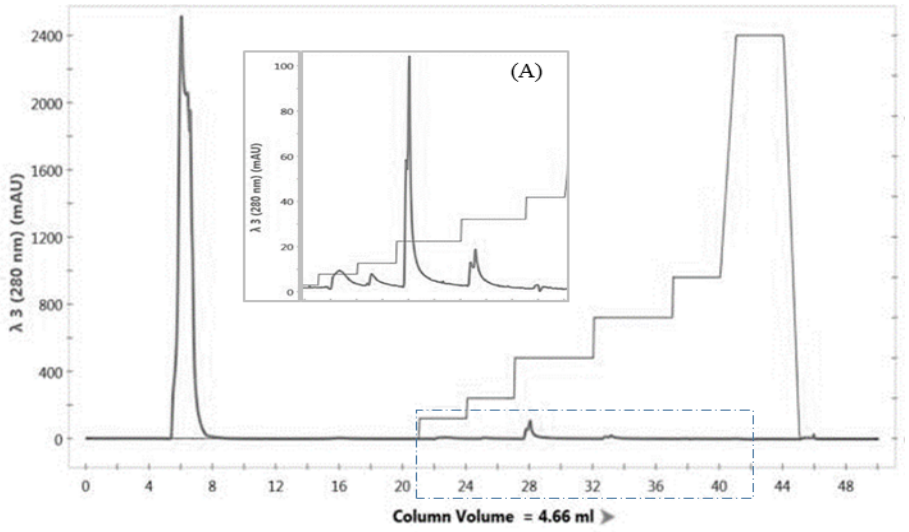

Figure 4.8 Chromatogram obtained with trial method 2

Shallow and long gradient

Shallow and long gradients of a known percentage of elution buffer are generally given to obtain the maximum resolution between the peaks (Biosciences, 2002). Hence, trial method 1 (linear gradient) and trial method 2 (step gradient) were carefully analysed, and the gradient between 0-30% B was made shallower and longer (Table 4.6). Initially, the gradient was made shallower for 10 CV and later it was varied to find the optimum CV required for the desired resolution.

Table 4.6 Chromatographic method steps (trial 3- shallow gradient)

Method step	Initial % B	Final % B	Column volume
Equilibration	0	0	2
Sample application	0	0	1
Column wash	0	0	5
Elution 1- linear	0	30	10
Elution 2-isocratic	30	30	5
Elution 3-linear	30	100	1
Regeneration	100	100	2

The chromatogram obtained with the shallow gradient is as given in Figure 4.9. SDS-PAGE (non-reducing) analysis (Figure 4.10) of the flowthrough (lane 2) and column wash fractions (lane 3) was observed to be similar to the previous trial methods. Whereas, unlike in the previous trials, the second peak (Figure 4.9 A) was seen to be

resolved in three different peaks. The second peak (Figure 4.9 A) started eluting when the conductivity of buffer reached from 0.8 mS to 3 mS (5% B). This fraction was observed to show the presence of the major impurities (IMW units of β-conglutin) as analysed by SDS-PAGE (lane 4).

When the conductivity of the process was reached to 7 mS (8% B), the second peak was observed to split into a third peak (Figure 4.9 A), which continued to elute until 13 mS (15% B). SDS-PAGE profile of the third peak (lane 5) represented traces of impurities along with the elution of γ-conglutin (48 kDa). At the end of 15% B, the fourth peak (Figure 4.9 A) started eluting until 20% B (16 mS). Though the fourth peak was observed to be smallest of all the peaks, it was found to have a more concentrated fraction of γ-conglutin with fewer impurities (lane 6) as compared to previous trials.

Shallower gradient enhanced the resolution between impurities and γ-conglutin peak (*Rs*=1.19). This indicates 98% separation between γ-conglutin peak and impurity peak with 2% overlap (Wilson & Walker, 2010). Though the elution peaks were resolved well with fewer impurities in the fourth peak, the loss of γ-conglutin in the third peak was higher as the change in the conductivity was drastic in this method gradient. To reduce the long separation times resulting from shallower and longer gradient, many screening trials of varying column volumes were performed. As a result, 7 column volumes of 0-30% B gradient was found to be optimum to provide the same results.

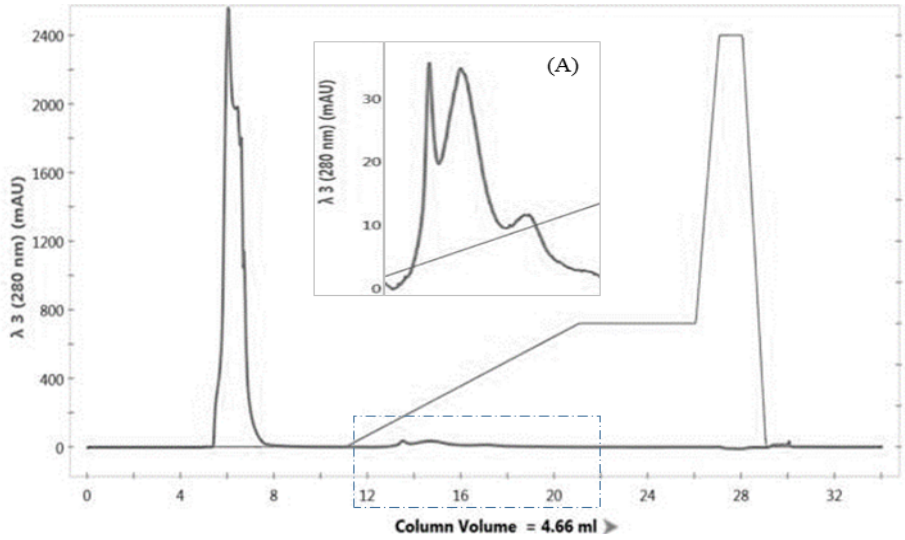

Figure 4.9 Chromatogram obtained with trial method 3

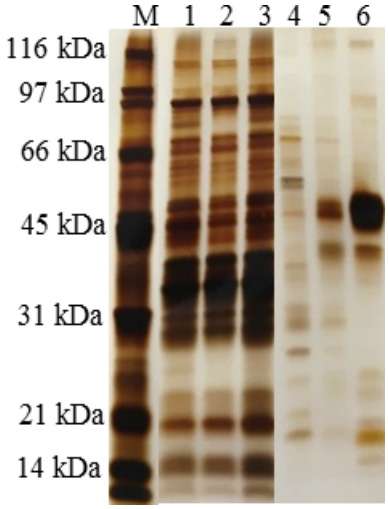

Figure 4.10 SDS-PAGE of fractions from trial method 3
(M: marker, L1: pH 6.5 altered extract, L2: flowthrough fraction, L3: column wash fraction, L4: second peak, L5: third peak, L6: fourth peak)

It was observed from the above gradient trials that, 20% B was enough to elute all the bound proteins from the adsorbent. Therefore, further optimization trials were performed with 0.5 M sodium chloride in buffer A instead of 1 M sodium chloride, so as to reduce the consumption of salt required for the process. To confirm the process with 0.5 M sodium chloride, trial method 3 was repeated with 0.5 M sodium chloride, and the obtained chromatogram and SDS-PAGE results were found to be similar to 1 M sodium chloride. Only the percentage of elution buffer required for displacing γ-conglutin from the adsorbent was increased (19-30% B required) by almost 1.5 times as compared to 1 M sodium chloride (13-20% B), which was due to a reduction in molarity of the elution buffer. The rest of all the parameters including conductivity required for elution of γ-conglutin were observed to be same as previous (fourth peak eluted at a conductivity of 13-16 mS, which was observed during 19-30% B). The gradient thus required further optimization to minimize the loss of γ-conglutin in earlier peaks and to increase the resolution between the target peaks.

Combination of step and linear gradient

The combination of step and the linear gradient was implemented to obtain faster separation times. A step gradient of 17% B was given for 5 CV to elute the impurities in a group and then the gradient was made linear from 17-30% B in 5 CV. This combination of step and the linear gradient was observed to less effective in increasing

resolution (*Rs*= 0.99), as γ-conglutin peak was seen to merge with the impurity peak. The reason for this merging of peaks could be the fact that step elution is technically simple but sharp changes in ionic strength can cause elution of the target protein along with the impurities (Biosciences, 2002). This indicates that a full linear gradient would be most effective for selective elution of γ-conglutin with minimum loss in early elution steps.

Optimized method gradient

The optimized method gradient was designed (Table 4.7) with all the required improvements in the previous trial methods incorporated. From the results of the earlier method trials, it was clear that the conductivity of 10.5 mS (18% B) is necessary to start the elution of γ-conglutin. Therefore, a steeper linear gradient was implemented in two steps, first from 0-17% B in 5 CV to elute bound impurities, and second from 17-30% B in 5 CV to selectively elute γ-conglutin.

Table 4.7 Optimized method gradient

Method step	**Initial % B**	**Final % B**	**Column volume**
Equilibration	0	0	2
Sample application	0	0	1
Column wash	0	0	5
Elution 1- linear	0	17	5
Elution 2- linear	17	30	5
Elution 3-linear	30	100	1
Regeneration	100	100	2

The chromatogram obtained with the optimized method is as shown in Figure 4.11. The nature of the first peak (flow thought and column wash) was found to be similar to previous trials. However, the second peak was observed to be different than previous trials (Figure 4.11 A). The second peak started eluting at 5% B (conductivity 3.2 mS) and continued till 16% B (condcutivity10 mS). Once the gradient of 0-17% B was completed, the third peak started eluting as conductivity was reached to 10.5 mS and it continued till 16 mS (30% B). The merging of impurity peak (second peak) and γ-conglutin peak (third peak) was seen to be reduced to 0.2% as the value for resolution

factor was improved to 1.59, which is good enough for achieving the desired separation between two molecules.

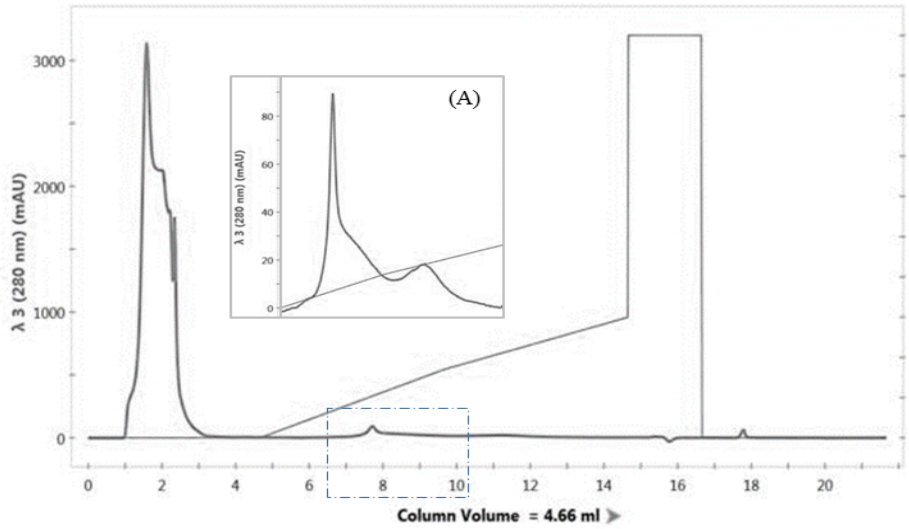

Figure 4.11 Chromatogram obtained with the optimized method gradient

SDS-PAGE profile (Figure 4.12) of the flowthrough fraction (lane 2) did not show any band at 48 kDa, which indicates the complete capture of γ-conglutin on the adsorbent at the flow rate of 0.5 ml/min. Also, the ionic interactions between γ-conglutin and ligands were strong enough to prevent any elution of γ-conglutin during the column washing step (lane 3). The protein profile of the second peak (lane 4) showed the major IMW and LMW β-conglutin impurities, which were competing with γ-conglutin for binding to the adsorbent. Also, this fraction did not show the presence of γ-conglutin at 48 kDa, which confirms that the optimized gradient is well suited for its selective elution. The third peak (Figure 4.11 A) as shown in lane 5, exhibited the purest γ-conglutin fraction with no impurities. The reducing SDS-PAGE and densitogram analysis of peak 3 fraction were also performed for confirming the identity of γ-conglutin (discussed in Chapter 5).

Further, the third peak fraction (Figure 4.11 A) was collected and desalted completely using 10 kDa membrane filtration (Minimate tangential flow filtration capsules, Pall Corporation, Perth, Western Australia). The membrane was connected to the peristaltic pump (Masterflex, John Morris Scientific, Perth, Western Australia) through respective inlet and outlet tubings (Masterflex, John Morris Scientific, Perth, Western Australia). The cross flow rate of 30 ml/min was maintained for the filtration process. Initially the membrane was washed with ultrapure water to remove the traces of the

preservatives. The sample (third peak fraction) was then subjected to buffer exchange through this membrane with ultrapure water. The process of buffer exchange was continued for four cycles until conductivity of the retentate became equal to the conductivity of ultrapure water (0 mS). The permeate was checked for protein content (BCA analysis) and protein profile (SDS-PAGE), which did not show the presence of γ-conglutin, indicating no loss of γ-conglutin in permeate during membrane filtration. The resulting retentate was filtered through 0.45 µm PVDF syringe filter and freeze-dried (Alpha 1-4 LDplus Freeze dryer, John Morris Scientific, Australia). Freeze drying cycle was set as -60^{0}C (12 hr), -30^{0}C (6 hr), -10^{0}C (4 hr) and 0^{0}C (4 hr). The freeze-dried product was then stored at -20ºC until further analysis.

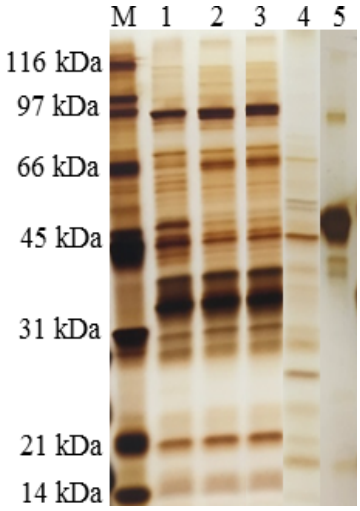

Figure 4.12 SDS-PAGE of fractions from the optimized method gradient (M: marker, L1: pH 6.5 modulated extract, L2: flowthrough fraction, L3: column wash fraction, L4: second peak, L5: third peak)

Freeze-dried γ-conglutin was first analysed for its protein content (by elemental analyser), which was observed to be 94.86% w/w. This indicates that the obtained freeze-dried fraction is ~95% w/w pure γ-conglutin. The remaining 5% w/w could be either moisture or bound carbohydrates (Marcello Duranti et al., 1981), the nature of which will be analysed in further studies. Freeze-dried γ-conglutin was further characterized (discussed in Chapter 5) by different analytical techniques such as RP-HPLC, reducing and non-reducing SDS-PAGE, FTIR, MS and western blotting for confirmation of its identity.

Thus, the developed process for purification of γ-conglutin from *Lupinus angustifolius* (Coromup variety) is the first method reported to produce the purest fraction of γ-

conglutin (95% w/w) with least possible process steps. The selective extraction at acidic pH and a simple intermediate step of pH modulation before cation exchange chromatography led to the reduction in overall steps in the conventional process of γ-conglutin purification as represented in Figure 4.13.

The yield and recovery of the process were calculated on % extraction (w/w) of the proteins at each stage of the process (Table 4.8). The calculations were done on total seed basis and on total globulin basis so as to compare with the reported value of γ-conglutin yield (~3-4% w/w of total globulins) (Blagrove et al., 1980; Marcello Duranti et al., 1981). The optimized process yielded ~2.20% (w/w of total globulins) γ-conglutin with highest reported purity of 95%, indicating 76.92% recovery of the process. The overall summary of the process and stages in the optimization of the process are represented in Table 4.9.

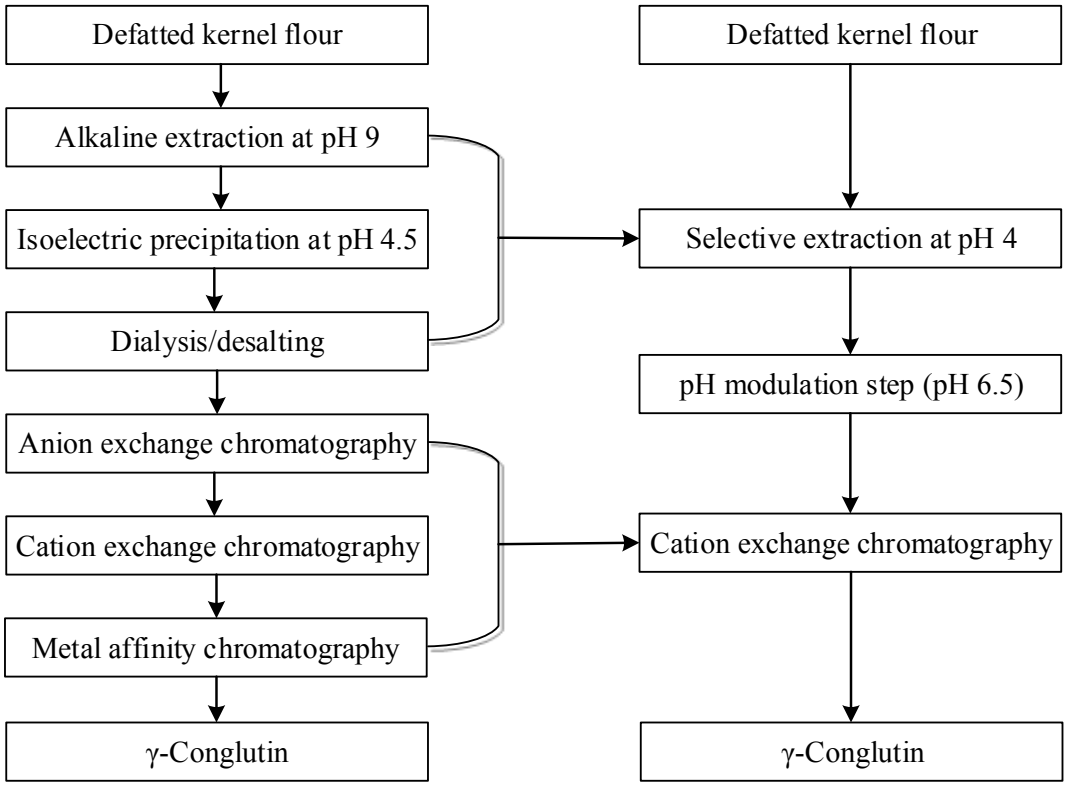

Figure 4.13 Comparison of conventional process (A) and developed process (B)

Table 4.8 % w/w extraction of protein at each step of the optimized process

Starting material	% w/w	Amount (gm)
Total seeds	100%	100.00
Kernels	75% w/w of seed	75.00
Kernel total proteins	42% w/w of kernels	31.50
Globulins	87% w/w of kernel proteins	27.40
pH 4 extract	15% w/w of globulins	4.11
pH 6.5 modulated extract	83% w/w of pH 4 extract	3.41
Cation exchange chromatography	18% w/w of sample loaded	0.60

Once the purification process was optimized, enough amount of γ-conglutin was produced for further studies, by increasing the volume of sample loaded on the column. Initially, pre-packed HiScreen Capto S column (Table 4.2) was used to check the effect of sample overloading on the purity of γ-conglutin obtained. The volume of sample loaded was decided based on the maximum binding capacity of Capto S (120 mg/ml) provided by the manufacturer (Table 4.1) and the concentration of pH 6.5 modulated extract load (1.23 mg/ml as analysed by BCA). As a result, 450 ml of pH 6.5 modulated extract was loaded on HiScreen Capto S, followed by the separation of γ-conglutin by the optimized method gradient (Table 4.7) with all the other parameters same as mentioned earlier (Section 4.5.3). The chromatogram and SDS-PAGE (non-reducing) obtained with the optimized method gradient are as shown in Figure 4.14 A and B respectively.

In Figure 4.14 A, F1 represents flowthrough fraction, which did not show the presence of γ-conglutin at 48 kDa when analysed by SDS-PAGE (lane 1, Figure 4.14 B). Similarly, the column wash step F2 (Figure 4.14 A) also did not wash bound γ-conglutin (lane 2, Figure 4.14 B), indicating no weaker interactions between γ-conglutin and functional groups on the adsorbent. The fraction F3 represents the elution of impurities between 0-17% B, which showed the major impurities of IMW β-conglutin as explained in the previous section. The fraction F4 which was eluted between 17-30% B (Figure 4.14 A), shows the presence of γ-conglutin predominantly at 48 kDa (lane 4, Figure 4.14 B).

Table 4.9 Summary of process optimization for purification of γ-conglutin

Process steps	**parameter study**	**Results**	
		Qualitative	**Quantitative**
Extraction	pH based extraction	Selective extraction of γ-conglutin was observed at acidic pH of 4 on account of its peculiar pI in the basic range.	~15% w/w γ-conglutin enriched fraction
Intermediary step	Modulating pH of γ-conglutin extract to pI of impurities	Adjusting the pH of γ-conglutin enriched fraction to 6.5 has reduced a load of impurities from the extract.	~17% reduction of impurities
Cation exchange chromatography (HiScreen Capto S)	Effect of conductivity	Maintaining conductivity of load and equilibration same did not improve binding of γ-conglutin to the adsorbent.	*Rs*=0.39*
	Effect of flow rate	Decreasing flow rate from 2.5 to 0.5 ml/min allowed γ-conglutin to diffuse through the pores well, increasing the residence time in the adsorbent.	*Rs*= 0.39*
	Linear gradient	Linear gradient predicted that 0.3 M sodium chloride is enough to elute all the bound proteins.	*Rs*=0.39*
	Step gradient	γ-conglutin starts eluting with impurities from 15% B and continues till 30% B.	*Rs*=0.44*
	shallow gradient	Shallowing the gradient between 17-30% B has reduced impurities from the final fraction.	*Rs*= 1.19*
	Step and linear gradient	Step gradient for 17% B elutes impurities along with γ-conglutin, due to a sudden change in conductivity.	*Rs*=0.99*
	Optimized gradient	This method gradient gives minimum separation time with a maximum possible resolution between peaks.	*Rs*=1.59*

*Resolution (*Rs*) is mentioned with reference to impurities and γ-conglutin peak.

SDS-PAGE profile of γ-conglutin produced by increasing the volume of the sample load was found to be similar to that of 5 ml load (lane 5, Figure 4.12). Once optimum conditions for γ-conglutin purity and yield were determined through HiScreen column experiments, the results were then used to design larger column (column volume 20 ml) for production process.

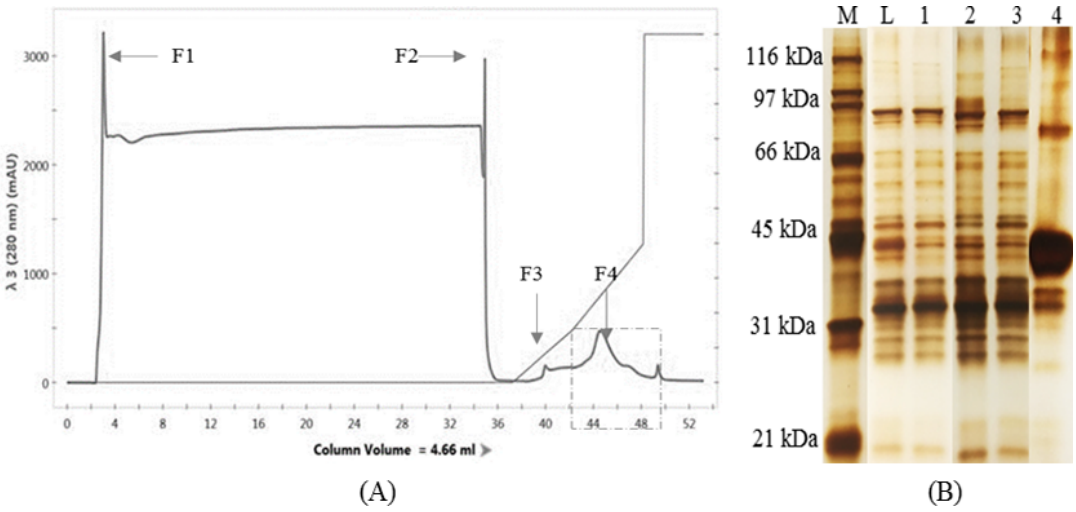

Figure 4.14 Chromatogram (A) and SDS-PAGE (B) of HiScreen Capto S column experiment

SDS-PAGE (B)- M: molecular weight marker, L: pH 6.5 load, L1: flowthrough fraction, L2: column wash fraction, L3: impurity peak, L4: γ-conglutin peak

In order to have successful scale up, it is desirable to maintain kinetic and dynamic parameters equivalence between columns of small scale and the large scale process. This can be achieved by using identical stationary phase, mobile phase, linear flow velocity, loading (mg of product/ml of adsorbent), feed concentrations, gradient length and gradient slope (Rathore & Velayudhan, 2003). To handle the increased volume at large scale, the most common procedure is to increase the column volume by increasing the column diameter (constant linear velocity scale-up) or by keeping the constant aspect ratio i.e (column length/diameter ratio) to maintain the constant residence time throughout the scale-up.

Therefore, XK 16/20 glass column was packed with Capto S manually as explained in the Section 4.2.2. The bed height was maintained at 20 cm so as to keep same aspect ratio as HiScreen Capto S column. The linear velocity (64.20 cm/hr) was also kept same as HiScreen column process to ensure constant process cycle time. As a result,

the flow rate was changed to 2.15 ml/min and the volume of sample loaded was increased up to 1930 ml. The separation was performed with the same optimized method gradient (Table 4.7) by keeping all the other parameters same as explained in the Section 4.5.3. The chromatogram and SDS-PAGE obtained with 20 ml column are as shown in Figure 4.15 A and B respectively.

As shown in Figure 4.15, the elution pattern (Figure 4.15 A) and SDS-PAGE (Figure 4.15 B) of γ-conglutin were observed to be as similar as HiScreen Capto S process (Figure 4.14). Thus, keeping same aspect ratio and linear velocity assisted in scaling up the process from 5 ml column volume to 20 ml column volume. The same column (20 ml) was then further used to produce γ-conglutin sample for further analysis.

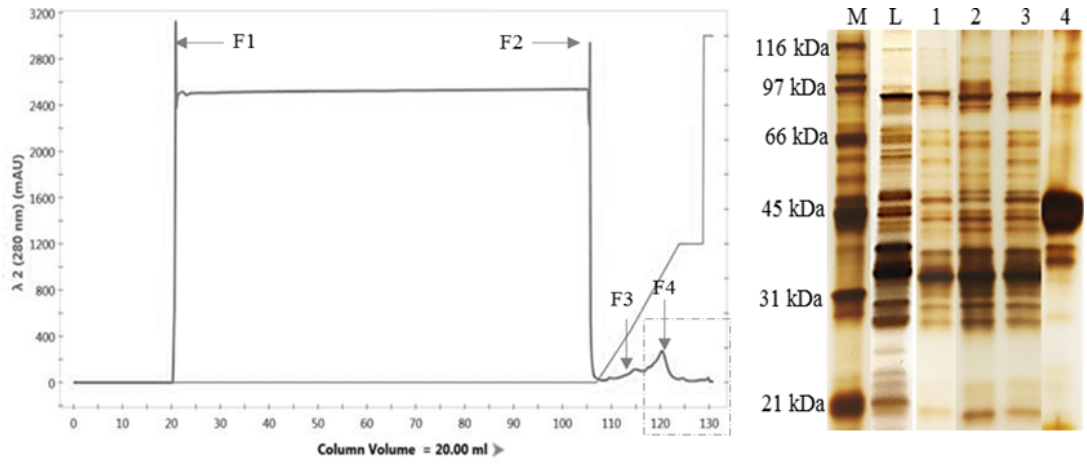

Figure 4.15 Chromatogram (A) and SDS-PAGE (B) of 20 ml column experiment SDS-PAGE (B)- M: molecular weight marker, L: pH 6.5 load, L1: flowthrough fraction, L2: column wash fraction, L3: impurity peak, L4: γ-conglutin peak

4.5.4 Adsorption isotherm analysis

Langmuir and Freundlich adsorption isotherm models are commonly used to evaluate adsorption processes for proteins (Freundlich, 1906; Langmuir, 1916). The Langmuir isotherm model is based on the equilibrium between adsorbed and free protein molecules during adsorption process and assumes no multilayer formation (Langmuir, 1916). The Freundlich isotherm assumes multilayer formation during adsorption process (Freundlich, 1906), and is used when the adsorption process behaviour can not be described by Langmuir isotherm model. The batch adsorption isotherm data for binding studies of γ-conglutin at different initial fluid phase concentrations on Capto

S is as shown in Figure 4.16. From Figure 4.16, the binding of γ-conglutin on Capto S was observed to follow Langmuir pattern, indicating favourable adsorption (Langmuir, 1916).

The adsorption isotherm fit to Langmuir and Freundlich isotherm model was evaluated by regression constant (R^2). For Langmuir isotherm model, '1/q' was plotted against '1/ C' (Figure 4.17 A), whereas 'Log q' was plotted against 'Log C' for Freundlich isotherm model (Figure 4.17 B). The regression constants greater than 0.95 indicate the suitability of both isotherm models for describing adsorption behaviour of γ-conglutin on Capto S. Isotherm parameters, such as maximum binding capacities and adsorption intensity (Table 4.10) were further analysed to highlight the physical significance of γ-conglutin binding on Capto S.

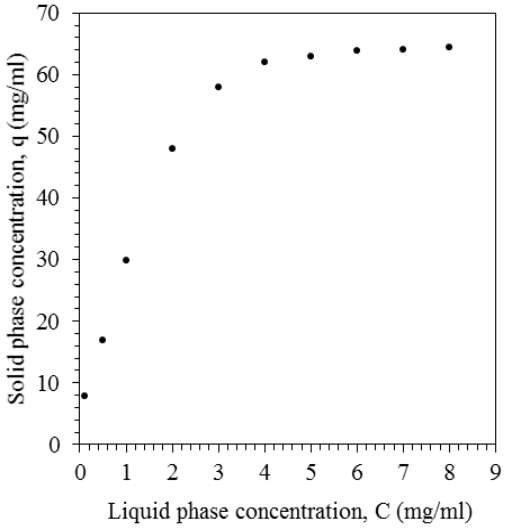

Figure 4.16 Batch equilibrium data for binding of γ-conglutin on Capto S

Initially, the Langmuir equilibrium constant (R_L) was calculated (equation 4.2) to decide the type of isotherm favoured for the adsorption of γ-conglutin on Capto S (Kumar et al., 2014). The isotherm is considered as favourable when $0< R_L<1$, whereas $R_L= 1$ indicates linear isotherm and $R_L> 1$ represents unfavourable isotherm (Giles, MacEwan, Nakhwa, & Smith, 1960). The equilibrium constants (R_L) obtained from the adsorption isotherm data were in the range of zero to one (Table 4.10) for all the initial fluid phase concentrations investigated, indicating favourable isotherm.

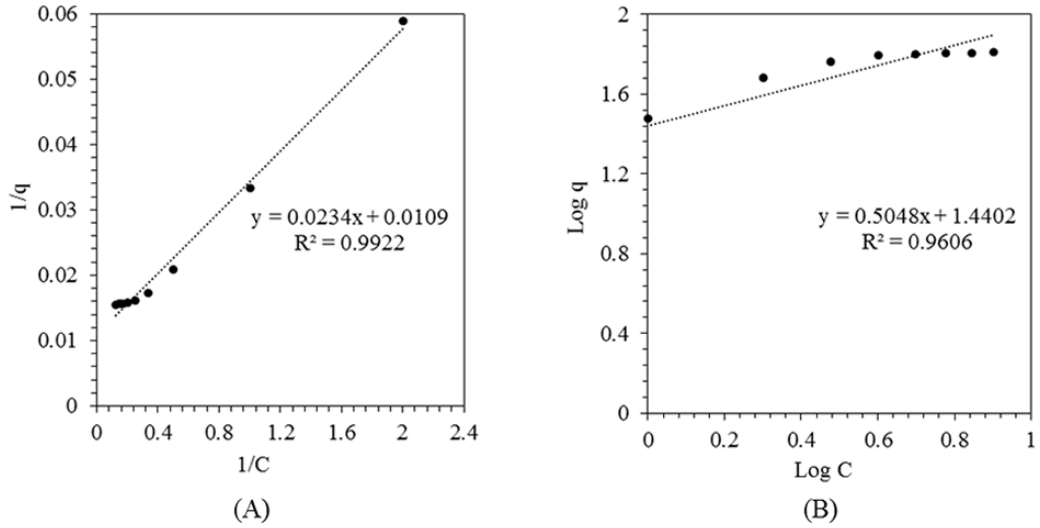

Figure 4.17 Fitting of batch equilibrium data to Langmuir (A) and Freundlich (B) isotherm models

The factor (1/n) from an equation (4.3) indicates the adsorption to be favourable if $0<1/n<1$. The value of slope (Figure 4.17 B) less than one represents favourable adsorption isotherm. The adsorption intensity (n) was also calculated (equation 4.3) to specify the adsorption mechanism (Schober, Bean, Tilley, Smith, & Ioerger, 2011). The value of 'n' greater than one represents monolayer adsorption (chemisorption), whereas 'n' less than one indicates multilayer adsorption (physisorption) (Schober et al., 2011). From the slope of the line of Freundlich isotherm model, the value of n was found to be greater than one (Table 4.10), specifying γ-conglutin on Capto S is a monolayer-chemisorption process. This further indicates that the Freundlich isotherm model failed to express maximum adsorption capacity of γ-conglutin on Capto S for monolayer adsorption process. Therefore, the Langmuir isotherm model was used to describe maximum binding capacity and adsorption equilibrium for γ-conglutin purification.

The maximum binding capacity, q_{max} indicates the quantity of γ-conglutin required to form a single monolayer on the surface of the adsorbent. Significant q_{max} of about 91.75 mg/ml of adsorbent was observed in the case of Capto S (Table 4.10), indicating a good amount of γ-conglutin adsorption due to the higher pore size of the adsorbent, enabling easier accessibility of the solute to ligands on the adsorbent surface. Another important parameter of the Langmuir isotherm model is the dissociation constant (K_d), which determines the strength of interaction between solute (γ-conglutin) and interacting ligands on adsorbent surface. The value of K_d should be typically between

10^{-8} M and 10^{-4} M (Yang, 2008) for effective separation of components under mild eluting conditions (Jagschies, Sofer, & Hagel, 2007). K_d higher than this indicates weaker interactions, whereas smaller K_d indicates stronger interactions which need harsh elution conditions (Yang, 2008). The equilibrium adsorption analysis data showed the value of K_d (Table 4.10) within the operating window stated above. In future, the dynamic binding capacity of Capto S for γ-conglutin will also be determined for further scale up purpose.

Table 4.10 Isotherm parameters from Langmuir and Freundlich isotherm models

Isotherm parameters	**Values**
Langmuir isotherm model	
Maximum adsorption capacity q_{max} (mg/ml Capto S)	91.75
Dissociation constant, K_d (M)	4.47×10^{-5}
R_L at C_0=1 mg/ml	0.60
R_L at C_0=10 mg/ml	0.21
R^2	0.99
Freundlich isotherm model	
Adsorption capacity, q_F (mg/ml Capto S)	27.55
Adsorption intensity, n	1.98
Adsorption intensity inverse, 1/n	0.50
R^2	0.96

4.6 CONCLUSION

The process for selective extraction and purification of γ-conglutin from *Lupinus angustifolius* seed (Coromup variety) was developed with a minimum number of process steps. The selective extraction of γ-conglutin enriched fraction (~15% w/w of total globulins) was attained by optimising pH of the medium (pH 4), solid to liquid ratio (1:30) and extraction time (30 min) by 'one factor at a time study'. The use of acidic extraction reduced major impurities of other conglutins in the initial stage itself, unlike conventional alkaline extraction. The key step of intermediary pH modulation of γ-conglutin enriched extract to 6.5 before loading on the adsorbent led to an efficient separation of γ-conglutin from other conglutins within the same range of pI.

The adsorbent screening experiments exhibited promising results with Capto S, which was further studied for its purification performance in dynamic conditions. The separation was found to be highly dependent on the flow rate and elution gradient. Strategic optimization of flow rate to 0.5 ml/min and finalised elution gradient was able to increase the resolution of γ-conglutin from rest of the impurities from 0.39 (normal linear gradient) to 1.59 (optimized gradient). The obtained γ-conglutin fraction was successfully confirmed its identity by SDS-PAGE (non-reducing). The Langmuir isotherm model described well the adsorption behaviour of γ-conglutin on Capto S, with a maximum binding capacity of 91.75 mg/ml and dissociation constant of 4.47×10^{-5} M. The optimized purification process was further scaled up from 5 ml column volume to 20 ml column volume by keeping constant aspect ratio and linear velocity. By performing optimization, the yield and purity obtained were 2.38% w/w γ-conglutin (of total globulins) and 95% respectively.

Chapter 5

Characteristic confirmation studies of γ-conglutin

Information in this Chapter has been published or will be submitted for publications as follows:

Mane S, Bringans S, Johnson S, Pareek V and Utikar R (2017). Reverse phase HPLC method for detection and quantification of lupin seed γ-conglutin. *Journal of Chromatography B*, 1063, 123-129.

Mane S, Hackett M, Johnson S, Pareek V and Utikar R (2017). FTIR spectroscopic characterization of lupin seed γ-conglutin. *Biochimica et Biophysica Acta (BBA)-Protein Structure* (to be submitted, June 2018).

5.1 INTRODUCTION

Before proceeding for further scale up, deeper insights into the characterization of γ-conglutin produced by the developed purification process was required. Therefore, different analytical techniques to characterize the identity of γ-conglutin were used. The percentage of γ-conglutin and its different oligomeric forms generated during purification process was analysed by SDS-PAGE along with scanning densitometry. Further, Western blotting was performed post SDS-PAGE as it is an efficient tool for immuno-detection of proteins, especially for proteins which are found at low concentrations in their original source (Kurien & Scofield, 2006). As a percentage of γ-conglutin in lupin seeds is lower than some of the other proteins (Blagrove, Gillespie, Lilley, & Woods, 1980), Western blotting was performed to identify γ-conglutin in its different oligomeric forms by the cross reactivity against specific polyclonal antibodies.

The structure of a protein can be modified unintentionally during processing due to exposure to high temperatures or highly acidic and alkaline conditions (Yada, 2004), which may have a direct impact on its bioactivity. Therefore, the study of the secondary structure of a protein is important to maintain its bioactivity. Few reports on secondary structure prediction of γ-conglutin by circular dichroism are available (Blagrove et al., 1980; Capraro, Spotti, Magni, Scarafoni, & Duranti, 2010; Marcello

Duranti, Gius, Sessa, & Vecchio, 1995). However, these reports did not present the detailed spectral analysis of γ-conglutin. In addition, conformational changes in the secondary structure of γ-conglutin during its purification process have not been reported so far. Therefore, FTIR analysis was carried out to analyse the secondary structure of the purified γ-conglutin and to investigate if process additives or conditions had affected the structural conformation of the purified protein.

Currently, very few reports are available on quantitation of γ-conglutin, such as indirect ELISA (enzyme linked immunosorbent assay) (Donzelli F., 2015) and HPLC-chip-multiple reaction monitoring (MRM) label-free method (Resta, Brambilla, & Arnoldi, 2012). However, the use of immuno-enzymatic methods such as ELISA have limitations of cross-reactivity or non-specific binding with other food proteins, which can lead to false results (Brambilla, Resta, Isak, Zanotti, & Arnoldi, 2009; Gan & Patel, 2013). Though HPLC-chip MRM method was observed to be promising for absolute quantitation of γ-conglutin (Resta et al., 2012), the possibility of adapting this method for routine analysis in the pharmaceutical industry is limited due to the high expenses involved in the mass spectrometry analysis. Therefore, there is a need to develop a sensitive, specific, rapid and easy method for detection and quantitation of γ-conglutin from lupin seed extract.

In recent years, HPLC with greater sensitivity and faster analysis time has emerged as one of the most important tools of analytical chemistry (Gupta, Jain, Gill, & Gupta, 2012; Snyder, 2012). In particular, reverse phase HPLC (RP-HPLC) has become the technique of choice for cost-effective analysis of bioactive proteins and peptides on account of its selectivity, excellent resolution and high accuracy in quantitation (Regnier, 1983). These features make RP-HPLC an ideal candidate for detection and quantitation of γ-conglutin. At present, only two reports are available on the use of RP-HPLC for analysis γ-conglutin. Duranti et al., have reported the use of RP-HPLC to separate glycosylated and deglycosylated forms of γ-conglutin (Marcello Duranti et al., 1995), whereas Garzon-de la Mora et al., used RP-HPLC to purify γ-conglutin and other conglutins from total globulin fraction (Garzon-de la Mora et al., 2008). As such, there is no any report available for quantitation of γ-conglutin by RP-HPLC.

Therefore, a simple, selective, sensitive and precise RP-HPLC method using a C-18 column and a linear gradient of water and acetonitrile was developed. In-house

preparation of γ-conglutin (purified as discussed in Chapter 4) was used as a standard for developing the analytical method. MS/MS analysis was performed to confirm the identity of the HPLC peak as γ-conglutin. The developed method was validated for its analytical characteristics such as specificity, linearity, sensitivity, reproducibility, precision and accuracy in accordance with guidelines provided by the Food and Drug Administration (FDA) programs for analytical methods (FDA, 2015), Association of Official Analytical Chemists (AOAC) International (AOAC, 2002) and International Conference on Harmonisation (ICH) (ICH, 2005). The method reported here is the first report on a validated RP-HPLC method for detection and quantitation of γ-conglutin from lupin seed extracts.

5.2 MATERIALS

5.2.1 Preparation of γ-conglutin standard solution

γ-Conglutin was purified from *Lupinus angustifolius* kernel flour by a combination of an acidic extraction and cation exchange chromatography as described in Chapter 4. Defatted kernel flour was extracted with 10 mM sodium acetate buffer (pH 4) for 30 min at room temperature. The suspension was centrifuged at 4700 rpm for 10 min at room temperature. The supernatant containing γ-conglutin enriched fraction was collected for further purification. The pH of obtained γ-conglutin enriched extract was modulated to pH 6.5 with 0.1 M Tris buffer under mild stirring conditions at room temperature. The resulting suspension was further centrifuged at 4700 rpm for 10 min at room temperature to separate precipitated impurities and positively charged γ-conglutin enriched supernatant. For further purification, pH modulated γ-conglutin enriched extract was loaded on Capto S packed in a column equilibrated with 10 mM sodium phosphate buffer (pH 6.5) and eluted selectively by the linear gradient of sodium chloride in equilibration buffer.

The purified protein was desalted by buffer exchange with ultrapure water through 10 kDa membrane filter (Minimate TFF capsule, Pall Corporations, Perth, WA) and freeze-dried. A stock solution of pure γ-conglutin was prepared by dissolving accurately weighed 100 mg of freeze-dried γ-conglutin in 10 ml ultrapure water (10 mg/ml) slowly under mild stirring conditions at room temperature. The resulting solution was centrifuged at 10,000 rpm for 10 min and the supernatant was filtered

through a 0.45 μm PVDF syringe filter. In-house standard solution of pure (95% w/w) γ-conglutin (10 mg/ml) was then stored at -20ºC until further analysis.

5.2.2 Chemicals and reagents

Acetonitrile and trifluoroacetic acid (TFA) of analytical grade were purchased from Sigma-Aldrich Pty Ltd (Perth, Western Australia). All solutions and buffers used for HPLC were filtered through 0.45 μm PVDF filter and degassed using a sonication bath for 10 min before use.

Nitrocellulose blot membranes for Western blotting were purchased from ThermoFisher Scientific, Perth, Western Australia. Polyclonal antibodies specific for γ-conglutin were kindly provided by Prof. Karam Singh, University of Western Australia. TBS (Tris-buffered saline/Tween) and goat anti-rabbit IgG horseradish peroxidise conjugate were purchased from Bio-Rad Laboratories Pty Ltd, Perth, Western Australia. Western Lightning Plus-ECL substrate was purchased from Perkin Elmer, Perth, Western Australia. Ponceau S staining solution was purchased from Sigma-Aldrich Pty Ltd, Perth, Western Australia.

5.3 CHARACTERIZATION METHODS

5.3.1 UV-Vis spectrometry

The UV-Vis absorption spectrum of in-house standard solution of γ-conglutin (1 mg/ml) was analysed using JASCO V-670 UV-Vis spectrophotometer (Perth, Western Australia) in the range of 200-800 nm wavelength so as to determine the absorption maxima (λmax) for γ-conglutin for further HPLC studies.

5.3.2 SDS-PAGE (reducing and non-reducing)

SDS-PAGE (reducing and non-reducing) of standard γ-conglutin solution and protein fractions generated during the process was performed as described in Chapter 3 (section 3.4.3). The obtained gels were stained with silver staining protocol and documented using gel imaging system (Versadoc, Bio-Rad Laboratories Pty Ltd, Perth, Western Australia). Image Lab 4 software associated with the imaging system was used to analyse gels and to determine the approximate percentage of γ-conglutin band in in pH 4 extract, pH 6.5 extract by studying the densitogram profile. In addition,

the percentage of different oligomeric forms of γ-conglutin produced during the purification process was predicted by scanning densitometry analysis.

5.3.3 Western blotting

Western blotting is an immunoblotting technique where specific antibodies are raised against the target protein (peptide), followed by incubating the sample containing protein and these antibodies together on the membrane. If the target protein is present in the sample, the specific antibodies bind to the target protein to form the conjugate, which is detected by chemiluminescence substrate. In the present study, Western blotting was performed to confirm the identity of the standard γ-conglutin solution.

The standard γ-conglutin solution under reducing and non-reducing conditions was subjected to SDS-PAGE as described in the Section 5.3.2, followed by transferring the unstained gel in a water in a transfer chamber. SDS-PAGE separated protein bands were transferred by dry blotting onto a nitrocellulose blot membrane (iBlot gel transfer stacks, ThermoFisher Scientific, Perth, Western Australia) using a iBlot 2 Dry Blotting System (ThermoFisher Scientific, Perth, Western Australia). The blotted membrane was then stained with Ponceau S solution for the reversible detection of protein bands on the membrane. After detection of bands, the membrane was washed with water till all staining solution wash out.

Western blots were then performed according to the method of Foley and Singh (Foley, 2002) with an overnight incubation of 1/1000 dilution of rabbit serum containing polyclonal antibodies (primary antibodies) at 4ºC under mild shaking. These primary antibodies were generated against a synthetic peptide of γ-conglutin from Agrisera (www.Agrisera.com) in a rabbit. γ-Conglutin peptide used in the production of polyclonal serum against γ-conglutin was synthesized by conjugating the peptide to keyhole limpet hemocyanin as a carrier with a maleimidocaproyl-N-hydroxysuccinimide linker (information provided by suppliers).

After incubation with primary antibodies, the membrane was washed with TBS for 30 min at room temperature, followed by 2 hr incubation with goat anti-rabbit IgG horseradish peroxidise conjugate (Bio-Rad Laboratories Pty Ltd, Perth, Western Australia). The membrane was then incubated with Western Lightning Plus-ECL substrate (Perkin Elmer, Perth, Western Australia), and the bands representing

reactivity for γ-conglutin were analysed by gel imaging system (Versadoc, Bio-Rad Laboratories Pty Ltd, Perth, Western Australia).

5.3.4 FTIR spectrometry

FTIR spectra of freeze-dried γ-conglutin standard and freeze-dried pH 4 extract and pH 6.5 modulated extract were recorded using attenuated total reflectance (ATR)-FTIR spectrometer as described in Chapter 3 (section 3.4.4). The analysis of spectra was done using 'Spectrum' software associated with the instrument. To analyse the secondary structure of the protein, second derivative spectra were determined using a 17 smoothing point savistky-golay function.

5.3.5 Reverse phase HPLC (RP-HPLC)

The HPLC system used was an Agilent Technologies 1200 series HPLC model equipped with quaternary pump module, vacuum degasser, autosampler with 100 µl injection loop, thermostatted column compartment and a diode-array detector (DAD). Data acquisition was performed by Agilent's ChemStation software. Reverse phase analysis was carried out with an Agilent Zorbax 300SB C-18 column (4.6 mm ID × 250 mm length, 5 µm particle size) at 25ºC. Mobile phase consisted of 0.1% v/v TFA in ultrapure water (phase A) and 0.1% v/v TFA in acetonitrile (phase B). Before adding TFA, both the phases were degassed using a sonication bath for 10 min.

Table 5.1 Reverse phase HPLC method for analysis of γ-conglutin

Time	**Mobile phase (%)**	
(min)	**Phase A**	**Phase B**
0	100	0
16	100	0
26	50	50
36	0	100
41	0	100
45	100	0
50	100	0

The reverse phase HPLC method used for analysis of standard γ-conglutin solution is presented in Table 5.1. A linear gradient of phase B was employed in 36 min, followed by regeneration and re-equilibration of the column in the next 14 min before further

injections. The injection volume for all samples was set to 50 µl and the flow rate of the method was maintained at 0.8 ml/min. The detection of protein was carried out at 280 nm.

Validation of reverse phase HPLC method

The performance of the RP-HPLC method for detection and quantitation analysis of γ-conglutin was validated as per FDA (FDA, 2015), AOAC International (AOAC, 2002) and ICH guidelines (ICH, 2005). The analytical characteristics validated were selectivity, linearity, quantitation and detection limits, precision, recovery and accuracy as stated further in this section.

Selectivity

Selectivity is the ability of a method to accurately assess the analyte in presence of other interfering components (matrices or potential impurities) that may be expected to be present in the sample (Gonzalez & Herrador, 2007). Specificity of a method is determined by adding specific interference (in this case, pH 4 extract) in the presence of target analyte (γ-conglutin) and evaluating the resolution of analyte peak from the nearest eluting peak in the matrix (Azilawati, Hashim, Jamilah, & Amin, 2014). Resolution (*Rs*) is expressed as the ratio of the difference in retention time (t_1 and t_2) between the two peaks to the mean of their base widths (W_1 and W_2) as given in an equation (5.1),

$$R_s = \frac{2\,(t_2 - t_1)}{W_1 + W_2} \qquad (5.1)$$

Linearity and range

Linearity study verifies the range of concentration where a response of the analyte is directly proportional to its concentration (Gupta et al., 2012). Linearity of a method was evaluated by plotting a calibration curve of peak area (Y) as a function of γ-conglutin concentration (X) using linear regression analysis. The calibration solutions were prepared in ultrapure water with ten different concentrations of γ-conglutin starting from quantitation limit, 8, 15, 25, 50, 100, 250, 500 and 1000 µg/ml. Each concentration was analysed in five independent replicates for validation of the analyte response (Green, 1996). The slope, s, intercept, c, correlation coefficient, R^2, and the respective variances were determined using the Excel linest function on peak area ratio at different calibration levels.

Sensitivity, detection limit and quantification limit

Sensitivity is the change in the analyte response divided by the corresponding change in the analyte concentration (Gonzalez & Herrador, 2007). Sensitivity of the analytical method was estimated in terms of the limit of detection (LOD) and limit of quantitation (LOQ). LOD is the lowest concentration of the analyte that can be detected and distinguished from the noise level of the system but not necessarily quantified as an exact value (Green, 1996). LOQ is the lowest concentration of the analyte which can be quantitatively determined with suitable precision and accuracy. LOD and LOQ were determined by the equations (5.2) and (5.3) respectively as suggested by ICH (ICH, 2005).

$$LOD = 3.3 \times \sigma / S \tag{5.2}$$

$$LOQ = 10 \times \sigma / S \tag{5.3}$$

Where σ is the standard deviation of the response and S is the slope of the calibration curve.

Precision

Precision is a measure of reproducibility of the analytical method and is expressed as the degree of agreement between a series of measurements obtained from the multiple sampling of the same homogeneous sample (ICH, 2005). Precision of the analytical method was determined by repeatability (intra-day) and intermediate precision (inter-day) (Sistla, Tata, Kashyap, Chandrasekar, & Diwan, 2005) at three different concentrations of γ-conglutin (0.08, 0.10, 0.12 mg/ml). Each concentration was analysed in five independent replicates. Repeatability of the method was determined by analysing the sample five times on the same day, whereas intermediate precision was assessed by analysing the sample on five different days over a period of one week (Gupta et al., 2012). The overall repeatability mean and standard deviation were calculated to determine the absolute value of the coefficient of variation (CV) which is the ratio of the repeatability, i.e. standard deviation, to its mean (Azilawati et al., 2014). The precision was expressed as the percentage of the relative standard deviation, % RSD, of responses at respective concentrations (Lindholm, 2004).

Accuracy and Recovery

The accuracy of the analytical method is the degree of closeness between the true value of an analyte in the sample and the value determined by the method (ICH, 2005). It is sometimes referred to as trueness (Lindholm, 2004). The accuracy of the method was determined by conducting a recovery experiment. Recovery was expressed as the percentage that was determined by comparing the responses of sample matrix in its original state (in this case, pH 4 extract) to the response after addition (spiking) of a known mass of analyte (γ-conglutin) (Azilawati et al., 2014; Jain, Jivani, Khatal, & Surana, 2011; Lindholm, 2004). To the pre-analysed sample matrix (pH 4 extract), a known amount of analyte (γ-conglutin) was added at 80, 100 and 120% level of the original amount and analysed by the proposed HPLC method (Jain et al., 2011).

5.3.6 Mass spectrometry (MS/MS spectrometry)

γ-Conglutin peak was collected post-HPLC (retention time 29.16 min) and dried in a SpeedVac (ThermoFisher Scientific, Perth, Western Australia). Dried γ-conglutin was digested with trypsin and generated peptides were extracted according to standard techniques (Bringans et al., 2008). Peptides were analysed by electrospray ionisation mass spectrometry using the Agilent 1260 Infinity HPLC system coupled to an Agilent 1260 Chipcube Nanospray interface on an Agilent 6540 mass spectrometer. Peptides were loaded onto a ProtIDChip-150 Agilent C-18 column and separated with a 10 min linear gradient of 5-40% acetonitrile and 0.1% (v/v) formic acid in water at a constant flow rate of 600 nL/min. A voltage of 2050 V at a temperature of 350ºC was applied between the needle and the source. An acquisition scan rate of 5 spectra/s with a collection rate of 200 ms/spectra was used for MS, whilst a scan rate of 1 spectra/s with a collection rate of 1000 ms/spectra was used for MS/MS respectively. MS data were collected in the range of 290–1700 m/z for MS and 60–1700 for MS/MS. Spectra were analysed to identify proteins of interest using Mascot sequence matching software (Matrix Science) with MSPnr100 database (Webb) and viridiplantae (Green plants) selected as the taxonomy. The MSPnr100 is a fasta file compiled from all known reference protein sequences including NCBI, Refseq, UniProt, EuPathDB and Ensembl.

5.4 RESULTS AND DISCUSSION

The characteristic analysis of γ-conglutin and starting material (pH 4 extract and pH 6.5 extract) by different techniques is as represented in the following section.

5.4.1 UV-Vis spectrum analysis

Identifying the wavelength at which maximum absorption of γ-conglutin takes place is essential parameter before setting the wavelength for post HPLC detection. Therefore, the UV absorption spectrum of pure γ-conglutin (1mg/ml) in the range of 200-800 nm wavelength was analysed as shown in Figure 5.1. The first absorption maxima for γ-conglutin was observed at 210 nm and, later it was observed at 280 nm. Proteins are generally known to absorb ultraviolet light with absorption maxima at 210 nm and 280 nm, due to the presence of peptide bond and amino acids with aromatic rings (Molina-Bolívar, Galisteo-González, Ruiz, Medina-O, & Parra, 2014).

The lesser absorption at 280 nm wavelength, could be due to the fact that γ-conglutin has the lesser percentage of aromatic amino acids (Blagrove & Gillespie, 1975). Therefore, HPLC was performed at both the wavelengths of 210 nm and 280 nm, which later was finalised to 280 nm so as to avoid the interference caused by acetonitrile in RP-HPLC at 210 nm (Snyder, 2012).

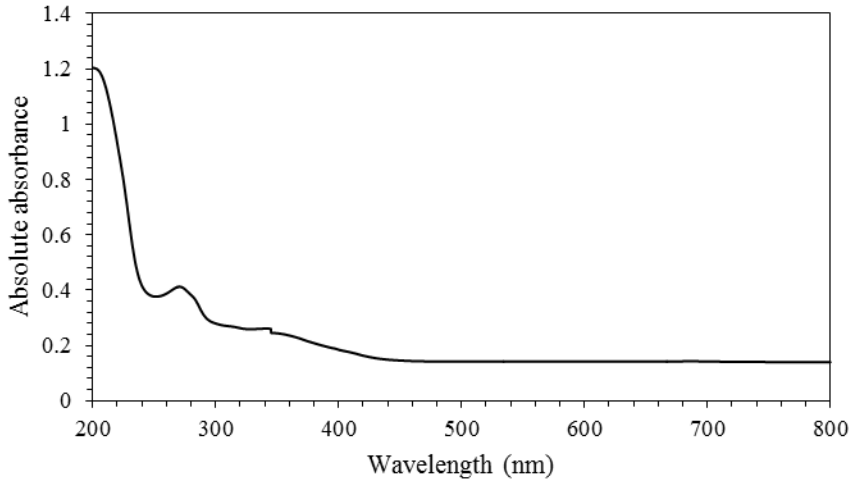

Figure 5.1 UV spectrum analysis of pure γ-conglutin (1 mg/ml)

5.4.2 SDS-PAGE (non-reducing and reducing) and densitogram analysis

The SDS-PAGE profile of pH 4 extract, pH 6.5 modulated extract and a standard γ-conglutin sample is as shown in Figure 5.2. The similar SDS-PAGE profile was

exhibited by pH 4 extract (lane 2) and pH 6.5 modulated extract (lane 3) with different conglutin fractions in the range of 10 to 100 kDa, along with the presence of γ-conglutin (48 kDa). As discussed earlier in Chapter 4 (section 4.5.2), these conglutins (impurities) can be IMW and LMW units of β-conglutin or acidic subunits of α-conglutin having their pI in the similar range as of γ-conglutin. With the implementation of the optimized cation exchange process (as discussed in section 4.5.3, Chapter 4), it was possible to purify γ-conglutin (lane 4) selectively from the extract.

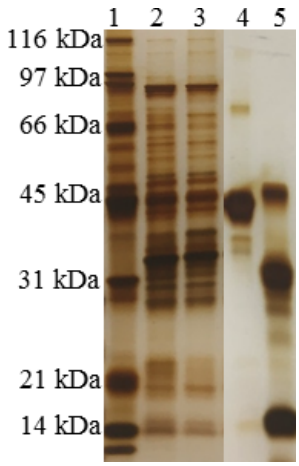

Figure 5.2 SDS-PAGE of initial extracts and standard γ-conglutin sample

(L1: marker, L2: pH 4 extract L3: pH 6.5 modulated extract, L4: γ-conglutin standard non-reduced profile, L5: γ-conglutin standard reduced profile)

The non-reducing SDS-PAGE profile of the purified γ-conglutin sample (lane 4) exhibited the presence of protein bands at 92 kDa and 48 kDa and 39 kDa. The presence of the protein band at 92 kDa and 48 kDa indicates γ-conglutin in its dimeric and monomeric form respectively, based on pH transitions during the process (Capraro et al., 2010). As pH of the optimized process was changed from the initial extraction stage and during the intermediate pH modulation step, γ-conglutin was seen to exist in its different oligomeric forms as reported by Capraro et al (Capraro et al., 2010). The details of the different polymeric forms of γ-conglutin during different stages of the developed purifcation process are represented in Table 5.2. The protein band at 39 kDa (lane 4) represents an impurity associated with purification process. This impurity can be IMW unit of β-conglutin, whose pI is very close to the pI of γ-conglutin.

SDS-PAGE (reducing) of γ-conglutin sample was also performed to confirm the identity of γ-conglutin and its polymeric forms. In reducing conditions (lane 5), the monomer (48 kDa) and dimer (92 kDa) were seen to reduce into its individual polypeptides at ~30 kDa (large chain) and ~18 kDa (small chain) through the breakage of disulphide linkage. Further, gel scanning densitometry analysis was performed to quantify the approximate percentage of γ-conglutin band in the pH 4 extract and pH 6.5 modulated extract (Kaur, Rutherfurd, Moughan, Drummond, & Boland, 2010), using Image Lab 4 software. The overlay of densitogram profile of pH 4 extract (lane 2), pH 6.5 modulated extract (lane 3), γ-conglutin in non-reduced form (lane 4) is as shown in Figure 5.3.

Table 5.2 Polymeric transitions of γ-conglutin during pH of the process

pH	**≤4**	**4.5**	**5.5**
Process	Extraction	after extraction	pH modulation
Structural transition	single polypeptide	monomer	Dimer
Molecular weight (kDa)	~30 and 18	~48	~92

Though SDS-PAGE profile of pH 4 extract and pH 6.5 modulated extract was found to be similar, the intensity at which proteins were present in these extracts was different, as analysed by densitogram profile (Figure 5.3). Both these extracts exhibited the dimeric and monomeric form γ-conglutin at 92 kDa (~3%) and 48 kDa (~15%) respectively, along with other conglutins (impurities) at different relative fronts (respective molecular weights). As stated earlier, the developed purification process was able to eliminate these conglutin impurities and produce an intense band (Figure 5.3) at 48 kDa (monomeric form), a minor band at 92 kDa (dimeric form), along with a minor impurity at 39 kDa (impurity).

For the confirmation of γ-conglutin oligomer reduction into its individual polypeptides, its densitogram profile in non-reduced and reduced conditions was compared (Figure 5.4). In non-reducing conditions, ~88% of a monomeric form (48 kDa) and ~10% of a dimeric form (~92 kDa) of γ-conglutin were observed, indicating ~98% purity of purified γ-conglutin fraction based on SDS-PAGE profile, with the presence of only 2% of impurity at 39 kDa.

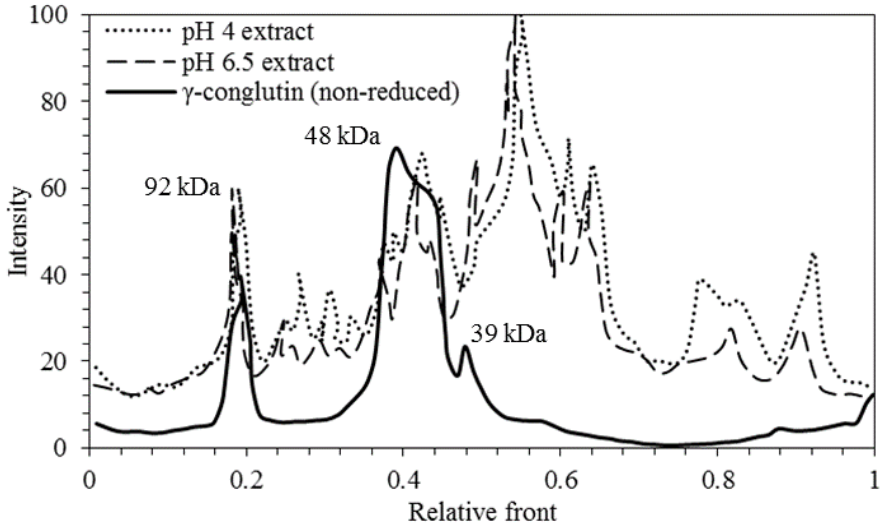

Figure 5.3 Overlay of densitogram profile of pH 4 extract, pH 6.5 modulated extract and purified γ-conglutin (non-reduced)

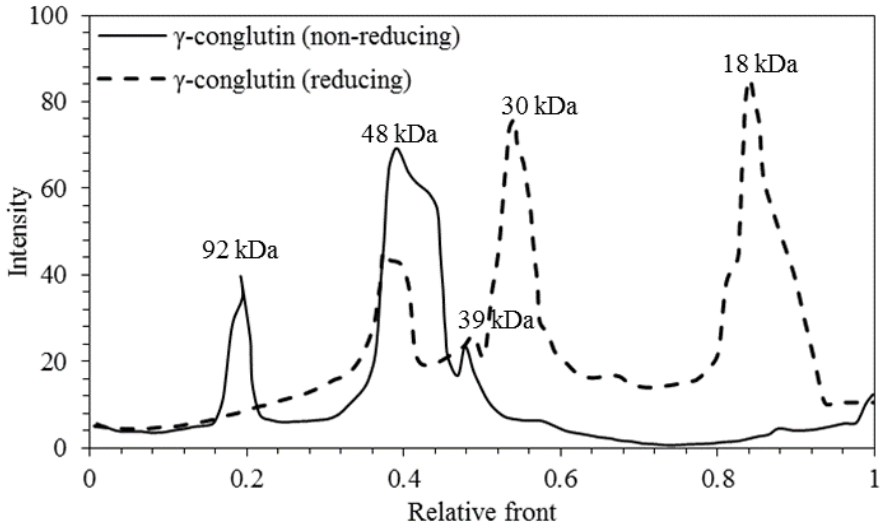

Figure 5.4 Overlay of densitogram profile of purified γ-conglutin (reduced and non-reduced form)

Under reducing conditions, densitogram profile of γ-conglutin (Figure 5.4) showed the complete reduction of its dimeric form into its disulphide bonded individual polypeptides at 30 kDa (~75%) and 18 kDa (~16%). Whereas the monomeric form of γ-conglutin was observed to reduce partially (~90% reduction) into its individual polypeptides, ~9% monomeric form was still observed under reducing conditions. The reason behind incomplete cleavage of γ-conglutin could be improper denaturation conditions or some resistance by γ-conglutin precursor to reducing conditions

(Terruzzi et al., 2011). This study of the uncleaved precursor of γ-conglutin will be carried out in the future work.

The same impurity at 39 kDa was also seen (Figure 5.4) in reduced form of γ-conglutin with the same intensity, which indicates the absence of disulphide linkages in this protein impurity. As a result, the identity of this protein impurity can be confirmed as one of the subunits of IMW β-conglutin, as β-conglutin is reported to show the absence of disulphide linkages (Melo, Ferreira, & Teixeira, 1994).

5.4.3 Western blotting analysis

Western blotting of the standard γ-conglutin sample was performed as mentioned in the Section 5.3.3. The staining of the blotted membrane with Ponceau S solution (Figure 5.5 A) exhibited the presence of γ-conglutin in its monomeric form at 48 kDa and its small polypeptide at 18 kDa, under non-reduced conditions (lane 1, Figure 5.5 A). In reduced condition (Figure 5.5 A, lane 2), γ-conglutin was reduced into its individual polypeptides at 30 kDa and 18 kDa through the breakage of the disulphide linkage. Western blotting of the same membrane (Figure 5.5 B), showed bands at 92 kDa and 48 kDa in non-reduced conditions (lane 1), whereas in reduced conditions (lane 2), bands were seen at 48 kDa and 30 kDa.

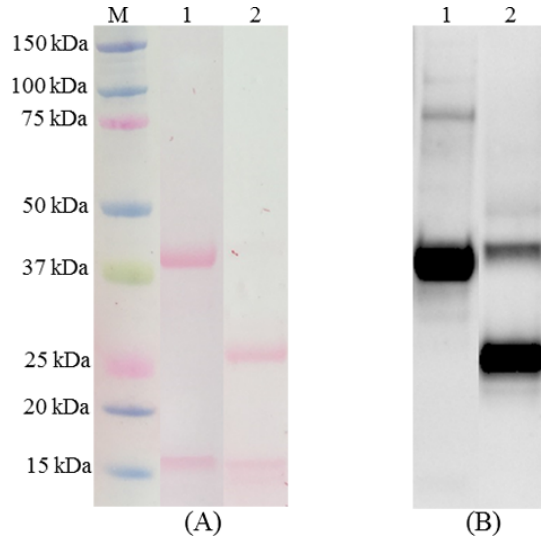

Figure 5.5 Ponceau S stained membrane (A) and Western blot (B) of γ-conglutin (M: precision plus kaleidoscope protein marker, L1: γ-conglutin non-reduced profile, L2: γ-conglutin reduced profile)

In the Figure 5.5 A, the oligomeric form of γ-conglutin at 92 kDa was not visible in non-reduced condition (lane 1), which might be due to less concentration of the protein

for its detection by Ponceau S stain. However, γ-conglutin antibody was able to detect this band even at a lesser concentration (lane 1, Figure 5.5 B). As per the information provided by the antibody supplier, γ-conglutin antibody is specific to a large subunit of γ-conglutin (30 kDa). Therefore, in western blots (Figure 5.5 B), the cross reactivity of γ-conglutin antibody was more intense with 30 kDa band and the oligomeric forms. The antibody did not show cross reactivity with 18 kDa polypeptide of γ-conglutin. Also, the uncleaved monomeric γ-conglutin at 48 kDa (lane 2, Figure 5.5 B) was detected by the specific antibody.

5.4.4 FTIR analysis

FTIR is the commonly used technique for studying the secondary structures of proteins by analysing the vibrational frequencies of protein components. The polypeptide and protein repeat units give rise to nine characteristic vibrational bands, namely, amide A, B, and I-VII (Fabian & Mäntele, 2002). Of all the amide bands, amide I is considered as the most important region for the analysis of protein secondary structure as it is related to the backbone structure of the protein (Barth, 2007). However, amide II (Oberg, Ruysschaert, & Goormaghtigh, 2004) and amide III (Xie & Liu, 2003) band have also been shown to be useful in predicting protein structure.

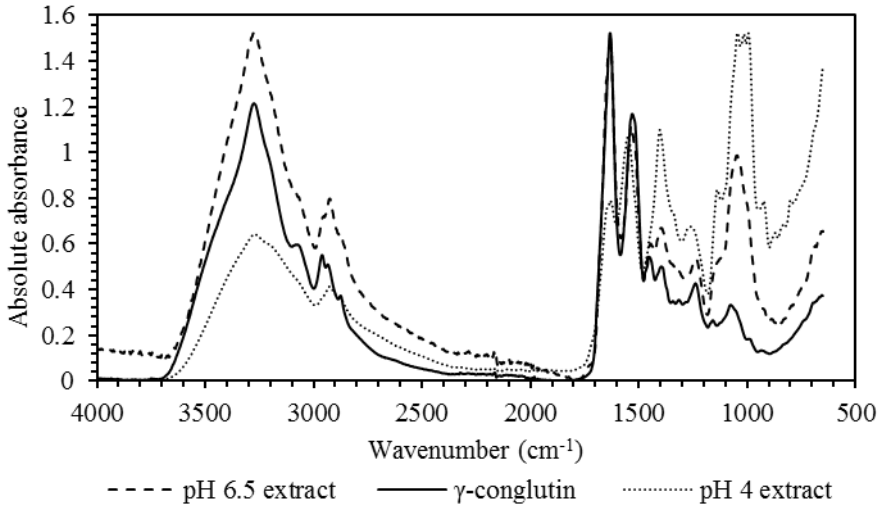

Figure 5.6 Overlay of FTIR spectra of freeze-dried extracts and γ-conglutin

FTIR spectrum of freeze-dried γ-conglutin standard was studied and compared with that of freeze-dried pH 4 extract and pH 6.5 modulated extract to identify structural changes in the purified protein (Figure 5.6). Several bands were observed in the mid-IR region (4000-400 cm^{-1}) as listed in Table 5.3. The occurrence of vibrational

frequencies was observed to be almost similar for both the extracts and purified γ-conglutin, except amide B (3071 cm^{-1}) band which was seen to be more prominent in the case of γ-conglutin (Figure 5.6).

The band at 3282 cm^{-1} represents amide A band which is mainly due to N-H stretch vibrations overlapped with O-H stretching vibrations of the hydrating H_2O molecule. This band is insensitive to the conformation of polypeptide backbone as it is localised on the N-H group in proteins (Barth, 2007). The part of amide A band has weakly absorbing frequency in the range of 3100-3000 cm^{-1}, which is recognised by amide B band due to the phenomenon of Fermi resonance (Barth, 2007).

C-H stretching vibrations of $–CH_2$, CH_3, CH and CHO functional groups are generally observed in the range of 2700-3000 cm^{-1}. This region can represent C-H stretching of either proteins or lipids (Howlett & Avery, 1997) or carbohydrates (Amir et al., 2013). In the case of pH 4 and pH 6.5 extract, C-H stretch vibrations were seen at 2950 cm^{-1}, whereas it was shifted to a higher wavenumber of 2970 cm^{-1} in the case of γ-conglutin (Figure 5.6).

Table 5.3 Major bands in FTIR spectra of extracts (pH 4 and pH 6.5) and γ-conglutin

Wavenumber (cm^{-1})	**Initial extracts**	**γ-conglutin**	**Amide region**
3282	✓	✓	Amide A
3071	×	✓	Amide B
2950-2970	✓	✓	--
1630	✓	✓	Amide I
1538	✓	✓	Amide II
1460	✓	✓	Amide II’
1396	✓	✓	Amide III
1243	✓	✓	Amide III
1052	✓	✓	--

A strong intense band observed at 1630 cm^{-1} (Figure 5.6) represents amide I band which is mainly due to C=O stretching vibrations with the minor contribution from out-of-phase C-N stretching vibrations and N-H in-plane bend. The nature of this band was similar in all the three samples, however, the intensity was observed to be less in the case of pH 4 extract. This might be due to less concentration of proteins in pH 4 extract as compare to other biomolecules. Amide I band depends upon the backbone

of the polypeptide chain and hence most commonly used for analysis of secondary structure (Barth, 2007). Amide I region of proteins consists of complex bands in terms of α-helix or β-sheets (parallel or antiparallel) or unordered structures (D'Souza, Devi, MP, & Naik, 2008). As amide I region decides the secondary structure of the protein, this region was further resolved by second derivative analysis (discussed in further section).

Amide I band was seen to be split into another major band at 1538 cm^{-1} representing amide II band (Figure 5.6). Amide II band is an out-of-phase combination of the N-H in-plane bend and the C-N stretching vibrations with smaller contributions from the C-O in-plane bend and the C-C and N-C stretching vibrations (Barth, 2007). Though there are reports on the prediction of secondary structure by amide II band alone (Oberg et al., 2004), the correlation between amide II frequency and protein secondary structure is less straightforward than that of amide I (Barth, 2007). N-deuteration converts amide II band to amide II' band by mixing with other modes in 1070-900 cm^{-1} region (Barth, 2007).

Amide III mode is the in-phase combination of N-H bending and C-N stretching vibrations with small contributions from the C-O in-plane bending and the C-C stretching vibrations (Barth, 2007). This mode is more complex as it depends on side chain structure of amino acid, unlike amide I and amide II bands. This region was observed at a very low intensity at two different wavenumbers of 1396 cm^{-1} and 1243 cm^{-1}, in the case of all the three samples.

The last band at the wavenumber of 1052 cm^{-1} (Figure 5.6) represents C-H stretching vibrations of carbohydrate group. This band was seen to be more intense in pH 4 and pH 6.5 extract, which might be due to the extraction of carbohydrate moieties in initial stages of extraction. This intensity was observed to be reduced in purified γ-conglutin. The reason for the occurrence of this band in purified γ-conglutin could be its glycoprotein nature, in which large subunit of γ-conglutin has carbohydrate moieties attached to it (Eaton-Mordas & Moore, 1979).

Analysis of second derivative spectra of freeze-dried extracts and pure γ-conglutin

As amide I region (1600-1700 cm^{-1}) decides the secondary structure of the protein, individual absorption in this region needs to be resolved completely so as to distinguish

the overlapped peaks. This is generally achieved by performing second derivative spectra so as to denoise the signals and to obtain precise calculations on band position in this region. Therefore, to study the secondary structure of the purified γ-conglutin, its amide I region was analysed by second derivative spectra and compared with that of pH 4 and pH 6.5 extract in order to track the structural changes during the purification process.

The overlay of second derivative spectra of freeze-dried pH 4 extract, pH 6.5 extract and γ-conglutin is as hown in Figure 5.7. One major (1630 cm^{-1}) and three minor (1642 cm^{-1}, 1656 cm^{-1} and 1685 cm^{-1}) bands were observed in amide I region in case of extracts and γ-conglutin (Table 5.4). In addition, the position of bands was found to be almost similar in all the three spectra, indicating no major structural damage to γ-conglutin during the purification process.

Table 5.4 Major bands in second derivative spectra of extracts and γ-conglutin

Wavenumber (cm^{-1})	**Assigned secondary structure**
1630	β-sheets
1642	β-sheets
1656	α-helix
1685	β-sheets

The presence of a major band at 1630 cm^{-1} in pH 6.5 extract and γ-conglutin represents the occurrence of β-sheets in larger amounts. However, this band was found to exist at higher intensity in γ-conglutin as compared to pH 6.5 extract. The lowest intensity of this band was observed in the case of pH 4 extract, which might be due to the fact that acetic acid used during adjusting the pH of extract to 4 might have converted β-sheets into α-helix (Blagrove & Gillespie, 1975). As a result, pH 4 extract was found to be rich in α-helical structures (Figure 5.7). The predominance of β-sheets in γ-conglutin analysed by FTIR in the present study is in close agreement with the previously reported results obtained by circular dichroism (Blagrove et al., 1980; Capraro et al., 2010; Marcello Duranti et al., 1995).

β-sheets (1620-1640 cm^{-1}) in the protein can be parallel or antiparallel depending on the alignment of polypeptides chains. Due to their presence at the same spectral position, it is experimentally difficult to distinguish between parallel and antiparallel β-sheets (Khurana & Fink, 2000). However, discrimination between parallel and

antiparallel β-sheets can be sometimes done by the absence of high wavenumber component (1685 cm^{-1}) in parallel β-sheets (Kubelka & Keiderling, 2001).

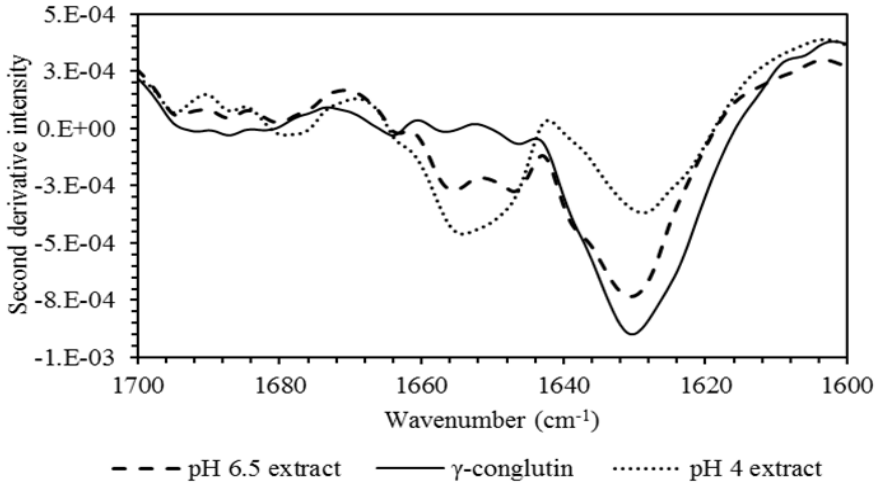

Figure 5.7 Overlay of second derivative spectra of freeze-dried extracts and γ-conglutin

Antiparallel β-sheets are known to exhibit a strong band near 1630 cm^{-1} and a weaker band at 1685 cm^{-1} (Wei, Jiao, & Xu, 2015). The position of β-sheets in spectra is more dependent on the number of strands in the protein and is hardly affected by the number of amide bands (Barth, 2007). The increase in the number of strands in β-sheets shifts the spectral position to lower wavenumber (1630 cm^{-1}), whereas their twisting shifts spectral position to higher wavenumber (1630 cm^{-1}) (Barth, 2007).

The minor band at 1656 cm^{-1} represents the occurrence of α-helix, however, the intensity was observed to be more in the case of pH 6.5 extract as compared to that of γ-conglutin. In addition to the bands mentioned in Table 5.4, few more bands were observed at very low intensity (Figure 5.7), which might be due to the fact that shorter α-helices produce several bands throughout amide I region (Torii & Tasumi, 1992). In the case of purified γ-conglutin, one more band at 1610 cm^{-1} was observed at very low intensity, however, this band was not seen in pH 4 and pH 6.5 extract (Figure 5.7). The reason behind the appearance of this band in γ-conglutin spectra could be due to the aggregation of protein to a lesser extent, which is a characteristic of intermolecular β-sheets (Arrondo & Goñi, 1999).

Thus, FTIR studies of pH 4 extract, pH 6.5 extract and γ-conglutin indicated no major changes in the structure of the protein during the purification process. All the spectra

indicated the presence of amide A, amide B, amide I-III. Second derivative spectra of amide I region showed the predominance of β-sheets in purified γ-conglutin sample.

5.4.5 RP-HPLC analysis

Pure γ-conglutin is freely soluble in ultrapure water at room temperature, therefore a simple linear gradient of water and acetonitrile was used to develop the method for its analysis. A C-18 column was selected for analysis to obtain optimal resolution of peaks in the sample (Snyder, 2012). Initially different trials with mobile phase gradients were performed, which resulted in an asymmetric peak with tailing effect. Therefore, an ion pairing agent (TFA) was added to the mobile phase as it is known to reduce tailing effects (Snyder, 2012). While developing a method, care was taken to provide sufficient equilibration and regeneration to avoid noise generated by the system. The run time of the developed method was 50 min, in which last 10 min were for regeneration and re-equilibration before further injections.

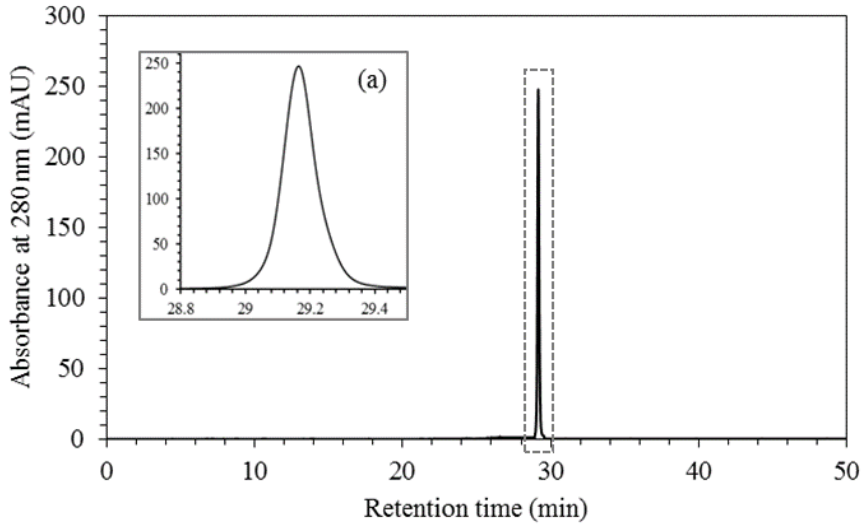

Figure 5.8 Spectral chromatogram of pure γ-conglutin (1 mg/ml)

The developed method (Table 5.1) resulted in a sharp peak with a retention time (RT) at 29.16 min, as represented in Figure 5.8. γ-Conglutin was seen to elute from the reverse phase column approximately between 60-70% v/v acetonitrile. The percentage area of the peak (Figure 5.8) was observed to be 97%, indicating the purity of the analyte (γ-conglutin) loaded on the column (Snyder, 2012). While developing method, the shape of the peak is considered as a performance characteristic for good quantitative results and is represented as peak asymmetry factor, *As*. Peak asymmetry factor is calculated as b/a where b is the distance from the peak midpoint to the trailing

edge of the peak measured at 10% of peak height and a is the distance from the leading edge of the peak to the peak midpoint measured at 10% of peak height (Snyder, 2012). Asymmetry factor of 0.95-1.10 is considered to be a symmetric peak with no tailing and fronting (Snyder, 2012). The asymmetric factor for the peak with RT of 29.16 min (Figure 5.8 a) was calculated to be 1.04, which represents an acceptable symmetric nature of peak for accurate quantification.

Thus the proposed method (Table 5.1) successfully produced a sharp and symmetric peak of γ-conglutin with RT at 29.16 min, with a simple mobile phase gradient. The developed method was then validated in accordance with guidelines provided by the FDA, AOAC International and ICH guidelines for its intended use as an analytical technique for detection and quantitation of γ-conglutin in lupin samples.

RP-HPLC method validation

Selectivity

The selectivity of RP-HPLC method for analysis of γ-conglutin was determined by observing the retention time of γ-conglutin after spiking with specific interference (pH 4 extract) and by calculating resolution between γ-conglutin peak and the nearest eluted peak in the extract. The original concentration of γ-conglutin in pH 4 extract was 0.1 mg/ml as calculated with the developed RP-HPLC method by comparing it with γ-conglutin in-house standard. Therefore, 1 mg/ml of pH 4 extract was added as interference to the standard γ-conglutin solution (0.1 mg/ml). The overlay of spectral chromatograms of standard γ-conglutin solution (0.1 mg/ml), original pH 4 extract and γ-conglutin (0.1 mg/ml) spiked with 1 ml of pH 4 extract is represented in Figure 5.9.

As seen from Figure 5.9, no change in the chromatogram pattern of original extract and the spiked extract was observed, except an increase in peak area at a retention time of 29.16 min (Figure 5.9 a). The area of γ-conglutin peak in the spiked extract was doubled as the concentration of γ-conglutin was doubled in the spiked extract. It was also observed from Figure 5.9 that the addition of pH 4 extract to standard γ-conglutin solution did not affect the retention time of γ-conglutin peak, which indicates the specificity of the developed RP-HPLC method for analysis of γ-conglutin.

The resolution between γ-conglutin and nearest eluting peak decides the degree at which the area of an analyte peak can be accurately measured for a perfectly symmetric

Gaussian peak (Azilawati et al., 2014). For minimum usable separation, *Rs*>1 is recommended by AOAC International (Azilawati et al., 2014; Verdon & Coueeder, 1999). The developed method demonstrated the chromatographic separation between γ-conglutin and the nearest eluting peak (Figure 5.10) with *Rs*=1.39. Similar results for selectivity of γ-conglutin was observed in the case of pH 6.5 extract (chromatogram not shown here). Thus the mobile phase composition and its gradient flow enabled separation of γ-conglutin in the presence of matrix/interference solution.

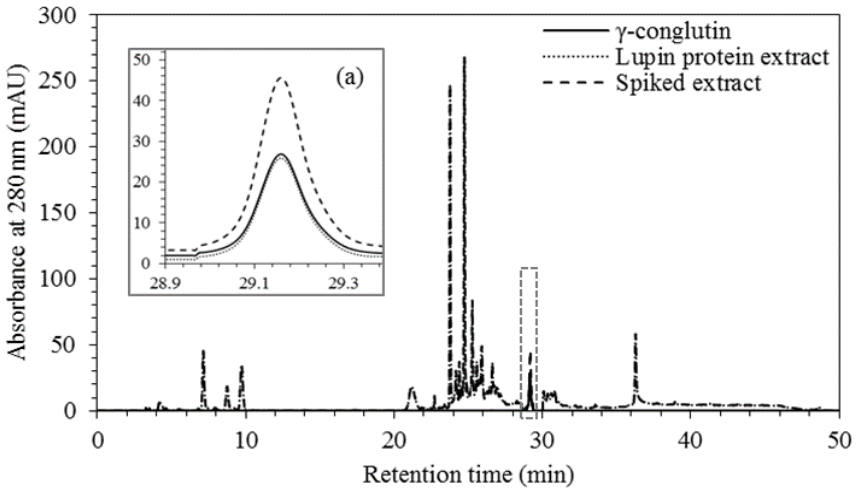

Figure 5.9 Overlay of spectral chromatograms of pure γ-conglutin (0.1 mg/ml), pH 4 extract and extract spiked with 0.1 mg/ml γ-conglutin

Linearity and range

Linearity of a method should be evaluated in the concentration span of 80-120% of the expected concentration range (Lindholm, 2004), starting from the lower limit of quantitation (Gonzalez & Herrador, 2007). Therefore, ten different concentrations of γ-conglutin ranging from 8, 15, 25, 50, 100, 250, 500 and 1000 µg/ml were analysed in five independent replicates. The overlay of spectral chromatograms of γ-conglutin at different concentrations is as represented in Figure 5.10. The height and area of the peak were observed to increase with an increase in the concentration of γ-conglutin (Figure 5.10 a). To determine the relationship between peak area and concentration of γ-conglutin, the calibration curve was constructed by plotting the mean response factor (peak area) against the respective concentration of γ-conglutin.

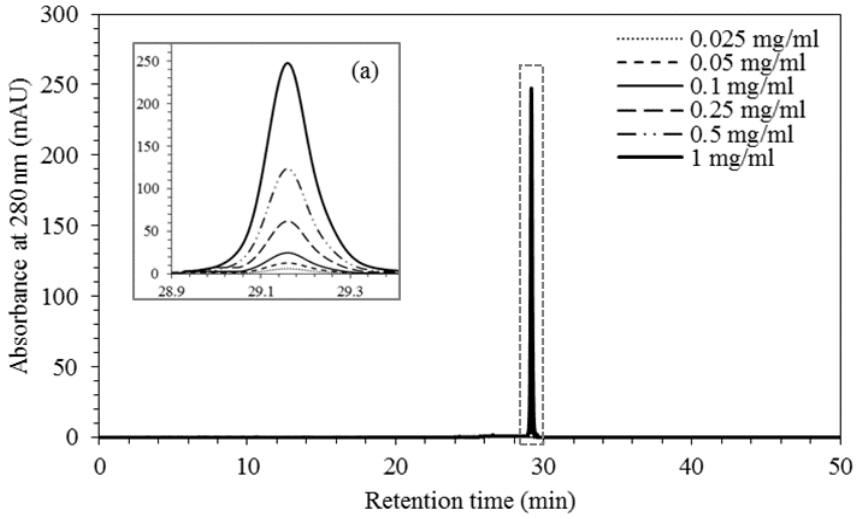

Figure 5.10 Overlay of spectral chromatograms of γ-conglutin at different concentrations

Linearity of the method was determined by correlation coefficient and intercept value of the calibration curve. A linear regression curve with a correlation coefficient of greater than 0.95 and an intercept not significantly different from zero is accepted as a standard curve (Lindholm, 2004). In the case of γ-conglutin, the linear relation with a good correlation coefficient of 0.999 was obtained (Figure 5.11), which shows the acceptable fit of the data to the regression line. From the standard equation of the calibration curve, values of slope and intercept were found to be 1957.2 and -2.7111 respectively.

Sensitivity, detection limit and quantitation limit

Sensitivity of a method for analysis of γ-conglutin was indicated in terms of the limit of detection (LOD) and limit of quantitation (LOQ). LOD and LOQ were calculated based on 3 times and 10 times the standard deviation of the response factor (peak area) over the slope, respectively (Azilawati et al., 2014; Lindholm, 2004). LOD and LOQ of γ-conglutin were estimated at 2.68 µg/ml and 8.12 µg/ml respectively.

Precision

Precision of the analytical method was expressed as the percentage of the relative standard deviation, % RSD, of responses at respective concentrations (Lindholm, 2004). The precision values are expected to yield the smallest variations among responses, generally RSD≤2 % at each concentration is required for developing highly precise analytical method (Lindholm, 2004). Intra-day and inter-day precision of the

developed method for analysis of γ-conglutin were found to be in the range of 0.38-0.49% (Table 5.5). Precision values at each concentration were within the acceptance criteria. The results indicated the high precision of the developed analytical method, with very less variability at different concentrations measured on different days.

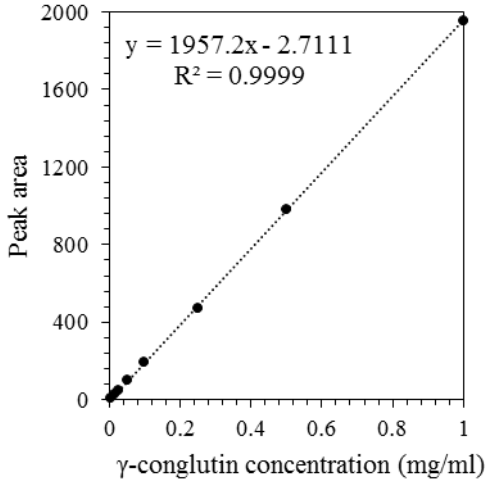

Figure 5.11 Calibration curve of γ-conglutin at different concentrations

Accuracy and Recovery

Accuracy of the proposed RP-HPLC method for analysis of γ-conglutin was expressed in terms of recovery at three different levels (80, 100 and 120%) in the range of expected concentration (five independent replicates). Recovery is calculated as the ratio of experimental concentration (concentration extrapolated from calibration curve) to theoretical concentration (added concentration for spiking) of the analyte (γ-conglutin) in the spiked extract (pH 4 extract). The average percentage recovery for γ-conglutin was found to be 97.77%, 97.01% and 98.63% for 0.08 mg/ml, 0.10 mg/ml and 0.12 mg/ml respectively (Table 5.6). The results indicated acceptable values of recovery at different concentration levels as recommended by AOAC Guidelines, which defines the high accuracy of the proposed RP-HPLC method for detection and quantitation of γ-conglutin (Horwitz, 2002).

Thus the performance characteristics of the developed RP-HPLC method were successfully validated based on guidelines of the known regulatory bodies. The method was found to be specific, reproducible and accurate for detection and quantitation of γ-conglutin for lupin seed extracts. The validated method can be suitably used for an effective and precise analysis of γ-conglutin in lupin extracts.

Table 5.5 Precision analysis (intra-day and inter-day) of γ-conglutin at three different concentrations

γ-conglutin added in extract (mg/ml)	**Intra-day studies***			**Inter-day studies***		
	R[a] ± SD[b]	**RT[c] ± SD[b]**	**% RSD[d]**	**R[a] ± SD[b]**	**RT[c] ± SD[b]**	**% RSD[d]**
0.08	330 ± 1.62	29.16 ± 0.07	0.49	333 ± 1.58	29.16 ± 0.08	0.47
0.10	378 ± 1.59	29.16 ± 0.08	0.42	382 ± 1.56	29.16 ± 0.09	0.41
0.12	417 ± 1.57	29.16 ± 0.06	0.38	423 ± 1.61	29.16 ± 0.07	0.38

*Average of five determinations

[a]Response (peak area)

[b]Standard deviation of five readings

[c]Retention time (min)

[d]Relative standard deviation

Table 5.6 Recovery and accuracy studies of γ-conglutin at three different concentrations

γ-conglutin in extract (mg/ml)	**R[a] ± SD[b]**	**RT[c] ± SD[b]**	**γ-conglutin added in extract (mg)**	**R[a] ± SD[b]**	**RT[c] ± SD[b]**	**γ-conglutin recovered (mg/ml)**	**Recovery %**
0.1	150 ± 1.52	29.16 ± 0.05	0.08	330 ± 1.52	29.16 ± 0.03	0.17 ± 0.006	97.77
0.1	150 ± 1.52	29.16 ± 0.06	0.10	378 ± 1.50	29.16 ± 0.02	0.19 ± 0.004	97.01
0.1	150 ± 1.52	29.16 ± 0.04	0.12	417 ± 1.48	29.16 ± 0.03	0.21 ± 0.007	98.63

[a]Response (peak area)

[b]Standard deviation of five readings

[c]Retention time (min)

[d]Relative standard deviation

5.4.6 MS/MS analysis

The same peak fraction (Figure 5.8 a) was analysed by mass spectrometry (MS/MS) to confirm its identity as γ-conglutin. The sample for MS/MS analysis was prepared and digested with trypsin as mentioned in section 5.3.6. The obtained MS/MS spectrum (Figure 5.12) was analysed using Mascot sequence matching software (Matrix Science) with MSPnr100 database to confirm the protein of interest. The database (MSPnr100) was a Viridiplantae (Green Plants) database downloaded in January 2017 with 5, 315, 975 sequences. A peptide tolerance of 0.2 and MS/MS tolerance of 0.2 with 1 missed cleavage was used as the default setting for protein identification. Individual ions scores > 48 indicate identity or extensive homology ($p<0.05$). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

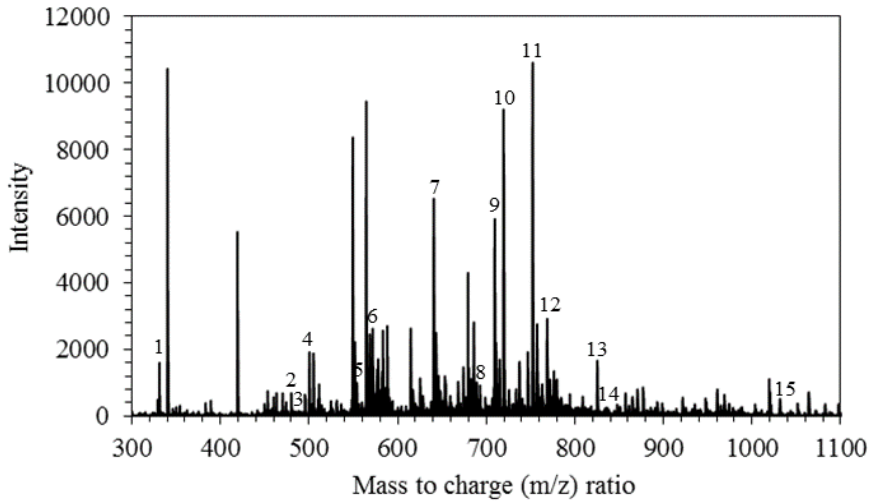

Figure 5.12 Mass spectrum of tryptic digest peptides of γ-conglutin HPLC peak (The numbers on peaks are peptides matched with those from γ-conglutin database, Q42369)

The protein hit with the maximum score of 540 was observed for γ-conglutin from *Lupinus angustifolius* (tr, Q42369) (Figure 5.13). In this case, 126 out of 449 amino acid residues from γ-conglutin database were observed to match with peptides generated from γ-conglutin peak, showing 28% coverage of peptide sequences. The second hit score of 42 was found for basic 7S globulin like protein from *Cicer arietinum* (NCBI, XP_004494958.1), which is reported to be homologous with γ-conglutin from *Lupinus albus* (M Duranti, Scarafoni, Di Cataldo, & Sessa, 2001), with 10% coverage.

1	MARNMAHILH	ILVISLSYSF	LFVSSSSQDS	QSLYHNSQPT	SSKPNLLVLP
51	VQEDASTGLH	WANIHKR**TPL**	**MQVPLLLDLN**	**GK**HLWVTCSQ	HYSSSTYQAP
101	FCHSTQCSRA	NTHQCFTCTD	STTTRPGCHN	NTCGLLSSNP	VTQESGLGEL
151	AQDVLAIHST	HGSK**LGPMVK**	VPQFLFSCAP	SFLAQK**GLPN**	**NVQGALGLGQ**
201	**APISLQNQLF**	**SHFGLK**RQFS	VCLSRYSTSN	GAILFGDIND	PNNNNYIHNS
251	LDVLHDLVYT	PLTISK**QGEY**	**FIQVNAIR**VN	KHLVIPTKNP	FISPSSTSYH
301	GSGEIGGALI	TTTHPYTVLS	HSIFEVFTQV	FANNMPKQAQ	VK**AVGPFGLC**
351	**YDSRKISGGA**	**PSVDLILDKN**	**DAVWR**ISSEN	FMVQAQDGVS	CLGFVDGGVH
401	AR**AGIALGAH**	**HLEENLVVFD**	**LER**SR**VGFNS**	**NSLK**SYGKTC	SNLFDLNNP

Figure 5.13 Protein view showing 28% peptide sequence coverage to Q42369 (matched peptides are shown in bold letters)

The characteristic details of the peptides (generated from γ-conglutin peak) confirming the identity of the protein and matching specific peptides from the database of γ-conglutin (Q42369) are represented in Table 5.7. This coverage coupled with the protein score substantiates the identity of γ-conglutin obtained by the proposed RP-HPLC method.

5.5 CONCLUSION

The structural characterization of γ-conglutin discussed in this Chapter shed light on the efficiency of the developed method to purify γ-conglutin with its intact structural aspects. SDS-PAGE and scanning densitometry analysis showed that the developed method purifies γ-conglutin mainly in its monomeric form, without its denaturation during the process. Western blotting confirmed the identity of γ-conglutin based on its reactivity against specific polyclonal antibodies. The developed process for purification of γ-conglutin was not leading to any major structural changes in the protein during purification process as analysed by FTIR study. The second derivative analysis of FTIR spectra revealed the presence of β-sheets in the purified γ-conglutin sample. RP-HPLC (C-18) method for detection and quantification of γ-conglutin from lupin seed extract was developed and validated. The mobile phase gradient and the flow rate of 0.8 ml/min were found to be appropriate to provide a sharp and symmetric peak (*As*=1.04) of γ-conglutin with retention time at 29.16 min. The identity of γ-conglutin was confirmed by MS/MS analysis, which showed 28% peptide sequence coverage of the γ-conglutin precursor (Q42369).

Table 5.7 Matching of masses of peptides generated from γ-conglutin peak (Figure 5.12) with the peptides available in the database of Q42369

Peak number	Peptide (m/z)	Peptide (Z)	Peptide score*	Peptide sequence	Start-end peptide sequence coverage
1	322.69	2	23	LGPMVK	165-170
2	479.92	3	19	QGEYFIQVNAIR	267-278
3	483.25	2	35	VGFNSNSLK	426-434
4	505.01	3	36	KISGGAPSVDLILDK	355-369
5	556.70	3	27	TPLMQVPLLLDLNGK	68-82
6	576.56	4	22	AGIALGAHHLEENLVVFDLER	403-423
7	642.87	2	28	AVGPFGLCYDSR	343-354
8	692.89	2	49	ISGGAPSVDLILDK	356-369
9	709.38	3	65	ISGGAPSVDLILDKNDAVWR	356-375
10	719.38	2	79	QGEYFIQVNAIR	267-278
11	757.02	2	9	KISGGAPSVDLILDK	355-369
12	768.41	3	61	AGIALGAHHLEENLVVFDLER	403-423
13	826.54	2	68	TPLMQVPLLLDLNGK	68-82
14	834.43	2	49	TPLMQVPLLLDLNGK	68-82
15	1040.25	3	60	GLPNNVQGALGLGQAPISLQNQLFSHFGLK	187-216

*Peptides with a score <48 have a < 95% confidence for identity but are useful for confirming the overall protein identity and add to the protein score of 540.

Chapter 6

Conclusions and future work

6.1 CONCLUSIONS

The findings presented in this thesis provide the first process for selective extraction and adsorptive capture of 'glucose controlling bioactive protein', γ-conglutin from lupin seeds with highest reported purity (95%) in minimum possible process steps. The process for the selective extraction of γ-conglutin was designed by studying solubility curve for lupin conglutins, followed by optimizing key parameters for extraction by one factor at a time approach. The optimized extraction conditions were able to extract γ-conglutin selectively from lupin kernel flour, along with few impurities, which were reduced partly by introducing an intermediate pH modulation step. This was then followed by a screening of different cation exchange adsorbents in a batch process to find the suitable adsorbent for adsorptive capture of γ-conglutin from the extract. The selected adsorbent was then checked for its performance in dynamic conditions and different process parameters were optimized in order to increase the resolution between γ-conglutin and an impurity peak. γ-Conglutin obtained by the optimized method gradient was then characterized by different analytical techniques for its identity confirmation. Conclusions from each individual chapter are summarized below.

6.1.1 Extraction optimization of lupin conglutins

The major conglutins (α and β-conglutin) of lupin seeds have potential applications in food industries on account of their emulsification and oil binding properties (Kohajdova, Karovicova, & Schmidt, 2011). Until now, the extraction of lupin conglutins has been performed by conventional alkaline extraction followed by isoelectric precipitation at acidic pH (Blagrove & Gillespie, 1975; Marcello Duranti, Restani, Poniatowska, & Cerletti, 1981; Melo, Ferreira, & Teixeira, 1994). Only one report (Ruiz & Hove, 1976) is available on the study of the effect of process parameters on conglutin extraction, however, it is based on one factor at a time approach, which does not consider the interaction between parameters.

Therefore, in this thesis, the statistical optimization of conglutin extraction was performed by DoE in order to study the interactions between process parameters and to find their optimal combination so as to increase the conglutin extraction yield and purity. Initially, the key parameters for conglutin extraction were investigated by one factor at a time approach, followed by the implementation of RSM CCD to find the optimal combination of these key parameters to achieve maximum conglutin extraction. The rate of conglutin extraction was also studied and the rate kinetics parameters were analysed by Peleg model. Further, to enhance the extraction efficiency, three different extraction approaches (magnetic stirring, ultrasonication and hydrodynamic cavitation) were used and optimized by RSM CCD. These extraction approaches were compared on the basis of conglutin yield, SDS-PAGE and FTIR profile. There was no significant difference observed in SDS-PAGE and FTIR of conglutins extracted by these extraction approaches.

The key factors and their optimized values by RSM were found to be pH of ultrapure water (9), the volume of water added (30 ml/gm) and extraction time (30 min), yielding almost 89% w/w conglutins by magnetic stirring, 91% w/w by ultrasonication and 92% w/w by hydrodynamic cavitation. SDS-PAGE and FTIR profile showed no difference in protein profile of conglutins extracted by three different extraction approaches. In future, the effect of these extraction approaches on emulsification and oil binding properties of extracted conglutins should be studied in order to select the suitable extraction approach for conglutin extraction for its intended use in food industries.

6.1.2 Development of cation exchange chromatography process

The available methods for purification of γ-conglutin are multistep (Blagrove & Gillespie, 1975; Czubinski et al., 2015; Melo et al., 1994) and involve the use of potentially toxic chemicals, such as zinc (M Duranti, Scarafoni, Di Cataldo, & Sessa, 2001; Sironi, Sessa, & Duranti, 2005), which makes these processes questionable for industrial scale production of γ-conglutin, for its use in nutraceutical and pharmaceutical applications. Therefore, this thesis was aimed to develop a prototype for an effective larger scale technology for production of γ-conglutin with least possible process steps. This was achieved by integrating selective extraction at acidic pH and adsorptive capture on cation exchange adsorbent.

In order to find suitable pH for selective extraction of γ-conglutin, the solubility curve for lupin conglutins was studied at different pH values. γ-Conglutin has a basic pI (6.9-8.0), unlike other conglutins whose pI are in acidic range (4-5) (Blagrove, Gillespie, Lilley, & Woods, 1980; Marcello Duranti et al., 1981; Sironi et al., 2005). Therefore, though the solubility study exhibited maximum conglutin extraction at alkaline pH, acidic pH of 4 demonstrated selective extraction of γ-conglutin as analysed by SDS-PAGE. As a result, the extraction was optimized at pH 4, which yielded 15% w/w γ-conglutin enriched fraction with optimized process parameters (solid to solvent ratio of 1:30 and extraction time of 30 min at room temperature). The selective extraction at acidic pH reduced the major impurities of other conglutin fractions in the extraction stage itself. γ-Conglutin enriched extract was observed to have the presence of few impurities from IMW units of β-conglutin, due to their pI in the range nearer to γ-conglutin. Therefore, the intermediate step of pH modulation was introduced, where pH of γ-conglutin enriched extract was modulated to pI of the impurities (pH 6.5). This key intermediate pH modulation step partly imparted neutral charges on the surface of impurities, therefore, impurities will not bind or loosely bind to the cation exchange adsorbent.

Different cation exchange adsorbents were screened for the adsorptive capture of γ-conglutin from the enriched extract in a batch process. SDS-PAGE analysis of batch screening experiment showed promising results with Capto S, which was further packed in a column and checked for its performance in dynamic conditions. The adsorption behaviour of γ-conglutin on Capto S in static conditions was explained by Langmuir isotherm model, with maximum binding capacity of 91.75 mg/ml and dissociation constant of 4.47×10^{-5} M. The effect of process parameters, such as conductivity, flow rate, pH and elution gradient on the resolution of γ-conglutin and an impurity peak was studied by one factor at a time approach. The method flow rate and elution gradient were found to be highly influential for the effective separation of γ-conglutin from impurities. The resolution between γ-conglutin and an impurity peak was increased from the value of 0.39 to 1.59 by using a flow rate of 0.5 ml/min and an optimized elution gradient. The yield and purity of γ-conglutin obtained with this integrated process were 2.38% w/w (of total globulins) and 95% respectively. Thus the overall steps in the conventional process for purification of γ-conglutin were reduced by integrating acidic extraction and chromatographic purification with an

intermediate pH modulation step. Clear advantages of this research of higher yield and purity in minimum process steps can supersede the methods reported earlier in the literature, making it an innovative and novel technology for designing and scale up of adsorptive chromatographic processes for commercial purification of γ-conglutin. The developed purification process for producing γ-conglutin is as shown in Figure 6.1.

6.1.3 Characterization of γ-conglutin

γ-Conglutin purified by the developed process was characterized by different analytical techniques as summarized below, to confirm its identity and to check the efficiency of the developed method to purify γ-conglutin without any structural denaturation.

SDS-PAGE and scanning densitometry

The purity of γ-conglutin obtained by the developed purification process was found to be 98% by SDS-PAGE and scanning densitometry analysis, indicating the presence γ-conglutin mainly in its monomeric form at 48 kDa (88%) and oligomeric form at 92 kDa (10%) under non-reduced conditions. Under reduced conditions, 92 kDa and 48 kDa γ-conglutin was reduced to its single polypeptides at 30 kDa (75%) and 18 kDa (16%) through breakage of the disulphide linkage. Around 9% of the monomeric form at 48 kDa was still observed under reducing conditions, which might be due to improper denaturation or resistance by some precursor for denaturation.

Western blotting

The cross reactivity of γ-conglutin against the specified polyclonal antibodies (generated against a synthetic peptide of γ-conglutin) in reducing and non-reducing conditions was demonstrated using western blotting. The western blot exhibited the bands at 92 kDa and 48 kDa in reducing conditions, indicating strong cross reactivity of the specified antibodies towards purified γ-conglutin. The antibodies are specific to large chain subunit (30 kDa) of γ-conglutin, therefore in reducing conditions, the antibodies showed reactivity only against 30 kDa unit.

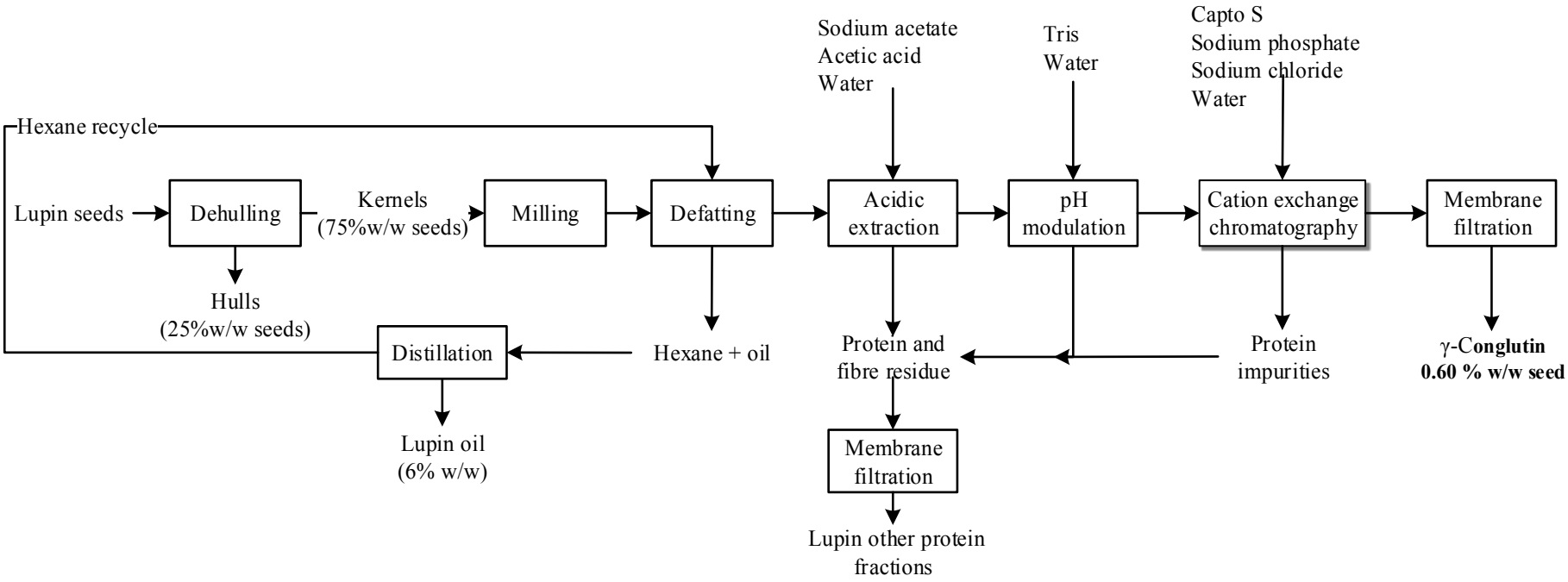

Figure 6.1 Developed purification process for producing γ-conglutin

FTIR

The analysis of the secondary structure of the purified γ-conglutin and the demonstration of the structural changes taking place in γ-conglutin during purification process was done using FTIR. The detailed spectral analysis of γ-conglutin, pH 4 extract and pH 6.5 modulated extract in the mid IR range (4000-400 cm^{-1}) showed no conformational changes in γ-conglutin structure during the purification process. For a precise analysis of the secondary structure of γ-conglutin, second derivative analysis of amide I region was performed, which revealed the predominance of β-sheets in the purified γ-conglutin sample.

RP-HPLC

A simple, selective and accurate reverse phase HPLC method was developed for detection and quantitation of γ-conglutin from lupin seed extract. A linear gradient of water and acetonitrile containing trifluoroacetic acid (TFA) on a reverse phase column (Agilent Zorbax 300SB C-18), with a flow rate of 0.8 ml/min was able to produce a sharp and symmetric peak of γ-conglutin with a retention time at 29.16 min. The proposed method was validated in terms of specificity, linearity, sensitivity, precision, recovery and accuracy as per the guidelines of ICH, FDA and AOAC. The percentage purity of γ-conglutin obtained by developed purification process was observed to be 97% as analysed from the peak area of standard γ-conglutin with the developed RP-HPLC method. The analytical parameters revealed that the validated method was capable of selectively performing a good chromatographic separation of γ-conglutin from the lupin seed extract with no interference of the matrix. The detection and quantitation limit of γ-conglutin were found to be 2.68 μg/ml and 8.12 μg/ml respectively. The accuracy (precision and recovery) analysis of the method was conducted under repeatable conditions on different days. Intra-day and inter-day precision values less than 0.5% and recovery greater than 96% indicated high precision and accuracy of the method for analysis of γ-conglutin. The method validation findings were reproducible and can be successfully applied for routine analysis of γ-conglutin from lupin seed extract.

MS/MS analysis

The identification of γ-conglutin was performed by MS analysis and the peptides generated were matched against the specified database using the software 'MASCOT'.

The peptide matching analysis demonstrated the maximum score of 540 with the available γ-conglutin precursor from *lupinus angustifolius* (Q42369). In this analysis, 126 out of 449 amino acid residues from γ-conglutin database were matched with the peptides generated from the purified γ-conglutin, indicating 28% coverage with the available γ-conglutin precursor.

Thus, γ-conglutin purified by the developed process was confirmed for its identity by necessary analytical techniques, so that the developed process can be further scaled up and implement for the production of nutraceutical and pharmaceutical grade γ-conglutin on a commercial scale. In conclusion, the findings presented in this thesis have a range of potential outcomes for several sectors of the community as follows:

- The nutraceutical/pharmaceutical industries could benefit from new industrially viable technology for efficient manufacturing of γ-conglutin as a glycaemic modulating bioactive.
- Health professionals could benefit from the availability of new nutraceutical and food products based on γ-conglutin with potent glycaemic regulation so as to recommend to clients for optimal health.
- The agricultural industry could benefit through greater demand and return on lupin crops grown specifically for γ-conglutin manufacture.
- Consumers could benefit from a reduced financial and quality of life burden that results from poor glucose control through the use of γ-conglutin.

6.2 RECOMMENDATIONS FOR FUTURE WORK

This thesis provides comprehensive details of the design of a scalable purification process for obtaining 95% pure γ-conglutin from lupin seeds, intended for future nutraceutical/pharmaceutical applications. Nevertheless, there are some areas where further research is needed.

6.2.1 Process improvement

Currently, the developed process has been successfully implemented at lab scale for producing 1 gm γ-conglutin. However, this process takes more than 40 hr due to high loading volume at low flow rates (64.20 cm/hr), as the feed stream belongs to low concentration-high volume category. The process can be made faster to produce 1gm

γ-conglutin per day by concentrating the feed stream by membrane filtration before loading on the column.

The recovery of the process can be increased to more than 90% by performing re-extraction of the remained residue at pH 4. The exact percentage of γ-conglutin in lupin seed is still questionable as there is no analytical method available for absolute quantification of γ-conglutin in lupin seed flour. The author and colleagues are currently working on developing isotope labelled mass spectroscopic analysis for absolute γ-conglutin quantitation with the data generated from this thesis.

6.2.2 Stability studies

The purified γ-conglutin will be studied for its stability at different temperatures and pH conditions. The samples will be studied for its stability at different time intervals and analysed by SDS-PAGE and the developed RP-HPLC method.

6.2.3 Scale up

The developed process can then be scaled up to produce 10 gm γ-conglutin per day (small scale pilot plant) by increasing the diameter of the column (diametrical scale up) to incorporate higher flow rates without affecting the efficiency of the separation.

Further, the process can be scaled up to large scale pilot plant (1 kg γ-conglutin per day) by converting batch process into continuous one (liquid solid circulating fluidized bed or by connecting cascade of columns in series).

6.2.4 Antidiabetic mechanism of γ-conglutin

There have been reports on the hypoglycemic activity of γ-conglutin in animal models (Magni et al., 2004), however, the detailed mechanism of its antidiabetic action has not yet been investigated. Therefore, the studies are under progress for substantiation of its antidiabetic mechanism by both insulinotropic (increase in insulin secretion) and insulin-mimetic (insulin like behaviour) ways in cell lines and in animal models.

6.2.5 Glycoprotein analysis

γ-Conglutin is a glycoprotein, in which the large subunit (30 kDa) has been reported to carry N-linked oligosaccharide moiety (Marcello Duranti et al., 1981). In future work, the profiling of the carbohydrate moieties covalently attached to γ-conglutin will

be performed using LC-MS. The information obtained will be beneficial in investigating the allergic potential of the bound carbohydrate moieties.

6.2.6 X-ray diffraction analysis

The crystallization of the purified γ-conglutin will be performed to obtain the crystals of pure γ-conglutin with desired specifications. This will be then followed by X-ray diffraction analysis to study the structural conformation of the purified γ-conglutin. The protein data file obtained after X-ray diffraction analysis will be used to create the three dimensional structural model of γ-conglutin, which then can be studied for its stability and reactivity with different molecules using molecular modelling software.

6.2.7 Interaction with flavonoids

γ-Conglutin has been reported to show resistance to digestion with pancreatin, recently this has been attributed to its interaction with flavonoids present in the lupin seed, either by hydrogen bonding to carbohydrate moiety or by electrostatic attraction on the surface of γ-conglutin (Czubinski et al., 2012). Therefore, the purified γ-conglutin will be analysed for its flavonoid content, followed by its digestion studies with pancreatic enzymes.

References

Aguilera, J., Gerngross, M., & Lusas, E. (1983). Aqueous processing of lupin seed. *International Journal of Food Science & Technology, 18*(3), 327-333.

Ahamed, T., Nfor, B. K., Verhaert, P. D., van Dedem, G. W., van der Wielen, L. A., Eppink, M. H., Van de Sandt, E. J., & Ottens, M. (2007). pH-gradient ion-exchange chromatography: An analytical tool for design and optimization of protein separations. *Journal of Chromatography A, 1164*(1), 181-188.

Amersham, B. (2004). *Stratergies for protein purification* (pp. 167).

Amir, R. M., Anjum, F. M., Khan, M. I., Khan, M. R., Pasha, I., & Nadeem, M. (2013). Application of Fourier transform infrared spectroscopy for the identification of wheat varieties. *Journal of food science and technology, 50*(5), 1018-1023.

AOAC guidelines for single laboratory validation of chemical methods for dietary supplements and botanicals, (2002).

Arnoldi, A. (2008). *Nutraceutical properties of white and narrow-leaved lupin.* Paper presented at the Proceedings of the 12th International Lupin Conference—Lupins for health and wealth.

Arrondo, J. L. R., & Goñi, F. M. (1999). Structure and dynamics of membrane proteins as studied by infrared spectroscopy. *Progress in biophysics and molecular biology, 72*(4), 367-405.

Azilawati, M., Hashim, D., Jamilah, B., & Amin, I. (2014). Validation of a reverse-phase HPLC method for the determination of amino acids in gelatins by application of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate reagent. *Journal of Chromatography A, 1353*, 49-56.

Bader, S., Czerny, M., Eisner, P., & Buettner, A. (2009). Characterisation of odour-active compounds in lupin flour. *Journal of the Science of Food and Agriculture, 89*(14), 2421-2427.

Bader, S., Oviedo, J. P., Pickardt, C., & Eisner, P. (2011). Influence of different organic solvents on the functional and sensory properties of lupin (Lupinus angustifolius L.) proteins. *LWT-Food Science and Technology, 44*(6), 1396-1404.

Barth, A. (2007). Infrared spectroscopy of proteins. *Biochimica et Biophysica Acta (BBA)-Bioenergetics, 1767*(9), 1073-1101.

Berghout, J., Boom, R., & van der Goot, A. (2014). The potential of aqueous fractionation of lupin seeds for high-protein foods. *Food chemistry, 159*, 64-70.

Bertoglio, J. C., Calvo, M. A., Hancke, J. L., Burgos, R. A., Riva, A., Morazzoni, P., Ponzone, C., Magni, C., & Duranti, M. (2011). Hypoglycemic effect of lupin seed γ-conglutin in experimental animals and healthy human subjects. *Fitoterapia, 82*(7), 933-938.

Bez, J., Schott, M., Knauf, U., Resta, D., Agostina, A., & Arnoldi, A. (2005). Protein isolates from sweet white lupin seeds: pilot production and techno-functional properties. *Optimized Processing for Preparing Healthy and Added Value Food Ingredients from Lupin Kernel, the European Protein-rich Grain Legume*, 21-35.

Biosciences, A. (2002). Ion Exchange Chromatography, Principles and Methods. *ISBN, 91*, 970490-970493.

Biotech, A. P. (1998). Gel filtration: Principles and Methods: Lund.

Blagrove, R., & Gillespie, J. (1975). Isolation, purification and characterization of the seed globulins of Lupinus angustifolius. *Functional Plant Biology, 2*(1), 13-27.

Blagrove, R., Gillespie, J., Lilley, G., & Woods, E. (1980). Physicochemical studies of conglutin γ, a storage globulin from seeds of Lupinus angustifolius. *Functional Plant Biology, 7*(1), 1-13.

Blaicher, F., Nolte, R., & Mukherjee, K. (1981). Lupin protein concentrates by extraction with aqueous alcohols. *Journal of the American Oil Chemists' Society, 58*(7), A761-A765.

Brambilla, F., Resta, D., Isak, I., Zanotti, M., & Arnoldi, A. (2009). A label-free internal standard method for the differential analysis of bioactive lupin proteins using nano HPLC-Chip coupled with Ion Trap mass spectrometry. *Proteomics, 9*(2), 272-286.

Bringans, S., Eriksen, S., Kendrick, T., Gopalakrishnakone, P., Livk, A., Lock, R., & Lipscombe, R. (2008). Proteomic analysis of the venom of Heterometrus longimanus (Asian black scorpion). *Proteomics, 8*(5), 1081-1096.

Caballero, B., Trugo, L., & Finglas, P. (2003). *Encyclopedia of food sciences and nutrition: Volumes 1-10*: Elsevier Science BV.

Capraro, J., Spotti, P., Magni, C., Scarafoni, A., & Duranti, M. (2010). Spectroscopic studies on the pH-dependent structural dynamics of γ-conglutin, the blood glucose-lowering protein of lupin seeds. *International journal of biological macromolecules, 47*(4), 502-507.

Casey, R. (1999). Distribution and some properties of seed globulins *Seed proteins* (pp. 159-169): Springer.

Chapleau, N., & de Lamballerie-Anton, M. (2003). Improvement of emulsifying properties of lupin proteins by high pressure induced aggregation. *Food Hydrocolloids, 17*(3), 273-280.

Cheung, R. C. F., Wong, J. H., & Ng, T. B. (2012). Immobilized metal ion affinity chromatography: a review on its applications. *Applied microbiology and biotechnology, 96*(6), 1411-1420.

Chew, P. G., Casey, A. J., & Johnson, S. K. (2003). Protein quality and physico-functionality of Australian sweet lupin (Lupinus angustifolius cv. Gungurru) protein concentrates prepared by isoelectric precipitation or ultrafiltration. *Food chemistry, 83*(4), 575-583.

Curtis, R., Prausnitz, J., & Blanch, H. (1998). Protein-protein and protein-salt interactions in aqueous protein solutions containing concentrated electrolytes. *Biotechnology and bioengineering, 57*(1), 11-21.

Czubinski, J., Barciszewski, J., Gilski, M., Szpotkowski, K., Debski, J., Lampart-Szczapa, E., & Jaskolski, M. (2015). Structure of γ-conglutin: insight into the quaternary structure of 7S basic globulins from legumes. *Acta Crystallographica Section D: Biological Crystallography, 71*(2), 224-238.

Czubinski, J., Dwiecki, K., Siger, A., Kachlicki, P., Neunert, G., Lampart-Szczapa, E., & Nogala-Kalucka, M. (2012). Release of flavonoids from lupin globulin proteins during digestion in a model system. *Journal of agricultural and food chemistry, 60*(7), 1830-1836.

Czubinski, J., Dwiecki, K., Siger, A., Neunert, G., & Lampart-Szczapa, E. (2014). Characterisation of different digestion susceptibility of lupin seed globulins. *Food chemistry, 143*, 418-426.

D'Agostina, A., Antonioni, C., Resta, D., Arnoldi, A., Bez, J., Knauf, U., & Wasche, A. (2006). Optimization of a pilot-scale process for producing lupin protein isolates with valuable technological properties and minimum thermal damage. *Journal of agricultural and food chemistry, 54*(1), 92-98.

D'Souza, L., Devi, P., MP, D. S., & Naik, C. G. (2008). Use of Fourier Transform Infrared (FTIR) spectroscopy to study cadmium-induced changes in Padina tetrastromatica (Hauck). *Analytical Chemistry Insights, 3*, 135.

DAFWA. (2017). Lupins in Western Australia farming. *Department of Agriculture and Food Australia*

Derbyshire, E., Wright, D., & Boulter, D. (1976). Legumin and vicilin, storage proteins of legume seeds. *Phytochemistry, 15*(1), 3-24.

Donzelli F., V. R., Andrea G and Duranti M. (2015). *Indirect ELISA test for quantitation of conglutin γ in Lupinus albus seeds and extracts (PRO-GAMMA ®)*. Paper presented at the XIV International Lupin Conference, Milan (Italy).

Drakos, A., Doxastakis, G., & Kiosseoglou, V. (2007). Functional effects of lupin proteins in comminuted meat and emulsion gels. *Food Chemistry, 100*(2), 650-655.

Duranti, M. (2006). Grain legume proteins and nutraceutical properties. *Fitoterapia, 77*(2), 67-82.

Duranti, M., Cerletti, P., Postiglione, L., & Lanza, A. (1990). Synthesis of storage proteins and seed development in Lupinus albus under different growth conditions. *Agricoltura Mediterranea, 120*(3), 262-267.

Duranti, M., Consonni, A., Magni, C., Sessa, F., & Scarafoni, A. (2008). The major proteins of lupin seed: characterisation and molecular properties for use as functional and nutraceutical ingredients. *Trends in Food Science & Technology, 19*(12), 624-633.

Duranti, M., Gius, C., Sessa, F., & Vecchio, G. (1995). The saccharide chain of lupin seed conglutin γ is not responsible for the protection of the native protein from degradation by trypsin, but facilitates the refolding of the acid-treated protein to the resistant conformation. *European journal of biochemistry, 230*(3), 886-891.

Duranti, M., Guerrieri, N., Takahashi, T., & Cerletti, P. (1988). The legumin-like storage protein of Lupinus albus seeds. *Phytochemistry, 27*(1), 15-23.

Duranti, M., Restani, P., Poniatowska, M., & Cerletti, P. (1981). The seed globulins of Lupinus albus. *Phytochemistry, 20*(9), 2071-2075.

Duranti, M., Scarafoni, A., Di Cataldo, A., & Sessa, F. (2001). Interaction of metal ions with lupin seed conglutin γ. *Phytochemistry, 56*(6), 529-533.

Dutta, D., Mahabir, S., & Pathak, Y. V. (2013). Background to Nutraceuticals and Human Health *Nutraceuticals and Health: Review of Human Evidence* (pp. 3-12): CRC Press.

Eaton-Mordas, C. A., & Moore, K. G. (1979). Subunit glycosylation of Lupinus angustifolius seed globulins. *Phytochemistry, 18*(11), 1775-1779.

El-Adawy, T., Rahma, E., El-Bedawey, A., & Gafar, A. (2001). Nutritional potential and functional properties of sweet and bitter lupin seed protein isolates. *Food Chemistry, 74*(4), 455-462.

Evans, A. J., Cheung, P. C. K., & Cheetham, N. W. (1993). The carbohydrate composition of cotyledons and hulls of cultivars of Lupinus angustifolius from Western Australia. *Journal of the Science of Food and Agriculture, 61*(2), 189-194.

Fabian, H., & Mäntele, W. (2002). Infrared spectroscopy of proteins. *Handbook of vibrational spectroscopy*.

FAO-STAT. (2017). Crops-Lupin production.

Guidelines for the validation of chemical methods for the FDA foods program, (2015).

Foley, R., Singh, K., Gilmartin, C. Bowler (Eds.). (2002). Western and Southwestern cDNA library screening. *Molecular plant biology volume one. A practical approach, Oxford University Press, Oxford*

Freundlich, H. (1906). Over the adsorption in solution. *J. Phys. Chem, 57*(385471), 1100-1107.

Gaberc-Porekar, V., & Menart, V. (2001). Perspectives of immobilized-metal affinity chromatography. *Journal of biochemical and biophysical methods, 49*(1), 335-360.

Gan, S. D., & Patel, K. R. (2013). Enzyme immunoassay and enzyme-linked immunosorbent assay. *Journal of Investigative Dermatology, 133*(9), 1-3.

Garzon-de la Mora, P., Avalos-Alcantara, G., Gurrola-Diaz, C., Lopez, J., Brune, D., Garcia-Lopez, J., Zamora-Natera, J., Garcia-Lopez, P., Palta, J., & Berger, J. (2008). *Isoltion and purity assessment of Lupin albus conglutines.* Paper presented at the Proceedings 12th International Lupin Conference.

GE_Healthcare. (2017). *GE Healthcare Life Sciences.*

Ghosh, R. (2006). *Principles of bioseparations engineering*: World Scientific Publishing Co Inc.

Gibbs, S. J., & Lightfoot, E. N. (1986). Scaling up gradient elution chromatography. *Industrial & Engineering Chemistry Fundamentals, 25*(4), 490-498.

Giles, C., MacEwan, T., Nakhwa, S., & Smith, D. (1960). 786. Studies in adsorption. Part XI. A system of classification of solution adsorption isotherms, and its use in diagnosis of adsorption mechanisms and in measurement of specific surface areas of solids. *Journal of the Chemical Society (Resumed)*, 3973-3993.

Glencross, B. (2008). *Harvesting the benefits of lupin meals in aquaculture feeds.* Paper presented at the Lupins for health and wealth. Proceedings of the 12th International Lupin Conference, Fremantle, Western Australia, 14-18 September 2008.

Glenng, L. (1999). 14. Lupin Globulins. *Seed Proteins*, 315.

Gogate, P. R., Tayal, R. K., & Pandit, A. B. (2006). Cavitation: a technology on the horizon. *Current Science, 91*(1), 35-46.

Gonzalez, A. G., & Herrador, M. A. (2007). A practical guide to analytical method validation, including measurement uncertainty and accuracy profiles. *TrAC Trends in Analytical Chemistry, 26*(3), 227-238.

Green, J. M. (1996). Peer reviewed: a practical guide to analytical method validation. *Analytical Chemistry, 68*(9), 305A-309A.

Gross, R., Von Baer, E., Koch, F., Marquard, R., Trugo, L., & Wink, M. (1988). Chemical composition of a new variety of the Andean lupin (Lupinus mutabilis cv. Inti) with low-alkaloid content. *Journal of food composition and analysis, 1*(4), 353-361.

Gupta, V., Jain, A. D. K., Gill, N., & Gupta, K. (2012). Development and validation of HPLC method-a review. *Int. Res J Pharm. App Sci, 2*(4), 17-25.

Hall, R., Johnson, S., Baxter, A., & Ball, M. (2005). Lupin kernel fibre-enriched foods beneficially modify serum lipids in men. *European journal of clinical nutrition, 59*(3), 325-333.

Harrison, R. G., Todd, P., Todd, P. W., Petrides, D. P., & Rudge, S. R. (2015). *Bioseparations science and engineering*: Oxford University Press, USA.

Horwitz, W. (2002). AOAC guidelines for single laboratory validation of chemical methods for dietary supplements and botanicals. *AOAC International, Gaithersburg, MD, USA*.

Hove, E. L. (1974). Composition and protein quality of sweet lupin seed. *Journal of the Science of Food and Agriculture, 25*(7), 851-859.

Howlett, N. G., & Avery, S. V. (1997). Induction of lipid peroxidation during heavy metal stress in Saccharomyces cerevisiae and influence of plasma membrane fatty acid unsaturation. *Applied and environmental microbiology, 63*(8), 2971-2976.

Hron, R., Koltun, S., & Graci, A. (1982). Biorenewable solvents for vegetable oil extraction. *Journal of the American Oil Chemists Society, 59*(9), 674A-684A.

Hughes, T. R., & Klotz, I. M. (1956). Analysis of Metal-Protein Complexes. *Methods of Biochemical Analysis, Volume 3*, 265-299.

Hunt, J. A., Ahmed, M., & Fierke, C. A. (1999). Metal binding specificity in carbonic anhydrase is influenced by conserved hydrophobic core residues. *Biochemistry, 38*(28), 9054-9062.

Guidelines for the validation of analytical procedures, (2005).

Institute, S. (2000). *JMP: Statistics and Graphics Guide*: Sas Inst.

Jagschies, G., Sofer, G. K., & Hagel, L. (2007). *Handbook of process chromatography: development, manufacturing, validation and economics*: Academic Press.

Jain, P., Jivani, H., Khatal, R., & Surana, S. (2011). Development and validation of RP-HPLC method for estimation of ethacridine lactate in bulk and in pharmaceutical formulation. *Pharmaceutical methods, 2*(2), 112-116.

Janson, J.-C. (2012). *Protein purification: principles, high resolution methods, and applications* (Vol. 151): John Wiley & Sons.

Janson, J. C., & Hedman, P. (1987). On the optimization of process chromatography of proteins. *Biotechnology progress, 3*(1), 9-13.

Jean-Marc, B., & Carre, B. (1989). Characterization of polysaccharides from white lupin. *Journal of the Science of Food and Agriculture, 34*, 861-865.

Jenkins, D. J., Wolever, T. M., & Jenkins, A. L. (1988). Starchy foods and glycemic index. *Diabetes care, 11*(2), 149-159.

Johansson, B.-L., Belew, M., Eriksson, S., Glad, G., Lind, O., Maloisel, J.-L., & Norrman, N. (2003). Preparation and characterization of prototypes for multi-modal separation aimed for capture of positively charged biomolecules at high-salt conditions. *Journal of Chromatography A, 1016*(1), 35-49.

Johnson, S. K., Clements, J., Villarino, C. B. J. and Coorey, R. . (in press). Lupins: Their unique nutritional and health-promoting attributes. In A. eds J. R. N. Taylor and J. M. Awika. Elsevier (Ed.), *The Gluten-free Ancient Grains: Cereals, Pseudocereals and Legumes- Sustainable, Nutritious and Health-promoting Foods for the 21st Century*.

Joubert, F. (1956). Lupin seed proteins III. A physico-chemical study of the proteins from white lupin seed (Lupinus albus). *Biochimica et biophysica acta, 19*, 172-173.

Kale, S., & Lali, A. (2011). Characterization of superporous cellulose matrix for high-throughput adsorptive purification of lysozyme. *Biotechnology progress, 27*(4), 1078-1090.

Karacabey, E., Bayindirli, L., Artik, N., & Mazza, G. (2013). Modelling solid-liquid extraction kinetics of trans-reseratrol and trans ε-viniferin from grape cane. *Journal of Food Process Engineering, 36*(1), 103-112.

Kaur, L., Rutherfurd, S. M., Moughan, P. J., Drummond, L., & Boland, M. J. (2010). Actinidin enhances gastric protein digestion as assessed using an in vitro gastric digestion model. *Journal of agricultural and food chemistry, 58*(8), 5068-5073.

Khurana, R., & Fink, A. L. (2000). Do parallel β-helix proteins have a unique Fourier transform infrared spectrum? *Biophysical journal, 78*(2), 994-1000.

Kohajdova, Z., Karovicova, J., & Schmidt, S. (2011). Lupin composition and possible use in bakery–a review. *Czech J. Food Sci, 29*(3), 203-211.

Komkova, E. (2010). Principles of Ion-Exchange Chromatography for Buffer Optimization. *Natrix Separations Inc., Burlington, ON, TN1001 rev, 610*.

Kopaciewicz, W., & Regnier, F. (1983). Mobile phase selection for the high-performance ion-exchange chromatography of proteins. *Analytical biochemistry, 133*(1), 251-259.

Kubelka, J., & Keiderling, T. A. (2001). Differentiation of β-sheet-forming structures: Ab initio-based simulations of IR absorption and vibrational CD for model peptide and protein β-sheets. *Journal of the American chemical society, 123*(48), 12048-12058.

Kumar, P., Lau, P. W., Kale, S., Johnson, S., Pareek, V., Utikar, R., & Lali, A. (2014). Kafirin adsorption on ion-exchange resins: Isotherm and kinetic studies. *Journal of chromatography A, 1356*, 105-116.

Kurien, B. T., & Scofield, R. H. (2006). Western blotting. *Methods, 38*(4), 283-293.

Langmuir, I. (1916). The constitution and fundamental properties of solids and liquids. Part I. Solids. *Journal of the American chemical society, 38*(11), 2221-2295.

Leterme, P. (2002). Recommendations by health organizations for pulse consumption. *British Journal of Nutrition, 88*(S3), 239-242.

Lindholm, J. (2004). *Development and validation of HPLC methods for analytical and preparative purposes.* Acta Universitatis Upsaliensis.

Liyana-Pathirana, C., & Shahidi, F. (2005). Optimization of extraction of phenolic compounds from wheat using response surface methodology. *Food chemistry, 93*(1), 47-56.

Lovati, M. R., Manzoni, C., Castiglioni, S., Parolari, A., Magni, C., & Duranti, M. (2012). Lupin seed γ-conglutin lowers blood glucose in hyperglycaemic rats and increases glucose consumption of HepG2 cells. *British Journal of Nutrition, 107*(01), 67-73.

Lqari, H., Vioque, J., Pedroche, J., & Millan, F. (2002). Lupinus angustifolius protein isolates: chemical composition, functional properties and protein characterization. *Food Chemistry, 76*(3), 349-356.

Lupins.org. (2015). Lupins Information Resource.

Magni, C., Sessa, F., Accardo, E., Vanoni, M., Morazzoni, P., Scarafoni, A., & Duranti, M. (2004). Conglutin γ, a lupin seed protein, binds insulin in vitro and reduces plasma glucose levels of hyperglycemic rats. *The Journal of nutritional biochemistry, 15*(11), 646-650.

Mandal, S., & Mandal, R. (2000). Seed storage proteins and approaches for improvement of their nutritional quality by genetic engineering. *Current Science-Bangalore, 79*(5), 576-589.

Mane, S., Agrawal, S., Utikar, R., & Kale, S. (2014). *Extraction of gamma-conglutin from lupin seeds.* Paper presented at the Chemeca 2014: Processing excellence; Powering our future.

Mane S, Johnson. S., Duranti M, Pareek V, Utikar R. (2018). Lupin seed γ-conglutin: extraction and purification methods- A review. *Trends in Food Science and Technology*, *73*, 1-11.

Marchesi, M., Parolini, C., Diani, E., Rigamonti, E., Cornelli, L., Arnoldi, A., Chiesa, G. (2008). Hypolipidaemic and anti-atherosclerotic effects of lupin proteins in a rabbit model. *British journal of nutrition, 100*(4), 707-710.

Melo, T. S., Ferreira, R. B., & Teixeira, A. N. (1994). The seed storage proteins from Lupinus albus. *Phytochemistry, 37*(3), 641-648.

Millan, F., Alaiz, M., Hernandez-Pinzon, I., Sanchez, R., & Bautista, J. (1994). Study of lipid components of Lupinus mutabilis meal and isolates. *Bioscience, biotechnology, and biochemistry, 58*(12), 2258-2260.

Mohamed, A., & Rayas-Duarte, P. (1995). Composition of Lupinus albus1. *Cereal Chem, 72*(6), 643-647.

Molina-Bolívar, J., Galisteo-González, F., Ruiz, C. C., Medina-O, M., & Parra, A. (2014). Spectroscopic investigation on the interaction of maslinic acid with bovine serum albumin. *Journal of Luminescence, 156*, 141-149.

Mujumdar, S., Kumar, P. S., & Pandit, A. (1997). Emulsification by ultrasound: Relation between intensity and emulsion quality.

Muranyi, I. S., Otto, C., Pickardt, C., Koehler, P., & Schweiggert-Weisz, U. (2013). Microscopic characterisation and composition of proteins from lupin seed (Lupinus angustifolius L.) as affected by the isolation procedure. *Food research international, 54*(2), 1419-1429.

Myers, R. H., Montgomery, D. C., Vining, G. G., Borror, C. M., & Kowalski, S. M. (2004). Response surface methodology: a retrospective and literature survey. *Journal of quality technology, 36*(1), 53.

Nfor, B. K., Ahamed, T., van Dedem, G. W., van der Wielen, L. A., van de Sandt, E. J., Eppink, M. H., & Ottens, M. (2008). Design strategies for integrated protein purification processes: challenges, progress and outlook. *Journal of chemical technology and biotechnology, 83*(2), 124-132.

Oberg, K. A., Ruysschaert, J. M., & Goormaghtigh, E. (2004). The optimization of protein secondary structure determination with infrared and circular dichroism spectra. *The FEBS Journal, 271*(14), 2937-2948.

Oomah, B. D., Tiger, N., Olson, M., & Balasubramanian, P. (2006). Phenolics and antioxidative activities in narrow-leafed lupins (Lupinus angustifolius L.). *Plant Foods for Human Nutrition (Formerly Qualitas Plantarum), 61*(2), 86-92.

Pilvi, T., Jauhiainen, T., Cheng, Z., Mervaala, E., Vapaatalo, H., & Korpela, R. (2006). Lupin protein attenuates the development. *Journal of physiology and pharmacology, 57*(2), 167-176.

Planinic, M., Velic, D., Tomas, S., Bilic, M., & Bucic, A. (2005). Modelling of drying and rehydration of carrots using Peleg's model. *European Food Research and Technology, 221*(3-4), 446-451.

Preece, K., Hooshyar, N., Krijgsman, A., Fryer, P., & Zuidam, N. (2017). Intensification of protein extraction from soybean processing materials using hydrodynamic cavitation. *Innovative Food Science & Emerging Technologies*.

Rathore, A., & Velayudhan, A. (2003). Guidelines for optimization and scale-up in preparative chromatography. *Biopharm international, 16*(1), 34-42.

Regnier, F. E. (1983). HPLC of proteins, peptides, and polynucleotides. *Analytical Chemistry, 55*(13), 1298A-1306A.

Reinhard, H., Rupp, H., Sager, F., Streule, M., & Zoller, O. (2006). Quinolizidine alkaloids and phomopsins in lupin seeds and lupin containing food. *Journal of Chromatography A, 1112*(1), 353-360.

Resta, D., Brambilla, F., & Arnoldi, A. (2012). HPLC-Chip-Multiple Reaction Monitoring (MRM) method for the label-free absolute quantification of γ-conglutin in lupin: Proteotypic peptides and standard addition method. *Food Chemistry, 131*(1), 126-133.

Restani, P., Duranti, M., Cerletti, P., & Simonetti, P. (1981). Subunit composition of the seed globulins of Lupinus albus. *Phytochemistry, 20*(9), 2077-2083.

Ritthausen, H. (1882). Mittheilungen des agriculturchemischen Laboratoriums der Universität Königsberg. 8. Ueber das Verhalten des Conglutins aus Lupinensamen zu Salzlösungen. *Advanced Synthesis & Catalysis, 26*(1), 422-440.

Rodrigues, I. M., Coelho, J. F., & Carvalho, M. G. V. (2012). Isolation and valorisation of vegetable proteins from oilseed plants: Methods, limitations and potential. *Journal of Food Engineering, 109*(3), 337-346.

Roe, S. (2001). *Protein purification applications*: Oxford University Press.

Rona, C., Vailati, F., & Berardesca, E. (2004). The cosmetic treatment of wrinkles. *Journal of cosmetic dermatology, 3*(1), 26-34.

Rowe, J., & Hargreave, G. (1988). *Nutritive value of lupin hulls.* Paper presented at the Proceedings of the Australian Society of Animal Production.

Ruiz, L. P., & Hove, E. L. (1976). Conditions affecting production of a protein isolate from lupin seed kernels. *Journal of the Science of Food and Agriculture, 27*(7), 667-674.

Scarafoni, A., Ronchi, A., & Duranti, M. (2009). A real-time PCR method for the detection and quantification of lupin flour in wheat flour-based matrices. *Food Chemistry, 115*(3), 1088-1093.

Schiarea, S., Arnoldi, L., Fanelli, R., De Combarieu, E., & Chiabrando, C. (2013). In-depth glycoproteomic characterization of γ-conglutin by high-resolution accurate mass spectrometry. *PloS one, 8*(9), e73906.

Schober, T. J., Bean, S. R., Tilley, M., Smith, B. M., & Ioerger, B. P. (2011). Impact of different isolation procedures on the functionality of zein and kafirin. *Journal of cereal science, 54*(2), 241-249.

Scientific, T. (2013). Pierce BCA protein assay kit. *Pierce BCA 449 Protein Assay Kit*.

Sipsas, S. (2008). *Lupin products—Concepts and reality*. Paper presented at the Proceedings of the 12th International Lupin Conference—Lupins for health and wealth.

Sipsas ,S. (2004). New Lupin Products: Final Report of Project DAW00069.Grains Research and Development Corporation, Barton, ACT, Australia.

Sironi, E., Sessa, F., & Duranti, M. (2005). A simple procedure of lupin seed protein fractionation for selective food applications. *European Food Research and Technology, 221*(1-2), 145-150.

Sirtori, C. R., Arnoldi, A., & Johnson, S. K. (2005). Phytoestrogens: end of a tale? *Annals of medicine, 37*(6), 423-438.

Sirtori, C. R., Lovati, M. R., Manzoni, C., Castiglioni, S., Duranti, M., Magni, C., Arnoldi, A. (2004). Proteins of white lupin seed, a naturally isoflavone-poor legume, reduce cholesterolemia in rats and increase LDL receptor activity in HepG2 cells. *The Journal of nutrition, 134*(1), 18-23.

Sistla, R., Tata, V., Kashyap, Y., Chandrasekar, D., & Diwan, P. (2005). Development and validation of a reversed-phase HPLC method for the determination of ezetimibe in pharmaceutical dosage forms. *Journal of pharmaceutical and biomedical analysis, 39*(3), 517-522.

Snyder, L. R. K., Joseph J Glajch, Joseph L. (2012). *Practical HPLC method development*: John Wiley and Sons,.

Sofer, G. (1995). Preparative chromatographic separations in pharmaceutical, diagnostic, and biotechnology industries: current and future trends. *Journal of Chromatography A, 707*(1), 23-28.

Speight, J. G. (2005). *Lange's handbook of chemistry* (Vol. 1): McGraw-Hill New York.

Stout, R., Sivakoff, S., Ricker, R., & Snyder, L. (1986). Separation of proteins by gradient elution from ion-exchange columns: optimizing experimental conditions. *Journal of Chromatography A, 353*, 439-463.

Sweetingham, M., & Kingwell, R. (2008). *Lupins-reflections and future possibilities.* Paper presented at the 12th International Lupin Conference; Fremantle, Western Australia.

Tek, H. N., Turhan, K. N., & Etzel, M. R. (2005). Effect of conductivity, pH, and elution buffer salinity on glycomacropeptide recovery from whey using anion exchange chromatography. *Journal of food science, 70*(4), E295-E300.

Terruzzi, I., Senesi, P., Magni, C., Montesano, A., Scarafoni, A., Luzi, L., & Duranti, M. (2011). Insulin-mimetic action of conglutin-γ, a lupin seed protein, in mouse myoblasts. *Nutrition, Metabolism and Cardiovascular Diseases, 21*(3), 197-205.

Torii, H., & Tasumi, M. (1992). Model calculations on the amide-I infrared bands of globular proteins. *The Journal of chemical physics, 96*(5), 3379-3387.

Uzun, B., Arslan, C., Karhan, M., & Toker, C. (2007). Fat and fatty acids of white lupin (Lupinus albus L.) in comparison to sesame (Sesamum indicum L.). *Food Chemistry, 102*(1), 45-49.

Verdon, E., & Coueeder, P. (1999). Multiresidue analytical method for the determination of eight penicillin antibiotics in muscle tissue by ion-pair reversed-phase HPLC after precolumn derivatization. *Journal of AOAC International, 82*, 1083-1096.

Villarino, C. B. J. (2014). *Maximising the nutritional and sensory quality of Lupin bread made using Western Australian bakers flour.*

Waksmundzka-Hajnos, M., Petruczynik, A., Dragan, A., Wianowska, D., & Dawidowicz, A. (2004). Effect of extraction method on the yield of furanocoumarins from fruits of Archangelica officinalis Hoffm. *Phytochemical Analysis, 15*(5), 313-319.

Wang, S., Errington, S., & Yap, H. H. (2008). *Studies on carotenoids from lupin seeds.* Paper presented at the Proceedings of 12th International Lupin Conference.

Wasche, A., Muller, K., & Knauf, U. (2001). New processing of lupin protein isolates and functional properties. *Food/Nahrung, 45*(6), 393-395.

Webb, D. A. Systems Biology Mascot Server. Retrieved from https://www.wehi.edu.au/people/andrew-webb/1295/andrew-webb-resources

Wei, Z., Jiao, D., & Xu, J. (2015). Using Fourier transform infrared spectroscopy to study effects of magnetic field treatment on wheat (Triticum aestivum l.) seedlings. *Journal of Spectroscopy, 2015*.

WHO. (2014). *Global status report on noncommunicable diseases 2014* *http://www.who.int/nmh/publications/ncd-status-report-2014/en/* (9241564857). Retrieved from

Widmann, M., Trodler, P., & Pleiss, J. (2010). The Isoelectric Region of Proteins: A Systematic Analysis. *PloS one, 5*(5), e10546.

Wilson, K., & Walker, J. (2010). *Principles and techniques of biochemistry and molecular biology*: Cambridge university press.

Wong, A., Pitts, K., Jayasena, V., & Johnson, S. (2013). Isolation and foaming functionality of acid-soluble protein from lupin (Lupinus angustifolius) kernels. *Journal of the Science of Food and Agriculture, 93*(15), 3755-3762.

Xiao, C. W. (2008). Health effects of soy protein and isoflavones in humans. *The Journal of nutrition, 138*(6), 1244S-1249S.

Xie, M.-X., & Liu, Y. (2003). Studies on Amide Ⅲ Infrared Bands for the Secondary Structure Determination of Proteins [J]. *Chemical Research In Chinese Universities, 2*, 011.

Yada, R. (2004). *Proteins in food processing*: Elsevier.

Yang, H. (2008). *Fc-binding hexamer peptide ligands for immunoglobulin purification*: North Carolina State University.

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